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# Genetic and ecological insights of Swiss morels (*Morchella* spp.), including an agricultural perspective

Blaise Hofer, *Morchella helvetica*

A dissertation submitted to the University of Neuchâtel,

for the degree of

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by

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Le Doyen, Prof. R. Bshary

*Thoughts bound and concentrated, burning like pyres*

*Open new windows in space, in time*

*Tides of Telepathy – Avatarium*

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# Summary

True morels (Ascomycota, *Pezizomycetes, Morchella*) are one of the rare edible mushrooms that can be collected in the wild and cultivated at commercial scales. The morel fruiting body is a healthy food appreciated worldwide as gourmet food and for its medicinal properties. *Morchella* have a complex taxonomy but are basically divided into three main clades (Rufobrunnea, Esculenta, Elata). *Morchella* also display a complicated lifecycle and trophic system, that remain partially unknown. Morels can reproduce asexually, and sexually by producing ascospores-bearing fruiting bodies. The mating type locus is the master regulator of sexual reproduction, and the genes it contains inform on the reproductive system of an organism. In *Morchella,* homothallism, pseudohomothallism, and heterothallism exist. Morels were domesticated in the early eighties, and since then the cultivation process has not stopped to improve. China is currently the only country to commercialize cultivated morels at large scale. In Switzerland, markets are provided with environmentally unsustainable and potentially socially unfair morel mushrooms. Harvesting morels in the wild cannot meet the demand, hence the development of cultivation in the Switzerland is needed. A native strain must be domesticated, because importing non-indigenous cultivars can cause biodiversity threats, namely invasiveness and hybridization. To do so, it is crucial to study Swiss morels. This thesis provides the first knowledge background necessary to develop morel cultivation in Switzerland. In section 2, the biodiversity of *Morchella* was assessed in nine cantons of the country. In this survey one species of the Esculenta clade (*Morchella esculenta*) and three described species of the Elata clade (*Morchella importuna, Morchella deliciosa/*Mel-13*, Morchella pulchella* species complex) were found. In addition, four new lineages were discovered, one of which was described as a new species (*Morchella helvetica* sp. nov.) in section 3. Section 4 evaluated an AI-based identification tool (the Centroid-based approach) that was able to identify 83% of the morel species based on genetic sequences of the internal transcribed spacer. The performance was comparable with four-locus phylogenetic analyses that were able to identify 84% of the *Morchella* at species level. In sections 5-6, the sexual reproduction system of Swiss *Morchella* was investigated. The reliability of four primer pairs was tested, the mating genotype of single-ascospore isolates of Swiss strains was assessed, and the genetic structure of the mating type locus was determined analyzing two whole genomes that were sequenced for this purpose. This revealed that Swiss morels were mainly heterothallic. The last sections investigated biologic interactions between *Morchella* and other organisms that it could encounter in soil. In section 7, tripartite interactions between *Morchella,* the bacterium *Pseudomonas koreensis*, and a bacteriophagous amoeba *Acanthamoeba castellanii* were investigated. This revealed an unexpected association between the fungus and the amoeba to thrive in the presence of the bacterium. In addition, it was demonstrated for the first time that *P. koreensis* can form biofilm around *Morchella* hyphae, and that amoeba can use hypha to facilitate their movement across unsaturated areas of the medium (i.e., fungal highways). Finally, in section 8, the mycophagous nematode *Aphelenchus avenae* and three fungal contaminants isolated from a fruiting body cultivation assay (*Penicillium* sp., *Cephalotrichum* sp., *Aspergillus westerdijkiae*). Overall, *Morchella* had a natural resistance against the predatory nematodes and outcompeted the fungal contaminants.

**Keywords:** true morels (*Morchella)*, cultivation, biodiversity, taxonomy, mating types, tripartite interactions.

# Résumé

Les morilles (Ascomycota, *Pezizomycetes, Morchella*) sont l'un des rares champignons comestibles que l'on peut ramasser dans la nature et cultiver à l'échelle commerciale. La fructification de la morille est un aliment sain apprécié dans le monde entier pour sa saveur et ses propriétés médicinales. La taxonomie de *Morchella* est complexe, essentiellement divisées en trois clades principaux (Rufobrunnea, Esculenta, Elata). Les morilles ont également un cycle de vie et un système trophique compliqués, restant partiellement inconnus. Les morilles peuvent se reproduire de manière asexuée, et sexuée en produisant des corps fructifères où se forment les ascospores. Le locus du type d'accouplement (MAT) est le régulateur principal de la reproduction sexuelle, et les gènes qu'il contient informent sur le système de reproduction. Les morilles peuvent être homothalliques, pseudohomothalliques ou hétérothalliques. Les morilles ont été domestiquées au début des années 80 et le processus de culture n'a cessé de s'améliorer. La Chine est actuellement le seul pays à commercialiser des morilles cultivées à grande échelle. En Suisse, les marchés sont approvisionnés en morilles non durables d'un point de vue environnemental et social. La récolte de morilles à l'état sauvage ne peut répondre à la demande, d'où la nécessité de développer la culture de morilles dans le pays. Une souche indigène doit être domestiquée, car l'importation de cultivars non natifs peut entraîner des menaces pour la biodiversité (invasions et hybridation). Il est ainsi essentiel d'étudier les morilles suisses. Cette thèse fournit les premières connaissances nécessaires au développement de la culture des morilles en Suisse. Dans la section 2, la biodiversité des morilles a été évaluée dans neuf cantons. Il en ressort qu'une espèce du clade Esculenta (*Morchella esculenta*) et trois espèces du clade Elata (*Morchella importuna, Morchella deliciosa*/Mel-13, complexe d'espèces *Morchella pulchella*) ont été trouvées. De plus, quatre nouvelles lignées ont été découvertes, l'une d'entre elles étant décrite comme une nouvelle espèce (*Morchella helvetica* sp. nov.) dans la section 3. La section 4 a évalué un outil d'identification basé sur l'IA (approche Centroïde) qui a permis d'identifier 83 % des espèces de morilles sur la base des séquences génétiques de l'espace transcrit interne (ITS). Cet outil concurrence les analyses phylogénétiques à quatre locus, permettant d'identifier 84% des espèces de morille. Dans les sections 5-6, la reproduction sexuelle des morilles suisses a été étudiée. La fiabilité de quatre paires d'amorces PCR a été testée, les génotypes de reproduction ont été évalués, et la structure génétique du locus MAT a été déterminée en analysant deux génomes entiers séquencés pour cette étude. Ceci a révélé que les morilles suisses étaient principalement hétérothalliques, conformément à la littérature. Les dernières sections ont étudié les interactions biologiques entre *Morchella* et d'autres organismes du sol. Dans la section 7, les interactions tripartites entre *Morchella*, *Pseudomonas koreensis* et une amibe bactériophage *Acanthamoeba castellanii* ont été étudiées. Cela a révélé une association inattendue entre le champignon et l'amibe. De plus, il a été démontré pour la première fois que *P. koreensis* peut former un biofilm autour des hyphes de *Morchella*, et que l'amibe peut utiliser les hyphes comme autoroutes fongiques. La section 8 portait sur le nématode mycophage *Aphelenchus avenae* et trois contaminants fongiques isolés à partir d'un essai de culture (*Penicillium* sp., *Cephalotrichum* sp., *Aspergillus westerdijkiae*). *Morchella* avait une résistance naturelle contre les nématodes prédateurs et était plus compétitive que les contaminants.

**Mots-clés :** morilles (*Morchella*), culture, biodiversité, taxonomie, reproduction, interactions tripartites.

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# Glossary

**Adsorption:** adhesion of gas or liquid to a surface.

**Antheridia:** male fertilizing organ in fungi.

**Ascogonia:** female reproductive organ in ascomycetes.

**Ascomycete:** or Ascomycota, phylum of Fungi constituting the Dikarya subkingdom with the basidiomycetes.

**Ascospore:** sexual spore produced by Ascomycetes.

**Ascus:** cell containing ascospores.

**Ataxia:** poor muscle control causing clumsy voluntary movements.

**Chlamydospore:** highly resistant asexual spore produced by fungi.

**Clade:** in *Morchella*, taxonomic unit corresponding to the “Section”, i.e., rank between the genus and the species.

**Commensalism:** association between two organisms, that is beneficial for one of them but neutral for the other.

**Conidiospore:** or conidia, asexual fugal spore.

**Continental endemism:** geographically restricted either to North America or Eurasia.

**Cyst:** dormant stage of an amoebal cell.

**Cultivar:** organism obtained by selective breeding in a cultivation process.

**Diagnosis (taxonomy):** distinctive characterization of a taxonomic unit.

**Dyspnea:** difficulty breathing or shortness of breath.

**Dysarthria:** difficult or unclear articulation of speech.

**Ectomycorrhiza:** symbiosis between a fungus and plant roots.

**Ectendomycorrhiza:** symbiosis between a fungus and plant roots, in which the fungal mycelium enters the root outer layer.

**Endophyte:** which lives inside a plant.

**Fruiting body:** multicellular structure arising from sexual reproduction in fungi.

**Hartig net:** characteristic structure of mycorrhiza, made by mycelium within plant roots.

**Heterothallism:** sexual reproduction system in which two organisms bearing opposite mating types must meet to reproduce.

**Homothallism:** self-fertile sexual reproduction system in which both mating types are contained in a single nucleus.

**Hybridization:** breeding with an individual of another species.

**Hymenium:** fertile tissue of the fruiting body. In ascomycetes, it bears asci.

**Hypha:** see “mycelium”.

**Macroconidia:** large multinucleate conidia.

**Mating type (MAT) locus:** genetic region that is the master regulator of the sexual reproduction in fungi. It contains *MAT* genes.

**Meiosis:** cell division used in sexual reproduction to produce gametes.

**Microconidia:** smaller conidia.

**Mitosis:** cell division producing clone cells.

**Monophyly:** common descent from a single ancestor.

**Mycelium:** filamentous structure constituting a fungus. Mycelium is composed by hyphae.

**Osmotrophy:** feeding strategy consisting in an enzymatic dissolution of organic compounds followed by an osmotic uptake.

**Parasite:** organism feeding by using a host.

**Phylospecies:** or phylogenetic species, is the smallest unit appropriate for phylogenetic analysis.

**Primordium:** earliest stage of development of the fruiting body.

**Provincialism:** geographically restricted either to eastern or western North America, Europe, or Asia.

**Pseudocryptic:** having diagnosis traits that have been underestimated.

**Pseudohomothallism:** self-fertile sexual reproduction system in which both mating types are contained in a single organism, but not in the same nucleus.

**Pyrophilic:** (organism) that needs fire to accomplish at least a part of its lifecycle.

**Saprotroph:** organism feeding by decaying dead organic matter.

**Sclerotia:** resistant and dormant mycelial mass containing nutritive reserves.

**Semicryptic:** having subtle/unstable/overlapping diagnosis traits.

**Septum:** permeable cloison present in the hyphae of some fungi.

**Spawn:** substrate colonized by mycelium used to “sow” fungi.

**Strain:** variety of a microorganism (taxonomic unit below the subspecies).

**Symbiotroph:** organism feeding thanks to a symbiotic partner (includes lichens, mycorrhizae, endophytes, and commensals).

**Thermolabile:** (substance) altered by temperature.

**Trichogyne:** female fertilizing organ in fungi.

**Trophozoite:** active stage of an amoebal cell.

**Umami:** pleasant savory taste, often defined as the “fifth taste”.

# 1. General introduction

## 1.1 Introducing fungi

### 1.1.1 Fungal biology

Fungi are eukaryotes that feed by osmotrophy and reproduce by forming spores (Money, 2016). In other words, fungi possess nuclei that contain DNA, obtain their energy by an enzymatic dissolution of organic compounds followed by an osmotic uptake, and produce sexual and asexual cells for their survival and dispersion. The kingdom Fungi was estimated to represent 2.2-3.8 million species worldwide (Hawksworth & Lücking, 2017). Fungi can be separated in two main groups: yeasts and filamentous fungi (Money, 2016). The latter produce filament-like structures called mycelium, itself composed of individual hyphae that can be compartmented by permeable septa, and that is involved in water and nutrient uptake and transport (Fricker et al., 2017). Filamentous fungi play key roles in ecosystems. While saprotrophs degrade the organic matter, symbiotrophs and parasites modulate host population dynamic (Bahram & Netherway, 2022). The immense ecological diversity of fungi has allow them to colonize virtually every ecological niche in the world, including terrestrial and aquatic habitats, animal bodies, and plants (Bahram & Netherway, 2022).

### 1.1.2 Consumption, domestication, and cultivation of fungi

While some fungal species can be a threat by causing human diseases or infecting crops and endangering food supplies, others are on the contrary crucial for humankind. Already in the prehistory, fungi were used by humans as food, ferment agents, in medicine, and for spiritual purposes (D.-W. Li et al., 2016). It has been estimated that at least 2000 fungal species were used for their medicinal properties (D.-W. Li et al., 2016), and that 820 varieties/subspecies were used as food worldwide (Boa, 2004). Mushrooms are healthy food as they are protein and fiber-rich, contain numerous essential amino acids and vitamins, and contain a healthy balance of fatty acids (Valverde et al., 2015). Other mushrooms are also prized for their rarity and unique organoleptic properties, as in the case of *Tuber magnatum* (white truffle) (Segelke et al., 2020), *Boletus edulis* (porcini), *Tricholoma matsutake* (matsutake), and wild *Morchella* spp. (true morel) (Mortimer et al., 2012). Morels are among the top five of the most sold edible fungi in Europe, regardless if they were cultivated or found in the wild (Lagrange & Vernoux, 2020), and their consumption is increasing every year (Xu et al., 2022).

The first fungi were domesticated to produce cheese 9500 years ago (Dupont et al. 2017). Domestication is a process of selection of particular traits of a given species for a human use (Ropars *et al.* 2020) that may create new species, as it is the case for *Penicillium camemberti* (Dupont et al. 2017). Domestication impacts species phenotypes and genotypes in multiple ways, as reducing fertility (e.g. in *Penicillium roqueforti* and *P. camemberti*), promoting hybridization (e.g., in *Saccharomyces cerevisiae*), reducing toxicity (e.g., in *Aspergillus oryzae*, the domesticated lineage of *Aspergillus flavus*), promoting horizontal gene transfer, and therefore increasing genome size (e.g., *A. oryzae,* cheese *Penicillium* spp.) (Dupont et al. 2017). Domestication also allowed to cultivate fungi producing edible fruiting bodies, as *Agaricus bisporus* (button mushroom), *Lentinus edodes* (shiitake), *Pleurotus* spp. (oyster mushroom), and *Flammulina velutipes* (enoki) (Valverde et al., 2015). *A. bisporus,* the most cultivated fungus worldwide, was domesticated 300 years ago in France, and the species is now divided into seven genetically distinct clades that diverge in their geographical origin and reproduction system (Zhang et al. 2023). Similarly, the 26 domesticated varieties of *L. edodes*, first domesticated 800 years ago in China, genetically diverge into three groups, that physiologically

differ by their growth temperature (Yu et al. 2022). These examples illustrate how domestication can rapidly impact species due to the continuous selection involved in this process.

## 1.2 The importance of studying morel mushrooms

### 1.2.1 Culinary and nutritional value

All true morel species are edible (Q. Liu et al., 2018). Mushroom guides generally consider morel mushrooms as “excellent edible” (Barone & Revil, 2021; Lemoine & Eyssartier, 2023). Morels are appreciated worldwide as food delicacy, for their unique taste, aroma, and texture (Tietel & Masaphy, 2018). The main flavor that provides the typical “mushroom taste” is umami, that is created in morels by a combination of adenosine-5’-monophophate, uridine-5’-monophosphate, L-glutamic acid, L-aspartic acid, and (S)-morelid (Rotzoll et al., 2006). The flavor profile of morels is completed by a meaty-like taste, produced by 5’-guanosine monophosphate (Tietel & Masaphy, 2018). In addition to their organoleptic value, morel mushrooms are healthy food. Other than the nutritional properties that are common between most of the edible mushrooms, morels contain especially high values of glutamic acid an alanine (Tietel & Masaphy, 2018), two amino acids involved in protein biosynthesis. In addition, morels contain significant amounts of vitamin E derivatives (tocopherols), vitamin $D_2$ and $D_4$, and antioxidative phenolic compounds (Tietel & Masaphy, 2018).

### 1.2.2 Commercial value

Given their culinary importance, morel mushrooms have an important economic value worldwide. Globally, the morel trade is a multi-billion dollar industry (Brown & Obst, 2016). Indeed, the price of morels is relatively high for a food product, with an average of $210 USD/kg in India (Kumar et al., 2022), $160 USD/kg in China (Q. Liu et al., 2018), $300 USD/kg in Michigan, USA (Malone et al., 2022), and $636 USD/kg in Switzerland (**Table 1**).

According to the Global Food Sourcing & Data Hub “Tridge”, China was the main morel exporter in 2022 (income of $218M USD/year), while the USA were the principal importer (*Morel Mushrooms Market, Tridge*, 2022). Switzerland imported morels for $28.32M USD in 2021, while the value dropped to $13.12M USD in 2022 (**Figure 1**), in correlation with the lower import volume. Switzerland exported morels for less than $7000 USD in 2022 (*Morel Mushrooms Market, Tridge*, 2022).

In Switzerland, morels can be found in almost any supermarket, delicatessens, or are collected in the wild as a hobby. Data collected in four Swiss supermarket chains revealed that dried morels mainly came from China, followed by Bosnia and Herzegovina (**Table 1**). Morels from Türkiye, Canada, and India were also found. Only the ones from Bosnia and Herzegovina were labelled as organic. Morels from China all came from cultivation, while the others were wild mushrooms.

**Table 1**. Survey of the origin and price of dried morel mushrooms sold in Swiss supermarkets. Data were collected in December 2023. The price for 100g was multiplied to obtain the equivalent for 1kg. The conversion of Swiss francs (CHF) in US dollar is provided (monetary rate of the 04.01.2024).

| ***Brand*** | ***Market*** | ***Origin*** | ***Organic*** | ***Cultivated*** | ***Price/kg in CHF and USD*** |
|---|---|---|---|---|---|
| *Migros Bio* | Migros | Bosnia and Herzegovina | Yes | No | CHF 698 =USD 821 |
| *Migros Fresca* | Migros | Türkiye | No | No | CHF 483 =USD 568 |
| *M Classic* | Migros | China | No | Yes | CHF 474 =USD 558 |
| *Naturaplan* | Coop | Bosnia and Herzegovina | Yes | No | Unknown |
| *Qualité & Prix* | Coop | Canada | No | No | Unknown |
| *Picosa* | Manor | India | No | No | CHF 580 =USD 682 |
| *Primess* | Denner | China | No | Yes | CHF 598 =USD 703 |
| *Denner* | Denner | China | No | Yes | CHF 525 =USD 617 |

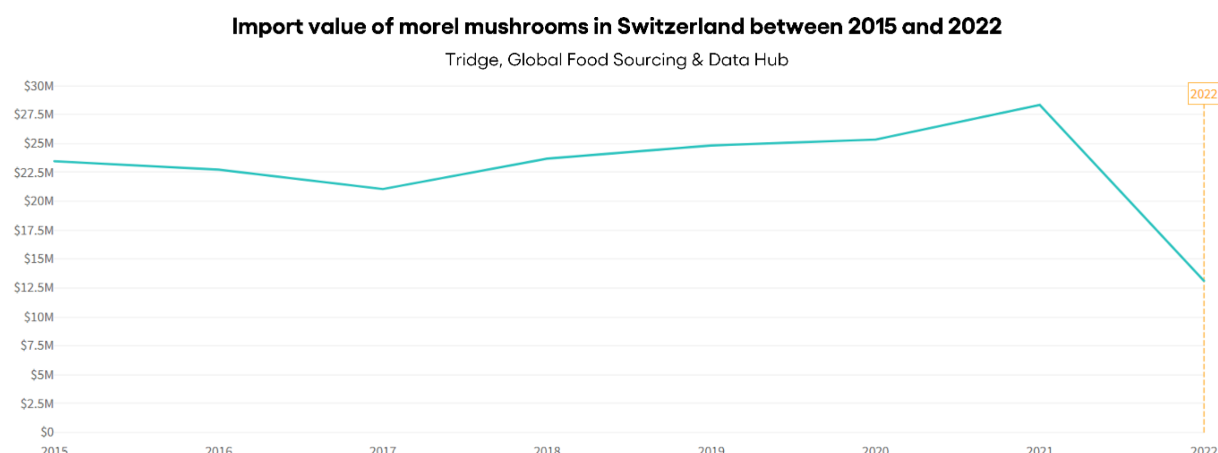


**Figure 1**. Import value of morel mushrooms (*Morchella* spp.) in Switzerland between 2015 and 2022. The graph was obtained on the Global Food Sourcing & Data Hub "Tridge", that was consulted the 04.01.2024.

### 1.2.3 Medicinal value

Traditionally, morels are used in Chinese Medicine to cure gastrointestinal symptoms, apathy, and dyspnea (Su et al., 2013). In Indian traditional medicines morels are used to treat gastrointestinal, respiratory, and cutaneous disorders (Thakur, 2021). For that, morels are prepared either in decoction, extracts, paste, or powder (Thakur, 2021). Although they are not currently used in modern medicine, morels have been widely investigated from the early 2000s for their medicinal properties. Between 2002 and 2016, more than 20 papers (reviewed in Tietel and Masaphy (2018)) demonstrated the bioactivity of health-promoting molecules found in morel fruiting bodies and mycelium (Tietel & Masaphy, 2018). These included anti-oxidant, anti-inflammatory, immunostimulatory, anti-mutagenic, anti-tumor, anti-hyperlipidemic, anti-atherosclerosis, anti-hepatotoxic, nephroprotective, hepatoprotective, gastroprotective, and immunostimulatory compounds (Tietel & Masaphy, 2018). Although these properties were mainly tested *in vitro*, experiments on mice showed promising results for the use of morel-extracted molecules in modern medicine, as cancer chemotherapy (Tietel & Masaphy, 2018). For instance, ethanolic extracts of

*Morchella esculenta* mycelium were effective on preventing and decreasing *in vivo* tumors, enhancing mice survival in a dose-dependent manner (Nitha et al., 2007).

### 1.2.4 Toxicity of morels

Although morels are healthy food that also has beneficial medicinal properties, they can cause intoxications, mostly when they are not handled properly by the consumers. To be edible, morel mushrooms need to be dried, rehydrated, and well-cooked to destroy the thermolabile toxins responsible for intoxication (Lagrange & Vernoux, 2020; Piqueras, 2021). In addition, less than 300g/person should be eaten in the same meal (Piqueras, 2021). In Switzerland, between 1995 and 2009, The Swiss Toxicological Information Centre was called 128 times to report symptomatic intoxications due to *Morchella* sp. (Schenk-Jaeger et al., 2012). The symptoms caused by morel intoxication include neurological disorders (tremor, dizziness, unsteadiness, inebriation, dysarthria, ataxia, i.e., the "cerebellar syndrome by morels, CBM"), gastrointestinal symptoms, hallucinations, cutaneous erythema, and cardiovascular disorders (palpitations, hypotension) (Piqueras, 2021).

In one case, severe gastrointestinal symptoms led to the decease of a woman (Piqueras, 2021). This occurred in 2019, after the ingestion of small quantities of morels prepared in a Spanish Michelin restaurant, that purchased mushrooms labelled as "wild mushrooms collected in Spain", but which actually corresponded to the cultivated species *Morchella sextelata* from China (genetically confirmed) (Piqueras, 2021). It was hypothesized that the presence of a pesticide (metaldehyde) used to preserve morels between Asia and Europe was the cause of the gastrointestinal symptoms and further dead of the person rather than any compound naturally present in the mushrooms (Piqueras, 2021). In 2020, two other deaths were reported after ingestion of morel mushrooms, in France. The details concerning the state and quantity of the ingested mushrooms were not provided, but the authors suggested that cultivated morels imported from China could have a different toxicological profile than that found in wild European morels (Le Visage et al., 2023). This is supported by the fact that severe intoxication symptoms have been increasing since 2014, which coincides with the large-scale commercialization of Chinese cultivated morels (Le Visage et al., 2023). However, investigations about the toxicity of the different morel species or type (wild/cultivated) would be needed to establish causality. The toxicity of imported morels could also come from the preservatives applied on the mushrooms for long-range travel, as suggested previously (Piqueras, 2021).

### 1.2.5 Other uses of morels

Besides being used as food or for their medicinal properties, morels can be used to recover soils polluted with heavy metals (Wang et al., 2021). More precisely, the mycelium of *M. sextelata* significantly reduced the bioavailable lead (Pb) content in soils by adsorption and fixation. As a result, plants grew better in soils inoculated with morel mycelium as it decreased the lead toxicity (Wang et al., 2021). It was also demonstrated that *Morchella* mycelium enhanced physicochemical soil quality (decreased soil bulk density, increased porosity, and resulted in augmented organic matter, nitrogen, phosphorus, and potassium contents) when intercropped with peach trees (*Prunus* sp.), which increased peach yield (Song et al., 2021).

## 1.3 From wild to domesticated morels

### 1.3.1 History of morel cultivation

As indicated previously, morels can be found in nature but are also cultivated. The history of morel cultivation began in 1982, with the indoor cultivation of fruiting bodies (R. Ower, 1982) of *Morchella*

*rufobrunnea*, misidentified as *M. esculenta in* Ower (1982) (Pilz et al., 2007). To do so, nutrient-rich mycelium and sclerotia (i.e., resistant mycelial masses containing nutrients) were generated in a substrate containing soil and wheat grains, and later used as spawn (R. D. Ower et al., 1989). Additional exogenous nutrients were added to the culture, then removed to trigger the formation of fruiting bodies (R. D. Ower et al., 1989). At the same time, soil was flooded with high quantities of water (R. D. Ower et al., 1989). Following this major breakthrough, indoor commercial cultivation started in the USA, but was rapidly abandoned because of yield instability (Q. Liu et al., 2018). In 2010, the indoor cultivation of *M. rufobrunnea* based on Ower's patent was also accomplished in Israel, for research purposes (Masaphy, 2010). In 2002, a new technology was developed to cultivate morels outdoors in China. It consisted in inoculating soil and morel mycelium in cavities of wood logs of *Populus bonatii*, from which fruiting bodies would emerge after overwintering (W. Liu et al., 2023). With an average yield of 330-3000 kg/ha, this technique was used to commercialize morels in 2004 but was rapidly stopped because of the unsustainable use of large amounts of wood (W. Liu et al., 2023). In parallel, Chinese researchers developed a new technique, based on Ower's findings that exogenous nutrition followed by decrease in nutrient availability was crucial for morel cultivation (Q. Liu et al., 2018; R. D. Ower et al., 1989). The novelties of this technology were the cultivation in fields, and the use of suitable exogenous nutrients (mostly wheat or rice grains/bran and sawdust) stored in bags that were deposited on the fields after mycelial colonization in the soil (W. Liu et al., 2023). The mycelium translocated the nutrients from the bags in the soil to form sclerotia that overwintered before producing fruiting bodies in Spring (W. Liu et al., 2023). After 2018, the land area cultivated using the latter technology (i.e., of exogenous bags) reached 10'000 hectares in China, with an average yield of 2250-4500 kg/ha (Xu et al., 2022). In 2018, the yield of fruiting bodies started to decline, or completely fail for two consecutive years (W. Liu et al., 2023). This was mainly caused by low spawn quality, cheap cultivation facilities that were unable to compensate climate variations, continuous cropping, in addition to unknown factors (W. Liu et al., 2023). The challenges facing the morel industry require an urgent need to better understand the morel biology to be able to stabilize the cultivation process.

### 1.3.2 Domestication and hybridization

Morels were domesticated from wild-collected fruiting bodies (Q. Liu et al., 2018). The domestication process implies (1) primary mycelial cultivation (from internal tissue of fruiting body) on chemically defined medium, (2) inoculation of young mycelium on a nutrient rich spawn substrate (mainly containing wheat/rice, sawdust, soil, calcium), (3) sowing of the spawn on soil to undergo the cultivation process (Kumar et al., 2022). If fruiting bodies emerge, the strain can be considered as domesticated (Kumar et al., 2022; Q. Liu et al., 2018). In 2022, nine morel species were domesticated: *M. sextelata*, *M. importuna*, *Morchella eximia*, *Morchella exuberans*, *Morchella oweri*, *M. rufobrunnea*, *Morchella tomentosa*, *Morchella* sp. Mel-13, and *Morchella* sp. Mel-21 (Xu et al., 2022). Only the three first cited species were used for large-scale cultivation, with *M. sextelata* accounting for 83.78% of the samples collected in morel farms between 2014 and 2021 (W. Liu et al., 2023).

A breeding program intended to produce high quality morel varieties accomplished an interspecific hybridization between domesticated *M. importuna* and *M. sextelata* by polyethylene glycol (PEG)-induced double inactivated protoplast fusion (He et al., 2020). The six hybrid progeny lines that were obtained displayed either parental types or intermediate morphologies. For instance, one of the hybrid lines had a *M. sextelata*-like stipe and a *M. importuna*-like cap (He et al., 2020). Hybridization also impacted the yield of produced ascocarps, amino acid content and mycelial growth rate of the progeny (He et al., 2020). This indicated that ideal cultivation characteristics can be selected by this method to increase the genetic diversity of the cultivars, produce higher yields, reduce strain aging,

and/or improve resistance. Hybridization events also occurred naturally, as demonstrated by Du et al. (2019): the domesticated strain M50 was a hybrid between *M. eximia* and *M. exuberans*, the domesticated strain M10 was a hybrid between *M. importuna* and *Morchella* sp. Mel-21, and the domesticated strain M124 was a hybrid between *M. sextelata* and Mel-21 (Du et al., 2019). It was not mentioned or known whether the parents of the hybrids were wild or cultivated morels. However, gene pools of domesticated morels were found in wild morels, in an interspecific manner (Du et al., 2019). These studies revealed that hybridization between different species of *Morchella* is artificially and naturally possible, and that cultivated morels are able to outcross with wild specimens. This point is critically important regarding biodiversity conservation and species invasiveness.

#### 1.3.3 Environmental and social issues in the morel market

Morel cultivation was started to meet the increasing demand for morel fruiting bodies that could not be reached in the wild (short harvest season of wild morels), and to restrict potential health risks such as the detection of noxious heavy metals in wild morel fruiting bodies (Q. Liu et al., 2018). In addition, the harvest of wild morels is associated with environmental and social issues.

As high yields of morel fruiting bodies are found after forest fires, intentional wildfires are triggered for this purpose in various parts of the world including Nepal (Devkota, 2008), Chile (Machuca et al., 2021) and USA (Larson et al., 2016). Although controlled fires can be used by the authorities to trigger the emergence of morel fruiting bodies (Larson et al., 2016), illegal fires cause important damage to ecosystems (Condé et al. 2019).

In addition, collecting and drying wild morels at a commercial scale is a demanding physical work, that is mostly done by poor illiterate women and children in some countries (Hamayun, 2006; Latif et al., 2003). For instance, there were 40% (Hamayun, 2006) to 54% of children that were employed to collect morels in Pakistan in the early 2000s (Latif et al. 2003). They received in average 3 USD/kg of fresh morels, that were processed and sold to the local market for 19 USD/kg, then to the middlemen for 24 USD/kg, and finally to the international market at 271 USD/kg (Latif et al. 2003). Switzerland was mentioned as one of the principal country importing wild morels from Pakistan (Latif et al., 2003).

Currently, the Swiss supermarkets mostly sell wild morels collected in Bosnia-Herzegovina, Türkiye, India, Canada, and cultivated morels from China (**Table 1**). Long-range export of food, as it is the case for Canadian, Indian and Chinese morels, are ecologically unsustainable as transport generates a high carbon footprint (M. Li et al., 2022). In addition, it cannot be excluded that the cultivated Chinese morels sold on the Swiss market are collected and processed in socially acceptable conditions. For instance, several morel farms are located in the region of Xinjiang (Du et al., 2019), a region that is unfortunately known for its wide oppression against the Uyghur population (Lehr & Bechrakis, 2019). There is therefore a crucial need to provide the Swiss market with socially and ecologically sustainable morels. To meet the high demand that cannot be reached with local wild morels that are not commercialized, it is hence needed to develop morel cultivation in Switzerland. However, to achieve this, key concepts and knowledge gaps related to *Morchella* research need to be stated.

## 1.4 Key concepts and knowledge gaps in *Morchella* studies

#### 1.4.1 Phylogeographic evolution of *Morchella*

True morels (Ascomycota, *Pezizomycetes, Morchella*) are divided into three main clades: *Morchella* Section *Rufobrunnea* (basal clade, “white” or “blushing” morels, or Rufobrunnea clade), *Morchella*

Section *Morchella* ("yellow" morels, or Esculenta clade), and *Morchella* Section *Distantes* ("black" morels, or Elata clade) (Richard et al., 2015). Overall, they comprise at least 70 phylogenetically accepted species (Loizides et al., 2022).

The divergence between *Morchella* and its most closely related sister genera (*Verpa* and *Disciotis*) was estimated to the Permian (274.06 Million years ago) (Du et al., 2012), but it might be overestimated as the divergence between *Morchellaceae* and the sister family *Tuberaceae* (containing *Tuber*, i.e., truffles) was determined in the Late Triassic (201.14 Mya) (W. Liu et al., 2018). *Morchella* probably originated from western North America (NA), spread in eastern NA in the early Cretaceous, and in Eurasia in Mid-Cretaceous (O'Donnell et al., 2011). The divergence between the Esculenta and Elata clades was most likely due to biogeographic isolation caused by the late Miocene desertification, that induced the colonization of divergent ecological niches (eastern temperate deciduous forests for the Esculenta, western boreal forests for the Elata) (O'Donnell et al., 2011). These factors contributed to marked continental endemism (i.e., species geographically restricted either to NA or Eurasia) and provincialism (i.e., species geographically restricted either to eastern NA, western NA, Europe, or Asia) (O'Donnell et al., 2011).

The distribution of currently accepted species of *Morchella*, reviewed by Loizides et al. (2022) comprises at least 44 countries from all continents (Loizides et al., 2022), but this is clearly underestimated as *Morchella* were not investigated in all countries. Although most of *Morchella* were found in the Northern Hemisphere, some species, such as *Morchella galilaea* (Rwanda, New Zealand, Java (Indonesia)) were described from the Southern Hemisphere (Loizides et al., 2022). Monitoring of *Morchella* biodiversity in geographic regions from which the genus was previously unknown usually led to the discovery of at least a new species, as it was the case in Australia (*Morchella australiana*) (Elliott et al., 2014), Peru (*Morchella peruviana*), Dominican Republic (*Morchella hispaniolensis*) (Baroni et al., 2018), or Chile (*Morchella andinensis* and *Morchella aysenina*) (Machuca et al., 2021). In Switzerland, the official repartition atlas of fungi from SwissFungi comprised 22 species/subspecies of *Morchella* (accessed the 09.01.2024), but only four of them are considered taxonomically valid (*Morchella semilibera, Morchella vulgaris, Morchella tridentina, M. esculenta*) (Loizides et al., 2022). Genetic sequences generated from Swiss samples revealed the presence of at least four *Morchella* species in Geneva, Switzerland: *Morchella crassipes* (invalid nomenclature, rather corresponding to *M. esculenta* (Richard et al., 2015)), *M. esculenta, M. semilibera*, *M. importuna, M. sextelata,* and unidentified *Morchella* sp. (Payre et al., unpublished). Another Swiss sample identified as "*M. crassipes*" (Pion et al., 2013) but rather corresponding to *Morchella* Sect. Rufobrunnea was also reported in the country. Loizides et al. (2022) reviewed that the species found in Switzerland comprised *Morchella americana*, *M. rufobrunnea*, *M. esculenta*, *M. importuna* and *Morchella norvegiensis* (Loizides et al., 2022). Otherwise, no studies investigated Swiss *Morchella* spp. before the present thesis.

### 1.4.2 Morphologic diversity of *Morchella*

Species identification in *Morchella* has for a long time been based on the morphology of the fruiting body. Although this is an important feature that is still consider nowadays, it must be combined with a genetic approach to encompass pseudocryptic (i.e., having diagnosis traits that have been underestimated) and semicryptic (i.e., having subtle/unstable/overlapping diagnosis traits) species (Loizides et al., 2022). Importantly, the morphology of the fruiting body can show high intraspecific variabilities induced by environmental factors such as temperature, light, humidity, and soil composition (Pilz et al., 2007). Pseudo- and semicryptic species mostly belong to the Elata clade. To better grasp the morphological variability that exists between and within Elata species, a review of the macro- and micromorphological characteristics of *Morchella* Sect. *Distantes*, and their

importance in species diagnosis, was performed and presented in **section 10.1** (summary and discussion) and **annex G1-2** (exhaustive list, to be found online on https://github.com/MorchellaThesis). This is a valuable tool for taxonomists, to compare the characteristics of potential new species with the ones already described.

### 1.4.3 Trophic modes in *Morchella*

Nutrition in *Morchella* has been controversial since 1890 (Buscot & Kottke, 1990) and currently remains partially unknown, mostly because not all the species share the same trophic modes, that can also be intraspecifically mixed (Loizides, 2017). In 1883, *Morchella* were described as parasites of Jerusalem artichoke (*Helianthus tuberosus*) rhizomes, because of the strong adherence between *Morchella* mycelium and the rootstock (Roze, 1883). It was imagined that the rhizomes, that overwinter in the field, provided nutrients for the overwintering survival of *Morchella* mycelium (Roze, 1883). In the 21th century, *Morchella* were mainly considered as saprotrophic (Dahlstrom et al., 2000; Q. Liu et al., 2018), as they can use dead organic matter as only food source *in vitro* (in chemically defined media) and in cultivation fields. However, many species were reported to form close interactions with plants. Morels from the Esculenta clade were often associated with broadleaf trees, while Elata morels were mostly associated with conifers (Loizides, 2017). Hartig nets proving ectomycorrhizal interactions between *Morchella* spp. (Esculenta and Elata clades) and *Picea abies* were observed in a natural environment, but the irreproducibility of such interaction indicated it was facultative and conditional (Buscot, 1994; Buscot & Kottke, 1990). More recently, these studies were reproduced, and the *in vitro* induction of mycorrhizal interactions was tested between several species of Pinaceae and *Morchella* (Dahlstrom et al., 2000). Ectomycorrhizal (partial or poor Hartig net) and ectendomycorrhizal (hyphal invasion between cells of the root outer layer) features were observed (Dahlstrom et al., 2000). Analysis of isotope patterns supported the ectomycorrhizal lifestyle of *Morchella* (Tedersoo et al., 2010). It was hypothesized that *Morchella* would use these interactions to obtain a constant amount of nutrients, and/or to position on the decaying/dying roots to feed by saprotrophy (Dahlstrom et al., 2000). It was not clear whether *Morchella* would impact the associated plant negatively (parasitism), neutrally (commensalism) or positively (symbiosis) (Dahlstrom et al., 2000). In *Bromus tectorum* however, endophytic *Morchella* mycelium was found in the stems and roots of the pyrophilic grass, offering beneficial thermoprotection to the seeds at high temperatures (>60°C) (Baynes et al., 2012). Several species from the Elata clade are obligate (*M. tomentosa*) or facultative (*M. sextelata, M. importuna, M. exuberans, M. eximia*) pyrohiles, that fruit after forest fires (Du & Yang, 2021). To conclude, *Morchella* can adapt to various ecological niches, and probably use different nutrition systems depending on the resources availability, and the species. Not all species could therefore be cultivable in bare fields.

### 1.4.4 *Morchella* reproduction

As in most filamentous ascomycetes (Burt, 2000), *Morchella* are facultatively asexual because they are able to multiply either by the mitotic generation of asexual spores (conidia or chlamydospores) (Yuan et al., 2020) or by the meiotic formation of sexual spores (ascospores) (Du & Yang, 2021).

In *Morchella*, conidia are believed to function as spermatia (i.e., fertilizing agent for sexual reproduction) and propagules for mycelial colonization (Yuan et al., 2020). They usually measure 2.5-5 µm in diameter (Yuan et al., 2020). In *Morchella galilaea*, conidia were produced by ascospores within dead (dry) or living (fresh) asci and after spore discharge (Du et al., 2023). One to six nuclei migrated from the ascospore to the conidia, which detached from ascospores once mature (2-10 µm in diameter) (Du et al., 2023). Another type of asexual spores, the chlamydospores (8-12 2.5-5 µm in diameter), were detected on conidia-derived mycelium (Yuan et al., 2020). They may serve as

resistant structures for long-term survival, however the link between conidia and chlamydospores remains unknown (Yuan et al., 2020).

Sexual reproduction is particularly important to study in the perspective of cultivation, because this process gives rise to the fruiting body. Sexual reproduction is a chromosomal recombination process aiming to increase variance in the progeny, and therefore improve fitness after selection (Burt, 2000). In *Morchella*, there are three principal modes of sexual reproduction: (1) homothallism, (2) pseudohomothallism and (3) heterothallism (Du & Yang, 2021). Sexual reproduction is governed by a unique genetic region called the mating type (MAT) (Coppin et al., 1997). The locus exists in two versions, MAT1-1 (that contains genes *MAT1-1-1, MAT1-1-10 and MAT1-1-11* in *Morchella*) and MAT1-2 (gene *MAT1-2-1*) (Chai et al., 2022). In homothallic species, as *M. rufobrunnea, M. peruviana,* and probably *Morchella* sp. Mes-15*,* both MAT are carried in their haploid genome (Chai et al., 2022). In pseudohomothallic organisms, as some isolates of *M. importuna*, single ascospores contain both mating types, but not in the same nucleus (Du & Yang, 2021). Homothallic and pseudohomothallic organisms are therefore self-fertile. Organisms in which different haploid individuals contain only one mating type are defined as sexually heterothallic, and this is the case for most of the *Morchella* species (Chai et al., 2017; Du et al., 2017, 2020; He et al., 2021; W. Liu et al., 2018).

Attraction of the complementary mating type is done by a hormonal mechanism usually regulated by the mating type locus (Coppin et al., 1997). The dominant genotype (i.e. the one involving the most in the reproduction) is usually called “maternal” while the complementary MAT is the “paternal” type (Du et al., 2017; Zou et al., 2019). Mating type system was not defined as male/female, because strains were not differentiated depending on the expressed MAT locus: receptors (ascogonia) and donors (microconidia, macroconidia or hyphae) structures are carried by each individual (Coppin et al., 1997).

In ascomycetes, fertilization is accomplished when the nucleus of the donor cell enters the receptor cell, but nuclei do not fuse directly; fertilized cells first multiply to initiate fruiting body development (Coppin et al., 1997). In *Morchella,* trichogyne and antheridia (i.e., fertilizing organs) were never observed (Du & Yang, 2021; He et al., 2017). Ascospores are produced within the asci, at least 20 days after the formation of the primordium in the cultivated *M. importuna* No.1 (He et al., 2017). In each ascus, one diploid nucleus resulting from karyogamy (illustrated in **Figure 2A**) first undergoes meiosis (I and II) (**Figure 2B**) to produce four haploid nuclei, that multiply by mitosis (**Figure 2C**). The ascospore is formed around each nucleus (**Figure 2D**). Each nucleus then undergoes one mitosis event (**Figure 2E**) followed by more mitoses (**Figure 2F**) resulting in up to 60 nuclei per ascospore, depending on the spore and species (He et al., 2017). Being homokaryotic, each ascospore (and resulting mycelium) therefore contain only one MAT, either MAT1-1 or MAT1-2 in heterothallic species (Du et al., 2017; He et al., 2017, 2021). In the hyphal cells of the ascocarp, however, nuclei of different MAT genotypes co-exist in an unbalanced state (He et al., 2021). In sterile tissues (stipe), MAT1-1 genotypes were dominant, while MAT1-1 and MAT1-2 genotypes were present in balances quantities in fertile tissues (hymenium) (He et al., 2021). This suggested a competitive advantage of the MAT1-1 genotype on the MAT1-2, as already stated by Du et al. (2017) (Du et al., 2017). In some strain, the genotypic competition led to the total disappearance of one of the mating types, and this could contribute to the instability or failure of morel cultivation (He et al., 2021). Loss of one of the MAT could be recovered by vegetative fusion of hyphae, as it was demonstrated that compatible homokaryons of opposite MAT could fuse and share their nuclei, resulting in heterokaryotic mycelium (Zhang et al., 2022).

The lifecycle of *Morchella* remains partially unknown. For instance, the role of conidia as potential fertilizing agents, the link between conidia-derived mycelium and chlamydospores (Yuan et al., 2020),

or the role of the rare conidia budding from ascospores remain unknown (Du et al., 2023). In addition, the typical structures involved in sexual reproduction of ascomycetes (i.e., ascogonia, antheridia, trichogynes, croziers) still remain unidentified in *Morchella* (Du & Yang, 2021). Finally, it was hypothesized that mycelium containing the two MAT would produce sclerotia, which might be the emergence point of the fruiting body primordium, but this has never been demonstrated (Du & Yang, 2021). The mating type analysis in sclerotia revealed that some of them contained both, or single MAT, rendering their role in the morel lifecycle even more unclear (Du & Yang, 2021). Similarly, the physicochemical or genetic factors triggering fruiting body formation remain unknown (Du & Yang, 2021). To stabilize the cultivation process involving the complete lifecycle of the fungus, a better understanding of all these developmental stages is therefore required.

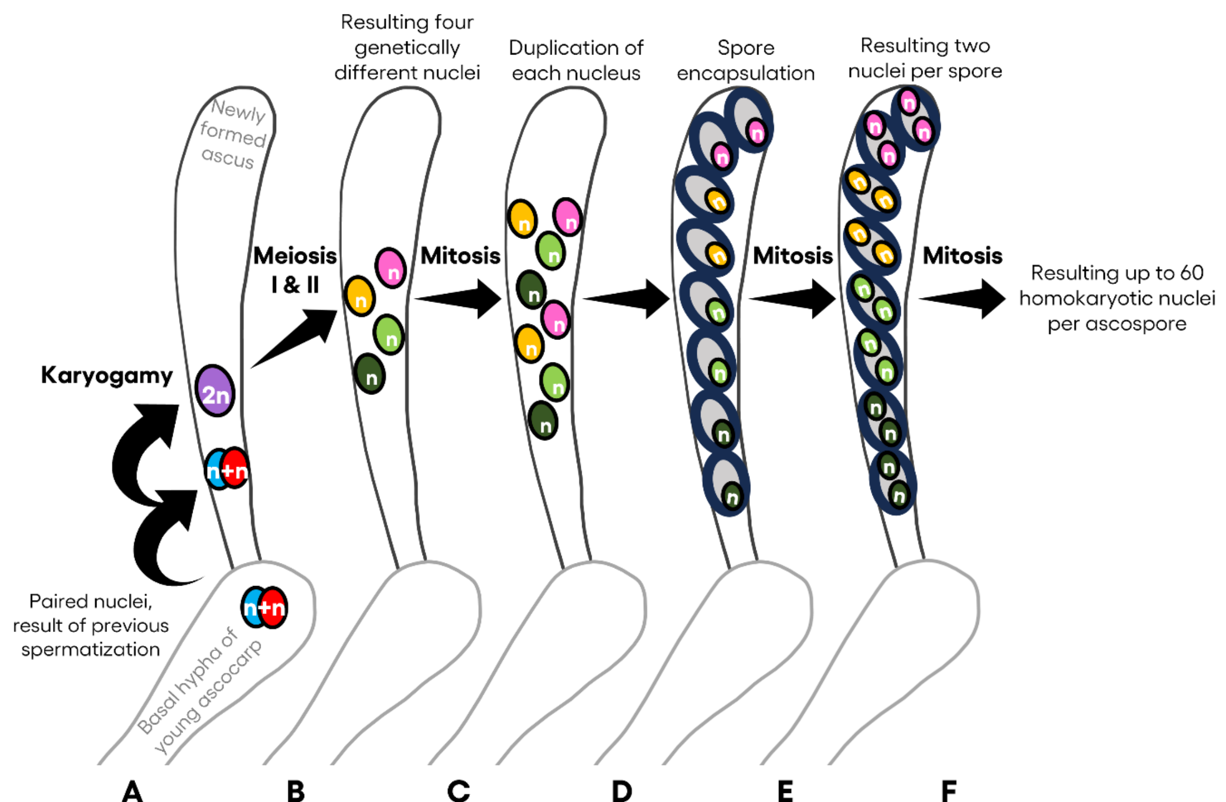


**Figure 2**. Ascosporogenesis in *Morchella*, made in this study to illustrate the results of He et al. (2017) (He et al., 2017). A= the paired nuclei resulting from previous spermatization undergo karyogamy within the young ascus; B= Diploid nucleus undergoes meiosis I and II to form four nuclei; C= Each nucleus duplicates by mitosis; D= Each nucleus is encapsulated in one ascospore; E= Within ascospores, nuclei undergo one mitosis event. The created nucleus migrates at the opposite side of the spore; F= multiple mitosis events form multinucleate homokaryotic mature spores.

## 1.5 Scope of the thesis

### 1.5.1 Motivations

As previously showed, the Swiss market does not sell environmentally sustainable morel mushrooms, and it cannot be excluded that socially unfair products are sold as well. In addition, the increasing severe intoxications due to cultivated Chinese morels, that led to the death of at least three people (Le Visage et al., 2023; Piqueras, 2021), is probably due to the chemical preservative applied for the long-range travel (Piqueras, 2021). Accordingly, this calls for precaution regarding imports. In addition, it was shown that cultivated morels have the ability to interspecifically hybridize with wild morels, potentially threatening the native biodiversity and structure of natural populations.

Domesticating a Swiss morel strain therefore appears crucial to protect our native ecosystems and to prevent potential invasiveness.

### 1.5.2 General aim

This thesis has the general aim to provide knowledge necessary for the domestication of Swiss morels. The goal in perspective is to provide the Swiss agriculture with a native cultivable strain, to furnish Swiss consumers a local, ecologically, and socially sustainable gourmet food product.

### 1.5.3 Research questions and general structure

This thesis was divided into three main research questions that altogether allowed a better understanding of the distribution, reproduction, and ecology of the Swiss morels, three topics that must be acknowledged in the perspective of cultivation.

The thesis was divided into nine sections: a general introduction, a general discussion, and seven chapters corresponding to a unique research area. **Sections 2** and **3** concerned the biodiversity of *Morchella* in Switzerland, **section 4** was related to genetic species identification tools, **sections 5** and **6** investigated sexual reproduction, and **sections 7** and **8** concerned biological interactions of *Morchella* in the soil.

The first research question, “what is the biodiversity of *Morchella* in Switzerland?”, aimed to evaluate which *Morchella* species were present in the country, their abundance, ecological requirements (altitude, associated plants, type of soil), and phylogenetic relationships. Monitoring of *Morchella* using genetic tools was never done before in Switzerland. This research question was answered in **sections 2** (monitoring of the biodiversity of *Morchella* in Switzerland) and **3** (description of a new morel species native to Switzerland). Directly linked to this research question, the thesis investigated “how to efficiently and routinely monitor *Morchella* species” in **section 4**, where a genetic method for rapid and reliable genetic identification of *Morchella* spp. was demonstrated.

The second research question, “how do Swiss morels sexually reproduce?”, aimed to investigate the reproductive system in native strains, as the fruiting bodies arise from sexual reproduction. More precisely, the mating type genes (*MAT*) were investigated. The first goal (1) was to find reliable genetic tools to detect the *MAT*, to then study its distribution (2), and therefore the reproductive systems of Swiss morels. The last goal (3) was to analyse the structure of the MAT loci in the genomes of Swiss strains. The first goal was accomplished in **sections 5** and **6**. Goals 2 and 3 were investigated in **section 6**.

The third research question, “how do *Morchella* spp. interact with other soilborne organisms?”, aimed to investigate bipartite and tripartite interactions with organisms that can be found in agricultural soils, namely the associated bacterium *Pseudomonas koreensis*, the amoeba *Acanthamoeba castellanii*, the mycophagous nematode *Aphelenchus avenae*, and the fungal competitors *Penicillium* sp., *Cephalotrichum* sp, and *Aspergillus westerdijkiae*. The amoeba and bacterium were investigated in **section 7**, while the others were studied in **section 8**.

In addition, this thesis was completed by a general discussion (**section 9**) that linked the findings of all the chapters in a global context of strain domestication. Limitations and perspectives of this thesis were also provided, along with a general conclusion that described the crucial and original advances that this research brought to the field of *Morchella* research.

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# 2. Biodiversity of *Morchella* in Switzerland: a study highlighting the diversity of native black morels and the discovery of new lineages

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### Foreword

This chapter presents a survey of *Morchella* in Switzerland using a polyphasic approach combining morphological, ecological, and genetic analyses. This study is in submission to the journal Mycologia. My personal contribution as first co-author was the generation and analysis of all data, main writing, and general review of the paper. This paper reports the presence of four described species of *Morchella*, in addition to four new lineages, one of them being published as a new species in section 3.

### Abstract

True morels (*Morchella* spp.) are a diverse fungal genus with at least 70 recognized species that show high endemism. The cultivation of Asian morel species in Europe is becoming more common in Switzerland and other European countries. However, it is currently unknown whether these species maintain endemic populations in Europe, and whether there is the potential for introgression with native morel populations. To address these questions, we sought to establish an initial inventory of the diversity of native morel species across Switzerland. The biodiversity of Swiss morels was assessed through a combination of field collection, ecological and morphological description, and single- and multi-locus (ITS, *RPB1, RPB2, TEF1-a, MAT1-1-1* and *MAT1-2-1* markers) phylogenetics. The analyses of a collection of 72 morel fruiting bodies revealed a high diversity within *Morchella* Section *Distantes*, and the discovery of three new lineages denoted here as *Morchella* sp. Mel-43, Mel-45, and Mel-47. *Morchella importuna, Morchella deliciosa, Morchella norvegiensis, Morchella pulchella* species complex and *Morchella esculenta* were also detected in Switzerland.

**Keywords:** true morels, integrative taxonomy, phylogenetics, ecology, morphology, species determination.

## 2.1 Introduction

True morels (*Morchella* spp.) are socially and economically important worldwide and are widely consumed as edible and medicinal fungi (Y. Xu et al. 2022). Morel taxonomy has been revised multiple times (O'Donnell et al. 2011; Richard et al. 2015; Loizides et al. 2022). Phylogenetic investigations have demonstrated that multiple morel species can share macromorphological traits, while closely related species may display distinct ecological or morphological features (Loizides et al. 2022). Moreover, fruiting body morphology, which has also been considered in species identification, is influenced by environmental factors (i.e., humidity, light, temperature, and soil) and also varies depending on the developmental stage (Pilz et al. 2007). In addition, different modes of sexual reproduction have been discovered even within a single species of morel. This is the case for *Morchella importuna* which includes secondary homothallic (self-fertile) and heterothallic (self-sterile) specimens (Du et Yang 2021). Finally, intraspecific ecological variation can also exist; for instance, *Morchella sextelata* and *Morchella importuna,* are both fire-adapted but can be cultivated independent of fire events (Du et Yang 2021). Therefore, the use of multiple phylogenetic markers (J. Xu 2020) and Genealogical Concordance Phylogenetic Species Recognition (GCPSR) (Taylor et al. 2000), in addition to morphological, developmental, and ecological data has shown to provide the most precise taxonomic classification of species in *Morchella* (Loizides et al. 2022). New morel species continue to be described from all continents using integrative phylogenetic approaches (Baroni et al. 2018; Du et al. 2019; Machuca et al. 2021). While many described species appear to be invalid based on phylogenetic and phenotypic criteria (e.g., *Morchella crassipes, Morchella costata, Morchella elata, Morchella conica*), the genus *Morchella* still includes at least 70 different recognized species (Loizides et al. 2022).

Another feature that has been highlighted in many taxonomic studies of *Morchella* is the endemic nature of populations from distinct geographical locations (O'Donnell et al. 2011; Taskin et al. 2010; Elliott et al. 2014). Therefore, describing the diversity of *Morchella* in specific geographic regions might be particularly valuable in fields such as conservation biology. While fungi are often not considered in conservation programs, fungal populations also experience pressures caused by habitat destruction, climate change, and pollution (Heilmann-Clausen et al. 2015). Although climate change might appear to have a positive effect on the distribution range of morels in China (Cao et al. 2022), it may have the opposite impact in other locations. The Global Fungal Red List Initiative includes *Morchella anatolica* as a critically endangered species, because of its very limited distribution range, rarity, overexploitation, and habitat loss due to the destruction of its natural habitat (i.e., eastern Mediterranean basin) (http://iucn.ekoo.se/, accessed the 13.12.2022). Studies of *M. anatolica* have been critical for understanding the evolution of morels, as members of this species represent basal lineages of *Morchella* (Loizides et al. 2021). Thus, a better understanding of the distribution range of morel species (as well as other fungi) is relevant in the context of the the current biodiversity crisis.

Invasive microbial species are those that colonize new geographic areas and impact local ecosystems, through over-competition for resources, ecological niches, or hybridization (Litchman 2010). The high level of regional endemism of *Morchella* spp. (O'Donnell et al. 2011; Loizides 2017) is a critical feature when considering the impact of potential invasive species. Less than ten morel species are reported to have a cosmopolitan distribution range (Loizides 2017), and this cosmopolitan range is likely, at least for some of them, due to the impact of human activities (Loizides et al. 2016). For instance, *Morchella galilaea* and *M. importuna* were likely introduced in non-native areas through human cultivation, as they are often reported from greenhouses, gardens, and landscaped areas (Loizides 2017). *Morchella americana* and *Morchella populiphila* were possibly introduced in Europe from North America in association to *Populus* spp. plantations (Richard et al. 2015). Similarly,

*Morchella eximia* and *Morchella tridentina* could have been introduced from the USA to South America along with North American trees (Pildain, Visnovsky, et Barroetaveña 2014). Morel species from the Esculenta clade form mycorrhizal-like (Dahlstrom, Smith, et Weber 2000) or endophytic interactions with plants, and can therefore be introduced inadvertently with the trade of plants (Loizides 2017).

Switzerland is one of the European countries in which the biodiversity of morel populations has never been assessed using genetic approaches. In the scientific literature, very few morels from Switzerland have been reported or analyzed. A specimen of *Morchella norvegiensis* (named *Morchella eohespera* strain M510) was reported from Davos (canton of Grisons, Switzerland) (Voitk et al. 2016). This specimen was held in the German Collection of Cell Cultures (DSMK), but the sampling date is not recorded (Voitk et al. 2016). From the same canton, specimens apparently belonging to the yet unresolved *Morchella inamoena ss* Clowez were collected under *Populus tremula* (Fatton 2016). Based on a morphological identification, Röllin and Anthoine (Röllin et Anthoine 2001) reported *M. tridentina* as a rare species found in Bex (canton of Vaud). A specimen of *Morchella esculenta* (strain PhC198) was collected in Malval along the Allondon River (canton of Geneva, Switzerland) in early May 2004 (Richard et al. 2015). *M. americana* and *Morchella rufobrunnea* were also reported from studies conducted in Switzerland (Loizides et al. 2022). In a book describing the macroscopic ascomycetes from Switzerland (Breitenbach et Kränzlin 1984), four species were described: *Morchella deliciosa* (named *Morchella conica* var. *deliciosa*), *M. esculenta, Morchella semilibera* (named *Mitrophora semilibera*), and a rare species named *M. elata* (the latter being considered as a synonym of *M. importuna* (Richard et al. 2015)), which could effectively correspond to *M. importuna* given its ecology, fruiting month, and morphology. In another book from the same year, several morels collected in Switzerland were described (Jacquetant 1984): *Morchella purpurascens* was collected at 2000 m on the canton of Valais; the postfire morel *M. eximia* was collected the canton of Jura near a fireplace; *Morchella dunalii* (named *Morchella rielana*) was apparently widespread in the alpine region of Switzerland; *Morchella atrotomentosa,* a possible synonym for *Morchella tomentosa* (according to morphological and ecological descriptions) or *M. importuna* (according to Clowez and Moreau (Clowez et Moreau 2018)), was collected in abundance after an exceptional forest fire in the canton of Valais; *M. esculenta* (named *Morchella pseudoumbrina*) was collected in Valais under *Corylus*; *M. esculenta* (named *Morchella hetieri*) was collected in a mixed forest in the canton of Vaud.

In this study, the biodiversity of Swiss morel populations was established by a combination of field sampling, ecological, morphological and genetic characterization of the specimens collected. Establishing a database describing the biodiversity of morels in Switzerland is crucial to be able to monitor species and their populations, to identify local strains suitable for cultivation, and to assess the risk of potential invasion by non-native species, which are used for morel cultivation in Switzerland. We hypothesized that morel species already reported from the surrounding countries (e.g., France, Italy, Germany), such as *M. esculenta, Morchella vulgaris, Morchella deliciosa,* or *M. eximia* would be found in Switzerland. Due to the high endemism of *Morchella* species (Elliott et al. 2014; Baroni et al. 2018), the discovery of previously undescribed lineages is also expected. Both hypotheses were met, as five described taxa (*M. esculenta, M. deliciosa, M. importuna, M. norvegiensis, M. pulchella* species complex) and three new lineages (*Morchella* sp. Mel-43, Mel-45 and Mel-47) were documented.

## 2.1 Material and methods

### 2.2.1 Sample collection

A wide-scale collection of morels in Switzerland was conducted during spring from 2019 to 2021 in collaboration with experienced morel foragers. Extensive sampling was conducted for the canton of Neuchâtel, where 46 specimens were collected between 2019 and 2021. In addition, morels were collected from cantons of Jura (1), Vaud (6), Fribourg (4), Valais (4), Bern (2), Luzern (6), Solothurn (1), and Ticino (2), for a total of 72 morels sampled in Switzerland. The fruiting bodies collected were photographed in the laboratory. A fraction of the carpophore (about 2 $cm^3$) was dissected in each fruiting body for DNA extraction. The fruiting bodies were frozen and kept at -20°C, except for four samples (M19-40, M19-43, M21-2 and M21-43) that were kept in plastic jars in a dark oven at 22°C for 2-3 days to trigger natural sporulation. Voucher specimens are preserved in the collection of the Laboratory of Microbiology of the University of Neuchâtel (LAMUN).

### 2.2.2 DNA extraction and sequencing

DNA was extracted from 2 $cm^3$ pieces of either fresh or dry hymenia, or by scrapping the mycelium in SAC, using Quick-DNA Fungal/Bacterial Miniprep Kits (Zymo Research, USA) following the protocol provided by the manufacturer. Eluted DNA was quantified with a Qubit kit (Invitrogen, USA) using the Broad Range buffer and reagent. DNA was then diluted with PCR-grade water to a concentration of 2 ng/µL to be used as template in polymerase chain reactions (PCR). Six regions were amplified: the internal transcribed spacer (ITS), including ITS1, the 5.8S ribosomal RNA gene, and ITS2 (Baynes et al. 2012); RNA polymerase II largest subunit (*RPB1*) (Du et al. 2012); and second largest subunit (*RPB2*) (Taskin et al. 2010); translation elongation factor 1 alpha (*TEF1-α*) (Rehner et Buckley 2005); and the coding regions of mating type genes *MAT1-1-1* and *MAT1-2-1* (Chai et al. 2022). Detailed primer information is available in **Supplementary S1**. For each sample, the PCR mix contained PCR-grade water, 2X ALLin™ Red Taq Mastermix (HighQu, Germany), 0.2 µM of both forward and reverse primers and 1 µL of 2 ng/µL DNA. Amplifications were performed in a Thermo Scientific Arktik thermal cycler. The following parameters were used: denaturation at 95°C for 1 min, 40 cycles of denaturation, annealing and elongation (95°C for 15 sec, 63°C [Elata MAT]; 62°C [ITS]; 60°C [Esculenta *MAT*]; 55°C [TEF1-α]; 50°C [RPB1]; 55°C [RPB2] for 15 sec, 72°C for 15 sec), final elongation at 72°C for 2 (non-mating regions) or 5 min (*MAT*), end at 15°C. PCR products were then loaded on a 1.2% agarose gel that underwent electrophoresis (100 mV, 30 min). Amplicons were visualized under UVs in a Genoplex VWR transilluminator. PCR products (i.e. single band at the expected size) were then purified with a MultiScreen® Filter Plates PCR µ96 (Millipore Corporation, USA) as follows: in each well, the PCR product and 50 µL of PCR-grade water was added; a vacuum of 20 bars was applied on the wells until they dry; 20 µL of PCR-grade water was added to each well; after 2 min, DNA contained in the membrane from each well was resuspended by pipetting up and down 20 times. Once purified, the PCR products were quantified by Qubit. Final DNA concentration was adjusted at 2-40 ng/µL and sent to Fasteris (Switzerland) for Sanger sequencing.

### 2.2.3 Phylogenetic analysis

Forward and reverse amplicon sequences obtained were trimmed and assembled using BioEdit v7.2.5 (Dagona 1999). Sequences that were generated in this study were deposited in the National Center for Biotechnology Information (NCBI) GenBank database (see **Supplementary S2A** for specific accession numbers). Reference sequences downloaded from NCBI were added to the datasets (see **Supplementary S2B** for strain references). Amplicon sequences were first aligned by single genetic region (ITS; *RPB1*; *RPB2*; *TEF1-α*; *MAT1-1-1; MAT1-2-1*) using MUSCLE (Edgar 2004) within the

software MEGA version 11 (Kumar et al. 2018). For multi-locus phylogenies, sequences were then concatenated (*ITS-RPB1-RPB2-TEF1-α; MAT1-1-1–MAT1-2-1*) and re-aligned. Gaps were automatically excluded using the online tool Gblocks 0.91b (Castresana 2000; Dereeper et al. 2008). The datasets that were used to generate the phylogenies can be found in **Supplementary S3A-D**. The best nucleotide substitution model was determined using the IQ-TREE web server's Model Selection (Kalyaanamoorthy et al. 2017) based on the Akaike information criterion (AIC) (Nguyen et al. 2015) (see models that were used in **Supplementary S4**). Maximum Likelihood (ML) phylogenies were generated with the best model in the IQ-TREE web server (Nguyen et al. 2015), with 10,000 ultrafast bootstrap (UFBoot) trees to assess node support values (Hoang et al. 2018). The phylogenies were visualized and annotated in FigTree v1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/).

#### 2.2.4 Microscopic analysis

Microscopic features were observed and photographed under a Leica DM4 B compound microscope (Leica Microsystems, Germany). Samples were prepared by cutting thin slices of hymenium (i.e., fertile part of alveoli), ridge and external layer of the stipe (i.e., ectal excipulum) that were immersed in physiological water (0.9% NaCl) on microscope slides. Asci (i.e., fertile elements of the hymenium), paraphyses (i.e., sterile elements of the hymenium), acroparaphyses (i.e., sterile elements of the ridges), sterile elements of the ectal excipulum and ascospores (from free spores or spore prints) were measured (length and width) from microscopic pictures with the software ImageJ v.1.53q (https://imagej.net/ij/index.html).

## 2.3 Results and Discussion

#### 2.3.1 Sampling

The aim of this study was to describe the biodiversity of *Morchella* in Switzerland. This was done by collecting 72 morel fruiting bodies (**Table 1**) across nine cantons (Neuchâtel, Jura, Vaud, Fribourg, Valais, Bern, Luzern, Solothurn, and Ticino) in the Springs of 2019, 2020 and 2021 (**Figure 1**). Diverse species already reported from other parts of Europe (e.g., *Morchella dunalii, M. pulchella, Morchella fluvialis*) or directly from Switzerland (e.g., *Morchella norvegiensis, Morchella americana*) in addition to cosmopolitan species (e.g., *M. rufobrunnea, M. importuna, M. esculenta*) were expected to be found in Switzerland. This hypothesis was supported with the phylogenetic analysis (see below) of the specimens belonging to *M. esculenta, M. importuna*, *M. norvegiensis, M. pulchella* species complex, and *M. deliciosa* collected. The absence of species diversity within the Esculenta clade was not expected, nor was the absence of specimens belonging to *M. rufobrunnea*. Except for *M. deliciosa* and *M. norvegiensis*, none of the black morels that were previously reported from Switzerland were collected in this study. However, as the collection effort was mostly conducted in the canton of Neuchâtel (**Figure 1**), they may be present in other regions. Further monitoring would be needed to expand the research area.

**Table 1.** List of the *Morchella* spp. that were collected in Switzerland for this study, and associated metadata.

| Species | Clade | Specimen | Canton | Collection date | Habitat: Altitude [m] | Habitat: Phytosociology |
|---|---|---|---|---|---|---|
| *Morchella* sp. Mel-47 | Sect. Distantes | M19-1 | Neuchâtel | 26-Mar-2019 | 730 | Bushy oak grove |
| *Morchella* sp. Mel-47 | Sect. Distantes | M19-5 | Neuchâtel | 26-Mar-2023 | 730 | Bushy oak grove |
| *Morchella* sp. Mel-47 | Sect. Distantes | M19-7 | Neuchâtel | 26-Mar-2025 | 589 | Bushy oak grove |
| *Morchella* sp. Mel-47 | Sect. Distantes | M19-8 | Neuchâtel | 26-Mar-2026 | 589 | Bushy oak grove |
| *Morchella* sp. Mel-47 | Sect. Distantes | M19-9 | Neuchâtel | 26-Mar-2027 | 589 | Bushy oak grove |
| *Morchella* sp. Mel-47 | Sect. Distantes | M19-10 | Neuchâtel | 26-Mar-2028 | 589 | Bushy oak grove |
| *Morchella deliciosa* | Sect. Distantes | M19-28 | Neuchâtel | 04-Apr-2019 | 723 | Bushy oak grove |
| *Morchella deliciosa* | Sect. Distantes | M19-29 | Neuchâtel | 04-Apr-2019 | 723 | Bushy oak grove |
| *Morchella* sp. Mel-47 | Sect. Distantes | M19-43 | Neuchâtel | 01-Apr-2019 | 758 | Bushy oak grove |
| *Morchella importuna* | Sect. Distantes | M20-3 | Valais | 10-Apr-2020 | N.A. | N.A. |
| *Morchella importuna* | Sect. Distantes | M20-4 | Valais | 10-Apr-2020 | N.A. | N.A. |
| *Morchella importuna* | Sect. Distantes | M20-5 | Valais | 10-Apr-2020 | N.A. | N.A. |
| *Morchella importuna* | Sect. Distantes | M20-6 | Valais | 10-Apr-2020 | N.A. | N.A. |
| *Morchella deliciosa* | Sect. Distantes | M20-8 | Jura | 10-Apr-2020 | N.A. | N.A. |
| *Morchella norvegiensis* | Sect. Distantes | M21-2 | Luzern | 25-Mar-2021 | 880 | Fir beech forest |
| *Morchella norvegiensis* | Sect. Distantes | M21-3 | Luzern | 25-Mar-2021 | 880 | Fir beech forest |
| *Morchella norvegiensis* | Sect. Distantes | M21-4 | Luzern | 25-Mar-2021 | 880 | Fir beech forest |
| *Morchella deliciosa* | Sect. Distantes | M21-8 | Neuchâtel | 27-Mar-2021 | 668 | Sedge beech grove |
| *Morchella* sp. Mel-47 | Sect. Distantes | M21-9 | Neuchâtel | 31-Mar-2021 | 689 | Luzule beech forest |
| *Morchella* sp. Mel-47 | Sect. Distantes | M21-13 | Neuchâtel | 31-Mar-2021 | 641 | Bushy oak grove |
| *Morchella deliciosa* | Sect. Distantes | M21-15 | Neuchâtel | 31-Mar-2021 | 723 | Bushy oak grove |
| *Morchella deliciosa* | Sect. Distantes | M21-16 | Neuchâtel | 31-Mar-2021 | 1063 | Fir beech forest |
| *Morchella deliciosa* | Sect. Distantes | M21-17 | Neuchâtel | 31-Mar-2021 | 1063 | Fir beech forest |
| *Morchella deliciosa* | Sect. Distantes | M21-18 | Neuchâtel | 31-Mar-2021 | 1063 | Fir beech forest |
| *Morchella deliciosa* | Sect. Distantes | M21-19 | Neuchâtel | 31-Mar-2021 | 1063 | Fir beech forest |
| *Morchella pulchella* | Sect. Distantes | M21-20 | Neuchâtel | 01-Apr-2021 | 715 | Bushy oak grove |
| *Morchella deliciosa* | Sect. Distantes | M21-22 | Luzern | 03-Apr-2021 | 933 | Fir beech forest |
| *Morchella deliciosa* | Sect. Distantes | M21-23 | Luzern | 03-Apr-2021 | 933 | Fir beech forest |
| *Morchella* sp. Mel-43 | Sect. Distantes | M21-33 | Ticino | 10-Apr-2021 | 1106 | N.A. |
| *Morchella* sp. Mel-43 | Sect. Distantes | M21-34 | Ticino | 10-Apr-2021 | 1106 | N.A. |
| *Morchella* sp. Mel-13 | Sect. Distantes | M21-35 | Neuchâtel | 08-Apr-2021 | 828 | Typical beech forest |
| *Morchella deliciosa* | Sect. Distantes | M21-37 | Neuchâtel | 11-Apr-2021 | 732 | Bushy oak grove |
| *Morchella deliciosa* | Sect. Distantes | M21-60 | Neuchâtel | 22-Apr-2021 | 1020 | Fir beech forest |
| *Morchella deliciosa* | Sect. Distantes | M21-63 | Neuchâtel | 22-Apr-2021 | 725 | Bushy oak grove |
| *Morchella deliciosa* | Sect. Distantes | M21-66 | Luzern | 20-Apr-2021 | 869 | Fir beech forest |
| *Morchella* sp. Mel-45 | Sect. Distantes | M21-82 | Fribourg | 04-May-2021 | 1127 | N.A. |
| *Morchella pulchella* | Sect. Distantes | M21-90 | Vaud | 20-Apr-2021 | N.A. | N.A. |
| *Morchella pulchella* | Sect. Distantes | M21-91 | Vaud | 20-Apr-2021 | N.A. | N.A. |
| *Morchella esculenta* | Sect. Morchella | M19-15 | Neuchâtel | 28-Mar-2019 | 1011 | Fir beech forest |
| *Morchella esculenta* | Sect. Morchella | M19-16 | Neuchâtel | 28-Mar-2019 | 1011 | Fir beech forest |
| *Morchella esculenta* | Sect. Morchella | M19-17 | Neuchâtel | 28-Mar-2019 | 1011 | Fir beech forest |
| *Morchella esculenta* | Sect. Morchella | M19-18 | Neuchâtel | 28-Mar-2019 | 760 | Bushy oak grove |
| *Morchella esculenta* | Sect. Morchella | M19-20 | Neuchâtel | 28-Mar-2019 | 761 | Bushy oak grove |
| *Morchella esculenta* | Sect. Morchella | M19-34 | Neuchâtel | 01-Apr-2019 | 759 | Bushy oak grove |
| *Morchella esculenta* | Sect. Morchella | M19-36 | Neuchâtel | 01-Apr-2019 | 759 | Bushy oak grove |
| *Morchella esculenta* | Sect. Morchella | M19-38 | Neuchâtel | 01-Apr-2019 | 1065 | Typical beech forest |
| *Morchella esculenta* | Sect. Morchella | M19-40 | Neuchâtel | 01-Apr-2019 | 541 | Bushy oak grove |
| *Morchella esculenta* | Sect. Morchella | M21-26 | Neuchâtel | 04-Apr-2021 | 1007 | Fir beech forest |
| *Morchella esculenta* | Sect. Morchella | M21-32 | Neuchâtel | 08-Apr-2021 | 722 | Bushy oak grove |
| *Morchella esculenta* | Sect. Morchella | M21-36 | Neuchâtel | 10-Apr-2021 | 1001 | Fir beech forest |
| *Morchella esculenta* | Sect. Morchella | M21-42 | Neuchâtel | 12-Apr-2021 | 732 | Bushy oak grove |
| *Morchella esculenta* | Sect. Morchella | M21-43 | Bern | 13-Apr-2021 | 756 | Typical beech forest |
| *Morchella esculenta* | Sect. Morchella | M21-46 | Neuchâtel | 15-Apr-2021 | 690 | Luzule beech forest |
| *Morchella esculenta* | Sect. Morchella | M21-54 | Neuchâtel | 18-Apr-2021 | 728 | Bushy oak grove |
| *Morchella esculenta* | Sect. Morchella | M21-55 | Neuchâtel | 18-Apr-2021 | 642 | Bushy oak grove |
| *Morchella esculenta* | Sect. Morchella | M21-58 | Neuchâtel | 18-Apr-2021 | 695 | Luzule beech forest |
| *Morchella esculenta* | Sect. Morchella | M21-59 | Neuchâtel | 19-Apr-2021 | 691 | Luzule beech forest |
| *Morchella esculenta* | Sect. Morchella | M21-61 | Neuchâtel | 22-Apr-2021 | 1048 | Fir beech forest |
| *Morchella esculenta* | Sect. Morchella | M21-62 | Neuchâtel | 22-Apr-2021 | 725 | Bushy oak grove |
| *Morchella esculenta* | Sect. Morchella | M21-64 | Neuchâtel | 22-Apr-2021 | 721 | Bushy oak grove |
| *Morchella esculenta* | Sect. Morchella | M21-67 | Vaud | 20-Apr-2021 | 804 | N.A. |
| *Morchella esculenta* | Sect. Morchella | M21-67 | Vaud | 20-Apr-2021 | 804 | N.A. |
| *Morchella esculenta* | Sect. Morchella | M21-68 | Vaud | 20-Apr-2021 | 804 | N.A. |
| *Morchella esculenta* | Sect. Morchella | M21-69 | Vaud | 20-Apr-2021 | 804 | N.A. |
| *Morchella esculenta* | Sect. Morchella | M21-70 | Vaud | 22-Apr-2021 | 804 | N.A. |
| *Morchella esculenta* | Sect. Morchella | M21-71 | Fribourg | 24-Apr-2021 | 1040 | N.A. |
| *Morchella esculenta* | Sect. Morchella | M21-72 | Fribourg | 24-Apr-2021 | 649 | N.A. |
| *Morchella esculenta* | Sect. Morchella | M21-74 | Fribourg | 24-Apr-2021 | 685 | N.A. |
| *Morchella esculenta* | Sect. Morchella | M21-75 | Bern | 27-Apr-2021 | 431 | N.A. |
| *Morchella esculenta* | Sect. Morchella | M21-76 | Neuchâtel | 29-Apr-2021 | 1061 | Fir beech forest |
| *Morchella esculenta* | Sect. Morchella | M21-77 | Neuchâtel | 29-Apr-2021 | 1061 | Fir beech forest |
| *Morchella esculenta* | Sect. Morchella | M21-78 | Neuchâtel | 29-Apr-2021 | 792 | Typical beech forest |
| *Morchella esculenta* | Sect. Morchella | M21-79 | Solothurn | 03-May-2021 | 452 | N.A. |

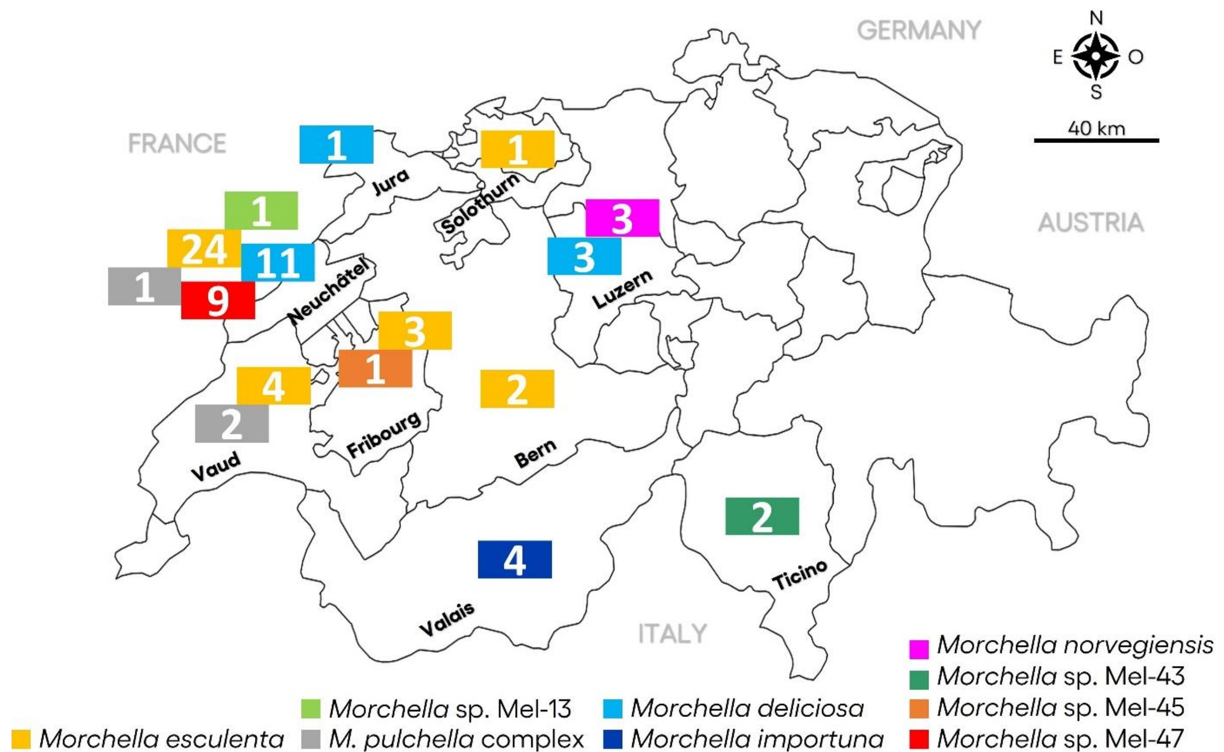


**Figure 1**. Map of Switzerland representing the sampling locations of each morel fruiting body that were collected between 2019 and 2021. The number of specimens per species and canton is provided.

### 2.3.2 Genetic identification of the Swiss *Morchella* using combined genetic, morphological, and ecological data

In the present study, different methods were applied to determine the biodiversity of *Morchella* in Switzerland. Genetically, multilocus phylogenies of concatenated ITS-*RPB1-RPB2-TEF1-*$\alpha$ were generated, in addition to single-locus (ITS; *RPB1; RPB2; TEF1-*$\alpha$) trees. To further investigate species from the Elata clade, an additional two-locus (*MAT1-1-1–MAT1-2-1*) phylogeny was produced. In *Morchella,* the concept of phylogenetic species as proposed by O'donnell et al. (O'Donnell et al. 2011) based on multilocus phylogenetics and the concept of genealogical concordance phylogenetic species recognition (GCPSR) stated by Taylor et al. (Taylor et al. 2000) has been widely used to delineate species (Baroni et al. 2018; Du et al. 2019; Taskin et al. 2010; Elliott et al. 2014; Du et al. 2012; Petrželová et Sochor 2019). For this, three criteria should be used: the "criterion of monophyly" (Petrželová et Sochor 2019), indicating that a species can be recognized if the monophyly from a multilocus tree is not contradicted by any of the single-locus trees (O'Donnell et al. 2011), and the "genealogical criterion" (Petrželová et Sochor 2019), indicating that genealogical exclusivity must be supported by bootstrap analysis in both the multilocus tree and in at least one of the single-locus trees (O'Donnell et al. 2011). Additionally, the genetic analysis could include the "criterion of distinctness", indicating that the genetic distance between a species and its sister taxa should be considered, and that it should reflect divergence by at least one single nucleotide polymorphism (SNP) in the used genetic markers (i.e., the "criterion of polygenic differentiation") (Petrželová et Sochor 2019). None of these concepts can be based on a single collection, as it does not encompass the genetic divergence that may exist intraspecifically (i.e., the "criterion of minimal sampling") (Petrželová et Sochor 2019).

These criteria were used as a guideline to investigate the new taxa found in Switzerland. As suggested by Loizides et al., to delineate *Morchella* species, genetic methods should be combined to ecological, morphological, and developmental descriptions (Loizides et al. 2022). GCPSR and the "criterion of monophyly" should also be considered carefully, as strict phylogenetic analysis may not reflect biological or ecological differences that can be revealed by a polyphasic approach (Loizides et al. 2022). In addition, the genetic markers that were used in the phylogenetic analysis (ITS; *RPB1; RPB2; TEF1-α*) have different resolution power. It was shown that the ITS, followed by the *TEF1-α,* had the highest resolution ability in *Morchella* (Du et al. 2012), which was concordant with our results. For the reason of this asymmetry, the GCPSR might not applied as originally described (Taylor et al. 2000), but rather as an additional tool, pondered for each genetic marker depending on its resolution power.

The analysis of each taxon is discussed in the paragraphs below. Although the new lineages Mel-43 and Mel-45 did not fulfill the criterion of monophyly, as the specimens all come from single populations represented by 1-3 fruiting bodies. However, we found important for future research in *Morchella* taxonomy to present the genetically divergent lineages found in the country, even though they may not represent true phylospecies. Therefore, these lineages were considered as "new lineages". Further sampling at the locations where they were found will be necessary to describe these lineages.

**2.3.3 Species identification in the Esculenta clade**

Swiss carpophore specimens were assigned to the Esculenta (*Morchella* Sect. *Morchella*, i.e., yellow morels) clade based on phylogenetic analyses conducted with reference species. Five phylogenies were constructed for the Esculenta clade: four single-locus phylogenies (ITS; *RPB1; RPB2; TEF1-α*) and one four-locus phylogeny (concatenated ITS-*RPB1*-*RPB2*-*TEF1-α*). The multilocus phylogeny (**Figure 2**) contained 34 Swiss specimens and 29 reference taxa, for a total of 63 sequences containing 349 parsimony-informative distinct patterns (PIDP), 352 singleton sites, and 2248 constant sites. The analysis revealed that all the Swiss morels from the Esculenta clade belonged to *M. esculenta*. The single-locus phylogenies aimed to verify the phylogenetic concordance of the four loci that were used in the multilocus analysis, following the concept of Genealogical Concordance Phylogenetic Species Recognition (GCPSR) (Taylor et al. 2000). The single-locus phylogenies had different taxonomic resolution power, the ITS being the most informative (with 301 PIDP), followed by *TEF1-α* (45 PIDP), *RPB1* (19 PIDP) and *RPB2* (18 PIDP). The datasets (aligned sequences) that were used to generate the Esculenta phylogenies can be found in **Supplementary S3A**.

The four-locus phylogeny revealed that all the yellow morels included in this study belonged to *M. esculenta* (**Figure 2**). In the single-locus phylogenies, the Swiss samples also grouped with *M. esculenta* references, but the taxa were not monophyletic for the *RPB1, RPB2* and *TEF1-α*, as they contained *Morchella* sp. Mes-20, *Morchella* sp. Mes-26, *Morchella* sp. Mes-27, *Morchella fluvialis* (*RPB1*, see **Supplementary S5A**), *Morchella vulgaris* and *M. fluvialis* (*RPB2*, see **Supplementary S5B**) or *M. fluvialis* (*TEF1-α*, see **Supplementary S5C**). *M. esculenta* were monophyletic in the single-locus ITS tree (**Supplementary S5D**). Therefore, the assignation to *M. esculenta* was confirmed based on the genealogical criterion, but not on the criterion of monophyly. *M. esculenta* was not monophyletic in all the single-locus phylogenies (**Supplementary S5A-C**), as *M. fluvialis* grouped with *M. esculenta* in the *RPB1, RPB2* and *TEF1-α* single-locus trees. However, as mentioned previously, the ITS is generally enough to resolve the taxonomy of *Morchella* Sect. *Morchella* (Clowez et al. 2014). In addition, as shown in the present study, the ITS had a much higher resolution than the other markers for the Esculenta clade. For this reason, we concluded that the Swiss samples presented here all belong to *M. esculenta.*

*M. esculenta* were collected in five cantons (Neuchâtel, Fribourg, Vaud, Bern, Solothurn) under *Abies, Fagus, Fraxinus,* or *Quercus* trees, at altitudes varying between 452-1065 m. This is in accordance with literature findings (Richard et al. 2015). Morphologically, the Swiss *M. esculenta* were highly variable in the shape of the cap, stipe, and alveoli, in color, and in size (**Figure 3A-B**). The caps were globose, elliptical, and ovoid to acute conical. The stipes could be cylindrical, clavate or concave, generally with ridges or folds at the base. The alveoli were irregular and varied in shape and depth. The alveoli were light yellow to grey or brown at maturity. The ridges were the same color than the alveoli, but were generally orange, brown, or grey. The primary ridges were generally undistinguishable from the secondary ridges. The immature specimens were usually grey or brown grey, the alveoli becoming yellow prior to the ridges. The dried specimens of *M. esculenta* displayed (dark) grey alveoli and yellow to brown or orange ridges. Ascospores were elliptical and measured in mean (n=50) 20.4 x 13.1 µm. Naturally released ascospores (from spore prints, see **Figure 3E**) were slightly longer and thinner than spores that were still contained within asci of mature specimens. Asci (232-297 x 6-18 µm) were cylindrical or sinuous, eight-spored (**Figure 3C**). Paraphyses were longer than the asci and various in width (11-20 µm) (**Figure 3D**). They often had one septum in the upper portion (forming a cylindrical terminal cell) or across the mid-point of the paraphysis. Paraphyses could be fasciculate or solitary. The sterile elements of the stipe were catenulate, uni- or bifurcate, with globose and/or rectangular cells; the terminal cell was often elongated. This was in accordance with literature findings (Clowez 2012).

In conclusion, all the Swiss samples of Esculenta morels belonged to *M. esculenta,* based on the genealogical criterion, ecology, and morphology. Yellow morels were thought to show greater levels of endemism, and lower species diversity compared to black morels (Du, Zhao, et Yang 2015). This could be due to the higher cold tolerance of black morels, that might have helped them to survive during the Quaternary Ice Age (Du, Zhao, et Yang 2015). This hypothesis was partially supported by the present study, because although only one yellow morel species was reported, *M. esculenta* is not endemic but widespread in at least 15 different countries across the Eurasia (Loizides et al. 2022).

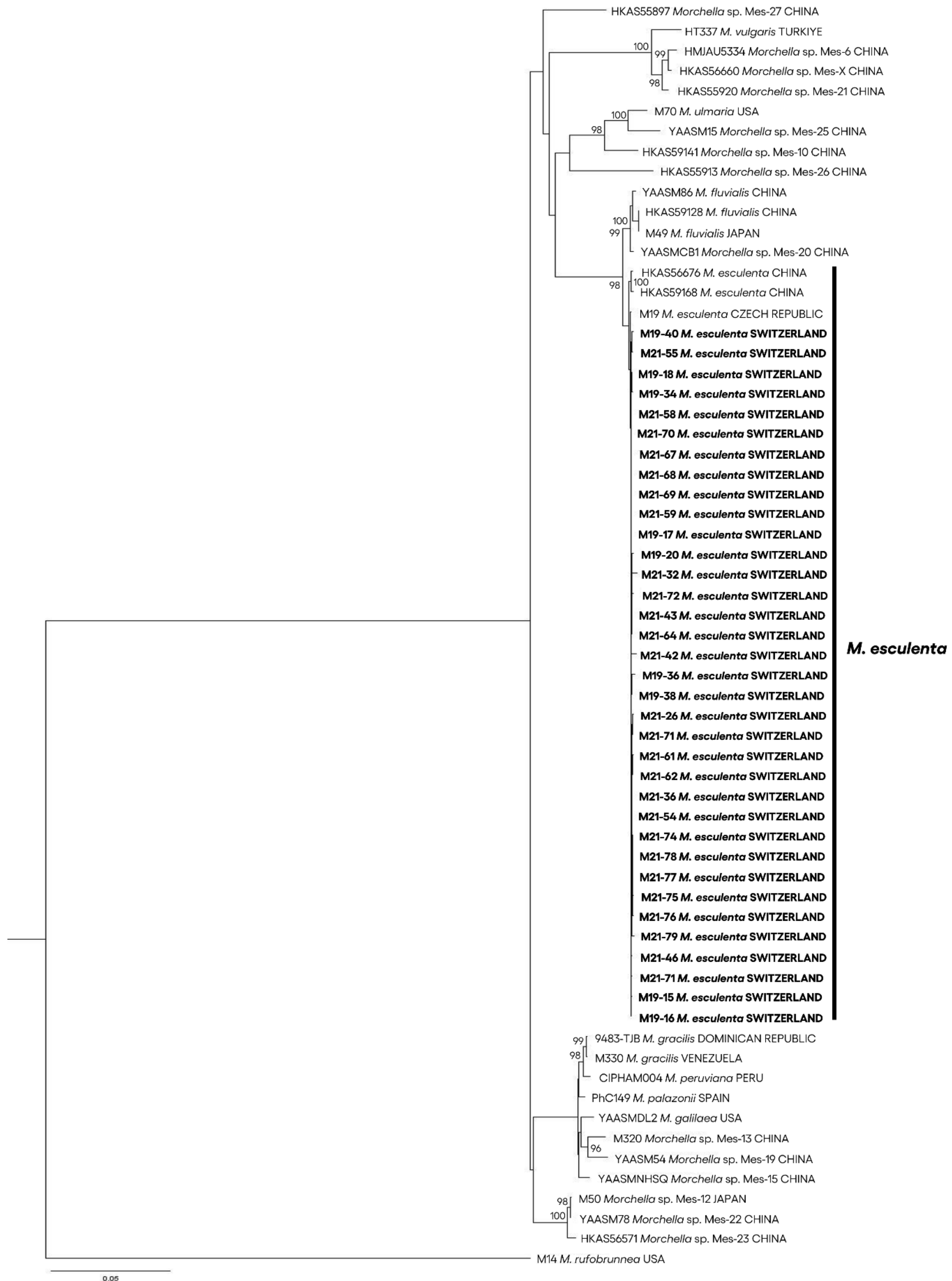


**Figure 2.** Multi-locus (ITS-*RPB1*-*RPB2*-*TEF1*-$\alpha$) ML phylogeny of *Morchella* Sect. *Morchella* with Swiss specimens (bold) and reference sequences. The strain ID and sampling location corresponding to each sequence is provided. The phylogeny was based on 10,000 ultrafast bootstrap values (UFBoot > 95% displayed at branch nodes). The tree was rooted with the basal clade *M. rufobrunnea.*

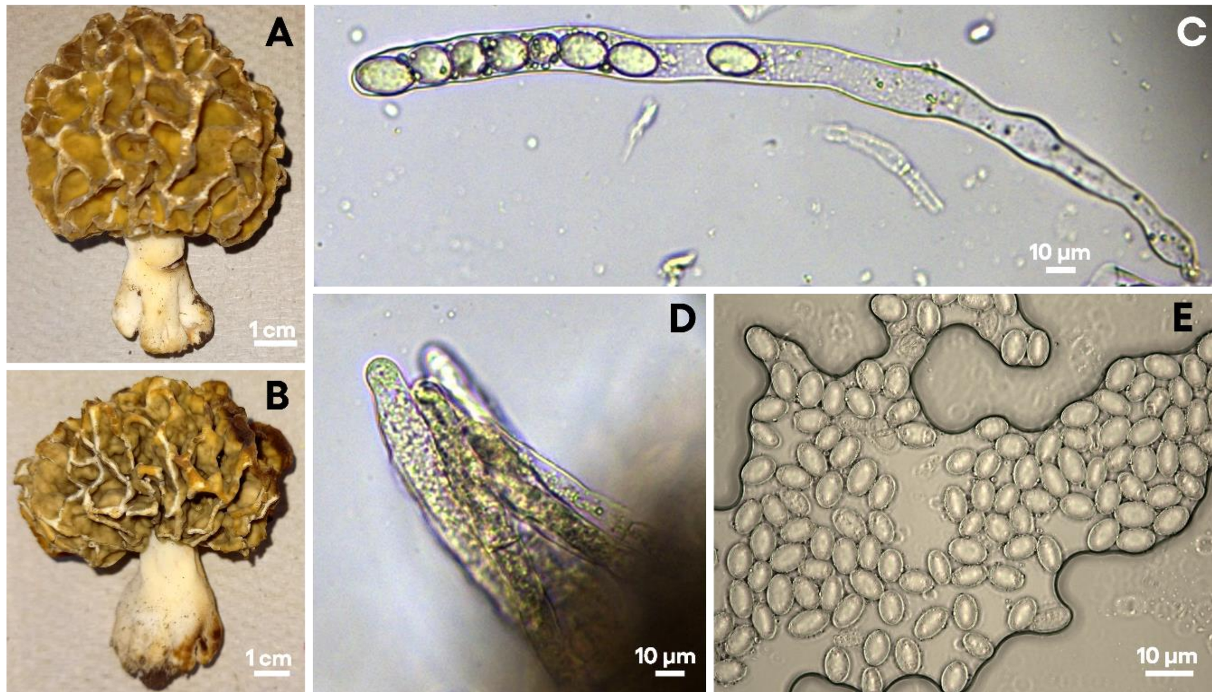


**Figure 3**. A= Fruiting body of *Morchella esculenta* M21-79; B= Fruiting body of *M. esculenta* M21-46; C= Ascus with eight ascospores, *M. esculenta* M21-55; D= Paraphyses of *M. esculenta* M21-55; E= Spore print from *M. esculenta* M19-40. Pictures C-E were taken with a light microscope, in physiological water, at 40x magnification.

### 2.3.4 Species identification in the Elata clade

Swiss carpophore specimens were assigned to the Elata (*Morchella* Sect. *Distantes*, i.e., black morels) clade based on phylogenetic analyses conducted with reference species. Six phylogenies were constructed for the Esculenta clade: four single-locus phylogenies (ITS; *RPB1; RPB2; TEF1-$\alpha$*), one two-locus phylogeny (concatenated *MAT1-1-1–MAT1-2-1*), and one four-locus phylogeny (concatenated ITS-*RPB1*-*RPB2*-*TEF1*-$\alpha$). The non-mating four-locus phylogeny (**Figure 4**) contained 38 Swiss specimens and 46 reference taxa, for a total of 84 sequences containing 391 parsimony-informative distinct patterns (PIDP), 231 singleton sites, and 1853 constant sites. It revealed that four Swiss specimens belonged to *M. importuna,* fifteen to *M. deliciosa*, one to *Morchella* sp. Mel-13, and three to the *M. pulchella* species complex, itself containing *M. pulchella, M. septentrionalis, M. conifericola*, and *Morchella* sp. Mel-23. In addition, several Swiss morels did not group with any known species and were then attributed to new lineages named *Morchella* sp. Mel-43, Mel-45, and Mel-47. As for the Esculenta clade, the single-locus phylogenies aimed to verify the phylogenetic concordance of the loci that were used in the four-locus analysis. Similarly, the single-locus ITS had the highest resolution power (154 PIDP), followed by *TEF1-$\alpha$* (113 PIDP), *RPB1* (101 PIDP) and *RPB2* (61 PIDP). The two additional genetic markers (mating type genes *MAT1-1-1* and *MAT1-2-1*) that were used to generate a two-locus phylogeny to further investigate the new lineages, displayed a high taxonomic resolution, with 338 PIDP, and a topology consistent with the non-mating phylogeny (**Figure 5**). The datasets (aligned sequences) that were used to generate the Elata phylogenies can be found in **Supplementary S3B** (non-mating loci) and **S3C** (mating loci). In the following sections, each species or lineage found in Switzerland was investigated genetically, ecologically and morphologically.

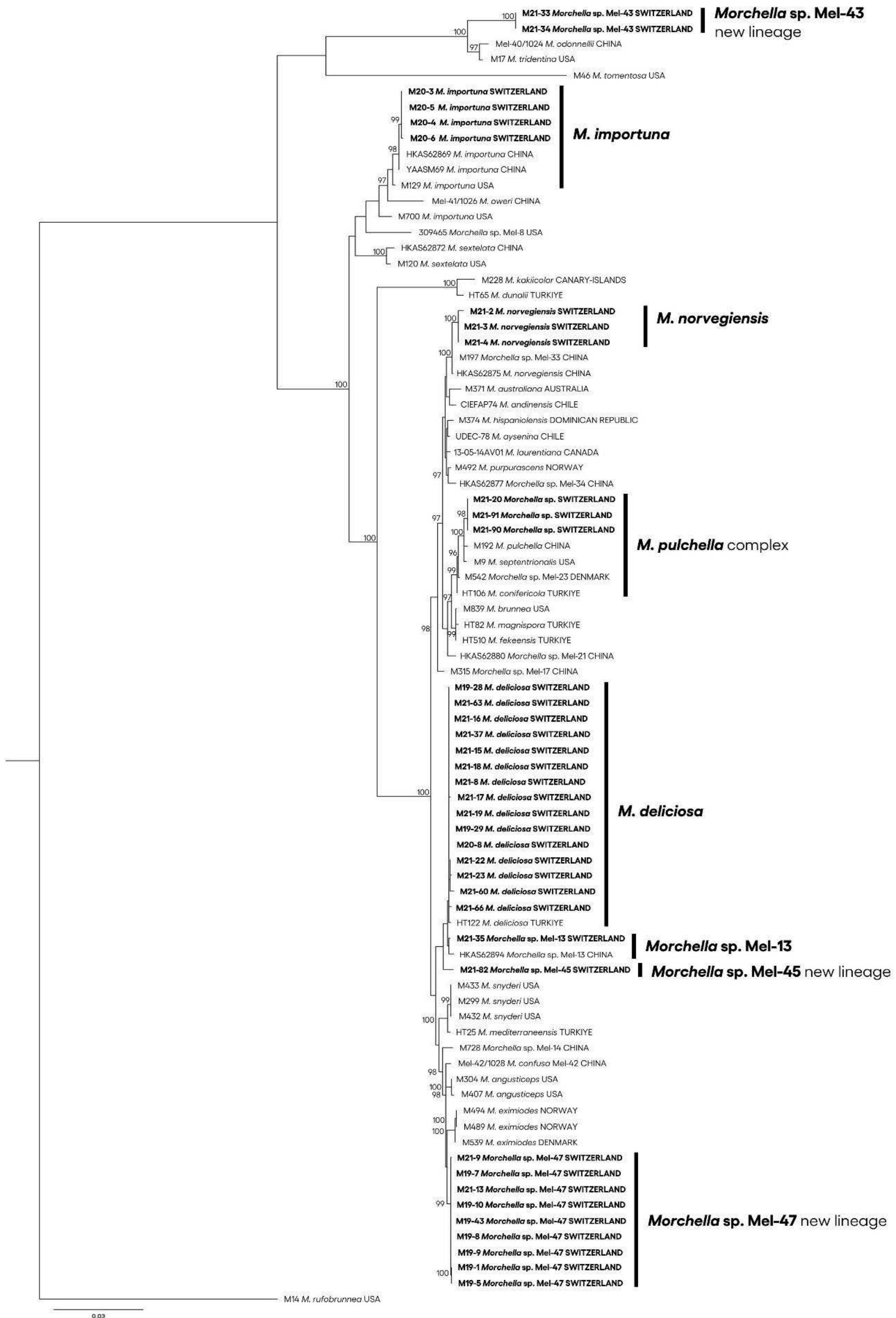


**Figure 4.** Multi-locus (ITS-RPB1-RPB2-TEF1-α) ML phylogeny of *Morchella* Sect. *Distantes* with Swiss specimens (bold) and reference sequences. The strain ID and sampling location corresponding to each sequence is provided. The phylogeny was based on 10,000 ultrafast bootstrap values (UFBoot > 95% displayed at branch nodes). The tree was rooted with the basal clade *M. rufobrunnea.*

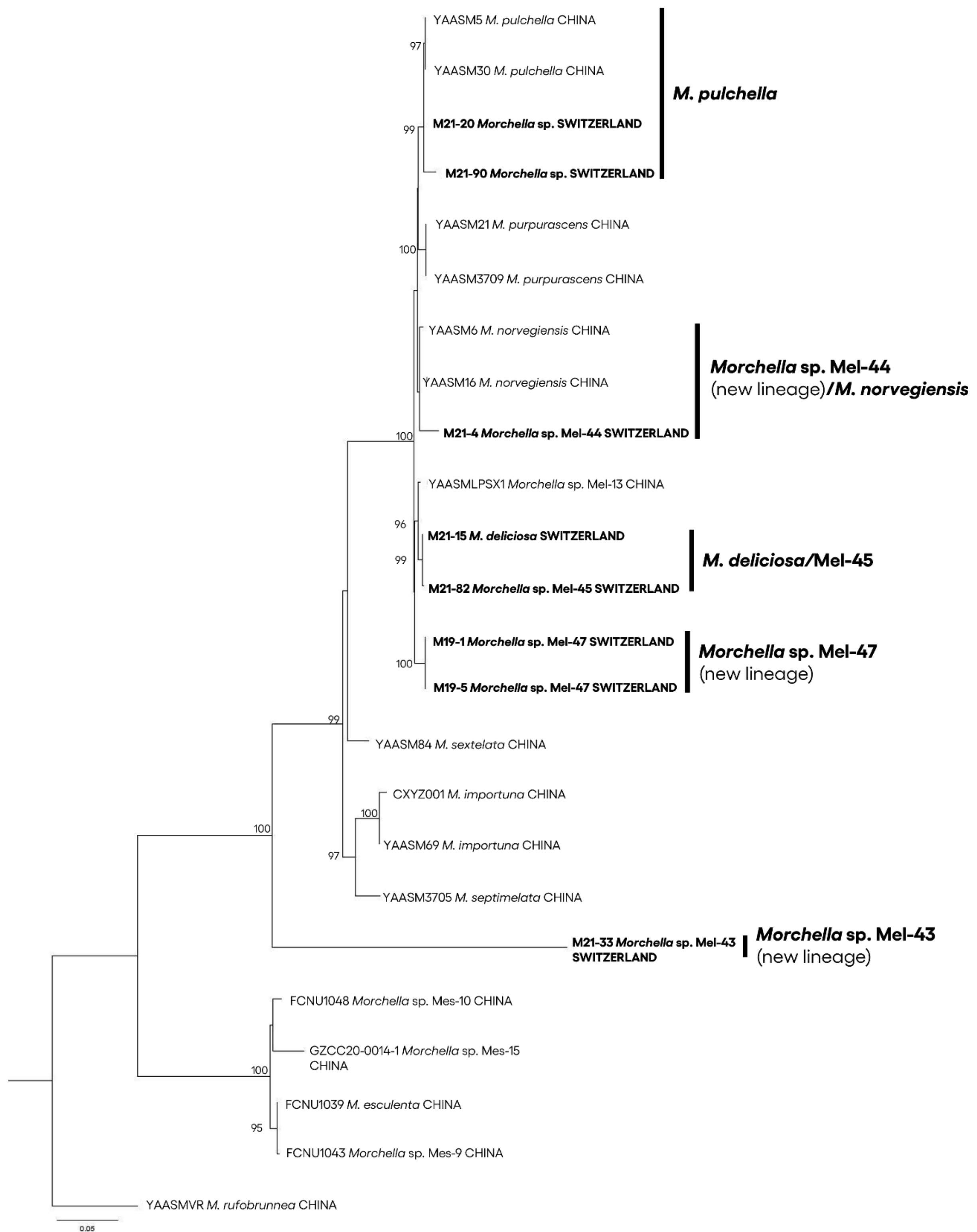


**Figure 5.** Multi-locus (*MAT1-1-1–MAT1-2-1*) ML phylogeny of *Morchella* with Swiss specimens (bold) and reference sequences. The strain ID and sampling location corresponding to each sequence is provided. The phylogeny was based on 10,000 ultrafast bootstrap values (UFBoot > 95% displayed at branch nodes). The tree was rooted with the basal clade *M. rufobrunnea*.

2.3.4.1 *Morchella importuna*

Four specimens of *M. importuna* (M20-3 to -6) were collected in Switzerland, in a single population in the canton of Valais in 2020. In the four-locus analysis, they were closely related to *M. importuna* from China. *M. importuna* were monophyletic in all single-locus trees (see **Supplementary S6A-D**). One of the reference specimens (*M. importuna* M700 from China) however grouped with *M. sextelata* based on the *TEF1-*$\alpha$ gene. The Swiss specimens were therefore attributed to *M. importuna* based on the genealogical criterion and the monophyly criterion. Unfortunately, no ecological data could be obtained on these samples. The stipes of the fruiting bodies were cut during collection and the specimens were received dry in the laboratory (see **Supplementary S7A**), impairing a reliable morphological description. Still, it could be observed that dry, the alveoli were lighter than the ridges. The primary ridges were organized in a laddered appearance with the numerous horizontally transversal secondary ridges. This typically corresponded to *M. importuna* (Kuo et al. 2012). Most of the asci were empty (**Supplementary S7C**), indicating sporulation had already occurred. The apices of the acroparaphyses were usually ophiomorphous (i.e., in a snake-like shape), or globose (**Supplementary S7B**). In summary, and despite the lack of ecological and morphological data, there is little doubt that specimens M20-3 to -6 belonged to *M. importuna*, based on the genealogical criterion, monophyly criterion, and macromorphology.

2.3.4.2 *Morchella deliciosa, Morchella* sp. Mel-13 and *Morchella* sp. Mel-45

These three taxa were evaluated together because they were closely related phylogenetically. In the four-locus analysis (**Figure 4**), 15 specimens (putative *M. deliciosa*) were closely related to *M. deliciosa* from Türkiye, *Morchella* sp. Mel-13 from China, and the Swiss specimen M21-35. The specimen M21-35 was attributed to *Morchella* sp. Mel-13 as they formed a monophyletic taxon in the multilocus tree. Another specimen that was genetically close to *M. deliciosa* and Mel-13 was the Swiss specimen M21-82. This sample formed a monophyletic group, sister taxon to *M. deliciosa/*Mel-13 (statistically not significant), and it was considered as a new lineage named *Morchella* sp. Mel-45. The single-locus phylogeny of ITS supported the fact that *M. deliciosa, Morchella* sp. Mel-13 and *Morchella* sp. Mel-45 are distinct species (**Supplementary S6A**), but it was not the case for *RPB1* and *RPB2,* where *M. deliciosa/*Mel-13/Mel-45 formed a single group (**Supplementary S6B-C**). In the *TEF1-*$\alpha$ tree, *Morchella* sp. Mel-17 was additionally included in the *M. deliciosa/*Mel-13/Mel-45 group (**Supplementary S6D**). In the *MAT* phylogeny, Mel-45 grouped with *M. deliciosa* (**Figure 5**). Thus, the criterion of genealogy could not be validated for Mel-45 as its separation from the sister taxa was not statistically supported (UFBoot = 71). Following the criterion of monophyly, none of these three taxa should be considered as distinct species, as they grouped together in the other single-locus phylogenies and in the *MAT* tree. It has already been indicated that *M. deliciosa* could include Mel-13 (Loizides et al. 2022), and the present study tends to supports this hypothesis.

*M. deliciosa* was the most abundant black morel found in Switzerland, as 15 specimens were collected across three cantons (Neuchâtel, Jura, Luzern). They were mostly found under or near *Fagus* and *Fraxinus* trees, between 668-1063 m altitude. M21-35 was collected in the same ecological niche, at 828 m altitude under *Fagus* and *Fraxinus* trees. M21-82 was collected in the canton of Fribourg in 2021, without further information concerning its ecology. The caps of the Swiss *M. deliciosa* were acute conical (**Figure 6A**), with irregular fusiform alveoli. The stipes were usually clavate, ridged and folded. The alveoli could be light brown with yellow, grey, or reddish tones. The ridges always darker than the alveoli, from brown to black. The secondary ridges were well defined, transversal horizontally or diagonally, sunken and concolorous with the pit, or with the same texture and color as the primary ridges. Asci (232-297 x 6-18 µm) were cylindrical (**Figure 6B**), eight-spored. Paraphyses were uni- or bicatenulate, sometimes septate at mid-point of the paraphysis, with an ophiomorphous

or cylindrical terminal cell. Acroparaphyses often had one septum at mid-point of the element, often forming an elongated globose terminal cell (**Figure 6C**). Ascospores were elliptical and measured in mean (n=50) 19.7 x 11.6 µm (**Figure 6D**).

The unique Swiss representative of *Morchella* sp. Mel-13 (M21-35) was received damaged; the stipe was cut, as well as the apical part of the cap (**Supplementary S8A**). Furthermore, it corresponded to an immature fruiting body (**Supplementary S8B**, immature ascus). The alveoli were narrow with round apices, darker than the primary ridges that were warm brown. Secondary ridges were horizontal, sunken, with the same color and texture than the primary ridges. Asci were immature and did not contain any ascospore. Acroparaphyses were not septate, cylindrical with a globose or ophiomorphous terminal cell (**Supplementary S8C**). Similarly, the unique representative of *Morchella* sp. Mel-45 (M21-82) was an immature fruiting body (**Supplementary S9A**). The cap was conical, with narrow pits that were concolorous with the ridges. Secondary ridges were present, sunken, transversal, and with the same color and texture than the primary ridges. The stipe was concave, with basal folds. Asci were immature and did not contain any ascospore (**Supplementary S9B**). Acroparaphyses were not septate, cylindrical (**Supplementary S9C**). No distinct terminal cell was observed.

In summary, the existence of genetic differences between *M. deliciosa*, Mel-13 and Mel-45, was supported by the ITS locus, which was the marker with the higher resolution power in the used dataset. However, lack of representatives of Mel-13 and Mel-45 impair a conclusion (genetically, ecologically, and morphologically) regarding their distinction from *M. deliciosa.*

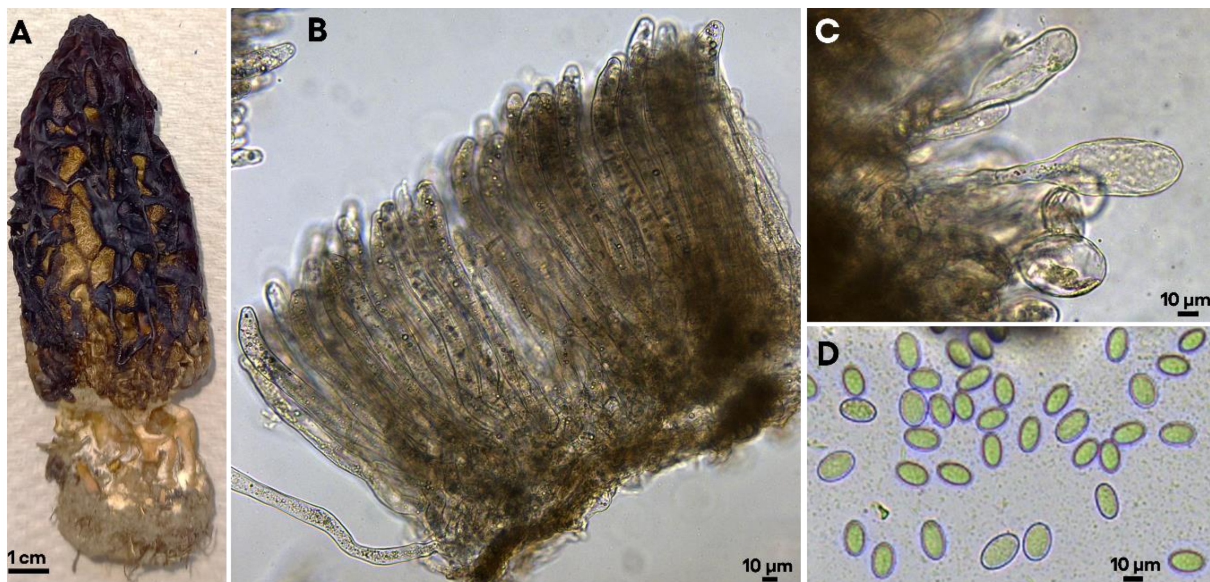


**Figure 6**. A= Aged fruiting body of *Morchella deliciosa* M21-66; B= Asci of *M. deliciosa* M21-8 (empty, no ascospores); C= Acroparaphyses of M. deliciosa M21-8; D= Spore print from *M. deliciosa* M21-8. Pictures B-D were taken with a light microscope, in physiological water, at 40x magnification.

### 2.3.4.3 *Morchella pulchella* species complex

This species complex, containing *M. pulchella, M. conifericola, M. septentrionalis, Morchella* sp. Mel-23 (Loizides et al. 2022; Petrželová et Sochor 2019), was investigated in this study to try to clarify the taxonomic status of the different species. Three Swiss samples attributed to this species complex by the multilocus tree were included in the analyses. Three Swiss specimens (M21-20, M21-90, M21-91) belonged to the *M. pulchella* species complex (*M. pulchella, M. conifericola, M. septentrionalis, Morchella* sp. Mel-23), according to the four-locus (**Figure 4**) and ITS phylogenies (**Supplementary**

**S6A**). In the *RPB1* tree, the *M. pulchella* reference (strain M192 from China) grouped with the three Swiss specimens (M21-20, M21-90, M21-91), *M. septentrionalis* (strain M9, USA), and *Morchella* sp. Mel-34 (strain HKAS62877, China), but not with *M. conifericola* (strain M542, Denmark), which was placed as the sister group (**Supplementary S6B**). In the *RPB2* phylogeny, the three Swiss strains grouped monophyletically as a sister taxon of the *M. pulchella* complex (**Supplementary S6C**). In the *TEF1-$\alpha$* tree, the *M. pulchella* species complex was not monophyletic, and also contained *M. brunnea, M. magnispora, M. fekeensis*, and *M. arbutiphila*, in addition to the three Swiss strains M21-20, M21-90 and M21-91 (**Supplementary S6D**). Mating type sequences were only available for strains annotated as *M. pulchella* (YAASM5 and YAASM30 from China), which grouped with the Swiss specimens of the *M. pulchella* complex (**Figure 5**).

To clarify the phylogenetic relationship of species comprised in the *M. pulchella* complex, single-locus phylogenies of the ITS, *RPB1, RPB2,* and *TEF1-$\alpha$* markers were generated, using the three Swiss specimens and all the sequences that were available on GenBank for *"M. pulchella"* (i.e., *Morchella* sp. Mel-31)*, "M. conifericola"* (i.e., *Morchella* sp. Mel-32)*, "M. septentrionalis"* (i.e., *Morchella* sp. Mel-24), and *"Morchella* sp. Mel-23". Sequences that were annotated as "*Morchella* sp. Mel-23/24/31/32" (e.g., isolate D115 from China) were also included. This analysis indicated that none of these species could be distinguished by the ITS and *TEF1-$\alpha$* markers (**Supplementary S10A-B**). In the *RPB1* phylogeny*, Morchella* sp. Mel-23 was monophyletic; *M. pulchella* could not be distinguished from *M. septentrionalis* and included the Swiss samples; *M. conifericola* was monophyletic, except that one sequence annotated as *M. pulchella* was present in the same taxon (**Supplementary S10C**). In the *RPB2* phylogeny two distinct groups were identified, one containing *M. pulchella/M. conifericola* and the Swiss samples, and the other containing *M. septentrionalis/*Mel-23 (**Supplementary S10D**).

In Taşkın et al., *M. conifericola* (i.e., Mel-32) and Mel-23 were both monophyletic and thus distinct from *M. pulchella* and *M. septentrionalis*. *M. pulchella* and *M. septentrionalis* were however not monophyletic and grouped together (Taskin et al. 2012). The multilocus analysis yielded the same results in the present study (**Figure 5**). However, the genealogical criterion, and the criterion of monophyly were not validated, as the tree topology was not the same in any single-locus phylogenies (**Supplementary S10A-D**). For instance, in the *RPB1* tree, *M. septentrionalis* was monophyletic with *M. pulchella*, but in the *RPB2* tree*, M. pulchella* grouped with *M. conifericola*.

The Swiss samples were collected in the cantons of Neuchâtel (M21-20, associated with *Quercus* sp. and *Fraxinus* sp., at 715 m altitude) and Vaud (M21-90 and M21-91, unknown ecology) in April 2021. Among the species of the *M. pulchella* complex, only *M. conifericola* differed in the phenology (fruiting in May (Taskin et al. 2012)) compared to the Swiss samples, *M. pulchella* and *M. septentrionalis* that fruit in April (Kuo et al. 2012; Badshah et al. 2018). A morphological comparison of macro- and micro characteristics of *M. pulchella, M. conifericola, M. septentrionalis* and the Swiss M21-20 (fresh specimen), M21-90 (dry, **Figure 7A**), M21-91 (dry, **Figure 7B**) was conducted (see **Supplementary S11** for the detailed comparative analysis). However, it did not allow a clear distinction because the Swiss samples shared traits with multiple species. The Swiss samples shared the stipe as described for *M. pulchella* i.e., convex with a conical wide base, tapered at apex (Badshah et al. 2018) (**Figure 7A-B**), but the presence of ridges and folds differed between all the descriptions. The literature lacked information concerning *M. pulchella* alveoli and ridges (color, texture, presence of secondary ridges), and the other species were similar to the Swiss samples. Based on the micromorphology, the Swiss samples corresponded to *M. septentrionalis*, as asci were shorter in *M. pulchella* and longer in *M. conifericola*, and the paraphyses larger in the same species. In addition, the shape of the paraphyses (**Figure 7E**) were similar in the Swiss samples and in *M. septentrionalis*.

Based on the morphology, the Swiss samples seem to correspond to *M. septentrionalis*, but this species was described as native from Northern America (Kuo et al. 2012), contrarily to *M. pulchella* and *M. conifericola* that were described from Eurasia (Loizides et al. 2016; Taskin et al. 2012; Badshah et al. 2018).

In summary, no clear distinction could be made between representatives of *Morchella pulchella*, as none of the criteria proposed previously (O'Donnell et al. 2011; Petrželová et Sochor 2019) were validated. In addition, a morphological distinction was not possible, as the shared traits are mixed between the species. Additional descriptions from samples collected on the different continents should be conducted to determine whether *M. septentrionalis* is a distinct species from America, or whether Mel-23/24/31/32 represents a single species present in multiple continents. The Swiss samples were then attributed to the *M. pulchella* species complex, which we considered as the lower taxonomic resolution level acceptable based on the current data.

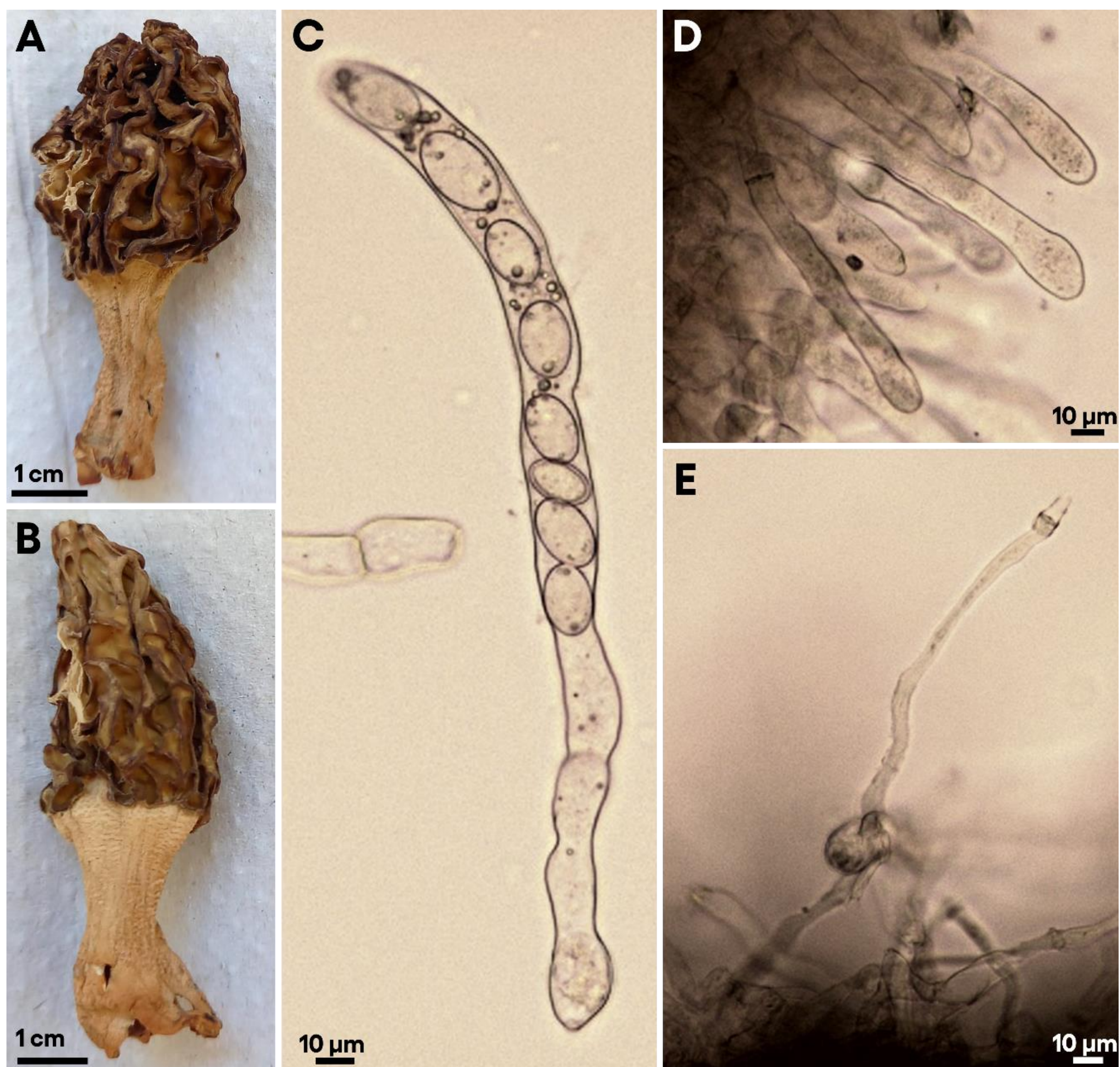


**Figure 7**. A= Dry fruiting body of M21-90, from the *Morchella pulchella* species complex; B= Dry fruiting body of M21-91, from the M. pulchella species complex; C= Ascus containing eight ascospores (M21-20, *M. pulchella* species complex); D= Acroparaphyses (M21-20, *M. pulchella* species complex); E= Paraphyse (M21-20, M. pulchella species complex). Pictures C-E were taken with a light microscope, in physiological water, at 40x magnification.

2.3.4.4 New lineage *Morchella* sp. Mel-43

*Morchella* sp. Mel-43 was represented by two specimens from a single population (M21-33 and M21-34) collected in the canton of Ticino in 2021. The four-locus analysis indicated that it was a monophyletic basal group of the Elata clade, siter taxon to *Morchella tridentina/Morchella odonnellii* (UFBoot = 100). In the single-locus phylogenies, Mel-43 was monophyletic for the ITS (**Supplementary S6A**), *RPB1* (**Supplementary S6B**), and *TEF1-α* (**Supplementary S6D**), but it contained *M. odonnellii* in the *RPB2* tree (**Supplementary S6C**). In the *MAT* phylogeny, Mel-43 was monophyletic, and located between the Esculenta and Elata clades (**Figure 5**). The genealogical criterion was therefore fulfilled, but not the criterion of monophyly for *RPB2*. However, as this was the sole incongruence, we concluded that Mel-43 represents a new phylospecies. Unfortunately, no ecological data could be obtained on these samples. The two representatives of *Morchella* sp. Mel-43 were received dry with a cut stipe at the laboratory (see **Supplementary S12A**) rendering impossible a reliable morphological description. In addition, the two specimens were immature as they were very small (<3 cm high) and contained no ascospore (**Supplementary S12B**). Although we are confident that Mel-43 represents a new phylospecies based on the genetic analysis, the criterion of minimal sampling was not fulfilled as Mel-43 was represented by only two specimens from a single population, that were furthermore immature.

2.*3.4.5 Morchella norvegiensis*

*Morchella norvegiensis* was represented by three specimens from a single population (M21-2, M21-3 and M21-4) collected in the canton of Luzern in 2021. The four-locus analysis indicated it was a monophyletic group in the Elata clade, siter taxon to *Morchella norvegiensis*/Mel-33 (ultrafast bootstrap = 100). In the single-locus phylogenies, the Swisss samples were monophyletic for *RPB2* and *TEF1-α* (Supplementary S4G-H), but it contained *M. laurentiana*/Mel-33 in the poorly resolved ITS tree (Supplementary S4E), and *M. norvegiensis*/Mel-33 in the RPB1 tree (Supplementary S4F). In the MAT phylogeny, M21-4 grouped with *M. norvegiensis* (Figure 5). Therefore, the genealogical criterion was met, but not the criterion of monophyly. To investigate this further, we compared DNA sequences from the specimen of *M. norvegiensis* that was previously reported from Switzerland (strain M510) (Richard et al. 2015) to M21-2, M21-3 and M21-4. This revealed 100% identities for ITS and *RPB1* and 99% id for *RPB2* (1 SNP A-G) and *TEF1-α* (1 SNP T-A), supporting that M21-2, M21-3 and M21-4 belong to *M. norvegiensis*. Moreover, the mating genotype was assessed in six single-ascospore cultures of M21-2. Four were heterothallic, with the *MAT1-2-1* gene present in each SAC, while two were homothallic, with both MAT genes present per SAC (Supplementary S5A).

The collection of Swiss *M. norvegiensis* was made late March, which is slightly earlier than what has been previously reported for *M. norvegiensis* (April-June, named "*M. eohespera*" in Voitk et al. (2016)) (Voitk et al. 2016). Other ecological data could not be obtained. The caps of the Swiss *M. norvegiensis* were oblong when immature (Figure 8B) or widely conical when mature (Figure 8A), with regular and vertically elongated alveoli. This corresponded with other descriptions of *M. norvegiensis* (subconical, conical, or ovoid cap with vertically elongated alveoli) (Voitk et al. 2016). The stipes of the Swiss samples were whitish with beige tones, widening basally, ridged and folded. This did not correspond with the stipe of previously reported *M. norvegiensis* that were cylindrical to tapered downwards, or subclavate (Voitk et al. 2016). The alveoli of the Swiss samples were dark brown when immature, and honey brown with yellow tones at maturity. The ridges were dark taupe with dark brownish tones when immature and light greyish-brown when mature. The number of vertical primary ridges (12-22) corresponded with *M. norvegiensis*. In *M. norvegiensis*, the young ridges were described as pale olive buff to pinkish buff, becoming fuscous black in older specimens, with sunken secondary ridges (Voitk et al. 2016). This was not the case in the Swiss samples, where

the young specimen was darker than the mature fruiting body, and the secondary ridges were well defined, transversal horizontally or diagonally, usually not sunken with the same texture and color as the primary ridges. The alveoli of the Swiss samples were vertically elongated, dark brown when young and becoming honey-brown, contrarily to other *M. norvegiensis* whose alveoli were pale olive buff to orange citrine when young, and snuff brown when mature (Voitk et al. 2016). In the Swiss *M. norvegiensis*, asci (210-240 x 15-22 µm) were cylindrical (Figure 8D), eight-spored. Paraphyses were versiform, thinner and longer than asci (Figure 8C), which corresponded to other *M. norvegiensis*. Acroparaphyses often had one septum before the terminal cell, which was usually globose or ophiomorphous (Figure 8E). This was not reported in other specimens of *M. norvegiensis* (Voitk et al. 2016).

In summary, the three specimens M21-2, M21-3 and M21-4 were clearly assigned to *M. norvegiensis* based on genetic data, mostly because the four sequences of the Swiss *M. norvegiensis* M510 was 99-100% identical with our specimens. However, morphological characters were slightly different from what has been reported before for *M. norvegiensis*, mainly by the color transition between young and mature specimens that are lightening in the Swiss samples and in the contrary darkening in other *M. norvegiensis* (Voitk et al. 2016). This could be a regional variation. In addition, *M. norvegiensis* from Northern Europe fruited a bit later (April-June) (Voitk et al. 2016) than the Swiss representatives that were collected late March, but this slight variation was attributed to climatic differences.

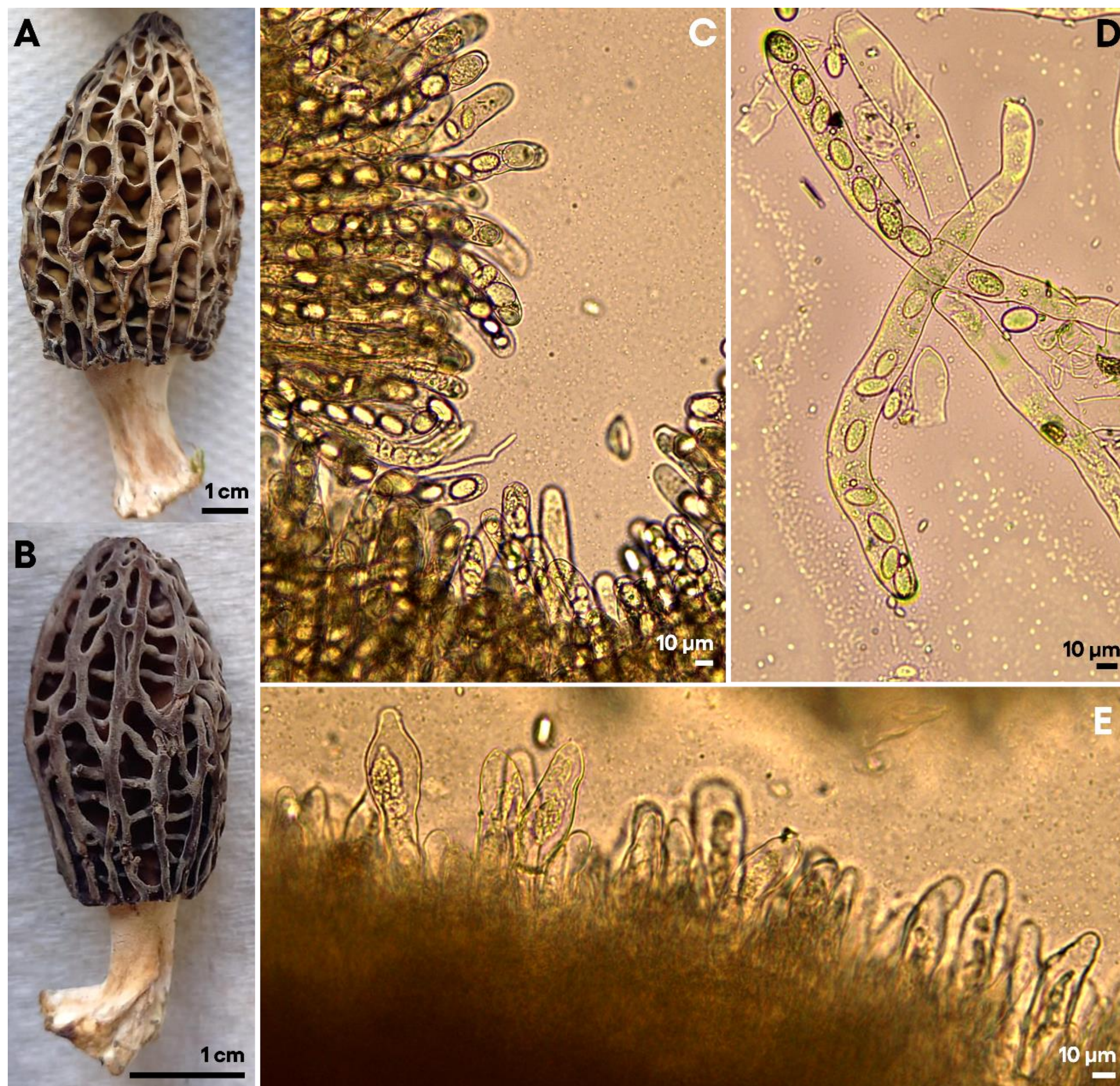


**Figure 8**. A= Fresh fruiting body of mature *Morchella norvegiensis*, specimen M21-2; B= Fresh fruiting body of immature *Morchella norvegiensis*, specimen M21-3; C= Asci containing ascospores, and one paraphysis of M21-2; D= Asci with ascospores of M21-2; E= Acroparaphyses of M21-2. Pictures C-E were taken with a light microscope, in physiological water, at 40x magnification.

2.3.4.6 New lineage *Morchella* sp. Mel-47

*Morchella* sp. Mel-47 was represented by nine specimens from five populations (M19-1, M19-5, M19-7 to -10, M19-43, M21-9, M21-13) collected in the canton of Neuchâtel in 2019 and 2021. The four-locus analysis indicated it was a monophyletic group of the Elata clade, siter taxon to *M. eximioides/M. angusticeps/M. confusa* (UFBoot = 100). In the single-locus phylogenies, Mel-47 was monophyletic for RPB2 (**Supplementary S6C**) and *TEF1-$\alpha$* (**Supplementary S6D**), but it contained *M. eximioides/M. angusticeps/M. confusa* in the ITS and *RPB1* trees (**Supplementary S6A-B**). In the *MAT* phylogeny, Mel-47 was monophyletic, and located near *M. deliciosa* (**Figure 5**). The genealogy criterion was met, but not the criterion of monophyly because the ITS and *RPB2* did not resolve the autonomy of Mel-47 from *M. eximioides, M. angusticeps* and *M. confusa*. The pair *M. eximioides/M. angusticeps* is one of the examples of two species that cannot be differentiated based on phylogenetic analyses only, but that display different distribution range (*M. angusticeps* is found in Northern America while *M. eximioides* is found in Eurasia) (Loizides et al. 2022), ecology (*M.*

*eximioides* is a fire adapted morel (Jacquetant 1984) while *M. angusticeps* is not (Kuo et al. 2012)), and morphology (Loizides et al. 2022). This tends to indicate that Mel-47 could therefore be a new phylogenetic species, closely related to the *M. angusticeps/M. eximioides* pair. Further sampling was done in 2023 to obtain further ecological and morphological data on specimens at different developmental stages. The preliminary data supported the fact that Mel-47 represents a new species. The formal description of Mel-47 was therefore done separately (Cravero et al. in revision).

**2.3.5 Species invasiveness of *Morchella* spp.**

Even though non-pathogenic fungi do not come into mind when considering invasive species, both the commercial exploitation of comestible fungi or the unintended import associated with the import of associated plant hosts, creates conditions in which these types of fungi might alter the structure of wild populations of phylogenetically related fungi or those with a similar ecological niche (Litchman 2010). Therefore, national or regional species lists are crucial to detect potential invasions of non-native species that could negatively affect the local environment, and to monitor the evolution (including the decline) of taxa. In Switzerland, the official list of fungal species (SwissFungi, WSL) contained data for 19 *Morchella* species, however only two of them corresponded to currently recognized species (*M. semilibera* and *M. esculenta*). The other entries corresponded to synonyms or illegitimate species names (Loizides et al. 2022). As a result of the present study, this list will be updated. Regarding invasiveness, our sampling in Switzerland revealed the presence of *M. importuna* that is presumed to be native to USA (Richard et al. 2015), but that is nowadays widely cultivated in China, and is starting to be cultivated in Europe (Y. Xu et al. 2022).

The species *M. importuna* was described in 2012 from the USA (Kuo et al. 2012), but specimens from Turkey (Taskin et al. 2010), Canada, France, Spain, and Switzerland have been known for some time (Kuo et al. 2012). This species may have been introduced from North America into Europe through horticultural or silvicultural materials (O'Donnell et al. 2011). Interestingly, only one population of *M. importuna* was detected in Switzerland in 2020. The specimens were collected in the canton of Valais, from which it was already reported in 1986 where it was named *Morchella hortensis* (Richard et al. 2015). Three records of *M. hortensis* were listed in SwissFungi, but the samples originated from the cantons of Neuchâtel, Ticino and Zürich, and not from Valais as described previously (Richard et al. 2015). The ITS sequence attached to the old specimen from Valais (KM588019.1) was available on GenBank under the name *M. elata* voucher 4609 (Richard et al. 2015). The comparison of that sequence with the ones from the *M. importuna* specimens collected in the same canton in 2020 revealed that the old specimen was highly divergent. The voucher sequence corresponded to *Morchella semilibera*. Thus, the specimen was likely misidentified and the corresponding sequence misannotated, or the wrong sequence was deposited on GenBank. Another mention of *M. hortensis* was made by Jacquetant (Jacquetant 1984) who collected it in the canton of Fribourg. He described it as impossible to confuse with other species due to the lack of sinus, short and densely tomentose stipe, numerous primary ridges (up to 30), and absence of primary alveoli. This description does not correspond with any of the specimens collected in Switzerland in this study. Thus, it is unclear whether specimens of *M. importuna* were really reported from Switzerland prior to the present study. Population genetics tools could help to determine whether this fungal species was introduced, as introduced populations are expected to show lower genetic diversity compared to native ones (Pringle et al. 2009). In the *TEF1-α* sequences of the Swiss *M. importuna*, no SNPs were detected within the population composed by six specimens. However, additional sampling would be required to evaluate and compare the population genetics of Swiss and North American *M. importuna.* Moreover, a population genetics study would require the development of specific population markers, something that was outside the scope of the present study.

The presence of *M. importuna* in Switzerland indicates that this species can adapt to the environmental conditions of the country, and this raises the question of the potential consequences of importing cultivars of *Morchella* spp. on the biodiversity of wild fungal populations. Due to recent geographical expansion of the outdoor cultivation of *Morchella* species to regions in which wild morels populations are present, establishing species lists is even more important to monitor the evolution of both native and imported populations, and to be able to propose restrictions if the non-native cultivars are shown to have a negative impact on the local ecosystems. Further, development of additional markers will help to distinguish native from non-native genotypes and the potential for introgression between native and introduced populations.

## 2.4 Conclusion

This study presents a survey of the biodiversity of *Morchella* spp. in Switzerland across eight cantons. The occurrence of *M. esculenta, M. importuna*, *M. norvegiensis, M. pulchella* species complex and *M. deliciosa* was reported. In addition, three new lineages (Mel-43, Mel-45, Mel-47) were discovered by concatenated four-locus analysis. The polyphasic approach combining genetic, developmental, morphological, and ecological data concluded that Mel-45 displayed genetic differences with *M. deliciosa/*Mel-13 but could unfortunately not be analysed morphologically to provide further support concerning its autonomy to the sister species. Mel-43 and Mel-47 are likely to represent new species, but additional collections are necessary to confirm this and to provide reliable morphoanatomical descriptions.

## 2.5 Declarations

**Availability of data and materials**

The datasets generated and/or analysed during the current study are available in the GenBank repository, under accession numbers OR482713-OR482845 (ITS); OR667829-OR667959 (*RPB1*); OR667965-OR668088 (*RPB2*); OR668096-OR668217 (*TEF1-a*); OR935869-OR935882 (*MAT*).

All data generated or analysed during this study are included in this published article and its supplementary information files Supplementary S1 to S12.

**Competing interests**

The authors declare that they have no competing interests.

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**Authors' contributions**

Melissa Cravero conducted the laboratory and bioinformatic work, analyzed the data and wrote the manuscript. Aaron J. Robinson and Gregory Bonito provided valuable comments concerning the bioinformatic analyses and reviewed the manuscript. Patrick Chain reviewed the manuscript. Saskia

Bindschedler and Pilar Junier designed the study and reviewed the manuscript. All authors read and approved the final manuscript.

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## Supplementary Material S1 - Primer sequences

| Primer | Orientation | Sequence 5'-3' | Target region | Reference |
|---|---|---|---|---|
| ITS1 | Forward | TCCGTAGGTGAACCTGCGG | ITS 5.8S | (White et al. 1990) |
| ITS4 | Reverse | TCCTCCGCTTATTGATATGC | ITS 5.8S | (Gardes and Bruns 1993) |
| RPB1B-F | Forward | AACCGGTATATCACGTYGGTAT | *RPB1* | (Du et al. 2012) |
| RPB1B-R | Reverse | GCCTCRAATTCGTTGACRACGT | *RPB1* | (Du et al. 2012) |
| RPB2-9F | Forward | CAAATGGGCRATTGTCATACG | *RPB2* | (Taskin et al. 2010) |
| RPB2-3R | Reverse | GCATYGGTATGCAGGTTGTGG | *RPB2* | (Taskin et al. 2010) |
| EF-2F | Forward | AACATGATSACTGGTACYTCC | *EF1-α* | (Rehner and Buckley 2005) |
| EF-2218R | Reverse | ATGACACCRACRGCRACRGTYTG | *EF1-α* | (Rehner and Buckley 2005) |
| p7-2f | Forward | CCGGTTTATCTTACTGGACTGGTTC | *MAT1-1-1* (Elata) | (Chai et al. 2022) |
| p8-5r | Reverse | TGGAATGTCTGTGATTGAGGCTGTG | *MAT1-1-1* (Elata) | (Chai et al. 2022) |
| p10-1f | Forward | GGCCAGAACAGATGCTCGAAGAAGC | *MAT1-2-1* (Elata) | (Chai et al. 2022) |
| p10-5r | Reverse | GTGGCAACTCCCAAAGCATGATCAA | *MAT1-2-1* (Elata) | (Chai et al. 2022) |
| p5-3f | Forward | ATGTCACTTCGCCCGGTTTATC | *MAT1-1-1* (Esculenta) | (Chai et al. 2022) |
| p5-3r | Reverse | CATCGCAATGTCGTGTGTTCTT | *MAT1-1-1* (Esculenta) | (Chai et al. 2022) |
| p4-3f | Forward | GACTACGATCGAATAATGGCTCCGC | *MAT1-2-1* (Esculenta) | (Chai et al. 2022) |
| p4-3r | Reverse | CGGTCTTAGCTTCGTCGACTTTAGT | *MAT1-2-1* (Esculenta) | (Chai et al. 2022) |

Symbols: A (Adénine), C (Cytosine), G (Guanine), T (Thymine), Y (C or T), S (G or C), R (A or G).

## Supplementary Material S2A – Swiss strains

| Strain | #Accession ITS | #Accession TEF1-a | #Accession RPB1 | #Accession RPB2 | #Accession *MAT1-1-1* | #Accession *MAT1-2-1* |
|---|---|---|---|---|---|---|
| M19-1 | OR482713 | OR667829 | OR667965 | OR668096 | OR935869 | OR935875 |
| M19-5 | OR482717 | OR667833 | OR667976 | OR668109 | OR935870 | OR935876 |
| M19-7 | OR482719 | OR667835 | OR667977 | OR668111 | - | - |
| M19-8 | OR482720 | OR667836 | OR667978 | OR668112 | - | - |
| M19-9 | OR482721 | OR667837 | OR667979 | OR668113 | - | - |
| M19-10 | OR482722 | OR667838 | OR667966 | OR668097 | - | - |
| M19-28 | OR482724 | OR667841 | OR667969 | OR668101 | - | - |
| M19-29 | OR482725 | OR667842 | OR667970 | OR668102 | - | - |
| M19-43 | OR482729 | OR667846 | OR667975 | OR668108 | - | - |
| M20-3 | OR482731 | OR667849 | OR667982 | OR668115 | - | - |
| M20-4 | OR482732 | OR667850 | OR667983 | OR668116 | - | - |
| M20-5 | OR482733 | OR667851 | OR667984 | OR668117 | - | - |
| M20-6 | OR482734 | OR667852 | OR667985 | OR668118 | - | - |
| M20-8 | OR482736 | OR667854 | OR667987 | OR668120 | - | - |
| M21-2 | OR482739 | OR667856 | OR667990 | OR668123 | - | - |
| M21-3 | OR482740 | OR667857 | OR667991 | OR668124 | - | - |
| M21-4 | OR482741 | OR667858 | OR667992 | OR668125 | - | - |
| M21-8 | OR482742 | OR667859 | OR667993 | OR668126 | - | - |
| M21-9 | OR482743 | OR667860 | OR667994 | OR668127 | - | - |
| M21-13 | OR482746 | OR667863 | OR667995 | OR668130 | - | - |
| M21-15 | OR482748 | OR667864 | OR667997 | OR668132 | OR935871 | OR935878 |
| M21-16 | OR482749 | OR667865 | OR667998 | OR668133 | - | - |
| M21-17 | OR482750 | OR667866 | OR667999 | OR668134 | - | - |
| M21-18 | OR482751 | OR667867 | OR668000 | OR668135 | - | - |

| M21-19 | OR482752 | OR667868 | OR668001 | OR668136 | - | - |
|---|---|---|---|---|---|---|
| M21-20 | OR482753 | OR667869 | OR668002 | OR668137 | OR935872 | OR935879 |
| M21-22 | OR482755 | OR667870 | OR668004 | OR668139 | - | - |
| M21-23 | OR482756 | OR667871 | OR668005 | OR668140 | - | - |
| M21-33 | OR482759 | OR667874 | OR668008 | OR668143 | - | - |
| M21-34 | OR482760 | OR667875 | OR668009 | OR668144 | - | - |
| M21-35 | OR482761 | OR667876 | OR668010 | OR668145 | - | - |
| M21-37 | OR482762 | OR667877 | OR668011 | OR668146 | - | - |
| M21-60 | OR482767 | OR667880 | OR668016 | OR668150 | - | - |
| M21-63 | OR482768 | OR667881 | OR668017 | OR668151 | - | - |
| M21-66 | OR482769 | OR667882 | OR668018 | OR668152 | - | - |
| M21-82 | OR482770 | OR667883 | OR668019 | OR668153 | OR935873 | OR935881 |
| M21-90 | OR482772 | OR667886 | OR668021 | OR668155 | OR935874 | OR935882 |
| M21-91 | OR482773 | OR667887 | OR668022 | OR668156 | - | - |
| M19-15 | OR482776 | OR667890 | OR668025 | OR668159 | - | - |
| M19-16 | OR482777 | OR667891 | OR668026 | OR668160 | - | - |
| M19-17 | OR482778 | OR667892 | OR668027 | OR668161 | - | - |
| M19-18 | OR482779 | OR667893 | OR668028 | OR668162 | - | - |
| M19-20 | OR482781 | OR667895 | OR668030 | OR668164 | - | - |
| M19-34 | OR482791 | OR667905 | OR668039 | OR668174 | - | - |
| M19-36 | OR482793 | OR667907 | OR668040 | OR668176 | - | - |
| M19-38 | OR482795 | OR667909 | OR668042 | OR668178 | - | - |
| M19-40 | OR482797 | OR667911 | OR668044 | OR668180 | - | - |
| M21-26 | OR482805 | OR667919 | OR668050 | OR668185 | - | - |
| M21-32 | OR482809 | OR667923 | OR668054 | OR668187 | - | - |
| M21-36 | OR482810 | OR667924 | OR668055 | OR668188 | - | - |
| M21-42 | OR482814 | OR667928 | OR668058 | OR668191 | - | - |
| M21-43 | OR482815 | OR667929 | OR668059 | OR668192 | - | - |
| M21-46 | OR482818 | OR667932 | OR668061 | OR668194 | - | - |
| M21-54 | OR482823 | OR667937 | OR668066 | OR668197 | - | - |
| M21-55 | OR482824 | OR667938 | OR668067 | OR668198 | - | - |
| M21-58 | OR482827 | OR667941 | OR668070 | OR668200 | - | - |
| M21-59 | OR482828 | OR667942 | OR668071 | OR668201 | - | - |
| M21-61 | OR482829 | OR667943 | OR668072 | OR668202 | - | - |
| M21-62 | OR482830 | OR667944 | OR668073 | OR668203 | - | - |
| M21-64 | OR482831 | OR667945 | OR668074 | OR668204 | - | - |
| M21-67 | OR482833 | OR667947 | OR668076 | OR668205 | - | - |
| M21-68 | OR482834 | OR667948 | OR668077 | OR668206 | - | - |
| M21-69 | OR482835 | OR667949 | OR668078 | OR668207 | - | - |
| M21-70 | OR482836 | OR667950 | OR668079 | OR668208 | - | - |
| M21-71 | OR482837 | OR667951 | OR668080 | OR668209 | - | - |
| M21-72 | OR482838 | OR667952 | OR668081 | OR668210 | - | - |
| M21-74 | OR482840 | OR667954 | OR668083 | OR668212 | - | - |
| M21-75 | OR482841 | OR667955 | OR668084 | OR668213 | - | - |
| M21-76 | OR482842 | OR667956 | OR668085 | OR668214 | - | - |
| M21-77 | OR482843 | OR667957 | OR668086 | OR668215 | - | - |
| M21-78 | OR482844 | OR667958 | OR668087 | OR668216 | - | - |
| M21-79 | OR482845 | OR667959 | OR668088 | OR668217 | - | - |

## Supplementary Material S2B – Reference strains

| Species | Clade | Strain | Country |
|---|---|---|---|
| *M. rufobrunnea* | Sect. Rufobrunnea | M14 | USA |
| Mes-27 | Sect. Morchella | HKAS55897 | China |
| *M. vulgaris* | Sect. Morchella | HT337 | Türkiye |
| Mes-6 | Sect. Morchella | HMJAU5334 | China |
| Mes-X | Sect. Morchella | HKAS56660 | China |
| Mes-21 | Sect. Morchella | HKAS55920 | China |
| *M. ulmaria* | Sect. Morchella | M70 | USA |
| Mes-25 | Sect. Morchella | YAASM15 | China |
| Mes-10 | Sect. Morchella | HKAS59141 | China |
| Mes-26 | Sect. Morchella | HKAS55913 | China |
| *M. fluvialis* | Sect. Morchella | YAASM86 | China |
| *M. fluvialis* | Sect. Morchella | HKAS59128 | China |
| *M. fluvialis* | Sect. Morchella | M49 | Japan |
| Mes-20 | Sect. Morchella | YAASMCB1 | China |
| *M. esculenta* | Sect. Morchella | HKAS56676 | China |
| *M. esculenta* | Sect. Morchella | HKAS59168 | China |
| *M. esculenta* | Sect. Morchella | M19 | Czech Republic |
| *M. gracilis* | Sect. Morchella | 9483-TJB | Dominican Republic |
| *M. gracilis* | Sect. Morchella | M330 | Venezuela |
| *M. peruviana* | Sect. Morchella | CIPHAM004 | Peru |
| *M. palazonii* | Sect. Morchella | PhC149 | Spain |
| *M. galilaea* | Sect. Morchella | YAASMLD2 | USA |
| Mes-13 | Sect. Morchella | M320 | China |
| Mes-19 | Sect. Morchella | YAASM54 | China |
| Mes-15 | Sect. Morchella | YAASMNHSQ | China |
| Mes-12 | Sect. Morchella | M50 | Japan |
| Mes-22 | Sect. Morchella | YAASM78 | China |
| Mes-23 | Sect. Morchella | HKAS56571 | China |
| *M. odonnellii* | Sect. Distantes | isolate 1024 | China |
| *M. tridentina* | Sect. Distantes | M17 | USA |
| *M. tomentosa* | Sect. Distantes | M46 | USA |
| *M. importuna* | Sect. Distantes | HKAS62869 | China |
| *M. importuna* | Sect. Distantes | YAASM69 | China |
| *M. importuna* | Sect. Distantes | M129 | USA |
| *M. oweri* | Sect. Distantes | isolate 1026 | China |
| *M. importuna* | Sect. Distantes | M700 | USA |
| Mel-8 | Sect. Distantes | 309465 | USA |
| *M. sextelata* | Sect. Distantes | HKAS562872 | China |
| *M. sextelata* | Sect. Distantes | M120 | USA |
| *M. kakiicolor* | Sect. Distantes | M228 | Canary Islands |
| *M. dunalii* | Sect. Distantes | HT65 | Türkiye |
| Mel-33 | Sect. Distantes | M197 | China |
| *M. norvegiensis* | Sect. Distantes | HKAS62875 | China |
| *M. australiana* | Sect. Distantes | M371 | Australia |
| *M. andinensis* | Sect. Distantes | CIEFAP74 | Chile |
| *M. hispaniolensis* | Sect. Distantes | M374 | Dominican Republic |
| *M. aysenina* | Sect. Distantes | UDEC-78 | Chile |
| *M. laurentiana* | Sect. Distantes | 13.05.14AV01 | Canada |
| *M. purpurascens* | Sect. Distantes | M492 | Norway |
| Mel-34 | Sect. Distantes | HKAS62877 | China |
| *M. pulchella* | Sect. Distantes | M192 | China |
| *M. septentrionalis* | Sect. Distantes | M9 | USA |
| Mel-23 | Sect. Distantes | M542 | Denmark |
| *M. conifericola* | Sect. Distantes | HT106 | Türkiye |
| *M. brunnea* | Sect. Distantes | M839 | USA |

| | | | |
|---|---|---|---|
| *M. magnispora* | Sect. Distantes | HT82 | Türkiye |
| *M. fekeensis* | Sect. Distantes | HT510 | Türkiye |
| Mel-21 | Sect. Distantes | HKAS62880 | China |
| Mel-17 | Sect. Distantes | M315 | China |
| *M. deliciosa* | Sect. Distantes | HT122 | Türkiye |
| Mel-13 | Sect. Distantes | HKAS62894 | China |
| *M. snyderi* | Sect. Distantes | M433 | USA |
| *M. snyderi* | Sect. Distantes | M299 | USA |
| *M. snyderi* | Sect. Distantes | M432 | USA |
| *M. mediterraneensis* | Sect. Distantes | HT25 | Türkiye |
| Mel-14 | Sect. Distantes | M728 | China |
| *M. confusa* | Sect. Distantes | isolate 1028 | China |
| *M. angusticeps* | Sect. Distantes | M304 | USA |
| *M. angusticeps* | Sect. Distantes | M407 | USA |
| *M. eximioides* | Sect. Distantes | M494 | Norway |
| *M. eximioides* | Sect. Distantes | M489 | Norway |
| *M. eximioides* | Sect. Distantes | M539 | Denmark |

## Supplementary Material S3A-D – Sequence alignments

Alignments downloadable on https://github.com/MorchellaThesis (Chapter1A_S3)

## Supplementary Material S4 – Best models

Best models based on Akaike Information Criterion

(Model Selection, http://iqtree.cibiv.univie.ac.at/):

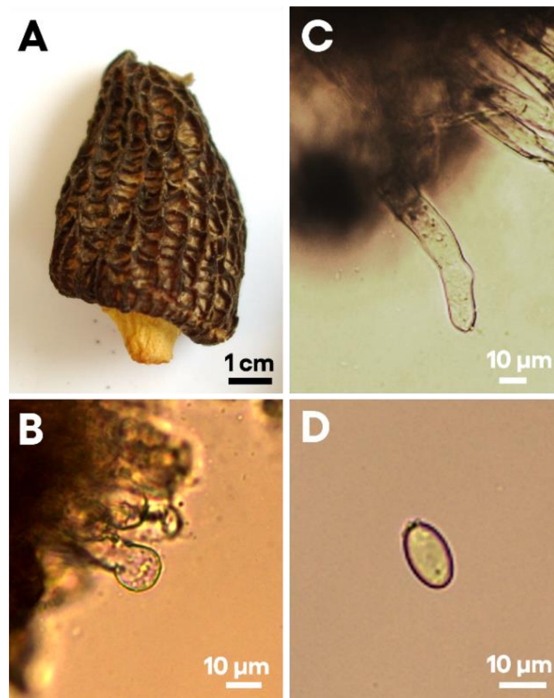
Single-locus Elata ITS: GTR+F+R4
Single-locus Elata RPB1: TN+F+G4
Single-locus Elata RPB2: K3P+G4
Single-locus Elata TEF1-a: SYM+R2
Single-locus Esculenta ITS: TIM3+F+R3
Single-locus Esculenta RPB1: TIM2+F+G4
Single-locus Esculenta RPB2: TIM+F+I
Single-locus Esculenta TEF1-a: TIM2e+G4

Four-locus Esculenta: GTR+F+R4
Four-locus Elata: TIM2+F+R4
Two-locus MAT: TIM3+F+G4
Single-locus Pulchella complex ITS: TVMe+I
Single-locus Pulchella complex RPB1: TIM2e+G4
Single-locus Pulchella complex RPB2: TNe+G4
Single-locus Pulchella complex TEF1-a: SYM+G4

## Supplementary Material S5A-D (Esculenta), S6A-D (Elata)– Single-locus

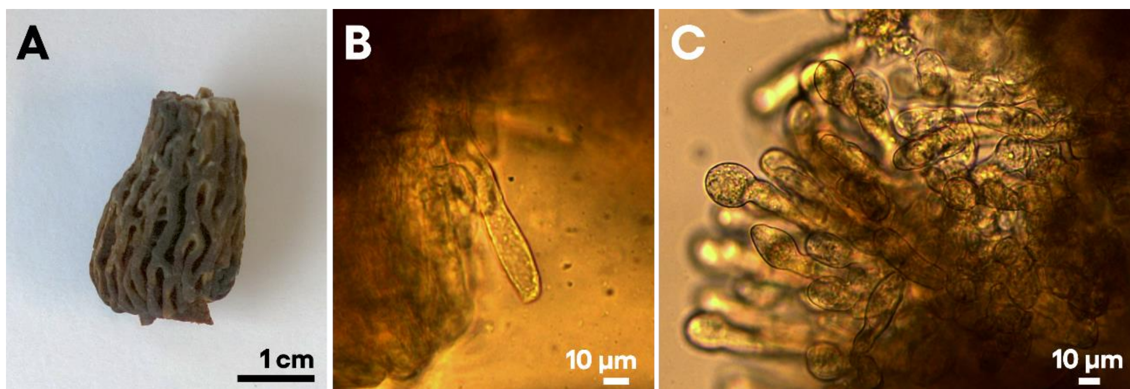
Phylogenies downloadable on https://github.com/MorchellaThesis (Chapter1A_S5, S6)

## Supplementary Material S7 – Morphology of *M. importuna*



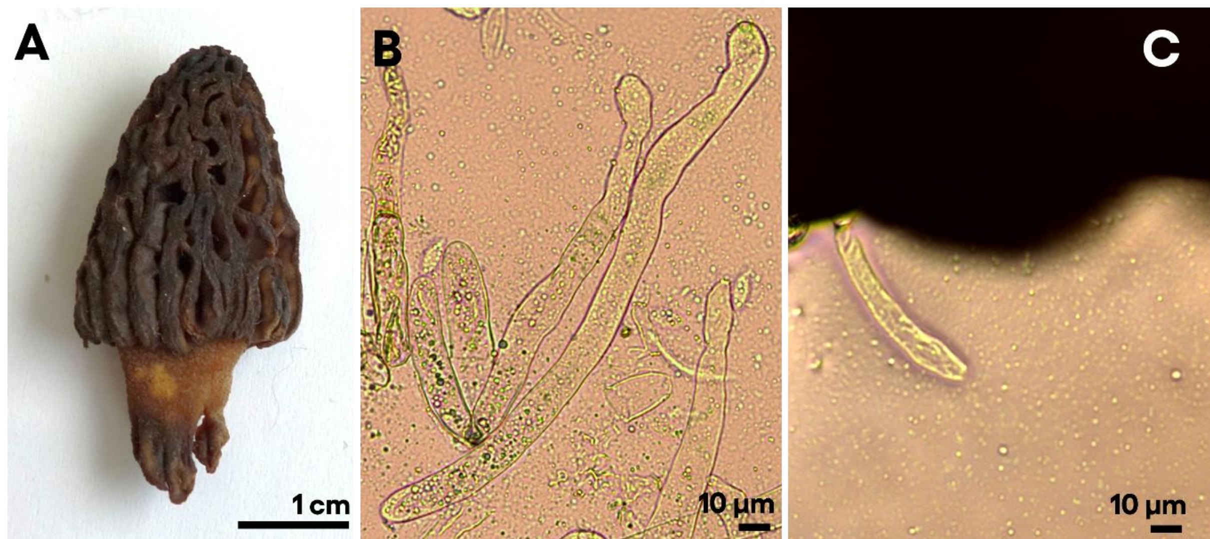

S7. Specimen M20-5; A= Fruiting body; B= Acroparaphyses; C= Ascus; D= Ascospore.

## Supplementary Material S8 – Morphology of Mel-13




S8. Specimen M21-35; A= Fruiting body; B= Ascus; C= Acroparaphyses.

## Supplementary Material S9 – Morphology of Mel-45




S9. Specimen M21-82; A= Fruiting body; B= Asci; C= Acroparaphysis.

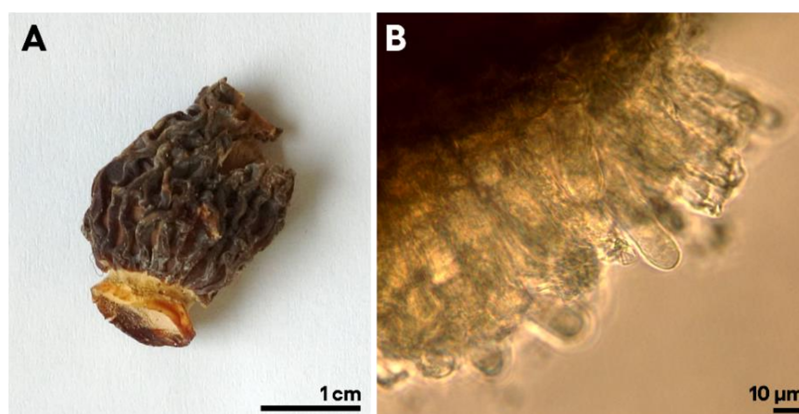
## Supplementary Material S10A-D – *M. pulchella* phylogenies

Phylogenies downloadable on https://github.com/MorchellaThesis (Chapter1A_S10)

## Supplementary Material S11 – *M. pulchella* morphological characteristics

Exhaustive list downloadable on https://github.com/MorchellaThesis (Chapter1A_S11)

## Supplementary Material S12 – Morphology of Mel-43




S12. Specimen M21-33; A= Fruiting body; B= Asci.

# 3. A new species of true morel from Switzerland: *Morchella helvetica* sp. nov.

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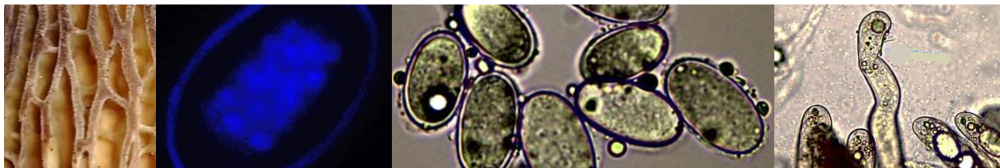

**Foreword**

This chapter describes a new species of black morels, namely *Morchella helvetica* sp. nov., that was discovered during the biodiversity survey presented in section 2. This study was submitted to the journal Mycologia. My personal contribution as first co-author was the generation and analysis of the data (genetic sequences, phylogenetic analyses, ecological and morphological descriptions), main writing, and general review of the paper.

**Abstract**

Previous sampling of *Morchella* in Switzerland revealed the presence of five new phylogenetic lineages within the Elata clade (*Morchella* sp. Mel-43, Mel-44, Mel-45, Mel-46 and Mel-47). In this study, the phylogenetic species *Morchella* sp. Mel-47 is formally described as *Morchella helvetica* sp. nov. (*Morchella* Section Distantes) using combined macro- and micromorphological, ecological, and genetic data from seven populations. *M. helvetica* is a sister species to *M. eximioides* but differs in the genetic analysis of *RPB2* and *TEF1-$\alpha$*, geographical distribution, and macromorphological features.

**Keywords:** *Morchella* spp., new taxon, phylogenetics, morphology, integrative taxonomy

## 3.1 Introduction

True morels (Ascomycota, Pezizales, *Morchella*) are edible and prized fungi that are distributed worldwide, particularly in the Northern Hemisphere (O'Donnell et al. 2011). Recent studies show *Morchella* is divided into three main clades: *Morchella* Section *Rufobrunnea* (Rufobrunnea clade), *Morchella* Section *Morchella* (Esculenta clade), and *Morchella* Section *Distantes* (Elata clade) (Richard et al. 2015; Loizides et al. 2022). The Esculenta and Elata clades comprise both formally described and undescribed (i.e., phylogenetic species denoted with Mes-# or Mel-#) species (Loizides et al. 2022). Current literature recognizes the existence of 59 valid morel species with a binomial (Machuca et al. 2021; Loizides et al. 2022; Clowez et al. 2022) in addition to 29 unnamed phylogenetic species (i.e., phylospecies) (Loizides et al. 2022; Cravero et al. submitted).

Before the advent of molecular tools *Morchella* taxonomy was particularly confusing because multiple names had been applied to different representatives of the same species based on the macromorphology of the morel fruiting body (Fatton 2016). *Morchella* taxonomy has since been revised multiple times using molecular data (Richard et al. 2015; Loizides et al. 2022). Reliable genetic identification based on multilocus phylogenetic inference (Sa et al. 2022) has identified phylogenetic species that require formally morphological and ecological description (Loizides et al. 2022). An integrative approach that combines genetic, morphological, and ecological data is necessary to reliably describe new morel species (Loizides et al. 2022). For this reason, in the present study, the description of the phylogenetic lineage Morchella sp. Mel-47 (Cravero et al. submitted) was made possible after sampling and analyzing fresh specimens at different stages of maturity. Ecological, macro- and micromorphological data indicated that the phylospecies Mel-47 corresponds to a new species, *Morchella helvetica* sp. nov. (*Morchella* Sect. *Distantes*) that is formally described in this paper based on seven different populations and multiple genetic markers. This is the first new *Morchella* species described from Switzerland using an integrative approach.

## 3.2 Material and methods

### 3.2.1 Samples collection

*Morchella helvetica* was identified through multigene phylogenetics as *Morchella* sp. Mel-47 in Cravero et al. (submitted) (Cravero et al. submitted). Fresh specimens were sampled in Spring 2023 (M23-#) to formally describe the species. Specimens of *M. helvetica* that were collected in 2019 and 2021 were also used for micromorphological characterization. In total, specimens from seven distinct populations were analyzed. The specimens of *M. helvetica* that were used in this study are listed in Table 1 below.

Table 1. Specimens of *Morchella helvetica* used in this study.

| Species | Strain | Date of collection | Location | Genetic marker | Accession number | Reference |
|---|---|---|---|---|---|---|
| *M. helvetica* | M19-4 | 26-Mar-2019 | Cressier, NE | ITS | OR482716 | Cravero et al., *submitted* |
| | | | | *TEF1-α* | OR667832 | |
| *M. helvetica* | M19-5 | 26-Mar-2019 | Cressier, NE | ITS | OR482717 | Cravero et al., *submitted* |
| | | | | *TEF1-α* | OR667833 | |
| | | | | *RPB1* | OR667976 | |
| | | | | *RPB2* | OR668109 | |
| *M. helvetica* | M19-7 | 26-Mar-2019 | St-Blaise, NE | ITS | OR482719 | Cravero et al., *submitted* |
| | | | | *TEF1-α* | OR667835 | |

| *M. helvetica* | M19-9 | 26-Mar-2019 | St-Blaise, NE | ITS | OR482721 | Cravero et al., *submitted* |
|---|---|---|---|---|---|---|
| | | | | *TEF1-α* | OR667837 | |
| *M. helvetica* | M19-12 | 26-Mar-2019 | Neuchâtel, NE | *TEF1-α* | OR667840 | Cravero et al., *submitted* |
| | | | | *RPB1* | OR667968 | |
| | | | | *RPB2* | OR668099 | |
| *M. helvetica* | M19-43 | 01-Apr-2019 | St-Blaise, NE | ITS | OR482729 | Cravero et al., *submitted* |
| | | | | *TEF1-α* | OR667846 | |
| | | | | *RPB1* | OR667975 | |
| | | | | *RPB2* | OR668108 | |
| *M. helvetica* | M21-10 | 31-Mar-2021 | Enges, NE | ITS | OR482744 | Cravero et al., *submitted* |
| | | | | *TEF1-α* | OR667861 | |
| *M. helvetica* | M21-12 | 31-Mar-2021 | St-Blaise, NE | ITS | OR482745 | Cravero et al., *submitted* |
| | | | | *TEF1-α* | OR667862 | |
| *M. helvetica* | M21-13 | 31-Mar-2021 | St-Blaise, NE | ITS | OR482746 | Cravero et al., *submitted* |
| | | | | *TEF1-α* | OR667863 | |
| | | | | *RPB1* | OR667995 | |
| | | | | *RPB2* | OR668130 | |
| *M. helvetica* | M23-1 | 21-Mar-2023 | Hauterive (pop. A), NE | ITS | OR539921 | This study |
| | | | | *TEF1-α* | OR757070 | |
| *M. helvetica* | M23-2 | 21-Mar-2023 | Hauterive (pop. A), NE | ITS | OR539922 | This study |
| | | | | *TEF1-α* | OR757071 | |
| *M. helvetica* | M23-3 | 21-Mar-2023 | Hauterive (pop. A), NE | ITS | OR539923 | This study |
| | | | | *TEF1-α* | OR757072 | |
| *M. helvetica* ***holotype*** | M23-4 | 21-Mar-2023 | Hauterive (pop. B), NE | ITS | OR539924 | This study |
| | | | | *TEF1-α* | OR757073 | |
| *M. helvetica* | M23-6 | 21-Mar-2023 | Hauterive (pop. B), NE | ITS | OR539925 | This study |
| | | | | *TEF1-α* | OR757074 | |
| *M. helvetica* | M23-10 | 28-Mar-2023 | Neuchâtel, NE | ITS | OR539926 | This study |
| | | | | *TEF1-α* | OR757075 | |
| *M. helvetica* | M23-11 | 07-Apr-2023 | St-Blaise, NE | ITS | OR539927 | This study |
| | | | | *TEF1-α* | OR757076 | |

All the strains are maintained in the collection of the Laboratory of Microbiology of the University of Neuchâtel, Switzerland. Accession numbers refer to GenBank.

### 3.2.2 Macroscopic analysis

Macroscopic pictures of fresh ascocarps were taken with a digital camera (Canon PowerShot SX230 HS, Japan). Fruiting bodies were measured (length, width, ratio cap:stipe). Primary and secondary ridges and alveoli were described (aspect, color), counted and measured. The color was subjectively determined by comparing the pictures with the color palette from Microsoft PowerPoint for which the hexadecimal code was noted. The sinus (i.e., the portion of the hymenophore that is attached to the stipe) was described (shape) and measured (depth and width). The fruiting bodies were kept in plastic jars in a dark oven at 22°C for 2-3 days to trigger natural sporulation. The spores were kept at room temperature and the dry fruiting bodies were frozen at -20°C.

### 3.2.3 Microscopic analysis

Microscopic features were observed and photographed under a Leica DM4 B (Leica Microsystems, Germany). Samples were prepared by cutting thin slices of hymenium (i.e., fertile part of alveoli), ridge and external layer of the stipe (i.e., ectal excipulum) that were immersed in physiological water (0.9% NaCl) on microscope slides. Asci (i.e., fertile elements of the hymenium), paraphyses (i.e., sterile elements of the hymenium), acroparaphyses (i.e., sterile elements of the ridges), sterile elements of the ectal excipulum and ascospores (144 spores from free spores or spore prints of three fruiting bodies from three populations) were measured (length and width) from microscopic pictures, using the software ImageJ v.1.53q (Schneider, Rasband, et Eliceiri 2012). In addition, ascospores of

*M. helvetica* M19-43 were stained with 4mM DAPI (4′,6-diamidino-2-phenylindole) and observed under a fluorescent light microscope Leica DM4 B (Leica Microsystems, Germany) to visualize and count the nuclei.

**3.2.4 DNA extraction and sequencing**

Presumed *M. helvetica* specimens collected in 2023 were genetically analysed to verify species determination. DNA was extracted from 2 cm3 pieces of fresh or dry hymenia using Quick-DNA Fungal/Bacterial Miniprep Kits (Zymo Research, USA) following the protocol provided by the manufacturer. Eluted DNA was quantified with a Qubit kit (Invitrogen, USA) using the Broad Range buffer and reagent. DNA was then diluted with PCR-grade water to a concentration of 2 ng/µL to be used as template in polymerase chain reactions (PCR). Two genetic regions were amplified: ITS1-2, including the 5.8S ribosomal RNA gene (ITS) (White et al. 1990; Gardes et Bruns 1993); *translation elongation factor 1 alpha* (*TEF1-α*) (Rehner et Buckley 2005). Detailed primer information is available in Supplementary S1. For each sample, the PCR mix contained PCR-grade water, 2X ALLin™ Red Taq Mastermix (HighQu, Germany), 0.2 µM forward and 0.2 µM reverse primer and 1 µL of 2 ng/µL DNA. Amplifications were performed in a Thermo Scientific Arktik thermal cycler. The following parameters were used: denaturation at 95°C for 1 min, 40 cycles of denaturation, annealing and elongation (95°C for 15 sec, 62°C [ITS]; 55°C [TEF1-α] for 15 sec, 72°C for 15 sec), final elongation at 72°C for 2 min, end at 15°C. PCR products were then loaded on a 1.2% agarose gel that underwent electrophoresis (100 mV, 30 min). Amplicons were visualized under UVs in a Genoplex VWR transilluminator. Positive PCR products (i.e. single band at the expected size) were then purified with a MultiScreen® Filter Plates PCR µ96 (Millipore Corporation, USA) as follows: in each well, the PCR product and 50 µL of PCR-grade water was added; a vacuum of 20 bars was applied on the wells until they dry; 20 µL of PCR-grade water was added to each well; after 2 min, DNA contained in the membrane from each well was resuspended by pipetting up and down 20 times. Once purified, the PCR products were quantified by Qubit. Final concentration was adjusted at 2-40 ng/µL and sent to Genesupport (Switzerland) for Sanger sequencing.

**3.2.5 Genetic analysis**

The identity of fresh specimens (M23-#) used for the descriptions of the phylospecies *Morchella* sp. Mel-47 as *Morchella helvetica* sp. nov., was confirmed through phylogenetic analysis of concatenated ITS-*TEF1-α*. The phylogeny was constructed with reference sequences of *Morchella* sp. Mel-47 and sister clades. The accession numbers are provided in Supplementary S2A. Forward and reverse sequences obtained from the sequencing were trimmed and assembled with Geneious Prime v.2022.2 (Biomatters, New Zealand). The direction of the sequences was checked, and the sequences were aligned with MUSCLE (Neighbor Joining cluster method) within the software Mega version 11 (Kumar et al. 2018). Sequences were concatenated (ITS-*TEF1-α*). Gaps were automatically deleted using the online tool Gblocks 0.91b (Castresana 2000; Dereeper et al. 2008). Gaps situated at the ends of the gene sequences were replaced by missing data ("N") with the software BioEdit v.7.2.5. (Dagona 1999). Nucleotide substitution models (GTR + I + G4 model) and maximum likelihood (ML) phylogeny was conducted within the IQ-TREE web server (Nguyen et al. 2015), generating 10'000 ultrafast bootstrap (UFBoot) values (Hoang et al. 2018). The phylogeny was displayed and annotated in FigTree v1.4.4 (Rambaut, 2010) and deposited in TreeBase (Submission 30784). In addition, the same tree-building approach was used to generate single-locus ITS, *RPB1, RPB2* and *TEF1-α* phylogenies of *Morchella* sp. Mel-47 and sister clades using reference sequences (Supplementary S2B) to evaluate the reliability of single gene markers to discriminate *Morchella* sp. Mel-47 from genetically close species.

## 3.3 Results

### 3.3.1 Phylogenetic analyses

To confirm that the specimens collected in 2023 in the canton on Neuchâtel belonged to the phylogenetic lineage *Morchella* sp. Mel-47, a phylogenetic analysis based on the genetic markers ITS and *TEF1-α* was performed (available on TreeBase, Submission 30784.). This revealed that six specimens from four populations (see Table 1 in section 2.1 for geographic information) clustered with that lineage (M23-1, M23-3, M23-4, M23-6, M23-10, M23-11) (Figure 1 below). These specimens were used to formally describe the lineage Mel-47 as *Morchella helvetica*. In addition, the single-locus phylogenies of the ITS, *RPB1*, *RPB2* and *TEF1-α* genetic markers indicated that ITS and *RPB1* were not reliable to discriminate *M. helvetica* from sister clades (Supplementary S3 and S4), however, *RPB2* and *TEF1-α* were able to discriminate these species (Supplementary S5 and S6).

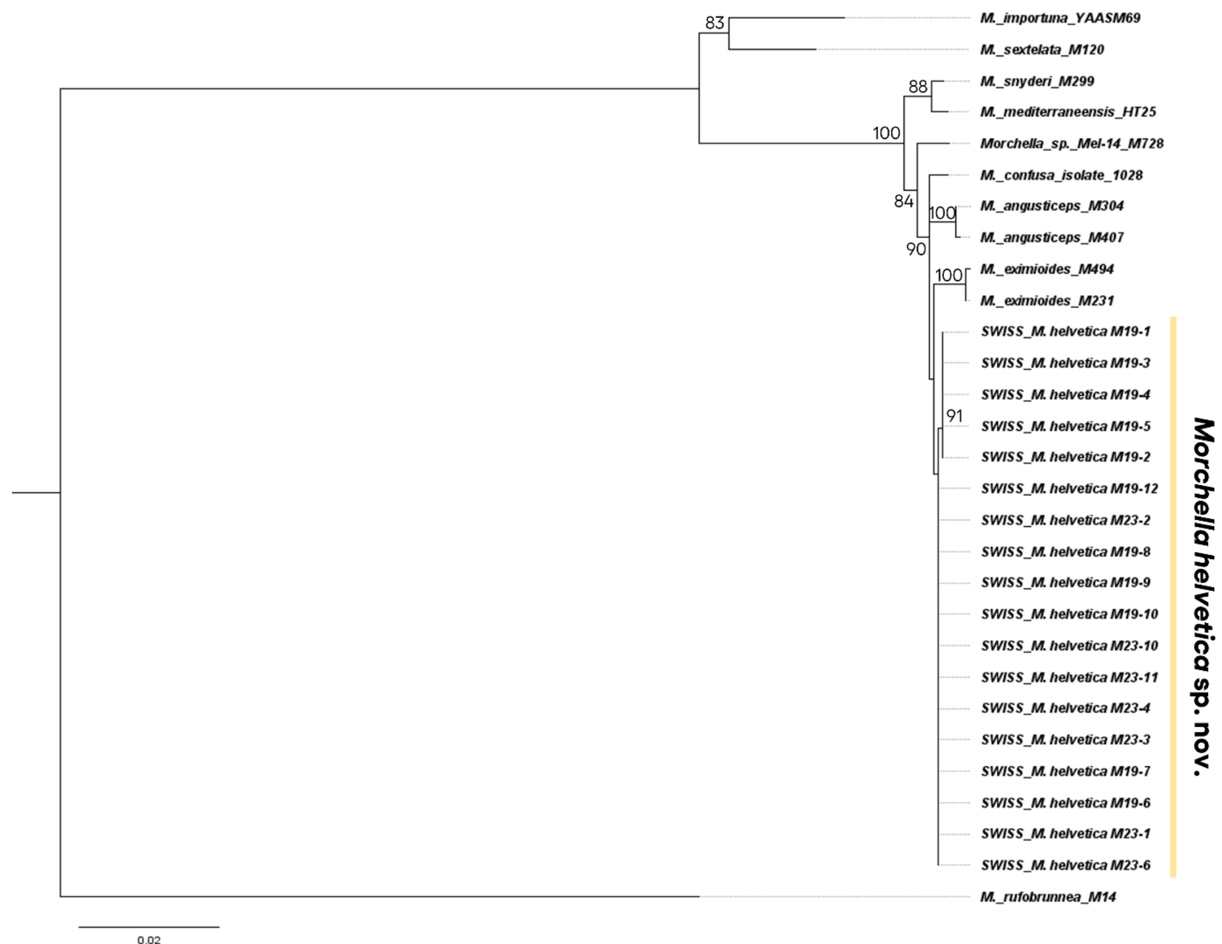


**Figure 1.** Multi-locus (ITS-*TEF1-α*) ML phylogeny of sister clades of *Morchella helvetica* (*Morchella* Sect. *Distantes*) with Swiss specimens (SWISS) and reference sequences. The phylogeny was based on 10'000 ultrafast bootstrap values (UFBoot > 90% displayed at branch nodes). The tree was rooted with the basal clade *M. rufobrunnea*.

### 3.3.2 Taxonomy

***Morchella helvetica*** Cravero, M., Bonito, G., Chain, P., Bindschedler, S. & Junier P., sp. nov.

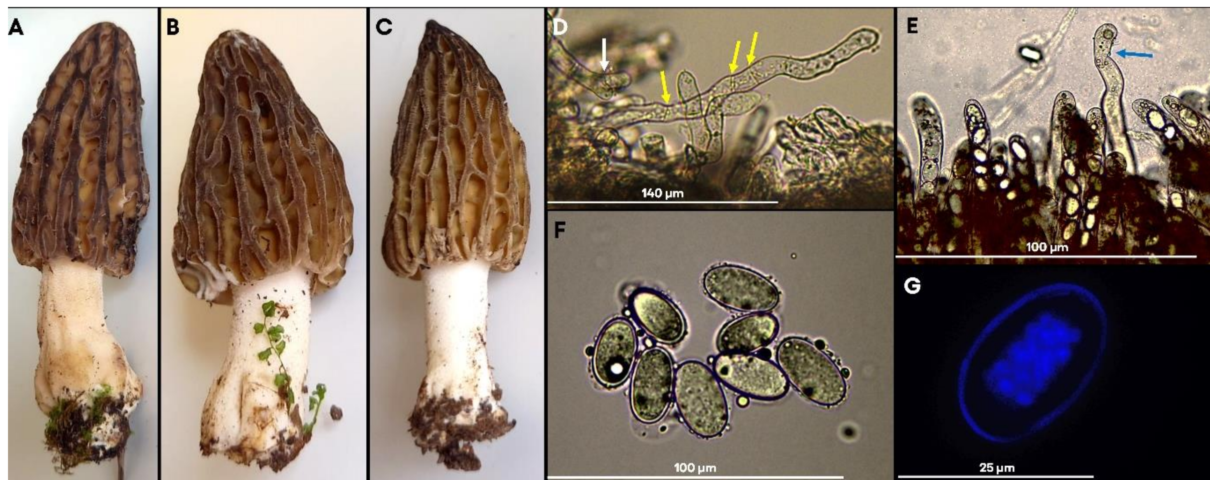


**Figure 2.** A= Mature fruiting body of *Morchella helvetica* M23-4 (Holotype); B= Young fruiting body of *M. helvetica* M23-2; C= Mature fruiting body of *M. helvetica* M23-3; D= Ectal excipulum (outer layer) of the stipe of *M. helvetica* M19-5, highlighting bipartite cylindrical paraphyses (white arrow) and septa (yellow arrows) (light microscope, φw, 20x); E= Asci and paraphyse (blue arrow) of *M. helvetica* M21-13 (light microscope, φw, 20x); F= Ascospores of *M. helvetica* M19-43 (light microscope, φw, 40x); G= Ascospore of *M. helvetica* M19-43 (light microscope, DAPI, 40x).

MycoBank MB[#]

*Typification:* Switzerland, canton of Neuchâtel, Hauterive, 47.01592337114174, 6.970745997089068, 568 m altitude, in a mixed forest under *Fraxinus excelsior* and *Quercus* spp., collected by Blaise Hofer the 21st of March 2023 (**holotype M23-4**). GenBank: ITS = [OR539924]; *TEF1-α* = [OR757073].

*Etymology: helvetica*, based on "Helvetia", the Latin word for "Switzerland", in reference to the country where the species was found.

*Diagnosis: Morchella helvetica* corresponds to the phylospecies *Morchella* sp. Mel-47 (Cravero et al. submitted). It belongs to *Morchella* Section *Distantes* and represents a sister taxon of the subgroup *M. angusticeps/M. eximioides/M. confusa*. *M. helvetica* is phylogenetically distinct from sister species by phylogenetic analysis of combined ITS-*TEF1-α* sequences. It is morphologically similar to *M. eximioides* but differs by its phenology (*M. eximioides* fruits between May-June (Weholt et al. 2020) while *M. helvetica* fruits between March-April) and morphologically by the stipe texture (which is tomentose and furfurasceous in *M. eximioides* (Jacquetant 1984) and mealy in *M. helvetica*) and darkening ridges when aging (absent feature in *M. eximioides* (Du et al. 2019). The presence of light dense hairs on ridges and alveoli seems to be typical of *M. helvetica* compared to other members of *Morchella* Sect. *Distantes*.

*Description:* **Macromorphology -** Ascomata 54-100 mm tall. Pileus ovoid-conical or oblong to sharply conical, 31-59 mm high and 24-36 mm wide (at widest point), attached to the stipe with a distinct, skirting sinus measuring 2-3 mm deep and 3-4 mm wide. Pileus pitted and ridged, with 16-22 vertically elongated primary ridges and numerous transecting, horizontal, sunken secondary ridges. No vertical secondary ridges. Primary ridges 1 mm thick, brown (around #70440F) and slightly rounded when young, blackening from the apex and becoming sharp or eroded with age. Primary

ridges finely tomentose with pale hairs. Secondary ridges concolorous with the pits, and rarely with the same texture and color as the primary ones. Primary alveoli vertically elongated, slightly irregular to regular with a laddered appearance. Alveoli lighter than the ridges with yellow tones when young, becoming brownish orange (around #BB7725) with light reddish tones (around #C8864E) with age, tomentose with pale hairs. Usually 0-9 secondary alveoli per pit. Stipe cylindrical, often basally enlarged, slightly to strongly channeled all along the stipe, often folded and perforated basally (even young), whitish and mealy, 20-55 x 9-27 mm. Ratio cap:stipe usually >1. Sterile inner surface whitish and granulose. Odor and taste unknown. **Micromorphology -** Ascospores elliptical, (17-)20-25(-29) x (13-)14-15(-16) µm, with 12-17 nuclei. Asci cylindrical, hyaline, eight-spored, (230-)265-325(-350) x (9-)15-19(-20) µm. Paraphyses cylindrical, with cylindrical, ophiomorphous, acute, or globular apices, septate, 6-7 µm wide. Acroparaphyses cylindrical, clavate, or ophiomorphous, uni- or bifurcate, hyaline, with 1-3 septa, occasionally with a small bud on the terminal cell. Sterile elements of the stipe cylindrical or ophiomorphous, uni- or bifurcate, hyaline, can be septate.

*Ecology and distribution:* In mixed forest under *Fraxinus excelsior* and *Quercus* spp., in calcareous soils or on mossy rocks. Fruiting between late March to early April, solitary, between 560 and 1050 m altitude in the Canton of Neuchâtel, Switzerland.

*Material examined:*

Switzerland, canton of Neuchâtel, Cressier, 47.05573674556411, 7.023590244777627, 730 m altitude, in mixed forests under *Fraxinus excelsior*, collected by Blaise Hofer the 26th of March 2019 (**M19-5**). GenBank: ITS = [OR482717]; *RPB1* = [OR667976]; *RPB2* = [OR668109]; *TEF1-α* = [OR667833].

Switzerland, canton of Neuchâtel, Neuchâtel, Forêt de Peseux, 47.02453041174021, 6.954939931272217, 1041 m altitude, in mixed forests under *Fraxinus excelsior*, collected by Blaise Hofer the 26th of March 2019 (**M19-12**). GenBank: *RPB1* = [OR667968]; *RPB2* = [OR668099]; *TEF1-α* = [OR667840].

Switzerland, canton of Neuchâtel, St-Blaise, Forêt du Chuffort, 47.02534711703331, 6.978415096361076 altitude, in mixed forests under *Fraxinus excelsior*, collected by Blaise Hofer the 1st of April 2019 (**M19-43**). GenBank: ITS = [OR482729]; *RPB1* = [OR667975]; *RPB2* = [OR668108]; *TEF1-α* = [OR667846].

Switzerland, canton of Neuchâtel, St-Blaise, Forêt du Chuffort, 47.02689102437238, 6.981460532006908, 641 m altitude, in mixed forests under *Fraxinus excelsior*, collected by Blaise Hofer the 31st of March 2021 (**M21-13**). GenBank: ITS = [OR482746]; *RPB1* = [OR667995]; *RPB2* = [OR668130]; *TEF1-α* = [OR667863].

Switzerland, canton of Neuchâtel, Hauterive, 47.01560153599948, 6.968675437293392, 600 m altitude, in mixed forests under *Fraxinus excelsior*, collected by Blaise Hofer the 21st of March 2023 (**M23-1**). GenBank: ITS = [OR539921]; *TEF1-α* = [OR757070].

Switzerland, canton of Neuchâtel, Neuchâtel, Forêt de Peseux, 47.014522661515834, 6.958775487149762, 662 m altitude, in mixed forests under *Fraxinus excelsior*, collected by Blaise Hofer the 28th of March 2023 (**M23-10**). GenBank: ITS = [OR539926]; *TEF1-α* = [OR757075].

Switzerland, canton of Neuchâtel, St-Blaise, Forêt du Chuffort, 47.02433227325016, 6.982521833106444, 586 m altitude, in mixed forests under *Fraxinus excelsior*, collected by Blaise Hofer the 07th of April 2023 (**M23-11**). GenBank: ITS = [OR539927]; *TEF1-α* = [OR757076].

*Comments: M. helvetica* is distinct from *M. angusticeps* and *M. confusa* by the absence of vertical secondary ridges, the orange to reddish tones of the aging primary alveoli, and the presence of hairs (i.e., tomentose) on the primary alveoli (Kuo et al. 2012; Du et al. 2019). Additionally, *M. helvetica* differs from *M. confusa* by the presence of folds and channels on the stipe, and by darkening edges with aging (Du et al. 2019). Concerning the maturation process, young specimens of *M. helvetica* have yellowish brown alveoli. They often turn reddish with age but remain lighter than the ridges that darken from the apex until becoming completely dark brown to black. The pileus becomes narrower with age, and alveoli become more regular. The secondary ridges are more apparent with aging. Genetically, the single ITS or *RPB1* markers do not distinguish it from *M. eximioides, M. angusticeps* or *M. confusa* (Supplementary S3 and S4), however, *RPB2* and *TEF1-$\alpha$* does distinguish these species (Supplementary S5 and S6).

## 3.4 Discussion

*Morchella helvetica* corresponds to the phylospecies *Morchella* sp. Mel-47, a new phylogenetic lineage that was reported in a previous study and the first described morel species from Switzerland (Cravero et al. submitted). To make a reliable morphological description of *M. helvetica*, fresh specimens were collected in spring 2023 at different maturity stages. The maturation process is an important feature in *Morchella* taxonomy (Loizides et al. 2016) that allowed here to distinguish *M. helvetica* from *M. confusa* and *M. eximioides*, the two latter lacking dark ridges with aging (Du et al. 2019). Fresh specimens were also necessary to correctly evaluate the color and presence of hairs on the fruiting body. *Morchella helvetica* is the first species to be described from Switzerland using genetic, morphological, and ecological data. A literature search was conducted to investigate whether *M. helvetica* could correspond to a species with an unresolved status and/or that has been abandoned in recent literature due to lack of representatives or genetic data. A particular attention was given to *Morchella autumnalis, Morchella canina, Morchella pratensis*, and *Morchella radicosa*, as they have been reported in Western Europe (Fatton 2016). Unfortunately, lack of descriptions, pictures, or references made impossible to potentially link those names with *M. helvetica*. None of the other unresolved species resembled *M. helvetica*. The sister species of *M. helvetica*, namely *M. angusticeps* from North America, *M. eximioides* (found in Europe and Asia) and *M. confusa* from China, have never been reported in Switzerland (Du et al. 2019; Loizides et al. 2022). *Morchella helvetica* has so far only been found in the canton of Neuchâtel, Switzerland, in mixed forests under ash and oak trees. *Morchella helvetica* does not appear to be a fire-adapted morel as no forest fires have been reported in its habitat. The species should be searched in other areas of the country to investigate the extent of its range of distribution.

## 3.5 Conclusion

This study presents the new species *Morchella helvetica*, a sister taxon of the subgroup *M. angusticeps/M. eximioides/M. confusa* from *Morchella* Section *Distantes*, from which it differs by phylogenetic, morphological and ecological data. This true morel has been exclusively found in the Canton of Neuchâtel, Switzerland, fruiting near ash and oak trees in early Spring.

## 3.6 Declarations

**Abbreviations**

ITS: internal transcribed spacer

TEF1-α: translation elongation factor 1 alpha

RPB1: RNA polymerase II subunit 1

RPB2: RNA polymerase II subunit 2

DAPI: 4′,6-diamidino-2-phenylindole

PCR: polymerase chain reaction

ML: maximum likelihood

UFBoot: ultrafast bootstrap

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**Disclosure statement**

The authors report there are no competing interests to declare.

**Author's contributions**

MC conducted the laboratory and bioinformatic work, analyzed the data, and wrote the manuscript. GB and PSC reviewed the manuscript. SB and PJ designed the study and reviewed the manuscript. All authors read and approved the final manuscript.

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**Availability of data and material**

The datasets supporting the conclusions of this article are available in the GenBank (Accession numbers OR539921-OR539927 [ITS] and OR757070- OR757076 [*TEF1-α*]) and TreeBase repositories (Submission 30784).

The datasets supporting the conclusions of this article are included within the article and its additional files Supplementary S1 to S6.

## 3.7 Literature


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## Supplementary S1 – Primer sequences

| Primer | Orientation | Sequence | Target | Reference |
|---|---|---|---|---|
| ITS1 | Forward | TCCGTAGGTGAACCTGCGG | ITS 5.8S | (White et al. 1990) |
| ITS4 | Reverse | TCCTCCGCTTATTGATATGC | ITS 5.8S | (Gardes and Bruns 1993) |
| *EF-2F* | Forward | AACATGATSACTGGTACYTCC | *EF1-α* | (Rehner and Buckley 2005) |
| *EF-2218R* | Reverse | ATGACACCRACRGCRACRGTYTG | *EF1-α* | (Rehner and Buckley 2005) |

## Supplementary S2A – Reference strains

| Species | Strain | Genetic marker | GenBank Accession number |
|---|---|---|---|
| *Morchella rufobrunnea* | M14 | ITS | JQ723124.1 |
| | | *TEF1-α* | GU550982.1 |
| *Morchella importuna* | YAASM69 | ITS | MG589675.1 |
| | | *TEF1-α* | MG589713.1 |
| *Morchella sextelata* | M120 | ITS | JQ723034.1 |
| | | *TEF1-α* | GU550988.1 |
| *Morchella snyderi* | M299 | ITS | GU551413.1 |
| | | *TEF1-α* | GU551376.1 |
| *Morchella snyderi* | ARD-04-02 | ITS | KM204677.1 |
| | | *TEF1-α* | KM034925.1 |
| *Morchella mediterraneensis* | HT 25 | ITS | HM056397.1 |
| | | *TEF1-α* | HM056357.1 |
| *Morchella* sp. Mel-14 | M728 | ITS | JQ723054.1 |
| | | *TEF1-α* | GU551565.1 |
| *Morchella confusa* | Mel-42/1028 | ITS | MK321849.1 |
| | | *TEF1-α* | MK321867.1 |
| *Morchella angusticeps* | M304 | ITS | JQ723055.1 |
| | | *TEF1-α* | GU551560.1 |
| *Morchella angusticeps* | M407 | ITS | GU551416.1 |
| | | *TEF1-α* | GU551379.1 |
| *Morchella eximioides* | M494 | ITS | JQ723056.1 |
| | | *TEF1-α* | GU551557.1 |
| *Morchella eximioides* | M231 | ITS | GU551428.1 |
| | | *TEF1-α* | GU551391.1 |

## Supplementary S2B – Swiss strains

| Species | Strain | Genetic marker | GenBank Accession number |
|---|---|---|---|
| *Morchella helvetica* | M19-1 | ITS | OR482713 |
| | | *RPB1* | OR667829 |
| | | *RPB2* | OR667965 |
| | | *TEF1-α* | OR668096 |
| *Morchella helvetica* | M19-2 | ITS | OR482714 |
| | | *RPB1* | OR667830 |
| | | *RPB2* | N.A. |
| | | *TEF1-α* | OR668100 |
| *Morchella helvetica* | M19-3 | ITS | OR482715 |
| | | *RPB1* | OR667831 |
| | | *RPB2* | OR667971 |
| | | *TEF1-α* | OR668103 |
| *Morchella helvetica* | M19-4 | ITS | OR482716 |
| | | *RPB1* | OR667832 |
| | | *RPB2* | N.A. |
| | | *TEF1-α* | OR668106 |
| *Morchella helvetica* | M19-5 | ITS | OR482717 |
| | | *RPB1* | OR667833 |
| | | *RPB2* | OR667976 |
| | | *TEF1-α* | OR668109 |
| *Morchella helvetica* | M19-6 | ITS | OR482718 |
| | | *RPB1* | OR667834 |
| | | *RPB2* | N.A. |
| | | *TEF1-α* | OR668110 |
| *Morchella helvetica* | M19-7 | ITS | OR482719 |
| | | *RPB1* | OR667835 |
| | | *RPB2* | OR667977 |
| | | *TEF1-α* | OR668111 |
| *Morchella helvetica* | M19-8 | ITS | OR482720 |
| | | *RPB1* | OR667836 |
| | | *RPB2* | OR667978 |
| | | *TEF1-α* | OR668112 |
| *Morchella helvetica* | M19-9 | ITS | OR482721 |
| | | *RPB1* | OR667837 |
| | | *RPB2* | OR667979 |
| | | *TEF1-α* | OR668113 |
| *Morchella helvetica* | M19-10 | ITS | OR482722 |
| | | *RPB1* | OR667838 |
| | | *RPB2* | OR667966 |
| | | *TEF1-α* | OR668097 |
| *Morchella helvetica* | M19-12 | ITS | N.A. |
| | | *RPB1* | OR667840 |
| | | *RPB2* | OR667968 |
| | | *TEF1-α* | OR668099 |
| *Morchella helvetica* | M19-43 | ITS | OR482729 |
| | | *RPB1* | OR667846 |
| | | *RPB2* | OR667975 |
| | | *TEF1-α* | OR668108 |
| *Morchella rufobrunnea* | M14 | *RPB1* | GU551064.1 |
| | | *RPB2* | GU551105.1 |
| *Morchella importuna* | YAASM69 | *RPB1* | MG598564.1 |
| | | *RPB2* | MG598606.1 |
| *Morchella sextelata* | M120 | *RPB1* | GU551070.1 |

| | | | |
|---|---|---|---|
| | | *RPB2* | GU551111.1 |
| *Morchella snyderi* | M299 | *RPB1* | GU551450.1 |
| | | *RPB2* | GU551490.1 |
| *Morchella snyderi* | ARD-04-02 | *RPB1* | KM204720.1 |
| | | *RPB2* | KM204753.1 |
| *Morchella mediterraneensis* | HT 25 | *RPB1* | HM056455.1 |
| | | *RPB2* | HM056517.1 |
| *Morchella* sp. Mel-14 | M728 | *RPB1* | GU551663.1 |
| | | *RPB2* | GU551712.1 |
| *Morchella confusa* | Mel-42/1028 | *RPB1* | MK321855.1 |
| | | *RPB2* | MK321861.1 |
| *Morchella angusticeps* | M304 | *RPB1* | GU551658.1 |
| | | *RPB2* | GU551707.1 |
| *Morchella angusticeps* | M407 | *RPB1* | GU551453.1 |
| | | *RPB2* | GU551493.1 |
| *Morchella eximioides* | M494 | *RPB1* | GU551655.1 |
| | | *RPB2* | GU551704.1 |
| *Morchella eximioides* | M231 | *RPB1* | GU551465.1 |
| | | *RPB2* | GU551508.1 |

## Supplementary S4-S6 – Single-locus phylogenies (ITS, *RPB1, RPB2, TEF1-a*)

Phylogenies downloadable on **https://github.com/MorchellaThesis** (Chapter1B_S4-S6)

# 4. Using a Centroid-based approach for a reliable identification of morels (*Morchella* spp.): a case study for food authentication

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AACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGG
AACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGG
AACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGG
AACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGG
AACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGG
AACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCTCTGGTATTCCGGGG

### Foreword

This chapter presents the comparison of three genetic tools to identify *Morchella* species. This study was submitted to the Journal of Food Science, in the topic Food Microbiology and Safety. My personal contribution as first co-author was the generation and analysis of the data (genetic sequences, phylogenetic analyses, methods comparisons), main writing, and general review of the paper. This paper reports the Centroid-based approach as a reliable and relevant genetic tool to routinely identify *Morchella* at species level.

### Abstract

Food fraud is a problematic but common phenomenon occurring in the food industry. It consists in providing false information on a food item either intentionally or unintentionally. Food fraud impacts numerous sectors, including the commercialization of edible mushrooms. Morel mushrooms are prized worldwide for their culinary and medicinal purposes. They represent a taxonomically complex group in which food fraud has already been reported. Multiple methods exist to evaluate fraud of food products. Some of these rely on comparisons of genetic sequences obtained from a sample to existing databases. However, their efficacy of the method is constrained by the nature of the comparison tool and the quality of the database used. In addition to this, such approaches are not intuitively accessible by non-specialists. The Centroid approach used by SmartGene is a proprietary AI-based curation method for the generation of reference databases databases which comes with a user-friendly interface. In this study, a Centroid database for the genus *Morchella* (true morels) was taxonomically reviewed and curated using both literature and phylogenetic analyses. This allowed to clarify the taxonomic status of some important species: *Morchella crassipes, Morchella elata, Morchella costata* and *Morchella conica.* Then, identification with the curated Centroid database based on the ribosomal internal transcribed spacer (ITS) was compared to two other identification tools (from UNITE and Mycobank). The Centroid database was accurate at 83% at species level. This was superior to the other two methods. Combined with the accompanying commercial software for the analysis, this Centroid-based approach constitutes a valuable asset in assuring the originality and quality of morel products on the market that can be used by non-experts.

**Keywords:** species identification, bioinformatic tool, internal transcribed spacer (ITS), IDNS software.

## 4.1 Introduction

Food fraud is a common phenomenon occurring in the food industry, where a product or its related information are intentionally modified for economic profits (Spink & Moyer, 2011). Food fraud is a problematic societal issue that flouts consumers' rights and may affect people health because it impacts food quality and safety (Dou et al., 2022). This is long-standing and global problem affecting virtually every type of food and beverage (Shears, 2010). The main categories of food fraud concern fake and/or wrong information regarding varieties, production systems, geographical origin, source, adulteration, and spoilage (Medina et al., 2019).

High-priced gourmet mushrooms are a common target for food fraud (Liu et al., 2022). Species such as *Boletus edulis, Cantharellus* spp.*, Tricholoma matsutake, Tuber melanosporum, Tuber magnatum* (Hall et al. 2003), and *Morchella* spp. (Pilz et al., 2007) are among the species for which information concerning the harvesting year, the geographic origin, the storage conditions, and species names can be fraudulently changed (Liu et al., 2022). This issue is well-known in *Tuber* spp., because the cheaper Asian truffles *Tuber sinense, Tuber indicum,* and *Tuber himalayense* are morphologically similar and have a strong truffle-like scent but are yet not as tasty as the expensive and flavorful *T. melanosporum* (Périgord truffle) (Segelke et al., 2020). As a result, Asian truffles are sold under a false name and origin for economic gain (Segelke et al., 2020). The same type of fraud could emerge with true morels (*Morchella* spp.) owing to a group with many morphologically similar species (Loizides, 2017) but with notable differences in taste supposedly related to the impact of soil composition or climate during the formation of the fruiting body (Pilz et al., 2007). Before reaching the market, those prized edible mushrooms are either collected in the wild or cultivated (Xu et al., 2022). Accordingly, the origin of both wild and cultivated morels could impact their taste. For cultivated morels, the varieties of cereals and vegetables used for intercropping have previously been shown to influence nutritional composition and quality (Su et al., 2022). As morels are traded under different forms (i.e., fresh, dried or incorporated to processed food items), it is not always easy to recognize their specific traits allowing to identify them to the species level. Mislabelling of some morel cultivars with a different name to increase their economic value has already been documented (Du et al., 2019). Therefore, this indicates a clear and timely need to develop tools able to differentiate morel species and/or varieties once they reach the market and whatever their conformation.

A first step to certify the origin of morels could be their identification at the species level, as from the about 80 currently accepted species in the genus, only a handful can be cultivated. In 2022, ten *Morchella* species were reported to be prone to cultivation: *Morchella rufobrunnea* (*Morchella* Section *Rufobrunnea*, "grey morels")*, Morchella sextelata, Morchella importuna, Morchella eximia, Morchella exuberans, Morchella oweri*, *Morchella tomentosa, Morchella* sp. Mel-13, and *Morchella* sp. Mel-21 (*Morchella* Section *Distantes*, "black morels") (Xu et al., 2022). None of the *Morchella* Section *Esculenta* ("yellow morels") could be cultivated yet. Although an integrative taxonomy approach combining ecological, phenotypic, geographical, and genetic data is the most reliable way to identify morels species (Loizides et al., 2022), some of those criteria (e.g., ecology and distribution) cannot be applied to domesticated species (i.e., cultivars). In addition, morphological identification requires expert knowledge, as the phenotype of fruiting bodies is intraspecifically variable and is affected by environmental factors (Pilz et al., 2007). Furthermore, a morphological identification cannot be applied to dried and/or processed fruiting bodies (Loizides et al., 2022).

An integrative genetic approach requires knowledge about the genus, technical skills, and also the generation of multi-locus datasets (internal transcribed spacer [ITS], RNA polymerase II subunit 1 [RPB1] and 2 [RPB2], translation elongation factor 1 alpha [TEF1-α]) for a reliable phylogenetic identification in *Morchella* spp. (Sa et al., 2022). These hurdles render such an approach unsuitable

and costly for food authentication. Instead, a suitable method should rely on a minimal set of genetic markers produced at low cost, require no or only minimal knowledge in taxonomy, and provide reliable results that are easy to interpret by non-specialists (i.e., agrifood industries, consumer services, and/or governmental agencies). Bioinformatic approaches that are currently used to identify morels (e.g., phylogenetics (O'donnell et al. 2011; Petrzelova and Sochor 2019) and ITS BLAST (Du et al. 2012)) do not meet these criteria. For this reason, in this study, we tested the Centroid-based approach, which is a patent-protected (#EP2215578) curation procedure based on annotation developed by SmartGene (Lausanne, Switzerland). A Centroid is a DNA sequence of a given genetic marker that is the most representative for a particular species given the observed natural intra-species diversity. An AI-based process defines the Centroids from profile- and quality-filtered entries of the public domain (e.g., GenBank) to constitute a reliable reference database of trusted sequences and annotations (**Figure 1**). This reduces the impact of misidentified sequences, a point that is particularly important in the case of morels, as about 66% of the ITS sequences of *Morchella* have been reported to be misannotated at species level in GenBank (Du et al., 2012). The Centroid-based approach can be used by customers within the environment of the SmartGene's IDNS® application, a user-friendly and secure cloud system where query sequences can be entered, annotated, and analyzed. This tool allows non bioinformaticians and non-taxonomists to generate understandable and reliable results for non-experts and is then appropriate for the field of food authentication.

The Centroid-based approach was initially developed to identify medically relevant microorganisms (Hiergeist et al., 2023), but is also applicable to detect fraud in meat and fish, by identifying the source organism at species level (S. Emler, personal communication, 2023). In this study, we tested the hypothesis that such a Centroid-based approach can be applied to the identification of edible mushrooms species, and we used the morphologically variable morels as a case-study for this. More specifically, we evaluated the reliability of the Centroid-based approach to identify morels at species level, with the aim of proposing a tool for non-specialists to detect potential fraud. The first step of this study consisted in evaluating the performance of three genetic markers available as Centroids, namely the internal transcribed spacer (ITS) including the 5.8S rDNA, the small subunit (SSU) 18S rDNA, and the large subunit (LSU) 28S rDNA. The Centroid approach was tested using reliable published sequences as reference. Preliminary results indicated that the database needed to be curated to adjust the nomenclature to the current taxonomy of the group. After expert curation, the tests using the Centroid database were repeated and compared to other alignment-based tools, using the public databases UNITE and *Morchella* MLST (multisequence typing). Finally, the Centroid-based approach was tested on *Morchella* samples obtained from the environment.

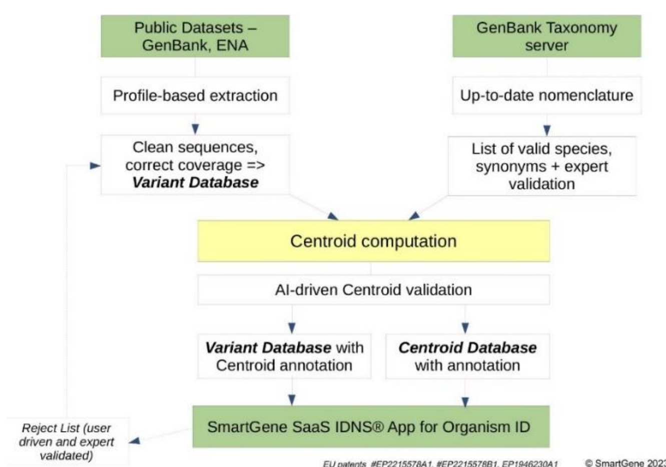


**Figure 1.** SmartGene's Centroid annotation process of fungal reference sequences.

## 4.2 Material and methods

### 4.2.1 Taxonomic value of ITS, 18S, and 28S

The taxonomic value of three genetic regions (ITS, 18S, 28S) that were available as Centroid databases was assessed using a phylogenetic analysis. The Centroid sequences of each genetic region were used to generate maximum likelihood (ML) phylogenies. Sequences were aligned using the algorithm MUSCLE (Neighbor Joining cluster method) within the software Mega version 11 (Kumar et al., 2018). The phylogenies were generated with the software IQ-TREE (Nguyen et al., 2015). The substitution model General Time Reversible (GTR) + Gamma (G) + Invariant sites (I) was used. 1000 trees were generated using an ultrafast bootstrap approximation (UFBoot) (Hoang et al., 2018).

### 4.2.2 Reference genetic material

Reliable ITS sequences with a proven expected result that were available on GenBank were used as references to test the different alignment-based tools that were compared in this study. This included type strains (e.g., MH014708.1), collection strains (e.g., MH857461.1), and voucher specimens (e.g., MG976050.1) (exhaustive list in **Supplementary S1A**).

### 4.2.3 Species determination using the Centroid-based approach

The reliability of using the Centroid-based approach to identify *Morchella* at species level was tested within the IDNS® software of SmartGene (version v3_12_0r1(r32526)) applying the following steps: (1) ITS sequences and metadata were uploaded to a personal archive; (2) query sequences from the archive were then searched against the ITS Centroid database with BLAST (Altschul et al., 1997), using the IDNS® tool “Search similar references” (option "IUPAC-aware" activated, to account for ambiguities); (3) the Centroid reference with the highest percent identities (% id) with regard to the query sequence was retained as identification. When multiple references had the same % id, the one with the highest BLAST score was retained (Altschul et al., 1997). In some cases where the reference with the highest score did not correspond to the one with the highest % id, the reference with the highest % id was given preference, as the BLAST score does not necessarily reflect fewer mismatches (Altschul et al., 1997). All ITS sequences (n= 224) listed in **Supplementary S1** were analyzed and thus identified with the Centroid-based approach using the aforementioned steps.

### 4.2.4 Taxonomic revision of the *Morchella* Centroid database

*Morchella* Centroids and Centroid annotations were generated by SmartGene using their patented annotation and curation procedure (#EP2215578) based on the sequences deposited in the public GenBank database (https://www.ncbi.nlm.nih.gov/genbank/). All morel species names were reviewed and a number of invalid names were eliminated or modified to reflect recent literature (e.g., Loizides et al. 2022). For this assessment, the species status of the unclear or invalid taxa *Morchella conica, Morchella costata, Morchella crassipes,* and *Morchella elata* were analyzed by phylogenetic analysis using datasets with sequences of the ITS of Centroid sequences, and of a number of published sequences from GenBank (see accession numbers on the phylogenies). The intraspecific nucleotide diversity (Diversity Index, DI) of the ITS sequences which were used by the SmartGene AI to generate the Centroids was represented graphically within a histogram.

### 4.2.5 Species determination using pairwise alignments with UNITE and *Morchella* MLST databases

The pairwise alignment tool from the Westerdijk Fungal Biodiversity Institute (https://wi.knaw.nl/Pairwise_alignment) was used to identify the reference ITS sequences listed in **Supplementary S1** at species level for the comparison with the results obtained with the Centroid-

based approach. The query sequences were searched within databases UNITE (https://unite.ut.ee/) and *Morchella* MLST (http://morchella.mycobank.org). The query sequence was identified using the same criteria (% id and score) as for the Centroid-based approach (**section 2.3**).

#### 4.2.6 Genetic material from cultivated and Swiss *Morchella* spp.

The Centroid-based approach was additionally tested on four types of morel samples: (A) wild fruiting bodies collected in Switzerland (sequences generated in Cravero et al. (submitted), see **Supplementary S1B** for accession numbers), (B) fruiting bodies cultivated in Asia (sequences retrieved from GenBank, see **Supplementary S1B** for accession numbers), (C) fruiting bodies cultivated in Switzerland (sequences produced in this study), and (D) in vitro-grown mycelium obtained from Chinese cultivars (sequences produced in this study). For sample types C and D, ITS sequences were produced using the following method: DNA was extracted from sporophores (PYB and PYC2) or from fresh mycelium (NEU142, NEU143), using Quick-DNA Fungal/Bacterial Miniprep Kits (Zymo Research, USA) and following the protocol provided by the manufacturer. Eluted DNA was quantified with a Qubit kit (Invitrogen, USA) using the Broad Range buffer and reagent. DNA was then diluted with PCR-grade water to a concentration of 2 ng/µL to be used as template in polymerase chain reactions (PCR). The following primer pair was used to amplify the whole ITS region including the 5.8S rDNA: ITS1-F (TCCGTAGGTGAACCTGCGG) (White et al., 1990) and ITS4 (TCCTCCGCTTATTGATATGC) (Gardes & Bruns, 1993). For each sample, the PCR mix contained PCR-grade water, 2X ALLin™ Red Taq Mastermix (HighQu, Germany), 0.2 µM forward and 0.2 µM reverse primer and 1 µL of 2 ng/µL DNA. Amplifications were performed in a Thermo Scientific Arktik thermal cycler. The following parameters were used: denaturation at 95°C for 1 min, 40 cycles of denaturation, annealing and elongation (95°C for 15 sec, 62°C for 15 sec, 72°C for 15 sec), final elongation at 72°C for 2 min, end at 15°C. PCR products were then loaded on a 1.2% agarose gel that underwent electrophoresis (100 mV, 30 min). Amplicons were visualized under UVs in a Genoplex VWR transilluminator. Positive PCR products (i.e. single band at the expected size) were then purified with a MultiScreen® Filter Plates PCR µ96 (Millipore Corporation, USA) as follows: in each well, the PCR product and 50 µL of PCR-grade water was added; a vacuum of 20 bars was applied on the wells until they dry; 20 µL of PCR-grade water was added to each well; after 2 min, DNA contained in the membrane from each well was resuspended by pipetting up and down 20 times. Once purified, the PCR products were quantified with a Qubit fluorometer as described before. Final DNA concentration was adjusted at 2-40 ng/µL and sent to Fasteris (Switzerland) for Sanger sequencing. Forward and reverse sequences were assembled and trimmed using BioEdit v.7.2 (Dagona, 1999) and resulting consensi were used for downstream analyses.

## 4.3 Results and Discussion

#### 4.3.1 Suitability of genetic markers ITS, 18S, 28S for *Morchella* species resolution

The three genetic markers for which Centroids were available (ITS, 18S and 28S) were tested for their suitability to identify *Morchella* species. The 18S was rapidly abandoned, because only two Centroid sequences were available for this marker. Currently, only four annotated 18S sequences with accepted species names are available in GenBank (AY544708.2, U42642.1, U42641.1, AY544709.2) (accessed the 10-Oct-2023). The 18S is a highly conserved gene present in all eukaryotes that can be used for genus assignment in fungi (Banos et al., 2018). A large number of 18S sequences are available for some fungal groups (for instance, aquatic fungi), because they are studied along with other organisms that also contain 18S (Panzer et al., 2015). A search for 18S sequences of

Ascomycetes in GenBank (accessed 10-Oct-2023) revealed that the taxa for which most of the 18S sequences were available all belonged to non-edible fungi, mostly budding yeasts (6774 sequences), phytopathogens (e.g., *Fusarium oxysporum*, 2875 sequences), powdery mildews (534 sequences), and lichens (e.g., *Xanthoparmelia chlorochroa*, 270 sequences). In Basidiomycetes, 18S sequences mainly belonged to rust fungi (437 sequences), smut fungi (339 sequences), and uncultured fungi. The number of 18S sequences available for edible mushrooms was limited (e.g., *Agaricus*, 40 sequences; *Cantharellus*, 20 sequences; *Boletus,* 11 sequences), and this was also the case for *Morchella* (4 sequences).

For the ITS and 28S, Centroid sequences were available for 48 and 34 species, respectively. These included the Centroids for four invalid species names: *M. crassipes, M. elata, M. costata* and *M. conica*, which are discussed later. Single-locus phylogenetic trees based on Centroid sequences of the two markers (ITS, Figure 2A; 28S, Figure 2B) were generated using IQ-TREE, and compared to the trees from literature (e.g., (Loizides et al., 2022)). This revealed that the ITS phylogeny corresponded to the currently accepted *Morchella*'s taxonomy, with high statistical support (i.e., UFBoot values > 95% (Hoang et al., 2018)) for most of the nodes. On the contrary, the phylogeny based on the 28S region was not reliable to discriminate morels at the clade level, because it grouped species of *Morchella* Section *Distantes* (Elata clade) within *Morchella* Section *Morchella* (Esculenta clade). It has already been demonstrated that phylogenetic resolution of Morchellaceae was not possible using the 28S genetic marker (Bunyard et al., 1995). This is concordant with our results. In addition, only four nodes were statistically relevant. For this reason, the 28S was not retained for further investigation. In conclusion, the results indicated that the suitability of a genetic marker for species identification and resolution depends on both the quantity and quality of available sequences in the databases, which can differ depending on the studied genus. In *Morchella*, ITS allowed a better species level identification than 18S and 28S.

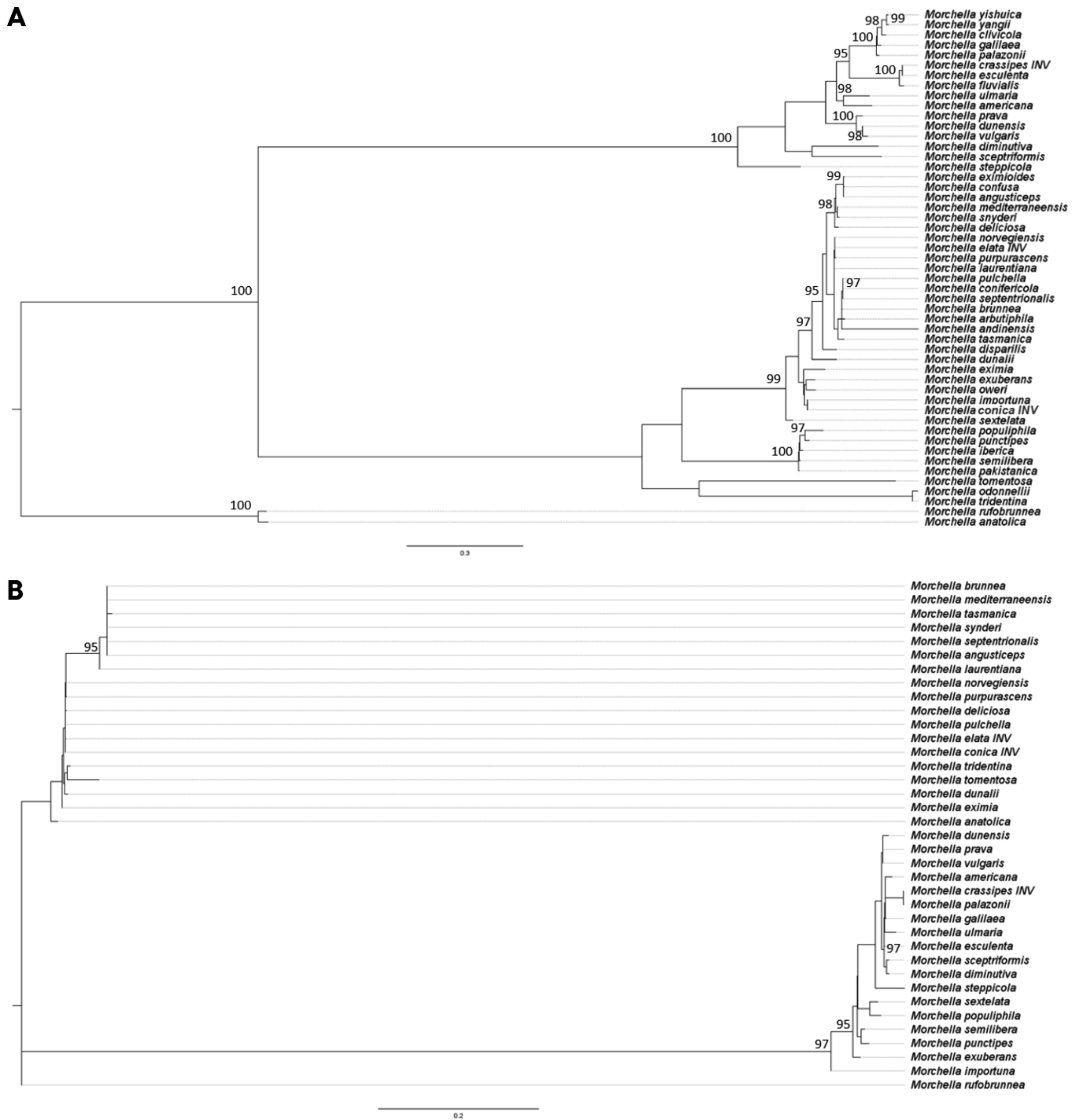


**Figure 2.** Maximum Likelihood phylogenetic trees for the ITS (A) and 28S (B) Centroids of *Morchella* spp. The support of the branches was calculated based on 1000 ultrafast bootstrap values. Invalid names are indicated in the tree as (INV). The trees were rooted with the basal species *M. rufobrunnea* and *M. anatolica*.

### 4.3.2 Nomenclature revision of *Morchella* ITS Centroid database

The primary source of current nomenclature for the generation of the morel Centroids is the NCBI Taxonomy Browser (https://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi); however, this source does not reflect the latest accepted morels' taxonomy. Preliminary tests with the *Morchella* ITS Centroid database indicated that the species nomenclature needed to be revised to correspond to the current taxonomy within the genus. In addition, the species *M. crassipes, M. elata, M. conica,* and *M. costata* for which Centroid sequences were generated required further investigation, since these names do not reflect current nomenclature (Loizides et al., 2022). After in depth verification of the taxonomy and nomenclature of *Morchella*, the nomenclature file used for the Centroid computations was updated. In addition, the DI (i.e., the intraspecific nucleotide diversity of publicly available ITS sequences) used to generate the Centroid of *Morchella vulgaris* indicated an intra-species diversity (Figure 3) that was higher in comparison to other valid species, which prompted further analysis of this taxon (see section 3.2.5).

In the next sections, results from the taxonomic analyses performed using the Centroid-based approach and phylogenetics are presented for each unresolved taxa for which a Centroid sequence existed (*M. crassipes, M. elata, M. conica*). In addition, the status of *M. costata* is clarified, as our findings did not correspond to the literature (Richard et al., 2015). Finally, *M. vulgaris* is investigated for the reason explained above.

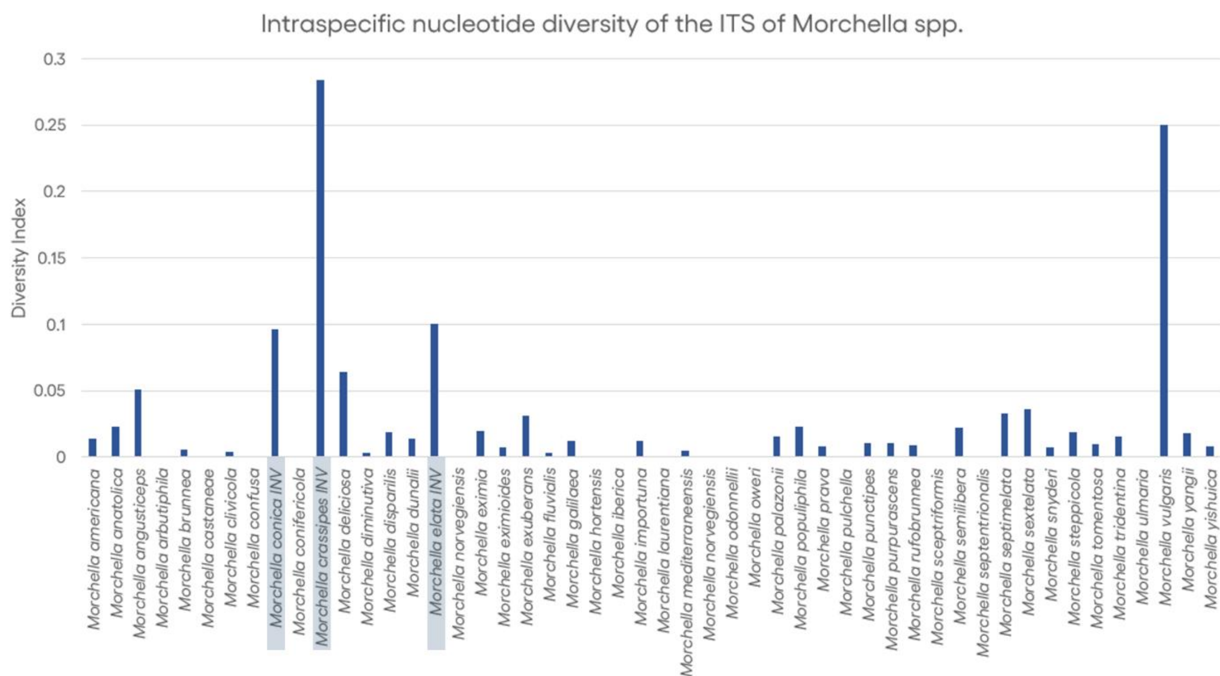


**Figure 3.** Diversity Index describing the intraspecific nucleotide diversity of the ITS sequences that were used to generate *Morchella* Centroids. Invalid names are highlighted in grey.

#### 4.3.2.1 *Morchella crassipes*

*M. crassipes* is a synonym of *Morchella semilibera* or a name that has been widely misapplied to large specimens (in size) of *Morchella esculenta* (Richard et al., 2015). The comparison of the ITS Centroid sequences revealed 100% similarity between *M. crassipes* and *M. esculenta*, the latter being shorter by just 68 bp. Unfortunately, no sequence of *RPB1, RPB2* or verified *TEF1-α* were available on NCBI for these species for further investigation.

#### 4.3.2.2 *Morchella elata*

*M. elata* has an undetermined species status (Loizides et al., 2022; Richard et al., 2015), but the name is still used in recent publications (Ramya et al., 2022; Wani et al., 2023). *M. elata* is considered as a synonym of *M. importuna*, but is also applied to specimens belonging to *M. eximioides, M. exuberans,* and *M. purpurascens* (Richard et al., 2015). *M. elata* is also hypothesized to represent the European lineage of *M. importuna* (Loizides et al., 2022). Interestingly, the ITS Centroid of *M. elata* appeared as closely related to *M. norvegiensis, M. laurentiana,* and *M. purpurascens,* but not to *M. importuna.* This suggests that most of the ITS sequences annotated as *M. elata* in GenBank do not belong to *M. importuna*. To investigate this further, an ML phylogeny based on ITS sequences of *M. elata, M. importuna, M. norvegiensis, M. laurentiana, M. eximioides, M. exuberans,* and *M. purpurascens* was generated. This showed that the ITS sequences annotated as "*M. elata*" on GenBank actually belonged to multiple species: *M. purpurascens, M. exuberans* and *M. importuna* (Supplementary S2A (phylogeny) and S2B (statistics)). In addition, 13 sequences formed a distinct taxon, sister to the subgroup of *M. purpurascens/M. norvegiensis/M. laurentiana*. In another phylogenetic analysis these distinct sequences grouped with specimens of *M. deliciosa* (see section 3.2.4). We concluded that *"M. elata*" comprised at least four distinct species of morels, *M. purpurascens, M. exuberans, M. importuna* and *M. deliciosa*, and decided not to use this name any further as designation of a single genetic taxon.

#### 4.3.2.3 *Morchella conica*

*M. conica* is an illegitimate species name that was used for specimens of *Morchella deliciosa, M. tridentina, M. purpurascens* (Richard et al., 2015), and *M. vulgaris* (Barseghyan et al., 2012). However, the ITS Centroid of *M. conica* was 100% identical to the Centroid of *M. importuna*. To investigate this further, a ML phylogeny based on ITS sequences of *M. conica, M. deliciosa, M. tridentina, M. purpurascens,* and *M. importuna* was generated (Figure 4, see Supplementary S3 for the statistics). The results indicated that the ITS sequences identified as "*M. conica*" in GenBank grouped with two distinct species: *M. deliciosa* and *M. importuna*. However, none of the *M. conica* was related to *M. purpurascens* or *M. tridentina*. This analysis supported the fact that *M. conica* is an illegitimate species name which has been used to annotate ITS sequence entries for *M. deliciosa* and *M. importuna* in GenBank.

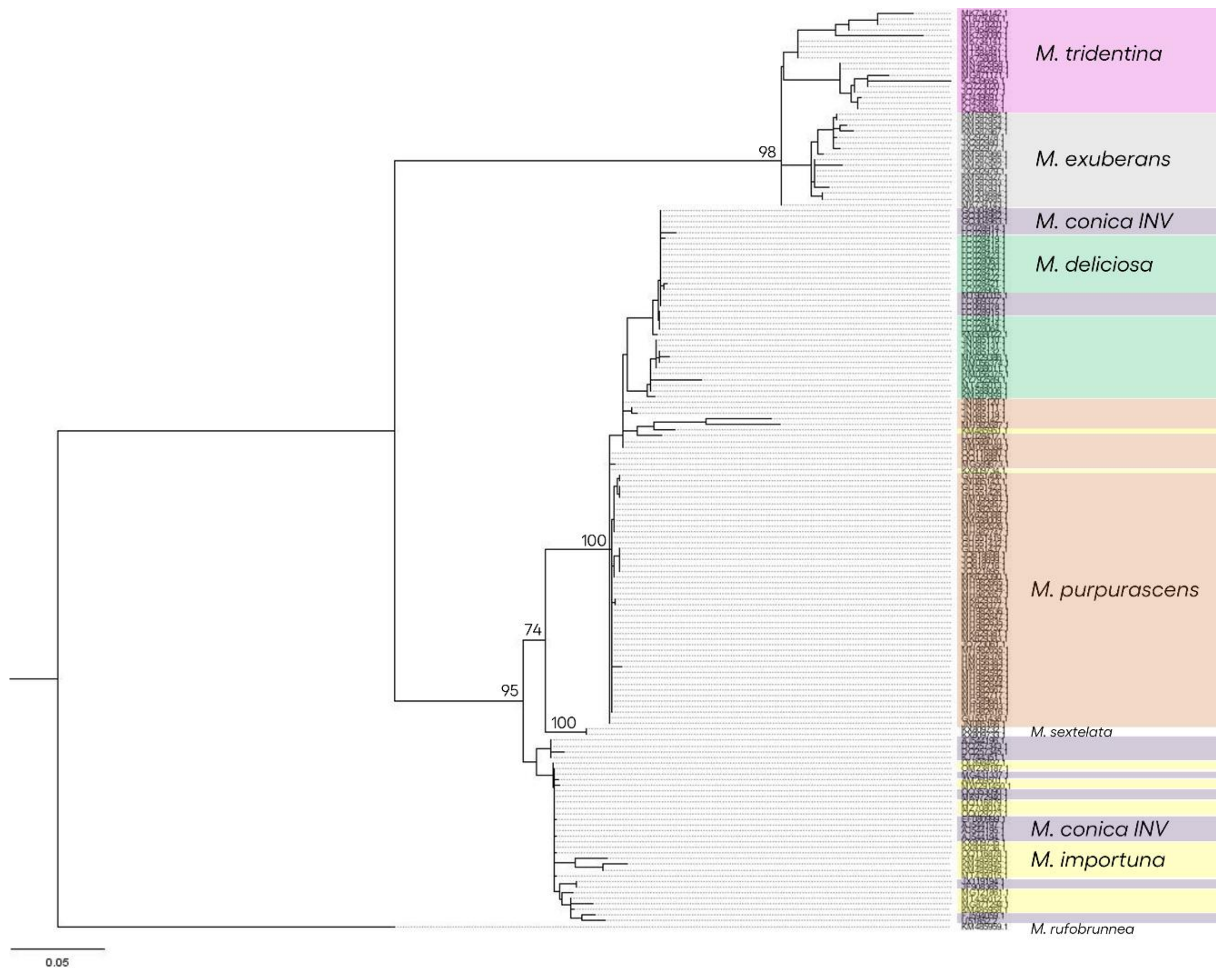


**Figure 4.** Maximum Likelihood phylogenetic tree based on ITS sequences of *Morchella conica* (invalid name) [purple], *Morchella tridentina* [pink], *Morchella importuna* [yellow], *Morchella purpurascens* [orange], *Morchella deliciosa* [green] and *Morchella sextelata* [white]. The tree was rooted with *M. rufobrunnea* [white]. The phylogeny was based on 1000 ultrafast bootstrap values. The sequences were represented by their accession number on GenBank and colored according to the annotations.

### 4.3.2.4 *Morchella costata*

*M. costata* is designated as a synonym of *M.* elata (Richard et al., 2015). According to the analysis performed before, *M. elata* designated specimens of *M. purpurascens, M. exuberans* and *M. importuna*. Therefore, *M. costata* is likely to correspond to a synonym of one of these species. However, the ITS Centroid of *M. costata* was closely related to *M. deliciosa* (>99% identity). To investigate this further, a ML phylogeny was constructed with ITS sequences of *M. costata, M. deliciosa, M. purpurascens, M. exuberans* and *M. importuna*. All the sequences annotated as "*M. costata*" belonged to *M. deliciosa*, except one (GQ265899.1) that belonged to *M. importuna* (Figure 5, see Supplementary S4 for the statistics). Accordingly, *M. costata likely* represents a synonym of *M. deliciosa*.

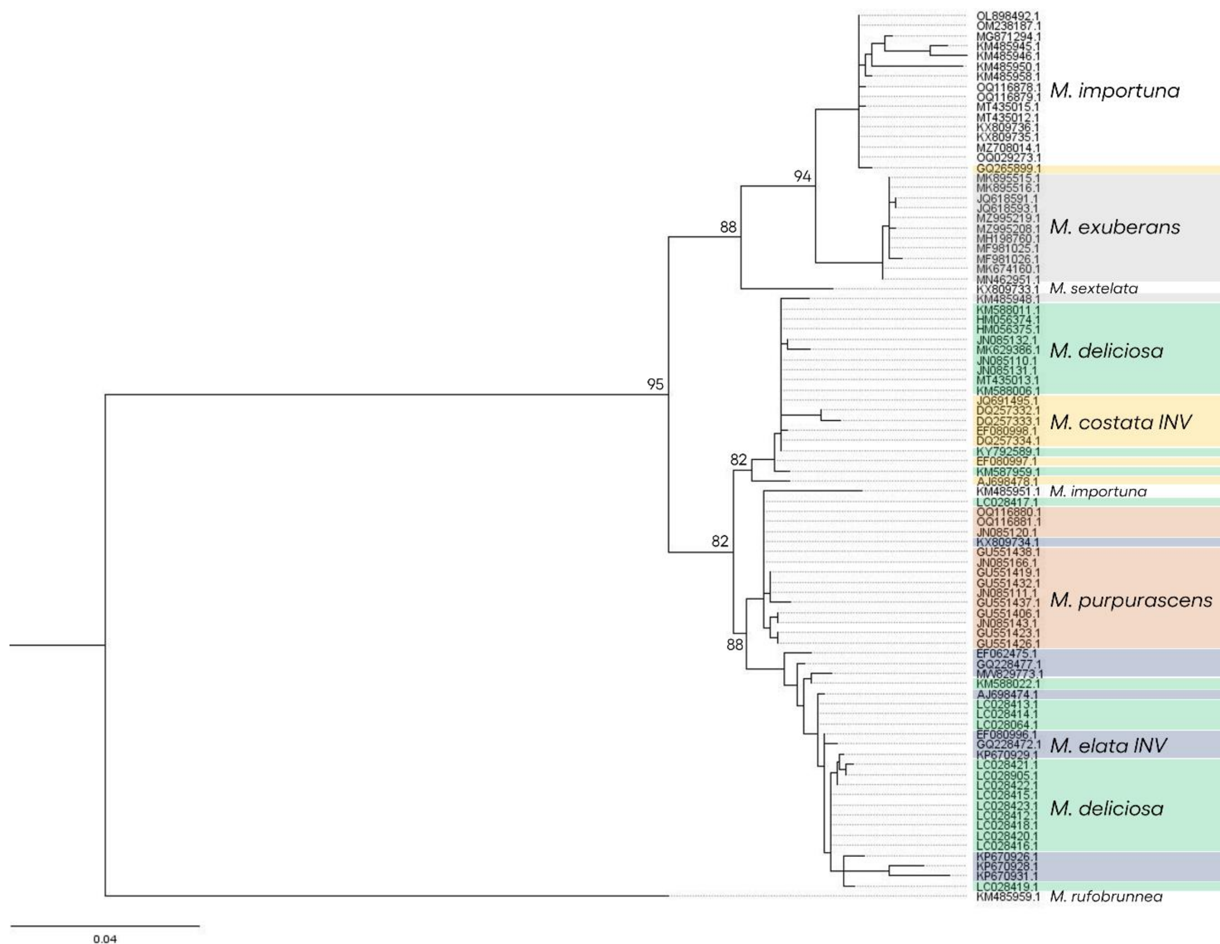


**Figure 5.** Maximum Likelihood phylogenetic tree based on ITS sequences of *Morchella costata* (invalid name) [yellow], *Morchella elata* (invalid name) [dark grey], *Morchella importuna* [white], *Morchella purpurascens* [orange], *Morchella deliciosa* [green], *Morchella exuberans* [light grey] and *Morchella sextelata* [white]. The tree was rooted with *M. rufobrunnea* [white]. The phylogeny was based on 1000 ultrafast bootstrap values. The sequences were represented by their accession number on GenBank and colored according to the annotations.

### 4.3.2.5 *Morchella vulgaris*

*M. vulgaris* can be considered as a variant of *Morchella dunensis* (Loizides et al., 2022). The high ITS intra specific variation revealed by the Centroid annotation (Figure 3) suggested that the name *M. vulgaris* actually represents different phylogenetic species. To test this, ITS sequences of *M. vulgaris* were used to construct a ML phylogeny of *Morchella* Sect. *Morchella* (Esculenta clade) along with the respective Centroid sequences. This revealed that *M. vulgaris* was divided into three phylogenetic subgroups: subgroup 1 contained 34 sequences including *Morchella* sp. Mes-17, *Morchella vulgaris* var. *dunensis* and the Centroid sequence of *M. vulgaris* (named *M. spongiola*, a synonym); subgroup 2 contained three sequences; subgroup 3 contained six sequences (Figure 6, see Supplementary S5 for the statistics). In addition, one sequence (MH857461.1, strain *M. vulgaris* CBS 231.55) was apparently misannotated as it grouped with *Morchella steppicola*. The most diverging taxa (subgroups 2 and 3) contained sequences that were all submitted by the same authors (Barseghyan et al., 2012), except for the sequence KJ741329.1 (direct submission, April 2014). In Barseghyan et al. (2012), the samples named "*M. vulgaris*" were considered as synonyms of *M. conica* and as belonging to *Morchella* Sect. *Distantes* (black morels) (Barseghyan et al., 2012), which it is now known to be erroneous, as *M. vulgaris* is assigned to *Morchella* Sect. *Morchella* (yellow morels) by integrative analyses (Loizides et

al., 2022). The sequences of *M. vulgaris* assigned to subgroups 2 and 3 were used to construct another phylogeny containing the Centroid sequences of *Morchella* Sect. *Distantes*. The results indicated that those sequences that were annotated as "*M. vulgaris*" belonged to black morels from different taxa: *Morchella dunalii, M. tridentina, M. importuna* and *M. eximioides* (Supplementary S6).

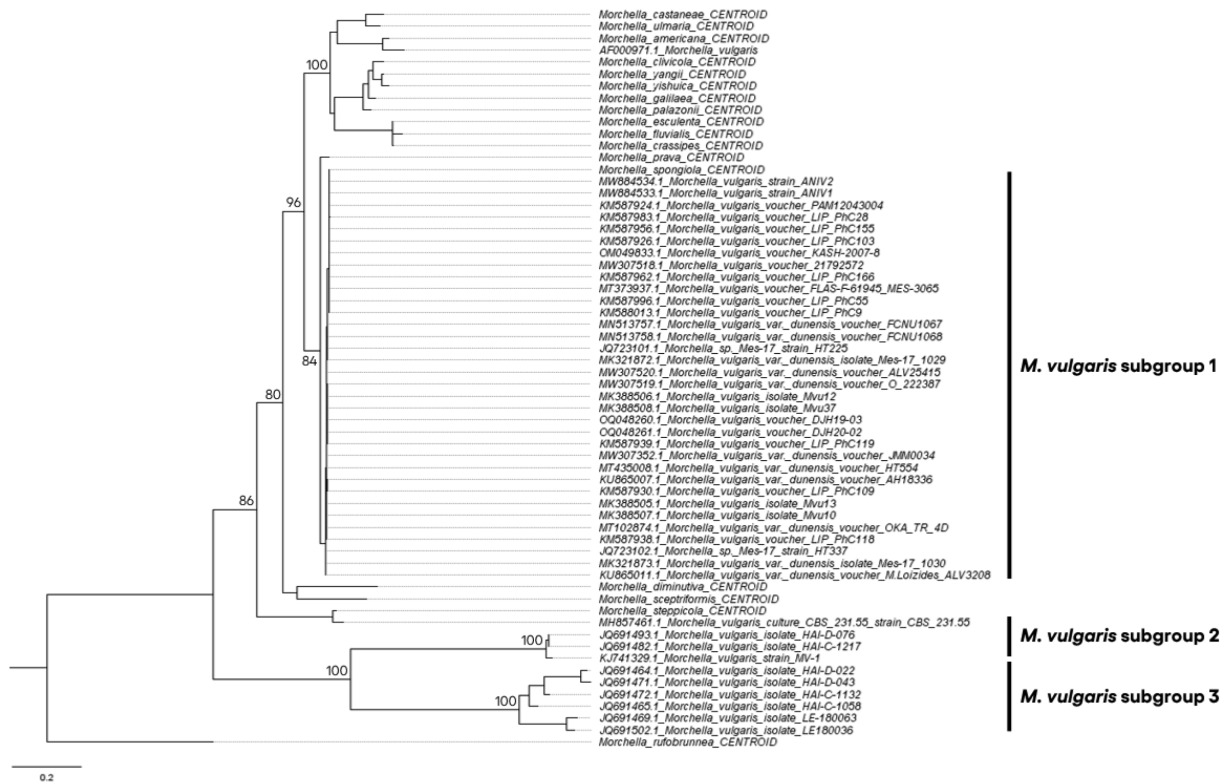


**Figure 6.** Maximum Likelihood phylogenetic tree based on ITS sequences of *Morchella vulgaris* and Centroid sequences of *Morchella* Sect. *Morchella*. The tree was rooted with *M. rufobrunnea*. The phylogeny was based on 1000 ultrafast bootstrap values. Centroid sequences are mentioned (CENTROID) while others are represented by their accession number on GenBank.

### 4.3.3 Reliability of the Centroid-based approach

4.3.3.1 Evaluation of the Centroid-based approach before and after taxonomic revision of the Centroid database

Before expert curation, 33/53 (= 62.3%) of the species were correctly identified by the Centroid-based approach (Table 1). These results indicated that the source for nomenclature, the NCBI Taxonomy Browser was not up to date with the latest taxonomic studies of *Morchella* (Loizides et al., 2022) and that a revision of nomenclature was needed. The revision of the nomenclature consisted of two types of corrections: (1) replacement of invalid/synonym species names by valid ones (e.g., *M. virginiana* became *M. sceptriformis*); (2) inclusion of valid species names that were absent in the earlier list (e.g., *M. dunensis*). The revision of the nomenclature file by expert curation improved the accuracy of the Centroid-based approach, since thereafter 44/53 (= 83.0%) of the species were correctly assigned (Table 1). The nine identifications that remained incorrect or inconclusive after the revision were caused by two situations: (1) no Centroid sequences was generated for a species because no sequence entry in GenBank matched the quality criteria imposed by SmartGene's profile

filter (e.g., *Morchella hispaniolensis*), or (2) the identification was inconclusive with closely related species that are undistinguishable using the ITS marker alone (e.g., *M. pulchella* and *M. septentrionalis*). These results were not surprising, because these subgroups have a poor genetic resolution due to the very closely related species they contain (Loizides et al., 2022). *M. conifericola* could even be considered as belonging to a single species merging *M. pulchella, M. septentrionalis, M. inamoena* ss Clowez and *Morchella* sp. Mel-23 (Loizides et al., 2022). Similarly, *M. odonnellii* could be a different geographical lineage of *M. tridentina* and not a distinct species (Loizides et al., 2022).

In this study, having 83% of correct identification with the ITS was unexpected, as Sa et al. (2022) reported that the tree-building method (with the P-distance model) of the single ITS was only able to discriminate up to 48.9% of the morel species (Sa et al., 2022). In addition, the best species resolution method that was reported by Sa et al. (2022) (i.e., four-locus neighbor-joining phylogeny with the Kimura 2-parameter model based on ITS-*RPB1-RPB2-TEF1-a*) was able to delineate 84.44% of the morel species (Sa et al., 2022), which is only slightly higher than the results from the Centroid-based approach. This indicates that the Centroid-based approach is a highly relevant method to discriminate *Morchella* at species level, using the universal ITS marker.

**Table 1**. Comparison of the identification performance of the ITS Centroid-based approach before and after nomenclature revision of the Centroid database. The sequences that were used as references for this analysis are described below (origin of the sample, strain ID, GenBank accession number). The expected result corresponds to the species that has been identified by the sequence authors. The grey boxes represent the sequences that were wrongly identified by the Centroid-based approach.

| Origin | Specimen ID | GenBank AC | Expected result | Identification before revision | Identification after revision | Cause of error |
|---|---|---|---|---|---|---|
| Greece | ML914MAK3 | MT036044.1 | *M. anatolica* | √ | √ | |
| Cyprus | ML91291M | MT036045.1 | *M. rufobrunnea* | √ | √ | |
| Spain | PhC114 | KM587934.1 | *M. castaneae* | √ | √ | |
| China | Mes-33/1022 | MK321876.1 | *M. clivicola* | √ | √ | |
| Czech R | IP96 | MH982813.1 | *M. americana* | √ | √ | |
| China | M780 | JQ723111.1 | *M. sceptriformis* | X (M. virginiana) | √ | Invalid name (synonym) |
| China | M42 | JQ723104.1 | *M. diminutiva* | √ | √ | |
| Czech R | IP233 | MH982821.1 | *M. esculenta* | X (M. crassipes) | √ | Invalid taxon |
| Spain | LIP 0000233 | KM252947.1 | *M. fluvialis* | √ | √ | |
| China | FCNU1063 | MN513699.1 | *M. galilaea* | √ | √ | |
| Venezuela | M686 | MH014707.1 | *M. gracilis* | X (M. palazonii) | X | No centroid for M. gracilis |
| Peru | CIPHAM004 | MH014708.1 | *M. peruviana* | X (M. palazonii) | X | No centroid for M. peruviana |
| China | M910 | JQ723100.1 | *M. prava* | √ | √ | |
| Serbia | PhC250 | KM587977.1 | *M. steppicola* | √ | √ | |
| Canada | PhC124 | KM587943.1 | *M. ulmaria* | X (M. cryptica) | √ | Invalid name (synonym) |
| China | FCNU1053 | MN513731.1 | *M. yangii* | √ | √ | |
| China | FCNU1105 | MN513751.1 | *M. yishuica* | √ | √ | |
| China | HT337 | JQ723102.1 | *M. dunensis* | X (M. spongiola) | √ | No centroid for M. dunensis |
| India | KASH-2007-8 | OM049833.1 | *M. vulgaris* | X (M. spongiola) | √ | Invalid name (synonym) |
| Spain | PhC211 | KT883900.1 | *M. palazonii* | √ | √ | |
| Cyprus | ML5133MD2 | KU865021.1 | *M. disparilis* | √ | √ | |
| Cyprus | ML51431MA | KU865023.1 | *M. arbutiphila* | √ | √ | |
| Australia | M337 | KC753471.1 | *M. australiana* | X (M. tasmanica) | X | No centroid for M. australiana |
| USA | MushroomObserver.org/237429 | MG976050.1 | *M. brunnea* | √ | √ | |
| China | Mel-42/1028 | MK321849.1 | *M. confusa* | √ | √ | |
| Turkey | OKA_TR_7D | MT102877.1 | *M. dunalii* | √ | √ | |
| USA | TENN-F-071527 | MK895515.1 | *M. exuberans* | √ | √ | |
| China | M375 | JQ723059.1 | *M. hispaniolensis* | X (M. laurentiana) | X | No centroid for M. hispaniolensis |
| USA | TAC 1376 | MH014727.1 | *M. kaibabensis* | X (M. laurentiana) | X | No centroid for M. kaibabensis |
| Spain | PhC335 | MN442340.1 | *M. iberica* | √ | √ | |
| China | M288 | JQ723065.1 | *M. kakiicolor* | X (M. dunalii) | X | No centroid for M. kakiicolor |
| Canada | 10.05.19AV02 | KT819376.1 | *M. laurentiana* | √ | √ | |
| Turkey | HT693 | MT435007.1 | *M. mediterraneensis* | √ | √ | |
| Norway | HS-Mo1-18 | MK629438.1 | *M. norvegiensis* | X (M. eohespera) | √ | Invalid name (synonym) |
| Norway | TJ-M1-16 | MK629388.1 | *M. purpurascens* | √ | √ | |
| China | Mel-41/1026 | MK321847.1 | *M. oweri* | √ | √ | |
| USA | JLF3769 | MH198763.1 | *M. populiphila* | √ | √ | |
| USA | MICH:352057 | MZ919243.1 | *M. punctipes* | √ | √ | |
| Czech R | VK15 | MH982769.1 | *M. semilibera* | X (M. gigas) | √ | Invalid name (synonym) |
| USA | JLF3291 | MH198766.1 | *M. snyderi* | √ | √ | |
| USA | Mushroom Observer 378284 | MT745593.1 | *M. tomentosa* | √ | √ | |
| USA | MICH:352047 | MZ919240.1 | *M. angusticeps* | √ | √ | |
| Norway | EE-M1-16 | MK629398.1 | *M. eximioides* | √ | √ | |
| Turkey | HT479 | JN085127.1 | *M. conifericola* | X (M. pulchella) | X | Closely related species |
| USA | iNat46132276 | MZ995216.1 | *M. pulchella* | √ | √ | |
| USA | MIN-890882 | MT373943.1 | *M. septentrionalis* | X (M. pulchella) | X | Closely related species |
| Norway | KV1-16 | MK629386.1 | *M. deliciosa* | X (M. costata) | √ | Invalid name (synonym) |
| India | ANI 1 | MW805426.1 | *M. importuna* | X (M. conica) | √ | Invalid taxon |
| Argentina | MES_1915 | MT952473.1 | *M. eximia* | X (M. septimelata) | √ | Invalid name (synonym) |
| Chile | FLAS:F:70540-MES-4014 | OP339584.1 | *M. andinensis* | X (M. pulchella) | √ | No centroid for M. andinensis |
| China | Mel-40/1023 | MK321850.1 | *M. odonnellii* | √ | √ | |
| USA | Mushroom Observer 429385 | MW989501.1 | *M. sextelata* | √ | √ | |
| Spain | HBIL-Fungi 2313 | MT712259.1 | *M. tridentina* | X (M. odonnellii) | X | Closely related species |

4.3.3.2 Comparison of three species determination tools

The comparison of three alignment-based identification methods for which the databases differed (*Morchella* MLST, UNITE, Centroid) was also part of this study. The pairwise-alignment tool provided by the Westerdijk Institute allows to search for sequence similarity between the user query and multiple databases, as Mycokey, FunCBS, UNITE and *Morchella* MLST (https://wi.knaw.nl/Pairwise_alignment, accessed the 16-Nov-2023). The database FunCBS provided

alignment results for *Morchella*, but it was not retained as it only contained five ITS sequences, one of them belonging to an invalid taxon (*Morchella hortensis* strain CBS 273.49). In addition, our data indicated that at least two of the four other sequences were wrongly identified at the species, or even clade level: *M. vulgaris* strain CBS 231.55 was closer to the Centroid sequence of *M. steppicola* (99.10% id) than *M. vulgaris* (88.32% id); *M. deliciosa* CBS 290.63 was closer to the Centroid of *M. americana* (99.44%) than *M. deliciosa* (85.81% id) (see Supplementary S7).

The database UNITE was chosen for comparison because it provided results for all the *Morchella* sequences that we tested. UNITE contains curated ITS sequences of Fungi that are retrieved from the International Nucleotide Sequence Database Collaboration (INSDC) and clustered into species-level operational taxonomic units (OTUs) named species hypotheses (SHs), which are represented by a reference sequence (Nilsson et al., 2019). It contains 106 SH for the genus *Morchella*, but 52 of them are not identified at the species level (https://unite.ut.ee/index.php, accessed the 16-Nov-23). The database *Morchella* MLST was chosen for comparison as it was specific to true morels. It is a public database including reference to voucher specimens and cultures. This database was implemented by Du et al. (2012) using data from multilocus phylogenetic analyses (Du et al., 2012).

To evaluate the performance of the Centroid-based approach to identify morels at species level, the method was therefore compared with the pairwise alignment (PA) tool from the Westerdijk Fungal Biodiversity Institute, using the databases UNITE and *Morchella* MLST. For this, at least one reference sequence per *Morchella* species for which a binomial name had been applied (listed in Supplementary S1A) was analyzed. The results are summarized in Table 2 (see Supplementary S7 for the complete analysis). This indicated that 8/53 species (= 15.1%) could not be properly identified by any of the three methods (i.e., Centroid-based approach, PA with UNITE as reference database, PA with *Morchella* MLST as reference database). This was either due to the absence of the species in the databases (*Morchella gracilis, Morchella peruviana, Morchella hispaniolensis, Morchella kaibabensis, Morchella kakiicolor, Morchella australiana*) or due to the high genetic resemblance of sister clades (*Morchella conifericola, Morchella septentrionalis*), affecting resolution. The PA tools using the databases *Morchella* MLST and UNITE allowed the identification of 19/53 (= 35.85%) and 25/53 (= 47.2%) of *Morchella* species, respectively. The Centroid-based approach allowed the determination of 44/53 (= 83%) of the species. UNITE PA was the only tool that allowed a correct identification of *M. tridentina*. The species that could not be identified by the UNITE PA were not present in the database. It must be noted that some SHs also used invalid species names, as *M. crassipes* and *Morchella spongiola* var. *dunensis*. The *Morchella* MLST was the less reliable method tested in the present study. This was not expected, but was explained by the fact that the MLST database lacked some of the *Morchella* species, as 105/279 of the strains contained in the database were named "*Morchella* sp." (https://www.mycobank.org/morchella-search, accessed the 16-Nov-2023).

**Table 2**. Reliability of three species identification tools using single ITS sequences: Centroid-based approach; UNITE pairwise alignment (PA); *Morchella* MLST PA. The numbers indicate the percent identity (% id) between the query and the result. The grey color indicates the species that were wrongly identified by the tested method.

| Origin | Specimen ID | GenBank AC | Expected result | ITS Centroid | ITS UNITE | ITS *Morchella* Mycobank |
|---|---|---|---|---|---|---|
| Greece | ML914MAK3 | MT036044.1 | *M. anatolica* | **99.64** | **100** | **X** |
| Cyprus | ML91291M | MT036045.1 | *M. rufobrunnea* | **99.88** | **100** | **X** |
| Spain | PhC114 | KM587934.1 | *M. castaneae* | **100** | **100** | **X** |
| China | Mes-33/1022 | MK321876.1 | *M. clivicola* | **100** | **X** | **X** |
| Czech R | IP96 | MH982813.1 | *M. americana* | **99.23** | **X** | **100** |
| China | M780 | JQ723111.1 | *M. sceptriformis* | **99.88** | **99.88** | **100** |
| China | M42 | JQ723104.1 | *M. diminutiva* | **100** | **100** | **100** |
| Czech R | IP233 | MH982821.1 | *M. esculenta* | **100** | **100** | **100** |
| Spain | LIP 0000233 | KM252947.1 | *M. fluvialis* | **100** | **100** | **X** |
| China | FCNU1063 | MN513699.1 | *M. galilaea* | **99.91** | **X** | **100** |
| Venezuela | M686 | MH014707.1 | *M. gracilis* | **X** | **X** | **X** |
| Peru | CIPHAM004 | MH014708.1 | *M. peruviana* | **X** | **X** | **X** |
| China | M910 | JQ723100.1 | *M. prava* | **100** | **100** | **100** |
| Serbia | PhC250 | KM587977.1 | *M. steppicola* | **99.64** | **100** | **100** |
| Canada | PhC124 | KM587943.1 | *M. ulmaria* | **100** | **100** | **X** |
| China | FCNU1053 | MN513731.1 | *M. yangii* | **99.44** | **X** | **X** |
| China | FCNU1105 | MN513751.1 | *M. yishuica* | **99.90** | **X** | **X** |
| China | HT337 | JQ723102.1 | *M. dunensis* | **99.91** | **X** | **X** |
| India | KASH-2007-8 | OM049833.1 | *M. vulgaris* | **100** | **X** | **100** |
| Spain | PhC211 | KT883900.1 | *M. palazonii* | **99.82** | **100** | **X** |
| Cyprus | ML5133MD2 | KU865021.1 | *M. disparilis* | **100** | **100** | **X** |
| Cyprus | ML51431MA | KU865023.1 | *M. arbutiphila* | **100** | **100** | **X** |
| Australia | M337 | KC753471.1 | *M. australiana* | **X** | **X** | **X** |
| USA | MushroomObserver.org/237429 | MG976050.1 | *M. brunnea* | **100** | **X** | **100** |
| China | Mel-42/1028 | MK321849.1 | *M. confusa* | **100** | **X** | **X** |
| Turkey | OKA_TR_7D | MT102877.1 | *M. dunalii* | **100** | **X** | **100** |
| USA | TENN-F-071527 | MK895515.1 | *M. exuberans* | **100** | **100** | **100** |
| China | M375 | JQ723059.1 | *M. hispaniolensis* | **X** | **X** | **X** |
| USA | TAC 1376 | MH014727.1 | *M. kaibabensis* | **X** | **X** | **X** |
| Spain | PhC335 | MN442340.1 | *M. iberica* | **100** | **X** | **X** |
| China | M288 | JQ723065.1 | *M. kakiicolor* | **X** | **X** | **X** |
| Canada | 10.05.19AV02 | KT819376.1 | *M. laurentiana* | **100** | **X** | **X** |
| Turkey | HT693 | MT435007.1 | *M. mediterraneensis* | **99.86** | **X** | **X** |
| Norway | HS-Mo1-18 | MK629438.1 | *M. norvegiensis* | **100** | **X** | **X** |
| Norway | TJ-M1-16 | MK629388.1 | *M. purpurascens* | **99.85** | **X** | **X** |
| China | Mel-41/1026 | MK321847.1 | *M. oweri* | **100** | **X** | **X** |
| USA | JLF3769 | MH198763.1 | *M. populiphila* | **99.57** | **100** | **100** |
| USA | MICH:352057 | MZ919243.1 | *M. punctipes* | **99.72** | **100** | **100** |
| Czech R | VK15 | MH982769.1 | *M. semilibera* | **100** | **100** | **100** |
| USA | JLF3291 | MH198766.1 | *M. snyderi* | **100** | **100** | **100** |
| USA | Mushroom Observer 378284 | MT745593.1 | *M. tomentosa* | **99.89** | **99.89** | **100** |
| USA | MICH:352047 | MZ919240.1 | *M. angusticeps* | **100** | **100** | **X** |
| Norway | EE-M1-16 | MK629398.1 | *M. eximioides* | **99.84** | **99.67** | **X** |
| Turkey | HT479 | JN085127.1 | *M. conifericola* | **X** | **X** | **X** |
| USA | iNat46132276 | MZ995216.1 | *M. pulchella* | **100** | **X** | **X** |
| USA | MIN-890882 | MT373943.1 | *M. septentrionalis* | **X** | **X** | **X** |
| Norway | KV1-16 | MK629386.1 | *M. deliciosa* | **99.25** | **99.56** | **99.61** |
| India | ANI 1 | MW805426.1 | *M. importuna* | **99.84** | **99.84** | **X** |
| Argentina | MES_1915 | MT952473.1 | *M. eximia* | **99.13** | **99.68** | **98.82** |
| Chile | FLAS:F:70540-MES-4014 | OP339584.1 | *M. andinensis* | **100** | **X** | **X** |
| China | Mel-40/1023 | MK321850.1 | *M. odonnellii* | **100** | **X** | **X** |
| USA | Mushroom Observer 429385 | MW989501.1 | *M. sextelata* | **96.89** | **X** | **100** |
| Spain | HBIL-Fungi 2313 | MT712259.1 | *M. tridentina* | **X** | **99.88** | **X** |

#### 4.3.3.3 Environmental samples

The Centroid-based approach was also tested on different type of *Morchella* samples consisting of wild fruiting bodies collected in Switzerland, fruiting bodies cultivated in Asia, fruiting bodies cultivated in Switzerland, and in-vitro grown mycelium of Chinese cultivars (Supplementary S8). Among the 142 specimens of *Morchella* originating from Switzerland, 111 of them could be uncontestably assigned to a valid species using the Centroid-based approach (79 *M. esculenta,* 23 *M. deliciosa,* 6 *M. importuna,* 2 *M. pulchella* and 1 *M. sextelata*). The 31 remaining specimens that could not be identified by the Centroid-based approach belonged to *M. pulchella*, and to the phylospecies *Morchella* sp. Mel-43, Mel-44, Mel-45, Mel-46, and Mel-47 according to multilocus phylogenetic analysis (Cravero et al., submitted).

For food authentication, emphasis should be given to commercialized (supposedly known) species. Therefore, ITS sequences of 22 Asian morel cultivars were analyzed with the Centroid-based approach. All the species that can be cultivated (except for the phylospecies *Morchella* sp. Mel-13, and *Morchella* sp. Mel-21 for which no Centroid exist as they remain undescribed) were readily identified by the Centroid-based approach. Three of them did not correspond to the authors' annotation: cultivar *M. sextelata* Gansu 2 (OM956135.1) was divergent from all species Centroids with only 97.72% identity with *Morchella eximioides*; cultivar *M. sextelata* Gansu 12 (OM948735.1) showed 98.71% identity with *Morchella norvegiensis*; cultivar *M. sextelata* Gansu 10 (OM956126.1) was identified as *M. importuna* (99.29% id) (Supplementary S8). These divergences between the strain annotation and the Centroid-based was not surprising, and was already reported by Du et al. (2019) where the cultivars *M. importuna* M79 and *M. importuna* M80 from Sichuan actually belonged to *M. sextelata*.

### 4.3.4 Using the Centroid-based approach as a routine tool for food authentication

The time needed from uploading the sequence in the IDNS software to obtaining the identification result was estimated by Kwiatkowski et al. (2012) to ten minutes (Kwiatkowski et al., 2012), which was concordant with our observations. Identifying morels with the expert-curated database and the Centroid-based approach can be then used as a routine tool, that is relevant at species level, but it can currently not be applied at a lower (e.g., strain) level. To do so, the Centroid-based approach should be expanded to strain-mapping gene targets, to be validated in a separate study. According to Du et al. (2019), the numerous cultivars that emerged within a single species could not be distinguished using the regular genetic markers (ITS, *RPB1*, *RPB2* and *TEF1-a*). Instead, microsatellites (single sequence repeats, SSRs) need to be used to highlight allelic variations within cultivars labelled with the same name (Du et al., 2019).

## 4.4 Conclusion

The Centroid-based approach embedded in SmartGene's IDNS® cloud software provides users with a tool that can be adapted for non-specialists to identify species of the genus *Morchella*. Given its simple use, it could be of interest for routine laboratories (e.g., of the food industry), food distributors or for public surveillance agencies to assure food authenticity to avoid fraud with highly priced morels (e.g., wild versus cultivated morels, local versus imported morels). In addition, the Centroid-based approach allowed us to rapidly verify the taxonomic status of *Morchella* taxa. To conclude, the Centroid-based approach could be used in routine laboratories as a screening method for non-specialists to identify morel species readily and rapidly, and could be a useful tool for specialists investigating the taxonomy of *Morchella*.

## 4.5 Declarations

**Availability of data and materials**

The datasets generated and/or analysed during the current study are available in the GenBank repository, under accession numbers OR482713-OR482851.

All data generated or analysed during this study are included in this published article and its supplementary information files S1 to S8.

**Competing interests**

JR and SE are employees of SmartGene. The other authors declare that they have no competing interests.

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**Authors' contributions**

Melissa Cravero generated and analyzed the data and wrote the manuscript. Jean Ruelle and Stefan Emler analyzed the data, improved the *Morchella* Centroid database, and reviewed the manuscript. Saskia Bindschedler and Pilar Junier designed the study and reviewed the manuscript. All authors read and approved the final manuscript.

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## 4.6 Literature

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## Supplementary Material S1A (reference strains) and S1B (environmental strains) – Genetic material

Tables downloadable on https://github.com/MorchellaThesis (Chapter2_S1A-B).

## Supplementary Material S2A (phylogeny) and S2B (statistics) – *Morchella elata* phylogeny

Phylogeny and statistics downloadable on https://github.com/MorchellaThesis (Chapter2_S2A-B).

## Supplementary Material S3 – *Morchella conica* statistics

Phylogeny and statistics downloadable on https://github.com/MorchellaThesis (Chapter2_S3).

## Supplementary Material S4 – *Morchella costata* statistics

Phylogeny and statistics downloadable on https://github.com/MorchellaThesis (Chapter2_S4).

## Supplementary Material S5 – *Morchella vulgaris* statistics

Phylogeny and statistics downloadable on https://github.com/MorchellaThesis (Chapter2_S5).

## Supplementary Material S6 – *Morchella vulgaris* in Sect. *Distantes*

Phylogeny and statistics downloadable on https://github.com/MorchellaThesis (Chapter2_S6).

## Supplementary Material S7 – Complete methods comparison

| Origin | Specimen ID | GenBank AC | Annotated as… | ITS Centroid [% id] | ITS UNITE [% id] | ITS *Morchella* Mycobank [% id] |
|---|---|---|---|---|---|---|
| Greece | ML914MAK3 | MT036044.1 | *M. anatolica* | *M. anatolica 99.64%* | *M. anatolica 100%* | *M. eximia 81.95%* |
| Cyprus | ML91291M | MT036045.1 | *M. rufobrunnea* | *M. rufobrunnea 99.88%* | *M. rufobrunnea 100%* | No sequences found satisfying the identification parameters |
| Spain | PhC114 | KM587934.1 | *M. castaneae* | *M. castaneae 100%* | *M. castaneae 100%* | *M. americana 95.38%* |
| China | Mes-33/1022 | MK321876.1 | *M. clivicola* | *M. clivicola 100%* | *M. esculenta 96.45%* | *M. americana 91.85%* |
| Czech R | IP96 | MH982813.1 | *M. americana* | *M. americana 99.23%* | *M. americana/M. esculenta/M. crassipes 100% (same score)* | *M. americana 100%* |
| China | M780 | JQ723111.1 | *M. virginiana* (syn. of *M. sceptriformis*) | *M. sceptriformis 99.88%* | *M. virginiana 99.88%* | *M. sceptriformis 100%* |
| China | M42 | JQ723104.1 | *M. diminutiva* | *M. diminutiva 100%* | *M. diminutiva 100%* | *M. diminutiva 100%* |
| Czech R | IP233 | MH982821.1 | *M. esculenta* | *M. esculenta 100%* | *M. esculenta/M. crassipes 100% (same score)* | *M. esculenta 100%* |
| Spain | LIP 0000233 | KM252947.1 | *M. fluvialis* | *M. fluvialis 100%* | *M. fluvialis 100%* | *M. esculenta 93.39%* |
| China | FCNU1063 | MN513699.1 | *M. galilaea* | *M. galilaea 99.91%* | *M. crassipes 100%* | *M. galilaea 100%* |
| Venezuela | M686 | MH014707.1 | *M. gracilis* | There is no centroid for M. gracilis | *M. palazonii 98.99%* | *M. galilaea 97.21%* |
| Peru | CIPHAM004 | MH014708.1 | *M. peruviana* | There is no centroid for M. peruviana | *M. palazonii 94.59%* | *M. galilaea 97.61%* |
| China | M910 | JQ723100.1 | *M. prava* | *M. prava 100%* | *M. prava 100%* | *M. prava 100%* |
| Serbia | PhC250 | KM587977.1 | *M. steppicola* | *M. steppicola 99.64%* | *M. steppicola 100%* | *M. steppicola 100%* |
| Canada | PhC124 | KM587943.1 | *M. ulmaria* | *M. ulmaria 100%* | *M. cyptica (syn. of M. ulmaria)/M. ulmaria 100% (same score)* | *M. americana 95.38%* |
| China | FCNU1053 | MN513731.1 | *M. yangii* | *M. yangii 99.44%* | *M. crassipes 99.67%* | *M. americana 89.32%* |
| China | FCNU1105 | MN513751.1 | *M. yishuica* | *M. yishuica 99.90%* | *M. crassipes 97.23%* | *M. americana 89.32%* |
| China | HT337 | JQ723102.1 | *Morchella* sp. Mes-17 (syn. of *M. dunensis)* | *M. dunensis 99.91%* | *M. esculenta 100%* | *M. vulgaris 100%* |
| China | Mes-17/1030 | MK321873.1 | *M. vulgaris* var. *dunensis* (syn. of *M. dunensis*) | *M. dunensis 99.91%* | *M. esculenta 100%* | *M. vulgaris 100%* |
| India | KASH-2007-8 | OM049833.1 | *M. vulgaris* | *M. vulgaris 100%* | *M. spongiola (syn. of M. vulgaris)/M. esculenta 100%* | *M. vulgaris 100%* |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | | | *(same score)* | |
| Hungary | CBS 231.55 | MH857461.1 | *M. vulgaris* | *M. steppicola 99.10% (M. vulgaris = 88.32% id)* | *M. steppicola 99.70%* | *M. steppicola 99.43%* |
| Spain | PhC211 | KT883900.1 | *M. palazonii* | *M. palazonii 99.82%* | *M. palazonii 99.82%* | *M. galilaea 96.97%* |
| Cyprus | ML5133MD2 | KU865021.1 | *M. disparilis* | *M. disparilis 100%* | *M. disparilis 100%* | *M. purpurascens 95.70%* |
| Cyprus | ML51431MA | KU865023.1 | *M. arbutiphila* | *M. arbutiphila 100%* | *M. arbutiphila 100%* | *M. brunnea 100%* |
| Australia | M337 | KC753471.1 | *M. australiana* | *There is no centroid for M. australiana* | *M. deliciosa 97.67%* | *M. brunnea 99.22%* |
| USA | Mushroom Observer.org/237429 | MG976050.1 | *M. brunnea* | *M. brunnea 100%* | *M. elata 98.13%* | *M. brunnea 100%* |
| China | Mel-42/1028 | MK321849.1 | *M. confusa* | *M. confusa 100%* | *M. angusticeps 99.71%* | *M. eximioides/M. angusticeps 99.61%* |
| Turkey | OKA_TR_7D | MT102877.1 | *M. dunalii* | *M. dunalii 100%* | *M. dunalii/M. elata/M. esculenta 100% (same score)* | *M. dunalii 100%* |
| USA | TENN-F-071527 | MK895515.1 | *M. exuberans* | *M. exuberans 100%* | *M. exuberans 100%* | *M. exuberans 100%* |
| China | M375 | JQ723059.1 | *M. hispaniolensis* | There is no centroid for M. hispaniolensis | *M. septentrionalis 98.31%* | *M. brunnea 99.61%* |
| USA | TAC 1376 | MH014727.1 | *M. kaibabensis* | There is no centroid for M. kaibabensis | *M. septentrionalis 99.08%* | *M. brunnea 99.61%* |
| Spain | PhC335 | MN442340.1 | *M. iberica* | *M. iberica 100%* | *M. semilibera 98.96%* | *M. semilibera 98.92%* |
| China | M288 | JQ723065.1 | *Morchella* sp. Mel-11 (syn. of *M. kakiicolor*) | *There is no centroid for M. kakiicolor* | *M. tridentina 100%* | *M. quercus-ilicis 100% (syn. of M. kakiicolor)* |
| Canada | 10.05.19AV02 | KT819376.1 | *M. laurentiana* | *M. laurentiana 100%* | *M. septentrionalis 98.93%* | *M. purpurascens 100%* |
| Turkey | HT693 | MT435007.1 | *M. mediterraneensis* | *M. mediterraneensis 99.86%* | *M. deliciosa 99.72%* | *M. snyderi 99.22%* |
| Norway | HS-Mo1-18 | MK629438.1 | *M. norvegiensis* | *M. norvegiensis 100%* | *M. importuna 100%* | *M. purpurascens 100%* |
| Norway | TJ-M1-16 | MK629388.1 | *M. purpurascens* | *M. purpurascens 99.85%* | *M. elata 100%* | *M. purpurascens 100%* |
| China | Mel-41/1026 | MK321847.1 | *M. oweri* (mispelled as *M. owneri*) | *M. oweri 100%* | *M. esculenta 99.70%* | *M. eximia 97.66%* |
| USA | JLF3769 | MH198763.1 | *M. populiphila* | *M. populiphila 99.57%* | *M. populiphila 100%* | *M. populiphila 100%* |

| USA | MICH:352057 | MZ919243.1 | *M. punctipes* | *M. punctipes 99.72%* | *M. punctipes 100%* | *M. punctipes 100%* |
|---|---|---|---|---|---|---|
| Czech R | VK15 | MH982769.1 | *M. semilibera* | *M. semilibera 100%* | *M. semilibera 100%* | *M. semilibera 100%* |
| USA | JLF3291 | MH198766.1 | *M. snyderi* | *M. snyderi 100%* | *M. snyderi 100%* | *M. snyderi 100%* |
| USA | Mushroom Observer 378284 | MT745593.1 | *M. tomentosa* | *M. tomentosa 99.89%* | *M. tomentosa 99.89%* | *M. tomentosa 100%* |
| USA | MICH:352047 | MZ919240.1 | *M. angusticeps* | *M. angusticeps 100%* | *M. angusticeps 100%* | *M. eximioides/M. angusticeps 100%* |
| Norway | EE-M1-16 | MK629398.1 | *M. eximioides* | *M. eximoides 99.84%* | *M. eximioides 99.67%* | *M. eximioides/M. angusticeps 99.56%* |
| Turkey | HT479 | JN085127.1 | *Morchella* sp. Mel-32 (syn. of *M. conifericola*) | *M. pulchella 100%* | *M. elata 100%* | *M. pulchella/M. septentrionalis 100%* |
| USA | iNat46132276 | MZ995216.1 | *M. pulchella* | *M. pulchella 100%* | *M. elata 100%* | *M. pulchella/M. septentrionalis 100%* |
| USA | MIN-890882 | MT373943.1 | *M. septentrionalis* | *M. pulchella/M. septentrionalis 100% (same score)* | *M. elata/M. esculenta 100%* | *M. pulchella/M. septentrionalis 100%* |
| Netherlands | CBS:290.63 | MH858286.1 | *M. deliciosa* | *M. americana 99.44% (M. deliciosa = 85.81% id)* | *M. esculentoides (syn of. M. americana) 99.52%* | *M. americana 99.22%* |
| Norway | KV1-16 | MK629386.1 | *M. deliciosa* | *M. deliciosa 99.25%* | *M. deliciosa 99.56%* | *M. deliciosa 99.61%* |
| Turkey | S F27848 | JN085110.1 | *Morchella* sp. Mel-26 (syn. of *M. deliciosa*) | *M. deliciosa 99.86%* | *M. deliciosa 100%* | *M. deliciosa 100%* |
| India | ANI 1 | MW805426.1 | *M. importuna* | *M. importuna 99.84%* | *M. importuna 99.84%* | *M. tomentosa/M. eximia/M. importuna 99.57%* |
| Argentina | MES_1915 | MT952473.1 | *M. eximia* | *M. eximia 99.13%* | *M. septimelata (syn. of M. eximia) 99.68%* | *M. eximia 98.82%* |
| France | PhC70 | KM588002.1 | *M. eximia* | *M. eximia 99.68%* | *M. eximia/M. septimelata/M. elata 100% (same score)* | *M. eximia 100%* |
| Chile | FLAS:F:70540-MES-4014 | OP339584.1 | *M. andinensis* | *M. andinensis 100%* | *M. elata 97.51%* | *M. brunnea 98.81%* |
| China | Mel-40/1023 | MK321850.1 | *M. odonnellii* | *M. odonnellii 100%* | *M. tridentina 100%* | *M. tridentina 98.83%* |
| USA | Mushroom Observer 429385 | MW989501.1 | *M. sextelata* | *M. sextelata 96.89%* | *M. exuberans 97.03%* | *M. sextelata 100%* |
| Pakistan | TS-05 | MT584841.1 | *M. tridentina* | *M. tridentina 99.89%* | *M. tridentina 100%* | *M. tridentina 98.83%* |
| Spain | HBIL-Fungi 2313 | MT712259.1 | *M. tridentina* | M. odonnellii 99.88% | M. tridentina 99.88% | M. tridentina 98.83% |

Grey= No output available; Orange= Wrong identification.

## Supplementary Material S8 – Analysis of environmental samples

| Origin | Specimen ID | Type | Annotated as… | ITS Centroid [% id] |
|---|---|---|---|---|
| Switzerland | M19-1 | A | *Morchella Mel-47* | M. angusticeps 99.86% |
| Switzerland | M19-2 | A | *Morchella Mel-47* | M. angusticeps 99.86% |
| Switzerland | M19-3 | A | *Morchella Mel-47* | M. angusticeps 99.82% |
| Switzerland | M19-4 | A | *Morchella Mel-47* | M. angusticeps 99.43% |
| Switzerland | M19-5 | A | *Morchella Mel-47* | M. eximioides 99.86% |
| Switzerland | M19-6 | A | *Morchella Mel-47* | M. angusticeps 96.84% |
| Switzerland | M19-7 | A | *Morchella Mel-47* | M. angusticeps 100% |
| Switzerland | M19-8 | A | *Morchella Mel-47* | M. angusticeps 100% |
| Switzerland | M19-9 | A | *Morchella Mel-47* | M. eximioides 99.86% |
| Switzerland | M19-10 | A | *Morchella Mel-47* | M. angusticeps 100% |
| Switzerland | M19-11 | A | *Morchella Mel-47* | M. angusticeps 99.39% |
| Switzerland | M19-12 | A | *Morchella Mel-47* | M. eximioides 99.85% |
| Switzerland | M19-13 | A | *M. esculenta* | M. esculenta 99.91% |
| Switzerland | M19-14 | A | *M. esculenta* | M. esculenta 100% |
| Switzerland | M19-15 | A | *M. esculenta* | M. esculenta 100% |
| Switzerland | M19-16 | A | *M. esculenta* | M. esculenta 100% |
| Switzerland | M19-17 | A | *M. esculenta* | M. esculenta 99.91% |
| Switzerland | M19-18 | A | *M. esculenta* | M. esculenta 100% |
| Switzerland | M19-19 | A | *M. esculenta* | M. esculenta 100% |
| Switzerland | M19-20 | A | *M. esculenta* | M. esculenta 99.73% |
| Switzerland | M19-21 | A | *M. esculenta* | M. esculenta 99.88% |
| Switzerland | M19-22 | A | *M. esculenta* | M. esculenta 96.98% |
| Switzerland | M19-23 | A | *M. esculenta* | M. esculenta 100% |
| Switzerland | M19-24 | A | *M. esculenta* | M. esculenta 100% |
| Switzerland | M19-25 | A | *M. esculenta* | M. esculenta 100% |
| Switzerland | M19-26 | A | *M. esculenta* | M. esculenta 99.89% |
| Switzerland | M19-27 | A | *M. esculenta* | M. esculenta 100% |
| Switzerland | M19-28 | A | *M. deliciosa* | M. deliciosa 100% |
| Switzerland | M19-29 | A | *M. deliciosa* | M. deliciosa 99.46% |
| Switzerland | M19-30 | A | *M. pulchella* | M. conifericola 100% |
| Switzerland | M19-31 | A | *M. pulchella* | M. conifericola 99.40% |
| Switzerland | M19-32 | A | *M. esculenta* | M. esculenta 99.51% |
| Switzerland | M19-33 | A | *M. esculenta* | M. esculenta 100% |
| Switzerland | M19-34 | A | *M. esculenta* | M. esculenta 99.91% |
| Switzerland | M19-35 | A | *M. esculenta* | M. esculenta 100% |
| Switzerland | M19-36 | A | *M. esculenta* | M. esculenta 99.56% |
| Switzerland | M19-37 | A | *M. esculenta* | N.A. (low quality sequence) |
| Switzerland | M19-38 | A | *M. esculenta* | M. esculenta 99.83% |
| Switzerland | M19-39 | A | *M. esculenta* | M. esculenta 100% |
| Switzerland | M19-40 | A | *M. esculenta* | M. esculenta 99.74% |
| Switzerland | M19-41 | A | *M. deliciosa* | M. deliciosa 100% |
| Switzerland | M19-42 | A | *M. esculenta* | M. esculenta 99.09% |
| Switzerland | M19-43 | A | *Morchella Mel-47* | M. angusticeps 100% |
| Switzerland | M20-1 | A | *M. importuna* | M. conica/M. importuna 99.86% |
| Switzerland | M20-2 | A | *M. importuna* | M. conica/M. importuna 100% |
| Switzerland | M20-3 | A | *M. importuna* | M conica/M. importuna 100% |
| Switzerland | M20-4 | A | *M. importuna* | M conica/M. importuna 100% |
| Switzerland | M20-5 | A | *M. importuna* | M conica/M. importuna 100% |
| Switzerland | M20-6 | A | *M. importuna* | M. conica/M. importuna 99.72% |
| Switzerland | M20-7 | A | *Morchella Mel-46* | M. laurentiana 99.72% |
| Switzerland | M20-8 | A | *M. deliciosa* | M. deliciosa 99.86% |
| Switzerland | M20-9 | A | *M. deliciosa* | M. deliciosa 99.72% |
| Switzerland | M21-1 | A | *M. deliciosa* | M. deliciosa 99.85% |
| Switzerland | M21-2 | A | *Morchella Mel-44* | M. norvegiensis 100% |
| Switzerland | M21-3 | A | *Morchella Mel-44* | M. norvegiensis 100% |

| | | | | |
|---|---|---|---|---|
| Switzerland | M21-4 | A | *Morchella Mel-44* | M. norvegiensis 100% |
| Switzerland | M21-5 | A | *M. esculenta* | M. esculenta 99.91% |
| Switzerland | M21-6 | A | *M. esculenta* | M. esculenta 99.83% |
| Switzerland | M21-7 | A | *M. esculenta* | M. esculenta 100% |
| Switzerland | M21-8 | A | *M. deliciosa* | M. deliciosa 99.86% |
| Switzerland | M21-9 | A | *Morchella Mel-47* | M. angusticeps 99.72% |
| Switzerland | M21-10 | A | *Morchella Mel-47* | M. angusticeps 100% |
| Switzerland | M21-11 | A | *M. esculenta* | M. esculenta 100% |
| Switzerland | M21-12 | A | *Morchella Mel-47* | M. angusticeps 100% |
| Switzerland | M21-13 | A | *Morchella Mel-47* | M. angusticeps 100% |
| Switzerland | M21-14 | A | *M. deliciosa* | M. deliciosa 99.86% |
| Switzerland | M21-15 | A | *M. deliciosa* | M. deliciosa 99.86% |
| Switzerland | M21-16 | A | *M. deliciosa* | M. deliciosa 99.86% |
| Switzerland | M21-17 | A | *M. deliciosa* | M. deliciosa 99.86% |
| Switzerland | M21-18 | A | *M. deliciosa* | M. deliciosa 99.86% |
| Switzerland | M21-19 | A | *M. deliciosa* | M. deliciosa 99.86% |
| Switzerland | M21-20 | A | *M. pulchella* | M. pulchella 100% |
| Switzerland | M21-21 | A | *M. deliciosa* | M. deliciosa 99.72% |
| Switzerland | M21-22 | A | *M. deliciosa* | M. deliciosa 99.86% |
| Switzerland | M21-23 | A | *M. deliciosa* | M. deliciosa 99.72% |
| Switzerland | M21-24 | A | *M. esculenta* | M. esculenta 99.91% |
| Switzerland | M21-25 | A | *M. esculenta* | M. esculenta 98.84% |
| Switzerland | M21-26 | A | *M. esculenta* | M. esculenta 99.48% |
| Switzerland | M21-27 | A | *M. esculenta* | M. esculenta 99.65% |
| Switzerland | M21-28 | A | *M. esculenta* | M. esculenta 100% |
| Switzerland | M21-29 | A | *Morchella Mel-47* | M. angusticeps 99.58% |
| Switzerland | M21-30 | A | *Morchella Mel-46* | M. angusticeps 99.43% |
| Switzerland | M21-31 | A | *M. esculenta* | M. esculenta 99.65% |
| Switzerland | M21-32 | A | *M. esculenta* | M. esculenta 99.83% |
| Switzerland | M21-33 | A | *Morchella Mel-43* | M. odonnellii 99.88% |
| Switzerland | M21-34 | A | *Morchella Mel-43* | M. odonnellii 99.65% |
| Switzerland | M21-35 | A | *M. deliciosa* | M. deliciosa 98.78% |
| Switzerland | M21-36 | A | *M. esculenta* | M. esculenta 100% |
| Switzerland | M21-37 | A | *M. deliciosa* | M. deliciosa 99.86% |
| Switzerland | M21-38 | A | *M. esculenta* | M. esculenta 99.91% |
| Switzerland | M21-39 | A | *M. esculenta* | M. esculenta 99.82% |
| Switzerland | M21-40 | A | *M. esculenta* | M. esculenta 99.91% |
| Switzerland | M21-41 | A | *M. deliciosa* | M. deliciosa 100% |
| Switzerland | M21-42 | A | *M. esculenta* | M. esculenta 99.47% |
| Switzerland | M21-43 | A | *M. esculenta* | M. esculenta 99.91% |
| Switzerland | M21-44 | A | *M. esculenta* | M. esculenta 99.82% |
| Switzerland | M21-45 | A | *M. esculenta* | M. esculenta 99.91% |
| Switzerland | M21-46 | A | *M. esculenta* | M. esculenta 99.65% |
| Switzerland | M21-47 | A | *M. esculenta* | M. esculenta 99.3% |
| Switzerland | M21-48 | A | *Morchella Mel-46* | M. pulchella 100% |
| Switzerland | M21-49 | A | *M. esculenta* | M. esculenta 99.74% |
| Switzerland | M21-50 | A | *Morchella Mel-46* | M. deliciosa 98.99% |
| Switzerland | M21-51 | A | *M. esculenta* | M. esculenta 99.82% |
| Switzerland | M21-52 | A | *M. esculenta* | M. esculenta 99.74% |
| Switzerland | M21-53 | A | *M. esculenta* | M. esculenta 99.91% |
| Switzerland | M21-54 | A | *M. esculenta* | M. esculenta 99.83% |
| Switzerland | M21-55 | A | *M. esculenta* | M. esculenta 99.83% |
| Switzerland | M21-56 | A | *M. esculenta* | M. esculenta 99.92% |
| Switzerland | M21-57 | A | *M. esculenta* | M. esculenta 99.91% |
| Switzerland | M21-58 | A | *M. esculenta* | M. esculenta 99.91% |
| Switzerland | M21-59 | A | *M. esculenta* | M. esculenta 99.92% |
| Switzerland | M21-60 | A | *M. deliciosa* | M. deliciosa 99.45% |
| Switzerland | M21-61 | A | *M. esculenta* | M. esculenta 99.91% |
| Switzerland | M21-62 | A | *M. esculenta* | M. esculenta 99.91% |

| | | | | |
|---|---|---|---|---|
| Switzerland | M21-63 | A | *M. deliciosa* | M. deliciosa 99.86% |
| Switzerland | M21-64 | A | *M. esculenta* | M. esculenta 99.83% |
| Switzerland | M21-65 | A | *M. esculenta* | M. esculenta 99.74% |
| Switzerland | M21-66 | A | *M. deliciosa* | M. deliciosa 99.72% |
| Switzerland | M21-67 | A | *M. esculenta* | M. esculenta 99.91% |
| Switzerland | M21-68 | A | *M. esculenta* | M. esculenta 99.82% |
| Switzerland | M21-69 | A | *M. esculenta* | M. esculenta 99.91% |
| Switzerland | M21-70 | A | *M. esculenta* | M. esculenta 99.91% |
| Switzerland | M21-71 | A | *M. esculenta* | M. esculenta 99.91% |
| Switzerland | M21-72 | A | *M. esculenta* | M. esculenta 99.83% |
| Switzerland | M21-73 | A | *M. esculenta* | M. esculenta 99.83% |
| Switzerland | M21-74 | A | *M. esculenta* | M. esculenta 99.91% |
| Switzerland | M21-75 | A | *M. esculenta* | M. esculenta 99.57% |
| Switzerland | M21-76 | A | *M. esculenta* | M. esculenta 99.74% |
| Switzerland | M21-77 | A | *M. esculenta* | M. esculenta 99.91% |
| Switzerland | M21-78 | A | *M. esculenta* | M. esculenta 99.91% |
| Switzerland | M21-79 | A | *M. esculenta* | M. esculenta 99.91% |
| Switzerland | M21-80 | A | *M. esculenta* | M. esculenta 99.56% |
| Switzerland | M21-81 | A | *M. esculenta* | M. esculenta 99.39% |
| Switzerland | M21-82 | A | *Morchella Mel-45* | M. pulchella 99.86% |
| Switzerland | M21-83 | A | *M. sextelata* | M. sextelata 99.86% |
| Switzerland | M21-85 | A | *M. esculenta* | M. esculenta 99.89% |
| Switzerland | M21-86 | A | *M. esculenta* | M. esculenta 99.91% |
| Switzerland | M21-87 | A | *M. esculenta* | M. esculenta 99.91% |
| Switzerland | M21-88 | A | *M. esculenta* | M. esculenta 100% |
| Switzerland | M21-89 | A | *M. esculenta* | M. esculenta 99.91% |
| Switzerland | M21-90 | A | *M. pulchella* | M. septentrionalis 100% |
| Switzerland | M21-91 | A | *M. pulchella* | M. pulchella 99.86% |
| **Cultivated strains** | | | | |
| China | Cultivar Henan 1 | B | *M. sextelata* | M. sextelata 99.86% |
| China | Cultivar Henan 2 | B | *M. sextelata* | M. sextelata 100% |
| China | Cultivar Henan 3 | B | *M. sextelata* | M. sextelata 99.85% |
| China | Cultivar Henan 4 | B | *M. sextelata* | M. sextelata 99.43% |
| China | Cultivar Gansu 1 | B | *M. sextelata* | M. sextelata 99.57% |
| China | Cultivar Gansu 2 | B | *M. sextelata* | M. eximioides 97.72%; divergent from all centroids |
| China | Cultivar Gansu 3 | B | *M. sextelata* | M. sextelata 100% |
| China | Cultivar Gansu 4 | B | *M. sextelata* | M. sextelata 99.86% |
| China | Cultivar Gansu 5 | B | *M. sextelata* | M. sextelata 99.71% |
| China | Cultivar Gansu 6 | B | *M. sextelata* | M. sextelata 99.57% |
| China | Cultivar Gansu 7 | B | *M. sextelata* | M. sextelata 100% |
| China | Cultivar Gansu 8 | B | *M. sextelata* | M. sextelata 99.72% |
| China | Cultivar Gansu 9 | B | *M. sextelata* | M. sextelata 99.86% |
| China | Cultiv Gansu 10 | B | *M. sextelata* | M. importuna 99.29% |
| China | Cultiv Gansu 11 | B | *M. sextelata* | M. sextelata 99.86% |
| China | Cultiv Gansu 12 | B | *M. sextelata* | M. norvegiensis 98.71%; divergent from all centroids |
| India | ANI1 | B | *M. importuna* | M. importuna 99.84% |
| India | ANI1 [2] | B | *M. importuna* | M. importuna 99.84% |
| China-Cult. in CH | PYC2 | C | *M. sextelata* | M. sextelata 99.46% |
| China-Cult. in CH | PYB | C | *M. sextelata* | M. sextelata 99.86% |
| China (Mycelium) | NEU142 | D | *M. sextelata* | M. sextelata 99.71% |
| China (Mycelium) | NEU143 | D | *M. importuna* | M. importuna 99.71% |

# 5. Importance of appropriate genome information for the design of mating type primers in black and yellow morel populations

**Melissa Cravero**[1], Aaron J. Robinson[2], Patrick Hilpisch[1], Patrick S. Chain[2], Saskia Bindschedler[1], Pilar Junier[1]

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**Foreword**

This chapter presents the analysis of the reliability of four PCR primer pairs designed to amplify mating types in *Morchella* Sect. *Morchella* and Sect. *Distantes*. This study was published as a commentary paper in the open access journal IMA Fungus. My personal contribution as first co-author was the laboratory testing of the primers, generation of genetic sequences, analysis of the sequences, main writing, and general review of the paper. This paper reports unreliable or wrong published primer sequences.

**Abstract**

Morels are highly prized edible fungi where sexual reproduction is essential for fruiting-body production. As a result, a comprehensive understanding of their sexual reproduction is of great interest. Central to this is the identification of the reproductive strategies used by morels. Sexual reproduction in fungi is controlled by mating-type (MAT) genes and morels are thought to be mainly heterothallic with two idiomorphs, MAT1-1 and MAT1-2. Genomic sequencing of black (Elata clade) and yellow (Esculenta clade) morel species has led to the development of PCR primers designed to amplify genes from the two idiomorphs for rapid genotyping of isolates from these two clades. To evaluate the design and theoretical performance of these primers we performed a thorough bioinformatic investigation, including the detection of the MAT region in publicly available Morchella genomes and in-silico PCR analyses. All examined genomes, including those used for primer design, appeared to be heterothallic. This indicates an inherent fault in the original primer design which utilized a single Morchella genome, as the use of two genomes with complementary mating types would be required to design accurate primers for both idiomorphs. Furthermore, potential off-targets were identified for some of the previously published primer sets, but verification was challenging due to lack of adequate genomic information and detailed methodologies for primer design. Examinations of the black morel specific primer pairs (MAT11L/R and MAT22L/R) indicated the MAT22 primers would correctly target and amplify the MAT1-2 idiomorph, but the MAT11 primers appear to be capable of amplifying incorrect of-targets within the genome. The yellow morel primer pairs (EMAT1-1 L/R and EMAT1-2 L/R) appear to have reporting errors, as the published primer sequences are dissimilar with reported amplicon sequences and the EMAT1-2 primers appear to amplify the *RNA polymerase II subunit* (*RPB2*) gene. The lack of the reference genome used in primer design and descriptive methodology made it challenging to fully assess the apparent issues with the primers for this clade. In conclusion, additional work is still required for the generation of reliable primers to investigate mating types in morels and to assess their performance on different clades and across multiple geographical regions.

**Keywords:** PCR, MAT idiomorphs, *Morchella*, heterothallic, homothallic, sexual reproduction, fruiting bodies, morels.

## 5.1 Background

True morels, belonging to the genus *Morchella*, are highly prized ascomycete fungi due to the exceptional organoleptic properties of their ascocarps. While morels can form ascocarps under appropriate environmental conditions in natural systems, production of these sexual structures through artificial cultivation remains a challenge (Liu et al. 2018a, b). This has motivated efforts to better understand the biological mechanisms that permit sexual reproduction in these fungi, with the aim of facilitating the cultivation of fruiting bodies. In heterothallic members of the Ascomycota, sexual reproduction is generally controlled by a bipolar mating system comprised of two mating idiomorphs most frequently identified as MAT1-1 and MAT1-2, although alternative identifiers are used in some cases (Robinson and Natvig 2019). Each idiomorph encodes a variable number of mating-type genes (Wilken et al. 2017) that produce unrelated proteins (Casselton 2002). Given that the genes at this locus have little homology, they are referred to as idiomorphs rather than alleles (Arie et al. 1997; Zheng et al. 2013; Chai et al. 2017). The genomes of monokaryotic individuals from heterothallic species possess only one of the two idiomorphs (either MAT1-1 or MAT1-2) and they must find a partner with the opposite mating-type idiomorph to complete the sexual part of their life cycle and to produce ascospores (Coppin et al. 1997). In contrast, individuals from homothallic species are self-fertile as they either possess both mating-type idiomorphs within a single haploid genome (primary homothallism) or produce multinucleated spores with both mating idiomorphs present, but each in an individual haploid nucleus (secondary homothallism) (Wilson et al. 2015). An often-defining characteristic of the mating type idiomorphs are genes that contain high mobility group (HMG) domains. The *MAT1-1-1* gene encodes an α1 protein belonging to the MATα_HMG family, while *MAT1-2-1* encodes a protein belonging to the MATA_HMG family (Arie et al. 1997; Zheng et al. 2013; Zou et al. 2019; Robinson and Natvig 2019). In addition to their importance in determining the mode of reproduction of ascomycete fungi, mating-type genes can be a useful tool to improve species determination and phylogenetic analyses (Du et al. 2005). This is due to the observation that *MAT1-1-1* and *MAT1-2-1* have a high interspecific and low intraspecific variability in several examined fungal lineages (Coppin et al. 1997). *Morchella* spp. are considered to primarily be heterothallic, but secondary homothallism (Du and Yang 2021) and primary homothallism (Chai et al. 2022) have also been observed. Furthermore, the mating strategies can be mixed within a single species. For instance, the black morel *Morchella* importuna can reproduce by three different mating systems: heterothallism, homothallism, and pseudohomothallism (Du and Yang 2021). However, no obvious morphological differences have been observed for fruiting-bodies and mycelia resulting from these various mating strategies. As a result, sequencing-based approaches and analyses are frequently utilized to characterize fungal mating strategies and genotypes. Once mating-type loci have been identified and sequenced for a particular fungal group or clade, it is quite common to design primers for the characterization of other closely related fungal isolates in a rapid and cost-efficient manner through the use of polymerase chain reaction (PCR) and gel electrophoresis (Du et al. 2017, 2020; Chai et al. 2017, 2019).Primers designed to amplify partial regions of two MAT genes in Morchella spp. of the Elata (black) clade have been previously published (MAT11L/R and MAT22L/R; Du et al. 2017). Given that black and yellow morels differ in their ecology and morphology (Pilz et al. 2007) and are phylogenetically divergent (O'Donnell et al. 2011), the loci contributing to sexual mating could also be expected to differ. This was the motivation for the development of additional specific primer pairs to examine species of the Esculenta (yellow) clade as well (EMAT1-1 L/R and EMAT1-2 L/R; Du et al. 2020). Subsequently, several differences were reported when comparing sequences obtained from representatives of each clade using these primer sets. Differences in the genetic structure of the partial *MAT1-1-1* and *MAT1-2-1* sequences obtained using these primers were observed between the black and yellow clades. The length of the genes differed

between both clades: MAT1-1-1 was 729–736 bp in length in black morels and 708 bp in yellow morels, while *MAT1-2-1* was 398–408 bp in black morels and 869–880 bp in yellow morels (Du et al. 2017, 2020). In addition, there are notable differences in the genetic architecture of the mating-type regions of black and yellow morels, as *MAT1-1-10* was observed in Mes-20 (Esculenta clade) (Chai et al. 2019) but both *MAT1-1-10* and *MAT1-1-11* were present within the mating region in *M. importuna* (Elata clade) (Chai et al. 2019). Until now, these previously published primers were mainly used to investigate mating types in morel strains from an Asian origin (Du et al. 2017, 2020). However, due to the diverging evolutionary histories of morel species from different geographical regions (O'Donnell et al. 2011), their performance in populations of other origins might vary and thus needs to be assessed. In thus study, we aimed at evaluating the performance of primers designed to amplify partial regions of *MAT1-1-1* and *MAT1-2-1* genes specifically in the Elata (Du et al. 2017) and Esculenta (Du et al. 2020) clades using in-silico methods. For this, custom hidden Markov model (HMM) profiles were designed to identify putative *MAT1-1-1* and *MAT1-2-1* genes in *Morchella* genomes. Additionally, our investigations considered genomic context when identifying putative mating-type regions, specifically the location of potential MAT genes relative to the APN2 and SLA2 genes, which often flank the mating-type region (Robinson et al. 2019). The NCBI Primer-BLAST tool and other alignment-based methods were used to estimate the efficiency of the previously published primer sets in amplifying the intended mating-type genes from Morchella genomes. These investigations provided further support that the Elata-specific *MAT1-2-1* primer pair would correctly amplify the proper MAT gene in the Elata clade and in the Esculenta clade. On the other hand, all other examined primers have the potential to amplify incorrect of-targets in diverse *Morchella* genomes and do not function as intended. This was further confirmed with PCR experiments utilizing the published primer sequences. This work highlights important considerations when designing and evaluating primers for the characterization of diverse fungi and provides insights as to how mating-type specific primers can be properly developed using available genomic resources.

## 5.2 Main text

### 5.2.1 *In silico* analysis of mating-type gene primers for *Morchella*

Two sets of Hidden Markov model (HMM) profiles were created for both *MAT1-1-1* and *MAT1-2-1*. One set was created using protein sequences from several *Tuber* and *Morchella* species, and the other set was created using non-Pezizomycetes sequences from diverse ascomycetes. Details of these HMM profiles and their summary statistics can be found in Additional file 1. Amino-acid alignments for the creation of the HMM profiles were generated using Clustal Omega (Sievers et al. 2011). the non-Pezizomycetes HMM profile included sequences from highly studied taxa that helped establish canonical models for mating-type genes, such as Neurospora and Magnaporthe, making them generally more trustworthy than largely unverified sequences from *Tuber* or *Morchella*. Additionally, published sequences for Morchella and Tuber were quite limited in number. These HMM profiles were used to search (hmmsearch) predicted protein sequences from M. importuna strain SCYDJ1-A1 (Morimp1) and M. importuna strain CCBAS932 (Morco1) (Additional file 2). The genome assembly and annotated protein sequences for these M. importuna isolates were obtained from the Joint Genome Institute (JGI) MycoCosm portal (https://mycocosm.jgi. doe.gov/mycocosm/home). Putative mating-type genes identified by these HMM profiles were further analyzed by comparing their position relative to the APN2 and SLA2 genes within the genome for each isolate examined. The APN2 and SLA2 genes were identified in the *M. importuna* genomes through homology-based searches using genes from Neurospora as a reference (NCBI accessions: XP_964240.1 and ESA43843.1).

Putative mating type genes identified in the HMM searches that were also located between APN2 and SLA2 loci were considered to have the strongest support and selected for all downstream analyses. Nucleotide and amino acid sequences for both the putative M. importuna mating-type genes and the region containing the mating-type locus and flanking genes can be found in Additional file 3 along with their coordinates in each genome assembly and other relevant identifiers. The nucleotide sequences for both the putative *MAT1-1-1* and *MAT1-2-1* genes from *M. importuna* were aligned to the genome assembly of *M. crassipes* strain M10 (NCBI accession: GCA_009192285.1) and *M. eximia* strain MG90 (GCA_003314645.1) using blastn. Nucleotide sequences of APN2 and SLA2 obtained from *M. importuna* were also used to identify homologs in these assemblies using blastn. The nucleotide sequence for the putative *MAT1-1-1* gene identified in the *M. crassipes* strain M10 assembly and the putative *MAT1-2-1* gene identified in the *M. eximia* strain MG90 can be found along with their coordinates in Additional file 3. The NCBI Primer-BLAST tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi) was used to screen the genome assemblies of *M. eximia* strain MG90 (GCA_003314645.1) and M. crassipes strain M10 (GCA_009192285.1). All default parameters were used in screens performed with Primer-BLAST and the primer pairs used in each screen were entered as the forward primer (L) and reverse primer (R). Previously published primers for mating-type genes were also aligned to the Morchella genome assemblies investigated in this work using blastn (-task blastn-short). All alignments involving nucleotide sequences of amplicons, putative mating-type genes, and/or *Morchella* genome assemblies were performed using blastn.

### 5.2.2 Evaluating the design of previously published mating-type primers for Elata clade

*In silico* analysis of the published PCR primers designed to amplify *MAT1-1-1* and *MAT1-2-1* genes in *Morchella* were performed. First, custom designed HMM profiles developed for this work were used to screen annotated genome assemblies of two M. importuna isolates (Elata clade) obtained from the JGI MycoCosm portal. These screens identified a putative *MAT1-1-1* gene in one of the isolates (CCBAS932/Morco1) and a putative *MAT1-2-1* gene in the other isolate (SCYDJ1-A1/Morimp1). The putative mating-type regions in these *M. importuna* genomes are highly conserved other than for the loci encoding the mating-type genes (Additional file 4). These putative mating-type gene sequences were then used to genotype the genome of *M. eximia* strain MG90 (GCA_003314645.1), which was the assembly used to generate the Elata-specific primers (Du et al. 2017). A high coverage (98%) and identity (94.77%) alignment was found between the putative MAT1-2-1 gene sequence and the *M. eximia* genome assembly, while the alignment with the putative *MAT1-1-1* gene sequence had lower coverage (2%) and identity (81.40%). These results appear to indicate that the *M. eximia* reference genome used to design both the *MAT1-1-1* and *MAT1-2-1* primers is heterothallic and harbors only the MAT1-2 idiomorph. Next, we aligned the primers against this *M. eximia* genome using the NCBI Primer-BLAST tool. Tree amplicon products were predicted for the MAT1-1 primer set (MAT11L/R), none of which overlap with the mating-type region identified in the genomic screen. Two amplicon products which both overlap with the region identified in the genomic screen were predicted for the MAT1-2 primer set (MAT22L/R), but the product size was highly variable (Additional file 5) (502 and 2248 bp). Alignments between the MAT1-2 primer set and the *M. eximia* and *M. importuna* (Morimp1) genome assemblies indicated the top alignments overlapped with the putative mating-type regions identified in our genomic examinations. However, similar alignments between the MAT11 primer set and the *M. importuna* (Morco1) genome assembly yielded a relatively low identity score (65–70%) and only the MAT11L primer aligned to the putative mating type region identified (Additional file 6). In addition to the *in silico* analysis, PCR experiments were performed with samples from a morel collection obtained in Switzerland. We compared amplicon sequences obtained from our Morchella isolates to those published by the authors of the primer sets (NCBI PopSet 1,213,383,383 and 1,213,383,466). The majority of our sequenced amplicon products obtained from

members of the Elata clade using the MAT11 primer set were highly similar (>98% coverage and identity) to those published by the authors. Alignments between our amplicon products and the putative *MAT1-1-1* sequence obtained from *M. importuna* also showed high sequence similarity (90–97% identity). This was repeated for the MAT22 primer set with similar results from comparisons with the author published amplicon sequences (>90% coverage and identity), but the comparisons with the putative MAT1-2-1 sequence from *M. importuna* revealed relatively lower sequence similarity (74–90% identity).

### 5.2.3 Evaluating the design of previously published MAT primers for Esculenta clade

In order to evaluate the primers for the Esculenta clade in a similar fashion, we first aligned the putative mating type gene sequences from *M. importuna* to the genome of *M. crassipes* strain M10. An alignment (92% coverage and 73.25% identity) was found for the *MAT1-1-1* sequence, while no alignment was found for the *MAT1-2-1* sequence. This putative *MAT1-1-1* gene was located between the APN2 and SLA2 gene annotations. In a similar fashion to the investigation of the Elata clade primers, the EMAT primer sets were aligned to *M. crassipes* strain M10 genome assembly. A total of three amplicon products were predicted for the EMAT1-2 primer set (EMAT1-2 L/R) and no amplicon products were predicted for the EMAT1-1 primer set (EMAT1-1 L/R; Additional file 5). One of the three predicted amplicon products overlapped with the genome region that aligned with the putative MAT1-1-1 sequence identified in the *M. importuna* alignment, while the other two were located on separate contigs (Additional file 5). Alignments between the amplicon sequences we obtained and the published amplicon sequences from Du et al. (2020) (NCBI PopSet 1,809,496,744 and 1,809,496,908) generated using the same primers found no significant alignments for either the EMAT1-1 or EMAT1-2 primer set. Alignments between our amplicon products obtained using the EMAT1-1 primers and the NCBI nucleotide database yielded no significant similarity with any sequence, while the amplicon sequences we obtained from the EMAT1-2 primer sets had DNA dependent *RNA polymerase II (RPB2)* sequences from *Morchella* as the top hits.

## 5.3 Conclusion

The importance of sexual reproduction in the life-cycle of numerous species used for human consumption (e.g., *Morchella* spp.) (Pilz et al. 2007), medicine, or as insect pests biocontrol agents (e.g., Cordyceps sp.) (Zheng et al. 2013; Zou et al. 2019) makes a general understanding of fungal sexual reproduction relevant to many fields. This knowledge is particularly essential in conservation biology, where the potential for species invasiveness and genetic recombination between geographically isolated populations depends strongly on the possibility of genetic exchange and the formation of viable hybrids. In addition, the study of mating types also contributes to phylogenetic studies that aid in understanding the evolution of fungi (Du et al. 2017; Zou et al. 2019), as mating genes generally evolve more rapidly than non-mating ones, and can delimit species (Chai et al. 2019). In the specific case of morels, even though it has been possible to cultivate morels, the exact mechanisms underlying fruiting body formation are still not completely understood. It is generally assumed that two aspects are essential for the formation of fruiting-bodies: the intricate ecological requirements of the fungus, which include climatic, edaphic and biotic factors; as well as the possibility to achieve a sexual cycle through the appropriate encounter of compatible participants (Liu et al. 2018a). Morels were long considered as strictly heterothallic and thus, encounters of mycelia carrying the two opposite idiomorphs was necessary to produce fruiting bodies. However, recently, it has been shown that some species such as M. importuna can produce ascospores containing both mating types. These pseudohomothallic sexual spores give rise to fertile ascocarps and are easier to

cultivate in comparison to heterothallic species or strains (Du et Yang 2021). Therefore, a reliable way to identify mating types in these fungi is necessary. For this purpose, primer pairs have been designed to rapidly and readily amplify by PCR mating-type genes *MAT1-1-1* and *MAT1-2-1* in black (Du et al. 2017) and yellow morels (Du et al. 2020). However, our analyses indicated that three out of four of those primer pairs are not reliable. The *in silico* analysis and the complementary *in vitro* confirmation presented here demonstrated that only the MAT22L/R was trustworthy as it did not lead to of-targets, which are problematic in the case of MAT11L/R. In contrast, the reported EMAT1-1 L/R and EMAT1-2 L/R primer sequences appear as inappropriate for assessing mating genotypes in *Morchella* since they target other genetic regions. For future studies, the in silico and experimental validation of other primer sets (Chai et al. 2017, 2019), or the design of new primer sets based on genomes from single-ascospore cultures genome is still needed to identify putative MAT idiomorphs and sexual reproduction strategies in Swiss morels.

## 5.4 Declarations

**Abbreviations**

HMM: Hidden Markov model; MAT: Mating type; *RPB2: RNA polymerase II subunit*; tntBLAST: Thermonucleotide BLAST.

**Supplementary material**

The online version contains supplementary material available at https://doi. org/10.1186/s43008-022-00101-6.

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**Authors' contributions**

MC conducted the lab experiments and wrote the manuscript. AJR performed the bioinformatic analyses and wrote the manuscript. PH helped processing morel collections. PSC reviewed the manuscript. PJ and SB designed the study, analyzed the data and reviewed the manuscript. All authors read and approved the final manuscript.

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**Availability of data and materials**

All data generated or analyzed during this study are included in this published article and its supplementary information files S1 and S6.

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# 6. Mating types (MAT) in Swiss morels (*Morchella* spp.)

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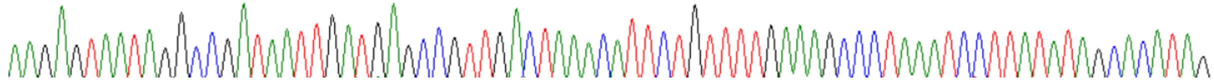

### Foreword

This chapter is the follow-up of section 5, that evaluates the reliability of four other PCR primer pairs designed to amplify mating type genes in *Morchella* Sect. *Morchella* and Sect. *Distantes*. This chapter also presents the mating genotype in Swiss morels, and the genetic structure of the mating type locus of two Swiss strains. Except the extraction of two genomes, and the *in silico* analysis of the primer pairs, I generated and analyzed all the data, redacted, and reviewed the text.

### Abstract

True morels (*Morchella*) are edible fungi that can be cultivated in crops. However, the cultures are not stable, mostly due to climatic variations and poor understanding of the reproduction system. Studying sexual reproduction in *Morchella* is then crucial for agriculture. In this study, the mating types of Swiss morels was investigated in two ways: (1) the MAT1-1:MAT1-2 ratio in single-ascospore cultures was evaluated, and (2) the genomic structure of the MAT1-1 locus was identified. For this purpose, the reliability of mating type primers was first evaluated. Although the *in silico* analysis confirmed that the primers targeted the expected genetic region with no off-targets, the PCR analysis indicated that weak signals corresponding to off-targets were present even after parameters optimization. 4/37 isolates were considered as (pseudo)homothallic, and 33/37 as heterothallic. The analysis of the MAT1-1 structure in two whole genomes revealed the presence of *MAT1-1-1, MAT1-1-10* and *MAT1-1-11* in the black morel isolate, while *MAT1-1-11* was absent in the yellow morel isolate.

**Keywords:** sexual reproduction, single-ascospore culture, phylogenetics, genomic analysis, mating type structure.

## 6.1 Introduction

Filamentous ascomycetes are characterized by their ability to sexually produce multicellular structures (fruiting bodies) that harbor specialized bag-shaped structures (asci) containing ascospores (Coppin et al., 1997). Specific genetic and environmental conditions have to be met for sexual reproduction to occur and fruiting body to form (Pöggeler et al., 2018). However, these processes remain unclear in the true morel (genus *Morchella*) (Du & Yang, 2021), a prized ascomycete collected in the wild worldwide for its culinary and medicinal properties (Tietel & Masaphy, 2018). To meet high demand, commercial crop cultivation of *Morchella* fruiting bodies occurs in China at large scale since 2012, where it reached 10’000 ha after 2018 (Xu et al., 2022). However, cultures are unstable (Du & Yang, 2021; Liu et al., 2018), mainly due to climatic variations and poor understanding of the biological processes responsible for fruiting body formation (Xu et al., 2022).

In ascomycetes, there are three principal modes of sexual reproduction that can all appear within a single genus: (1) homothallism, (2) pseudohomothallism (or secondary homothallism) and (3) heterothallism (Witthuhn et al., 2000; Zheng et al., 2013). The discovery of the third mode led to find the mating type (MAT) locus, a unique genetic region existing in two versions (MAT1-1 and MAT1-2) and governing sexual reproduction in ascomycetes (Coppin et al., 1997). Usually, the combination of both mating types is necessary to accomplish sexual reproduction (Coppin et al., 1997; Zou et al., 2019). In homothallic species, both MAT are carried in a single haploid genome, making them self-fertile. In pseudohomothallic species, single ascospores and vegetative hyphal cells usually contain two haploid nuclei, each for one mating type. In heterothallic species, individuals carrying opposite MAT must meet to reproduce sexually (Coppin et al. 1997). The dominant genotype (i.e., the one involving the most in the reproduction) is usually called “maternal” while the complementary MAT is the “paternal” type (Du et al., 2017; Zou et al., 2019). Receptor (ascogonia) and donor (microconidia, macroconidia or hyphae) structures are carried by each individual (Coppin et al., 1997). Fertilization is accomplished when the nucleus of the donor cell enters the receptor cell, multiplying (without fusing) to initiate fruiting body development. Then, both nuclei migrate into the ascogenous hyphae to undergo meiotic divisions that will give rise to asci. Finally, karyogamy occurs and is followed by meiosis and mitosis to produce ascospores (Coppin et al., 1997).

The three modes of reproduction presented in the previous paragraph exist in the genus *Morchella*, although heterothallism remains dominant (Du & Yang, 2021). Pseudohomothallism was found in wild fruiting bodies *Morchella sextelata* (10%), *Morchella importuna* (12.1%), and *Morchella* sp. Mes-15 (1.2%) (Du & Yang, 2021). Based on the genetic analysis of the MAT structure, it has been hypothesized that *Morchella* sp. Mes-15 could be a true homothallic species (Chai et al., 2022). Homothallism (also named unisexual reproduction or haploid meiosis) was detected in *M. importuna*, *Morchella eximia*, *Morchella eximioides*, *Morchella norvegiensis* and *Morchella* sp. Mel-13, but the resulting fruiting bodies were sterile (i.e., did not produce any ascospore) and it was thus suggested to be the result of failed sexual reproduction (Du et al., 2017). However, the genetic organization of the mating type genes *MAT1-1-10, MAT1-1-1* (representative of MAT1-1 locus) and *MAT1-2-1* (representative of MAT1-2 locus) indicated that *Morchella rufobrunnea* and *Morchella peruviana* were truly homothallic, and could represent the ancestral sexual reproduction mode in *Morchella* (Chai et al., 2022).

Understanding sexual reproduction is crucial for morel cultivation and to understand *Morchella* evolution, the present study aimed to investigate the sexual reproduction modes in Swiss morels. For this, single-ascospore mycelial cultures were generated, and the presence of *MAT1-1-1* and *MAT1-2-1* was assessed by polymerase chain reaction (PCR). To further study the genetic organization of the MAT loci, the full genomes of two single-ascospore (*Morchella* sp. M21-48-1 and M21-48-2) and two

multi-ascospore (*Morchella helvetica* M19-43 and *Morchella esculenta* M19-34) isolates were generated. The mating type locus of two of the genomes was analyzed.

## 6.2 Material and methods

### 6.2.1 Fungal material

*Morchella* fruiting bodies that were collected in Switzerland in 2019, 2020 and 2021 (**Table 1**) were used this this study. Two types of mycelial cultures were generated: single-ascospore cultures (SAC), and hymenium-derived cultures (HYM). SAC were generated by inoculating ascospores (obtained by spore print from mature fruiting bodies) on potato dextrose agar (PDA, 39 g/L, Sigma-Aldrich) that were let 1 day at 22-23°C in darkness. Under an H600L stereo microscope (Nikon, Japan), germinating single-ascospores were picked with a sterile Fine-ject® needle (26Gx1, 0.45x25mm) and transferred on new PDA plates, and incubated as mentioned before. HYM cultures were generated by inoculation of a piece (about 1 $cm^3$) of dry or frozen hymenium of *Morchella* on PDA. If needed, cultures were purified after one day to eliminate bacterial or fungal contaminants. For the extraction of high-quality genomic DNA and RNA (see **section 2.5**), strain M19-43 (HYM) was additionally cultured in liquid medium (Malt Broth, MB; Sigma-Aldrich) within glass flasks, and in malt-agar plates on EDTA (ethylenediamine tetraacetic acid)-treated cellophane to ensure convenient removal of mycelium, as detailed in Casarrubia et al. (2016) (Casarrubia et al., 2016).

**Table 1**. *Morchella* strains that were used in this study. The analysis in which they were involved is indicated. SAC= single-ascospore culture; HYM= hymenium-derived culture; gDNA= extraction of high-quality genomic DNA.

| Strain | Species | Origin of the mycelial culture | Purpose of analysis |
|---|---|---|---|
| M19-34 | *M. esculenta* | HYM | gDNA |
| M19-43 | *M. helvetica* | SAC, 7 cultures; HYM | MAT determination in SAC; gDNA (HYM) |
| M21-43 | *M. esculenta* | SAC, 6 cultures | MAT determination in SAC |
| M21-2 | *Morchella* sp. Mel-44 | SAC, 6 cultures | MAT determination in SAC |
| M21-48 | *Morchella* sp. | SAC, 18 cultures | MAT determination in SAC; gDNA |

### 6.2.2 DNA extraction and sequencing of MAT genes

DNA was extracted from mycelium that was scraped from 7-days old mycelial cultures, using Quick-DNA Fungal/Bacterial Miniprep Kits (Zymo Research, USA) and following the protocol provided by the manufacturer. Eluted DNA was quantified with a Qubit kit (Invitrogen, USA) using the Broad Range buffer and reagent. DNA was then diluted with PCR-grade water to a concentration of 2 ng/µL to be used as template in polymerase chain reactions (PCR). In each sample, coding regions of the mating-type genes *MAT1-1-1* and *MAT1-2-1* were amplified (see **Table 2** for detailed primer information). For each sample, the PCR mix contained PCR-grade water, 2X Genie Fusion Ultra High-Fidelity (Assay Genie, Ireland), 0.4 µM of both forward and reverse primers and 1 µL of 2 ng/µL DNA. Amplifications were performed in a Thermo Scientific Arktik thermal cycler. The following parameters were used: denaturation at 95°C for 2 min, 30 cycles of denaturation, annealing and elongation (95°C for 15 sec, 60°C [yellow]; 63°C [black] for 15 sec, 72°C for 30 sec), final elongation at 72°C for 5 min, end at 15°C. PCR products were then loaded on a 1.2% agarose gel that underwent electrophoresis (100 mV, 30 min). Amplicons were visualized under UVs in a Genoplex VWR transilluminator.

For samples used in the phylogenetic analysis, positive PCR products (i.e. single band at the expected size) were then purified with a MultiScreen® Filter Plates PCR µ96 (Millipore Corporation, USA) as follows: in each well, the PCR product and 50 µL of PCR-grade water was added; a vacuum of 20 bars was applied on the wells until they dry; 20 µL of PCR-grade water was added to each well; after 2 min, DNA contained in the membrane from each well was resuspended by pipetting up and down 20 times. Once purified, the PCR products were quantified by Qubit. Final DNA concentration was adjusted at 2-40 ng/µL and sent to Fasteris (Switzerland) for Sanger sequencing.

**Table 2**. Primers that were used to amplify the coding regions of gene *MAT1-1-1* and *MAT1-2-1* in black (Elata) and yellow (Esculenta) *Morchella* species.

| Clade | Primer | Target region | Sequence 5'-3' | Reference |
|---|---|---|---|---|
| Elata | p7-2f | *MAT1-1-1* | CCGGTTTATCTTACTGGACTGGTTC | (Chai et al., 2022) |
| Elata | p8-5r | *MAT1-1-1* | TGGAATGTCTGTGATTGAGGCTGTG | (Chai et al., 2022) |
| Elata | p10-1f | *MAT1-2-1* | GGCCAGAACAGATGCTCGAAGAAGC | (Chai et al., 2022) |
| Elata | p10-5r | *MAT1-2-1* | GTGGCAACTCCCAAAGCATGATCAA | (Chai et al., 2022) |
| Esculenta | p5-3f | *MAT1-1-1* | ATGTCACTTCGCCCGGTTTATC | (Chai et al., 2022) |
| Esculenta | p5-3r | *MAT1-1-1* | CATCGCAATGTCGTGTGTTCTT | (Chai et al., 2022) |
| Esculenta | p4-3f | *MAT1-2-1* | GACTACGATCGAATAATGGCTCCGC | (Chai et al., 2022) |
| Esculenta | p4-3r | *MAT1-2-1* | CGGTCTTAGCTTCGTCGACTTTAGT | (Chai et al., 2022) |

### 6.2.3 *In silico* verification of the MAT genes sequences

To verify that the *MAT1-1-1* and *MAT1-2-1* sequences that were generated did correspond to *Morchella* mating type genes, they were aligned against genomes of *Morchella* that have been generated within the framework of projects 1000 Fungal Genomes (1KFG) and CSP1974 by the DOE Joint Genome Institute (JGI). This was performed by Aaron J. Robinson (Bioscience Division of Los Alamos National Laboratory, USA). The percent identity between the MAT sequences and the annotated MAT genes was analysed.

### 6.2.4 Extraction of high-quality genomic DNA and RNA

The full genomes of two single-ascospore cultures (*Morchella* sp. M21-48-1, *MAT1-1-1* predominant; M21-48-2, *MAT1-2-1*) and one hymenium-derived culture (*M. esculenta* M19-34) were extracted using an SDS (sodium dodecyl sulfate)-based DNA extraction procedure (Zhou et al., 1996). RNA was extracted using *Quick*-RNA Miniprep Kit (Zymo Research, USA) following the manufacturer's protocol. Genomes sequenced with PacBio (Pacific Biosciences, USA), assembled with Flye, and annotated with the JGI Annotation Pipeline (Joint Genome Institute, USA). The transcriptomes were sequenced with Illumina (Illumina Inc., USA) and assembled with Trinity. The DNA and RNA extractions of M19-34, M21-48-1 and M21-48-2 were performed by La Verne Gallegos-Graves (Bioscience Division of Los Alamos National Laboratory, USA). Genomic annotations were conducted by Richard Hayes (JGI Science Department of Joint Genome Institute, USA).

The full genome of *M. helvetica* M19-43 (HYM) was extracted using a CTAB (cetyltrimethylammonium bromide)-based DNA extraction procedure, that has been optimized by Laure Fauchery (Ecogenomic team of INRAE Nancy, France) and detailed in **Supplementary S1**. RNA was extracted using RNeasy Mini Kit (Qiagen, Germany) following a modified protocol detailed in **Supplementary S2**. Sequencing and annotation were performed by the JGI as explained in the previous paragraph. Library creation is still under processing.

### 6.2.5 Genome analysis

Genomes of *Morchella* sp. M21-48-1 and *M. esculenta* M19-34 were first analyzed within the MycoCosm JGI platform (https://mycocosm.jgi.doe.gov/mycocosm/home), where mating type genes (*MAT1-1-1, MAT1-1-10, MAT1-1-11, MAT1-2-1*) were searched in the genomes using BLAST (Altschul et al., 1990). When the MAT loci were identified, the hypothetical proteins contained in the MAT region and the flanking hypothetical proteins were identified using the tool protein BLAST from NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The structure of the MAT loci was displayed by the synteny tool of the MycoCosm JGI platform.

## 6.3 Results

### 6.3.1 Reliability of the *MAT* gene primers

The *in silico* verification of the MAT genes sequences revealed that each primer pair used in this study was able to correctly amplify *Morchella* mating types. The top percent identify between the MAT sequences and the annotated MAT genes from the full genomes varied between 95.882-99.554% for *MAT1-1-1* and 84.858-99.777% for *MAT1-2-1*.

### 6.3.2 *MAT* genes distribution in single-ascospore cultures of *Morchella* spp.

The mating types of 37 single-ascospore cultures was determined by PCR amplification of the coding regions of gene *MAT1-1-1* and *MAT1-2-1* (**Figure 3**). The results are listed below in **Table 3**. The DNA signal at gel electrophoresis was classified into three categories: strong signal (++), weak signal (+) and no signal (-). The sequencing of the PCR products indicated that only the strong (++) signals corresponded to *MAT* genes. The PCR products that displayed a weak (+) signal at gel electrophoresis resulted in sequences that did not correspond to any gene after BLAST search and the sequences were always of poor quality (i.e., contained unidentified nucleotides, see **Supplementary S3**).

This analysis showed four types of MAT distribution in single-ascospore cultures: (I) a strong signal of MAT1-2; (II) a strong MAT1-1 + a weak MAT1-2; (III) a weak MAT1-1 + a strong MAT1-2; (IV) a strong MAT1-1 + a strong MAT1-2 (**Table 3**).

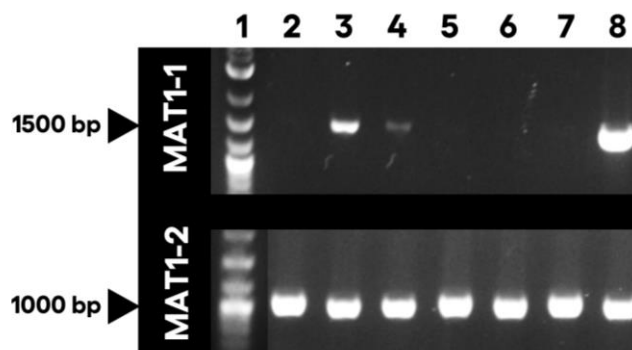


**Figure 3**. Example of a gel electrophoresis of PCR products of the amplification of the coding regions of *MAT1-1-1* (top) and *MAT1-2-1* (bottom) in *Morchella*. Lane 1= Quick-load® Purple 1kb plus DNA ladder; Lane 2-8= *Morchella helvetica* M19-43-1 to M19-43-7. In this example, lanes 2, 5, 6 and 7 represent inexistant (-) MAT1-1 and strong (++) MAT1-2 signals, lane 8 represents strong (++) MAT1-1 and MAT1-2 signals, and lanes 3 and 4 represents weak (+) MAT1-1 and strong (++) MAT1-2 signals.

**Table 3**. Mating-type determination in single-ascospore cultures of *Morchella* spp. The intensity of the DNA signal at gel electrophoresis was noted as strong (++), weak (+) and inexistant (-). The PCR products verified by sequencing and corresponding to *MAT* genes are highlighted in green. The weak signals (+) correspond to off-targets.

| Isolate | Species | Type | MAT1-1 | MAT1-2 | Isolate | Species | Type | MAT1-1 | MAT1-2 |
|---|---|---|---|---|---|---|---|---|---|
| M19-43-1 | *M. helvetica* M19-43 | I | - | ++ | M21-48-1 | *Morchella* sp. M21-48 | II | ++ | + |
| M19-43-2 | | III | + | ++ | M21-48-2 | | I | - | ++ |
| M19-43-3 | | III | + | ++ | M21-48-3 | | I | - | ++ |
| M19-43-4 | | I | - | ++ | M21-48-4 | | I | - | ++ |
| M19-43-5 | | I | - | ++ | M21-48-5 | | II | ++ | + |
| M19-43-6 | | I | - | ++ | M21-48-6 | | I | - | ++ |
| M19-43-7 | | IV | ++ | ++ | M21-48-7 | | I | - | ++ |
| M21-43-1 | *M. esculenta* M21-43 | III | + | ++ | M21-48-8 | | I | - | ++ |
| M21-43-2 | | III | + | ++ | M21-48-11 | | III | + | ++ |
| M21-43-3 | | III | + | ++ | M21-48-12 | | I | - | ++ |
| M21-43-4 | | III | + | ++ | M21-48-13 | | I | - | ++ |
| M21-43-5 | | III | + | ++ | M21-48-14 | | II | ++ | + |
| M21-43-6 | | III | + | ++ | M21-48-15 | | I | - | ++ |
| M21-2-1 | *Morchella* sp. Mel-44 M21-2 | III | + | ++ | M21-48-16 | | III | + | ++ |
| M21-2-2 | | IV | ++ | ++ | M21-48-17 | | II | ++ | + |
| M21-2-3 | | IV | ++ | ++ | M21-48-18 | | II | ++ | + |
| M21-2-4 | | III | + | ++ | M21-48-19 | | I | - | ++ |
| M21-2-5 | | III | + | ++ | M21-48-20 | | II | ++ | + |
| M21-2-6 | | III | + | ++ | | | | | |

### 6.3.3 Genomic analysis

#### 6.3.3.1 *Morchella* sp. M21-48-1

The full genome of isolate *Morchella* sp. M21-48-1 (putative MAT1-1 isolate) was annotated ("*Morchella* sp. M21-48-1 v1.0"). Genome assembly size was 54.07 Mbp, with a sequencing read coverage depth of 70.37x. The 120 contigs could be assembled with zero remaining gaps. A total of 11'265 genes were predicted.

One mating type region was annotated (MAT1-1 locus protein, four exons). It was located on scaffold 6, between 501600-503367 bp, for a total length of 1767 bp. A BLAST search of *MAT1-1-1* (query *M. pulchella* YAASM30 (MG680978.1)) yielded 99.9% identity with scaffold 6:501803-503284. The search

of other *MAT* genes yielded no result, indicating that the annotated MAT1-1 only contained this one gene. The comparison of this region with the genome of *M. pulchella* Mel-31 (v1.0) however indicated that the MAT1-1 locus was larger than suggested by the automatic annotation. Analysis of the flanking coding regions of *MAT1-1-1* revealed that hypothetical protein 410721 corresponded to MAT1-1-10 (98.1% identity with MAT1-1-10 of *Morchella purpurascens* (QQL94643.1)), and hypothetical protein 353363 corresponded to MAT1-1-11 (99.46% identity with MAT1-1-11 of *M. pulchella* (WLS48977.1)). The 5' flanking region of the MAT genes (hypothetical protein 159509) corresponded to the mitochondrial inner membrane MBA1 (91.35% identity with MBA1 of *M. importuna* (AVI60815.1)), while the 3' flanking region corresponded to the succinate dehydrogenase 2 (SDH2) (69.04% identity with SDH2 of *M. importuna* (AVI60819.1)). The complete MAT1-1 idiomorph, including *MBA1* and *SDH2*, therefore measured 14'041 bp (scaffold6:498514-512555) (**Figure 4**).

No MAT1-2 was annotated, and further verification indicated there was no match for *MAT1-2-1* in the genome of M21-48-1.

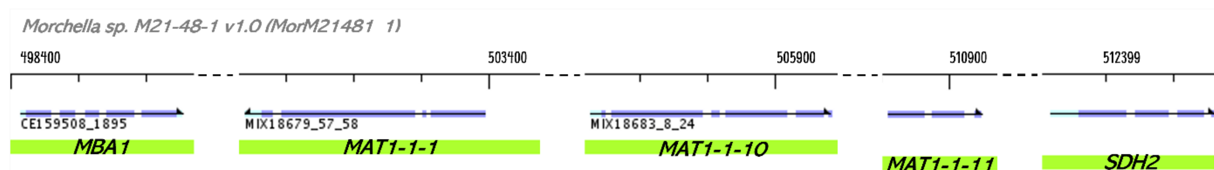


**Figure 4**. Structure (5'-3') of the mating type locus MAT1-1 of *Morchella* sp. M21-48-1. It contains three genes, *MAT1-1-1, MAT1-1-10* and *MAT1-1-11.* The flanking regions correspond to the mitochondrial inner membrane 1 (MBA1) and succinate dehydrogenase 2 (SDH2).

6.3.3.2 *Morchella esculenta* M19-34

The full genome of *M. esculenta* M19-34 was annotated ("*Morchella* sp. M19-34m"). Genome assembly size was 51.98 Mbp. The 62 contigs were assembled with 32 remaining gaps. A total of 11'219 genes were predicted.

Two mating type proteins were annotated, MAT1-1-1 (protein 450057) and MAT1-1-10 (protein 450053). BLAST search indicated both genes were located on scaffold 11 between 1179373-1181019 bp (*MAT1-1-10*) and 1183037-1184793 bp (*MAT1-1-1*). There was no match with *MAT1-1-11*. BLAST search in the NCBI database confirmed the proteins were correctly annotated (99.16% identity for MAT1-1-10 with *Morchella* sp. Mes-20 (AVI61143.1); 98.71% identity for MAT1-1-1 with *Morchella* sp. Mes-20 (AVI61084.1)). As for M21-48-1, the 5' flanking region corresponded to *SDH2*, and the 3' flanking region to *MBA1*. The complete MAT1-1 locus therefore measured 12'579 bp (scaffold 11:1175350-1187929) (**Figure 5**).

No MAT1-2 was annotated, and further verification indicated there was no match for *MAT1-2-1* in the genome of M19-34.

Genomes of *Morchella* sp. M21-48-2 (putative MAT1-2 isolate) and *M. helvetica* M19-43 were not annotated yet and could therefore not be analyzed.

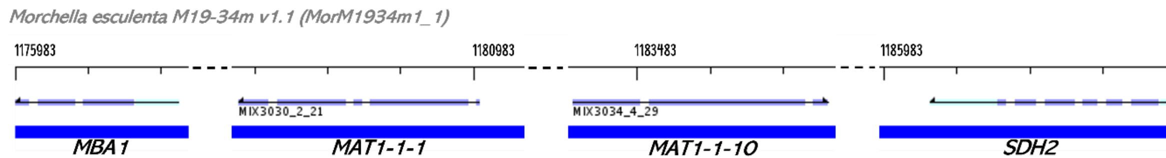


**Figure 5**. Structure (5'-3') of the mating type locus MAT1-1 of *Morchella esculenta* M19-34. It contains two genes, *MAT1-1-1* and *MAT1-1-10*. The flanking regions correspond to the mitochondrial inner membrane 1 (MBA1) and succinate dehydrogenase 2 (SDH2).

## 6.4 Discussion

Analyzing mating type genes in *Morchella* is crucial for the selection of reliable strain for cultivation, as the encountering of opposite mating types (*MAT1-1-1* and *MAT1-2-1*) is required in most of the *Morchella* species or isolates to produce fruiting bodies resulting from sexual reproduction (Du & Yang, 2021). In addition, recent studies revealed that mating systems in *Morchella* could be more complex than previously acknowledged, unveiling the presence of true homothallic specimens (Chai et al., 2022; Du & Yang, 2021). Better understanding mating systems in *Morchella* is crucial to improve the stability of *Morchella* cultivation, as genetic loss of one mating type may happen during cultivation. Having consistent genetic tools to detect both mating types in *Morchella* is therefore important for agricultural research. For this reason, the principal goal of this study was to verify the reliability of primers amplifying mating type genes in both *Morchella* Section *Morchella* (Esculenta clade) and Sect. *Distantes* (Elata clade). The four pairs of mating type gene primers developed by Chai et al. (Chai et al., 2022) (see **Table 2**) were confirmed to be reliable by comparing the *MAT1-1-1* and *MAT1-2-1* sequences generated in this study with full genomes of *Morchella* species. This revealed high percent identities (>84%) an no off-targets, contrarily to previous analysis of MAT11L/R (Du et al., 2017), EMAT1-1L/R and EMAT1-2L/R (Du et al., 2020) primer pairs (Cravero et al., 2022).

MAT sequences of *Morchella* were generated in this chapter to determine MAT genes distribution in SAC of *Morchella*. In addition, the analysis of the whole genomes allowed to determine the genetic structure of the MAT loci, and further verify the reliability of the MAT primers.

### 6.4.1 Reliability of mating type primers and MAT distribution in Swiss morels

The first goal was achieved as the reliability of the MAT primers from Chai et al. was assessed. The *in silico* analysis revealed that the primers were reliable as they did target the MAT region. It was unexpected that the PCR analysis revealed frequent off-targets for both primer pairs (**Table 3**). These were represented by weak signals at gel electrophoresis, that corresponded to the expected length of the MAT products (i.e., 1500 bp for *MAT1-1-1* and 1000 bp for *MAT1-2-1,* **Figure 3**). This was confirmed by the sequencing of the products, which indicated that only the products producing strong signals at gel electrophoresis corresponded to *MAT* gene sequences. More importantly, the genomic analysis of the isolate *Morchella* sp. M21-48-1, that displayed PCR signals for *MAT1-1-1* (strong) and *MAT1-2-1* (weak), revealed that only the MAT1-1 locus was present in the full genome (**Figure 4**).

It was anticipated that the SAC would display only one MAT and have a MAT1-1:MAT1-2 ratio not significantly diverging from 1:1, as it is the case in heterothallic strains (Chai et al., 2017, 2019). Also, it was expected that most of the Swiss specimens would be heterothallic, because this is the main mode of reproduction that has been reported in *Morchella* Sect. *Distantes* (Chai et al., 2017; Pagliaccia et al., 2011) and Sect. *Morchella* (Chai et al., 2019). However, as indicated prevously,

pseudohomothallic (*M. importuna, M. sextelata, Morchella* sp. Mes-15) (Du & Yang, 2021) and homothallic (*M. importuna* (Du & Yang, 2021), *M. rufobrunnea* (Chai et al., 2022)) species have been reported. In these cases, SAC display two MAT. The prevalence of this phenomenon was measured between 1.2%-12.1% depending on the species (Du & Yang, 2021).

In the Swiss strains that were investigated, there were only three SAC displaying two (*MAT1-1-1* and *MAT1-2-1*) simultaneously strong signals (type IV, see **Table 3**), i.e., representing (pseudo)homothallic isolates: *M. helvetica* M19-43-7, *Morchella* sp. Mel-44 M21-2-2, *Morchella* sp. Mel-44 M21-2-3. The results from the PCR analysis are hence in concordance with the literature cited above and our expected results, as the majority of the *Morchella* single-ascospore cultures displayed only one mating type, this suggesting a dominantly heterothallic reproductive system.

To distinguish between homothallism and pseudohomothallism in the three isolates cited above, the genetic structure of the complete MAT loci should be investigated. In true homothallic organisms, the haploid genome would contain linked *MAT1-1-1* and *MAT1-2-1* genes that would be flanked by *SDH2* and *MBA1* (Chai et al., 2022).

### 6.4.2 Genetic structure of MAT1-1 in *Morchella* sp. and *Morchella esculenta*

The genomic analysis of one black morel (M21-48-1) and one yellow morel (M19-34) suggested that both isolates are heterothallic, as they only contained the MAT1-1 locus which was flanked by the highly conserved genes *MBA1* and *SDH2* (Chai et al., 2022). The MAT1-1 locus of M21-48-1 contained three genes (*MAT1-1-1, MAT1-1-10, MAT1-1-11*) (**Figure 4**). This was concordant with the structure of five other black morels (*Morchella eximia, M. importuna, M. sextelata, M. pulchella, M. purpurascens*) (Chai et al., 2022). Further analysis of the genes indicated that *MAT1-1-11* of M21-48-1 only measured 555 bp. In the genomes of close relatives of *Morchella* sp. M21-48-1, *M. purpurascens* and *M. pulchella* (both heterothallic), a truncated *MAT1-1-11* (about 900 bp) was present in MAT1-1 (Chai et al., 2022). The complete *MAT1-1-11* (about 1800 bp) was present only in the MAT1-2 isolates, next to *MAT1-2-1* (Chai et al., 2022). It has been proposed that the *MAT1-1-11* originated from MAT1-1, and unconventionally integrated into MAT1-2 in some species (Chai et al., 2022). In *Morchella* sp. M21-48-1, the short fragment of *MAT1-1-11* present in the MAT1-1 locus is likely to be truncated as well. Accordingly, it is also expected to find a complete *MAT1-1-11* in MAT1-2 isolates of *Morchella* sp. M21-48. This will be verified when the genomic annotation of M21-48-2 will be completed.

In the yellow morel isolate (M19-34), *MAT1-1-11* was completely absent from the MAT1-1 locus (**Figure 5**). This was concordant with the study of Chai et al. (2022), where the four yellow morels that were studied (*Morchella* sp. Mes-6, Mes-15, Mes-19, Mes-20). All of these only presented *MAT1-1-1* and *MAT1-1-10* in their MAT1-1 locus (Chai et al., 2022). It was hypothesized that *MAT1-1-11* is the most recent mating gene that appeared during evolution, contrarily to *MAT1-1-1, MAT1-1-10* and *MAT1-2-1* that already existed in the basal species *M. rufobrunnea* (Chai et al., 2022).

## 6.5 Conclusion

The *in silico* analysis indicated that the PCR primers that were used were reliable (i.e., produced no off-targets), but the PCR yielded unexpected results, as more than a half of the samples displayed off-target signals in addition to the expected *MAT*. This was confirmed by sequencing of the products and by the whole genome sequencing of one of the isolates. This suggests that bioinformatic analysis of the primers should be deepened to identify the source of the off-targets. However, thanks to

sequencing, the mating type distribution in the Swiss samples could nevertheless be reliably assessed. It revealed that 3/37 single-ascospore isolates displayed (pseudo)homothallism, this being in accordance with the literature suggesting heterothallism as the main reproduction strategy in *Morchella*.

## 6.6 Acknowledgements

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## Supplementary material S1 - Extraction of high-quality DNA for Genome Sequencing

From the protocol of Laure Fauchery, INRAe Nancy, Ecogenomic team.

Melissa Cravero, Laboratory of microbiology of the University of Neuchâtel, 19.04.2023.

ALL THE STEPS ARE PERFORMED IN A NON-STERILE ENVIRONMENT. USE A FUME HOOD WHEN PERFORMING THE DNA AND RNA EXTRACTIONS. WEAR GLOVES AND COAT ANYTIME.

### Required reagents

To prepare in stock solutions. Keep at room temperature (RT).

Warning: the stock solution may not correspond to the final solution needed in the protocol.

- Potassium Acetate (KAc) 5M, pH 7.5
- Sodium Acetate (NaAc) 3M
- RNAse A, 10 mg/mL
- Proteinase K, 20 mg/mL
- PVP 1%
- Chlorom:Isoamyl alcohol (24:1)
- Isopropanol 100%
- Ethanol 70%
- BUFFER A: 0.35M Sorbitol

0.1M Tris-HCl, pH 9
5 mM EDTA, pH 8

- BUFFER B: 0.2M Tris-HCl, pH 9

50 mM EDTA, pH 8
2M NaCl
2% CTAB

- BUFFER C: 5% Sarkosyl (N-lauroylsarcosine sodium salt SIGMA L5125)

### Preparation of the lysis buffer

To make the same day as the extraction. These volumes can be measured with a Falcon tube.

➔ Lysis Buffer For 17.5 mL (per 500-700 mg of starting material)

- 2.5 V of Buffer A 6.5 mL
- 2.5 V of Buffer B 6.5 mL
- V of Buffer C 2.6 mL
- PVP 0.1% 1.75 mL
- Proteinase K 125 µL

### Additional material

▪ Qiagen Genome-tip 500/G, Qiagen QC, QBT and QF Buffers

▪ 50 mL Falcon tubes

▪ Pestle and mortar

▪ Ice

▪ Liquid nitrogen

**Preparation of the starting material**

Weeks (or days) before the extraction, grow the mycelium in solid (over a sterile cellophane membrane) or liquid culture to get at least 4g of mycelium (also needed for the RNA extraction).

Warning: the samples must never thaw once flash-frozen. Always preliminarily place the material (tubes, pestle and mortar, etc.) on liquid nitrogen. Always keep the samples in liquid nitrogen when not used.

1. Place a Falcon tube in liquid nitrogen (LN), then place it on ice. Fill the tube with LN. During step 2, add LN regularly to keep the tube filled up.
2. Harvest the mycelium with a scalpel blade and put it in the Falcon tube (flash-freeze). Fill the tube entirely by regularly ramming the frozen mycelium with the blade. Warning: harvest the younger mycelium as possible because accumulated secondary metabolites can interfere with the extraction. Do not harvest any piece of agar because it interferes with the extraction.
3. When the tube is filled, let evaporate the LN from the tube.
4. Proceed to grinding using a pestle and mortar or a laboratory grinder. Warning: this step is very important and impacts the final yield and quality of the gDNA. The powder must be as fine as possible and must never thaw.

**Protocol of extraction**

Warning: never vortex the samples, except at step 4. Always poor the liquids from the opposite side of the pellet to avoid losing it. Mix well each reagent before use. Perform the extraction with at least 2 samples (2 x 500-700 mg). Proceed under a fume hood.

1. Prewarm Buffer B at 65°C. Prewarm a heating bath at 65°C. Prepare the centrifuge at 4°C.
2. Prepare the Lysis Buffer (17.5 mL per 500-700 mg of sample).
3. Transfer 500-700 mg of sample in a 50 mL Falcon tube.
4. Add 17.5 mL of Lysis Buffer per tube and mix by vortexing until it is completely homogenous.
5. Homogenize the Lysis Buffer between each sample.
6. Incubate for 30-60 min at 65°C. Invert the tubes to mix the solution every 5 min.
7. Open the tubes and check for the presence of white filaments of polysaccharides. They can also be absent depending on the species.
8. Prewarm TE or DNAse-free water at 65°C.
9. Add 0.33 V (5.75 mL) of KAc 5M, invert the tubes once to mix the solution, and incubate for 30-60 min on ice. The samples can be let overnight at 4°C t improve yield.
10. Centrifuge for 20 min at 5000g at 4°C.
11. Transfer the supernatant in a new 50 mL Falcon tube, add 1 V (15 mL) of Chlorom:Isoamyl alcohol (24:1). This step can be repeated to eliminate all the contaminants.
12. Centrifuge for 10 min at 4000g at 4°C.
13. Transfer the aqueous phase in a 50 mL Nalgene tube (chloroform-resistant).
14. Add 1/10 V (1.25 mL) of NaAc and 0.8-1 V (12.5 mL) of Isopropanol (RT). Incubate 5 min at RT.

15. Centrifuge for 30 min at 10’000g at 4°C.
16. Carefully discard the supernatant.
17. Wash the pellet by adding 2 mL of Ethanol 70% directly on the pellet. Try to take it off the wall of the tube by shaking gently by hand.
18. The pellets from multiple tubes can be pooled in a single one.
19. Centrifuge for 10 min at 10’000g at 4°C.
20. Carefully discard the supernatant and dry the pellet by flipping the open tube on an absorbent paper. Let dry exactly 5 min at RT.
21. Resuspend the pellet in 500 µL (per sample; adapt to the final volume if the samples were pooled) of TE or DNAse-free water at 65°C. To help the DNA resuspend, poor the tube in a bath at 55°C and shake gently by hand.
22. Store at -80°C overnight. This may help dissolving the remaining solid pellet.

**RNAse treatment**

Warning: proceed under a fume hood as RNase should not be inhaled.

1. When completely dissolved, transfer the DNA solution (by pipetting) from the Falcon to a 1.5 mL microcentrifuge tube.
2. Add 2 µL of RNAse (10 mg/mL) per 100 µL of diluted sample.
3. Incubate 1h30 at 37°C.

**Purification of the gDNA using the Qiagen genomic-tip columns**

This step also removes the RNAse. Warning: use one 500/G Column for maximum 2 g of starting material (= 4 extractions).

1. Prewarm QF Buffer at 50°C.
2. Defrost DNA solution on ice.
3. Place the column on a new Falcon tube.
4. Equilibrate the column with 10 mL of QBT Buffer. Discard the flowthrough.
5. In a Falcon tube, mix 1 mL of DNA with 4 mL of QBT Buffer.
6. Load the mix in the column. Discard the flowthrough.
7. Wash the column with 15 mL of QC Buffer. Discard the flowthrough.
8. Repeat step 7.
9. Place the column on a new Falcon tube and elute DNA with 20 mL of QF Buffer (50°C).
10. Prewarm TE or DNAse-free water at 65°C.
11. Add 1/10 V of NaAc and 1 V of Isopropanol and incubate 5 min at RT. The sample can also be let overnight to increase yield.
12. Centrifuge for 30 min at 10’000g at 4°C.
13. Carefully discard the supernatant.
14. Wash the pellet by adding 2 mL of Ethanol 70% directly on the pellet. Try to take it off the wall of the tube by shaking gently by hand.
15. Centrifuge for 5 min at 10’000g at 4°C.
16. Carefully discard the supernatant and dry the pellet by flipping the open tube on an absorbent paper. Let dry exactly 5 min at RT.

17. Keep the tube horizontal and add 80 µL of TE or DNAse-free water at 65°C directly on the pellet and shake gently by hand to dissolve it. Once dissolved, transfer (by pipetting) the gDNA in a new 1.5 mL microcentrifuge tube. Warning: avoid mixing the DNA with ethanol droplets remaining on the walls of the tube; this will cause contaminations.

The purification in columns can be repeated a second time if contaminations remained, but this will reduce the DNA concentration.

**Quality controls**

▪ Nanodrop: check for contaminations. A260/280 ratio should be between 1.6 and 2.0. A260/230 ratio should be between 1.9 and 2.2 (JGI Guidelines).

▪ Agarose gel: prepare an 1% agarose gel and load 1 to 5 µL of gDNA. The gDNA should measure around 14’000 bp. No smear must appear. No RNA contaminations (= 200 bp fragments) must appear.

▪ Qubit: measure the quantity of dsDNA. The concentration should range between 25-1000 ng/µL.

▪ Volume: 25-400 µL are required.

## Supplementary material S2 - Extraction of RNA for Genome Sequencing

From the protocol of Laure Fauchery, INRAe Nancy, Ecogenomic team.

Melissa Cravero, Laboratory of microbiology of the University of Neuchâtel, 19.04.2023.

ALL THE STEPS ARE PERFORMED IN A NON-STERILE ENVIRONMENT. USE A FUME HOOD WHEN PERFORMING THE RNA EXTRACTIONS. WEAR GLOVES AND COAT ANYTIME. CLEAN WELL THE PLACE AND MATERIAL USED FOR THE RNA EXTRACTION WITH RNASE-ZAP.

**RNA extraction with RNeasy Mini Kit Qiagen**

Warning: the samples must never thaw once flash-frozen. Always preliminarily place the material (tubes) on liquid nitrogen. Always keep the samples in liquid nitrogen when not used. The extraction was adapted from the manufacturer’s protocol.

1. Prewarm RNAse-free water at 56°C.
2. Wash the working place and material with RNase-ZAP.
3. Place 2 x 2 mL microcentrifuge tubes in liquid nitrogen.
4. Fill the tubes with about 50 mg of powdered sample (see gDNA extraction protocol for sample preparation). If the samples defrost, discard them and and start with new tubes.
5. Mix 1 mL of RLC Buffer and 10 µL of beta-mercaptoethanol (for 2 samples).
6. Add 450 µL of the mix to each sample. Vortex well and place the tubes at 56°C for 3 min.
7. Transfer the lysates in the lilac columns.
8. Centrifuge for 2 min at 13’000 g.
9. Transfer the supernatant in a new microcentrifuge tube.

10. Add 0.5 V (200 µL) of ethanol (96-100%) and pipet up and down until homogenous. Directly transfer in the pink columns.
11. Centrifuge for 30 sec at 10’000 g. Discard the flowthrough.
12. Add 700 µl of RW1 Buffer and centrifuge for 30 sec at 10’000 g. Discard the flowthrough.
13. Add 500 µl of RPE Buffer and centrifuge for 30 sec at 10’000 g. Discard the flowthrough. Warning: ensure that ethanol was added to RPE Buffer before use.
14. Repeat step 12.
15. Place the RNeasy spin column in a new 2 ml collection tube and discard the old collection tube with the flowthrough. Centrifuge for 1 min at 13’000 g.
16. For the elution, add 40 µL RNAse-free water (56°C) directly by touching the membrane with the pipet tip.
17. Incubate 1 min at RT.
18. Centrifuge for 1 min at 10’000 g. Rapidly put the samples on ice. They can be let on ice for 1h. Warning: never let RNA samples at RT after elution.
19. Proceed with the DNAse protocol (DNA-free Kit user guide, Invitrogen) following the manufacturer’s protocol, then perform a quality control.

## Supplementary material S3 - Off-targets sequencing

>M21-48-11_MAT1-1 weak signal

NNNNNNNNNNNNCNGANCTGTTNTGAACGNNNATGCATTTNCTAGANNNNNANGNNGTCGTACAATCCTTGGGGTNNNCCCTCTATCTCTCNNACTGN
CTCTATGCCTCANAATGAGGCTCCGAGNTTTNGGATTCTGGGGATTANNNNCCCNGNCGATATATGGNACCCCCNGCTTGCGGGTGGGNATTTCTGGGGC
TNGGGANACCCCTCCCGTTCCAGANATGGCTCACTTTTATGCNTCGACTCATACGTCNTGAAGGAAGGGTTGAGCTANATCCTAAGGTCCCGTACAANTGTG
CANCCCAAAGGCTTTATGGNGAACACNNCANGAGNANTNGGCCGATAATTGATGAGCCGTTGACTAAACNTTCGGTTTTGAAAGCCCTACATCCGTATNNN
GCACATANANNTAAGGAANGAANAATCCCTTTTCTAATTAATATCNNATTTAATTTGTCTCTTTTNNCTTGNATATCNNAGTNCCNGGNAGGGCTANGATTTACN
NNNNTGCTTNTNNCNNNACTCACCANANGNGNNTGGCNACNANAGAANANNAAAGCCNTGTNNTCNAATATANCAAGANTGTNCGCNTNNNACCGNN
ATCNAGGANNGCNCACNTCNNCTCTCNAANATTTTATCNNAGCNCANCTTGNCNAGAATAAACAANATCCCTCCNCTANNTCGNNCCTACACCACTGCN
GTATAAAACTTGANNATGNGAGAAAACNAATGANNNCCACTTGNACTCCANCTGATGCTCNNCCNTGNCTATTGNGACAGTGGNGCCCNTGGANAAGACT
TANTNNGAAANTCATGATATTCCNAATGTNCACTTTCAAGCGCCTACNNTTCCTGACTCTANCACCGNGGAGNATANNNGAGATGGAGNCNATTNNANTGT
CANNCGCATCNNTNANNANATCGAAAGATCNTACNNTNGANCCNNNNNCNNNGGNNGAGCNNNANCANNNNNNCGNTCNAANTAANNNNATTNNT
CNNTATCGNCNCNNCGCTNNNANNNCNCGNCNNNCNNNNCCNATNNANGNTNTAGATCNNCCTNANNNNGGNNTTNNNACCTNNNNNCNTACNN
NNNNNGGNNANNNNGNNCGANNNNACTTCNNNNNNNAATCNNNNNCNNGGAGNNNNNNNNNNCAANCNNCGNANNNNNNANNCCNNNCNNN
NNNNNNNNNNNNNNNNNNNNNNNNTNCCNNNTNNTCNACNNGNNNNN

>M21-48-1_MAT1-2 weak signal

NNNNNNNNNNNNCNNGTTCTGGGCAGNTGGNTCTTGTACCCNGATGTTGGTCAGATCATCAAGATTGANGTAACCNGGGCTCGGGCNGCGAATTGNCAA
TGAGGGTGGAGCTAGCTGTGGATGTGGAGGTGGAGCTGGAGGCGGANGCGGAAGTGGGGCTTGANGTGGANGTGGANACGGAATCNCCAAACTACTACT
GCGATGAANANCGGGGTTACNGATATGCTCCNTATTATCACCAACCGGAACTGACTTCTTACGGCGGCNAACAACATCTTCAGGTTTTCGAGGTGTATACTTAT
ACCCAGGATGCTTTACGTGGTGCTCTCGTGCANATTTTGCGGCCAAATCCTTTTCTANATTANCAATATGAGCATATTTACTTGAAGAAGTAATTATTATTTTTAATTC
NCCTTATAATGCTCTCTGATCTTANGANCCNGCTCCCGCCACCGTTGCCCGANGATCCGCGCTATAACGTAAATTGNCAATAAAANNNTCAAAAAGTTTAAAA
TTNANNCNTATGATCATATTTTGAGGGACAAATGAGAAACAAAAAAACTTACNAACATCGNTGTTCCCAATCCCTGGATATTTCTGGCGAACAGTCTTCATCTCT
GTGTTGCGATAGATTATAAAGCTATCANCATTTATGGTTAGCATCTCNNATCACCAAAAGGATTATACCAATGAANTTACNCATTCATANGACGAGGAACATGCTC
TAATATATTCTTCTTGNTAACTCNACTCANGTCTTNCGCTAATANCTGCGGGTCCACNAAACTTTCATGGCCTTGATAAATTGTTTCTGCNNAANTGACNNNANA
NGTCNTANACNAANACCAGATCACCCTCATTGGNNCTAANANNAGGGTAACCGTTCACATGANTGGGTTTTTCTGAAATAGTAAATGTNTGGNNCCCATGNN
CANCACNNCTTTCCCTAATCCTCTNGATNNNNANANANACCNTGNNNNTCANNATATAGCNGNCTGATCNNGTGAANCGANGANGNGNNNNNCGGNN
GGGNTTNNNNCNNNNNNNCNATCCNANGNGNCNTNNNANTNNNNNNCAANNTCTGANNNNNTGACNNNTNNGTCNNNNGNNNNNNN

# 7. Tripartite interactions between *Morchella* spp., *seudomonas koreensis* and *Acanthamoeba castellanii* reveal a beneficial fungal-amoebal association

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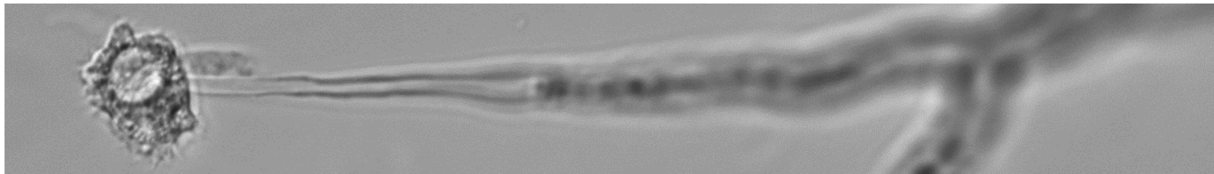


**Foreword**

This chapter investigates bi- and tripartite interactions between *Morchella* spp., *Pseudomonas koreensis* and *Acanthamoeba castellanii* in various *in vitro* experimental systems. This research article is under submission to Current Biology. My personal contribution as first co-author was the generation and analysis of all data, redaction and revision of the main text. This paper presents for the first time the formation of bacterial biofilms on *Morchella* hyphae, the ability of *P. koreensis* to form biofilms, toxicity of *Morchella* ascospores, and ability of *A. castellanii* to use fungal highways to move.

**Abstract**

*Morchella* spp. interact in soil with various organisms, but these interactions have rarely been investigated. In this study, the relationship between *Morchella* and its bacterial associate *Pseudomonas koreensis* was investigated under the predatory pressure of *Acanthamoeba castellanii*. This free-living amoeba that can be found in soils is both bacteriophagous and mycophagous. In this study, the first hypothesis tested was that *P. koreensis* uses *Morchella* to evade predation by hiding intracellularly in the hyphae. Accordingly, first whether *A. castellanii* could feed on *P. koreensis* and/or *Morchella*, and the impact of *A. castellanii* on mycelium were tested. Then, tripartite interactions between *A. castellanii, P. koreensis* and *Morchella* were studied by observational and quantitative experiments. The results from the bipartite interactions indicated that *A. castellanii* was able to feed on *P. koreensis,* but not on *Morchella*, and that *A. castellanii* used the mycelium as fungal highways to disperse across unsaturated areas to reach its bacterial prey. Given that *P. koreensis* inhibits the growth of *Morchella*, bacterial predation by *A. castellanii* was expected to be beneficial to *Morchella*. This was confirmed by the results from the tripartite competition assay. Moreover, the hypothesis that *P. koreensis* would hide within *Morchella* hyphae under predatory pressure was rejected. On the contrary, in the presence of the amoeba, *P. koreensis* formed biofilms around *Morchella* hyphae, which were more easily grazed by *A. castellanii*. The association between *Morchella* and *A. castellanii* was therefore beneficial to both organisms, as it enhanced the predation of *A. castellanii* against *P. koreensis,* reducing the nutrient competition.

**Keywords:** true morel, pseudomonad, amoeba, protozoa, bacterial-fungal interactions (BFI), biofilms, fungal highways

## 7.1 Introduction

*Morchella* spp. (true morels) are prized ascomycetes that produced gourmet fruiting bodies as the result of sexual reproduction (Pilz et al. 2007). In soils in the wild and in agricultural systems, *Morchella* interact with various organisms such as bacteria, fungi, and animals (slugs, insect larvae, springtails) (Liu et al. 2018). However, these interactions are poorly characterized. Most of the studies so far have been focus on the monitoring of the microbial communities associated with *Morchella* at different life stages (Zhang et al. 2019; Longley et al. 2019), or in different environments including greenhouse (Benucci et al. 2019), crops (Tan et al. 2021), peach-*Morchella* intercropping (Song et al. 2021), or its natural habitat (Longley et al. 2019), rather than studying microbial interactions at a closer level.

*Pseudomonas koreensis* is a soil bacterium commonly found in agricultural soils (Kwon et al. 2003; Rafikova et al. 2016). In a recent study, a culturable representative of this species (*P. koreensis* strain NEU1358=B33.4) was isolated from mycelium of *Morchella esculenta* M19-34 and it was shown to be closely affiliated to bacteria belonging to the bacteriome of *Morchella* spp. (Cailleau et al. 2023). *P. koreensis* is a mobile, non-spore forming and rod-shaped Gram-negative bacterium that can use sugars, alcohols and organic acids as carbon source (Rafikova et al. 2016). *P. koreensis* is also reported as a plant-growth promoting rhizobacterium (PGPR), capable of fixing nitrogen and of producing phytohormones including auxin Indole-3-acetic acid (IAA) and cytokinin-like substances (Rafikova et al. 2016; Guo et al. 2020). Additionally, some strains (*P. koreensis* IB-4) have antagonistic effects on phytopathogens such as *Fusarium* spp., *Alternaria alternata,* and *Bipolaris sorokiniana* by producing fungicides that impair cell-wall formation (Rafikova et al. 2016).

The genomic analysis of strain NEU1358 showed that its genome contains a combination of genes typical of free-living bacteria, but also of intracellular bacteria (chitinases, fimbriae, toxin/antitoxin system, insecticidal toxins) (Cailleau et al. 2023). For this reason, it was hypothesized that *P. koreensis* could persist in *Morchella* hyphae intracellularly. As this phenomenon was not observed in bipartite interactions (Cailleau et al. 2023), it was considered that predation could trigger cell invasion. The bacteriophagous amoeba *Acanthamoeba castellanii* was used to test this hypothesis. *A. castellanii* is a free-living amoeba commonly found in soils (Klink et al. 1992) that has a strong ability to predate bacteria (Huws, McBain, and Gilbert 2005). It is also a model species to study amoebal-fungal interactions because of its ability to predate yeast (Gonçalves et al. 2019) and conidia (Steenbergen et al. 2004; Nunes et al. 2016).

In this study, the relationship between *Morchella* and its associated bacterium *P. koreensis* was investigated under biotic stress, i.e., predation of *A. castellanii*. For this, experiments in bipartite interactions were conducted to establish the effect of each organism on one another and to predict the outcome of tripartite assays. *A. castellanii* was predicted to feed on *P. koreensis*, because it is known to feed on other *Pseudomonas* (e.g., *Pseudomonas fluorescens* (Huws, McBain, and Gilbert 2005)). However, it was not expected that the amoeba could feed on *Morchella* mycelium, as only a few species of amoeba are able to do so (e.g., *Thecamoeba granifera,* Leptomyxida species (Chakraborty and Old 1982), *Saccamoeba* sp. (Chakraborty, Old, and Warcup 1983)). Secondly, the impact of *P. koreensis* and *A. castellanii* on *Morchella* was investigated. It has already been demonstrated that *P. koreensis* could inhibit the growth of *Morchella* (Cailleau et al. 2023), but the same was unknown in the case of *A. castellanii*. It was hypothesized that if it did not use the fungus as food, *A. castellanii* would not be detrimental to the fungus. Finally, tripartite interactions were established, to investigate the initial hypothesis that *P. koreensis* would enter *Morchella* hyphae to evade predation.

## 7.2 Material and methods

### 7.2.1 *Acanthamoeba castellanii* cultivation

*Acanthamoeba castellanii* strain ATCC 30234 was kindly provided by Prof. Hubert Hilbi from the University of Zurich. The amoeba was cultured in cell cultivation flasks in Peptone Yeast Broth (PYB; 20g/L Peptone, 10g/L Yeast extract) for a maximum of three weeks before being used in the experiments. Cultures were maintained at room temperature (20-21°C) in darkness. Concentrations of *A. castellanii* were recorded by counting the number of cells contained in a volume of 1 µL under an EVOSM5000 inverted light microscope (Invitrogen, USA) and diluted or concentrated (by 5 min centrifugation at 1100 rpm followed by removal of supernatant) at the required concentration for the experiments described below.

### 7.2.2 *Pseudomonas koreensis* cultivation

*Pseudomonas koreensis* strain NEU1358 (=B33.4) (Cailleau et al. 2023) was maintained on Nutrient Agar plates (NA; 5 g/L Peptone, 3 g/L Yeast extract, 5 g/L NaCl, 12 g/L Agar) in an incubator at 20°C in darkness. The strain used (GFP-NEU1358) was previously tagged with the Green Fluorescent Protein (GFP) by electroporation, using the plasmid pUX-BF13 and the transposon Mini-Tn7 GFP Kanamycin-resistant (Cailleau et al. 2023). When needed, fresh colonies (i.e., less than three days old) were suspended in physiological water (0.9% NaCl). Bacterial concentration was measured either using a BD Accuri™ C6 Plus Flow Cytometer (BD Biosciences, USA) or by direct count using a Neubauer improved cell counting chamber with an inverted light microscope and diluted at the required concentrations.

### 7.2.3 *Morchella* spp. cultivation

*Morchella esculenta* M19-34 (Esculenta), *Morchella helvetica* M19-43 (Elata), and *Morchella* sp. M21-48 (Elata) were previously sampled in Switzerland (Cravero et al. submitted). Mycelial cultures were either hymenium-derived (i.e., cultured by inoculating a piece of hymenium on potato dextrose agar (PDA; Potato dextrose 26 g/L, Agar 13 g/L, Sigma-Aldrich) and reculturing the resulting mycelium) or ascospore-derived (i.e., by spreading *Morchella* ascospores on PDA). *Morchella* mycelia were maintained in an oven at 20°C in darkness.

### 7.2.4 Bipartite assays

#### 7.2.4.1 Feeding assay between *A. castellanii* and *P. koreensis*

To investigate whether *A. castellanii* could feed on *P. koreensis,* the cultivation system consists in inoculating small amounts of bacterial, fungal, or amoebal cells in drops of liquid medium (Malt Broth; MB 12 g/L, Sigma-Aldrich) or physiological water (0.9% NaCl) deposited in cell culture plates (Buffi et al. 2023). Each drop was observed with an inverted light microscope without disrupting the experiment. The system was incubated at room temperature in darkness (20-21°C). Interactions between *A. castellanii* and *P. koreensis* were observed and photographed in MB with an EVOSM5000 inverted light microscope.

#### 7.2.4.2 Feeding assay between *A. castellanii* and *Morchella*

To investigate whether *A. castellanii* could feed on *Morchella* mycelium, both organisms were co-cultured in a drop system in MB, as explained above. Two types of mycelial inoculation were tested: (1) mycelium containing mechanically cut hyphae obtained from a primary mycelial culture of M21-48 in solid medium that was cut (about 1 $mm^3$); and (2) starting from ascospores inoculated in a drop for

two days before adding *A. castellanii*. This mycelium contained only intact hyphae. Interactions between *A. castellanii* and *Morchella* were observed as described above.

To investigate whether *A. castellanii* could feed on *Morchella* ascospores, amoebae were inoculated in drops of physiological water with various food sources at room temperature (20-21°C) in darkness for five days. The food sources corresponded to ascospores of *Morchella* (strains *M. esculenta* M19-42, *M. helvetica* M19-43, and *Morchella* sp. M21-48, 500 ascospores/mL), conidia of *Aspergillus westerdijkiae* strain U2 (2000/mL), and bacterial cells (*P. koreensis* GFP-NEU1358) as a positive control. The initial (200 cells/mL) and final concentrations of *A. castellanii* were recorded as explained previously.

In addition, trophozoites of *A. castellanii* and ascospores of *Morchella* sp. M21-48 were placed in the same MB drop and observed microscopically. Timelapse movies were recorded.

7.2.4.3 Impact of *P. koreensis* and *A. castellanii* on *Morchella*

To evaluate the impacts of *P. koreensis* and *A. castellanii* on *Morchella* hyphal growth, co-cultures of *M. esculenta* M19-34 or *Morchella* sp. M21-48 with either *A. castellanii* or *P. koreensis* were established on PDA. *Morchella* were inoculated at the same time as *A. castellanii* or *P. koreensis* and let to grow at room temperature (20-21°C) in darkness for nine days. Pictures were taken at 3 and 9 dpi, to measure the radial growth of *Morchella,* evaluate the mycelial density, and record stress characteristics such as melanization of the mycelium, presence of inhibition zones, or formation of aerial hyphae. Each co-culture was prepared in triplicate. As a control, *Morchella* were also cultivated in pure cultures. The statistical significance of differences between the groups was calculated with a One-way ANOVA test using the Microsoft Excel Data Analysis ToolPak.

**7.2.5 Microscopic observation of tripartite interactions**

To observe tripartite interactions between *A. castellanii, P. koreensis* and *Morchella,* the organisms were co-cultured in a drop system and observed with an inverted microscope, as explained previously. The three organisms were inoculated at the same time.

**7.2.6 Fungal highways assay**

To test whether *A. castellanii* could use *Morchella* hyphae as fungal highways, PDA plates from which 5 mm thick agar bands were removed were prepared (Bravo et al. 2013). In the left part, *M. esculenta* M19-34 or *Morchella* sp. M21-48 were inoculated. The system was incubated at room temperature (20-21°C) in darkness. Five days later, when *Morchella* mycelium reached the right part of the plate, *A. castellanii* were inoculated in the left part of the plate. Food was placed in the right part (*P. koreensis* GFP-NEU1358). Three days later, mycelium in the right part of the plate (at about 1-2 cm of the *A. castellanii* inoculum) was scratched with a sterile loop and deposited on a physiological water drop for microscopic observations. As a control, mycelium was scratched in the left part of the Petri dish, at 1-2 cm of the *A. castellanii* inoculum as well and observed microscopically. The presence of *A. castellanii* in the mycelial samples was monitored.

**7.2.7 Quantification of tripartite interactions**

To quantify the effect of co-culturing *A. castellanii, P. koreensis* and *Morchella* on growth, each combination of organism was inoculated in 12-well plates. Each well contained 1 mL of MB. Ascospores of *Morchella* (concentration of 1.4 ascospores/µL) were first inoculated. The plates were incubated at 20°C in darkness. Two days later, *A. castellanii* (concentration of 1.7 cells/µL) and *P. koreensis* (concentration of 15 cells/µL) were inoculated in the wells. After five days of incubation,

200 mL of dispersive agent (1% sodium hexametaphosphate, $(NaPO_3)_6$) was added in each well to detach bacteria and amoeba that could be attached to the hyphae. The content of each well was transferred in Eppendorf tubes and vortexed to homogenize. The concentrations of *A. castellanii* and *P. koreensis* were recorded as explained previously (Neubauer cell). *Morchella* was quantified using pictures of the wells with the software ImageJ v1.54d (Schneider, Rasband, and Eliceiri 2012) by measuring the area of the well that was occupied by the mycelium. The statistical significance of the difference between the groups was calculated with a One-way ANOVA test using the Microsoft Excel Data Analysis ToolPak.

## 7.3 Results

### 7.3.1 Bipartite interactions

7.3.1.1 *A. castellanii* can feed on free-living cells of *P. koreensis*

Microscopic observations confirmed that *A. castellanii* could feed on free-living cells of *P. koreensis.* Phagocytosis was observed (**Figure 1A**) and confirmed by the presence of fluorescence (emitted by the GFP-tagged bacteria) in food vacuoles (**Figure 1B**). Moreover, “bacterial clusters” around *A. castellanii* were also observed (**Figure 1C**).

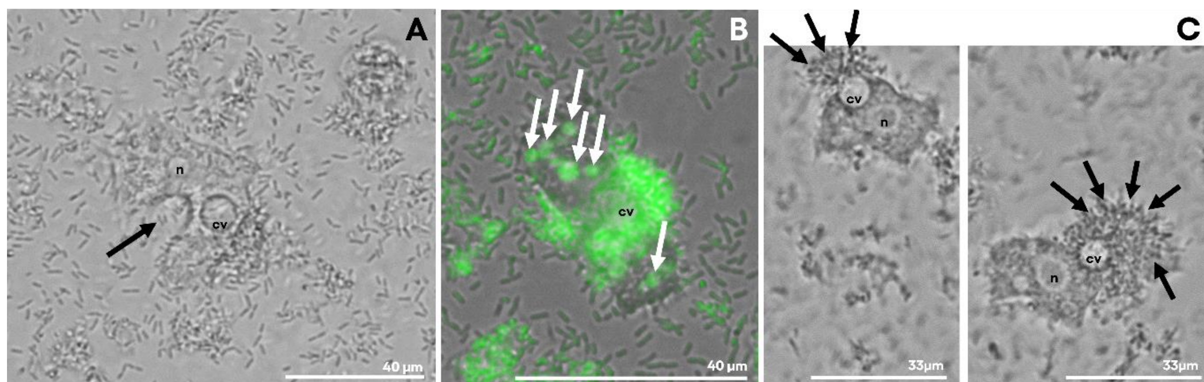



**Figure 1**. A= Phagocytosis of *Acanthamoeba castellanii* ATCC 30234 on *Pseudomonas koreensis* GFP-NEU1358 (black arrow). B= presence of green fluorescent bacteria (GFP-NEU1358) in food vacuoles of A. castellanii. C= bacterial clusters of GFP-NEU1358 around two trophozoites of A. castellanii. The nucleus (n) and contractile vacuole (cv) of A. castellanii are indicated when visible. Pictures were taken with an inverted microscope 18 hours post inoculation at magnification 400x.

7.3.1.2 *A. castellanii* is not able to feed on *Morchella* mycelium

In the co-inoculation of *A. castellanii* and *Morchella* the amoeba was not observed to feed on the fungus as no phagocytosis was observed. The hyphae did not seem damaged (perforated or cut). Frequently, *A. castellanii* were located at the top of hyphal tips, gliding or even surrounding them over 10-20 µm (**Figure 2A-C**). However, after a change in the position of the amoeba, hyphae were always visually intact (usually after 5-30 min of observation). Unusual immobile round cells of *A. castellanii* were also frequently observed at hyphal tips (**Figure 2D-E**). In the cultures where mechanically cut hyphae were present, trophozoites of *A. castellanii* were frequently observed partially entering the hyphae (**Figure 2F-G**).

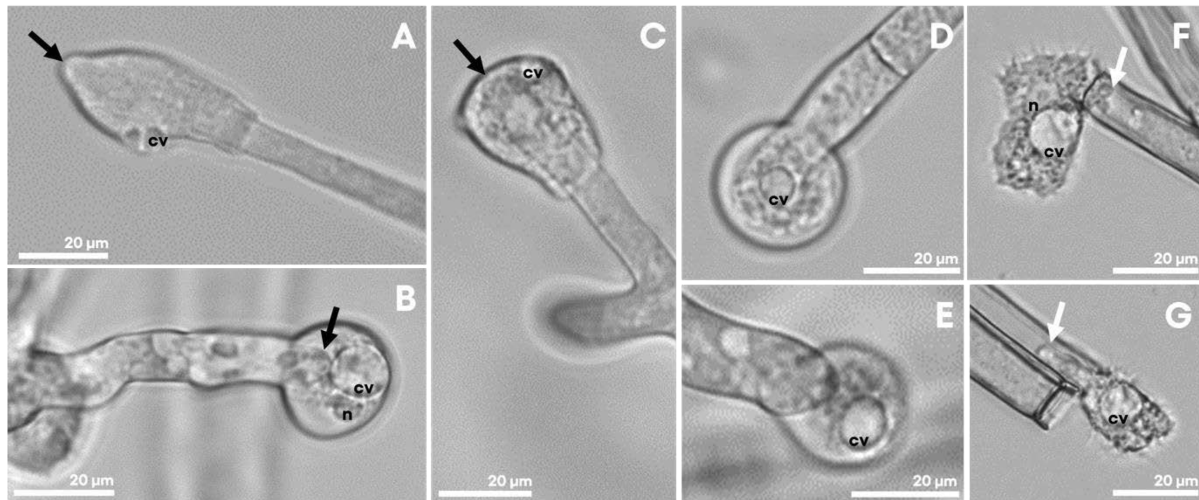



**Figure 2**. Trophozoites of *Acanthamoeba castellanii* ATCC 30234 interacting with hyphae of *Morchella helvetica* M19-43 (A-E) or *Morchella* sp. M21-48 (F-G). A-C= Intact hyphal tips surrounded by *A. castellanii*; the apical part of the hyphae can be seen inside the amoeba (black arrows). D-E= *A. castellanii* round trophozoites attached at the top of intact hyphal tips. F-G= *A. castellanii* trophozoites entering mechanically cut hyphal cells; amoeba pseudopods can be seen inside the hyphae (white arrows). The nucleus (n) and contractile vacuole (cv) of *A. castellanii* are indicated when visible. Pictures were taken with an inverted microscope 72 (F-G) and 96 (A-E) hours post inoculation at magnification 400x.

### 7.3.1.3 *Morchella* ascospores are toxic to *A. castellanii*

Ascospores of three *Morchella* strains (M19-42, M19-43, M21-48), conidiospores of *A. westerdijkiae* and bacteria GFP-NEU1358 (positive control) were inoculated with *A. castellanii* in physiological water, to determine whether the amoeba could survive by feeding on spores. Initially, trophozoites of *A. castellanii* were inoculated at a concentration of 2 cells/µL. The number of cysts and trophozoites was recorded in each drop after five days (**Table 1**). In the negative controls without a food source, only cysts of *A. castellanii* were found (**Figure 3A**). In the positive control (i.e., *P. koreensis* as a food source), about a half of the *A. castellanii* survived and appeared healthier (i.e., active movement, measured > 20 µm; see **Figure 3B**). In presence of *A. westerdijkiae* conidiospores, the trophozoites that survived had low mobility, irregular shape, and a dark appearance (**Figure 3C**). No *A. castellanii* was found in the drops inoculated with *Morchella* ascospores. Further microscopic observations of *A. castellanii* incubated with *Morchella* ascospores in MB drops revealed that amoeba rapidly (10 min post inoculation) started to interact with the spores, attaching and gliding on the spore surface. Observations 28 hours post inoculation (hpi) revealed that *Morchella* ascospores could be phagocytosed by *A. castellanii* (**Figure 3D**). Some of the ingested spores had an unusual irregular and thick outer cell wall, which has never been observed in other conditions. To elucidate the fate of the ingested ascospores, a time-lapse movie ($t_0$ = 28 hpi) of a trophozoite that had already ingested a fungal spore was recorded (**Figure 3E**). The amoeba started to reject the ascospore within 30 min after the ingestion. Then, the amoeba remained in physical contact with the ejected ascospore. The trophozoite had an unusual dense, dark, and granular structure, and its shape was round. One hour and 30 min later, the amoeba exploded.

**Table 1**. Mean concentration of *Acanthamoeba castellanii* (trophozoites or cysts) recorded in triplicates after five days of incubation in physiological water with spores of four fungi.

| Food source | Concentration of trophozoites [cells/10µL] | Concentration of cysts [cells/10µL] |
|---|---|---|
| None | 0 | 1.2 |
| *Pseudomonas koreensis* GFP-NEU1358 | 0.9 | 0 |
| *Morchella esculenta* M19-42, ascospores | 0 | 0 |
| *Morchella helvetica* M19-43, ascospores | 0 | 0 |
| *Morchella* sp. M21-48, ascospores | 0 | 0 |
| *Aspergillus westerdijkiae* U4, conidiospores | 0.6 | 0 |

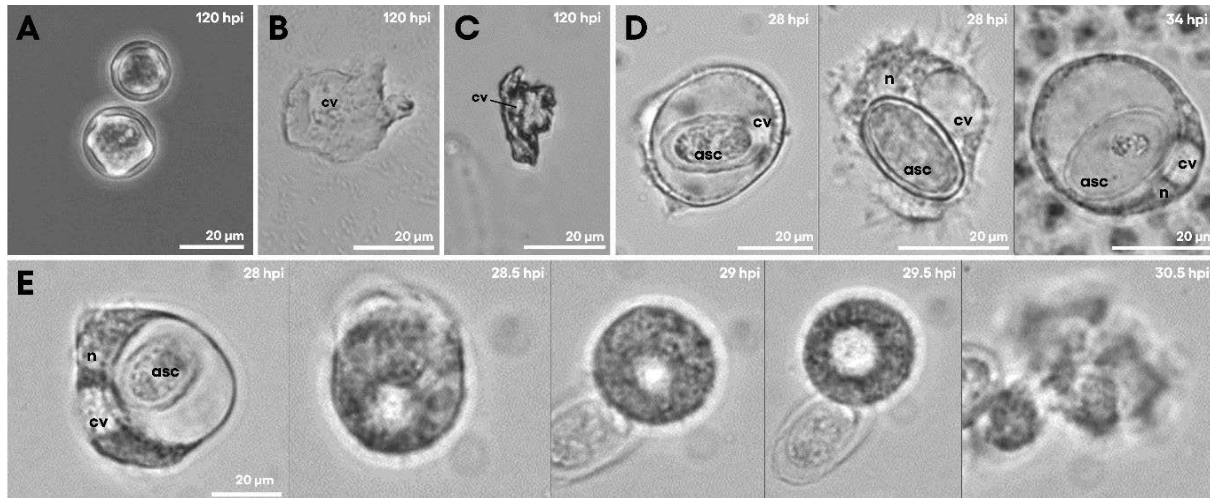



**Figure 3**. Feeding assay of *Acanthamoeba castellanii* ATCC 30234 with *Morchella* ascospores. A= cysts of *Acanthamoeba castellanii* in physiological water (negative control, 120 hours post inoculation; hpi). B= Trophozoite of *A. castellanii* with *Pseudomonas koreensis* GFP-NEU1358 as a food source (positive control, 120 hpi). C= Trophozoite of *A. castellanii* with conidia of *A. westerdijkiae* as a food source (120 hpi). D= Three different trophozoites of *A. castellanii* having ingested an ascospore (asc) of *Morchella helvetica* M19-43 (28-34 hpi). E= One trophozoite of *A. castellanii* interacting with an ascospore of *M. helvetica* M19-43: A. castellanii ingested an ascospore (28 hpi), but starts ejecting it (28.5 hpi). The ejected ascospore remains in contact with the amoeba (29-29.5 hpi), followed by apoptosis of *A. castellanii* (30.5 hpi). The nucleus (n) and contractile vacuole (cv) of A. castellanii are indicated when visible. Pictures were taken with an inverted microscope at magnification 400x.

### 7.3.2 *P. koreensis* does not hide within *Morchella* hyphae to avoid predation but forms biofilms around them

The hypothesized mechanism of *P. koreensis* hiding intracellularly in *Morchella* mycelium under the pressure of grazing by the amoeba was never observed. In contrast, *P. koreensis* formed a biofilm around the hyphae (**Figure 4**). After 18 hours, dense bacterial biofilms (up to 42 µm thick) were observed around *Morchella* hyphae (**Figure 4G**). Trophozoites of *A. castellanii* were observed at the surface of the bacterial biofilms. Without *A. castellanii,* biofilms were not observed (**Figure 4C**). After 54 hours, biofilms were not present anymore (**Figure 4H**).

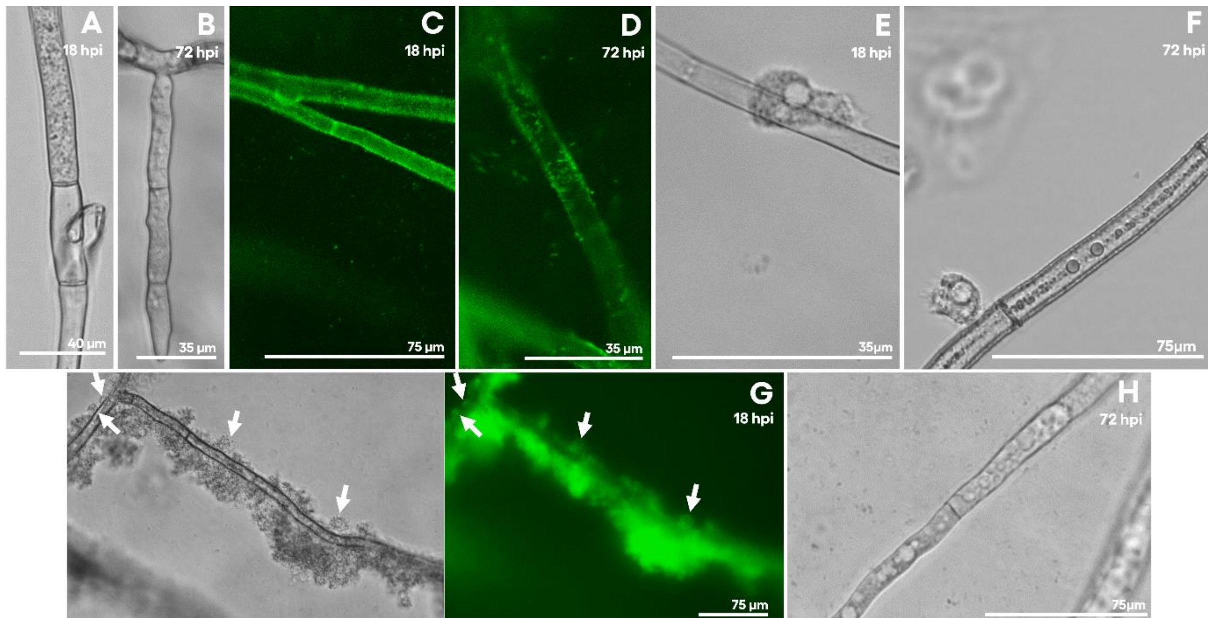
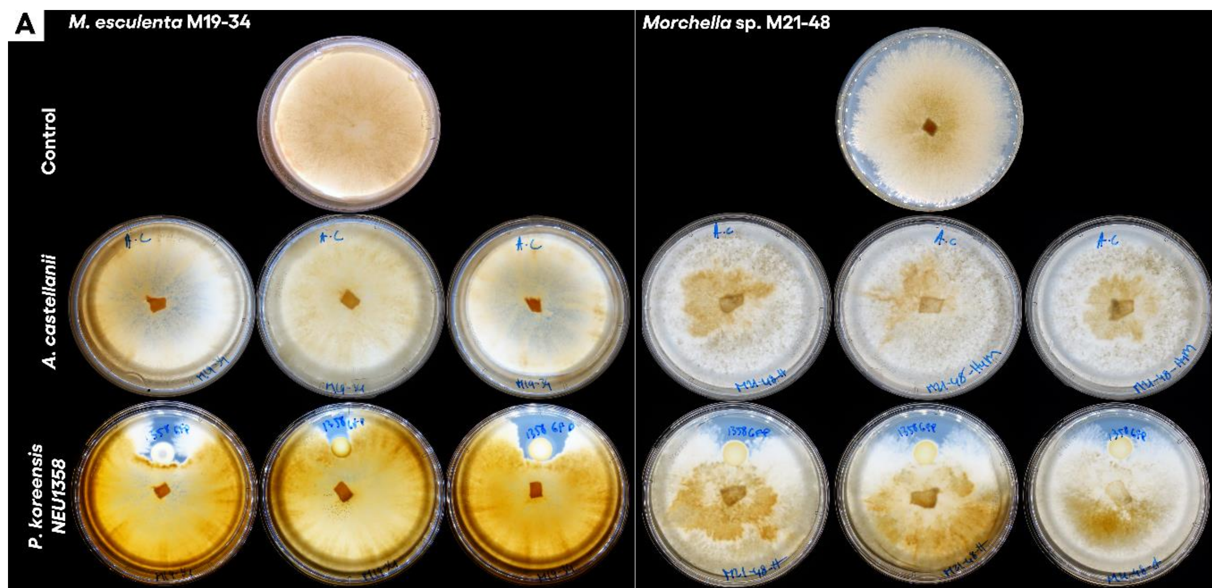



**Figure 4**. Microbial interactions with *Morchella* sp. M21-48 at 18 hpi (A, C, E, G) and 72 hpi (B, D, F, H). A-B= control with hyphae in pure culture; C-D= in co-culture with *P. koreensis*; E-F= in co-culture with *A. castellanii*; G-H= co-culture with *P. koreensis* and *A. castellanii* simultaneously (biofilms at 18 hpi). Bright field and fluorescent (C, D, G right) pictures were taken with an inverted microscope at magnification 400x.

### 7.3.3 Impact of *A. castellanii* and *P. koreensis* on *Morchella* hyphal growth

To evaluate the potential negative effect of either *A. castellanii* or *P. koreensis* on *Morchella,* co-cultures were established in solid medium (PDA). The mycelium of *M. esculenta* M19-34 showed signs of stress in the presence of *P. koreensis*, as indicated by the darkening of the mycelium and a growth inhibition zone in the contact area (**Figure 5A**). *A. castellanii* did not seem to have any visible effect on *Morchella* (**Figure 5A**). Radial growth of mycelium was measured in the opposite direction of the co-inoculated organism (as a control, see **Figure 5B-I**), and towards the co-inoculated organism (**Figure 5B-II**). The difference between the groups was not statistically significant (*p*-value= 0,065). In the control and in the co-cultures with *A. castellanii*, mycelium grew at a similar rate (35-36 mm for M19-34 and 25-26 mm for M21-48). In the co-cultures with *P. koreensis*, mycelium grew at lower rates (maximum 18.2 mm).



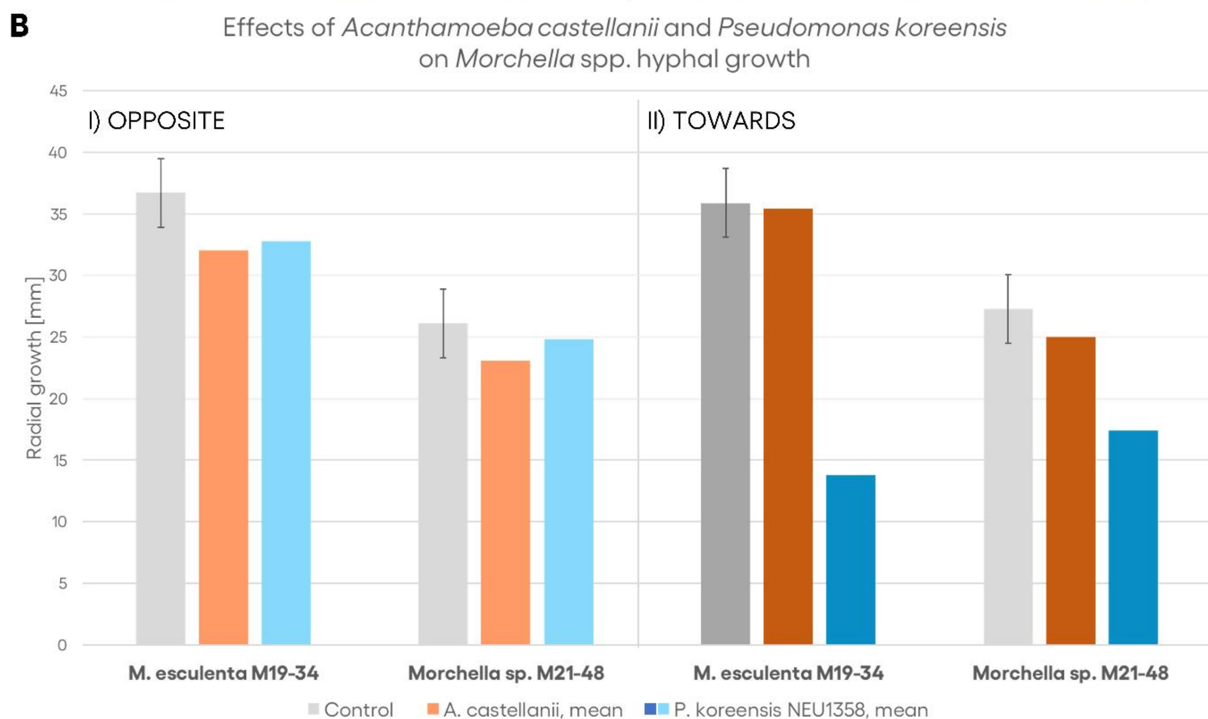




**Figure 5**. A= Co-cultures of *Morchella esculenta* M19-34 or *Morchella* sp. M21-48 in pure culture (control, first line), with *A. castellanii* (2nd line) or *P. koreensis* (3rd line) 9 days post inoculation (dpi). Diameter of the Petri dish was 90 mm in the co-cultures and 120 mm in the controls. B= Histogram representing radial growth (3 dpi) of *Morchella* mycelium in presence of *A. castellanii* or *P. koreensis*. I)= mycelial growth measured at opposite of the co-inoculated organism; II)= mycelial growth measured towards the co-inoculated organism. The difference between the groups is not statistically significant (p-value > 0.05). Error bars represent standard deviation.

### 7.3.4 *A. castellanii* can use *Morchella* hyphae as fungal highways

As *A. castellanii* were observed gliding on *Morchella* hyphae, and as it did not impair hyphal growth, a fungal highways assay was made to test the hypothesis that the amoeba could use the hyphae to move. *A. castellanii* was inoculated in the left part of a gap created in Petri dishes with agar medium separated by the removal of a 5 mm thick agar band. The sampling and microscopic observation of the mycelium situated in the right part of the gap allowed to determine whether *A. castellanii* could

use *Morchella* hyphae to reach food (i.e., *P. koreensis*). The experimental setup is shown in **Figure 6A-B**. The observations revealed that trophozoites of *A. castellanii* were found in the part of the plate separated by a gap but bridged by *Morchella* hyphae of both tested species (**Figure 6C-D**).

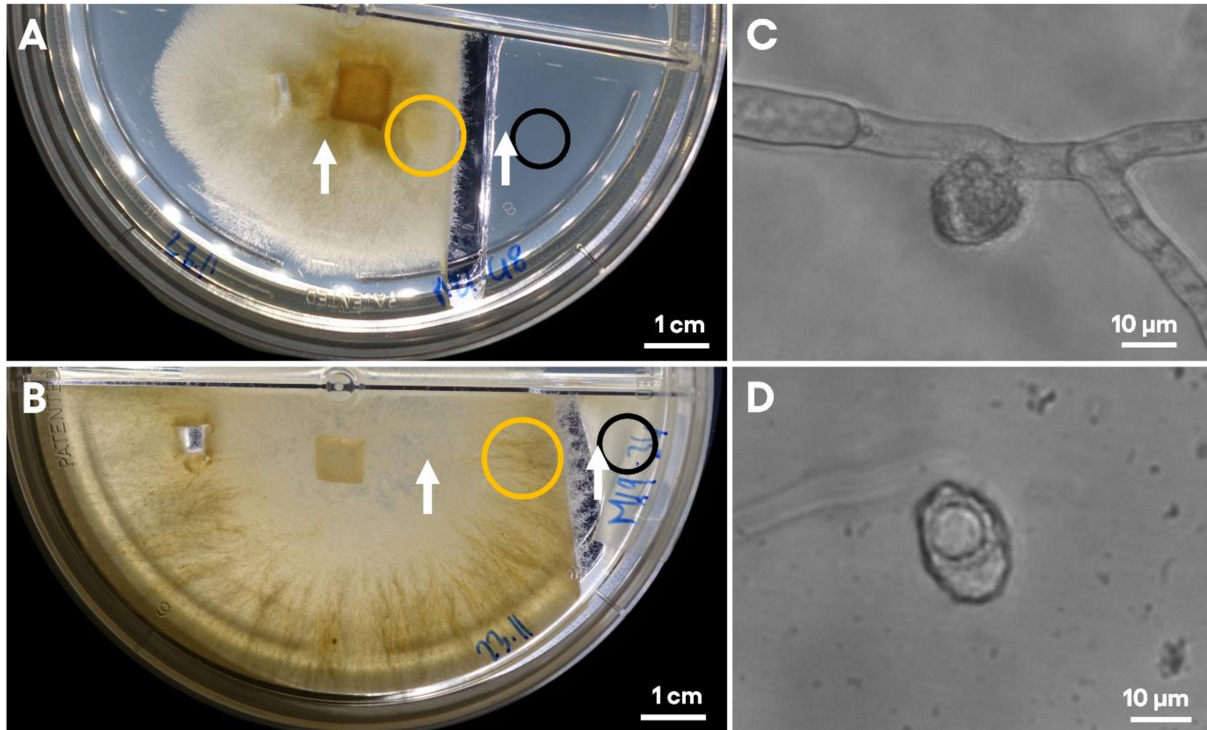



**Figure 6**. Experimental setup for the fungal highway assay (A= mycelium of *Morchella* sp. M21-48 on PDA; B= mycelium of *Morchella esculenta* M19-34 on PDA). The yellow circles indicate the location of the *Acanthamoeba castellanii* inocula. The black circles indicate the location of the *Pseudomonas koreensis* inocula. The white arrows indicate where mycelium was sampled 8 days post inoculation. Pictures A and B were taken 3 dpi. Pictures C and D show two trophozoites of *A. castellanii* that could cross the gap shown between the yellow and black circles in pictures A-B, using Morchella hyphae to reach the food *P. koreensis.* They were taken within an inverted light microscope at magnification 400x in physiological water.

### 7.3.5 Quantification of tripartite interactions

Different combinations of the three organisms were co-cultured in triplicates in MB in 12-wells plates. Five of the cultures could not be included in the quantification of growth because one of the organisms did not grow (images in brackets in **Supplementary S1**). For *A. castellanii* and *P. koreensis,* growth rate was expressed in concentration of cells/µL five days post inoculation. For *Morchella* sp. M21-48, that has been inoculated two days before, growth rate was expressed as the surface area of the well that was occupied by the mycelium seven days post inoculation. The results indicated that *A. castellanii* was not able to grow in the sole presence of *Morchella*, whereas their population increased by almost 200 times in the control (**Figure 7A**). *A. castellanii* grew better in presence of *P. koreensis*, and even more when *Morchella* was also present. Concerning *Morchella*, its growth rate was relatively similar in all culture conditions (**Figure 7B**). However, the fungus grew better alone, and the lowest growth was recorded in presence of the bacterium or the combination *A. castellanii* + bacterium. Finally, the abundance of *P. koreensis* was higher in co-culture with either *A. castellanii* or *Morchella* as compared to the control (i.e., cultivated in pure culture; **Figure 7C**). In contrast, in the

presence of the fungus and the amoeba, the abundance of bacteria decreased. The difference between the groups was statistically significant (*p*-value= 0,014).

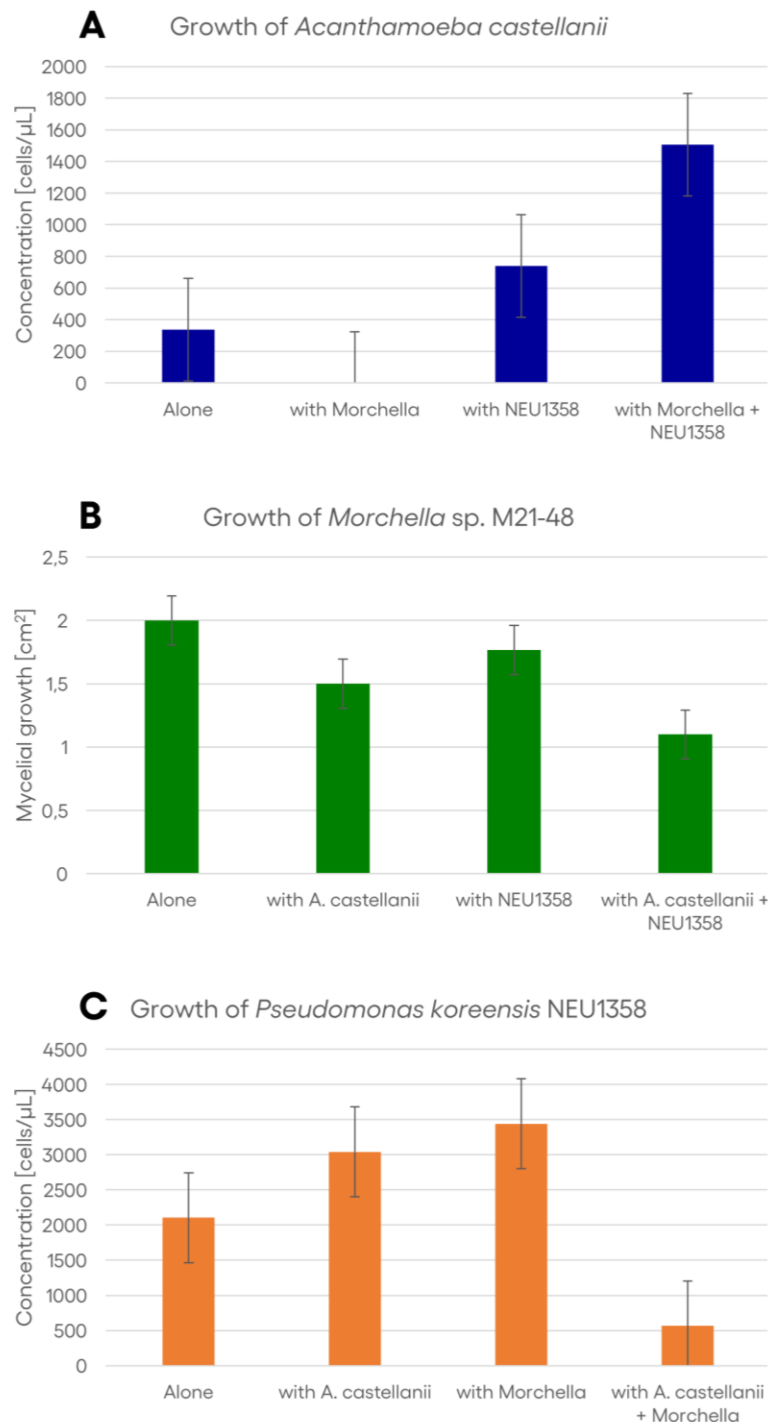



**Figure 7**. Quantification of *Acanthamoeba castellanii* ATCC 30234 (A), *Morchella* sp. M21-48 (B), and *Pseudomonas koreensis* GFP-NEU1358 (C) when co-cultured with other organisms (see horizontal labels). For *A. castellanii* and *P. koreensis*, the growth rate was expressed in concentration of cells/µL. For *Morchella* sp., growth was expressed in surface area colonized by mycelium in $cm^2$. Measures were taken five days post inoculation of *A. castellanii* and *P. koreensis*, and seven dpi of *Morchella* sp. The difference between the groups was statistically significant ($p$-value $< 0.05$). Errors bars represent standard deviations.

## 7.4 Discussion

In this study, bi- and tripartite interactions between the predatory amoeba *A. castellanii*, its prey *P. koreensis* and *Morchella* were investigated. The aim was to verify the hypothesis that *P. koreensis* would hide intracellularly in *Morchella* hyphae under predatory pressure. This hypothesis was not validated, as this defense behavior was not observed. However, other phenomena were observed, and hence discussed below.

### 7.4.1. *A. castellanii* can feed on free-living cells and biofilms of *P. koreensis*

Microscopic observations indicated that *A. castellanii* was able to predate free-living cells of *P. koreensis* by phagocytosis (**Figure 1A-B**). In addition, *A. castellanii* could feed by grazing the biofilms that *P. koreensis* formed around *Morchella* hyphae (**Figure 4G**). Bacterial biofilms are an aggregation of bacteria within self-produced extracellular polymeric substances that can form on abiotic surfaces, but also on living structures as fungal hyphae (Miquel Guennoc et al. 2018). The formation of bacterial biofilms has been reported on hyphae of filamentous ascomycetes (e.g., on *Candida albicans, Aspergillus nidulans, A. alternata, Neurospora crassa* (Hogan, Wargo, and Beck 2009), *Aspergillus niger* (Kjeldgaard et al. 2019)), but this is the first time this is observed for *Morchella*. Biofilms are beneficial for bacteria in multiple ways, including the use of the fungus as a source of nutrients or for dissemination, enhancing symbiosis or antagonist interactions and as step preparing bacterial-fungal endosymbiont interactions (Hogan, Wargo, and Beck 2009), or by improving resistance to biotic and abiotic stresses (Miquel Guennoc et al. 2018). Biofilms were only formed when the amoeba were present, suggesting that *P. koreensis* could have formed biofilms as a protection mechanism. However, this was not effective as the bacterium grew better growth in the absence of *Morchella* (and thus could not form biofilms; **Figure 7C**). In contrast, the growth rate of *A. castellanii* increased when co-cultured with *P. koreensis* in presence of *Morchella* (and thus biofilms) (**Figure 7A**). This further supports that biofilms were not beneficial for *P. koreensis*. Biofilms can be particularly detrimental to bacteria in presence of biofilm-grazing specialists (Matz 2009). It has been demonstrated that *A. castellanii* CCAP 1534/2 could also feed on bacterial biofilms of *Klebsiella pneumoniae, Pseudomonas fluorescens* and *Staphylococcus epidermidis* without any preference (Huws, McBain, and Gilbert 2005). This amoebal strain maintained bacterial biofilms to ensure future predation (Huws, McBain, and Gilbert 2005), a phenomenon that was observed in the present study where biofilms completely disappeared 72 hpi (**Figure 4H**).

A second phenomena observed in addition to biofilms was the formation of bacterial clusters surrounding *A. castellanii* (**Figure 1C**). Amoebae are known to initiate the formation of these bacterial clusters and used them as recipients for future food uptake. Thin filaments originating from the amoeba (filopodia) could be responsible for the maintenance of the bacterial aggregates (Doyscher et al. 2013). Amoeba induce the formation of these clusters to feed more easily taking advantage of bacterial appendages (Matz 2009; Hochstrasser et al. 2022). It was shown that *A. castellanii* binds motile *Pseudomonas* spp. at specific sites, using their flagella (Doyscher et al. 2013). Our previous work indicated both the presence of flagella and adhesive fimbria in *P. koreensis* NEU1358 (= 33.4) (Cailleau et al. 2023). Both structures could contribute to the formation of bacterial clusters on *A. castellanii*. The formation of bacterial clusters (or backpack aggregates) on *Acanthamoeba* has been observed in numerous species including *Listeria* spp., *Bacillus* spp., *Erwinia amylovora, Cronobacter sakazakii, Salmonella typhimiurium, Pseudomonas aeruginosa* (Doyscher et al. 2013), and *Legionella pneumophila* (Hochstrasser et al. 2022). In *L. pneumophila,* Flagellin A (FlaA) was the principal factor allowing the binding of bacteria to *A. castellanii* (Hochstrasser et al. 2022). In that case, however, bacteria could survive within the amoebae, replicate, consume the amoeba, and spread in the environment (Hochstrasser et al. 2022). In other words, the formation of clusters was beneficial for *L.*

*pneumophila*. This was not the case for *P. koreensis*. These examples indicated that bacterial aggregation may be beneficial (nutrition) (Doyscher et al. 2013) or not (host cell infection) (Hochstrasser et al. 2022) for amoeba. In our experiments, it is more likely that *A. castellanii* promoted the formation of bacterial clusters to better feed on *P. koreensis*, as demonstrated by the very active grazing of biofilms (**Figure 7A**) that are on itself a sort of bacterial aggregate.

It has been shown that bacterial biofilms can be beneficial or detrimental for the fungal host. For instance, biofilms of *Burkholderia terrae* protect fungal partners against antagonistic bacteria (*P. fluorescens*) and a fungicide (cycloheximide), potentially by shielding, sorption, or detoxification mechanisms (Nazir, Tazetdinova, and van Elsas 2014). On the contrary, the *in vitro* formation of a *Pseudomonas aeruginosa* biofilm on *C. albicans* hyphae resulted in the death of the hyphae (Hogan, Wargo, and Beck 2009). For this reason, some fungi developed strategies to counter bacterial colonization of their hyphae. For instance, living hyphae of *T. melanosporum* inhibit *P. fluorescens* biofilm formation possibly by secreting DNAse, as eDNA production seems required to form biofilms (Miquel Guennoc et al. 2018). The specificity of biofilm-forming bacteria is low (i.e., multiple bacterial species can form a biofilm on the same fungus), but the specificity towards the fungal host is high (i.e., bacteria cannot form biofilm on all fungi) (Hogan, Wargo, and Beck 2009; Miquel Guennoc et al. 2018). Before the present study, biofilms have never been reported occurring on *Morchella* tissues. The fungal partner is crucial in the establishment of bacterial biofilms (Miquel Guennoc et al. 2018), which leads to two hypotheses: (1) *Morchella* could have promoted biofilm formation due to the presence of *A. castellanii* in order to protect its associate bacteria; or (2) *Morchella* could have promoted biofilms formation to help the bacteriophagous amoeba to decrease *P. koreensis* population. The second hypothesis seems to be more plausible as the bacterium inhibited fungal growth (**Figure 6)**, while the amoeba did not and predation would be beneficial to reduce nutritional competition.

### 7.4.2 *A. castellanii* is not able to feed on *Morchella* but uses its hyphae as fungal highways

In the literature, we found only one report of *A. castellanii* interacting with fungal hyphae. When co-cultured with the filamentous fungus *Rhizoctonia solani, A. castellanii* encysted rapidly (24h-48h) and cysts aggregated around fungal hyphae (Long et al. 2019). This lead to the hypothesis that *R. solani* produced anti-amoeba compounds that stimulated encystation when amoeba tried to feed on hyphae and it was supported by the fact that hyphae remained intact, with no wall perforation nor nucleus alteration (Long et al. 2019). In the present experiments, no cysts were observed around *Morchella* hyphae. Amoeba are known to feed on fungal hyphae. To initiate this behavior, amoeba generally attach to the fungus at hyphal tip (Chakraborty, Old, and Warcup 1983). This was frequently observed in the present study (**Figure 2A-E**). To ingest hyphal cells, *Saccamoeba* sp. and *T. granifera* formed a loop on the hyphal tip, formed a granulated round mass, and uptake the hyphal cell within 10-15 min, leaving an empty and deformed hyphal tip (Chakraborty, Old, and Warcup 1983). Such empty apical hyphal cells were observed in *Acanthamoeba-Morchella* co-cultures containing mechanically cut hyphae (**Figure 2F-G**), suggesting that *A. castellanii* could have been feeding on damaged *Morchella* hyphae. In cultures with intact mycelium, however, no hyphal ingestion was observed even after numerous microscopic observations and timelapses recording. Trophozoites of *A. castellanii* were observed gliding on *Morchella* hyphal tips or even surrounding them (**Figure 5A-C**), but they remained intact even after being left by the amoebae. No phagocytosis of *A. castellanii* on mycelial fragments was observed either. Hence, it was concluded that *A. castellanii* was not able to feed on *Morchella* mycelium. On the contrary, *A. castellanii* used the mycelium to move and reach food, as demonstrated by the fungal highways assay (**Figure 6**). To our knowledge, this interaction is shown for the first time in the present study.

### 7.4.3 *Morchella* ascospores are toxic to *A. castellanii*

It has been demonstrated that *A. castellanii* could phagocyte conidia of *H. capsulatum* (Steenbergen et al. 2004) and *Fusarium solani* (Nunes et al. 2016). Conidia of *F. solani* that have been phagocytosed by *A. castellanii* were able to germinate and perforate the amoeba to be released in the environment, without killing the amoeba (Nunes et al. 2016). In the present work, *A. castellanii* was able to ingest *Morchella* ascospores (**Figure 3D**), but the spores did not germinate. All the ascospores that have been observed after ingestion by *A. castellanii* were later ejected, indicating the amoebae were not able to perforate the spore wall and feed on them. The amoebae even exploded (two observations) after the ejection of the fungal spore (**Figure 3E**), suggesting that *Morchella* ascospores could be toxic for *A. castellanii* and maybe other predators. Toxins (e.g., in *Amanita phalloides*) (Kaya et al. 2015), and anti-cell wall lysis agents like melanin (Kuo and Alexander 1967) have been detected in fungal ascospores. In addition, conidia of *H. capsulatum* were cytotoxic against *A. castellanii*, that were used as food by the fungus (Steenbergen et al. 2004). The fact that *Morchella* ascospores could kill *A. castellanii* to get a nutrient source is not likely, as no germination occurred. However, it is likely that ascospores, which are highly resistant structures meant for dispersion, are cytotoxic to evade predation. The toxicity of *Morchella* ascospores was further demonstrated by the spore feeding assay, where *A. castellanii* could not survive when *Morchella* ascospores (from three different strains) were the only food source (**Table 3**).

## 7.5 Conclusion

This study investigated the interactions between the bacteriophagous and mycophagous amoeba *A. castellanii* with a prey, *P. koreensis,* and the prey's host *Morchella.* As *P. koreensis* formed biofilms around *Morchella* hyphae, *A. castellanii* benefited from the presence of the fungus as it used biofilms to feed. *Morchella* was not impaired by the presence of *A. castellanii*, and even benefited from its presence as the enhanced predation reduced the nutritional competitivity between *Morchella* and *P. koreensis*. Although the hypothesis that *P. koreensis* would hide within *Morchella* hyphae under predatory pressure was not validated, the experiments and literature findings indicated that it is likely that *Morchella* promoted biofilm formation to help *A. castellanii* to feed on *P. koreensis*, hence reducing the nutrient competition. The tripartite interactions tested in the present study are therefore beneficial for *Morchella* and *A. castellanii*, and detrimental to *P. koreensis* as predation is increased (**Figure 8**). This study revealed an unexpected beneficial association between *Morchella* and *A. castellanii*, that illustrates the complexity of microbial interactions.

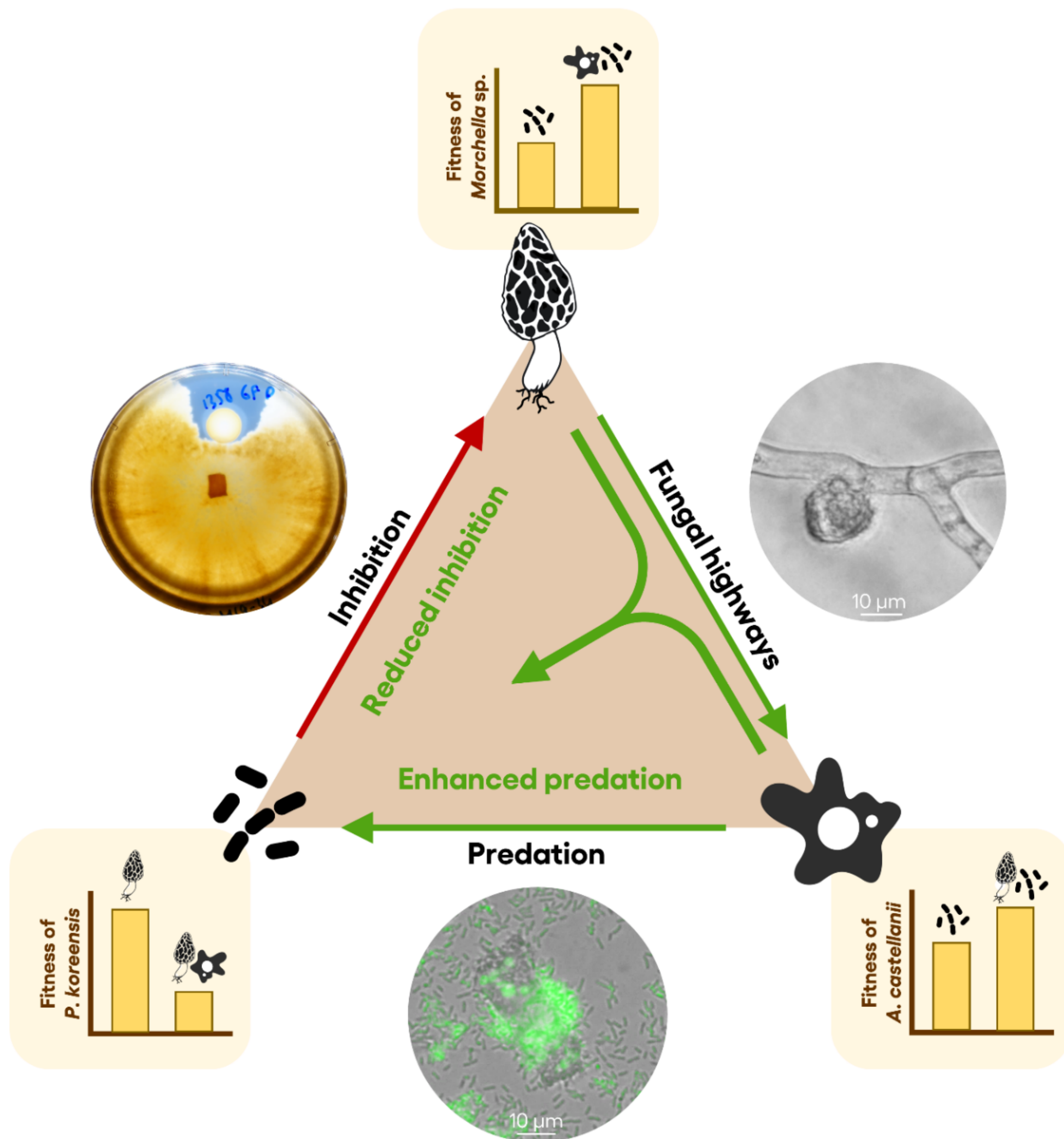



**Figure 8.** Summary of the bi- and tripartite interactions between the predatory amoeba *Acathamoeba castellanii*, its prey *Pseudomonas koreensis*, and the prey's host *Morchella* sp. The red arrow indicates a negative effect (i.e., growth inhibition of Morchella in presence of P. koreensis) while the green arrows show positive effects (i.e., predation and fungal highways benefit *A. castellanii*; the association between *Morchella* and *A. castellanii* are beneficial for both organisms). The histograms are a schematic representation of the fitness (represented by the growth) of each organism in presence of the two others.

## 7.6 Acknowledgment

We are very grateful to Prof. Dr. Hubert Hilbi (UZH Zürich) and Dr. Anaisa Moreno (UZH Zürich) for giving us the opportunity to learn about and work with amoeba, and for their useful comments on the experiments. This study was funded by a Science Focus Area Grant from the U.S. Department of Energy (DOE), Biological and Environmental Research (BER), Biological System Science Division (BSSD) under grant number LANLF59T.

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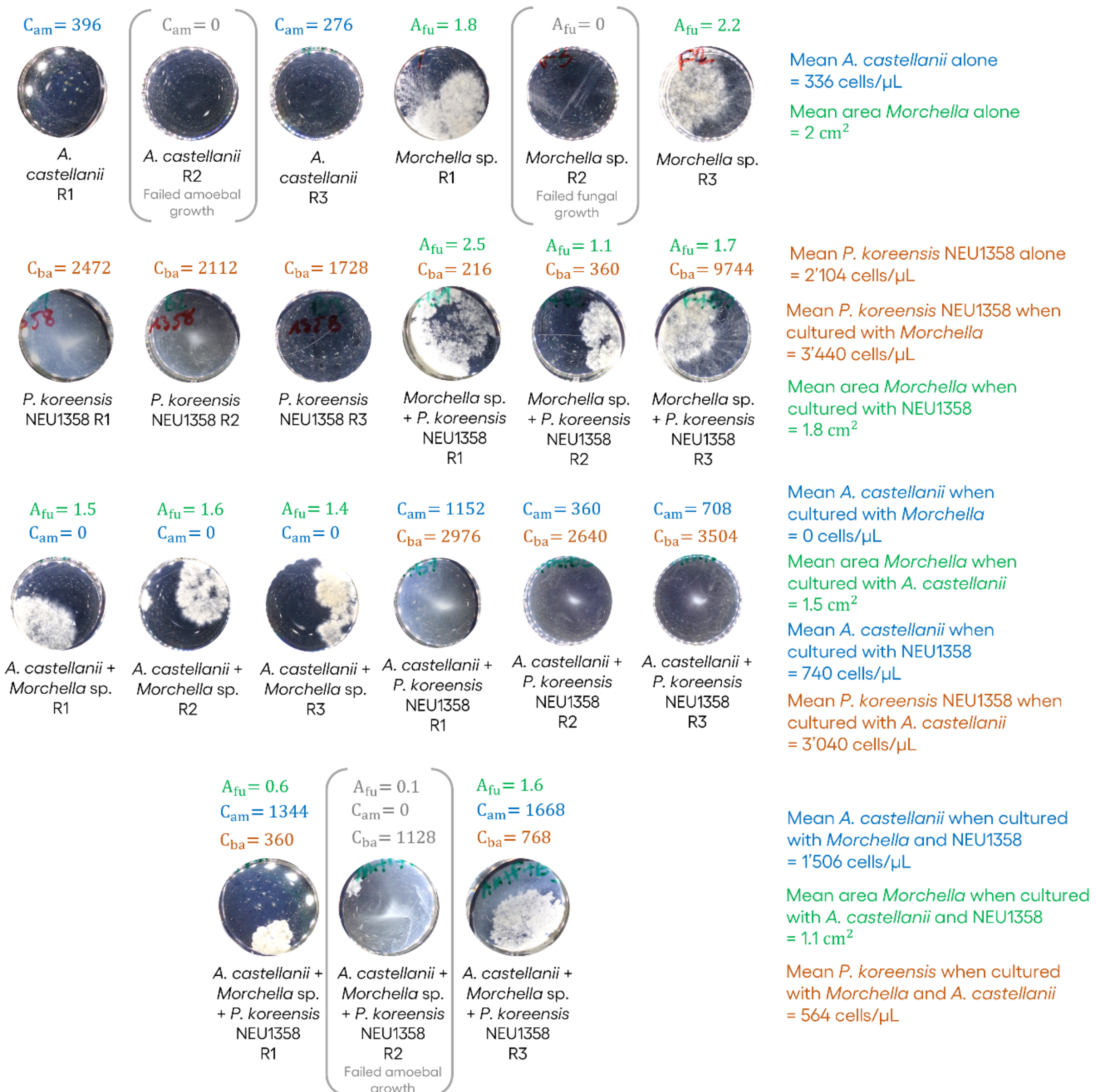
## Supplementary material S1 – Phenotypic characteristics from the quantitative experiment




***S1.*** *Quantification of different combinations of co-cultures between* Morchella *sp. M21-48 [green],* Acanthamoeba castellanii *[blue], and* Pseudomonas koreensis *GFP-NEU1358 [orange] in 12-well plates. Each combination was tested in triplicate. The quantification results corresponding to each well is indicated in color: $A_{fu}$= Area of the well occupied by* Morchella *mycelium, in $cm^2$; $C_{am}$= concentration of amoeba in the well, in cells/µL; $C_{ba}$= concentration of bacteria in the well, in cells/µL. Grey color corresponds to unreliable concentrations, because one of the organisms did not grew in one of the replicates. Pictures were taken five days post inoculation of* A. castellanii *and* P. koreensis *and seven dpi of* Morchella *sp. Diameter of each well= 2.1 cm.*

# 8. Biological interactions between *Morchella* and other soilborne organisms

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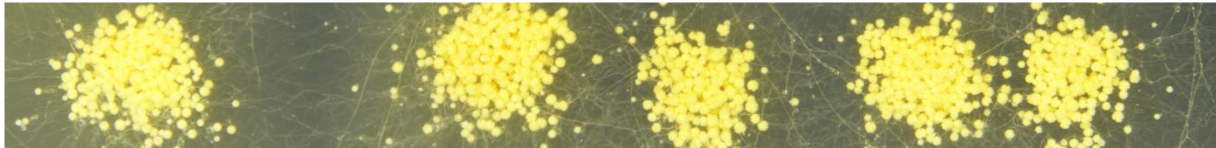

**Foreword**

This chapter investigated interactions between *Morchella* and soil microorganisms, namely the mycophagous nematode *Aphelenchus avenae* and three filamentous fungi (*Penicillium* sp., *Cephalotrichum* sp., *Aspergillus westerdijkiae*). I generated and analyzed all the data, redacted, and reviewed the text. This study shows that *Morchella* have a natural ability to resist predation and to outcompete fungal competitors.

**Abstract**

Although *Morchella* interact with various organisms in the soil such as bacteria, fungi, and animals, these interactions have been rarely studied. This chapter focused on several types of biological interactions. First, the relationship between the mycophagous nematode *Aphelenchus avenae* and *Morchella* was analyzed. It was hypothesized that *Morchella* mycelium containing a native bacteriome would be better protected from predation than a bacteriome-free mycelium. This assumption was not validated, as *A. avenae* had a higher propagation rate and survivability on the bacteriome-containing mycelium. *Morchella* sp. from the Esculenta clade was not a suitable food source for the mycophagous nematode. Secondly, this chapter studied the capacity of *Morchella* spp. to compete with fungal contaminants occurring in fruiting body cultivation assays, i.e., *Penicillium* sp., *Cephalotrichum* sp., and *Aspergillus westerdijkiae*. In addition, the ability of *Pseudomonas koreensis* to act as a biocontrol agent was tested against the latter contaminants. *P. koreensis* had no beneficial effect for *Morchella,* which naturally outcompeted the contaminants.

**Keywords:** bacterial-fungal interactions (BFI), co-cultures, *Pseudomonas koreensis*, *Aphelenchus avenae*, mycophagy

## 8.1 Introduction

*Morchella* spp. form edible fruiting bodies (morel mushrooms) that are sought after worldwide for culinary and medicinal purposes (Y. Xu et al. 2022). *Morchella* spp. interact in the soil with various organisms (bacteria, fungi, animals) that can have a significant impact on morel cultures (Liu et al. 2018). In morel crops, bacteria can act both as competitors or beneficial agents for fungal fructification (Liu et al. 2018). In contrast, soilborne fungi, such as *Trichoderma, Aspergillus, Rhizopus, Mucor, Neurospora, Coprinus*, are major competitors to *Morchella* (Liu et al. 2018). Competing species can inhibit mushroom growth and fructification (Choi et al. 2010). For instance, *Trichoderma* could cause up to 100% crop loss in *Agaricus bisporus* (Ghimire et al. 2021). The primary infection source of *Trichoderma* is usually the fungal substrate, especially if it contains seed grain (Gea et al. 2021), as it is often the case in *Morchella* cultivation (Liu et al. 2018; Y. Xu et al. 2022). Concerning animals, flies (mostly Cecidomyiidae) can directly damage the fruiting bodies by feeding on them, or indirectly by being vectors of fungal pathogens (Ghimire et al. 2021). Other animals such as slugs, springtails and worms were also reported as pest in morel crops (Liu et al. 2018). However, the interactions between *Morchella* and those problematic organisms has never been studied. For this reason, this chapter focused on studying *in vitro* the interactions between *Morchella* and relevant soilborne microorganisms, namely the mycophagous nematode *Aphelenchus avenae* and fungal competitors isolated from soil used in fruiting body cultivation assays.

*A. avenae* is a generalist mycophagous nematode that can also be a facultative plant parasite (Walker 1984). Its interactions with *Morchella* have never been investigated before the present study. We hypothesized that bacteria-free mycelium would be a better host for *A. avenae* compared to bacteria-containing mycelium, because symbiotic bacteria can produce antinematode toxins to protect their fungal associate against predation (Büttner et al. 2021). To test this, bacteria-free mycelial cultures (generated from ascospores) and mycelium harboring its native bacteriome (generated from hymenium) were used in a feeding assay.

During a failed *Morchella* fruiting body cultivation assay in climatic chamber, we isolated three fungal contaminants that had colonized the soil. These fungi were identified in the present study. They were co-cultivated with *Morchella* in presence or absence of *Pseudomonas koreensis,* a potential biocontrol bacterium. *P. koreensis* is found in agricultural soils (Kwon et al. 2003; Rafikova et al. 2016) and was shown to be closely associated to wild *Morchella* spp. (Cailleau et al. 2023). *P. koreensis* strains NEU1358 (= B33.4) and NEU1362 (=VD-NE white) were isolated from mycelial cultures of *M. esculenta* M19-34 and *Morchella* sp. M20-7, respectively (Cailleau et al. 2023). They were previously tagged with green fluorescent protein (GFP) in order to facilitate their microscopic observation (Cailleau et al. 2023). In the conditions tested until now, the bacterial-fungal association between *Morchella* and *P. koreensis* was not beneficial for the fungus (Cailleau et al. 2023; Cravero et al. in preparation), probably because of competition for nutrients. As *P. koreensis* belongs to the core bacteriome of *Morchella* (Cailleau et al. 2023), it was hypothesized that the association was maintained by the fungus because it was beneficial under certain conditions, for instance biotic stress. It is well known that some bacterial strains have antagonistic/inhibitory effects on fungal phytopathogens' growth, thus being already used in agriculture as biocontrol agents (Mardanova et al. 2016). It was hypothesized that *Morchella* would have an enhanced competitivity against fungal competitors when supplemented with *P. koreensis.* To test this, confrontations were established between *Morchella* spp. (host strains of *P. koreensis* and non-hosts) and the three filamentous fungi isolated from fruiting body cultivation assay.

## 8.2 Material and methods

### 8.2.1 Bacteriome-free ascospore-derived mycelial cultures

8.2.1.1 Fungal material

*Morchella deliciosa* M19-41, *Morchella esculenta* M19-42 and *Morchella helvetica* M19-43 were sampled in the canton of Neuchâtel in 2019 and previously identified (Cravero et al. submitted). Fruiting bodies were incubated in plastic jars in an oven at 22°C for 2-3 days to trigger natural sporulation. Spores were kept dry in plastic boxes. Mycelial cultures were generated by spreading ascospores on Potato Dextrose Agar (PDA; Potato dextrose 26 g/L, Agar 13 g/L, Sigma-Aldrich). Mycelium was maintained for seven days at 22-23°C in darkness before DNA extraction.

8.2.1.2 DNA extraction and 16S amplification by PCR

Mycelium from pure cultures of ascospore-derived mycelium was scrapped and used for DNA extraction using the Quick-DNA™ Fungal/Bacterial Miniprep Kit and following the instructions provided by the manufacturer (Zymo Research, USA). Using an amplification method previously described (Robinson et al. 2021), the 16S rDNA was amplified from ascospore-derived mycelium to evaluate the presence of vertically transmitted bacteria.

### 8.2.2 Feeding experiments between *Aphelenchus avenae* and *Morchella* spp.

8.2.2.1 Co-cultivation of *Morchella* spp. and *Aphelenchus avenae*

*Morchella* sp. M21-48 (Elata clade) and *Morchella* sp. M22-6 (Esculenta clade) were sampled in the canton of Neuchâtel in 2021 and 2022, respectively. Primary mycelial cultures were generated as explained in **section 2.1.1.** Secondary cultures were prepared on Corn Meal Agar (CMA; Corn Meal 9 g/L, Agar 12g/L, Sigma-Aldrich) and let grown for seven days at 23°C in darkness before adding the nematodes. The mycophagous nematode *Aphelenchus avenae* was kindly provided by Prof. Markus Künzler (Künzler Lab, ETH Zürich, Switzerland). Nematodes were primarily cultivated on a sporulation-deficient strain (BC-3) of *Botrytis cinerea* (Tayyrov et al. 2018) on CMA at 23°C in darkness, and transferred on *Morchella* by chunking (i.e., cutting and inoculating a 8 x 8 $mm^2$ piece of agar from an active culture).

8.2.2.2 Viability assay and evaluation of the nematodes' propagation rate

To count dead and living nematodes, we collected them from solid cultures as follows: pieces of agar (1 $cm^3$) from active cultures were soaked for 10 sec (gently shaking) in 100 µL of physiological water (0,9% NaCl) deposited beforehand in an empty Petri dish; 100 5 nM of SYTOX Dead Cells Stain (Invitrogen, USA) was added to the water drop and incubate in darkness for at least 15 min; cover slides were deposited on the drops to determine the dead:living ratio of nematodes by counting them with an inverted light microscope. For each co-culture, we counted nematodes from three sampling sites (**Figure 1**). Six sampling sites were evaluated in the control co-cultures (*Botrytis-Aphelenchus*), and nine sampling sites were evaluated in the *Morchella* co-cultures (**Table 1**). The statistical significance of the difference between the groups was calculated with a One-way ANOVA test using the Microsoft Excel Data Analysis ToolPak.

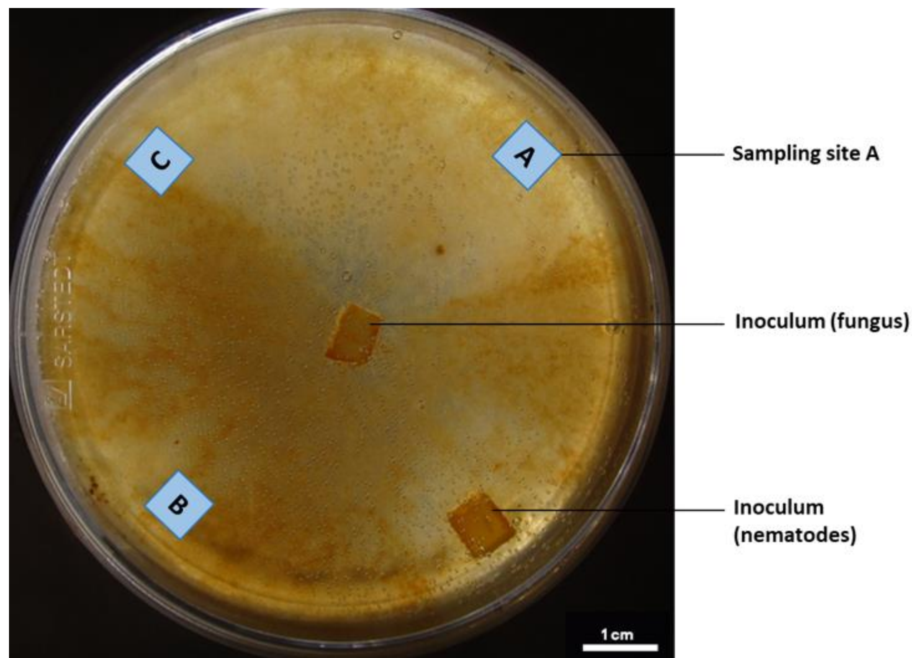


**Figure 1**. Sampling sites A, B and C in the co-cultures *Morchella-Aphelenchus* from which the mortality and propagation rates of the nematodes were evaluated.

**Table 1**. Fungal strains that were co-cultured with *Aphelenchus avenae* for the feeding experiments. The presence of bacteria in the mycelial cultures and the numbers of replicates that were used for each treatment are indicated.

| Fungus | Bacteria | Nematode | Replicates |
|---|---|---|---|
| *Botrytis cinerea* BC-3 | Native bacteriome | *Aphelenchus avenae* | 2x |
| *Morchella* sp. M21-48-Asc | None | *Aphelenchus avenae* | 3x |
| *Morchella* sp. M21-48-Hym | Native bacteriome | *Aphelenchus avenae* | 3x |
| *Morchella* sp. M22-6-Asc | None | *Aphelenchus avenae* | 3x |
| *Morchella* sp. M22-6-Hym | Native bacteriome | *Aphelenchus avenae* | 3x |

### 8.2.3 Effect of *Pseudomonas koreensis* against competitive fungi

#### 8.2.3.1 Bacterial and fungal material

*Morchella* sp. M23-16 (Elata clade) was sampled in the canton of Neuchâtel in 2023. To generate mycelial cultures, we inoculated a piece (about 1 $cm^3$) of fresh hymenium on PDA. If needed, we purified the cultures after one day to eliminate bacterial or fungal contaminants. We maintained mycelium for 5-10 days at 22-23°C in darkness before using it in the experiments.

We previously isolated *Pseudomonas koreensis* strains NEU1358 and NEU1362 from *Morchella* spp. and tagged them with green fluorescent protein (GFP) (Cailleau et al. 2023). For the experiments, we prepared overnight cultures of *P. koreensis* GFP-NEU1358 and GFP-NEU1362 in Luria Bertani broth (LB; 10 g/L Tryptone, 10 g/L NaCl, 5 g/L Yeast extract, Sigma Aldrich).

We isolated three fungal contaminants (strains U1, U2 and U4) from an indoor morel fruiting body cultivation trial of strain *Morchella* sp. M21-48. Contaminants appeared at soil surface or coating wheat grains from culture substrate, after one month when parameters were set as follows: 18°C, 80% relative humidity, 10h light/14h dark. We sampled the contaminants and inoculated them on PDA with a platinum loop. We purified the cultures if necessary, and maintained them for seven days at 20°C in darkness before DNA extraction.

8.2.3.2 Identification of the fungal contaminants

To identify the fungal contaminants described above, we scrapped mycelium from pure cultures and used it for DNA extraction, which was performed with a Quick-DNA™ Fungal/Bacterial Miniprep Kit and following the instructions provided by the manufacturer (Zymo Research, USA). For the PCR, the following primers were used to amplify the ITS portion 1 and 2, including the 5.8S ribosomal rDNA: ITS1-F (TCCGTAGGTGAACCTGCGG) (White et al. 1990) and ITS4-R (TCCTCCGCTTATTGATATGC) (Gardes and Bruns 1993). For each sample, the PCR mix contained PCR-grade water, 2X ALLin™ Red Taq Mastermix (HighQu, Germany), 0.2 µM forward and 0.2 µM reverse primer and 1 µL of 2 ng/µL DNA. Amplifications were performed in a Thermo Scientific Arktik thermal cycler with the following parameters: denaturation at 95°C for 1 min, 40 cycles of denaturation, annealing and elongation (95°C for 15 sec, 62°C for 15 sec, 72°C for 15 sec), final elongation at 72°C for 2 min, end at 15°C. PCR products were then loaded on a 1.2% agarose gel that underwent electrophoresis (100 mV, 30 min). Amplicons were visualized under UVs in a Genoplex VWR transilluminator. Positive PCR products (i.e., single band at the expected size) were then purified with a MultiScreen® Filter Plates PCR µ96 (Millipore Corporation, USA) as follows: in each well, the PCR product and 50 µL of PCR-grade water was added; a vacuum of 20 bars was applied on the wells until they dry; 20 µL of PCR-grade water was added to each well; after 2 min, DNA contained in the membrane from each well was resuspended by pipetting up and down 20 times. Once purified, the PCR products were quantified by Qubit. Final concentration was adjusted at 2-40 ng/µL and sent to Fasteris (Switzerland) for Sanger sequencing. The sequences were trimmed and assembled with BioEdit 7.2. and entered in nBLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) for identification.

We observed mycelium and sporulating structures under an H600L stereo microscope (Nikon, Japan) directly in the PDA plates, or on microscope slides with an EVOSM5000 inverted light microscope (Invitrogen, USA).

8.2.3.3 Co-cultivation (*Morchella-Pseudomonas*-fungal contaminant) in solid medium

To evaluate *in vitro* the ability of *Morchella* to compete against contaminants, we established co-cultures on malt agar (MA; malt 12 g/L, agar 12 g/L, Sigma-Aldrich) by inoculating a piece (1 $cm^3$) of actively growing mycelium of *Morchella* and a contaminant at opposite sides of petri dishes. After two days of growth at 19°C in darkness, we added sterile physiological water (0.9% NaCl) or a bacterial suspension of *P. koreensis* GFP-NEU1358 at about 2 cm of the *Morchella* inoculum. A total of 72 confrontations were established. We recorded radial mycelial growth of both *Morchella* and contaminants, as well as the length of exclusion zones in each co-culture at 8 days post inoculation (dpi) using the software ImageJ v1.54d (Schneider, Rasband, and Eliceiri 2012). We made histograms to visualize the data. In addition, the presence of sclerotia, and bacterial colonies was recorded. We calculated the statistical significance of the difference between the treatments with One-way ANOVA from the Microsoft Excel Data Analysis ToolPak.

8.2.3.4 Co-cultivation (*Morchella-Pseudomonas*-fungal contaminant) in culture substrates

We tested two substrates in this experiment: wheat culture substrate (CS) and spent beer brewery grains (SBBG). One kilogram of CS contained organic wheat grains (560 g; Biofarm), organic wheat bran (280 g; Biofarm), woodchips (100 g, Migros), CaSO4 (10 g; Sigma-Aldrich), Precipitated Calcium Carbonate (PCC, 10 g; Sigma-Aldrich), organic soil (40 g, Migros) and tap water (450 mL). SBBG was obtained from the organic brewery Bières d'OZ (Switzerland). Substrates were autoclaved twice for 1h at 121°C.

At the bottom of sterile 160 x 29 mm glass tubes filled with substrate, we deposited pieces of agar with actively growing mycelium (1 $cm^3$, either *Morchella* or contaminants, see below). Then, we added 3mL of bacterial suspension (0.9% NaCl with $10^6$ cells/mL of GFP-NEU1358 or GFP-NEU1362) or sterile physiological water (control) to the tube. After 7 days, we added a second mycelial inoculum (either *Morchella* or contaminants) at the top of the tube. In the CS, *Morchella* was inoculated at the top, while it was inoculated at the bottom in SBBG. We sealed glass tubes with Parafilm and maintained them at 19°C in darkness for one month. A graphic representation of the cultures is shown in **Figure 2**.

We measured mycelial growth (in mm) of *Morchella* and the contaminants from the inoculum to the most recent hyphal tips, at 21 and 28 dpi (count since the inoculation of the first mycelium). We also measured the interval of the glass tube that was filled with substrate to calculate the portion of the tube (in %) that was colonized by *Morchella* and the contaminant. Histograms were made to visualize the data. In addition, we recorded the presence of spores, sclerotia, exclusion zone, or contaminations. We calculated the statistical significance of the difference between the treatments with One-way ANOVA from the Microsoft Excel Data Analysis ToolPak.

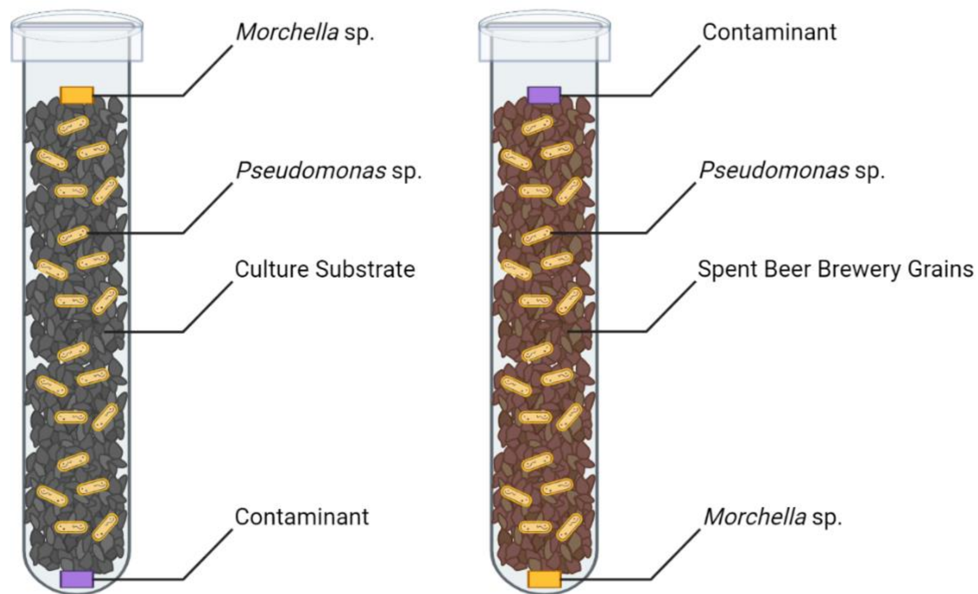


**Figure 2**. Culturing method used to evaluate the ability of P. koreensis GFP-NEU1358 and GFP-NEU1362 to enhance competitivity of *Morchella* sp. against fungal contaminants. Two types of substrates were tested: Culture Substrate (left) and Spent Beer Brewery Grains (right). Inoculum at the top was placed 7 days after the inoculum at the bottom and bacteria.

## 8.3 Results

### 8.3.1 Anti-predator effect of the *Morchella* bacteriome against *Aphelenchus avenae*

8.3.1.1 Ascospore-derived mycelium is bacteria-free

The 16S rDNA amplification in the ascospore-derived mycelial cultures revealed that no bacteria were present in any of the tested *Morchella* strains. In other words, the ascospore-derived mycelia were bacteria-free.

8.3.1.2 Feeding assay

We evaluated the mortality and propagation rates of *A. avenae* on *Morchella,* and compared it with the control (co-culture *Botrytis-Aphelenchus*). The initial concentration of nematodes was not known. For this experiment, we used two types of *Morchella* mycelia to evaluate the anti-predatory ability of *Morchella*'s bacteriome: bacteria-free mycelium (ascospore-derived) and mycelium with native bacteriome (hymenium-derived) (see **Table 1** in **section 2.2.1** for a summary of the tested combinations). In the control co-culture (*Botrytis-Aphelenchus*), each sampling site contained in average 93.83 nematodes, with a proportion of 1.78% of dead nematodes (**Figure 3**). In the hymenium-derived mycelial cultures of *Morchella,* there was an average of 17.83 nematodes per sampling site, with 1.56% of them being dead. In the cultures of bacteria-free *Morchella* (i.e., ascospore-derived mycelium), there was an average of 9.5 nematodes, for 8.19% of them being dead (**Figure 4**). The difference between the three groups was statistically significant (*p*-value= 0.041). in addition, we made a detailed histogram to compare the average number of nematodes per sampling site, culture replicate, and co-inoculated mycelium (**Figure 5**). This revealed that less nematodes were able to live on *Morchella* sp. M22-6 (average per site= 0.28) as compared to *Morchella* sp. M21-48 (average per site= 27.06). Similarly, the proportion of dead nematodes in *Morchella* sp. M22-6 and *Morchella* sp. M21-48 were 20% and 3.7%, respectively. The different sampling sites (A, B or C) had no influence on the average number of nematodes. To summarize, this experiment showed that *A. avenae* were less able to propagate on *Morchella* mycelium (even more if it was bacterial-free) compared to the control *B. cinerea*. Within *Morchella*, the strain from the Elata clade (M21-48) was more suitable for the propagation of *A. avenae* than the strain from the Esculenta clade (M22-6).

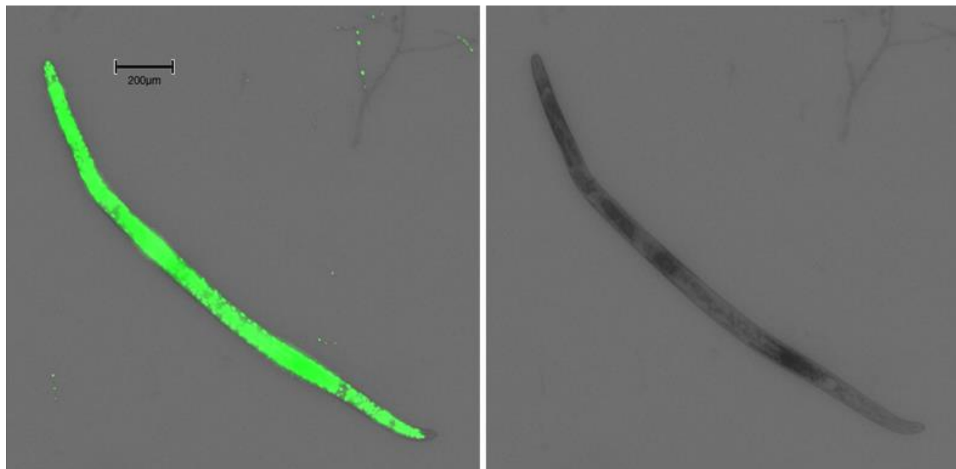


**Figure 3**. Dead nematode (*Aphelenchus avenae*) stained by SYTOX and magnified 20x with an inverted light microscope (left= bright field + green fluorescence; right= bright field, DI water).

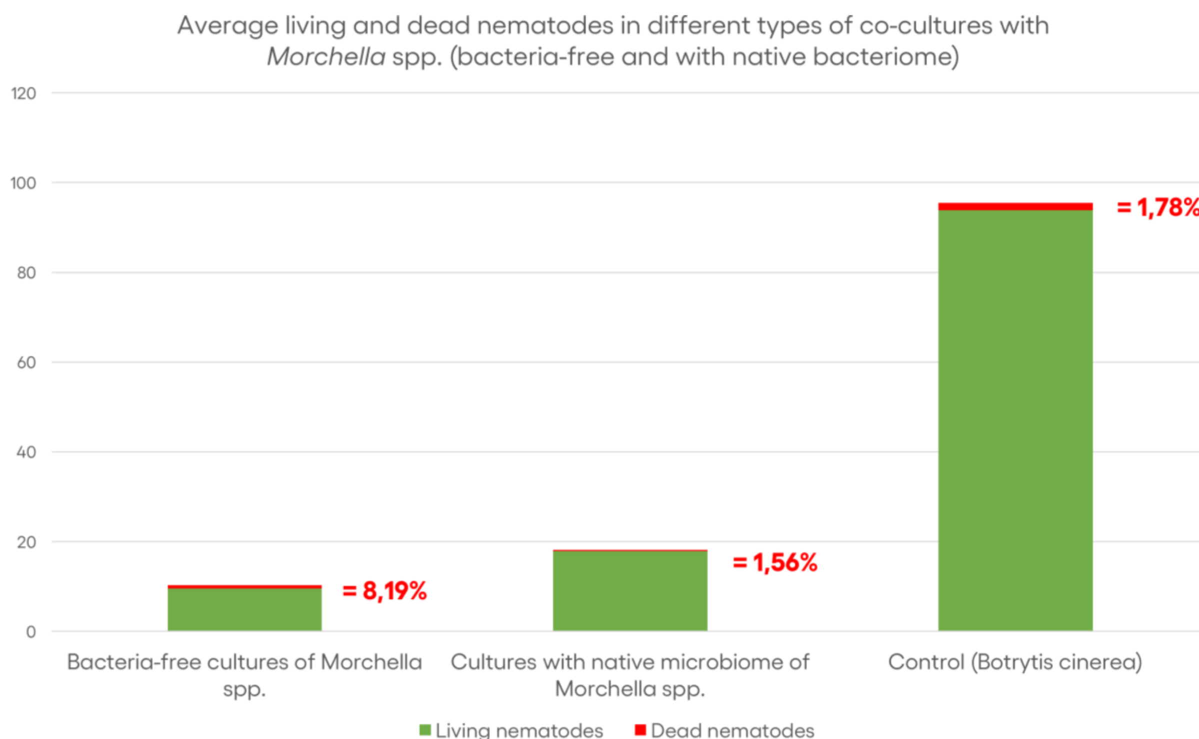


**Figure 4**. Histogram representing the number of nematodes (alive in green; dead in red) in average per sampling sites (n=18 for *Morchella*; n=6 for *Botrytis;* also see Figure 2) of ascospore-derived (bacteria-free) and hymenium-derived (native bacteriome) mycelial cultures of Morchella spp. and control Botrytis cinerea. The percentages of dead nematodes per treatment are written in red. The difference between the three treatments is significant (p-value < 0.005).

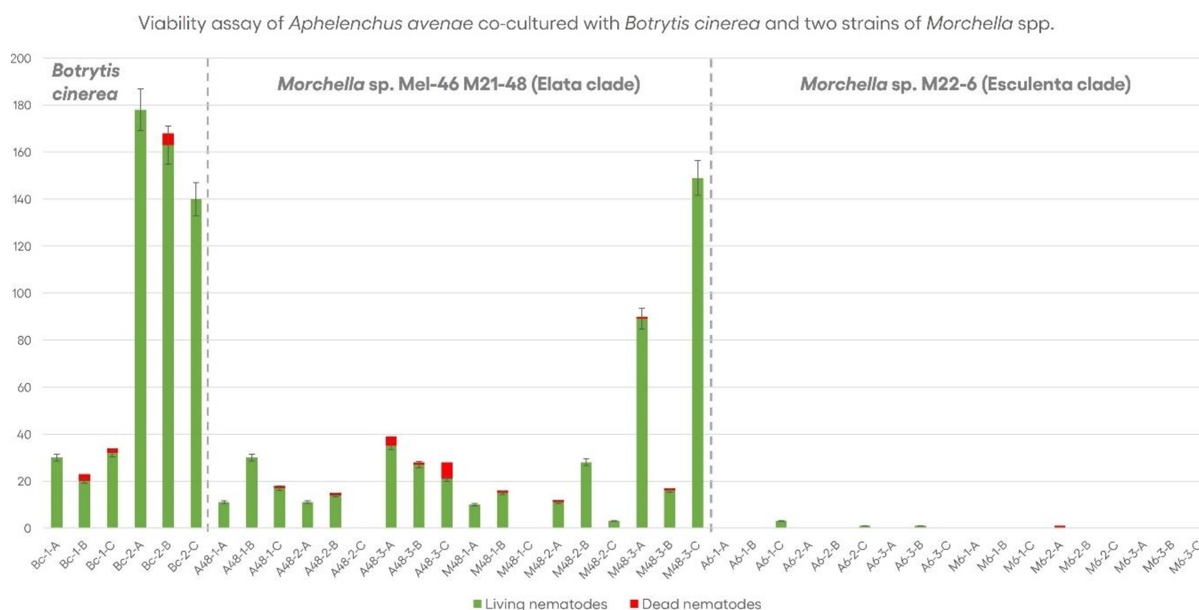


**Figure 5**. Histogram representing the number of nematodes (alive in green; dead in red) per sampling site (A, B and C), culture replicate (1, 2 and 3) and co-inoculated fungus (*Botrytis cinerea* [Bc, control]; *Morchella* sp. M21-48 [48]; Morchella sp. M22-6 [6]) originating from ascospores (A; bacteria-free) or hymenium (M; natural bacteriome). The difference between the five groups is significant (p-value < 0.0002).

### 8.3.2 Effects of *Pseudomonas koreensis* against competitive fungi

8.3.2.1 Identification of the fungal contaminants

Using NCBI BLAST, the ITS of strain U1 had 100% identity (ID) and 100% query cover (QC) with two species (*Penicillium vanbeymae* [teleomorph = *Penicillium baarnense*] and *P. turbatum*); U2 had 100% ID and QC with two species (*Cephalotrichum microsporum* and *Cephalotrichum gorgonifer* [synonym = *Trichurus spiralis*]); U4 had 100% ID and QC with two species (*Aspergillus ochraceus* and *A. westerdijkiae*). For strain U1 (**Figure 6A**), further distinction between *P. vanbeymae* and *P. turbatum* was not possible due to lack of documentation regarding the morphology or the ecology of these fungi. Strain U1 was then attributed to *Penicillium* sp. at genus level. Based on the morphology of the mycelial mass and the conidiophores, U2 did not correspond to *C. microsporum* nor *C. gorgonifer.* (Sandoval-Denis et al. 2016), as the conidia of U2 were white and not brown-black (**Figure 6D-E**). None of the *Cephalotrichum* species had white conidia as U2, but the morphology of the conidiophores and the shape of the conidia (**Figure 6F**) do match with this genus (Sandoval-Denis et al. 2016). For this reason, according to both genetics and mycelium micromorphology, U2 was assigned to *Cephalotrichum* sp. at genus level. Finally, morphological comparison of *A. ochraceus* and *A. westerdijkiae* allowed to assign the contaminant U4 to *A. westerdijkiae* (Visagie et al. 2014) (**Figure 6B**). The structure represented in **Figure 6C** is a diminutive conidiophore (Samson et al. 2014) that appeared in physiological water.

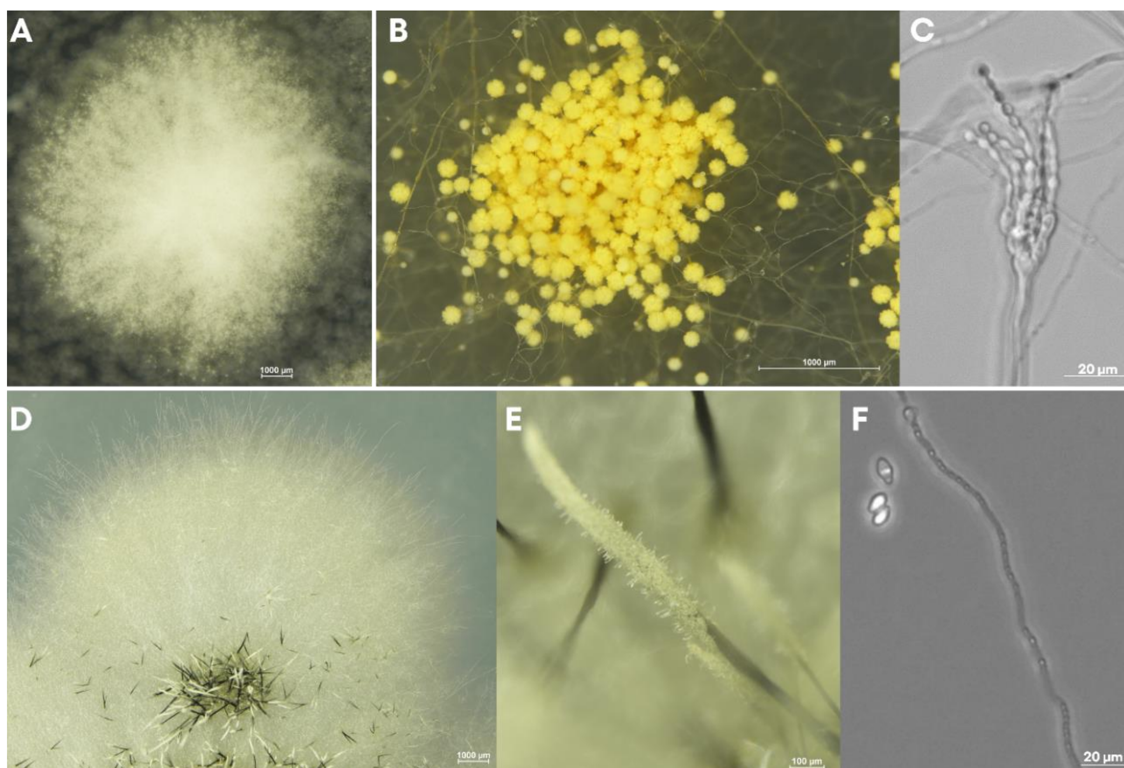


**Figure 6**. Microscopic pictures of the fungal contaminants *Penicillium* sp. U1 (A), *Aspergillus westerdijkiae* U4 (B-C), *Cephalotrichum* sp. U2 (D-F). Fungal colonies A, B, D and E were grown on malt agar. Fungal colonies C and F were grown in physiological water. Pictures were taken directly on malt-agar medium at magnification 0.75x (A-B), 3x (D) and 8x (C) with a stereo microscope or in physiological water (C, F) with an inverted light microscope at magnification 400x.

8.3.2.2 Co-cultures of *Morchella*, fungal contaminants and *Pseudomonas koreensis* in solid medium

To evaluate the impact of associate bacteria (*P. koreensis* GFP-NEU1358) on host (*M. esculenta* M19-34) and non-host (*Morchella* sp. M21-48) confronted to fungal contaminants (*Penicillium* sp. U1, *Cephalotrichum* sp. U2, *A. westerdijkiae* U4), tripartite confrontations were established on MA. It was expected that *P. koreensis* would enhance the competitivity of the host fungus (M19-34) by promoting its mycelial growth or inhibiting competitors' growth.

The two treatment groups (with/without added *P. koreensis*) differed significantly (*p*-value= 0.016). Mycelial growth of *Morchella* with *P. koreensis* was lower (total growth= 1298.58 mm) compared to the treatments with physiological water (mean growth= 1477.83 mm) (**Figure 7**). The hymenium-derived strains grew better (total growth of M19-34 and M21-48-Hym= 503.77 and 514.72 mm, respectively) compared to the ascospore-derived mycelium (total growth of M21-48-Asc= 459.54 mm). With the bacterial treatment, host mycelium (total growth of M19-34-Hym= 503.65 mm) grew better than non-host mycelia (total growth of M21-48-Hym and M21-48-Asc= 412.97 and 381.96 mm, respectively).

All contaminants performed better in the presence of *P. koreensis* (total growth= 581.62 mm) compared to the water treatment (542.08 mm) (**Figure 8**).

In addition, exclusion zone was larger with the bacterial treatment in each confrontation, except in the controls (*Morchella* vs *Morchella*) (**Figure 9A**). Finally, we evaluated the ratio of the *Morchella:Contaminant* radial growth (**Figure 9B**). Ratio >1 indicated that *Morchella* had a better growth than the contaminant, which was the case in all confrontations, excepted controls. Ratio was higher in the water treatment compared to the bacterial treatment, excepted in M21-48-Hym vs *A. westerdijkiae* U4.

Various phenotypic features were observed on *Morchella*: melanization (spreading from *Morchella* inoculum or at hyphal tips) (**Figure 10A**), small sclerotia (**Figure 10B**), aerial hyphae, presence of additional bacterial colonies (spreading from the primary bacterial inoculum or located at hyphal tips) (**Figure 10C**), and mycelial "bridges" spreading from *Morchella* hyphal tips directly towards the contaminant (**Figure 10D-E**). These features were recorded for each co-culture, but not patterns could be established.

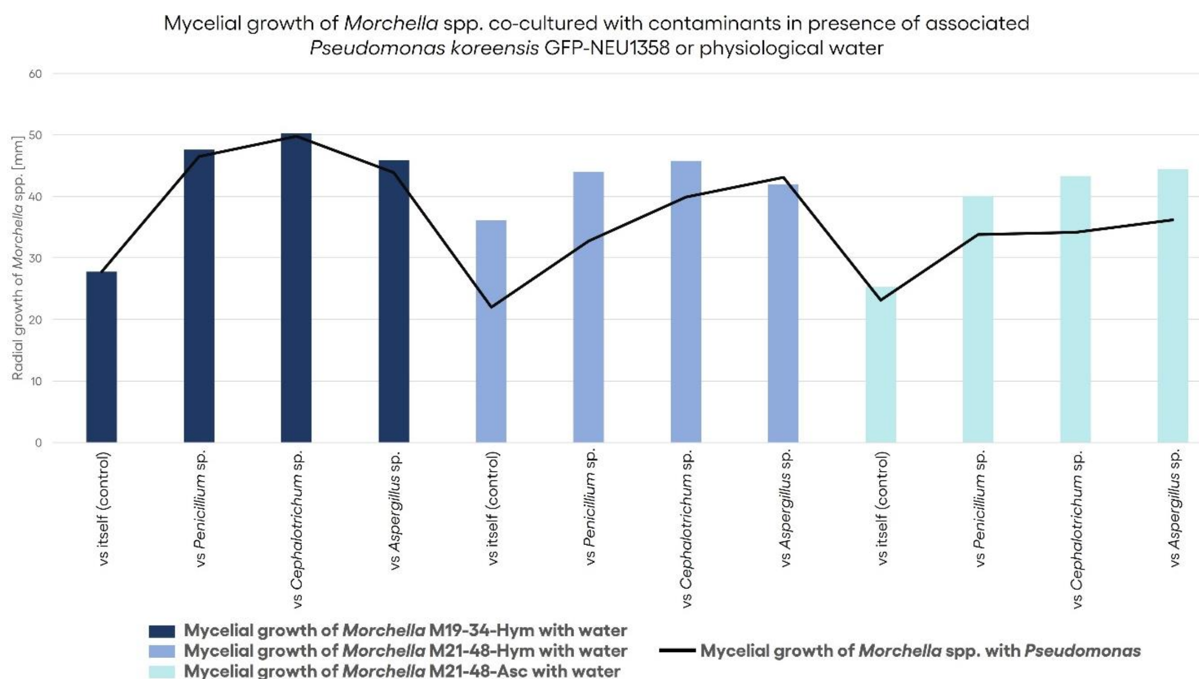


**Figure 7**. Combined histogram representing the radial mycelial growth of *Morchella esculenta* M19-34 (hymenium-derived [dark blue]), *Morchella* sp. M21-48 (hymenium- [medium blue] and ascospore-derived [light blue]) confronted with themselves (control), *Penicillium* sp. U1, *Cephalotrichum* sp. U2, or *A. westerdijkiae* U4. The co-cultures were either supplemented with physiological water [blue columns] or with *P. koreensis* GFP-NEU1358 [black line]. Growth was recorded on malt-agar at 8 dpi.

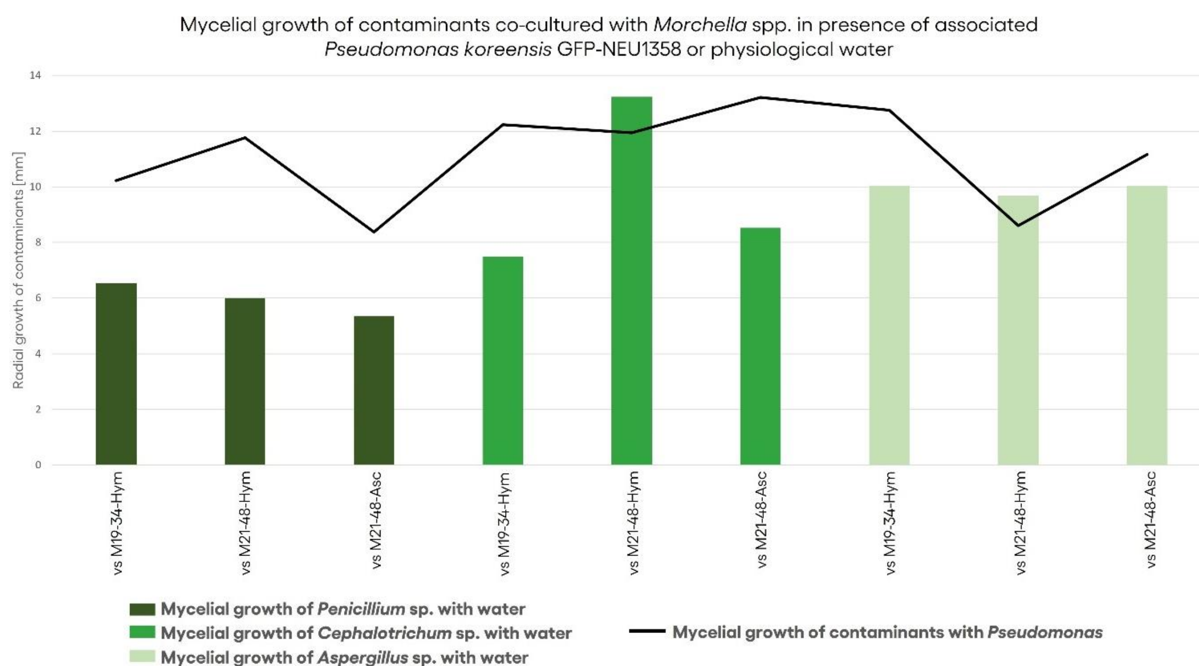


**Figure 8**. Combined histogram representing the radial mycelial growth of three fungal contaminants *(Penicillium* sp. U1 [dark green], *Cephalotrichum* sp. U2 [medium green], and *A. westerdijkiae* U4 [light green]) in confrontation with *Morchella esculenta* M19-34 (hymenium-derived), *Morchella* sp. M21-48 (hymenium- and ascospore-derived). The co-cultures were either supplemented with physiological water [blue columns] or with *P. koreensis* GFP-NEU1358 [black line]. Growth was recorded on malt-agar at 8 dpi.

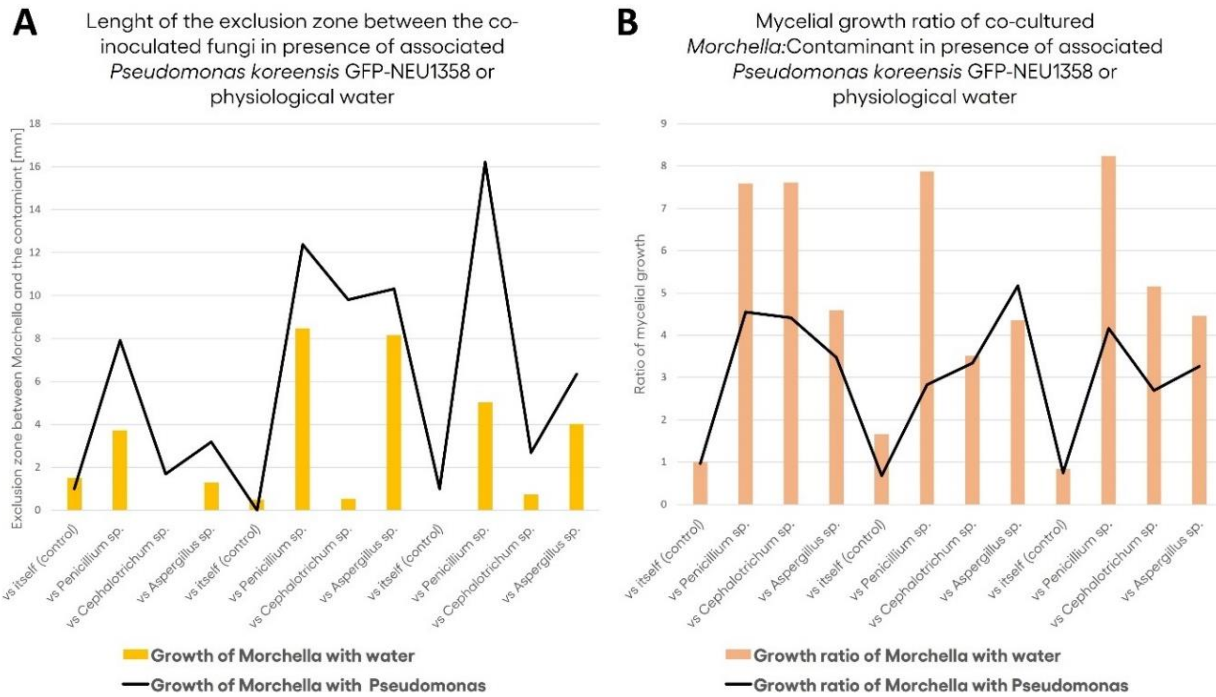


**Figure 9**. Combined histograms representing the length of the exclusion zone between the co-inoculated Morchella spp. and fungal contaminants (A) and the growth ratio of co-cultured *Morchella*:Contaminant (B). Ratio >1 means that *Morchella* had a better growth than the contaminant. In both graphs, the colored bars represent the treatment with physiological water while the black line represents the bacterial treatment. Growth was recorded on malt-agar at 8 dpi.

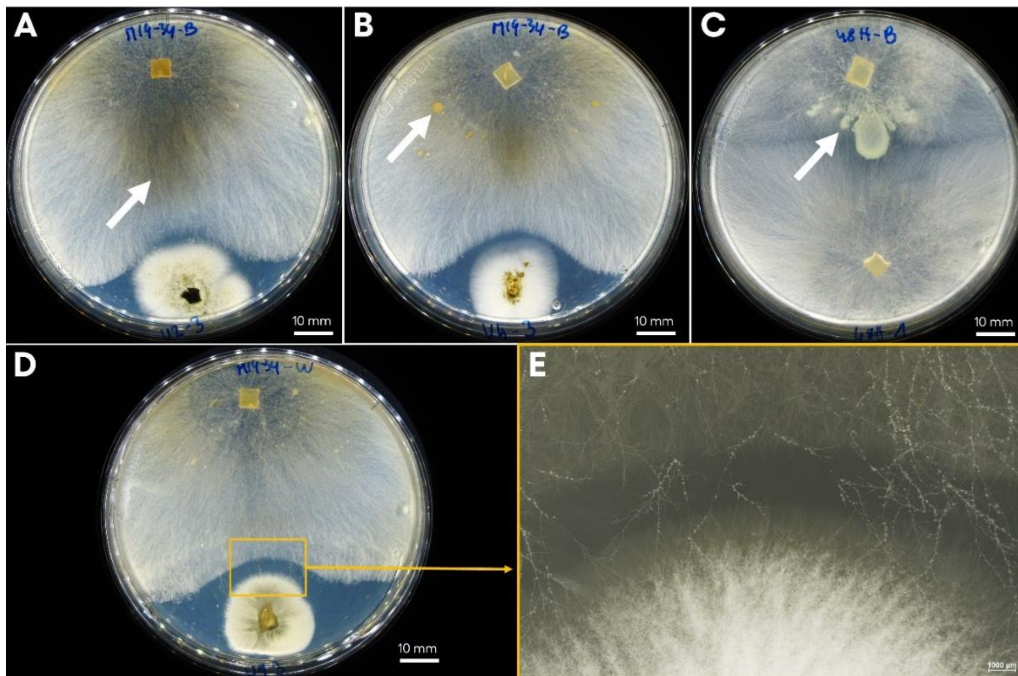


**Figure 10**. Pictures showing various features (white arrows) observed in tripartite confrontations between *Morchella* spp., *Pseudomonas koreensis* and fungal contaminants: (A)= melanization spreading from *Morchella* inoculum, mostly around the bacterial inoculum (M19-34-Hym + P. koreensis vs *Cephalotrichum* sp. U2); (B)= small sclerotia (M19-34-Hym + *P. koreensis* vs *A. westerdijkiae* U4); (C)= additional bacterial colonies spreading from the primary bacterial inoculum (M21-48-Hym + P. koreensis vs itself); (D)= mycelial “bridges” spreading from *Morchella* hyphal tips directly towards the contaminant (M19-34-Hym + water vs *Penicillium* sp. U1); (E)= microscopic magnification of (D) (0.75x). Pictures were taken at 8 dpi on malt-agar.

8.3.2.3 Co-cultures of *Morchella*, fungal contaminants and *P. koreensis* in culture substrates

We confronted *Morchella* sp. M23-16 with the three contaminants (*Penicillium* sp. U1, *Cephalotrichum* sp. U2 and *A. westerdijkiae* U4) in glass tubes supplemented with either sterile water or *P. koreensis* suspensions (strains GFP-NEU1358 or GFP-NEU1362). We tested this system in two substrates: Culture Substrate (CS) and Spent Beer Brewery Grains (SBBG). We did not retain the results concerning CS because the majority of the cultures presented secondary contaminations (mostly green mold and *A. westerdijkiae*) growing from wheat grains. In SBBG, green mold was only observed in two cultures (*Morchella-Penicillium* + water [replicate 1]; *Morchella-Penicillium* + GFP-NEU1358 [replicate 1]).

In SBBG, the difference of mycelial growth between the three treatments ($H_2O$, GFP-NEU1358, GFP-NEU1362) was not statistically significant (p-value = 0.865), but we observed tendencies. *Morchella* grew less with *P. koreensis* GFP-NEU1362 (mean growth = 27.75 µm) and with sterile water (32.17 µm) than with *P. koreensis* GFP-NEU1358 (37.17 µm). The same trend was seen for *Penicillium* sp. and *Cephalotrichum* sp. However, *A. westerdijkiae* grew more with sterile water than with bacteria.

Overall, *Morchella* outcompeted all contaminants in SBBG, as it grew faster and was able to colonize a higher portion of the substrate, regardless of the presence or not of *P. koreensis* (**Figure 11**).

*Penicillium* sp. U1 was in physical contact with *Morchella* at 28 dpi, in three cultures (one with water and two with GFP-NEU1358). We observed an inhibition zone (i.e., a zone where the mycelium is less dense or absent) in 2/3 of the cultures. *A. westerdijkiae* U4 produced a large quantity of spores in each treatment.

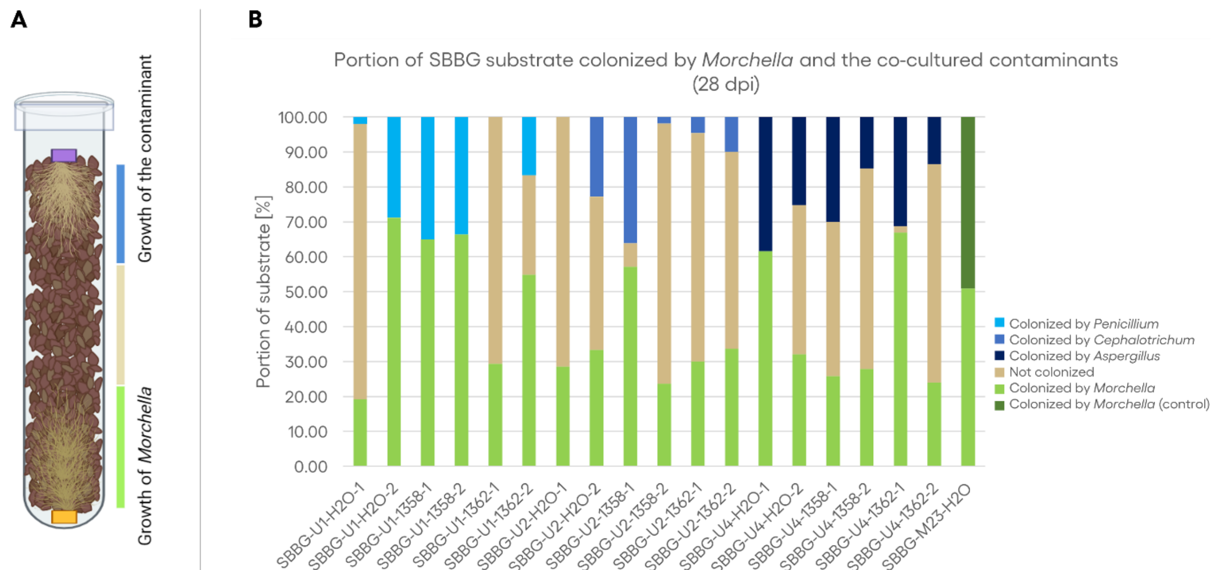


**Figure 11**. Co-cultures of fungal contaminants and *Morchella* in Spent Brewery Beer Grains (SBBG). A= Schematic representation of the cultivation system; B= Histogram representing the proportion of colonization [%] of the SBBG substrate by the contaminants *Penicillium* sp. U1, *Cephalotrichum* sp. U2 and *Aspergillus westerdijkiae* U4 (in three tones of blue) in confrontation with *Morchella* sp. M23-16 (in green), supplemented with deionized water [-H2O], P. koreensis GFP-NEU1358 [-1358] and GFP-NEU1362 [-1362]. The control in dark green represents the co-cultivation of M23-16 with itself. The measurements were done at 28 dpi.

## 8.4 Discussion

In this chapter, we demonstrated that *Morchella* spp. have a natural ability to resist predation from the mycophagous nematode *A. avenae* and to outcompete fungal contaminant. In addition, we showed that *P. koreensis* did not enhance *Morchella* competitivity. We further discuss these findings in the next sections.

### 8.4.1 *Morchella* spp. have a natural ability to defend against *Aphelenchus avenae* predation

Büttner et al. compared the production of antinematode compounds (benzolactone necroximes) between mycelial cultures of *Mortierella verticillata* NRRL 6337 containing native endobacteria, and the same strain that has been antibiotically cured from any endosymbiont (Büttner et al. 2021). This revealed that the *Mycoavidus* endosymbionts were responsible for the production of the cytotoxic compounds. The mycophagous predator *A. avenae* was able to live in cultures of cured *M. verticillata* but not in presence of the endosymbiotic strain (Büttner et al. 2021). In *Morchella*, we obtained bacteria-free mycelium by cultivation of dry ascospores (see **section 3.1.1**). Therefore, we explored the resistance of bacteria-free *versus* bacteriome-containing *Morchella* mycelium in presence of *A. avenae,* similarly to Büttner et al. (Büttner et al. 2021). Our initial hypothesis was that bacteria-free mycelium would be less resistant to *A. avenae* predation, but this was not the case, as nematodes propagation and survivability rates were significatively higher in cultures where bacteria were present (**Figure 4**). *A. avenae* was not able to survive on the yellow morel M22-6, as only 0.28 living nematodes was recorded per sampling site (**Figure 5**). The black morel M21-48 was a better host, with 27.06 average *A. avenae* per sampling site. This was still more than three times less than in the *B. cinerea* control. These findings showed a high interspecific disparity in the response of *Morchella* towards predation. In the literature, no report of co-cultures nor interactions between *Morchella* and *A. avenae* (or other nematodes) were found. However, it has been demonstrated that numerous fungi (besides *B. cinerea*) were reliable food sources for *A. avenae*. Those included *Fusarium oxysporum, Chaetomium cochlioides, Chaetomium funicola, Chaetomium globosum* (Ascomycota)*, Rhizoctonia solani, Agaricus bisporus, Coprinopsis cinereus* (Basidiomycota) (Okada and Kadota 2003), *Alternaria alternata, Stemphylium* sp., *Thielaviopsis basicola* and *Verticillium dahliae* (Ascomycota) (Chen and Ferris 2000). Interspecific differences existed within *Chaetomium* hosts (Okada and Kadota 2003), but they were less important than what has been observed between *Morchella* spp. M21-48 and M22-6. From literature review, unreliable fungal hosts for *A. avenae* included *Aspergillus niger* (Javed and Khan 2021), *Penicillium* sp. (Chen and Ferris 2000) and *Pleurotus ostreatus* (Okada and Kadota 2003). No hypothesis has been formulated concerning the non-suitability of *A. niger* (Javed and Khan 2021) and *Penicillium* sp. to host *A. avenae* (Chen and Ferris 2000). Concerning *P. ostreatus*, its mycelium trapped and prayed *A. avenae* thanks to ostreatin, a paralyzing toxic chemical allowing the digestion of the nematode by the fungus (Barron and Thorn 1987). As *A. avenae* is found in higher abundances in crop fields and grassland, but less in forest soils, it has been hypothesized that its inability to survive with the forest fungus *P. ostreatus* could be due the lack of co-occurrence in natural environments. Alternatively, the presence of predatory *P. ostreatus* in forest soils could have naturally excluded *A. avenae* from this ecological niche (Okada and Kadota 2003). *Morchella* M21-48 and M22-6 have been isolated in forest soils and would have thus probably poorly co-existed naturally with *A. avenae*. This could explain why *Morchella* was an unreliable source of food for the mycophagous nematode, similarly to the findings in *P. ostreatus* (Okada and Kadota 2003). This would also indicate that domesticated strains of *Morchella* could be or become sensitive to *A. avenae* in case of co-occurrence in crop fields. Further studies should therefore evaluate the natural resistance to mycophagous predators potentially occurring between cultivated and non-cultivated strains of *Morchella.*

### 8.4.2 *Morchella* outcompetes the fungal competitors that were isolated from a fruiting body cultivation assay

The moulds *Penicillium* sp*., Cephalotrichum* sp*.* and *A. westerdijkiae* appeared in the soil surface or coating wheat grains of a failed indoor morel fruiting body cultivation trial. While the impact of fungal contaminants (competitors) in *Morchella* cultures remains unknown, the presence of molds in cultures of *Agaricus bisporus* has been widely studied. *Penicillium* sp. could induce a complete failure of fruiting body formation when it is present in the mushroom compost, while other moulds such as *Fusarium* sp. did not cause any yield reduction (Grogan, Scruby, and Harvey 2000). Low yields associated with the presence of mould could also be linked with the presence of viruses that increased along with the occurrence of moulds (Grogan, Scruby, and Harvey 2000). *Penicillium* is not only a competitor, but also a well-known fungal pathogen affecting *Morchella*. *Penicillium raperi* causes morel rot, a fast-evolving disease affecting *Morchella sextelata* fruiting bodies (X. Xu et al. 2024), while *Penicillium kongii* was isolated from morel ascocarp lesions (Shi et al. 2022). Similarly, *Aspergillus* sp. can cause white mildew of *Morchella* (Wei-Ye et al. 2022). *A. westerdijkiae* is commonly infecting agricultural crops and human food (Han et al. 2016). It is a producer of ochratoxin A (OTA), a neurotoxic and potential carcinogenic polyketide secondary metabolite for humans (Han et al. 2016). This phytopathogen possesses plant polysaccharide-degrading enzymes and proteases (Han et al. 2016) and can therefore infect various food products including cereals, coffee, chocolate, fresh and dried fruits, vegetables, and spices (Gil-Serna et al. 2015). However, there was not report of *A. westerdijkiae* infecting fungal fruiting bodies or mushroom crops. The third fungal contaminant, *Cephalotrichum,* was the most abundant fungal genus in the soil substrate through the complete and successful cultivation cycle of *Morchella rufobrunnea* (i.e., until fruiting body production), but also in failed crop assays (Longley et al. 2019), indicating that its presence was not the reason of the cultivation failure.

The competition assays indicated that *Morchella* mycelium outcompeted the co-cultured fungi in chemically defined medium and in culture substrates, suggesting that alone, they should not be problematic competitors in future cultivation assays. However, it may be possible that the combination of the three contaminants would be harder to outcompete for *Morchella*, and this should be tested in future experiments.

### 8.4.3 Pseudomonas koreensis *does not have a beneficial impact on* Morchella *during fungal-fungal competition*

*P. koreensis* IB-4 is known to have an antagonistic effect on phytopathogens *Fusarium* spp., *A. alternata* and *B. sorokiniana* (Rafikova et al. 2016). We therefore hypothesized that *P. koreensis* GFP-NEU1358 and/or GFP-NEU1362 could have a negative effect on the fungal contaminants *Penicillium* sp., *Cephalotrichum* sp*.* and *A. westerdijkiae*, thus being beneficial for *Morchella*. We expected that *Morchella'*s growth rate would be higher in cultures supplemented with *P. koreensis*, and/or that the growth of the contaminants would be decreased compared to the water treatment. The opposite was observed, as mycelial growth of *Morchella* was higher in both systems when *P. koreensis* was not present (**Figures 7** and **11**). Similarly, the contaminants performed better in presence of *P. koreensis* compared to the water treatment (**Figure 8**), except for *A. westerdijkiae* in SBBG but this result was not statistically significant (**Figure 11**). In Petri dishes, ascospore-derived mycelium of *Morchella* sp. M21-48 performed the worst, while hymenium-derived mycelium of M21-48 performed the best. This demonstrated that two isolates originating from the same fruiting body can behave very differently, but this was not surprising as significant differences between those two isolates (M21-48-Asc and M21-48-Hym) were already observed in the *A. avenae* feeding assays (**Figure 4**). Isolate M21-

48-Asc repelled the most *A. avenae*, but had the worst growth ability against fungal contaminants, indicating that each isolate has unique capacities to cope with different stresses.

It was not expected that *P. koreensis* would decrease mycelial growth of *Morchella,* but we can explain that by at least two elements. First, *P. koreensis* utilized the media as food source, thus competing for nutrients. This was mostly important in Petri dishes, where bacterial suspensions were added near the *Morchella* inoculum. Second, as shown by Uehling et al., maintaining associate bacteria can decrease the fitness of the fungus (Uehling et al. 2017), causing it to be less competitive. *P. koreensis* did not produce high antagonistic effects on *Morchella*, as no inhibition zone or reduction of mycelial density was noticed. However, melanization was frequently observed at bacterial inoculum on *M. esculenta* M19-34 (**Figure 10**), this being considered as a stress response from the fungus (Cordero and Casadevall 2017).

## 8.5 Conclusion

In this study, we investigated the interactions between *Morchella* and four soilborne organisms that may have adverse effects on the fungus. *Morchella* had a natural ability to defend itself against the mycophagous nematode *A. avenae*. *Morchella* were also more competitive than the three fungal contaminants (*Penicillium* sp., *Cephalotrichum* sp., *A. westerdijkiae*), regardless of the presence of the associated bacterium *P. koreensis*. The association of *Morchella* spp. and *P. koreensis* was not beneficial for the fungus in the tested conditions, but it could advantage the fungus in other ways that have not been tested, for instance by promoting resistance to abiotic stresses (e.g., extreme temperatures, high salinity, low humidity) or toxic compounds (e.g., fungicides, heavy metals).

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# 9. General discussion, perspectives & conclusion

## 9.1 General discussion

### 9.1.1 Research background

True morels (*Morchella* spp.) are gourmet edible mushrooms that are prized for their unique taste and texture. Morels are healthy food that are also used for their medicinal properties (Tietel and Masaphy 2018). Morel fruiting bodies are harvested in the wild worldwide, before being sold on local or international markets (Pilz et al. 2007). In China, morels are cultivated as crops at commercial scale (Xu et al. 2022). In Switzerland, supermarkets only sell non-native morels that are either imported from China (cultivated) or other countries including Chile, Türkiye, or Pakistan (wild). The ecological cost of importing this valuable mushroom is therefore high, as long-range transport of food generates elevated carbon emissions (Li et al. 2022). In addition, in some countries as Pakistan, children are employed to forage morels that are thereafter sold in Europe and the USA (Latif, Shinwari, and Begum 2003; Hamayun 2006). Forced labor for morel harvest or cultivation can also not be excluded in some regions. For instance, Xinjiang, which is one of the main morel cultivation province (Du et al. 2019), is sadly known for its oppression against the Uyghur population (Lehr and Bechrakis 2019).

Hence, there is a crucial need to provide the Swiss market with socially and environmentally sustainable morel mushrooms. Morel cultivation in Switzerland currently exists but at an extremely low scale. Morels that are cultivated are Chinese cultivars of *M. sextelata*, *M. importuna* and *M. eximia*. Importing foreign species, whether animals, plants, or fungi, can be a threat for the native ecosystems (Litchman 2010). Our previous work demonstrated that the Chinese cultivars that were imported in Switzerland for cropping were more competitive than native *Morchella* strains in chemically defined media and in soil-like substrates (Junier et al. 2022). This, in addition to the fact that *M. importuna* and *M. sextelata* were found in the wild in Switzerland, indicated that the Chinese cultivars can colonize Swiss ecosystems, with the potential ability to outcompete indigenous strains. There is therefore a need to develop native cultivars for the Swiss market. However, the lack of knowledge concerning Swiss morels rendered this very difficult, as domestication requires to understand the sexual reproduction and ecology of the desired strain (Xu et al. 2022). Indeed, cultivating fungi require to understand the factors that trigger a change from the vegetative into the reproductive form (Chang 2008), which have not been completely understood in *Morchella*. In *A. bisporus,* primordia formation is triggered on casing soil (nutrient poor substrate) by decreasing air temperature and atmospheric $CO_2$ (Baars et al. 2020). Bacterial associates were also responsible to consume volatile compounds that have inhibitory effects on primordia formation (Baars et al. 2020). In other fungi, light, nitrogen (Baars et al. 2020) soil temperature and soil moisture also often impact fruiting (Pinna et al. 2010).

Only a few species of morels from the Elata (*M. importuna, M. sextelata, M. eximia, M. exuberans, M. oweri, M. tomentosa, Morchella* sp. Mel-13, *Morchella* sp. Mel-21 (Xu et al. 2022)) and Rufobrunnea (*M. rufobrunnea* (Masaphy 2010)) clades have been cultivated so far. Domesticated morels are easy-fruiting species that do not share the same sexual reproduction strategies, mating genes, nor ecology (Du and Yang 2021). For instance, *M. importuna* is a fire-adapted species that can reproduce by heterothallism, pseudohomothallism, and homothallism, and that contains the mating type genes *MAT1-1-1, MAT1-1-10, MAT1-1-11* and *MAT1-2-1* (Du and Yang 2021). *Morchella* sp. Mel-21 is a non-fire-adapted species that can only reproduce by heterothallism and does not contain *MAT1-1-11* (Du and Yang 2021). Interestingly, five out of nine cultivable species belong to the same taxonomic subgroup containing *M. sextelata, M. exuberans, M. importuna, M. oweri, M. eximia, Morchella* sp.

Mel-8 (Du and Yang). Secondary or primary homothallic species could be easier to cultivate, as they do not require the meeting of opposite partners for sexual reproduction (Coppin et al. 1997). In *A. bisporus*, the three cultivated varieties display different main reproduction systems: primary homothallism in var. *eurotetrasporus*, secondary homothallism in var. *bisporus*, and heterothallism in var. *burnettii*, although in the two latter heterothallic and secondary homothallic life cycles occur (Kamzolkina et al. 2006). The first variety to be cultivated was *A. bisporus* var. *bisporus*, and along with *A. bisporus* var. *eurotetrasporus*, they are considered the most interesting varieties for cultivation due to their haploid fruiting ability (Kamzolkina et al. 2006).

To find a domesticable Swiss strain, it is therefore particularly important to investigate the phylogenetics, sexual reproduction, and ecology of the Swiss morels. These three research areas were studied in this thesis.

### 9.1.2 Biodiversity of *Morchella* in Switzerland

In the first chapter, I presented the first survey of *Morchella* biodiversity in Switzerland using a phylogenetic approach (**section 2**). In addition to the molecular tools, ecological and morphological characteristics were used for a polyphasic species identification. This allowed to make an evaluation of the biodiversity of Swiss morels, for two main purposes: (1) monitor *Morchella* species present in Switzerland to track potential species invasiveness and biodiversity loss, and (2) identify the native species that may be cultivable. It was hypothesized that species that were already described from limitrophe countries would be found in Switzerland. As endemism is common in *Morchella* (Taskin et al. 2012), new native species were expected to be discovered.

The first goal was achieved, and both hypotheses were validated. The analysis revealed that one Esculenta species (*M. esculenta*) and four Elata (*M. deliciosa*/Mel-13, *M. importuna, M. pulchella* species complex) species were found in Switzerland, in addition to four new lineages of the Elata clade. Two of these lineages (*Morchella* sp. Mel-43 and Mel-44) were very likely to correspond to new phylogenetic species, but more samples are needed to confirm this assumption. The distinction between Mel-45 and *M. deliciosa*/Mel-13 could not be reliably determined as the new lineage was only represented by a single specimen. The last lineage, Mel-47, was described as a new species (*Morchella helvetica* sp. nov.) in a separate paper that constituted **section 3**. The presence in the country of *M. importuna,* a domesticated species widely cultivated in China (Xu et al. 2022), confirmed that non-native species can fructify in Switzerland, as we previously hypothesized (Junier et al. 2022).

The second goal was also accomplished, as the phylogenetic analysis allowed to identify the best candidate strains to be cultivated. Among the species that were found in Switzerland, one of them (*Morchella* sp. Mel-13) was already domesticated (Du et al. 2019). The 15 strains of *M. deliciosa* could also be cultivable, as it probably represents the same species as *Morchella* sp. Mel-13 (Loizides et al. 2022). As indicated in the previous section, none of the new Swiss lineages were related to the taxonomic subgroup containing most of the domesticated *Morchella* species, indicating they may not be relevant candidates for cultivation. To conclude, the monitoring of the biodiversity of *Morchella* in Switzerland revealed that at least 16 of the collected strains are interesting candidates for domestication.

### 9.1.3 Using a Centroid-based approach for *Morchella* spp. identification

The biodiversity analysis of **section 2** required the amplification of four genetic regions and the generation of multiple phylogenies, which was costly, required time, skills, and knowledge about the genus *Morchella*. However, as mentioned earlier, monitoring biodiversity on a regular basis is

important to track species invasiveness and biodiversity loss. Therefore, in **section 4**, we compared three methods of genetic identification that required only one marker (the internal transcribed spacer ITS), and that needed less time and knowledge to generate a species identification. Although it is well known that the Esculenta clade can be resolved using the single ITS, this is much more complex for the Elata clade (Du et al. 2012). This was exemplified in **section 2** by the pair *M. deliciosa/*Mel-13 that could not be resolved yet. Similarly, the *M. pulchella* species complex that contained *M. pulchella*, *M. conifericola, M. septentrionalis* and *Morchella* sp. Mel-23 could not be genetically resolved, even using four genetic markers as in **section 2**. Indeed, these species or subspecies are closely related and may represent geographically divergent lineages of a single species (Loizides et al. 2022). Another issue in *Morchella* taxonomy has been the important presence of misidentified genetic sequences in public databases, as it was reported that about 60% of the *Morchella* ITS sequences were misidentified in GenBank (Du et al. 2012). The Centroid-based approach that was tested in **section 4** is hence particularly relevant for the genus *Morchella* because it minimizes the intraspecific diversity and the presence of misidentified sequences in the public databases. This method is an AI-based process that defines a Centroid sequence for each species, assuming that this sequence will be the most representative of all the sequences annotated for the same species in the public databases. It can be used with the ITS, 18S and 28S genetic markers.

The goals of this study were therefore to (1) evaluate the reliability of three markers (ITS, 18S, 28S) to identify *Morchella* at species level, and (2) evaluate the reliability of the Centroid-based approach for the same purpose, by comparing the results with two other pairwise-alignment based methods (identification tools from UNITE and *Morchella* Mycobank) and the literature. The research hypothesis was that the Centroid-based approach was the most reliable tool to identify morels at species level on the sole basis of the ITS marker.

The first goal was achieved and indicated that the ITS had the best resolution. The second goal was also accomplished, and the results supported the research hypothesis. The Centroid-based approach allowed a better species identification than the two other tested methods. Importantly, based on the ITS, the Centroid-based approach performed as well as the four-locus phylogenetic analysis, as these methods were able to identify 83% and 84% (Sa 2022) of *Morchella* species, respectively.

To conclude, the Centroid-based approach is a reliable tool that can be used routinely to monitor the presence of invasive species, or food fraud. Additionally, this method can be used to rapidly identify *Morchella* strains that are good candidates for domestication based on their taxonomy.

### 9.1.4 Sexual reproduction in Swiss morels

The sexual reproduction of Swiss morels was investigated through the analysis of the mating type genes. This is a crucial aspect of the *Morchella* life cycle that needs to be understood for domestication. This research was separated in three main goals: (1) find reliable PCR primers to amplify *MAT1-1-1* and *MAT1-2-1* genes, (2) use these primers to evaluate the presence of the *MAT* genes in single-ascospore isolates to determine their reproductive system and (3) analyze the structure of the MAT loci in whole genomes of Swiss strains. It was expected that the published *MAT* primers would be reliable to rapidly amplify and detect *MAT* genes, and that the results from this analysis would indicate that the Swiss morels would be mainly heterothallic (i.e., contain a single MAT per ascospore-derived culture). In addition, it was expected to find (pseudo)homothallic isolates that would be good candidates for cultivation as they would not require to meet an opposite partner to undergo sexual reproduction, and thus produce fruiting bodies.

The first hypothesis was not validated, because 7/8 of the tested primer pairs yielded unsatisfying results. In **section 5**, the four primer pairs that were tested both in the laboratory and *in silico* were

not reliable as they produced off-targets (Elata MAT1-1 (Du et al. 2017)) or amplified wrong genetic regions (Esculenta EMAT1-1 and EMAT1-2 (Du et al. 2020)). In **section 6**, four other MAT primer pairs that were published by another research group (Chai et al. 2022) were tested. The results indicated that, although the primers were reliable based on the bioinformatic analysis, the PCR produced off-targets even after optimization of the cycling parameters. These off-targets corresponded to weak PCR signals of same length as those expected for the mating types, i.e., 1500 bp for *MAT1-1-1* and 1000 bp for *MAT1-2-1*. The sequencing of these products confirmed that they did not correspond to a known gene. Furthermore, the analysis of the whole genome of one of the isolates for which one strong (MAT1-1) and one weak (MAT1-2) PCR signals were present indicated that only the MAT1-1 existed in the genome. The analysis of single ascospore cultures showed that four samples were (pseudo)homothallic, while only one MAT was found in the 33 remaining isolates that were thus heterothallic. Isolates *M. helvetica* M19-43-2, M19-43-7, *Morchella* sp. Mel-44 M21-2-2 and M21-2-3 are therefore interesting for domestication.

The analysis of the mating type structure in whole genomes revealed that the single-ascospore isolate (M21-48-1, Elata) contained genes *MAT1-1-1, MAT1-1-10, MAT1-1-11* flanked by the conserved genes *MBA1* and *SDH2*. Isolate M19-34 (Esculenta) did not contain *MAT1-1-11*. This was in accordance with literature findings and supporting the fact that mating type genes have a taxonomic significance (Chai et al. 2022). The *MAT* sequences generated for this chapter were besides used as additional phylogenetic markers in **section 2**.

To conclude, the **section 5** and **6** investigated the distribution and genetic structure of mating types in Swiss *Morchella*, and this indicated that four (pseudo)homothallic specimens are interesting candidates for domestication.

#### 9.1.5 Interactions between Swiss morels and other soilborne organisms

The last chapter focused on the ecology of Swiss morels, and more particularly on how they interact with various soilborne organisms that could be present in agricultural fields. **Section 7** was dedicated to the interactions between *Morchella*, its associate bacterium *Pseudomonas koreensis* and the mycophagous and bacteriophagous amoeba *Acanthamoeba castellanii*. The goal of this research was to evaluate the benefits or disadvantages resulting from the interactions, for each of the three organisms. The principal hypothesis was that the *Morchella-Pseudomonas* interaction would be beneficial to *P. koreensis* as it would use the hyphae to evade *A. castellanii* predation. This was not validated, as *P. koreensis* formed an unexpected biofilm around *Morchella* hyphae, that was more readily grazed by *A. castellanii*. The association between *Morchella* and *A. castellanii* was positive for both, as this also reduced the competition between *Morchella* and *P. koreensis* that was detrimental to the fungus.

**Section 8** focused on the interactions between *Morchella* and the mycophagous nematode *Aphelenchus avenae,* and with three fungi (*Penicillium* sp., *Cephalotrichum* sp., *Aspergillus westerdijkiae*) that were isolated from an indoor cultivation assay. Two main hypotheses guided this research: (1) bacteria-free *Morchella* mycelium is a better food source for *A. avenae* compared to mycelium containing a native bacteriome; (2) *P. koreensis* can be used as a biocontrol agent to enhance *Morchella* competitivity against fungal competitors.

None of the hypotheses were validated. Concerning the first research question, the nematodes unexpectedly performed better on the bacteria-containing mycelium. Regarding the second research question, *P. koreensis* was more detrimental than beneficial because its presence reduced hyphal growth of *Morchella*, probably due to nutritional competition as indicated previously.

Overall, the combined observational and quantitative experiments conducted in **section 8** revealed that *Morchella* displayed a natural ability to resist predation and to outcompete fungal contaminants. The propagation rate and survivability of *A. avenae* were lower on *Morchella* compared to the control *Botrytis cinerea*, especially on *M. esculenta* that led to the death of almost all the nematodes. *A. castellanii* was also not able to feed on *Morchella* mycelium. Furthermore, ascospores were toxic to the amoeba that tried to feed on it but resulted dead a few hours after the ingestion of a spore. *Morchella* was also more competitive than *Penicillium* sp., *Cephalotrichum* sp. and *A. westerdijkiae* in chemically defined media and in culture substrates. Globally, these results are encouraging in the perspective of cultivation, as *Morchella* could be exposed to these predators and competitors in crops.

### 9.1.6 Additional work

Globally, this thesis provided crucial knowledge that paved the way for the cultivation of native strains in Switzerland. In parallel to the research, three trials of fruiting body cultivation were performed, indoors in a cultivation chamber (**section 10.2**), outdoors in association with *Abies* sp. (**section 10.3**) and outdoors in agricultural crops with the help of a morel farmer that was able to cultivate the Chinese strains in Switzerland. Unfortunately, none of the cultivation assays was successful. The stage of conidiation was obtained in the three systems, but fruiting bodies never appeared after wintering. In field cultivation assay, Chinese cultivars were sown in parallel but also did not produce any fruiting body. The causes of the failure remain unknown and is unfortunately not rare in the morel industry (Xu et al. 2022). Cultures are often unstable because the morel life cycle is still not completely understood. For instance, the requirements to switch from a vegetative asexual stage to a primordium remain partially unknown, and probably vary depending on the species or strain (Xu et al. 2022). In *A. bisporus*, reduced yields were noticed at large scale due to a phenomenon called "sectored morphology", which is a change in the vegetative mycelium phenotype that creates dense patches of hyphae, and which produce only few fruiting bodies (Li et al. 1994). Studies revealed that the sector phenotype was transmitted and frequent in the domesticated lines, while almost absent in wild strains, suggesting that introducing regularly wild genetic material into domesticated strains to recover this irreversible and transmissible instability (Li et al. 1994). A similar phenomenon of inherited instability could occur in *Morchella* as well.

To be able to cultivate native *Morchella*, more time and facilities (e.g., fields) are needed. For this reason, I co-authored a proposal that was submitted to the Swiss Federal Office for the Agriculture (FOAG) (**section 10.4**). It was however rejected, because it was considered there were too many unclear points regarding the transferability to the Swiss agriculture (e.g., who would produce the spawn, who would sell the final product), and that the project was too risky as morels were still considered as a niche product.

As an alternative to the fruiting bodies that require the access to fields, I imagined a food product based on *Morchella* mycelium. The concept consisted in cultivating morel mycelium in (semi)-liquid medium using recoverable waste from the agriculture and forestry as nutrient source, harvesting, lyophilizing, and reducing the mycelium into a flavorful powder that could be used as a nutritional supplement in various food as bread, pasta, and cheese. I elaborated a proof-of-concept bridge project to test the feasibility of the idea (**section 10.5**), but it was rejected because more proofs from laboratory experiments were needed beforehand.

## 9.2 Perspectives

The goals of this research were (1) to monitor the biodiversity of *Morchella* in Switzerland and investigate (2) the sexual reproduction and (3) ecology of the collected specimens to select candidates for domestication.

The first goal was successfully achieved, as more than 70 fruiting bodies were collected across nine Swiss cantons between 2019 and 2023. They were phylogenetically identified and described morphologically and ecologically (Cravero, Bonito, et al. submitted). The new lineages that were discovered, but for which more representatives were needed (i.e., *Morchella* sp. Mel-43, Mel-44, Mel-45 (Cravero, Bonito, et al. submitted)) should be further described morphologically and ecologically by sampling of additional representatives. The first genetic identification of the potential representatives of the new lineages could be done using the Centroid-based approach that was validated for routine identification (Cravero, Ruelle, et al. submitted). Then, multilocus phylogenetic analysis should be conducted to describe the specimens of the new lineages. For the morphological and ecological analysis, it will be necessary to understand the important characteristics before the sampling campaign. This includes to know at least the precise sampling location (longitude, latitude, altitude), the soil/substrate characteristics and the associated vegetation of the sample. A picture of the fruiting body should be taken *in situ*. Then, morels must be collected entirely (i.e., not cutting the stipe) and if possible, at various developmental stages. In the laboratory, fruiting bodies must be described fresh, mostly for the macro characteristics as size, color, and texture.

Additionally, in **section 2** it was shown that the cultivated species *M. importuna* was able to naturally fructify in Switzerland. As this species could be invasive (Junier et al. 2022), it will be important to monitor it in the future to evaluate its impact on the local biodiversity. To do so, the ITS Centroid-based approach could be used for the reason mentioned as it is a reliable tool for routine identification.

The second goal of the thesis was to study the sexual reproduction of *Morchella*. The analysis of four unreliable primer pairs that have been designed to amplify *MAT* genes in *Morchella* resulted in a commentary paper (Cravero et al. 2022). Four other *MAT* primer pairs were investigated and also provided unsatisfactory results due the presence of off-target amplifications in more than a half of the samples. These off-targets should be further investigated to understand which region of the genome was amplified instead of the *MAT* genes. For this, patterns corresponding to the primer sequences should be searched in full genomes. Two out of four of the full genomes of *Morchella* that were generated could be analysed, because they were already annotated. This allowed to determine the structure of the MAT locus in one Elata (*Morchella* sp. Mel-48) and one Esculenta (*M. esculenta* M19-34) species. In the future, the MAT structure in the other genomes should be studied as well.

The third goal of the thesis was the investigation of *Morchella* ecology. It more precisely focused on an area that has been poorly studied, which concerns the interactions between *Morchella* and other organisms that could be encountered in agricultural crops. Unexpected phenomena were observed, mainly in the tripartite interactions between *Morchella* spp., *P. koreensis* and *A. castellanii*. The observational experiments were complemented by quantitative analyses to constitute a research paper (Cravero et al. in preparation). In the future, these tripartite interactions should be further explored, for instance to better understand how biofilms of *P. koreensis* are formed, characterize their composition, and investigate whether they would form in the soil. Overall, this thesis supported that *P. koreensis* has neutral or negative impacts on *Morchella*, a fact that we already demonstrated (Cailleau et al. 2023). Understanding why this bacterium is a stable component of the core bacteriome of *Morchella* is one of the main perspectives of the research about biotic interactions of

*Morchella*. To do so, the fitness of the pair *Morchella/P. koreensis* versus *Morchella* alone should be compared in conditions that were not tested in this thesis, namely environmental stresses (humidity, temperature, salinity, nutritional depletion) or chemical stresses (heavy metals, fungicides). Another aspect that should be investigated is the difference between both strains of *P. koreensis* that were isolated from *Morchella* spp., because they provided divergent results depending on the experiment. For instance, their growth rate was highly divergent, as well as their cell size, but their impact on *Morchella* was similar in most of the experiments. It would be interesting to focus on transcriptomics to better understand these divergences. Another crucial point that remains unknown is the role of associated bacteria in the fruiting body formation (Xu et al. 2022).

Overall, this thesis aimed to provide knowledge background for the cultivation of native strains in Switzerland. Naturally, future research should focus on reiterating the cultivation assays with the candidate strains mentioned previously.

## 9.3 Conclusion

The general aim of this thesis was to study true morels in Switzerland, in order to provide a strong research background to develop in the future a native cultivar of *Morchella* for cropping in Switzerland. In addition, it was aimed to select candidate for domestication, based on genetic and ecological analyses. The goals of this research were therefore to monitor the biodiversity of *Morchella* in Switzerland, and to investigate the sexual reproduction and biotic interactions of the collected specimens. The general aim of the thesis was successfully achieved, as crucial knowledge was produced in the fields of biodiversity, sexual reproduction, and ecology. The biodiversity of Swiss morels was evaluated for the first time using genetic data. This offers a background to evaluate future species evolution, biodiversity loss, hybridization, and invasiveness. This monitoring revealed the presence of at least one morel species unknown to science, *Morchella helvetica* sp. nov., that was described in this thesis. To ease future species determination, three genetic methods were evaluated and compared. This showed that the Centroid-based approach was particularly relevant to identify *Morchella* at species level using the ITS marker. This will be important for routine identification, notably in the fields of biodiversity conservation, cultivation, and food fraud. In addition, the reproductive strategies (i.e., heterothallism or (pseudo)homothallism) of Swiss morel isolates were investigated for the first time in this thesis, as well as the structure of the mating type loci of one black and one yellow morel from Switzerland. Original research was conducted on the biotic interactions between *Morchella* and soilborne organisms, namely *P. koreensis, A. castellanii, A. avenae, Penicillium* sp., *Cephalotrichum* sp. and *A. westerdijkiae.* This notably revealed unknown phenomena, such as biofilm formation by *P. koreensis* on *Morchella* hyphae in presence of predator amoeba, the ability of *A. castellanii* to use *Morchella* hyphae as fungal highways, and the toxicity of *Morchella* ascospores towards *A. castellanii*.

Taken altogether, the analyses from all chapters indicated that at least 20 strains of *Morchella* that are preserved in the fungal collection of the Laboratory of Microbiology of the University of Neuchâtel are interesting candidates for the development of a native cultivar for the Swiss agriculture, based either on taxonomic or reproductive criteria. Although this thesis brought essential knowledge about Swiss *Morchella* spp., there is still a lot of aspects of the morel biology that needs to be understood to be able to domesticate new strains (e.g., the trophic strategies) and to stabilize the fruiting body cultivation (e.g., pest control).

To conclude, this thesis provides for the first time a knowledge background for *Morchella* cultivation in Switzerland, but also to track potential invasiveness of Chinese cultivars imported in the country.

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# 10.1 Annex - Ecological and morphological variability of *Morchella* Section *Distantes*

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## 10.1.1 Ecology

The morphological and ecological characteristics of 35 different species of black morels were compiled in **Annex G1B** (with the references). Their distribution, reviewed by Loizides et al. (2022) for most of them, expanded to 23 countries from all continents: Argentina, Armenia, Australia, Canada, Chile, China, Cyprus, Czech Republic, Denmark, Dominican Republic, Estonia, Finland, France, Germany, Greece, India, Israel, Italy, Japan, Kazakhstan, Lithuania, Mexico, Norway, Netherlands, Pakistan, Poland, Portugal, Slovakia, Spain, Sweden, Switzerland, Türkiye, and USA.

Two subgroups diverging from the typical black morels were identified: the half-free morels, that have a distinctively long sinus that is free on a large portion of the stipe (*M. iberica, M. populiphila, M. punctipes, M. semilibera*); the postfire morels, that occasionally or exclusively fruit after forest fires (*M. eximia, M. eximioides, M. exuberans, M. importuna, M. sextelata, M. tomentosa*). 6/35 of these morel species could be cultivated (Xu et al., 2022), and only one of them was not a pyrophilic species (*M. oweri,* firstly orthographized *"M. owneri*") (Du et al., 2019).

The fruiting season of morels generally occurred between March and June. Some species, as *Morchella dunalii*, already fruited late February in the Northern Hemisphere (NH). *M. sextelata* and *M. eximia* fruited until July in the USA, which was a relatively long period compared to most of the black morels that emerged during only two months (generally, April-May). *M. tomentosa*, one of the basal black morels, fruited until August in Northern America. In other countries, as Dominican Republic (DR), there are only two seasons, running between December and April (dry season) and between May and November (wet season). The one morel species from DR, *M. hispaniolensis,* fruited in January. Only one morel species was known to produce ascocarps in Autumn: the yellow morel *Morchella galilaea* (Taşkin et al. 2015).

Black morels were reported from various types of substrates, including bare soil, grasslands, sand dunes, swamps, burned bark, industrial waste, or deadwood. Soils were either well-drained and dry (*M. arbutiphila, M. laurentiana*), or wet (*M. purpurascens, M. semilibera*). Some species, as *M. importuna, M. eximioides* and*, M.* norvegiensis, were often associated with anthropologically disturbed environments like gardens, parcs, roadsides, mushroom farms, waste, or imported trees. All the species fruited in forests, that could be deciduous, with conifers, or mixed. A third of the black morels were associated with *Pinus* spp. or *Picea* spp., while they were also often found under *Populus* and *Fraxinus* species.

## 10.1.2 Macromorphology

The size of morel ascocarps was highly divergent inter- (e.g., 25-180 mm, *M. punctipes*) and intraspecifically (20-200 mm high). However, the smaller specimens could correspond to immature ascocarps. The ratio between the cap and the stipe height was considered of a taxonomic importance (Loizides et al., 2016), but it was rarely provided. In the half-morels, the stipe at maturity is always higher than the hymenophore, and this can also be the case for *Morchella andinensis*. In *M. arbutiphila, M. aysenina, M. deliciosa,* and *M. disparilis*, the cap was higher or equal to the stipe.

The shape of the hymenophore was found not to be discriminant, because it varied importantly intraspecifically. The maturity stage influenced the shape of the cap, often conical or ovoid when young and elongating and sharpening with age.

The shape of the stipe was a conserved feature, not taxonomically relevant, being mostly cylindrical with a wide base. As noted by Loizides et al. (2016), the presence of ridges, channels, folds, perforations, or chambers on the stipe surface was sometimes distinctive enough to have a taxonomic importance. Some species as *M. confusa, M. conifericola, M. sextelata* and *M. tomentosa*, did not display any of these features, even at maturity. Although most of the black morels presented basal ridges or folds at maturity, *M. hispaniolensis, M. kaibabensis* and *M. laurentiana* typically displayed ridges from the sinus to the base. In *M. septentrionalis*, these characteristics were occasional. The stipe of *M. exuberans* was considerably chambered internally. Concerning the color of the stipe, it was only distinctive for *M. tomentosa*, that had a typically grey to black stipe when young. Others were white to pale brownish, although pinkish tones (rufescence) sometimes appearing in *M. arbutiphila, M. disparilis, M. kaibabensis* and *M. purpurascens* could be taxonomically important. Most of the stipes were mealy, furfurasceous, or covered with whitish to brown granules.

The sinus, considered important to distinguish for instance *M. disparilis* (Loizides et al., 2022), was actually not described in the half of the species. The depth and width that were sometimes provided varied importantly intraspecifically (e.g., 1-7 mm deep and wide in *M. exuberans*), but globally varied between 1-25 mm deep (maximum reached in the half-morels) and 1-10 mm wide (maximum reached in *M. kaibabensis*).

The number of primary ridges on the hymenophore was not found to be taxonomically important for most of the species where it varied between ten and twenty, but the extremes were characteristic (e.g., only up to 6 ridges in *M. hispaniolensis*, and up to 28 in *M. exuberans*). The primary ridges were always vertical, more or less sinuous and/or anastomosed. Their thickness was rarely provided but varied between 1-6 mm. Except for *M. tomentosa* in which the sterile ridges were densely tomentose, the interspecific textural variations (from glabrous to tomentose, or finely granulose) rendered this feature poorly informative. However, the color, and mostly the darkening process, was distinctive for most species, although interspecific variations existed: in *M. andinensis, M. australiana, M. arbutiphila, M. aysenina, M. conifericola, M. disparilis, M. dunalii, M. exuberans, M. iberica, M. importuna, M. kakiicolor, M. oweri* and *M. snyderi*, the ridges were concolorous with the alveoli in young specimens, but became darker (and with a distinct color) with age; in *M. tridentina* and *M. tomentosa,* the primary ridges became lighter with age; in *M. arbutiphila, M. deliciosa, M. dunalii, M. kaibabensis, M. magnispora, M. mediterraneensis,* and *M. norvegiensis*, pinkish or purplish tones appear when young or at maturity. Concerning the shape of the primary ridges, they were often first flattened or rounded and became sharp or eroded with age. The secondary ridges of black morels could be vertical (often few and short) but mostly horizontal (transecting, often sunken and concolorous with the pits but could also have the same aspect as the primary ridges). In some

species, the horizontal ridges formed a typical laddered appearance (occasional in *M. deliciosa, M. dunalii* and *M. kakiicolor,* and usual in *M. importuna* and *M. oweri*).

The primary alveoli were mostly vertically elongated and often more or less irregular. Their shape was often poorly described. Their texture was often glabrous or granulose, but was finely tomentose in *M. brunnea, M. kaibabensis, M. norvegiensis,* and occasionally in *M. oweri*, indicating this feature could be taxonomically important. The secondary alveoli were very rarely described.

The context (i.e., the sterile internal tissue) was sometimes described (thickness, color, presence of chambers or layers) but was not distinctive because of the high intraspecific variations. The same conclusion was made for the sterile inner surface of the ascocarp.

### 10.1.3 Micromorphology

The sexual spores (ascospores) play roles in fungal survival and dispersal, and their size and shape influence the propagation abilities (Quijada et al., 2022). Their morphology is under strong evolutionary pressure, because they vary in shape, size, and ornamentation depending on the ecological niche or trophic mode (Calhim et al., 2018). Size and ornamentation of mature ascospores was considered taxonomically valuable in *Morchella* (Loizides et al., 2022). The average size of ascospores in black morels was 20.02-25.56 x 11.78-15.74 µm. Spore size could be related with climatic adaptation, as larger spores would be less prone to desiccation and ultraviolet radiation (Calhim et al., 2018), but such correlation was not found in black morels. The species with the smallest spores was *M. oweri* (14-20 x 9-12 µm). *M. confusa, M. kakiicolor* and *M. tomentosa* also had relatively small spores, rarely exceeding 20 x 13 µm. *M. magnispora,* according to its name, was one of the species with the largest spores (24-31 x 13-21 µm), along with *M. norvegiensis* (that could reach 40 x 24 µm) and *M. brunnea* (that could reach 40 x 25 µm). The intraspecific variation in spore size was in average 7.57 x 5.31 µm. This variation may be dependent of the number and/(or age of the studied specimens. The length/width ratio (Qm) was not provided, as it was considered poorly discriminative (Loizides et al., 2016). Concerning the shape, the ascospores of black morels were always elliptical to oblong or ovoid. Such shapes generally do not allow a long-range dispersal and are more effective to reach the soil or plant roots belowground (Calhim et al., 2018). Those spores are generally ornamented to ease the arthropods-mediated propagation within the soil (Calhim et al., 2018). In *Morchella*, the ornamentation of the spore surface variated intraspecifically but not (or slightly) interspecifically. Ascospores could be smooth, crackled, wrinkled, longitudinally striated by straight or sinuous ridges or crests, the latter being sometimes transversally connected and/or converging at poles. Morel spores were hyaline, indicating a poor resistance ability due to lack of dark pigments (Quijada et al., 2022).

The ascospores were contained in asci, which are cylindrical to clavate, aseptate and operculate structures each containing eight spores in *Morchella*. Their length was relatively similar between morel species, generally ranging between 220-300 µm. *M. australiana* was distinct by its short asci (140-165 µm). Paraphyses are sterile elements of the hymenium that probably help the ejection of ascospores from the asci by applying a lateral pressure (Quijada et al., 2022). Morel’s paraphyses were generally shorter and thinner than asci. Their size should be considered carefully, as it increases with age, but still rarely exceed the length of the asci (Jacquetant, 1984). They were cylindrical, sometimes bifurcate, and septate. The number, and most importantly, the location of the septa are taxonomically important (Loizides et al., 2016). These precisions were only provided for a half of the species, in which up to 5 septa per paraphyse were detected. They were either located in the lower

half/third (*M. andinensis, M. arbutiphila, M. aysenina, M. disparilis*), or randomly distributed (*M. dunalii, M. importuna, M. tridentina*). The shape of the paraphyses' apices was highly variable, both inter- and intraspecifically, and even within a same individual: they could be cylindrical, rounded, (sub)clavate, (sub)capitate, subacute, subfusoid, lageniform, or fusiform. Enlarged paraphyse apices could help maintaining humidity in the hymenium to support the developping asci (Quijada et al., 2022). The acroparaphyses (i.e., sterile elements of the ridges) had the same size as the paraphyses or were slightly shorter. However, they were often larger. Up to 3 septa per acroparaphyse could be present. The shapes of the terminal cells were also highly variable between and within species, but some of them were distinct because they lacked capitate elements (*M. arbutiphila, M. fekeensis, M. laurentiana, M. magnispora, M. mediterraneensis*). The aspect of the paraphyses in KOH (2-5%) was often provided and revealed hyaline or brown(ish) contents, that are probably protective pigments (Quijada et al., 2022). Finally, the hyphoid hairs or other elements of the ectal excipulum were only documented for a half of the black morels. Their sizes and shapes were various, more or less differentiated depending on the species, could be septate, and were often catenulate.

### 10.1.4 Literature


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## 10.2 Annex - Fruiting body cultivation assay in climatic chamber

Melissa Cravero, Laboratory of Microbiology of the University of Neuchâtel

### 10.2.1 General indications and notes about *Morchella* cultivation

These indications were obtained by a Swiss morel farmer that cultivated the Chinese cultivar PYL in agricultural fields.

- A complete cultivation cycle can be performed in 75-85 days.
- Soil must be 15 cm deep minimum. Common garden soil can be use. Soil humidity must be maintained between 50-70% (use film mulching).
- Spawn should be inoculated from mycelium originating from spores to be more productive. The mycelium should be young. 500g of spawn should be sown on 1.5 $m^2$ of soil, at 19°C. Cover with 2-3 cm of soil.
- Composition of spawn substrate: 46% wheat grains, 38% wheat bran, 10% sawdust, 1% $CaSO_4$, 1% precipitated calcium carbonate (PCC), 4% humus.
- When conidia appear (10-15 days), nutrient bags can be put on the soil surface (cut two openings on the bag and put it in contact with the soil). Nutrient bags contain the same ingredients as the spawn. They are sterile. One bag is needed every 50 cm. After 15-20 days, the mycelium should have colonized the bags. Nutrient bags can be removed once depleted (= about 45 days).
- When temperature is going up (6-8°C), intensive flooding must be done near the mycelium (soil humidity 65-75%, air humidity 85-90%).
- *Morchella* fructify at low temperature (6–16 °C) and moderate relative humidity (around 85%).

### 10.2.2 Tested strains

*M. importuna* PYL (Chinese cultivar, **Figure 1**), *M. deliciosa* M19-29 (wild Swiss strain, **Figure 1**), *Morchella* sp. M21-48 (wild Swiss strain).

### 10.2.3 Production of *Morchella* spawn

1. Inoculate morel ascospores in PDA for 15 days at 15°C (Inoculum 1).
2. Prepare the substrate: wheat grain 56%, wheat husk 28%, wood 10%, $CaSO_4$ 1%, precipitated calcium carbonate (PCC) 1%, humus 4%; Soak the substrate, mix to homogenize and autoclave it in a bottle/plastic bag.
3. Inoculate the substrate with a piece (quarter of Petri dish) of I1. Incubate 20 days at 15°C (Inoculum 2).
4. Inoculate I2 **first removing the top layer** in a new bottle containing morel substrate from step 2. Incubate 20 days at 15°C (Inoculum 3).
5. Homogenize I3 **first removing the top layer** and inoculate on soil. Cover with 2cm of soil.

**Figure 1.** Spawn (first inoculation) colonized by *Morchella deliciosa* M19-28 (left) and the Chinese cultivar *Morchella importuna* PYL (right). Picture was taken seven days post inoculation.

## 10.2.4 Planning

This cultivation planning was made on the basis of the information indicated previously, and to mimic natural winter-spring seasons of Switzerland.

| Days | Task | Location | T° | Humidity | Light/Dark |
|---|---|---|---|---|---|
| 1-8 | 1st inoculation, mycelial growth | Incubation room | RT | natural | Dark |
| 9-25 | Stock at 4°C, latency | Cold room | 4°C | natural | Dark |
| 26-45 | 2nd inoculation, mycelial growth | Incubation room | 19°C | natural | Dark |
| 46-74 | 3rd inoculation, mycelial growth | Incubation room | 19°C | natural | Dark |
| 87-107 | Soil inoculation, mycelial colonization | Climatic chamber | 19°C | 60% | 14h dark/10h light |
| 108 | Addition of nutrients | Climatic chamber | 19°C | 60% | 14h dark/10h light |
| 109-160 | Regular T° and RH decrease up to 0° | Climatic chamber | 15-0° | -> 35% | Increase darkness |
| 161-190 | Regular T° and RH increase up to 10° | Climatic chamber | 0-10°C | -> 50% | Increase light |
| 191-210 | Watering every three days | Climatic chamber | 10°C | -> 85% | 12h dark/12h light |
| 211 | Regular T° increase up to 15° | Climatic chamber | 10-15°C | 85-90% | 12h dark/12h light |

# 10.3 Annex - Fruiting body cultivation assay of *Morchella* in the Botanical Garden of Neuchâtel

Melissa Cravero, Laboratory of Microbiology of the University of Neuchâtel

## 10.3.1 Material and Methods

- *Morchella* spawn was produced as indicated in Annex P1, with strains *M. deliciosa* M19-41 and *M. helvetica* M19-43
- Spawn was inoculated in the Botanical Garden in pots that contained garden soil or garden soil and pines (with/without pines treatment). Some of the cultures were covered with black plastic bags (sun/shade treatment).
- 20 days later, exogenous nutrient bags were added in some cultures (with/without nutrients treatment).
- 15 days later, plastic bags were removed to allow soil to freeze and promote fructification formation. Pictures were taken (26.01.2021).
- 24 days later, new pictures were taken (19.02.2021). Mycelium was almost not visible at the surface of the soil but aggregated in tissue-like structures under the nutrient bags.

## 10.3.2 Results

Cultures with pines (**Figure 1A**) were followed-up until March, but no fruiting body appeared, although mycelium heavily colonized the soil (**Figure 1C**) and the exogenous nutrient bags (**Figure 1B**). Unfortunately, the experiment should be interrupted because invasive insects were found on the pines that had to be destroyed. In the cultures without pines, no morel fruiting bodies appeared during the follow-up that was made until July.

**Figure 1.** *Morchella* fruiting body cultivation assay with pines (A). Mycelium colonized the exogenous nutrient bags (B, *M. helvetica* M19-43, 59 days post inoculation) and the soil (C, *M. deliciosa* M19-41, 35 dpi).

## 10.4 Annex - OFAG Proposal for fruiting bodies cultivation

**Summary**

Core statements on topic, context, research questions, methods and potential benefits (max. 1'500 characters incl. spaces)

This project aims to develop morel mushroom (*Morchella* spp.) cultivation in Switzerland by proposing cultivars originating from Swiss morel strains and by adapting the method developed in China to the Swiss climatic and socioeconomic context. Morels are well-known gourmet mushroom, prized for their exceptional taste and texture. Besides truffles, they are among the most expensive mushrooms in the market. A large fraction comes from wild harvests, some of them being induced through forest fires. Recently, China has developed their commercial cropping. This method is available in France and Switzerland through the company France morilles. Morels represent a very interesting crop for vegetables growers since sowing occurs in fall and harvest during Spring (generally March to June), hence filling a usually low-productive period for them. Moreover, a research mandate recently performed for the FOEN demonstrated that the Chinese cultivars may have an adverse effect on Swiss morel populations. As a result, it is timely to develop a cropping method adapted to the Swiss context. For this, we will assess which Swiss *Morchella* spp. are amenable to cultivation, from cultivar trait stability and spawn production using agro-food waste, all the way to fruiting-body yield. Different climatic zones in Switzerland will be considered by running field trials with different partners. Eventually, this will allow proposing a sustainable morel cropping method to Swiss farmers.

**Initial situation**

Subject, political context, scientific context (current state of knowledge, ongoing research in Switzerland and abroad, status of own research)

**Introduction**

True morels (*Morchella* spp.) are edible mushrooms highly prized worldwide for their unique taste and texture[1]. As other mushrooms, morels are nutritionally interesting thanks to their high levels of protein, fibers, and minerals[2]. In addition, they contain different health-beneficial compounds as antioxidants (e.g. vitamin D), anti-inflammatory molecules (e.g. sterols), and immunostimulants (e.g. heteropolysaccharides)[3]. The fruiting-body, composed by a stipe and a honeycomb-like cap, emerges as a result of sexual reproduction[4], generally in Spring[5]. Current integrative taxonomies suggest the existence of about 80 different morel species worldwide[6]. All of them can be consumed, if well-cooked and/or previously dried[7]. Worldwide, morels are harvested in the wild before being consumed or sold on the market[8]. In addition, morel cultivation has been achieved in different countries (e.g. China[9], Denmark[10], India[11], Turkey[12] and France[13]). In Switzerland, the culture of Chinese morel cultivars was achieved at a very small scale[14] between 2018 and 2022. However, the cultivation at an industrial scale (i.e., with a yield that allows commercialization) was only achieved in China, since 2012[15]. China is the main morel exporter, representing 28.04% of a global market and reaching 963.40 million USD for 121.66 million tons of dried morels in 2021[16]. In 2021, Switzerland imported morel mushrooms for 28.37 million USD, mainly from Italy, Belarus, and Lithuania[17]. The cost of imported morel ascocarps is very high: in 2022, dried ascocarps (from China, Pakistan, India, Chile, or Montenegro) cost in average 55 CHF/100g in Swiss supermarkets (Coop and Migros; personal observation). As a comparison, dried boletes cost 8-15 CHF/100g in the same vendors. However, the ecological and social cost for importing these products is high. First, transport accounts for 1/5 of total carbon footprint of the food industry[18]. In addition, imported Chinese morels contain chemical additives that are deleterious to health, to preserve their integrity during the long travel[19]. In the wild, morel yield may increase after habitat disturbances such as forest fires[20]. As a result, intentional forest fires are triggered for wild morel harvesting (e.g. in Nepal[21], USA[22], and Chile[23]). Although controlled forest fires can be triggered by the authorities[24], illegal fires cause important damage to ecosystems[25]. Socially, the trade of wild morels is often highly unfair. For instance, the Pakistani collectors are mostly poor illiterate women and children that conduct the hard physical work of harvesting and drying morels but receive very low salaries[26]. In Pakistan, 54% of the morel collectors were children[27]. Children received in average 3 USD/kg of fresh morels, that are processed and sold to

the local market for 19 USD/kg, then to the middlemen for 24 USD/Kg, and finally to the international market at 271 USD/kg in 2003[28]. For these reasons, it is crucial to develop morel cultivation in Switzerland, to be able to provide the Swiss consumers with high quality, environmentally and socially sustainable morel mushrooms. To remain free from importation constraints, in particular regarding the Convention on Biological Diversity (Nagoya protocol)[29], we aim to select Swiss morel strains that can be reliably cultivated outdoors and indoors. Swiss morel cultivars will have additional competing advantages on the market, for the consumers (organic certification; food control and authenticity) and for the cultivators (added economical value for "terroir" or local products; high yield thanks to the use of strains adapted to the Swiss climate and pathogens; additional revenue stream during a low-activity period (November-April); proximity with the strains/spawn providers). Finally, concerning potential risks of invasiveness of non-native fungal strains in Switzerland[30], it is important to provide farmers with strains that can safely propagate in the environment without any adverse effect on indigenous populations.

**Morel cultivation**

The edible fruiting-body (or ascocarp) is the result of sexual reproduction. Morels can reproduce sexually by three different modes: heterothallism, primary homothallism, and secondary homothallism[31]. While primary and secondary homothallic individuals are self-fertile, heterothallism requires the presence of two complementary partners (i.e. carrying the complementary version of the mating type gene, *MAT1-1* and *MAT1-2*) to accomplish sexual reproduction[32]. These three modes can appear within a single *Morchella* species[33]. This is important to consider regarding cultivation because self-fertile individuals may be easier to reproduce, but at the same time would induce a loss of genetic diversity that could explain the reduced yield observed after a few years of cultivation. Indeed, morel cultures are unstable, yields vary between 2250-4500 kg/ha in China[34], and can collapse after a few years of consecutive cultivation in the same field. The reproduction and life cycle of *Morchella* is still not completely understood. However, the techniques developed in China to cultivate morels outdoors apear well established. First, the morel spawn must be generated by inoculating fresh mycelium in an organic-based substrate, for instance, consisting in wheat, sawdust, calcium carbonate and soil[35]. Substrate composition are variable and may impact final yield. After substrate colonization, the spawn can be sown directly on soil in Autumn and covered by a thin layer of soil. Mycelium will then propagate and the adjunction of nutrient bags, basically containing the same compounds as the spawn substrate, will allow the storing of nutrients. Further, sclerotia (i.e. resistant structures) will be formed from these nutrients to overwinter. In Spring, the primordia formation is triggered when the temperature, light and humidity increase. The primordia finally develop into fruiting-bodies between March and April. Culturing morel ascocarps is highly valuable for vegetable producers because this is an additional revenue stream occurring in a low-activity period (November-April). Intercropping systems with cereals, vegetables and morels were already implemented in China to improve yields of each culture and maintain soil health[36]. Additionally, the cultivation of morel fruiting-bodies is also possible indoors, under controlled conditions[37]. This allows to cultivate mushrooms year-round, for instance in a climatic chamber.

**Own research**

The Laboratory of Microbiology of the University of Neuchâtel (LAMUN) has been working on *Morchella* spp. since 2012. While we initially focused on their interaction with soil bacteria[38,39], we started investigating the biology of these fungi into more details since 2018. Between 2019 and 2022, we investigated the ecology of Chinese and Swiss morels for the Federal Office for the Environment (FOEN). This has led to the publication of three reports ("Microbial invasions, with focus on Morels (*Morchella* spp.)"[40] in Appendix 1; "Analysis of the invasive potential of Morels" in Appendix 2; "Analysis of and compatibility between morel strains of different origins" in Appendix 3). Through these mandates, the LAMUN has acquired a very large expertise on the biology of Morchella spp., including a clear understanding of their ecological requirements and a thorough comprehension of the factors governing their sexual reproduction. The latter has recently led to the publication of a paper concerning the sexual reproduction of *Morchella*[41]. Importantly, our analyses have revealed that the Chinese cultivars have the capacity to become invasive over the Swiss environment. For this reason, we have started collaborating with a vegetable producer that has the skills to grow Chinese morel cultivars in Switzerland, with the aim to

select Swiss strains amenable to cultivation. With him, we have run a pilot experiment between Fall 21 and Spring 22 consisting in cultivating Swiss strains from our collection in Fribourg and Vaud. However, we did not manage to obtain fruiting-bodies, probably due to unfavorable climatic conditions in the period considered. The experiment could not be reiterated in Fall 2022 due to lack of funding. In 2022, we have collaborated with a Swiss company (SmartGene) to establish a reliable database to genetically identify *Morchella* spp., aimed for food authentication for the Swiss market (funded by an Innovation cheque from Innosuisse). This small project has highlighted the difficulty of defining a species in the microbial world, advocating for being very cautious with the invasive potential of alien species for instance (Summary of the project available in Appendix 4). Most importantly for the proposed project, we currently have a collection of 152 Swiss morels. From those, 40 strains represent black morels (Elata clade), which is the clade most amenable to cultivation. In addition, we also have four Chinese cultivars that can be used as controls.

**Research questions**

**1) Which substrate is optimal for morel spawn production?**
A high-quality spawn is crucial to be able to eventually obtain morel fruiting-bodies. It also ensures qualitative multiplication of the inoculants. For sustainable agricultural practices, it is also recommended to use a substrate with a low-carbon footprint. For this reason, agricultural and agroforestry waste products will be prioritized for the substrate optimization tests. This will be done in WP1.

**2) Which *Morchella* spp. strains from the Elata clade (black morels) native from Switzerland are amenable to cultivation?**
About ten morel species are currently cultivable worldwide (e.g., *M. sextelata, M. importuna*). All belong to the Elata clade. The reasons for the inability to cultivate other species are unknown. We propose to test which species/strains originating from Switzerland are amenable to cultivation. This will be assessed in WP2 (outdoor) and WP4 (indoor).

**3) Does a consortium of multiple *Morchella* spp. strains used as a single inoculant improve genetic diversity and resistance to pathogens?**
Along to single strains, we will also test whether consortia consisting of a combination of 2 to 4 strains allow obtaining more stable and vigorous cultivars by maintaining an active genetic pool. This will also allow testing whether this brings a better resilience against morel pathogens (mostly molds and insects). Indeed, monocultures are known to be poorly resistant to environmental stresses. For this reason, consortia consisting of >3 strains will be compared to single strain inoculant in WP3 (outdoor) and WP4 (indoor).

**4) What are the cultivars and agricultural practices that are optimal for the Swiss agricultural context?**
After the development of actual morel cultivars in WP2, we will test different environmental conditions and agricultural practices (including intercropped vegetables or cereal species) that are optimal for Swiss producers. This will be achieved in WP3 with the help of local institutions active in the field of agricultural practice training.

**Methods**

Planned quantitative and qualitative methods of data collection

**WP1 – Production of high-quality spawn (UniNE)**
Different spawn substrates will be tested using in priority agricultural and agroforestry waste and their by-products to promote a circular economy based on waste valorization. For each substrate, the propagation rate of the morel mycelium in the spawn substrate is important to consider because this will ensure the rapid propagation of the mycelium in the soil. This step is crucial to store enough nutrients to eventually form the sclerotia and in turn the fruiting-bodies. The rapid browning of the mycelium (melanization caused by stress/aging) appears to be associated with lower yields. On the other hand,

the formation of multiple sclerotia may be an indicator of high-quality spawn. Thus, these characteristics will be considered to optimize spawn production.

**WP2 – Development of a Swiss cultivar (Unine and FRI)**
Developing Swiss morel cultivars will allow avoiding the current dependency on a foreign cultivar. This situation is also not in compliance with the Nagoya protocol. In addition to this, using a cultivar adapted to Swiss soils and climatic conditions should lead to productive yields on the long term for Swiss farmers. For this, the mycelium of 40 different black morel strains from the LAMUN collection will be propagated in spawn substrate from WP1, singly or as consortia of multiple strains. Their development on the spawn substrate will be monitored to eliminate unreliable strains/consortia. The spawn of a selection of the most promising strains/consortia will be sown in fields for a complete cultivation cycle to determine the strains amenable to outdoors cultivation.

**WP3 – Field trials in different environmental conditions (FRI and Unine)**
Identifying and understanding the ecological parameters that allow obtaining high morels yields (≥ 200 g/m$^2$) is crucial for long term profitability for the farmers. For this, field experiments at different locations (Jura, Prealps, Plateau) will be conducted. The environmental conditions (climate, soil), agricultural practices, and intercropped cultures will be evaluated to determine the most productive and sustainable crops. In addition, resistance and yield of strain consortia will be compared to monocultures. This will ultimately lead to define parameters of utmost importance to guarantee productive yields, to safeguard soil protection, and establish good agricultural practices for morel crop growth.

**WP4 – Indoor trials (UniNE)**
Along to outdoor cropping, we aim at investigating the possibility to develop a method of growing morels year-round indoors. A benefit of indoor cultures is that it maximizes the surface for food production. This may be unvaluable not only for mushroom producers, but also for scientific research. Among the strains/consortia selected in WP2, we will select the most promising for indoor culture. Then, the environmental conditions necessary to grow morels outdoors (light, temperature, humidity and their cyclicity) will be mimicked and applied in a climatic chamber.

**Division of labor (for cooperative projects)**

Description incl. justification

**Main partner - LAMUN (UniNE):** The LAMUN will act as project manager. It is the reference laboratory for morel research in Switzerland due to its expertise of more than 10 years with this fungal genus and its recognized expertise in mycology. The collection of more than 150 *Morchella* strains constitutes the foundation of WP2. The equipment required to manipulate and test mycelial cultures is available at the LAMUN (WP1 and WP2), as well as the climatic chamber necessary for indoor trials (WP4). Prof. Pilar Junier is the head of the LAMUN and is an expert in microbial ecology, including bacterial-fungal interactions. She has promoted the research on *Morchella* at the LAMUN. Dr Saskia Bindschedler is an expert in mycology and has been involved in the *Morchella* research since its beginning. Melissa Cravero has been working on various aspects of the biology of *Morchella* spp. since 2019 and will be involved as researcher in this project. In addition to this, a technician from the laboratory will be able to assist Ms Cravero for all the tasks that involve strain maintenance, spawn preparation and inoculation, and field experiment preparation.

**Additional partners – Institutions with a link to practice (FRI and landowners)**
Field trials for WP2 and 3 will be performed at different locations in Switzerland through collaboration with the Fondation Rurale Interjurassienne (FRI) as well as with landowners through our already established network.

Jura trials will be done with the help of the FRI, which is the main instrument for rural development in the canton of Jura and the Bernese Jura. Its main tasks focus on education, training, research and development in the area of agriculture and rural development. Field facilities at Courtemelon have space available for morel assays. FRI will be involved in field trials for both WP2 and 3 and their involvement will be essential to define the primary parameters for all further field trials (year 2 to 4).

Then, Prealps and Plateau trials will be done by selecting directly landowners through our already existing network. For instance, Prealps trials will be done with the vegetable grower with whom we had

collaborated during the FOEN research mandate. Furthermore, we have contacts at Grangeneuve (competence center of the canton of Fribourg for training, advice, and execution in the field of agriculture) and OTM (institution based in the cantons of Vaud and Geneva which provides advice to vegetable growers that are part of the association) in order to reach a wider network of vegetable growers willing and motivated to implement new practices.

To note, all field trials will be primarily coordinated by the LAMUN and the input of institutions/landowners will be to provide access to their field facilities (including field preparation and monitoring for FRI) and/or their network of producers in which to select for on-farm trials (Grangeneuve, OTM).

**Potential benefit**

Potential benefits for a sustainable food system and/or agricultural policy

Morels that are currently sold on the Swiss market are considered socially and environmentally unsustainable. Indeed, they mostly come from wild harvests in low-income countries or from cropping in China. Their cropping in Switzerland is possible in practice[42,43,44] but still relies on strains originating from China, thereby, coming with a threat in terms of environmental invasiveness. As a result, developing the cultivation of morels in Switzerland using native strains of *Morchella* appears as a logical move towards a more sustainable of agricultural production. In addition to this, morel crops occur in a low-activity period and can be used in intercropping with vegetables and cereals. Therefore, developing a Swiss morel cropping method will be highly valuable in terms of economic income and stability for Swiss growers. At the same time, this will allow Swiss agriculture to provide consumers with a local, environmentally sustainable, and socially controlled product. Finally, investigating the feasibility of indoor morel culture will be a great asset for producers interested in implementing innovative approaches in their practice, such as in the case of mushroom producers, a field that is currently developing at a high pace given the consumer willingness to decrease meat consumption.

**Risks**

Risk analysis (situations or events that may affect the project), risk management (strategies or measures to avoid risks)

Morel cropping appears as a challenging task and we are aware of this. This proposal is based on several years of experience working with this fungal genus and we have run several preliminary experiences from which we will be able to construct the proposed research plan as well as implementing the appropriate mitigation strategy in case of negative results. For each WP, we have identified the main risks, the likelihood that they happen and the strategy to mitigate each of these risks. The matrix is presented as Appendix 5.

**Arrangements for optimal valorisation of the results**

e.g. involvement of potential direct beneficiaries and other stakeholders (education, counselling, other multipliers), target group-oriented communication of results, etc.

Once Swiss morel cultivars will be successfully obtained and a cropping method established, direct beneficiaries will be numerous. The first and obvious beneficiaries will be farmers. Morels indeed represent a local and organic product of great value on the market, including restaurants, grocery shops and food markets. The cropping method will be advertised through dedicated workshops, in order to help producers to implement this new practice in their culture scheme. As already mentioned, morel production represents an additional revenue stream since it is a new intercrop occurring in a usually low-activity period. The impact of morel cropping on soil quality for the following will also be assessed in this project, in order to allow for an optimal crop rotation scheme. The consumers will be other directly beneficiaries by accessing sustainable and healthy fresh products of local origin. In addition, if the indoor culture is successful, mushroom growers will also benefit by expanding their product range. Finally, the Swiss Confederation will indirectly benefit from the development of a Swiss cultivar, as the current Chinese cultivars do not comply with the Nagoya Protocol and could become invasive and affect our local environment.

## 10.5 Annex - BRIDGE Proof-of-concept “Development of a mycelial morel powder for the food industry”

Melissa Cravero, Laboratory of Microbiology of the University of Neuchâtel

### 10.5.1 Summary

Morel mushrooms (*Morchella* spp.) are highly prized worldwide as a delicacy food and for their medicinal properties. Morel fruiting bodies are provided to the market via two ways: wild harvests and commercial cropping. Wild harvests consist of fruiting-bodies collected in the wild in Spring before being commercialized. Commercial cropping has been developed in China since a few years, where morel mushrooms are produced as a crop at an industrial scale. Several significant economical, ecological, and social issues are linked to the commercial exploitation of these mushrooms. Wild morel harvesting is sometimes linked to illegal forest fires that are used to trigger morels’ emergence in the wild, and child-labour is also commonly used in Asia for the harvesting of morels. For cropped morels, their yield is highly variable, and this instability implies high economical risks for morel farmers.

Worldwide, different initiatives have investigated how to cultivate morels. However, up to now, all of them failed to propose a method with a constant and consistent yield. Therefore, in this project, I propose to develop an alternative to fruiting-body cultivation inspired from batch cultivation of fungal mycelia in the mycelial fermentation industry. This idea emerged when I remarked a very pleasant smell, similar to the one of actual fruiting-bodies, emerging from substrate colonized by morel mycelia. Therefore, the goals of this project are: (1) to identify a Swiss morel strain for the development of a reliable mycelial product. For this, a collection of native morel strains available in the host laboratory is already available; (2) to evaluate multiple substrates for mycelial production. A waste valorization approach will be considered by prioritizing by-products or waste products from agriculture and/or agroforestry; (3) to produce a mycelial powder and (4) characterize its organoleptic properties and (5) nutritional profile.

A literature review revealed that a food-flavoring mycelial morel powder product was already considered and developed at an industrial scale in the 1950s, but although the flavor was considered pleasant and “mushroom-like”, it was rapidly retired from the market because it could not compete with the cost of the fruiting bodies. Nowadays, the average cost of dried imported morels in the Swiss supermarkets is 55 CHF/100g. As a comparison, dried boletes cost 8-15 CHF/100g. Annual imports of dried morels in Switzerland average 28 million USD. A new food product based on mycelium will have great competitive advantages on the market of imported morel fruiting bodies, in terms of flavor-cost balance, ecological and social sustainability. In Spring 2022, a Chinese research group tested and demonstrated the safety of using morel mycelial powder as food in rats and indicated aiming to conduct safety trials in humans. In addition, a comparison of the nutritional profile of mycelial powder and fruiting bodies indicated that the mycelium contains more lipids, proteins, and energy than fruiting-bodies, highlighting another competitive advantage of using morel mycelium instead of fruiting bodies. In contrast to 1950 when economic considerations killed the development of such a product, nowadays, the arguments of a local supply, sustainability, and ethical production, together with improved yield and nutritional value, are all strong motivations for the development of this new product.

By demonstrating the feasibility of producing a morel powder that has great organoleptic properties at the laboratory-scale, this proof-of-concept project will generate the basis for the future development of a commercial product. Further development to propose the product on the Swiss market will involve the assessment of the safety of the product for human consumption, and the implementation of the process at an industrial scale. For all these steps, I have already identified key potential partners to bring the idea further. The mycelial morel powder will be a 100% Swiss-made product based on Swiss morels costing less than morel ascocarps, and being then a good flavoring alternative in various food preparations such as cheese, soups, sauces, and risotto. As the product is nutritionally more interesting than fruiting bodies, it can additionally be used as (e.g., protein) supplement in pasta or bread. The product will not rely on imports and can then be certified by Swiss labels. Finally, contrarily to morel ascocarps for which culture is unstable and requires large parcels of crops, morel mycelium can be easily and stably cultivated in narrow areas. In conclusion, the production of a mycelial morel powder will generate a highly profitable, ecologically and socially responsible economic enterprise.

## 10.5.2 Project description

### 10.5.2.1 Research background - Biology of morels

True morels (Ascomycota, Pezizomycetes, *Morchella*) are edible mushrooms prized worldwide for the intense and unique flavor of their fruiting body (also known as ascocarp or fructification) and their health-beneficial properties (e.g., antioxidant, anti-inflammatory, immunomodulatory) (Tietel and Masaphy 2018). The fruiting-body, composed by a stipe and a honeycomb-like cap, emerges as a result of sexual reproduction (Coppin et al. 1997). Morel fruiting-bodies generally appear in Spring, in a wide range of habitats (e.g., forests, meadows, orchards, disturbed areas) and substrates (i.e., soils, sand, ashes) (Pilz et al. 2007). They are often associated with dead or living trees such as oak, elm, ash, cherry trees, and conifers (Pilz et al. 2007), and some species appear post fire (Du and Yang 2021). Morels are mostly present in the Northern Hemisphere, but can also be found in Central and South America, Southern Africa, and Oceania (Gbif, accessed the 08.11.2022). Current integrative taxonomies suggest the existence of about 80 different morel species worldwide (Loizides et al. 2022), which are divided into three main clades: the basal *Morchella* Section *Rufobrunnea* (Rufobrunnea or white morels), *Morchella* Section *Morchella* (Esculenta or yellow morels) and *Morchella* Section *Distantes* (Elata or black morels) (Loizides et al. 2022).

### 10.5.2.2 Market review: cultivated and wild morels

Worldwide, morel mushrooms are harvested in the wild before being sold on the market (Boa et al. 2004). In addition, morel cultivation has been achieved in different countries (e.g., Denmark (https://thedanishmorelproject.com/the-morel-project/), India (Kumar et al. 2022), Turkey (Masaphy 2010), France (https://francemorilles.com/fr/), and at an extremely small scale in Switzerland. However, the cultivation at an industrial scale (i.e. with a yield that allows commercialization) was only achieved in China (Liu et al. 2018). Indoors, the cultivation of morel ascocarps was established in the USA in 2005, but only lasted three years because of contamination, and yield issues (Xu et al. 2022). In addition, soilless culture was achieved in the laboratory, but has never been expanded to industrial scale (Masaphy 2010). China is the main morel exporter, representing 28.04% of a global market and reaching 963.40 million USD for 121.66 million tons of dried morels in 2021 (https://www.tridge.com/). In 2021, Switzerland imported morel mushrooms for 28.37 million USD, mainly from Italy, Belarus, and Lithuania (https://www.tridge.com/).

The main issue with the cultivation of morel fruiting bodies appears to be yield instability, which is influenced by environmental conditions and other unknown factors (Xu et al. 2022). Indeed, the biology and mostly the sexual reproduction of *Morchella* are not yet completely understood (Du and Yang 2021). This results in high economical risks for the producers (Du et al. 2017), because the yield varies between 2250-4500 kg/ha, but can reach exceptionally 15’000 kg/ha under certain conditions (Xu et al. 2022). Therefore, for morel farmers, there is an urgent need to address the issues of morel’s yield instability, in order to reduce the economic risks related to *Morchella*’s production (Du et al. 2017).

In the wild, morel yield may increase after habitat disturbances such as forest fires (Larson et al. 2016). As a result, intentional forest fires are triggered for wild morel harvesting (e.g., in Nepal (Devkota 2008), USA (Larson et al. 2016), and Chile (Machuca et al. 2021)). Although controlled forest fires can be triggered by the authorities (Larson et al. 2016), illegal fires cause important damage to the ecosystems (Condé et al. 2019). Socially, the trade of wild morels is often highly unfair. For instance, the Pakistani collectors are mostly poor illiterate women and children that conduct the hard physical work of harvesting and drying morels but receive very low salaries (Hamayun 2006). In Pakistan, 54% of the morel collectors were children (Latif et al. 2003). Children received in average 3 USD/kg of fresh morels, that are processed and sold to the local market for 19 USD/kg, then to the middlemen for 24 USD/Kg, and finally to the international market at 271 USD/kg in 2003 (Latif et al. 2003).

#### 10.5.2.3 Aim of the project

The aim of this proposal is to demonstrate the feasibility to develop, at a laboratory scale, a food product consisting in a mycelial powder that can be used as a “morel flavor” seasoning for numerous food products such as cheeses, sauces, soups, and risotto. This product will represent an alternative to morel fruiting-bodies and could be highly valuable for the Swiss food industry because the cultivation of fungal mycelium is stable, almost unlimited once the proper conditions are set, and can be done in relatively small surfaces. In addition, it might offer a more sustainable alternative to the imported morel ascocarps sold in Swiss supermarkets, as it will be based on Swiss cultivars and produced in the country. Indeed, transport accounts for 1/5 of total carbon footprint of the food industry (M. Li et al. 2022), and thus a locally produced and distributed food flavoring product will have a positive impact on the carbon footprint of the product. Finally, a recent study has shown that morel mycelium has higher nutritional values than dried morel ascocarps (**Table 1**) (I.-C. Li et al. 2022), indicating it could be used as a supplement in various food preparations such as bread or pasta. The steps to produce the morel mycelial powder are depicted in **Figure 1**. If this process is a success at laboratory scale, the steps stemming from the proposal will be to implement the production of morel mycelial powder at industrial scale as a future development of this proof-of-concept.

**Table 1.** Nutrition profiles of morel mycelial powder and dry ascocarp (data from Li et al. 2022).

| | Lipids (%) | Proteins (%) | Carbohydrates (%) | Energy (kcal/100g) |
|---|---|---|---|---|
| Mycelial powder | 17.17 ± 0.07 | 39.35 ± 0.35 | 38.96 ± 4.60 | 467.77 ± 0.21 |
| Ascocarp | 2.54 ± 0.22 | 11.31 ± 0.64 | 78.33 ± 1.01 | 386.72 ± 0.72 |

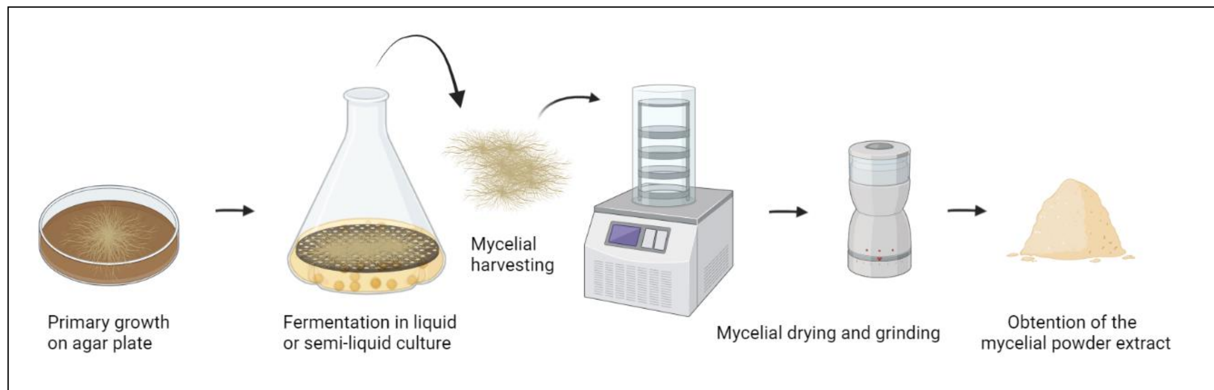


**Figure 1.** Steps to produce morel mycelial powder (picture generated with Biorender).

## 10.5.3 My background as a researcher

Before further evaluating the potential of the product to be developed in this project, the context in which the idea emerged and my contribution as researcher will be presented. As explained previously, morels can nowadays be cultivated in fields. A few years ago, Chinese cultivars (i.e., morel strains that could be cultivated) began to be grown in France and Switzerland. This raised the concern of species invasiveness and hybridization as a risk to the biodiversity of native morels in Switzerland. For this reason, the Federal Office for the Environment (FOEN) mandated the Laboratory of Microbiology of the University of Neuchâtel (LAMUN) to carry out a proper investigation on the invasive potential of morel cultivars/strains originating from China. During my master's studies, I worked in the frame of a first FOEN mandate that aimed to review the knowledge about microbial invasions and morel's biology (Etter et al. 2019). The first mandate was then extended to experimentally investigate the invasion and hybridization potential of the Chinese cultivars, assess morel's biodiversity in Switzerland, and to study bacterial-fungal interactions (BFI) between *Morchella* and their associated bacteria. Two reports were generated to present the results from this research. In parallel to the FOEN mandates, my master thesis entitled "Mating types: a tool for identification and phylogenetic analysis of morels (*Morchella* spp.)" focused on the study of morel mating locus (MAT). This led to a first peer-reviewed publication (Cravero et al. 2022). Since the completion of my MSc, I have been working on morel biology at the LAMUN as a scientific collaborator under the Science Focus Area Grant from the U.S. Department of Energy (DOE). I participated in the study of the morel's bacteriome (Cailleau et al., *in preparation*), and more deeply investigated BFI between Swiss morels and their associated bacteria. I also investigated melanin production by morels, and its potential to act as an antimicrobial agent. Finally, I am currently assessing the genetic diversity of morels present in Switzerland.

Since 2021, I have been collaborating with a Swiss farmer who was able to cultivate Chinese morels in canton Fribourg, to try to develop a Swiss cultivar from morel strains isolated from specimens collected in the wild in Switzerland. At present, we still did not manage to obtain any fruiting-bodies from either outdoor or indoor cultures. One of the steps to crop morels includes the preparation of a mycelial spawn, which is a solid substrate colonized by a dense mycelial network. During the inoculation of this morel spawn in cultivation beds, I noticed a very pleasant morel-like aroma coming from the spawn. Interestingly, the aroma differed between strains, from an herbal to an earthy-smoky smell. This was an intriguing observation and was the inception of my innovative idea to develop a food-flavoring product based on mycelial morel powder. To test the validity of the idea, I deconstructed the substrate (wheat grains, wheat bran, sawdust, and calcium carbonate (Liu et al.

2018)) and used the elements alone and combined, for the inoculation of one Swiss morel strain. Mycelial growth is currently in progress. The next steps will be to compare the aroma profile of the liquid cultures using gas chromatography-mass spectrophotometry (GC-MS).

## 10.5.4 Innovation potential and market review

The review of the literature and market products surprisingly revealed that morel mycelium-based food products are currently inexistent. In the 1950s, a "Morel mushroom flavoring" industrial product was developed in the USA, but it was rapidly removed because it could not compete with the low cost of dry imported ascocarps (I.-C. Li et al. 2022). Before 1982, the culture of morel ascocarps was not possible (Ower 1982), and numerous scientists tested the potential of using morel mycelium for the food industry (Litchfield 1967, Sanderson 1969). However, the aromas were highly divergent depending on the strain, substrate, and culture conditions, and the research field was abandoned (Sanderson 1969). In the early 2000s, several publications described again the potential of the morel mycelium for food industry (Mau et al. 2004; Tsai et al. 2006). It is only very recently that the idea has been considered once more: in two publications in 2020 and 2022, Li and colleagues confirmed the safety of using *Morchella esculenta* mycelial powder as food in rats, and reported the plan to conduct safety trials in humans (I.-C. Li et al. 2022).

The health benefits of mushroom consumption, including morels, is undeniable and mushrooms can replace meat and fish in a balanced diet (Tietel and Masaphy 2018). However, the cost of morel ascocarps is very high: in 2022 imported dried ascocarps (from China, Pakistan, India, Chile, or Montenegro) cost in average 55 CHF/100g in Swiss supermarkets (Coop and Migros) (personal observation). As a comparison, dried boletes cost 8-15 CHF/100g in the same vendors. In addition, the ecological and social cost for importing these products is high, as described in the previous section. Thus, developing a Swiss-made food product containing high nutritional and organoleptic values has a vast industrial, ecological, and social potential. These three topics are nowadays at the core of many consumer's concerns. Indeed, the taste of the product will be the main determinant of its success, but equally important selling arguments will be that: (1) this is a Swiss-made product based on Swiss morels, (2) morels are gourmet food with a unique flavor, (3) the product will cost less than morel ascocarps and is then a good flavoring alternative in food preparation, (4) the product is nutritionally more interesting than ascocarps, (5) the product can be used as protein supplement in pasta, bread or other preparations, (6) the product does not rely on foreign imports, and can then be certified by Swiss labels (e.g., BioSuisse), and (7) contrarily to morel ascocarps for which culture is unstable and requires large parcels of crops, morel mycelium can be easily and stably cultivated in restricted areas.

### 10.5.4.1 Description of the project and implementation strategy

This project aims to develop a new food product consisting of a Swiss-made morel mycelial powder, produced from a Swiss strain, that will have significant competitive advantages on the market of imported morel fruiting bodies, in terms of flavor-cost balance, nutrient content, and ecological and social sustainability. The goals of this project proposal are the following: (1) to identify a Swiss morel strain for the developing of a reliable mycelial product, for this a collection of native morel strains available in the host laboratory is already available; (2) to evaluate multiple substrates for mycelial production. A waste valorization approach will be considered by prioritizing by-products or waste products from agriculture and/or agroforestry; (3) to produce the mycelial powder and (4) characterize its organoleptic properties and (5) nutritional profile.

To achieve these goals, Swiss *Morchella* strains will be selected among a collection of 143 strains, according to its organoleptic properties and ability to grow *in vitro* rapidly and qualitatively (i.e., high mycelial branching and density). Different cultivation media will be tested for this purpose as well. By-products of the agriculture and agroforestry that are safe for human consumption will be prioritized. Drying and grinding processes will be optimized to keep or improve the flavor of the product. The organoleptic properties and the nutritional profile of the product will be chemically defined.

Stemming from the proposal, if the development of a laboratory-scale product is a success, the next step will be to assess the safety of the product for human consumption. Then, upscaling biomass production and product development at an industrial scale will be considered. A business plan on how to implement production most efficiently will be prepared with the aim to sell a license or to create a start-up dedicated to the production of the mycelial powder. Liquid fermentation devices aiming to produce pure mycelium in large quantities already exist and could be applied for morel mycelium production. To my knowledge, there is no company producing fungal mycelium in fermenters at an industrial scale in Switzerland, which is a great opportunity to develop the field using the morel mycelium as a pioneering product. Regarding substrates and waste valorization, the LAMUN is already in contact with Mycrobez, a young start-up aiming to produce sustainable mycelium composites (https://mycrobez.ch/), that could be a good partner to both identify adequate waste streams and implement the industrial liquid culture of mycelium in Switzerland. Drying and grinding of the biomass at an industrial scale will be less challenging than the mycelial culture itself, as those procedures are already used in various food industries worldwide (e.g., for drying: https://liophilizador.com; for grinding: https://www.alpapowder.com/). The packaging for the final product will also be thought to be adapted to the product (i.e., long-term conservation in a dry environment) and ecologically sustainable. For this purpose, the company Servoartpack based in Switzerland could be a potential partner (https://www.servoartpackeurope.com/).

### 10.5.5 Project plan, milestones and deliverables

To achieve the goals of this proposal, the tasks that will be conducted have been distributed into three work packages: WP1= Optimization of the production of mycelium (selection of morel strains and growth media); WP2= Production of the mycelial powder; WP3= Product analysis (determination of the organoleptic properties and evaluation of the nutritional profile). The milestones for achieving the corresponding goals are described in **Table 2**. The planned tasks of the proof of concept are described in a Gantt Chart in **Table 3**. In **Table 4**, a risk management matrix is provided to demonstrate the feasibility of the idea.

**Table 2.** Milestones for achieving the goals of the proof of concept.

| Work Packages | Goals | Milestones | Description |
|---|---|---|---|
| WP1: Optimization of the production of mycelium | (1) Strains selection | Month 2 | The strains with the best growth abilities and producing pleasant aroma will be first selected to be cultivated in different media. Then, the strain-medium combination(s) with the best growth abilities and (subjectively) aroma can be selected. |
| | (2) Medium selection | Month 4 | |

| WP2:<br>Production of the mycelial powder | (3) Production of the powder | Month 5 | The selected strain grown in the selected medium can be used to produce the mycelial powder. |
|---|---|---|---|
| WP3:<br>Product analysis | (4) Organoleptic properties | Month 8 | The organoleptic properties and nutritional profile of the mycelium powder will be objectively measured by chemical analyses. |
| | (5) Nutritional profile | Month 11 | |

**Table 3.** Gantt Chart for the development of a mycelial morel powder for the food industry.

| | WP | Tasks | Month 1 | Month 2 | Month 3 | Month 4 | Month 5 | Month 6 | Month 7 | Month 8 | Month 9 | Month 10 | Month 11 | Month 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | WP 1 | Obtaining valorizable waste/by-products of the agriculture/agroforestry | | | | | | | | | | | | |
| 2 | WP 1 | Preparation of the starting mycelial cultures (primary inoculation + purification) | | | | | | | | | | | | |
| 3 | WP 1 | Setting the experimental controls, culture conditions and first strain selection | | | | | | | | | | | | |
| 4 | WP 1 | Testing the mycelial growth rate and quality in simple (controls) and complex media | | | | | | | | | | | | |
| 5 | WP 1 | Selection and optimization of media to enhance the organoleptic properties of the mycelia | | | | | | | | | | | | |
| 6 | WP 1 | Optimization of mycelial harvesting in the final media in large cultures | | | | | | | | | | | | |
| 7 | WP 2 | Production of morel powder extracts from fresh biomass (testing drying and grinding processes) | | | | | | | | | | | | |
| 8 | WP 3 | Analysis of the organoleptic profile of the morel powder extracts | | | | | | | | | | | | |
| 9 | WP 3 | Determination of the nutritional profile of the morel powder extracts | | | | | | | | | | | | |

**Table 4.** Risk management and mitigation strategy of tasks 1-9 referring to Table 3.

| Task | Risk | Risk Potential | Risk Impact | Mitigation strategy |
|---|---|---|---|---|
| 1 | No waste/by-product can be obtained | Remote (<10%) | Minor | Use of non-waste substrates. |
| 2 | A failure is not foreseen as this is part of routine laboratory work. | | | |
| 3 | A failure is not foreseen as this is part of routine laboratory work. | | | |
| 4 | A failure is not foreseen as this is part of routine laboratory work. | | | |
| 5 | None of the tested media enhance the aroma of the mycelium | Unlikely (10-35%) | Moderate | Other substrates must be tested; The powder can be made without medium optimization for the further steps. |
| 6 | The harvesting process is unreliable. | Unlikely (10-35%) | Major | The growth media and/or culturing methods must be adapted to be able to reliably harvest the mycelium. |
| 7 | A fine powder cannot be obtained from dried mycelium. | Remote (<10%) | Critical | The form of the product must be changed, for instance by extracting the aroma to create a flavored oil. |
| 8 | A failure is not foreseen as this is part of routine laboratory work. | | | |
| 9 | A failure is not foreseen as this is part of routine laboratory work. | | | |

### 10.5.6 Commitment to a sustainable development

The morel mycelium powder product proposed in this project will be a local all year-round alternative to dried morel ascocarps coming from foreign countries, providing to consumers the choice to buy a Swiss made merchandise produced within a short food supply chain and with agricultural waste that is undeniably more sustainable than imported food products (M. Li et al. 2022). In addition, the working conditions of the staff involved to produce 100% Swiss made items can be controlled and will be fair. In addition to this, imported morel ascocarps are generally not organic, even though they have been wild-picked or cultivated without synthetic pesticide. Indeed, chemical additives are necessary to preserve the fruiting bodies during transportation (Piqueras, 2021). To the contrary, the product that will be developed in this project will be able to meet criteria to obtain organic certifications. Finally, the product will be produced and implemented within a circular economy, preferentially using by-products from the agriculture and/or agroforestry as growth substrate and eventually using the substrate after mycelial harvest as a high-quality fertilizer.

## 10.6 Annex – Poster, Swiss Society of Microbiology 2023

# Using a Centroid-based approach for a reliable identification of morels (*Morchella* spp.): a case-of-study for food authentication

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### INTRODUCTION

The genus ***Morchella*** is taxonomically complex and recent data show that differentiation to the species level using only morphology is inaccurate. In a context of **commercial-cropping** with Chinese cultivars and of commercial trade, proper **taxonomic identification** appears crucial. **Food fraud** is type of criminal activity in which cheaper products are sold under false names or origin for financial profit. As morels are gourmet mushrooms with a high price on the market, there is a clear need for an accessible approach for food authentication. For this, we tested a molecular-based method to easily identify morels at the species level using the **SmartGene Centroid-based approach**.

*Morchella* taxonomy in brief

**What is a Centroid?** It is a DNA sequence of a given genetic marker that is the most similar to all the other sequences annotated as belonging to a same species (**Fig. 1**). The algorithm that defines the SmartGene Centroids (EU#2215578) uses and curates the public GenBank database to produce relevant data considering interspecific diversity. The generation and maintenance of the Centroid dataset is an automated AI-based process.

**How does the Centroid-based approach work?**

1. Centroid sequences are generated by SmartGene.
2. Users upload DNA sequences in the SmartGene IDNS software.
3. Queries are compared to the Centroid database (using BLAST).
4. A conclusive identification is provided.

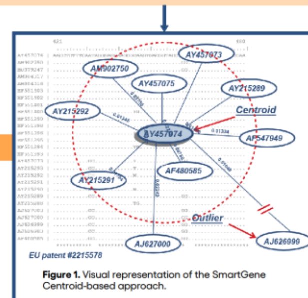


**Figure 1.** Visual representation of the SmartGene Centroid-based approach.

### MATERIAL & METHODS

**Expert curation**: The Centroid database was first curated to correspond to actual taxonomy reviewing the literature.

**Reliability of the Centroid genetic markers**: ITS, 25-28S, and 18S *Morchella* Centroid sequences were used to produce phylogenies (ML tree, GTR + I + G4 model) that were compared to the literature (e.g., Loizides et al. 2022) to assess their reliability in *Morchella*.

**Query sequences**: Reference sequences were retrieved from GenBank.

**Reliability of species determination**: Reference sequences were analyzed using the Centroid-based approach within the IDNS software to test the reliability of the method in *Morchella*.

### RESULTS & DISCUSSION

**Reliability of the genetic markers**: Only the ITS marker was retained (see **Fig.2**).

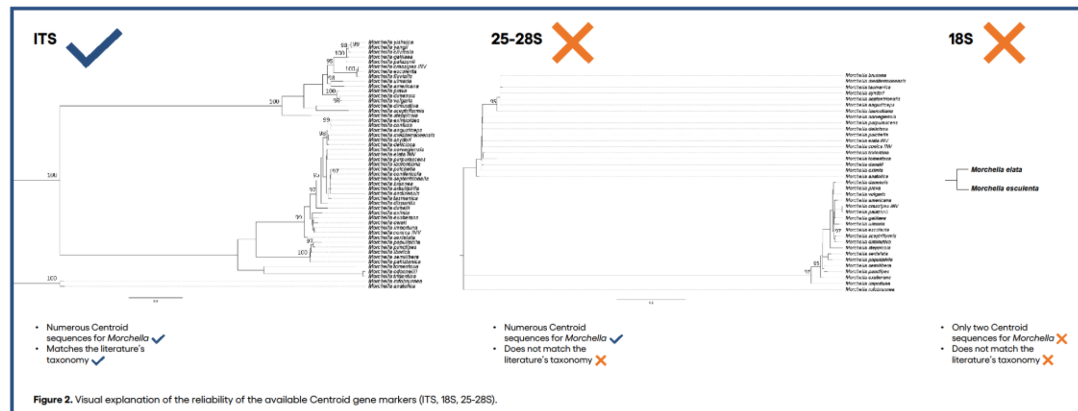


**Figure 2.** Visual explanation of the reliability of the available Centroid gene markers (ITS, 18S, 25-28S).

**Reliability of the Centroid-based approach**: The identification results provided by the **ITS Centroid-based approach** corresponded to the reference sequences' annotations for **83.6% species of *Morchella***. In comparison, Sa et al. (2022) demonstrated that the tree-building method (P-distance model) with ITS sequences was only able to identify 48.89% of the morel species.

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This is the number of morel species that could be genetically defined using the ITS Centroid-based approach.

### Conclusion

The Centroid method appears as an appropriate tool for the molecular identification within the genus *Morchella*. This approach is suitable for non-specialist such as actors of the food industry interested in tracking frauds in the morel market or environmental biologists interested in detecting species invasiveness.

### REFERENCES

Loizides et al. (2022): Loizides, Michael, Pablo Alvarado, Pierre-Arthur Moreau, Boris Assyov, Viktorie Halasů, Marc Stadler, Andrea Rinaldi, et al. 2022. "Has Taxonomic Vandalism Gone Too Far? A Case Study, the Rise of the Pay-to-Publish Model and the Pitfalls of Morchella Systematics." Mycological Progress 21 (1): 7–38.

Sa et al. (2022): Sa, Wei, Jinxia Qiao, Qiyuan Gao, Zhonghu Li, and Qianhan Shang. 2022. "DNA Barcoding and Species Classification of Morchella." Genes 13 (10): 1806.

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## 10.7 – *Curriculum Vitae*


**Melissa CRAVERO**

**Microbiologist**

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### Profile

Background in academic research in microbiology requiring strong autonomy, polyvalence, and adaptability. High meticulosity and analytical qualities.

### Work history

**2021-present** PhD thesis in microbiology, at the Laboratory of Microbiology of the University of Neuchâtel (LAMUN).

**2019-2021** Scientific collaborator at the LAMUN.

### Education

**2019-2021** Master of Science in Biology in Conservation biology and Sustainable agriculture at the University of Neuchâtel.

**2018-2019** Master of Science in Biology in Animal molecular life sciences at the University of Fribourg, one semester.

**2015-2018** Bachelor of Science in Biology at the University of Neuchâtel.

### Skills

**Molecular biology**

DNA and RNA extraction and quantification – PCR – Bacterial tagging

**Microbiology**

Experimentation with bacteria, fungi, nematodes, amoeba – Microscopy – Flow cytometry

**Bioinformatics**

DNA sequences analysis (BioEdit, GeneiousPrime) – Phylogenetic and taxonomic analysis (MegaX, TreeGraph, IQ-Tree, Mesquite) – Programming (R)

**Languages**

French (first language), English (C1), Italian (B2), German (B1). Good writing and oral presentation abilities in French and English.

## Projects

**Innovation Cheque with SmartGene**

Between 2022 and 2023, I conducted an Innovation Cheque with the Swiss company SmartGene to investigate the potential of the patented Centroid-based approach to identify morels at the species level for food authentication. The project was successful and led to a paper (see below) and further collaboration.

**Teaching**

Since 2021, I have been involved in multiple teaching activities at the University of Neuchâtel: in two fall semesters, I was teaching lab work for Master students in Biogeosciences (Molecular Biology class); in 2023, I supervised the lab work of five students doing their Bachelor Thesis in microbiology, which led to a publication (see below); I was the mentor of a high school student doing her Maturity Diploma with the Provoc program; I was teaching practical work in microbiology during one semester in 2023; between 2023-2024, I was coaching a Master student for her diploma about morel cultivation.

**FOEN mandates**

Between 2020 and 2022, I was the principal scientist involved in the accomplishment of two mandates commissioned by the Federal Office for the Environment (FOEN). The aim was to investigate the invasive and hybridization potential of non-native morels in Switzerland, assess the biodiversity of Swiss morels and study the interactions between morels and associated bacteria. In 2019, I was involved in another FOEN mandate about microbial invasions. The three mandates were successfully accomplished and published.

## Publications

**Cravero, M**., Robinson, A.J., Hilpisch, P. *et al.* Importance of appropriate genome information for the design of mating type primers in black and yellow morel populations. *IMA Fungus* 13, 14 (2022). https://doi.org/10.1186/s43008-022-00101-6

**Cravero, M**., Bonito G., Robinson A. J., Chain P. S., Bindschedler S., and Junier P. "Biodiversity of Morchella in Switzerland: A Study Highlighting the Diversity of Native Black Morels and the Discovery of New Lineages." *FEMS Microbiology ecology*, submitted.

**Cravero, M**., Bonito G., Chain P. S., Bindschedler S., and Junier P. "A new species of true morel from Switzerland: *Morchella helvetica* sp. nov." *Mycologia*, in revision.

**Cravero, M**., Ruelle J., Emler S., Bindschedler S., and Junier P. "Using a Centroid-Based Approach for a Reliable Identification of Morels (Morchella Spp.): A Case Study for Food Authentication." *Journal of Food Science*, in revision.

**Cravero, M**., Robinson A. J., Chain P. S., Bindschedler S., and Junier P. "Tripartite Interactions between *Morchella* spp., *Pseudomonas koreensis* and *Acanthamoeba castellanii* Reveal a Beneficial Fungal-Amoebal Association," *iScience,* submitted.

Cailleau, G., Hanson, B.T., **Cravero, M**. *et al.* Associated bacterial communities, confrontation studies, and comparative genomics reveal important interactions between *Morchella* with *Pseudomonas* spp. Frontiers, Sec. Fungal Physiology and metabolism, Vol. 4 (2023). doi: 10.3389/ffunb.2023.1285531

Estoppey, A., Tinguely, C., **Cravero M**., *et al.* Gamification as a tool to teach key concepts in microbiology to bachelor students in biology: a case-study using microbial interactions and soil functioning. *Microbiology Society* (2023). https://doi.org/10.1099/acmi.0.000699.v1