

University of Neuchâtel – Faculty of Sciences

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**From fundamental questions to practical conservation
actions: a study of the ecological niche (soil and vegetation),
the pollination system, population genetics, population
demography and mycorrhizal associations of *Cypripedium
calceolus* (Orchidaceae)**

PhD Thesis

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Titre :

**“From fundamental questions to practical conservation
actions : a study of the ecological niche (soil and
vegetation), the pollination system, population genetics,
population demography and mycorrhizal associations of
Cypripedium calceolus (Orchidaceae)”**

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« On n'est pas riche de ce qu'on prend mais de ce qu'on laisse. »

Robert Hainard

« The need for orchid conservation is paramount if we are to leave to future generations the rich and wildly fascinating orchid legacy we all enjoy today. Without effective conservation actions..., threatening process will continue to militate against the survival of rare orchids, resulting in their continued degradation and inevitable extinction. »

Swart and Dixon, 2017 in *Conservation methods for terrestrial orchids*, J. Ross Publishing

A mon papa, qui m'a appris à suivre le chemin de la connaissance.

Abstract

The current epoch, the Anthropocene, is witnessing a generalized and rapid extinction of species worldwide. As the causes of such extinctions, often related to global change drivers (e.g., habitat destruction, pollution, climate change), might not be hampered immediately, conservation actions should focus on preserving, restoring and regenerating the disappearing species through concrete action plans. Rare and endangered plant species, for instance, can be preserved by reintroducing seedlings in the same habitat, or introducing them into yet unoccupied habitats to generate novel populations. While this approach holds several good promises, it often generates mixed results, likely due to the fact that the current or novel habitat do not actually offer the optimal conditions for the target species to grow and thrive. With this thesis, we have tackled the problem of how to better characterize the ecological requirements of an endangered plant species in order to increase the success rate of future (re)introduction actions and to develop more efficient and targeted conservation measures. By focusing on the iconic and endangered orchid species *Cypripedium calceolus* (Lady's Slipper) as a model species, we have taken a broad-range perspective, including both geomatics and field work to study the links between multiple ecological factors (soil properties, climate, association with local vegetation and health of 34 *C. calceolus* populations across Switzerland).

In the **first chapter**, we propose a two-step approach to identify (re)introductions sites for endangered plant species using *C. calceolus* as a model species. The first step involves modelling its niche and its distribution with bioclimatic and topographical predictors. The second step consists in refining these bioclimatic predictions by analysing stationary ecological factors, such as edaphic conditions, and relating them to populations-level fitness values. The first results show that climatic predictions alone were not precise enough, but nevertheless could highlight a likely decline of the species range during the next 50 years due to predicted climate warming. Moreover, when incorporating topographical layers for the modelling approach at the regional scale, we show that the species is more likely to occur near forest edges. Finally, by analysing in situ soil factors, we show that soil organic matter, cation-exchange capacity and pH correlated most strongly with *C. calceolus* population fitness variables as described by multivariate function trait space. We thus advocate for the combination of modelling tools with fine scale on-site ecological surveys to identify suitable reintroduction sites for this, and potentially other, endangered plant species.

In the **second chapter**, we aimed to determine the relationships between the performance of *C. calceolus* populations and soil and vegetation factors, to improve this species conservation and but also to advance the theoretical underpinning of which facets of an ecosystem most influence this species fitness. By studying *C. calceolus* functional traits of 34 sites across Switzerland, we found that large (>20 individuals) populations of *C. calceolus* displayed a specific assemblage of measurable characteristics that discriminate them from small (<10 individuals) populations, indicating that it is possible to assess the health of a population of a rare plant species by measuring a specific set of traits. While we could not directly predict population health status from vegetation (phytosociological alliances) and soil types, we show that a unique combination of companion plants and several edaphic

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variables, such as soil organic matter (SOM), CaCO_3 , pH, and P could be used to potentially assess the optimal sites to implement (re)-introduction actions for this emblematic and patrimonial orchid species.

In the **third chapter**, we addressed the pollination ecology of *C. calceolus* by studying the links between plant vegetative and floral traits and local pollinator biodiversity. Moreover, we studied potential variation at the population level of how plants attract their pollinators and subsequently how this variation relates to the overall reproductive effort of *C. calceolus* individuals. We found that small *C. calceolus* populations occur in less diverse sites, both in terms of surrounding plants and insects. However, while plants from larger populations were physically larger and produced more flowers per capita, they produce the same number of seeds per capita (i.e., seed set) as small populations. This occurred despite the lower pollinator diversity present at the small population sites, meaning that plants from small populations must compensate by providing a stronger attraction to pollinators. Indeed, we discovered that floral volatile organic compounds are produced in higher quantity from the flowers of plants from small populations, suggesting that small populations compensate for low pollinator diversity by producing a stronger floral scent.

Two additional perspective chapters deal with the genetic structure of the Swiss *C. calceolus* populations and their association with mycorrhizal fungi. While these data opens new interesting venues, these topics merits further investigations in the future.

In summary, our results do not only increase the fundamental knowledge about *C. calceolus* biology and ecology but also allow to establish concrete conservation measures and to select more appropriate translocation sites. Moreover, this thesis emphasizes the complexity of orchid ecology and supports the use of integrated research and practical protection measures for this family of plants, and likely others. In a context of global climatic changes and due to the fragility of orchid ecological needs, this kind of approach is more relevant than ever. From a broader perspective, the method we developed is transposable to other plant taxa. Therefore, we advocate for the use of comprehensive methodologies based on multiple aspects of plant biology and ecology, as presented here, to study and protect a broad range rare and endangered plants.

Résumé

Notre époque actuelle, l'Anthropocène, est la scène d'extinctions rapides et généralisées des espèces dans le monde entier. Leurs causes, liées la plupart du temps à des facteurs de changements globaux tels que la destruction des habitats, la pollution, ou encore la modification du climat, ne peuvent pas être résolues à court terme. Par conséquent, des actions concrètes de conservation sont indispensables pour assurer la préservation, la restauration et la régénération des espèces en voie de disparition. Les espèces végétales rares et menacées notamment peuvent être sujettes à la réintroduction d'individus dans leur habitat d'origine, voire dans des habitats encore inoccupés afin d'initier de nouvelles populations. Bien que cette approche soit prometteuse, elle génère souvent des résultats mitigés. En effet, l'habitat actuel tout comme le nouvel habitat n'offrent pas toujours les conditions optimales pour l'espèce cible. Dans le cadre de cette thèse, nous avons développé une méthodologie permettant d'augmenter le taux de succès de futures (ré)introductions. L'objectif est de mettre en place des mesures de conservation concrètes pour des espèces végétales menacées en se basant sur une meilleure connaissance leurs exigences écologiques. En utilisant l'emblématique orchidée *Cypripedium calceolus* (Sabot de Vénus) comme espèce modèle, nous avons choisi une approche qui inclut à la fois la géomatique et le travail de terrain afin d'étudier les liens entre de multiples facteurs écologiques (propriétés du sol, climat, association avec la végétation locale et santé de 34 populations de *C. calceolus* à travers la Suisse).

Dans le **premier chapitre**, nous proposons une approche en deux étapes pour identifier des sites de (ré)introduction pour des espèces végétales menacées. Pour ce faire, *C. calceolus* est utilisée comme espèce modèle. La première étape consiste à modéliser sa niche et sa distribution par le biais de prédicteurs bioclimatiques et topographiques. La deuxième étape permet d'affiner ces prédictions bioclimatiques en analysant les facteurs écologiques stationnels, tels que les conditions édaphiques, et en les reliant aux valeurs de vitalité des populations de *C. calceolus*. Les premiers résultats montrent que les prédictions climatiques seules ne sont pas assez précises, mais qu'elles peuvent néanmoins mettre en évidence un déclin probable de l'aire de répartition de l'espèce au cours des 50 prochaines années en raison du réchauffement climatique. De plus, en incorporant à l'échelle régionale des couches topographiques dans le processus de modélisation, nous montrons que l'espèce est plus susceptible d'être présente près des lisières de forêt. Enfin, en analysant les facteurs pédologiques *in situ*, il apparaît que la matière organique du sol, la capacité d'échange cationique et le pH sont les variables les plus fortement corrélées avec celles de vitalité de la population de *C. calceolus*. Nous préconisons donc la combinaison d'outils de modélisation et d'études écologiques de terrain à petite échelle afin d'identifier des sites de réintroduction appropriés pour cette espèce végétale menacée, et potentiellement pour d'autres.

Dans le **deuxième chapitre**, nous avons cherché à déterminer les relations entre la vitalité des populations de *C. calceolus* et les facteurs inhérents au sol et à la végétation, afin d'améliorer la conservation de cette espèce. En étudiant les traits fonctionnels de *C. calceolus* à travers la Suisse, nous avons constaté que les grandes populations (>20 individus) présentaient un ensemble spécifique

de caractéristiques qui les distinguaient des petites populations (<10 individus). Ces résultats indiquent qu'il est possible d'évaluer la vitalité d'une population d'une espèce végétale rare en mesurant un ensemble spécifique de traits fonctionnels. Bien que nous n'ayons pas pu prédire directement l'état de santé des populations de *C. calceolus* à partir de la végétation alentour (alliances phytosociologiques) et des types de sol, nous avons montré qu'une combinaison unique de plantes compagnes et de plusieurs variables édaphiques, telles que la matière organique du sol, le CaCO_3 , le pH et la teneur en phosphore, pouvait être utilisée pour évaluer des sites propices aux actions de (ré)-introduction pour cette espèce menacée.

Dans le **troisième chapitre**, nous nous sommes focalisés sur la pollinisation de *C. calceolus* en étudiant les liens entre les caractères végétatifs et floraux des plantes ainsi que la biodiversité locale des pollinisateurs. De plus, nous avons investigué la variation des moyens d'attraction mis en place par les plantes et destinés aux pollinisateurs à l'échelle de la population. Nous avons constaté que les petites populations de *C. calceolus* se trouvent dans des sites moins diversifiés, tant en termes de plantes environnantes que d'insectes. De plus, les plantes présentes dans les grandes populations sont morphologiquement plus grandes et produisent plus de fleurs *per capita* ; ces dernières génèrent toutefois le même nombre de graines *per capita* que les plantes des petites populations. Ceci signifie que les plantes issues de petites populations semblent compenser la faible diversité d'insectes en augmentant leur attractivité pour les pollinisateurs. En effet, nous avons découvert que les plantes de petites populations produisent de plus grandes quantités de composés organiques volatiles floraux : elles compenseraient ainsi la faible diversité des pollinisateurs en libérant un parfum floral plus fort.

Deux autres chapitres, encore à l'état d'ébauche, traitent de la structure génétique des populations suisses de *C. calceolus* et de leur association avec les champignons mycorhiziens. Ces données ouvrent de nouvelles perspectives intéressantes. Ces sujets méritent d'être approfondis à l'avenir.

Finalement, nos résultats incrémentent les connaissances fondamentales sur la biologie et l'écologie de *C. calceolus* mais permettent également d'établir des mesures de conservation concrètes et de sélectionner des sites de translocation plus appropriés. De plus, ce travail de recherche souligne la complexité de l'écologie des orchidées et soutient une approche de recherche intégrée ainsi que l'utilisation de mesures de protection pratiques pour cette famille de plantes. En effet, dans un contexte de changements climatiques globaux et en raison de la fragilité des besoins écologiques des orchidées, ce type d'approche est plus pertinent que jamais. Dans une perspective plus large, la méthode que nous avons développée est transposable à d'autres taxons végétaux. Par conséquent, nous préconisons l'utilisation de méthodologies globales basées sur de multiples aspects de la biologie et de l'écologie des espèces végétales, telles que présentées ici, afin d'étudier et de protéger un large éventail de plantes rares et menacées.

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General introduction

It is now commonly accepted that we are currently facing the Earth's sixth mass extinction of living species (Ceballos et al. 2017; Cordier et al. 2021; Primack 2014). Contrary to previous mass extinctions, the current one is caused by anthropogenic factors (Primack 2014). The combined effects of natural habitat destruction, over-exploitation of natural resources and other global change factors cause a disheartening loss of the extraordinary diversity of life (Van Dyke and Lamb 2020). In their 2017-2020 Report, the IUCN Red List (<http://www.iucnredlist.org>) reported 128,918 species from all taxa falling into one of the categories of threat (IUCN 2021). In response to this menace, the field of Conservation biology has developed over the last 40 years with three main objectives : (i) catalogue biodiversity, (ii) study the causes of biodiversity decline and (iii) develop practical approaches to prevent species extinction (Primack 2014). Although these three targets are well defined, they are not straightforward to be put into practice (Van Dyke and Lamb 2020).

Plants are sessile organisms that have limited abilities to migrate and are therefore particularly sensitive to current environmental changes (Hamann et al. 2021). As a result, plants critically need protection measures (Heywood and Iriondo 2003). Focusing on vegetation conservation, Swarts and Dixon (2009) argued that protection through the reserves' system is not enough to comprehensively protect all plant species. Indeed, due to the loss, fragmentation and change of habitats combined with loss of species, the restoration of viable populations can rarely be achieved by natural recruitment and dispersal alone (Seddon 2010). Therefore, an effective maintenance of endangered plant species is needed to ensure the persistence of populations, including among others (i) identification of factors threatening them (ii) systematic collection of baseline data on species natural history (i.e., identification of species ecological niche, etc.) and (iii) study of population demography and genetics (Heywood and Iriondo 2003; Pavlik 1994). Fundamental research needs to focus on these topics to develop practical conservation methods for plants; and this is even more important in the context of current and future near term changes in landscape and climate (Maschinski and Haskins 2012).

In the field of effective maintenance of plant species, assisted migration of individuals to new sites has increasingly been used in the last decades to overcome barriers to dispersal (Menges 2008; Seddon 2010; Silcock et al. 2019; Swan et al. 2018; Swarts and Dixon 2009; Zimmer et al. 2019). Conservation translocation, as a deliberated movement of living organisms from one area to another, consists in (i) reinforcement and reintroduction, and (ii) introduction procedures (IUCN/SSC 2013). Reinforcement and reintroduction are the transplantation of new individuals inside their indigenous range, while introduction consists of moving a species to areas where it has not been originally observed (Bottin et al. 2007; IUCN/SSC 2013). Plant translocations aim to establish resilient, self-sustaining populations that have sufficient genetic resources to bear adaptive evolutionary changes (Guerrant Jr and Kaye 2007; Zimmer et al. 2019). In order to complete reintroduction and introduction task successfully, several steps need to be fulfilled (Kaye 2008). They consist in (i) planning and identification of objectives, (ii) finding source material, (iii) propagation, (iv) site selection, (v) site preparation, (vi) outplanting, (vii) monitoring, (viii) evaluation and interpretation, (ix) feedback to improve protocols,

(x) communication with others, (xi) habitat maintenance and (xii) repeated actions if necessary to meet objectives (Kaye 2008). In addition, it is crucial to consider genetic and demographic factors before any reintroduction or introduction (Menges 2008). While some plant translocation attempts succeeded (Bottin et al. 2007; Cogoni et al. 2013; Maschinski et al. 2004; Maschinski and Duquesnel 2007; Ramsay and Stewart 1998), many others failed, despite the existence of a theoretical framework of plant translocation (Bottin et al. 2007; Drayton and Primack 2012; Godefroid et al. 2011; Heywood and Iriondo 2003; Silcock et al. 2019). According to Godefroid et al. (2011), the flowering, the fruiting and the recruitment rate of reintroduced plants species are often low (on average 52%, 19% and 16% respectively). The five most often-mentioned reasons that led to failure are: (i) the lack of understanding of reasons causing decline in remaining plant populations (Godefroid et al. 2011; Heywood and Iriondo 2003); (ii) the deficit of biological and ecological understanding of species (Bottin et al. 2007; Godefroid et al. 2016; Godefroid et al. 2011; Guerrant Jr and Kaye 2007; Heywood and Iriondo 2003; Menges 2008); (iii) the insufficient monitoring procedures following reintroduction attempts (Armstrong and Seddon 2008; Drayton and Primack 2012; Godefroid et al. 2011); (iv) the lack of experimental, scientific and integrated approaches (Armstrong and Seddon 2008; Heywood and Iriondo 2003; Seddon et al. 2007); and (v) the insufficient quantity of documentation and peer-reviewed articles that results in a deficit to understand the factors that may lead to successes, or even more importantly, to failures (Drayton and Primack 2012; Godefroid et al. 2011; Godefroid and Vanderborgh 2011; Silcock et al. 2019; Swan et al. 2018). Indeed, reporting these failures is crucial because it leads scientists to think of better methods and strategies for plant conservation, reintroduction and introduction. Some of them are for instance the utilization of ecological niche-based modelling tools (Guisan et al. 2013), the study of biological and ecological factors limiting the establishment of species (Drayton and Primack 2012; Godefroid et al. 2011; Guerrant Jr and Kaye 2007), a better use of comparison with reference populations as well as a better evaluation of sites prior to reintroduction (Drayton and Primack 2012; Menges 2008). Even if the theoretical framework for plant translocations is well known, comprehensive and practical approaches leading them to success are still missing.

Focusing on the selection of suitable sites for plant translocations, Guerrant Jr and Kaye (2007) argued that it is a particularly complex topic. Indeed, the choice of a suitable area requires a highly sophisticated understanding of species ecology and biology (Falk et al. 1996; Maschinski and Haskins 2012). As plants are sessile organisms, survival of seeds and seedlings can vary on a scale of less than 1 m² (Maschinski and Haskins 2012). Fiedler and Laven (1996) proposed four classes of site-selection criteria. The first class, physical criteria, involves both large-scale geomorphic factors and finer-scale parameters such as soil properties. The second one, biological criteria, includes autoecological and synecological parameters such as targeted species natural history, pollination interactions or mycorrhizal symbiosis (Swarts and Dixon 2017). The third and the fourth criteria include logistical and historical factors (Fiedler and Laven 1996; Guerrant Jr and Kaye 2007). Even if these criteria are well-known on paper, integrated and practical approaches including all these aspects to select suitable sites for plant translocation still need to be developed.

Among the plant kingdom, some families are particularly vulnerable and need a special conservation focus such as Orchidaceae, which is potentially one of the largest and threatened one (Fay 2016; Fay et al. 2018). Indeed, the complexity of their ecological interactions with pollinators and mycorrhizal fungi makes them particularly vulnerable to environmental changes and challenges conservationists. In order to protect them despite these difficulties, integrated conservation research has to be conducted to understand how all relevant aspects of their complex ecology intersect. In addition, translating scientific insights about orchids into practical conservation measures is urgently needed (Dirzo et al. 2014; Fay 2016; Fay and Chase 2009; Fay et al. 2018; Fay et al. 2015; Gargiulo et al. 2021a; Swarts and Dixon 2017; Swarts and Dixon 2009). Additional information such as (i) demography and natural history of pollinators, (ii) identification, phenology, demography and biochemistry of sympatric flora, (iii) genetic variability within and among orchid populations, (iv) the number of genotypes that have reached flowering size vs. the number of plants that bloom annually and (v) pollination rates and fruit set vs. the impact of climatic cycles is crucially required to monitor and conserve populations of threatened orchids (Bernhardt and Edens-Meier 2010).

In this study, we aim to develop a comprehensive practical conservation approach for endangered plant species based on fundamental knowledge about the natural history, biology, demography and genetics. For that purpose, we focused on the Lady's slipper orchid *Cypripedium calceolus* L. (Orchidaceae), an iconic species of high relevance. Due to its complex ecological requirements, it is a perfect model species for testing and developing conservation strategies (Gargiulo et al. 2021b; Swarts and Dixon 2009). Furthermore, because of its particular flowers and its exotic look, it is one of the most outstanding plants in Europe (Devillers-Terschuren 1999) (Figure 1).

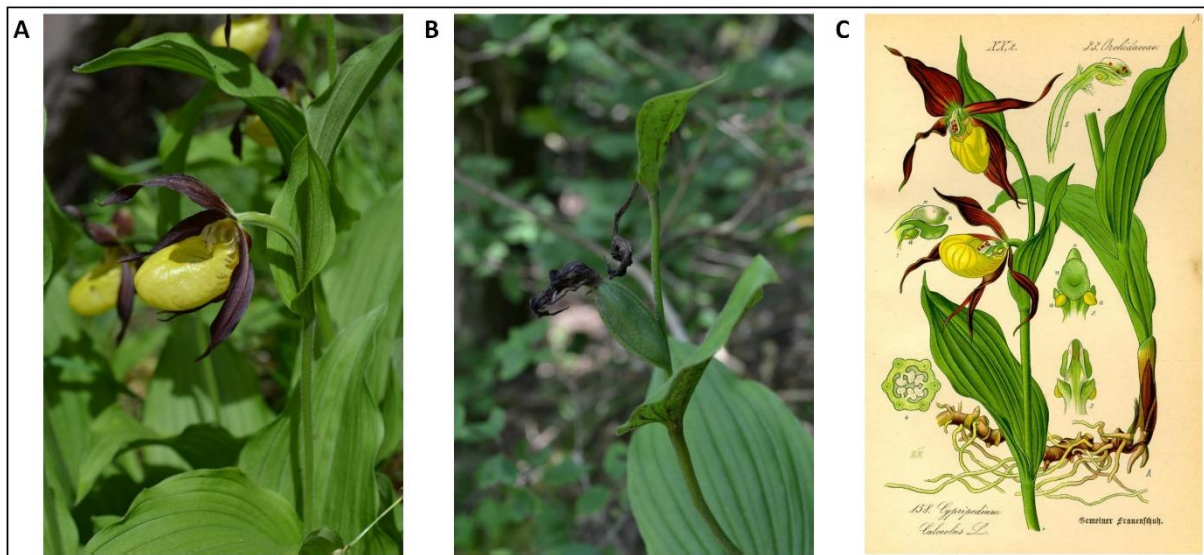


Figure 1: Anatomy of *Cypripedium calceolus*. A. Typical yellow clog-shaped labellum; B. *C. calceolus* fruit and C. (a) *C. calceolus* general anatomy (Source: Thomé 1885).

This Eurasian boreal species is distributed from northern England to eastern China and from northern Finland to the Spanish Pyrenees and the Balkans (Fay and Taylor 2015) (Figure 2).

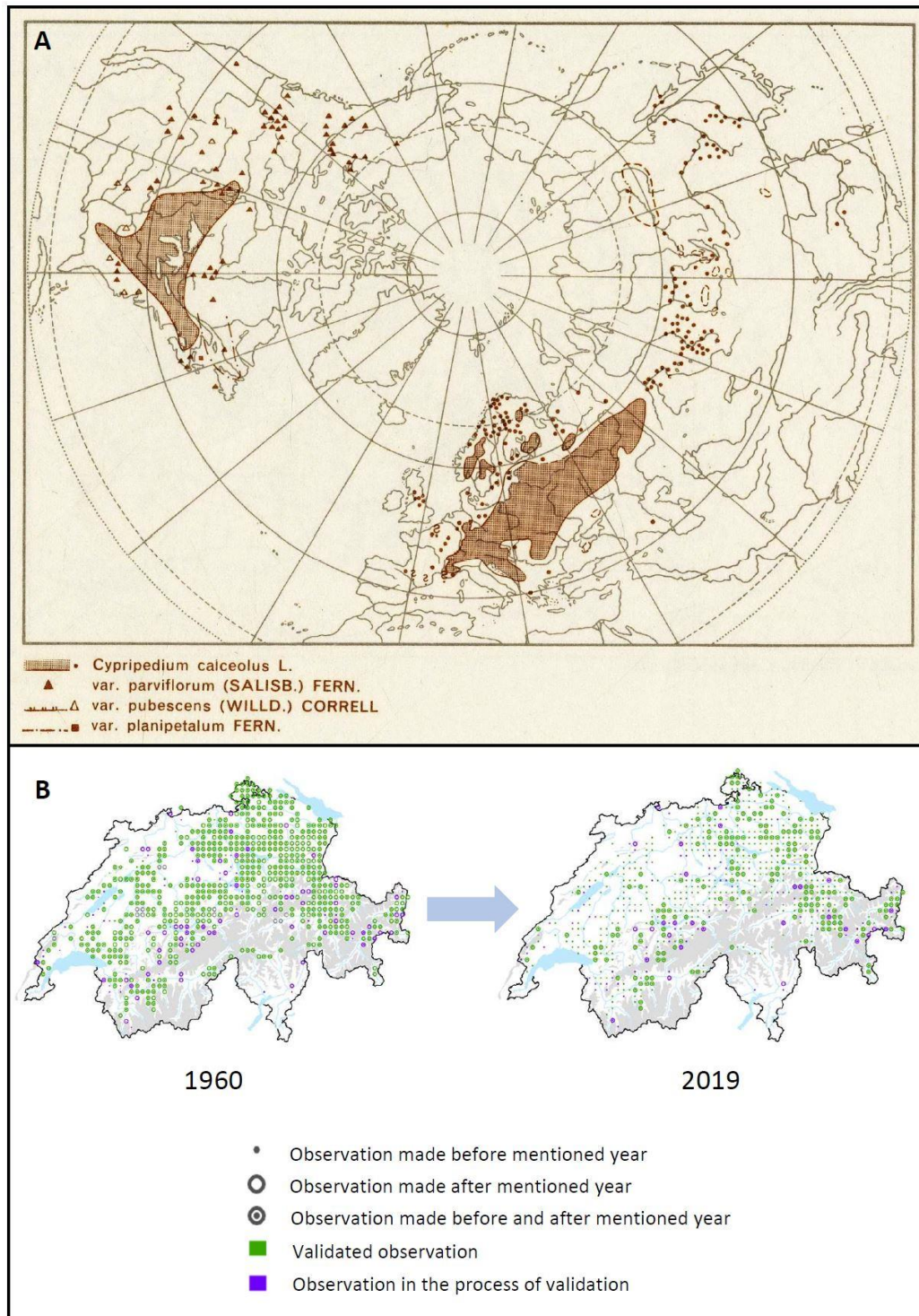


Figure 2: Eurasian distribution of *Cypripedium calceolus*. A. Map showing *C. calceolus* Eurasian distribution (Source: Meusel et al. (1978)), and B. Example of *C. calceolus* population decline in Switzerland. between the years 1960 and 2019. These maps don't show the actual populations of *C. calceolus* for a given year but the observations that were made before and after the focus year (Source: www.infoflora.ch.)

This represents a large range, while actual populations show a scattered distribution (Devillers-Terschuren 1999). The number and size of these populations have drastically and globally declined over the last decades making it a threatened plant species (Devillers-Terschuren 1999; Jakubska-Busse et al. 2021; Kolanowska and Jakubska-Busse 2020; Kull 1999) (Figure 2). Its population decrease is mainly attributed to three factors: (i) loss of habitats, (ii) narrow and specific ecological requirements and (iii) survival and reproduction capacities (e.g.: Devillers-Terschuren 1999; Hurskainen et al. 2017; Käsermann and Moser 1999; Minasiewicz et al. 2018; Ramsay and Stewart 1998; Wood et al. 1984) (Table 1).

Table 1: Identified decline causes of *Cypripedium calceolus* complied from Devillers-Terschuren 1999; Hurskainen et al. 2017; Käsermann and Moser 1999; Ramsay and Stewart 1998 and Wood et al. 1984.

Decline causes	
Habitat loss	Forest canopy closure, shadow, bushland expansion
	Forest roads construction
	Pastures in forest
	Intensive forestry
	Change in water regime and lowering of groundwater table
	Trampling
	Eutrophication
Narrow and specific ecological requirements	Lack of knowledge about ecological requirements
	Lack of knowledge about population ecology
Survival and reproduction capacities	Picking
	Digging up of rhizomes
	Small and scattered populations
	Grazing
	Lack of pollinator insects
	Weak rate of pollination success
	Weak recruitment rate

In term of conservation status *C. calceolus* has different statuses across its distribution; as Least Concern (LC) at the global scale, Vulnerable (VU) in Switzerland, and Critically Endangered (CR) in the United Kingdom (Bornand et al. 2016; Gargiulo et al. 2018; Rankou and Bilz 2014; Stroh et al. 2014). Due to its fast decline, this emblematic species has been the target of several conservation plans, with varying degrees of success (e.g.: Devillers-Terschuren 1999; Fay et al. 2018; Ramsay and Stewart 1998). Hence, numerous studies have focused on its complex biology (e.g.: Antonelli et al. 2009; Barsberg et al. 2013; Blinova 2002; Braunschmid et al. 2021; Hurskainen et al. 2017). Even though knowledge about

C. calceolus is constantly increasing, several fundamental and applied questions about its conservation remain open (Gargiulo et al. 2021a)(Figure 3) and some facets of its complex ecological requirements still need to be understood (Gargiulo et al. 2021b; Swarts and Dixon 2009).

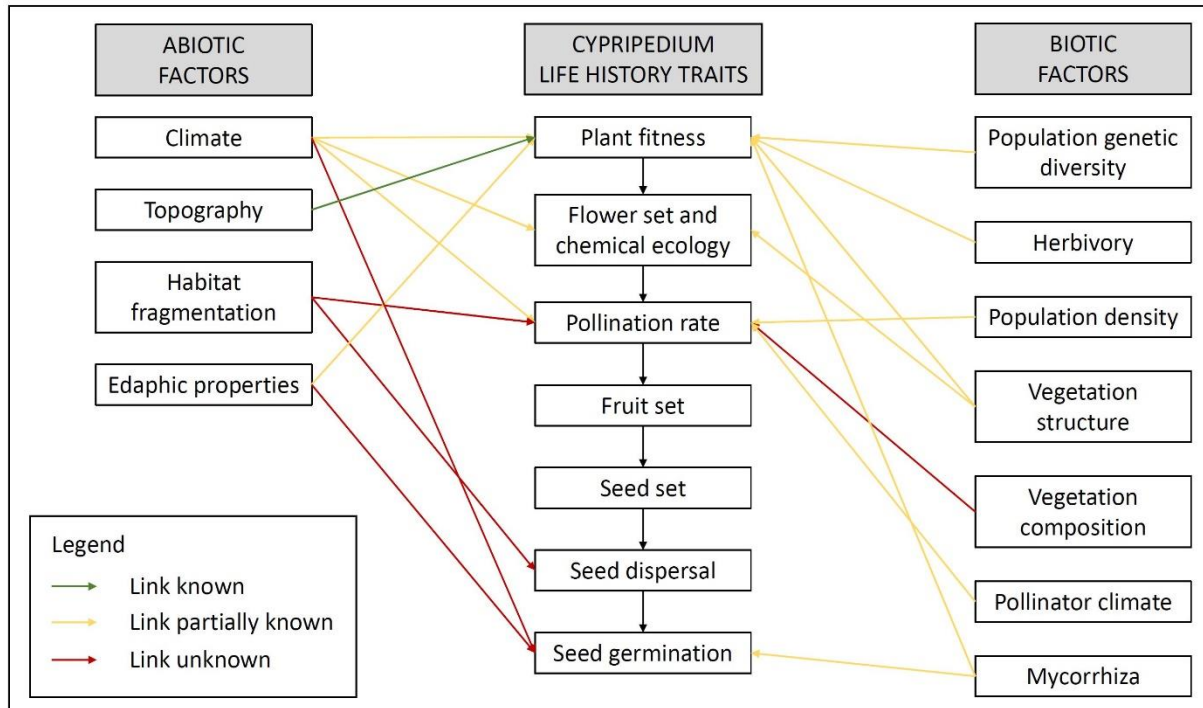


Figure 3: Links between *Cypripedium calceolus* life history traits and biotic and abiotic factors. Green, yellow and red arrows highlight state of knowledge according to the current understanding.

Based on natural history observations, the ecological requirements of *C. calceolus*' habitat are principally defined by three factors: (i) light, (ii) soil moisture and (iii) soil bases richness; and practitioners have generally postulated that suitable sites for the growth of this species consists on a restricted combination of these factors (Devillers-Terschuren 1999). Particularly, due to its high sensitivity to light conditions, this plant suffers from competition with a dense bush layer or with taller plants in terms of survival, growth and reproduction (Hurskainen et al. 2017; Kirillova and Kirillov 2019). On the other hand, studies showed that with more sunlight, plants exhibited shorter leaves and ramets (Kull 1999). Therefore, *C. calceolus* has been thought to prefer shady areas without direct concurrence for light but its precise light requirements are still unknown (Devillers-Terschuren 1999; Kirillova and Kirillov 2019). *Cypripedium calceolus* is mainly found in shady deciduous and mixed woodland and sometimes in stone-strew slopes (Foremnik et al. 2021; Kull 1999). At higher elevation, it can grow in full sunlight (Devillers-Terschuren 1999; Kull 1999). That said, in Switzerland, it is identified in 14 different vegetation alliances with an optimum in *Cephalanthero-Fagenion* (xerothermophilous beech forest) and *Erico-Pinion* (basophilic subcontinental pine forest) (Delarze et al. 2015; Käsermann and Moser 1999). Regarding edaphic requirements, *C. calceolus* appears to grow better in soils containing calcium carbonates and preferentially should be rich in bases which usually occur over limestone or dolomite bedrock (Käsermann and Moser 1999; Kull 1999). According to Käsermann and Moser (1999), soil pH requirements range from neutral to moderately acidic, while

texture needs to contain fine particles such as clay or silt. It is interesting to notice that this plant will grow on richer substrates when in shade, as compared to sunnier conditions, likely due to the competition effect with other plant species (Käsermann and Moser 1999). Concerning soil moisture, according to Devillers-Terschuren (1999), *C. calceolus* grows on moderately moist soils that are quite dry during summer. To conclude with pedological information, this orchid usually grows on Moder humus form (Rusconi 2017). Despite decades of research on the habitat preferences of *C. calceolus*, important gaps remain making its conservation a path full of pitfalls.

Regarding the ecology of *C. calceolus*, this orchid has a very slow development and a long life-expectancy, potentially living more than 30 years (Kull 1999). The first green leaves appear 1 to 4 years after germination and flowering occurs 6 to 16 years after germination (Fast 1985; Kull 1988; Kull and Tuulik 1994; Rasmussen 1995). *Cypripedium calceolus* is either able to reproduce vegetatively or sexually and vegetative reproduction occurs through the production of tufts of clones from rhizome ramification (Kull 1988). A study demonstrates that clones patches considered as an individual are rarely larger than 70 cm in diameter (Kull 1997, 1999). Regarding sexual reproduction, *C. calceolus* is most frequently pollinated by short-tongued solitary bees belonging to the genera *Andrena*, *Lasioglossum* and *Halictus* (e.g.: Antonelli et al. 2009; Braunschmid et al. 2021; Braunschmid et al. 2017; Nilsson 1979; Westrich 2019). Insects enter the front opening of the labellum, fall in it and cannot leave by the same way (Braunschmid et al. 2017). They have to cross the labellum, allowing them to touch the stigma and then the anthers before finding the exit (Braunschmid et al. 2017). This plant does not produce nectar and its pollination system is therefore deceptive and as often with such pollination systems, pollination success and fructification rates are weak and vary among populations (Devillers-Terschuren 1999). Despite its deceptive pollination system, *C. calceolus* attract pollinators through its yellow colour and volatile organic compounds (Nilsson 1979). While the scents emitted by *C. calceolus* to attract pollinators have been studied for over 40 years, the mechanisms of scent communication are still not fully understood (Przybyłowicz et al. 2012). *Cypripedium calceolus* seeds develop during sixteen to twenty weeks and then, capsules separate during wet weather and dust-like seeds are dispersed by the wind (Figure 1) (Bockel 1972; Kull 1998). No precise data about germination success seems to exist. Kull and Tuulik (1994) and Kull (1998) observed a range from 1 to 10 seedlings out of one seed emission (5940 to 16700 seeds) (Kull 1997). During first growing seasons, germination and growth of *C. calceolus* seems to be tightly dependent on orchidaceous mycorrhizal fungi (Kull 1999) (Kull 1999), particularly of the genus *Tulasnella* or *Rhizoctonia* (Fay et al. 2018; Oja et al. 2015; Shefferson et al. 2007). In addition, Fay et al. (2018) demonstrated through stable isotopes analyses that *C. calceolus* has also mycorrhizal relationships during its adult phase. However, a great gap remains in fundamental knowledge about symbiotic interactions with mycorrhizal fungi. Population genetic studies performed in Europe showed that this species has a relatively high genetic diversity compared with rare taxa and taxa with the same life history (e.g.: Brzosko et al. 2002; Fay et al. 2009; Minasiewicz et al. 2018). On the other hand, signatures of a bottleneck effect and recent founder events were identified in Estonia, and genetic and genotypic diversity variables were significantly correlated with population size in Poland (Brzosko et al. 2011; Gargiulo et al. 2018). A better knowledge

and understanding of the biotic interactions and population genetics of *C. calceolus* would be crucial for its conservation.

To reach our general target to develop efficient comprehensive conservation strategies for endangered plants using *C. calceolus* as a model species, the aims of this PhD thesis are to characterize (i) its climatic and topographic niche thanks to spatial modelling techniques (**Chapter 1**) and (ii) its habitat requirements (soil and vegetation type and structure) through observational and experimental approaches (**Chapter 2**). In addition, we aimed to understand better its (iii) pollination biology and underlying chemical ecology (**Chapter 3**), (iv) population genetics and demography (**Chapter 4** – presented as Perspective Chapter), (v) relationship with mycorrhiza (**Chapter 5** – presented as Perspective Chapter) (Figure 4).

To address our aims, we randomly sampled 34 populations of *C. calceolus* across Switzerland in eight cantons: Aargau, Basel-Landschaft, Bern, Graubünden, Neuchâtel, Valais, Vaud and Ticino in 2018, 2019 and 2020 (Figure 5 and Table 2). Occurrences were provided by Info Flora, the cantonal administrations and naturalists. These 34 populations cover a 1500 m elevation gradient from 400 m to 1900 m. Selection criteria included site accessibility, possibility of obtaining a visitation permit by the cantonal offices and population size. For this latter criterion, we categorized populations as being small (i.e., less than 10 inventoried individuals per population), or large (i.e., more than 20 individuals per population). In each population, data for each of the different chapters were collected. Among other data, we collected population plant functional traits on *C. calceolus* directly related to population fitness and reproduction (Adler et al. 2014; Díaz et al. 2016). These functional traits data were transversally used in each chapter of this thesis. We visited each site twice: once during the flowering period of *C. calceolus* (between April and July depending on the elevation and the weather of the year) and once during the fruiting period (between August and September).

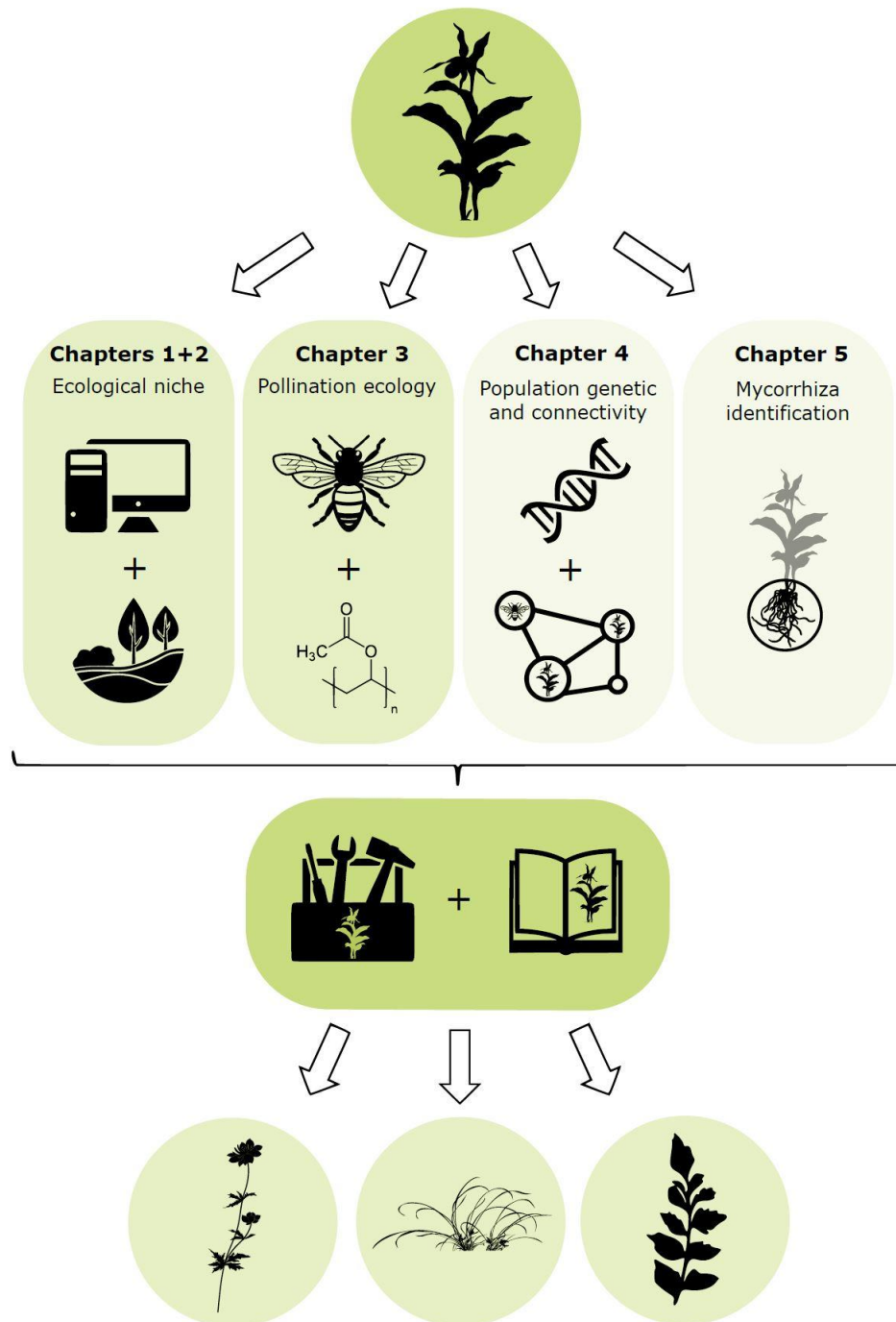


Figure 4: Aims and main axes of this thesis. Focusing on *Cypripedium calceolus*, the aim of this thesis is to (i) describe the climatic and topographic niche thanks to spatial modelling technique (**Chapter 1**), (ii) characterize its edaphic niche and the vegetation surrounding its populations (**Chapter 2**), (iii) understand better its pollination system and the underlying chemical mechanism (**Chapter 3**), (iv) study its population genetics and connectivity (**Chapter 4** – presented as Perspective Chapter I) and (v) identify its mycorrhizal symbiont (**Chapter 5** – presented as Perspective Chapter II). Thanks to these findings, the next step is to convert fundamental knowledge into practical conservation action. Finally, the tools developed in this study can be adapted for other plant species.

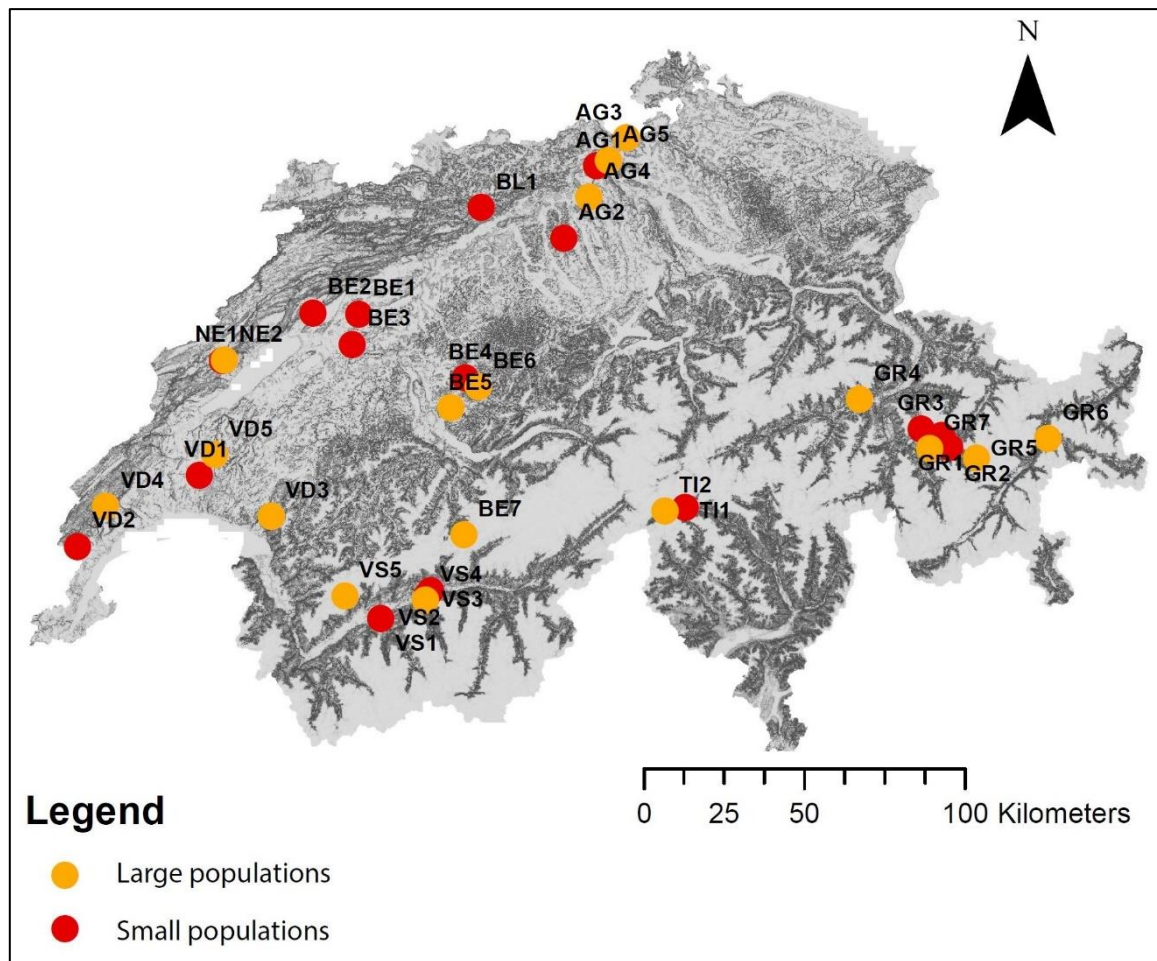


Figure 5: The 34 populations of *Cypripedium calceolus* visited in the scope of this PhD thesis between 2018 and 2021. We categorized populations as being small (i.e., less than 10 inventoried individuals per population, red dots), or large (i.e., more than 20 individuals per population, orange dots). The complete name of each population is given in Table 2. On the background map, shades of grey indicate the habitat suitability for *C. calceolus* based on a climatic niche model with light grey representing low suitability and dark grey high suitability (Chapter 1).

Table 2: The 34 Swiss *Cypripedium calceolus* populations visited in the scope of the PhD. Site names are abbreviations of the canton where the population stands and the site number (Aargau: AG, Bern: BE, BL: Basel-Landschaft, GR: Graubünden, NE: Neuchâtel, VD: Vaud, VS: Valais and TI: Ticino). Populations were categorized as being small (i.e., less than 10 inventoried individuals per population), or large (i.e., more than 20 individuals per population).

Site name	Canton	Municipality	Small or large population
AG1	Aargau	Turgi	Small
AG2	Aargau	Gontenschwil	Small
AG3	Aargau	Rümikon	Large
AG4	Aargau	Dottikon	Large
AG5	Aargau	Freienwil	Large
BE1	Bern	Kappelen	Small
BE2	Bern	La Neuveville	Small
BE3	Bern	Radelfingen	Small
BE4	Bern	Bowil	Small
BE5	Bern	Fahrni	Large
BE6	Bern	Röthenbach	Large
BE7	Bern	Kandersteg	Large
BL1	Basel-Landschaft	Langenbruck	Small
GR1	Graubünden	Albula/Alvra	Small
GR2	Graubünden	Bergün Filisur	Small
GR3	Graubünden	Vaz/Obervaz	Small
GR4	Graubünden	Trin	Large
GR5	Graubünden	Bergün Filisur	Large
GR6	Graubünden	Zernez	Large
GR7	Graubünden	Albula/Alvra	Large
NE1	Neuchâtel	La Grande Béroche	Small
NE2	Neuchâtel	La Grande Béroche	Large
VD1	Vaud	Bretigny-sur-Morrens	Small
VD2	Vaud	Chésérèx	Small
VD3	Vaud	Saint-Légier-La Chiésaz	Large
VD4	Vaud	Saint-George	Large
VD5	Vaud	Fey	Large
VS1	Valais	Sion	Small
VS2	Valais	Salgesch	Small
VS3	Valais	Sierre	Small
VS4	Valais	Anniviers	Large
VS5	Valais	Conthey	Large
TI1	Ticino	Airolo	Small
TI2	Ticino	Bedretto	Large

In the **first chapter**, we proposed a two-step approach to identify (re)introductions sites for endangered plant species using *C. calceolus* as model species. The first step involved modelling its niche and its distribution with bioclimatic and topographical predictors. The second step consisted of refining these bioclimatic predictions by analysing stationary ecological parameters, such as edaphic parameters, and relating them to populations-level fitness values. Moreover, we compared edaphic properties of natural sites of *C. calceolus* and (re) introduction sites to investigate the potential edaphic mechanisms behind (re)introduction failures. Our approach was based on the hypothesis that successful (re)introductions depend on refining bioclimatic model predictions with indepth knowledge of which ecological parameters correlate most strongly with the highest population fitness. This chapter thus aims to increase the probability of success for conservation plans of endangered plant species that require finding suitable habitats for replenishing or creating novel viable populations.

In the **second chapter**, we aimed to determine the relationships between the performance of *C. calceolus* populations and soil and vegetation factors, in order to improve this species conservation and also to advance the theoretical underpinning of which facets of an ecosystem most influence its fitness. To this end, we measured functional traits related to plant and population performance, realized vegetation surveys, and soil profiles analyses, and tested for relationships between plant traits and the surrounding vegetation structure or soil physicochemical parameters. Specifically, we asked: 1) which plant functional traits best describe differences between small and large populations? 2) Is there a specific or recurrent pattern in vegetation composition or/and structure in relation to *C. calceolus* presence and population size? 3) Are there combinations of companion plant species that specifically dictate *C. calceolus* population size? 4) Is the population size of *C. calceolus* dependent on local edaphic properties? We hypothesized that while this plant species has broad ecological preferences, some facets of the vegetation or soil physicochemical properties can be used to explain why some populations perform better than others (Devillers-Terschuren 1999; Kłaviņa and Osvalde 2017; Kull 1999; Onuch and Skwaryło-Bednarz 2014), and therefore, plant species performance, estimated using specific plant functional traits, could be linked to unique combinations of vegetation and soil properties, which ultimately would be used to inform more efficient conservation efforts.

In the **third chapter**, we aimed to understand potential variation at the population level of how plants attract their pollinators and subsequently how this variation relates to the overall reproductive effort of *C. calceolus* individuals. First, we performed vegetation inventories and insect diversity surveys, both for those directly visiting the orchids' flowers and for those found within the surrounding vegetation to assess ecosystem-level biodiversity-*C. calceolus* population fitness relationship. We measured population-level functional plant traits related to growth and reproduction. We sampled flower volatile organic compounds (VOCs) on multiple individuals across all populations. To understand potential flower odour mimicry, we also sampled VOCs on the most common yellow melliferous flower, *Hieracium murorum* aggr. (Asteraceae), which is a principal nectar source for solitary bees in woodlands (Westrich 2019). We specifically asked if population level diversity of pollinators, flower scents, and the number of fruits produced are correlated to each other, to plant functional traits, or to the size of each population of *C. calceolus*. We hypothesized a direct link between the diversity of

pollinators and *C. calceolus* population size and reproduction, that flowers VOCs vary across populations, that there is a link between VOCs blend and reproductive success, and that the most representative VOC blends mimics the VOCs blend of the co-flowering yellow melliferous species (*H. murorum*). We specifically predicted that (i) bigger and fitter *C. calceolus* populations grow in more diverse vegetation types, and that they have higher pollination rates because they are visited by a wider diversity of pollinators than smaller populations; and (ii) that flowers in larger populations produce more VOCs to attract more pollinators, also because they mimic odours from the surrounding co-flowering vegetation. We propose that the identification and characterization of the pollinator community of *C. calceolus*, as well as a better understanding of mechanisms driving pollination success should lead to more precise and accurate conservation actions and management measures for this species.

In the **fourth** and the **fifth chapters**, we focused respectively on *C. calceolus* population genetic and the mycorrhizal symbiosis of this orchid. In the fourth one, we aim to study the population genetics of *C. calceolus* in Switzerland, to compare the fitness of plants with their genetic diversity and to assess the effect of habitat fragmentation and connectivity of the landscape on genetic differentiation of the species. For that purpose, we sampled *C. calceolus* leaves on twenty individuals per population and we genotyped all individuals at 17 microsatellite loci. We specifically predicted that (i) Swiss metapopulations of *C. calceolus* are no more genetically connected; (ii) smaller populations of *C. calceolus* have a smaller genetic pool and (iii) *C. calceolus* populations with a reduced genetic pool present functional traits translating lower population fitness. In the fifth chapter we asked whether *C. calceolus* plants are associated with one or more species of OM throughout their life cycle. We hypothesized that *C. calceolus*, as most orchids, rely on a suite of OM to successfully germinate, grow, and reproduce and that the narrow ecological requirements of *C. calceolus* are determined by the ecological requirements of its symbiotic fungi. We sampled soil in *C. calceolus* populations and roots on three individuals per population. From soil and roots, first, we extracted and amplified DNA with a general primer for fungi ITS86F/ITS4 and with a primer designed for Tulasnellaceae 5.8S-Tulngs/ITS4-Tul2 and secondly, sequenced it with Illumina method. All data for the fourth and the fifth chapters were collected and analysed in the lab (genotyping, Illumina sequencing, etc.). The final statistical analysis and the redaction of the manuscripts are still in progress and are presented as Perspective Chapters I and II in this PhD report.

This PhD project originated from a collaboration between the University of Neuchâtel (Laboratory of functional Ecology), the botanical garden of Neuchâtel and the Canton of Neuchâtel in 2016. In this canton, numerous actions and reintroduction plans to conserve this orchid were developed, without reaching the expected outcomes (Storrier 2016). In 2016, a new reintroduction project lead by a private foundation was planned in the north-west of Switzerland including the cantons of Neuchâtel, Bern, Basel-Landschaft, Aargau, Zurich and the Parc Chasseral which is a natural park located at the border between the cantons of Neuchâtel and Bern (De Jong and Springer 2020). The canton de Neuchâtel, the Parc Chasseral and the University of Neuchâtel worked together to identify appropriate sites for *C. calceolus* for the above-mentioned reintroductions as a part of a master thesis in 2016 and 2017

(Rusconi 2017). At the end of 2017, the Swiss Academies of Art and Sciences (SCNAT) contacted the Laboratory of Functional Ecology through the Swiss Biodiversity Forum letting them know that practitioners from different cantons were interested in more fundamental research on *C. calceolus* in order to protect this species more efficiently in the field. As a result, the Laboratory of functional Ecology decided in January 2018 to start this PhD thesis focusing on fundamental research on *C. calceolus* ecology to develop practical tools to protect it. At the request of the Forum Biodiversity Swiss, several people and organizations responded that they were interested in joining this project: the cantons of Neuchâtel, Aargau and Bern, the Swiss Federal Institute for Forest, Snow and Landscape research (WSL), the National Data and Information Centre on the Swiss Flora (Info Flora), the Botanical Garden of Neuchâtel and the Parc Chasseral. In the early months of this PhD, a meeting with all actors of this project was organized by the Swiss biodiversity Forum and the Laboratory of functional Ecology. The aim of this meeting was to understand the needs of the practitioners and to identify the grey areas of the ecology of *C. calceolus* to draw the main lines of this project which became the five chapters of this PhD.

Parallely to the *C. calceolus* project, we present in the appendices of this PhD report a side project focusing on the distribution of the testate amoeba *Apodera vas*, a morphologically conspicuous and model soil microbe in microbial biogeography. The aim of this study was to predict the present, past (Last Glacial Maximum - LGM) and future (2061-80) potential distribution of *A. vas*. We especially aimed to assess if the well-documented distribution of *A. vas* across the Southern Hemisphere and inter-tropical zone could be explained by its climatic niche rather than palaeogeography and its vulnerability in the face of ongoing climate change due to potential dispersal limitation. We validated 391 georeferenced records for *A. vas* and developed a climatic niche model to predict its present, past, and future potential distributions in responses to climate by relating to climatic and topographic predictors. The modelling techniques used in this project were the same used for *C. calceolus* in the first chapter but at another scale. In both projects, spatial modelling was used for conservation purpose.

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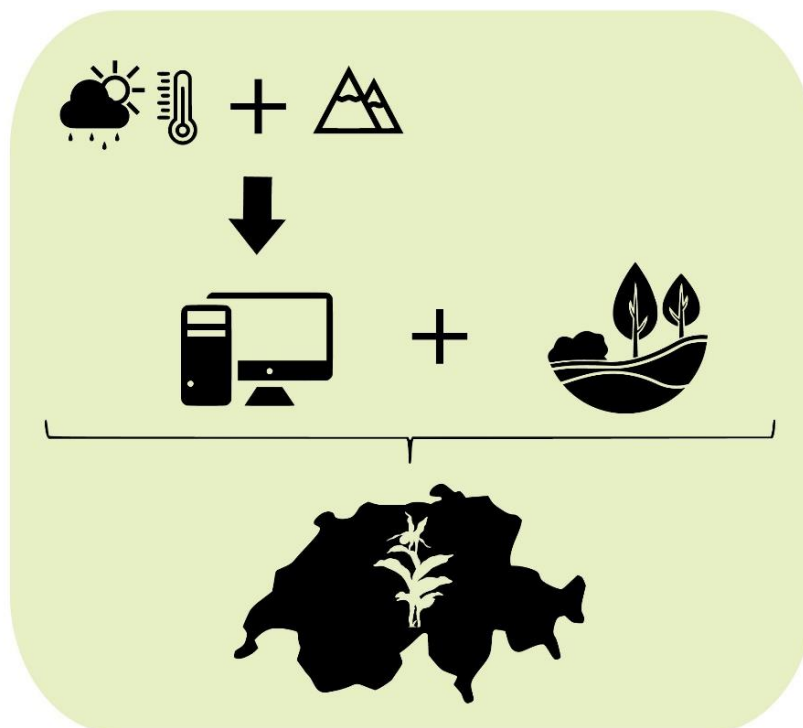
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CHAPTER I: DETECTING PRESERVATION AND REINTRODUCTION SITES FOR ENDANGERED PLANT SPECIES USING A TWO-STEP MODELLING AND FIELD APPROACH



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AUTHOR CONTRIBUTIONS

OR and SR developed the ideas and the sampling design. OR, YS, and RCLB collected the data. OB and AG provided the data and know-how for spatial modelling. OR, OB, and SR analysed the data. OR and SR wrote the first draft of the manuscript, and all co-authors contributed to editing.

Abstract

To withstand the surge of species loss worldwide, (re)introduction of endangered plant species has become an increasingly common approach in conservation biology. Successful (re)introduction plans, however, require identifying sites that provide the optimal ecological conditions for the target species to thrive. In this study, we propose a two-step approach to identify appropriate (re)introduction sites. The first step involves modelling the niche and distribution of the species with bioclimatic and topographical predictors, both at continental and at national scales. The second step consists of refining these bioclimatic predictions by analysing stationary ecological factors, such as soil conditions, and relating them to population-level fitness values. We tested this methodology using Swiss populations of the lady's slipper orchid (*Cypripedium calceolus* L., Orchidaceae), for which conservation plans have existed for years but have generally been unfruitful. Our workflow identified sites for future (re)introductions based on the species requirements for mid-to-sunny light conditions and specific soil physicochemical properties, such as basic to neutral pH and low soil organic matter content. Our findings show that by combining wide-scale bioclimatic modelling with fine scale field measurements it is possible to carefully identify the ecological requirements of a target species for successful (re)introductions.

Keywords: Endangered plants, ecological niche, ensemble forecasting, population fitness, red list, restoration, soil properties

1. Introduction

Earth is experiencing the sixth mass extinction of living species (Barnosky et al. 2011; Ceballos et al. 2017; Cordier et al. 2021; IUCN/SSC 2008; Primack 2014). To fight against species disappearance, the field of conservation biology has developed during the last 40 years and has guided the creation of a substantial number of nature reserves and conservation sites, where species can be maintained or reintroduced (Primack 2014). Nonetheless, many conservation plans are failing and species disappearance is continuing (Gaston and Fuller 2008; Pimm et al. 2014), suggesting that habitat protection alone is not enough to fully protect all species (Swarts and Dixon 2009). Indeed, because of habitat loss, fragmentation and change, the restoration of viable populations can rarely be achieved by unaided natural recruitment and dispersal (Seddon 2010). As a consequence, assisted migration to new sites has been proposed in order to overcome barriers to dispersal and recruitment (Menges 2008; Seddon 2010; Swarts and Dixon 2009).

Conservation translocation is defined as the deliberate movement of living organisms from one area to another and consists of either reinforcement plus reintroduction or introduction plans (IUCN/SSC 2013). Reinforcement and reintroduction plans entail the translocation of new individuals inside a species indigenous range, while introduction involves moving a species to areas where it is currently not observed (Bottin et al. 2007; IUCN/SSC 2013). While many translocation attempts have been successful (Bottin et al. 2007; Maschinski et al. 2004; Maschinski and Duquesnel 2007; Ramsay and Stewart 1998), they also commonly do not reach the expected results (Bottin et al. 2007; Drayton and Primack 2012; Heywood and Iriondo 2003). For instance, according to Godefroid et al. (2011), who analysed 249 plant species reintroduction plans worldwide, survival, flowering, and fruiting rates are often low following reintroduction, on average 52%, 19%, and 16%, respectively. The reasons that led to these failures include: i) a lack of understanding of primary ecological and biological grounds that caused the decline in population sizes in the first place (Godefroid and Vanderborght 2011; Heywood and Iriondo 2003); ii) a deficit of biological and ecological understanding of the species under scrutiny (Bottin et al. 2007; Guerrant Jr and Kaye 2007; Menges 2008); iii) a lack of experimental, scientific and integrated approaches (Seddon et al. 2007); and iv) an insufficient quantitative overview of the parameters that lead to failures (Drayton and Primack 2012; Godefroid et al. 2011).

Within the framework of plant species translocation efforts, the selection of suitable sites is a particularly complex endeavour. As plants are sessile organisms, survival of seeds, and seedlings can vary on a scale of $<1 \text{ m}^2$ (Falk et al. 1996; Guerrant Jr and Kaye 2007; Maschinski and Haskins 2012). Accordingly, while in the context of current and future changes in the landscape and climate it is important to select habitats for (re)introduction efforts by modelling current and future bioclimatic envelopes and their geographic projection (Guisan et al. 2006; Maschinski and Haskins 2012), the choice of a suitable area for (re)introduction requires a highly sophisticated understanding of the species ecology and biology (Falk et al. 1996; Maschinski and Haskins 2012). Within this framework, Fiedler and Laven (1996) proposed four classes of site-selection criteria for the (re)introduction of rare plants: 1) physical criteria (including both large-scale geomorphic factors and finer-scale parameters,

such as soil types); 2) biological criteria (including autoecological and synecological parameters); 3) logistic criteria (including accessibility, and feasibility); and 4) historical development of the site (Fiedler and Laven 1996; Guerrant Jr and Kaye 2007).

The selection of translocation areas has therefore taken two complementary routes. The first route merges physical and biological selection criteria through the use of a spatially implicit habitat suitability modelling approaches (Guisan et al. 2013; Pecchi et al. 2019; Prasad et al. 2016). For instance, Brown et al. (2008) developed a multi-scale analysis for the management of the tree *Syzygium jambos* that included both a landscape- and a population-scale model of the tree distribution. A second route advocates for the use of a finer-scale multidimensional approach for building precise ecological requirements for the target species (Prasad et al. 2016; Richardson et al. 2009). For instance, Vittoz et al. (2006) performed an integrated study of multiple ecological factors, including soil physicochemical properties, hydrology, microtopography, and vegetation community analysis, for identifying the optimal niche of the rare species *Saxifraga hirculus*. Edaphic and environmental factors have also been used to evaluate the ecology of the endangered plant species *Primulina tabacum* (Ren et al. 2010). We here sustain that a more valuable approach would be to combine the two routes and select suitable translocation sites for plants by combining predictions or habitat suitability models with stationary analysis of ecological factors that cannot be retrieved from standard/available raster layers (Seddon et al. 2007). While this has been done to some extent for animals (Borgo 2003; Cook et al. 2010), to our knowledge this has rarely been attempted for plants.

In this study, we aimed to further exemplify such a multi-step approach that allows selecting the most appropriate (re)introduction sites (Figure 1). We specifically analysed naturally growing and (re)introduced Swiss populations of the orchid species *Cypripedium calceolus* L. (Orchidaceae). In term of conservation status *Cypripedium calceolus* is characterized with different statuses across its distribution; as Least Concern (LC) at global scale, but as Vulnerable (VU) in Switzerland and as Critically Endangered (CR) in the United Kingdom (Bornand et al. 2016; Gargiulo et al. 2018; Rankou and Bilz 2014; Stroh et al. 2014). Its complex ecological requirements made it a perfect model species for testing and developing conservation strategies (Gargiulo et al. 2021b; Swarts and Dixon 2009). While having a Eurasian distribution (Figure S1a), this species is disappearing rapidly in numerous countries, including in Switzerland (Le Lay et al. 2010). For instance, while in the 1960s, when occurrence data started to be consistently gathered, this species was observed over an area of approximately 400 km², in 2019 the area covered by the populations of *C. calceolus* in Switzerland shrunk by almost 50% (Source: National Data and Information Centre on the Swiss Flora, www.infoflora.ch, Figure S1b). Because of such a fast decline, this emblematic species has been the target of several conservation plans, with varying degrees of success (e.g., Devillers-Terschuren 1999; Fay et al. 2018; Ramsay and Stewart 1998). Its spatial distribution in Switzerland was also modelled based on various environmental maps with the aim to support the detection of new (unsurveyed) populations, but the attempt was unsuccessful (Le Lay et al. 2010).

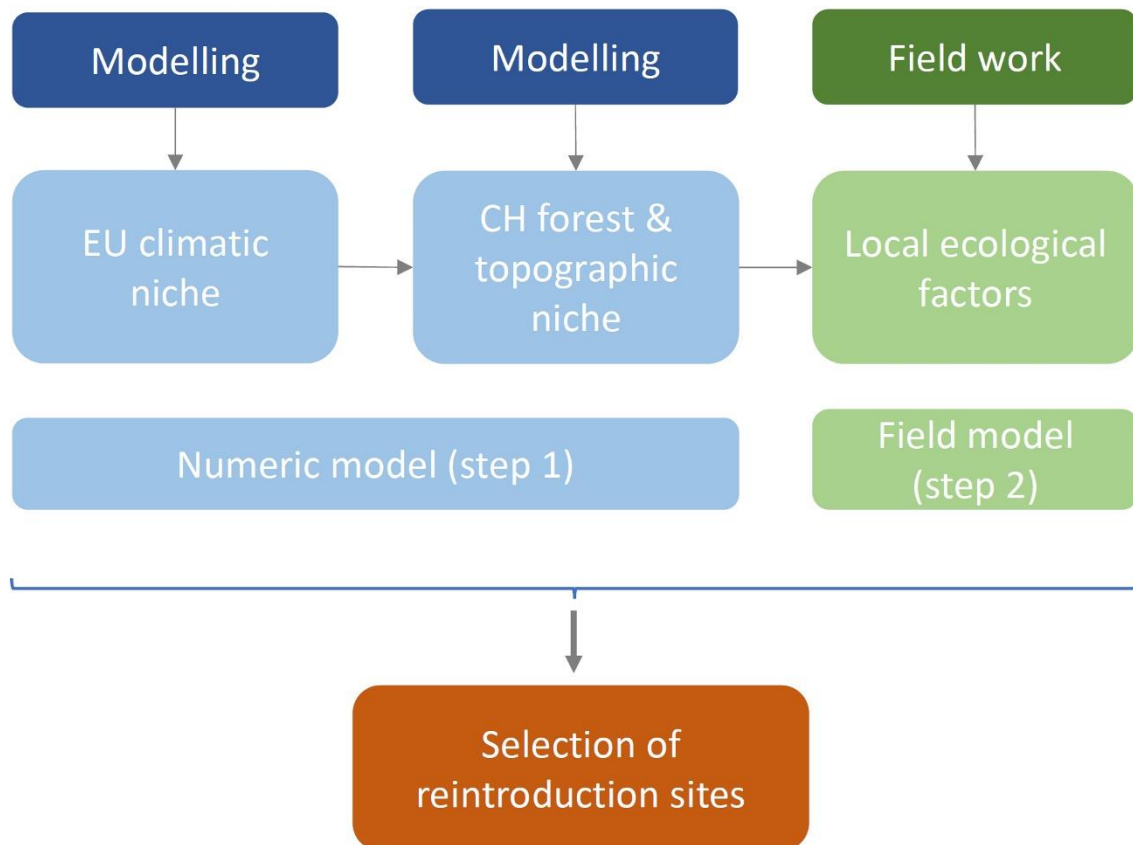


Figure 1. Hierarchical workflow for selecting appropriate reintroduction and introduction sites for endangered plant species. The workflow starts by modelling the potential occurrence of target species at the continental scale (numeric model, step 1, EU scale for *Cypripedium calceolus*). Following this, the potential distribution model of the target species can be refined at the national scale using additional layers (e.g., forest edges and topographic factors; numeric model, step 1, national scale). The continental bioclimatic niche is then integrated into the national niche modelling. The modelling step is followed by field observations (field model, step 2), which allows refining the niche of the target species by including additional fine-scale ecological factors that are not available in spatially explicit form, such as soil variables. The merging of step 1 and step 2 should yield a more refined selection of suitable (re)introduction sites for conservation plans.

Based on natural history observations, the ecological requirements of *C. calceolus* appear to be centred around the three factors of light, soil moisture and soil bases richness, and practitioners have generally postulated that suitable sites for the growth of this species consist on a limited combination of these parameters range (Devillers-Terschuren 1999). For instance, *C. calceolus* tends to be mainly found in shady deciduous mixed woodland and sometimes on stone-strewn slopes (Foremnik et al. 2021; Kull 1999). Regarding edaphic requirements, *C. calceolus* seems to be predominantly associated with basic soils containing calcium carbonates (Kull 1999) and with moderate soil moisture (Devillers-Terschuren 1999). Nonetheless, field observations show that the species ecological requirements can be broader. For instance, soil pH requirements for *C. calceolus* have been shown to range from neutral to moderately acidic (Käsermann and Moser 1999) and, in Switzerland, the species can be found in 14

different habitat types, with a preference for *Cephalanthero-Fagenion* (xerothermophilous beech forest) and *Erico-Pinion* (basophilic subcontinental pine forest) (Delarze et al. 2015; Käsermann and Moser 1999). Therefore, at first sight, the specific biotic and abiotic requirements for this species tend to be broad and widespread. Accordingly, because field observations highlight strong variation in *C. calceolus* populations size and health condition, we can hypothesize that the performance of the species across habitats vary depending on the local ecological conditions.

To address our aim, we first developed a spatial distribution model that included both current and future climatic scenarios. Second, we performed a field-based sampling approach to correlate stationary edaphic parameters with population fitness variables. Moreover, we compared edaphic properties of natural sites of *C. calceolus* and (re)introduction sites to investigate the potential edaphic mechanisms behind (re)introduction failures. Our approach was based on the hypothesis that successful (re)introductions depend on refining bioclimatic model predictions with in-depth knowledge of which ecological parameters correlate most strongly with the highest population fitness. Our work thus aims to increase the probability of success for conservation plans of endangered plant species that require finding suitable habitats for replenishing or creating novel viable populations.

2. Material and methods

2.1. Step 1: spatial modelling of *C. calceolus* populations at two geographical scales

We developed a hierarchical spatial distribution model based on two scales (Eurasian and Swiss occurrences) to avoid biases related to niche truncation (Chevalier et al. 2021; Mateo et al. 2019).

2.1.1. Eurasian-scale models

To estimate the current and future bioclimatic niche of *C. calceolus* specifically for Switzerland, we first calibrated Eurasian models with Eurasian occurrences and bioclimatic variables at a 30'' resolution (~1 km²). We used 2854 occurrence points obtained from the GBIF database (<https://gbif.org>), and also found in Averyanov (1999) and Gargiulo et al. (2019). We disaggregated occurrences to avoid spatial autocorrelation (grid resolution = 5', occurrences kept: n = 1613) and we selected 100,000 background points in Eurasia (Barbet-Massin et al. 2012; Barve et al. 2011). We used 5 out of 19 bioclimatic (<https://chelsa-climate.org>) descriptors to characterize the current climatic niche; (i) bioclim1: mean annual air temperature, (ii) bioclim2: mean diurnal air temperature range (mean of monthly [max. temperature–min. temperature]), (iii) bioclim5: mean daily air temperature of the warmest month, (iv) bioclim12: annual precipitation amount, and (v) bioclim15: precipitation seasonality (Table S1). Those descriptors were the most consistent with species ecology and had a low pair-wise correlation (computation of matrix of Pearson and Spearman rank-correlation <0.7 (Dormann et al. 2013). For future climatic scenarios (2061–2080), we kept the same five Bioclim variables at 1 km² resolution, and we selected two Global Circulation Models (GCMs): IPSL-CM5A-LR (Dufresne et al. 2013) and HadGEM2-AO (Collins et al. 2008) together with two Representative Concentration Pathway (RCP): RCP4.5 and RCP8.5 (Van Vuuren et al. 2011). We took the conservative decision to use data from GCMs

from CMIP5, as studies for comparing CMIP5 and the more recent CMIP6 are still ongoing (e.g., Shu et al. 2020). To model Eurasian niches of *C. calceolus*, we processed three algorithms included in biomod2 (version 3.5.1, Georges and Thuiller 2013; Thuiller et al. 2016) in R, version 4.1.1 (R Core Development Team 2021): (i) Generalized Linear Model (GLM), (ii) Boosted Regression Trees (GBM) and (iii) Generalized Additive Model (GAM) (Gallien et al. 2012; Thuiller et al. 2016). For each set of pseudo-absences (1), each modelling technique (3) was run 33 times. To avoid circular reasoning in model evaluations, we used split-sample cross-validation procedure (Gallien et al. 2012). Models were calibrated on 80% of the initial data and evaluated on the 20% left with Probability of detection (POD, Wehling et al. 2011), Receiver operating statistics (ROC, Hanley and McNeil 1982) and with the maximization of the true skill statistic (hereafter TSS, Allouche et al. 2006; Guisan et al. 2017). We draw response curves for each variable and for each model to show how predicted values change along each variable gradient while keeping all other variable at their mean value (Elith et al. 2005). Finally, we projected Eurasian models on Switzerland using the same bioclimatic variables as the ones used for the calibration at the European scale, but this time from the CHclim25 dataset at a scale of 25 m (Broennimann 2018). We used Ensemble Forecasting to create final prediction maps for Switzerland. Two maps were produced for each time period; a map showing mean prediction weighted by model quality (TSS), and a map illustrating coefficients of variation between different models. Ensemble Forecasting was also evaluated with TSS. Those maps were used as covariates in the Swiss-scale models described below (Table S1).

2.1.2. Swiss-scale models

To describe a more detailed climatic and topographic niche of the species at regional scale (Switzerland), we calibrated another model with 1796 Swiss occurrences, which were obtained from the National Data and Information Centre on the Swiss Flora (www.infoflora.ch), from the Arbeitsgruppe Einheimische Orchideen Aargau (AGEO, <https://ageo.ch/>) and from the canton of Fribourg. In this case, we post-processed data in order to suppress records with a precision <25 m, those that were older than 1960 (when occurrence data started to be consistently gathered and scrutinized), and those who were irrelevant (e.g., botanical gardens, urban areas). We also disaggregated data at a grid resolution of 250 m to finally obtain a set of 528 occurrences. As for the European scale, 100,000 background points were selected, but this time within the Swiss territory. We selected six descriptors corresponding to *C. calceolus* ecology to define the Swiss-wide niche at a scale of 25 m²: (i) the climatic niche obtained at Eurasian scale used as covariate, (ii) the topographic aspect (aspval, corresponding to a sin-transformation of aspect, Aspect Tools of Spatial Analyst, ArcGIS 10.4), (iii) the slope (slp25, Slope Tools of Spatial Analyst, ArcGIS 10.4), (iv) the topographic position (topos, Zimmermann and Roberts 2001), (v) the maximum height of the canopy (Ginzler and Hobi 2015), and (vi) the distance between occurrences of *C. calceolus* populations and forest borders calculated with ArcGIS (ArcGIS 10.4). We included those two latter variables because the canopy height gives information about the light reaching the soil, and because digital map observations highlighted that *C. calceolus* populations are frequently located near forest borders (i.e., forest distance smaller than 150 m for 1243 over 1423 occurrences) (Table S1). We next modelled a prediction map for each

climatic scenario. To model Swiss niches of *C. calceolus*, we used the same process as at Eurasian scale. For Ensemble Forecasting at Swiss scale, we kept only models with a TSS value higher than 0.5. Models were projected on the same variables used for calibration at a resolution of 25 m. Two maps were produced for each time period; a map showing mean prediction weighted by model quality (TSS), and a map illustrating coefficients of variation between different models. Ensemble Forecasting was also evaluated with TSS. To select potential reintroduction sites on the final current prediction map, we used a threshold of 0.5 as it is the weight given to presence and pseudo-absences when fitting the models (Liu et al. 2013).

2.2. Step 2: field study of *C. calceolus* populations

2.2.1. Study sites

To conduct field-based surveys of *C. calceolus* populations, we selected 11 naturally occurring sites along the Swiss Jura Mountains (Tables S2 and S3). This region was selected based on the fact that the Jura mountains are predominantly built of limestone, thus likely the optimal substrate for this species, that fit the broader climatic requirements of *C. calceolus*, and that the Jura region has been the subject of multiple (re)introduction attempts since more than 15 years (Figure 2a). In addition, we surveyed five sites selected for reintroduction attempts between 2010 and 2014 (Tables S2 and S3). These reintroduction sites were chosen on the basis of vegetation similarities with historically known forested sites that hosted *C. calceolus* in the past, and the presence of indicator species for mountain spruce forests (*Carex flacca* and *Luzula sylvatica*). Source material for the reintroduced plants came from local seeds to avoid outbreeding depression (e.g., Frankham et al. 2017). Nonetheless, no genetic analyses were performed on these source populations. Plants were cultivated by private plant growers and were reintroduced when they were 4 years old, with a size of about 4 cm, and with two leaves and no flower.

2.2.2. Edaphic parameters

To assess the edaphic requirements of *C. calceolus* populations, at each site, we dug a soil profile until bedrock, minimum 40 cm away of the studied plant in order to not damage roots. All soil layers were described and soils and humus forms classified according to the international taxonomic keys (Baize and Girard 2009; IUSS Working Group 2015; Zanella et al. 2018). Next, in the topsoil layers (OL, OF, OH, and A layers), which corresponded to a depth of 15.5 ± 8.1 cm, and where the rooting of *C. calceolus* takes place, we quantified several physicochemical parameters, including i) water content, obtained by desiccation at 40°C and weighing, ii) organic carbon relative to total nitrogen (C/N ratio; quantified with an elemental analyser, Flash 2000, CHN-O Analyzer, Thermo Scientific), iii) total organic matter content (OM), quantified through the ignition loss after heating the samples at 450°C for 2 h, iv) water pH (Metrohm 827 pH-meter, Metrohm AG), v) cationic exchange capacity (CEC), quantified using the “cobalt hexamine trichloride” method (Ciesielski & Sterckeman, 1997), and vi) total carbonates (CaCO_3), quantified following the dissolution with HCl 6 M and using The Bernard calcimeter test (Dreimanis 1962).

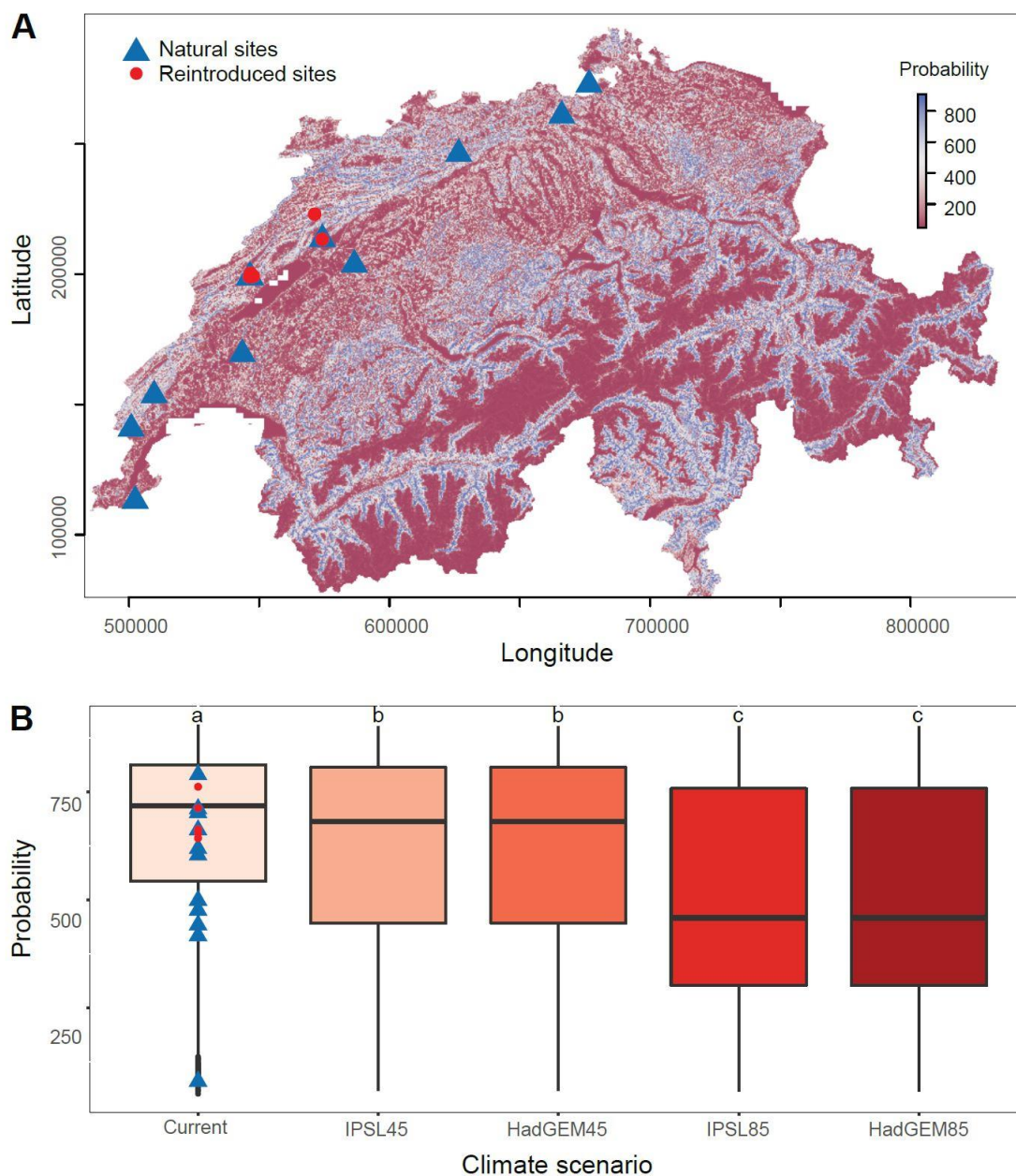


Figure 2. *Cypripedium calceolus* populations and probability of occurrence in Switzerland. A. The map is coloured based on the spatial modelling integrating Swiss and Eurasian populations, whereby red indicates low probability of occurrence and blue indicates high probability of occurrence based on the climatic and topographic variables at 25 m resolution scale. Also shown are the 11 natural populations (blue triangles) and five reintroduction sites (red dots) chosen for field work. B. Boxplots showing medians and quantiles of probability of occurrence values for current climate scenarios and the two GCMs: IPSL-CM5A-LR (IPSL45 and IPSL85) and HadGEM2-AO (HadGEM45 and HadGEM85) together with two RCP scenarios: RCP4.5 and RCP8.5 (Figure S3). Letters above boxplots indicate significant differences among climatic scenarios (Tuckey HSD, $p < .05$). GCM, global circulation model; RCP, representative concentration pathway.

2.3. *Cypripedium calceolus* population parameters

In order to understand the relationship between *C. calceolus* populations health status and the edaphic parameters, we measured several plant and population functional traits using non-intrusive methods. *C. calceolus* functional traits (Pérez-Harguindeguy et al. 2013) included plant growth parameters, such as: 1) plant size (measured in centimetres, from the ground to the top of the highest leaf), 2) number of leaves per plant, 3) leaf area (measured as the median leaf area of the highest stem, calculated using the ellipse area formula), 4) photosynthetic activity (i.e., the median leaf photosynthetic activity, measured with a SPAD chlorophyllometer, Konica Minolta), and reproduction-related parameters, such as: 5) number of flowers per plant, 6) number of pods per plant, and 7) number of stems per plant (Table S2). Each stem or group of stems spaced with 70 cm from another group of stems were considered as a separated individual (Kull 1988; Kull 1999). Finally, we assessed 8) a “vitality” parameter (scale 0–3) for scoring an integrative vitality value of each population, where 0 indicates that the population is practically extinct, and 3 indicates that the population is big, plants are healthy, and produce a lot of flowers and fruits. We analysed traits on 10 randomly chosen plants within 40 m radius and separated by a minimum of 2 m from each other.

2.4. Statistical analyses

Based on the SDM analyses, we first assessed the effect of climate warming on the probability of population presence within Switzerland by performing one-way ANOVA on the probability predictions of each population under the four climate warming scenarios and the current predictions.

Next, to assess the correlation between soil quality and *C. calceolus* population health status, we first calculated the horizon depth-weighted, summed, depth-segment-aligned dissimilarity across the 11 soil pedological profiles based on pH, CEC, CaCO₃, OM, and CN using the `profile_compare` function in the package `aqp` (version 1.40, Beaudette et al. 2013). Second, we calculated the Euclidean distance across *C. calceolus* populations along the two first axes of a principal component analysis based on the growth and reproductive traits measured. Third, we measured the correlation between the two matrices (dissimilarity and distance matrices, respectively) using a mantel test (function `mantel.test` in the package `vegan`, version 2.5.7). Finally, because the mantel test revealed a significant correlation (Section 3), we performed a multivariate correlation between the measured average values of the soil parameters (pH, CEC, CaCO₃, OM, and CN) found in the first 20 cm of each pedological profile, and the *C. calceolus* growth and reproduction traits using the `envfit` function in `vegan`. We performed this analysis in order to highlight the soil parameters with the best fitting correlation with the measured *C. calceolus* functional traits.

Finally, we compared the soil physicochemical parameters (pH, CEC, CaCO₃, OM, and CN) of the 11 natural population sites with the 5 reintroduction sites across aggregated variables within horizon depth (`slab` function in the `aqp` package, Beaudette et al. 2013).

3. Results

3.1. Step 1: spatial modelling of *C. calceolus* populations at two geographical scales

3.1.1. Bioclimatic spatial modelling

We built species distribution models for *C. calceolus* and generated maps of probability of occurrences for this species (Figures 2a and S2). At both Eurasian and Swiss scales, model performance was based on TSS (Eurasian scale (EU): mean: 0.75, SD: 0.04; Swiss scale (CH): mean: 0.56, SD: 0.04), POD (EU: mean: 0.99, SD: 0.001; CH: mean: 0.99, SD: 0.007), and ROC (EU: mean: 0.92, SD: 0.03; CH: mean: 0.83, SD: 0.03). At the Eurasian scale, variable importance analysis highlighted mean annual air temperature (bioclim1), mean diurnal air temperature range (bioclim2) and mean daily air temperature of the warmest month (bioclim5) as the main drivers of this species distribution. At the Swiss scale, distance of *C. calceolus* populations to a forest border and canopy height were the two variables that mostly influenced model accuracy (Figure S3). Accordingly, in Switzerland, this species is preferentially found growing up to 40 m from the forest border and under a canopy measuring between 20 and 50 m. Finally, we combined best performing models to create final maps displaying the probability of occurrence for each climatic scenario (evaluation with TSS:EU: 0.774, CH: 0.61). Under a current climatic scenario, the areas predicted to be most favourable for *C. calceolus* are the Jura, the lower alpine valleys and the southern Swiss Alps (Figure 2a). When compared to current climate, future global circulation models RCP4.5 (IPSL45 HadGEM45) and RCP8.5 (IPSL85 HadGEM85) decrease the overall average probability of population occurrence by 6% and by 22%, respectively (Figure 2b; $F_{4,6505} = 105.5$, $p < .001$). Such a decline was the strongest for the RCP8.5 model, particularly in the low altitude areas and the southern Swiss Alps (Figure S4).

3.2. Step 2: field study of *C. calceolus* populations

3.2.1. Edaphic parameters

We found variation across *C. calceolus* populations in terms of soil physico-chemical properties (Figures 3a and S5). Nonetheless, the studied natural populations were only present on calcareous soils: 11 CALCOSOLS (Calcaric Cambisols) and 3 RENDOSOLS (Calcaric Leptosols). We characterized humus forms as Oligomull (2), Dysmull (5), Hemimoder (2), and Dysmoder (2). In reintroduction sites, we identified 3 CALCOSOLS (Calcaric Cambisols), 1 RENDOSOL (Calcaric Leptosol), and 1 BRUNISOL (Cambisol). Humus forms were Dysmull (4) and Oligomull (1) (see Supplementary data D1 for soil profile descriptions). Analyses of the first organo-mineral horizons showed that in natural populations, soil pH ranged from 7.4 to 7.83, CEC from 23.9 to 70.8 cmolc/kg, CaCO_3 from 4.43% to 63.4%, organic matter from 7.4% to 45.6% and C/N ratio from 10.4 to 29.6. In reintroduction sites, pH ranged from 5.77 to 8.18, CEC from 11.4 to 70.5 cmolc/kg, CaCO_3 from 0% to 67.7%, OM from 13.5% to 65.8%, and C/N ratio from 2.3 to 33.6 (Figure 3 and Table S3).

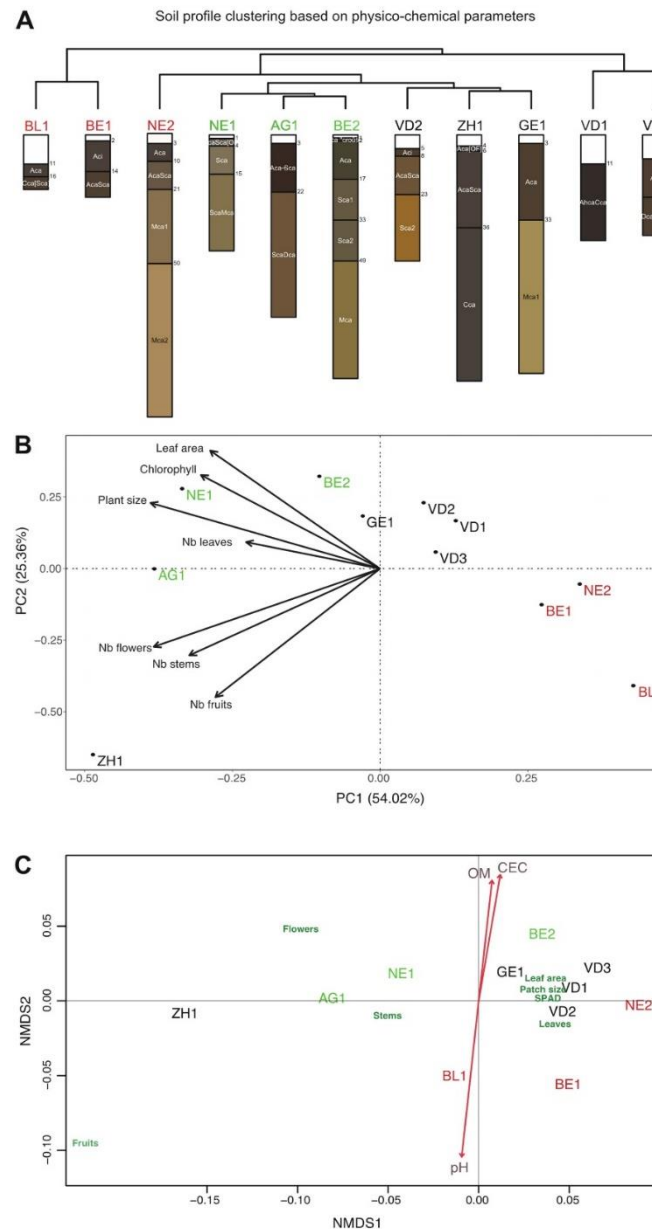


Figure 3. Effect of local edaphic factors on *Cypripedium calceolus* population growth- and reproduction-related parameters. A. Variation in soil colour profiles of the 11 natural field populations, aggregated based on soil physicochemical properties. Soil horizon colours are based on the Munsell code (Munsell 2015). B. Principal Component Analysis (PCA) of population fitness parameters, based on the following traits: Number of fruits, number of stems, number of flowers, number of leaves, plant size, chlorophyll, and leaf area in multidimensional space. C. Results of the envfit analysis for correlating population fitness traits with soil properties in the first organo-mineral horizon (a horizon). Red arrows indicate the three soil factors (total OM, CEC, and pH = soil pH) that correlated most strongly with the population trait matrix, $p < .05$ for pH and CEC, and $p < .1$ for OM). Populations are color-coded based on population trait similarity (panel b). Letter codes for *C. calceolus* populations are described in supplementary data D1. CEC, cation exchange capacity; OM, organic matter.

3.2.2. *Cypripedium calceolus* population parameters and correlation with soil parameters

We measured plant traits only in natural populations because no reintroduced plants survived through the years. The number of individuals per population varied from 1 to 50. Per individual, the number of stems varied from 1 to 60, the number of flowers from 1 to 38, the number of pods from 1 to 15, and the median leaf area from 29 to 140 cm² (Table S2). We found that across *C. calceolus* populations soil physicochemical parameters within pedological profiles (Figure 3a) correlated with growth- and reproduction-related plant traits (Figure 3b; mantel test based on 1000 permutations, $p = .03$). We also found that the soil properties that mostly discriminated *C. calceolus* populations were pH ($r = 0.84$, $p = .002$), CEC ($r = 0.56$, $p = .04$), and OM ($r = 0.72$, $p = .07$) (Table S4, and Figure 3c). Generally, we found that fitter and bigger populations occur on soils that with moderate pH, OM, and CEC values (Figure 3c; pH: 7–8, CEC: 30–40 cmolc/kg, and total organic matter: 15%–20%). Finally, we observed that the average horizon depth-weighted pH values for the natural population soils was 7.9, while the soil pH for the reintroduction site was 7.2 (i.e., 10% lower; Figure 4). We also found higher variability in CaCO₃ values for the reintroduction sites than for the natural population sites, and that reintroduction sites had 8% higher soil CN than the natural populations (Figure 4).

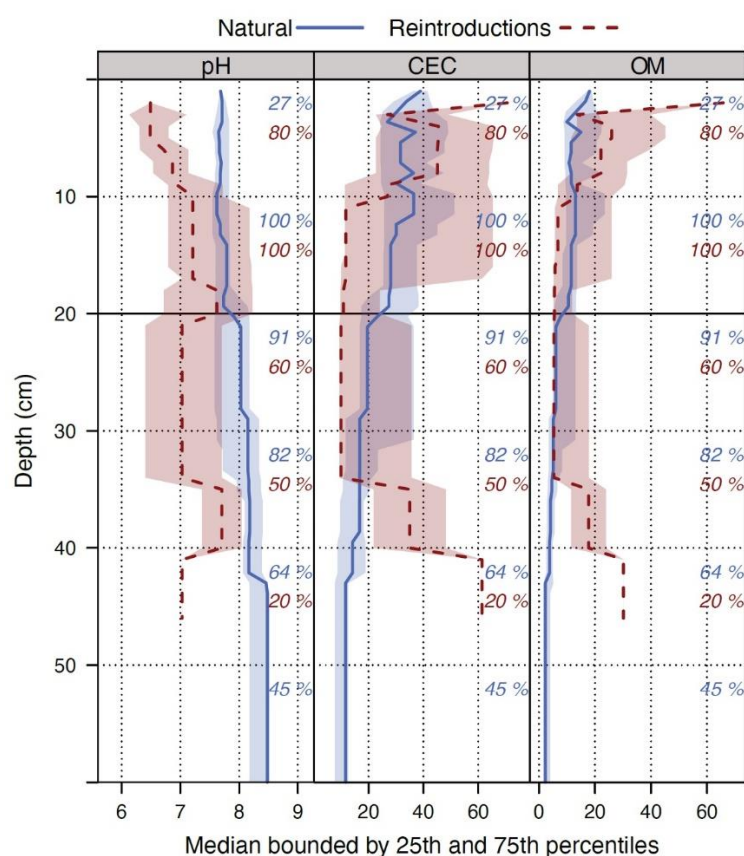


Figure 4. Soil profile comparisons across *Cypripedium calceolus* populations. The profiles show average changes in soil parameters (pH, CEC, and OM content) with soil depth for natural sites (blue lines; $n = 11$) and reintroduction sites (red lines; $n = 5$) of *C. calceolus* populations. Shaded areas around the median lines represent 75% confidence intervals. CEC, cation exchange capacity; OM, organic matter.

4. Discussion

We developed a two-step approach for identifying suitable sites for the (re)introduction of *C. calceolus* populations. First, we found that climatic predictions alone were rather broad in precision, but nevertheless could highlight a likely decline of the species range during the next 50 years of climate warming. Next, when incorporating topographical layers for the modelling approach at the regional scale, we found that the species was more likely to occur near forest edges. Finally, by analysing in situ soil parameters, we found that soil organic matter, cation-exchange capacity and pH correlated most strongly with *C. calceolus* population fitness variables as described by multivariate function trait space. We thus advocate for the combination of modelling tools with fine-scale ecological surveys to identify suitable reintroduction sites for this, and potentially other, endangered plant species.

4.1. Spatial niche modelling for conservation efforts

When modelling a current climate scenario, the continent-wide model parameterized with only bioclimatic variables did not give sufficient information, in that it did not exclude many areas of the Swiss map. Indeed, according to GBIF occurrences and the literature (e.g., Braunschmid et al. 2017; Käsermann and Moser 1999; Kull 1999), *C. calceolus* does appear to be adapted to a wide range of climatic conditions, from England to Japan and from Finland to Spain and Bulgaria (Devillers-Terschuren 1999). Therefore, it is perhaps not surprising that the climatic niche modelling alone shows little predictive power in relation to the population health status of this species (Kull 1999). By contrast, the regional scale model, which included bioclimatic as well as topographical and forest edge and forest height variables, was much more informative, highlighting that the highest probability of finding this species is tightly linked to how far the population is from forest edges and depends on canopy height (Foremnik et al. 2021; Hurskainen et al. 2017). A previous modelling study of the species in part of Switzerland, which included topographic, climatic and soil variables and a measure of vegetation productivity but no distance to forest edge, could successfully predict the species distribution but failed to support the identification of any new population in the field (Le Lay et al. 2010). Indeed, several authors have suggested that the requirements of this species in terms of light conditions correspond to open forest gaps (Fay and Taylor 2015; Hurskainen et al. 2017; Käsermann and Moser 1999; Kull 1999; Ramsay and Stewart 1998). Therefore, from a general point of view, our results corroborate previous theory suggesting that rare species geographic range distributions are less linked to macroclimatic patterns but more to microclimatic conditions, such as those that are created by the forest-open space ecotone, or in forest gaps (Wamelink et al. 2014).

Nonetheless, when modelling future climate scenarios, which considered both medium (RCP4.5) and high (RCP8.5) annual total greenhouse gas emission predictions, our modelling predicted declines in suitable future areas of *C. calceolus* population growth. This result suggests that while this species has broad climatic requirements, when growing on the warmer boundary of its climate envelope (e.g., the Mediterranean regions, or bottom of valleys), populations may already be growing at their maximum temperature tolerance. In this case, an average increase of 2°C–4°C is likely to be sufficient to bring multiple populations to the brink of collapse (Blinova and Chmielewski 2008; Kolanowska and

Jakubská-Busse 2020). This, coupled with the long-life expectancy and slow life cycle of *C. calceolus*, makes the inclusion of future prediction maps when selecting reintroduction and introduction sites particularly relevant in the context of climate change (Guisan et al. 2013; Maschinski and Haskins 2012; Primack 2014). However, it is important to underline that the association of old occurrences (from 1960) and more recent climatic data could underestimate the response to the species to climate change.

4.2. Ecological study and field parameters analyses

We based our study on the hypothesis that examining local ecological factors to identify suitable reintroduction and introduction sites increases the probability of plant establishment success (Maschinski and Haskins 2012). Indeed, it has been argued that failures in reintroductions and introductions are often the consequence of a lack of ecological data (Heywood and Iriondo 2003). In our case, soil variables showed a narrower range of values in native sites (e.g., pH, soil organic matter, CN ratio) than reintroduced population sites and were also correlated to changes in population fitness traits. These results highlight the importance of considering soil variables to make selection of translocation sites more accurate. Accordingly, the studied reintroduction sites for this species have historically been chosen based solely on vegetation and on plant companion species observations (Storrier 2016). Indeed, of the two indicator species used for the selection on reintroduction sites, *C. flacca* generally grows on calcareous soils, while *L. sylvatica* typically grows on acidic soils (Landolt et al. 2010). Therefore, one of the factors most likely to explain reintroduction failures is the difference in soil composition between natural and reintroduced sites. Moreover, by introducing small (4 cm tall) juvenile (4 years old) *C. calceolus* plants has probably had a negative impact on the success of these reintroductions (Gargiulo et al. 2021a). Indeed, Fay and Taylor (2015) advocate for reintroducing *C. calceolus* plants of an already sufficient large size to reduce risks of premature death of the seedlings. That said, our results do not fully correspond to what was previously argued in the classical literature. For instance, Käsermann and Moser (1999) suggested that the optimal pH requirements for this species range from neutral to moderately acidic, whereas our measurements of the A soil layer in native stations were neutral to moderately alkaline (pH 7.6). Secondly, Devillers-Terschuren (1999) described *C. calceolus* sites as being dry during summer months, while native sites studies here were often north-exposed and rather cool. Thirdly, as mentioned above, *C. calceolus* is thought to need full sun conditions for developing well (Devillers-Terschuren 1999). On the contrary, we observed that for best population performance, solar radiation reaching the plants should be indirect (i.e., close to forest edges, but not in the forest *per se*). We therefore could propose that the recruitment and establishment phases of this species might require full sun conditions, whereas further growth and flowering conditions is more appropriate under indirect light conditions. If true, *C. calceolus* would thus need pioneer conditions, such as after natural disturbance by tree felling, during early stages of development, and then a more mature ecosystem without a dense bushy understory for the rest of its development (Nicole et al. 2005). If this hypothesis were to be experimentally confirmed, such dynamic ecological requirements would further complicate the selection process of sites for conservation, reintroduction and introduction. Despite these findings, other ecological parameters found in the

literature correspond to those we observed. Notably, soils of native populations always contained calcium carbonates (Käsermann and Moser 1999; Kļaviņa and Osvalde 2017; Kull 1999). In summary, we observe that *C. calceolus* needs soils with a neutral to basic pH containing calcium carbonates, and requires a delicate balance of light, moisture, and bases richness (Devillers-Terschuren 1999). Such ecological requirements must therefore be taken into account in future reintroduction and introduction plans.

4.3. *Merging spatial niche modelling with ecological surveys*

This study empirically confirms what Maschinski & Hankins (2012) previously hypothesized: that it is essential to consider both broad- and fine-scale environmental qualities when choosing reintroduction and introduction sites. Indeed, heterogeneity in environmental conditions can influence reintroduction and introduction success on scales $<1 \text{ m}^2$ (Maschinski and Haskins 2012). Ideally, one would thus integrate field data with the broad-scale climate-based prediction maps to make identification of reintroduction and introduction sites more accurate (i.e., the first modelling approach step should already reveal high- and low-priority sites, whose suitability should then be assessed in later field-based data collection campaigns) (Chevalier et al. 2021; Mateo et al. 2019). Such a modelling approach is moreover currently limited by the fact that few variables exist in raster layer format, particularly those describing edaphic properties for large areas (Cianfrani et al. 2018); and see Descombes et al. (2020) and Hengl et al. (2017) for recent efforts to interpolate soil properties in geographic space. Moreover, considering future bioclimatic scenarios is likely to improve the success rate of reintroduction and introduction in the context of a rapidly changing climate (Marini et al. 2009). While promising, this methodological approach must now be confirmed by testing it across a large range of different species and by implementing an experimental design allowing long-term comparisons between reference sites and those selected by such a method (Seddon et al. 2007). Finally, all conservation attempts, regardless of whether they are successful or not, should be published in open-source databases, which would allow comparing and evaluating the relevance and success of different reintroduction and introduction attempts worldwide (Gargiulo et al. 2021a; Godefroid et al. 2011; Godefroid and Vanderborcht 2011; Heywood and Iriondo 2003; Seddon et al. 2007).

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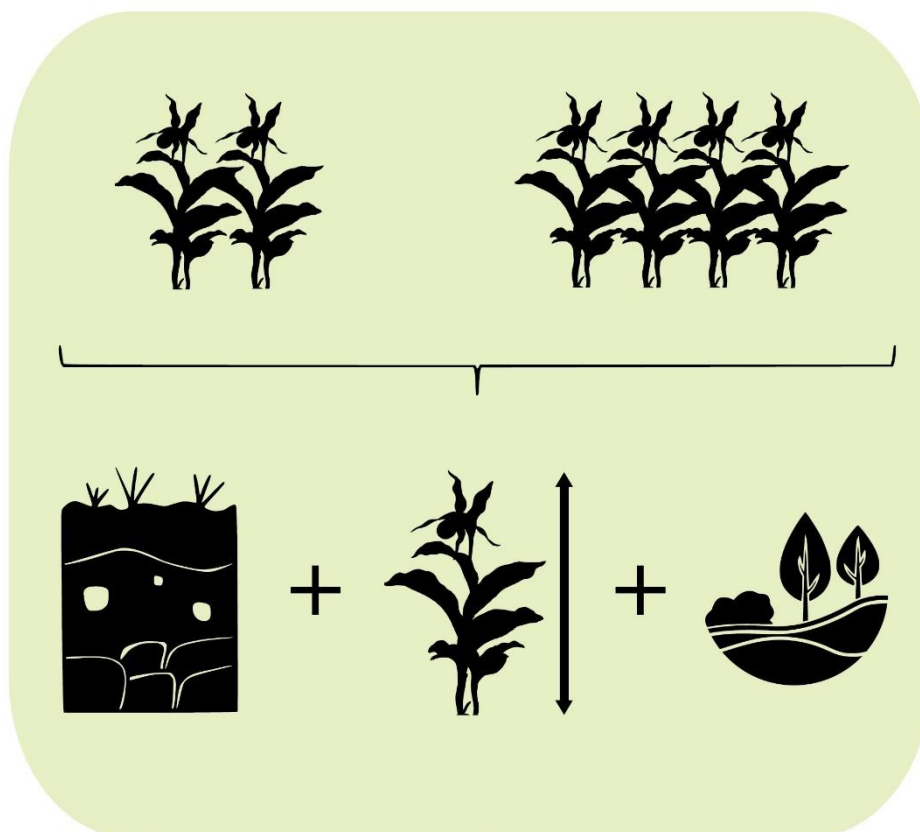
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CHAPTER II : SOIL PROPERTIES AND PLANT SPECIES CAN PREDICT POPULATION HEALTH AND POTENTIAL INTRODUCTION SITES OF THE ENDANGERED ORCHID *CYPRIPEDIUM CALCEOLUS*



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AUTHOR CONTRIBUTIONS

OR and SR conceived the ideas and designed the experiments, OR, TS, and CLB collected the data, SR, OR and CLB analysed the data. The first draft of the manuscript was written by SR and OR and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Abstract

Background and Aims

To counteract the ongoing worldwide biodiversity loss, conservation actions are required to re-establish and regenerate populations of threatened species. Two key factors predominantly involved in finding the most suitable habitats for endangered plant species are the surrounding plant community composition and the physicochemical parameters of the soil rooting zone. However, such factors are likely to be context- and species-dependent, so it remains unclear to what extent they influence the performance of the target species.

Methods

Here, we studied 34 Swiss populations of the highly endangered Lady's Slipper orchid *Cypripedium calceolus*. We measured functional traits related to plant and population performance, realized vegetation surveys, and soil profiles analyses, and tested for relationships between plant traits and the surrounding vegetation structure or soil physicochemical parameters.

Results

We found that neither vegetation alliances nor soil classes *per se* could predict *C. calceolus* functional traits and population size. However, we found that *C. calceolus* functional traits explaining population performance and size were related to specific soil parameters, namely soil organic matter content, pH and phosphorus, in addition to a combination of presence-absence of six plant indicator species, relating to ecotones between forests and forest gaps.

Conclusion

We show that, even for species that can grow across a wide range of vegetation groups, such as the orchid, both indicator species and specific soil parameters can be used to assess the optimal sites to implement (re)-introduction actions.

Keywords: edaphic properties, indicator species, Orchidaceae, plant conservation biology, red-list species, vegetation alliances.

1. Introduction

One-fifth of plant species worldwide are estimated to be at risk of extinction (Willis 2017) due to a number of factors, notably human habitat perturbation and climate change (Barnosky et al. 2011; Ceballos et al. 2017). Plant species loss has led to an urgent need for conservation actions. Despite this, habitat loss, fragmentation and change mean that the survival and persistence of rare plant populations can seldom be achieved by natural recruitment and dispersal alone. Instead, species conservation must rely on active reintroduction efforts in previously selected and appropriate habitat types (Seddon 2010). Consequently, to develop efficient conservation strategies for plants, a necessary prerequisite is to gather the best knowledge of the most important ecological factors that might influence the survival and the vitality of a target species (Godefroid et al. 2011; Heywood and Iriondo 2003; Seddon et al. 2007). Hence, the field of conservation science aims to act rapidly to understand the causes of each species disappearance and find suitable habitats for implementing successful (re)-introduction plans (Pimm et al. 2014; Primack 2014; Swarts and Dixon 2009).

For plant establishment and perpetuation, soil (edaphic) properties and the composition of surrounding vegetation communities are critical parameters that need to be evaluated (Antonovics and Bradshaw 1970; Rusconi et al. 2022). Accordingly, it is crucial to identify the potential links between soil properties, vegetation communities and the success of the focal endangered plant species (Godefroid et al. 2011). For instance, Vittoz et al. (2006), by analysing the edaphic conditions, hydrology, microtopography, and vegetation types of the niche of the endangered herbaceous plant *Saxifraga hirculus*, identified four major themes of conservation activities, namely grazing, mowing, reintroduction and substrate management. Similarly, through field surveys and herbarium analyses, Ren et al. (2010) were able to decipher the ecological requirements of the endangered *Primulina tabacum* in China and to identify suitable reintroduction sites. Moreover, factors determining the survival of the endangered endemic limestone-associated *Purshia subintegra* were uncovered by measuring 16 environmental factors, including soil and vegetation variables, in natural and experimental reintroduction sites (Maschinski et al. 2004). Therefore, the best chance of finding suitable habitats for endangered species (re)-introductions plans is to take a holistic approach combining measures of two of the predominant facets of terrestrial ecosystems: soil and vegetation properties.

The orchids (Orchidaceae) are among the most threatened plant families. Orchids have been subjected to intense collection because of their intrinsic beauty, and their complex and delicate life cycle has led to slow the recovery, or even disappearance, of populations (Hinsley et al. 2017; Thomas 2006). A well-known emblematic and patrimonial orchid growing throughout Eurasia is the Lady's slipper orchid *Cypripedium calceolus* L. (Fay and Taylor 2015). Although *C. calceolus* has a wide Eurasian distribution, ranging from the United Kingdom to the Pacific Ocean (Kull 1999), populations are in fact very scattered throughout its range (Devillers-Terschuren 1999). *C. calceolus* also has different conservation statuses across its distribution, being, for example, of Least Concern (LC) at a global scale, but marked as Vulnerable (VU) in Switzerland and Critically Endangered (CR) in the UK (Bornand et al. 2016;

Gargiulo et al. 2018; Rankou and Bilz 2014; Stroh et al. 2014). Accordingly, the number and size of *C. calceolus* populations have drastically declined over the last decades in several European countries (Devillers-Terschuren 1999; Kull 1999).

The complexity and the ecological particularities of this orchid, in addition to its patrimonial significance (Devillers-Terschuren 1999), have contributed to this species general decline (Gargiulo et al. 2021; Swarts and Dixon 2009). The physiological reasons for this decline might be that *C. calceolus* development is slow, and its life expectancy is long, theoretically up to more than 30 years (Käsermann and Moser 1999). From an ecological point of view, *C. calceolus* is endangered because its ecological requirements are mainly supposed to depend on an integrated sum of multiple parameters (Swarts and Dixon 2017). It is thought that *C. calceolus* requires a combination of three key ecological factors to thrive: (i) light intensity, (ii) soil moisture and (iii) soil base richness (Devillers-Terschuren 1999). For light intensity, *C. calceolus* populations should be primarily found in shady, deciduous, and mixed woodland forest types with a high canopy and very few bushes. This allows solar radiation to reach this species indirectly (Kull 1999; Rusconi et al. 2022). However, *C. calceolus* populations have also been shown to thrive in full sunlight at high elevations (Devillers-Terschuren 1999; Kull 1999). In Switzerland, *C. calceolus* has been found growing in as many as up to 14 different vegetation alliances, with a preference for the *Cephalanthero-Fagenion* (xerothermophilous beech forest) and the *Erico-Pinion* (basophilic subcontinental pine forest) alliances (Delarze et al. 2015; Käsermann and Moser 1999), suggesting that woodland type is not so constraining as previously thought. Concerning soil properties, it has been shown that *C. calceolus* can grow on a large variety of soil types (Klaviņa and Osvalde 2017; Rusconi et al. 2022) – with some caveats. First, *C. calceolus* appears to grow better in base-rich soils containing calcium carbonates (Kull 1999), which usually occur over limestone or dolomite bedrock (Käsermann and Moser 1999; Kull 1999). On the one hand, according to Käsermann and Moser (1999) soil pH requirements of this species range from neutral to moderately acidic. On the other hand, Rusconi et al. (2022) described pH requirements from neutral to alkaline. Moreover, *C. calceolus* seems to prefer richer substrates when in the shade compared to when growing in sunnier conditions, which may be due to the competition effect with other plant species (Käsermann and Moser 1999). Indeed, Klaviņa and Osvalde (2017) noticed a positive relationship between the population size of *C. calceolus* and soil organic matter concentration. Finally, Devillers-Terschuren (1999) showed that *C. calceolus* grow better on moderately moist soils. In short, while theory suggests that a narrow window of biotic and abiotic factors is required for *C. calceolus* to thrive, the ecological requirements for this species tend to be broad, widespread, and context-dependent making the selection of (re)introduction sites, at least at first sight, very complex and hard to predict.

With this study, we aimed to determine the relationships between the performance of *C. calceolus* populations and soil and vegetation parameters in order to improve this species conservation but also to advance the theoretical underpinning of which facets of an ecosystem most influence a species fitness. To this end, we characterised the vegetation communities and edaphic properties in 34 Swiss populations of *C. calceolus* (17 small and 17 large populations) and measured functional traits related to plant growth and reproduction. Specifically, we assumed that several unique functional traits are

indicators of plant fitness and population health, as highlighted by Adler et al. (2014). Specifically, we asked: 1) which plant functional traits best describe differences between small and large populations? 2) Is there a specific or recurrent pattern in vegetation composition or/and structure in relation to *C. calceolus* presence and population size? 3) Are there combinations of companion plant species that specifically dictate *C. calceolus* population size? 4) Is the population size of *C. calceolus* dependent on local edaphic properties? We hypothesised that while this plant species has broad ecological preferences, some facets of the vegetation or soil physicochemical properties can be used to explain why some populations perform better than others (Devillers-Terschuren 1999; Kļaviņa and Osvalde 2017; Kull 1999; Onuch and Skwaryło-Bednarz 2014). Therefore, plant species performance, estimated using specific plant functional traits, could be linked to unique combinations of vegetation and soil properties, which ultimately would be used to inform more efficient conservation efforts.

2. Material and methods

2.1. Population selection criteria

We chose *C. calceolus* populations of varying sizes from throughout Switzerland based on known occurrences of viable populations (data provided by www.infoflora.ch). We selected a total of 34 populations, 17 small (i.e., less than 10 individuals per population) and 17 large (i.e., more than 20 individuals per population) (Figure S1), encompassing the six major biogeographic regions of Switzerland (Figure 1A). Each population was visited between 2017 and 2021, once during the peak flowering period, between April and June, and once after the production of fruits (i.e., seed pods), between July and August.

2.2. Vegetation and plant traits

During the first visit to each *C. calceolus* population, we performed a comprehensive vegetation survey on a 10 x 10 m² plot comprehending the most homogenous vegetation type of each site following the Braun-Blanquet method (Braun-Blanquet 1964). We used measured vegetation community structure (characteristic and most abundant species) to assign an alliance name to each vegetation type based on phytosociological nomenclature (Barkman et al. 1986), and according to Delarze et al. (2015) (Table 1).

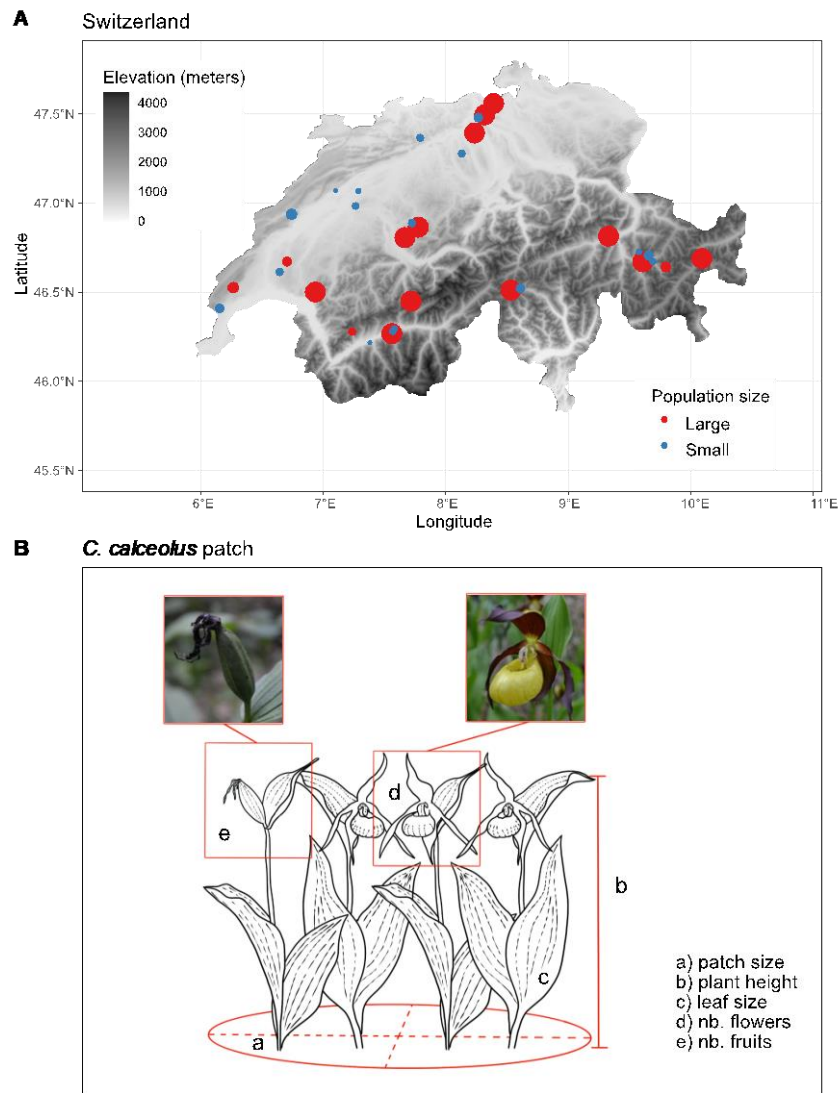


Figure 1. Sampling design. Shown are A. An elevation map of Switzerland including the 34 populations of *C. calceolus* surveyed during this study. Dots are coloured based on the population being small (blue dots; < 10 individuals), or large (red dots; > 20 individuals). The size of the dots is scaled according to the clone patch size of each *C. calceolus* individual. The average percent cover of the *C. calceolus* populations in the survey vegetation patches were 6.6%, and 3.1% for large and small populations, respectively (Figure S1). B. Plant traits measured on *C. calceolus* individuals: a) clonal patch area calculated as an ellipse, b) plant height taken from the soil to the top of the highest leaf (foliaceous bract) on the highest stem of the patch, c) leaf area calculated on the median leaf using the ellipse formula on the highest stem of the patch, d) number of flowers per patch and e) number of fruits (pods) per patch. Sampling of flowers and fruits were done at two time points, separated in average by a period of about two months. Other measures on the plants, but not shown here include number of leaves, leaf photosynthetic activity, specific leaf area (SLA), number of stems per patch and fruit volume.

Table 1. Soil and vegetation types measured across 34 populations of *C. calceolus*. Columns, from left to right, correspond to the name of the 34 sites as shown in Figure 3 and 5. The two-level factor of populations being either small (< 10 individuals), or large (> 20 individuals), the elevation in meters, the names of the soil types according to IUSS Working Group (2015), the Latin name of the vegetation alliance at each site (according to Delarze et al. 2015), the corresponding vegetation type, the most abundant plant species at each site, and the number of species found at each site.

Site	Size	Elevation	Soil_type	Alliance	Vegetation type	Dominant species	Richness
AG1	Small	501	Calcaric Cambisol (Colluvic)	<i>Galio-Fagenion</i>	Low altitude mesophyll beech forest	<i>Petasites_albus</i>	39
AG2	Small	600	Calcaric Cambisol	<i>Galio-Fagenion</i>	Low altitude mesophyll beech forest	<i>Acer_pseudoplatanus</i>	44
AG3	Large	450	Calcaric Cambisol	<i>Cephalanthero-Fagenion</i>	Xerothermophile beech forest	<i>Brachypodium_sylvaticum</i>	47
AG4	Large	467	Calcaric Cambisol	<i>Galio-Fagenion</i>	Low altitude mesophyll beech forest	<i>Brachypodium_sylvaticum</i>	48
AG5	Large	564	Calcaric Cambisol (Colluvic)	<i>Galio-Fagenion</i>	Low altitude mesophyll beech forest	<i>Frangula_alnus</i>	60
BE1	Small	440	Calcaric Cambisol	<i>Cephalanthero-Fagenion</i>	Xerothermophile beech forest	<i>Corylus_avellana</i>	34
BE2	Small	606	Calcaric Cambisol	<i>Cephalanthero-Fagenion</i>	Xerothermophile beech forest	<i>Fraxinus_excelsior</i>	16
BE3	Small	500	Calcaric Cambisol (Colluvic)	<i>Cephalanthero-Fagenion</i>	Xerothermophile beech forest	<i>Fagus_sylvatica</i>	37
BE4	Small	870	Calcaric Cambisol (Colluvic)	<i>Lonicro-Fagenion</i>	Beech forest of lower montane level	<i>Picea_abies</i>	36
BE5	Large	780	Calcaric Cambisol	<i>Lonicro-Fagenion</i>	Beech forest of lower montane level	<i>Brachypodium_sylvaticum</i>	41
BE6	Large	915	Calcaric Cambisol (Colluvic)	<i>Lonicro-Fagenion</i>	Beech forest of lower montane level	<i>Bromus_benekenii</i>	39
BE7	Large	1540	Calcaric Cambisol (Colluvic, Humic)	<i>Vaccinio-Piceion</i>	Spruce forest	<i>Calamagrostis_varia</i>	49
BL1	Small	933	Rendzic Calcaric Leptosol (Colluvic)	<i>Abieti-Fagenion</i>	Beech-fir forest of montane level	<i>Picea_abies</i>	17
GR1	Small	1923	Dolomitic Cambisol	<i>Erico-Pinion uncinatae</i>	Mountain pine forest	<i>Larix decidua</i>	35
GR2	Small	1128	Gypsic Dolomitic Cambisol	<i>Cephalanthero-Fagenion</i>	Xerothermophile beech forest	<i>Carex_alba</i>	44
GR3	Small	1915	Gypsic Dolomitic Cambisol	<i>Erico-Pinion sylvestris</i>	Basophilic subcontinental pine forest	<i>Pinus mugo</i>	37
GR4	Large	880	Calcaric Cambisol (Humic)	<i>Cephalanthero-Fagenion</i>	Xerothermophile beech forest	<i>Picea_abies</i>	31
GR5	Small	1700	Gypsic Dolomitic Cambisol	<i>Vaccinio-Piceion</i>	Spruce forest	<i>Deschampsia_cespitosa</i>	40
GR6	Large	1527	Dolomitic Cambisol	<i>Vaccinio-Piceion</i>	Spruce forest	<i>Larix decidua</i>	41
GR7	Large	1153	Calcaric Cambisol (Colluvic)	<i>Erico-Pinion sylvestris</i>	Basophilic subcontinental pine forest	<i>Picea_abies</i>	26
NE1	Small	1166	Calcaric Cambisol (Colluvic)	<i>Lonicro-Fagenion</i>	Beech forest of lower montane level	<i>Carex sempervirens</i>	32
NE2	Small	1187	Rendzic Calcaric Leptosol (Colluvic)	<i>Abieti-Fagenion</i>	Beech-fir forest of montane level	<i>Calamagrostis_varia</i>	29
TI1	Small	1265	Dolomitic Cambisol	<i>Sambuco-Salicion</i>	Pre-forest shrub stage	<i>Calamagrostis varia</i>	51
TI2	Large	1348	Dolomitic Cambisol	<i>Vaccinio-Piceion</i>	Spruce forest	<i>Brachypodium pinatum</i>	52
VD1	Small	658	Calcaric Cambisol (Colluvic)	<i>Galio-Fagenion</i>	Low altitude mesophyll beech forest	<i>Carex flacca</i>	29
VD2	Small	669	Calcaric Cambisol (Humic)	<i>Molinio-Pinion</i>	Subatlantic pine forest on marl slopes	<i>Acer_platanoides</i>	43
VD3	Large	994	Calcaric Cambisol	<i>Abieti-Fagenion</i>	Beech-fir forest of montane level	<i>Calamagrostis_varia</i>	35
VD4	Large	1117	Calcaric Cambisol	<i>Abieti-Fagenion</i>	Beech-fir forest of montane level	<i>Hordelymus_europaeus</i>	48
VD5	Large	621	Rendzic Calcaric Leptosol	<i>Galio-Fagenion</i>	Low altitude mesophyll beech forest	<i>Picea_abies</i>	34
VS1	Small	731	Leptic Calcaric Cambisol	<i>Cephalanthero-Fagenion</i>	Xerothermophile beech forest	<i>Sesleria_albicans</i>	34
VS2	Small	571	Stagnic Calcaric Cambisol	<i>Cephalanthero-Fagenion</i>	Xerothermophile beech forest	<i>Pinus_sylvestris</i>	29
VS3	Small	1075	Calcaric Cambisol	<i>Abieti-Piceion</i>	Spruce-fir forest	<i>Salix_caprea</i>	47
VS4	Large	1040	Calcaric Leptosol (Colluvic)	<i>Cephalanthero-Fagenion</i>	Xerothermophile beech forest	<i>Betula_pendula</i>	25
VS5	Large	1450	Calcaric Cambisol	<i>Erico-Pinion uncinatae</i>	Mountain pine forest	<i>Erica_carnea</i>	46

We next measured ten functional traits with known relationships with plant life history parameters (Adler et al. 2014; Díaz et al. 2016; Pérez-Harguindeguy et al. 2016) that could be quantified using non-intrusive methods. At each site, we sampled traits on a maximum of 10 randomly chosen individuals growing within a 100 m² area and separated by a minimum of 2 m from each other (Figure 1B). Each stem or group of stems (patch), separated by a minimum of 70 cm from each other, was considered as an individual (i.e., a clonal patch; Figure 1B) (Kull 1999). For growth-related traits, we measured clonal patch area (cm²), the vegetative plant height (cm), leaf number and median leaf area (cm², ellipse area formula) of the highest stem in a clonal patch and the median leaf photosynthetic activity (SPAD chlorophyllometer, Konica Minolta, Osaka Japan) and specific leaf area (SLA, mm²/mg) of 1 leaf per patch. For reproductive traits, we counted the number of stems and the number of flowers per patch on the first visit, and on the second visit we counted, per patch, the number fruits (pods) and estimated median fruit volume (approximated as a cylinder; Figure 1B).

2.3. Site and soil parameters

During the second visit, in each site, we dug a soil profile from the surface to the bedrock or parental material at a minimum distance of 40 cm from the nearest plant. We identified organic, organo-mineral and mineral horizons to characterise the soil type (Baize and Girard 2009; IUSS Working Group 2015) and corresponding humus forms (Zanella et al. 2018). Then, within the 100 m² area of each site, we sampled approximately 300 g of the organo-mineral horizon (A) three times, corresponding to a depth of 20.9 +/- 8.5 cm – i.e., the rooting zone of *C. calceolus*. From the A horizons, we measured eight soil physicochemical parameters, including; 1) relative humidity (HR), obtained by subsequent desiccation at 105 °C and weighing. 2) Total organic carbon (Corg) to total nitrogen (Ntot), and subsequent carbon-to-nitrogen ratio (CN), which were measured using an elemental analyzer (Flash 2000, CHN-O Analyzer, Thermo Scientific, Waltham, Massachusetts, United States). 3) Total soil organic matter content (SOM), measured via the loss of ignition (LOI) method, by heating the samples at 450°C for two hours. 4) pH, measured in distilled water with a Metrohm 827 pH meter (Metrohm AG, Herisa, Switzerland). 5) Soil total cationic exchange capacity (CEC), using the “cobalt hexamine trichloride” method (Ciesielski and Sterckeman 1997). 6) Total carbonates, estimated by CaCO₃ dissolution after HCl 6M addition, and according to the Calcimeter Bernard method (Dreimanis 1962). 7) Bioavailable phosphorus (P) estimated by extraction with sodium bicarbonate (Olsen 1954). 8) Nitrate (NO₃⁻) quantified after an extraction with potassium sulphate (Bremner and Shaw 1955). In addition, on soils that developed on a dolomitic substrate (geological information obtained from www.mapgeo.admin.ch), we performed dolomite quantification using the Calcimeter Bernard method (Dreimanis 1962) and gypsum quantification using a method with successive weighing after a passage in the desiccator and in the oven (105 °C) (Lebron et al. 2009).

2.4. Statistical analyses

All analyses were performed in R (R Core Development Team 2021).

2.4.1. *C. calceolus* population functional traits

We performed a principal component analysis (PCA) to visualize the multivariate trait space of all *C. calceolus* populations (*dudi.pca* function in the package *ade4*, (Dray and Dufour 2007)). We next performed a Regularized Discriminant Analysis (RDA) with the *rda* function in the package *vegan* (Oksanen et al. 2013), and a one-way ANOVA on the first axis of the PCA to assess the effect of population size (two levels; small and large) on *C. calceolus* functional traits. Site was included as a blocking factor in the analyses.

2.4.2. Vegetation community relationship with *C. calceolus* population size and functional traits

First, we performed a clustering analysis on the entire vegetation matrix to potentially detect clusters of similar vegetation types that best explain *C. calceolus* population size and plant traits. For this, we calculated a distance matrix of the vegetation communities using the *vegdist* function in *vegan* (Bray-Curtis distance) and then calculated the optimal number of groups using pairwise dissimilarities (distances) between communities in the vegetation data set (*daisy* function metric = “gower” in the *cluster* package (Maechler et al. 2013)). To assess a potential correlation between vegetation structure and the *C. calceolus* functional traits we performed a Mantel test between the trait distance matrix (Euclidean distance) and the obtained vegetation distance matrix (Bray-Curtis distance) (*mantel.test* function in *vegan*). Using a phylogenetic signal approach, we also calculated a potential correlation between dendrogram branch length and values for the number of fruits (a trait most representative of *C. calceolus* fitness) and the first principal component (PC1; as shown in Figure 2) using the *phyloSignal* function in the package *phylobase* (Hackathon 2020). Next, to assess the effect of *C. calceolus* population size (two levels; small and large) on the plant community structure of each site, we used a permutational ANOVA (PERMANOVA), using the *adonis* function in the *vegan* package. The Bray-Curtis metric was used to calculate dissimilarity among vegetation communities. Finally, independently of the vegetation community structure, we assessed potential indicator species associated with large or small *C. calceolus* populations species using the *multipatt* function in the *indicspecies* package (De Caceres et al. 2016).

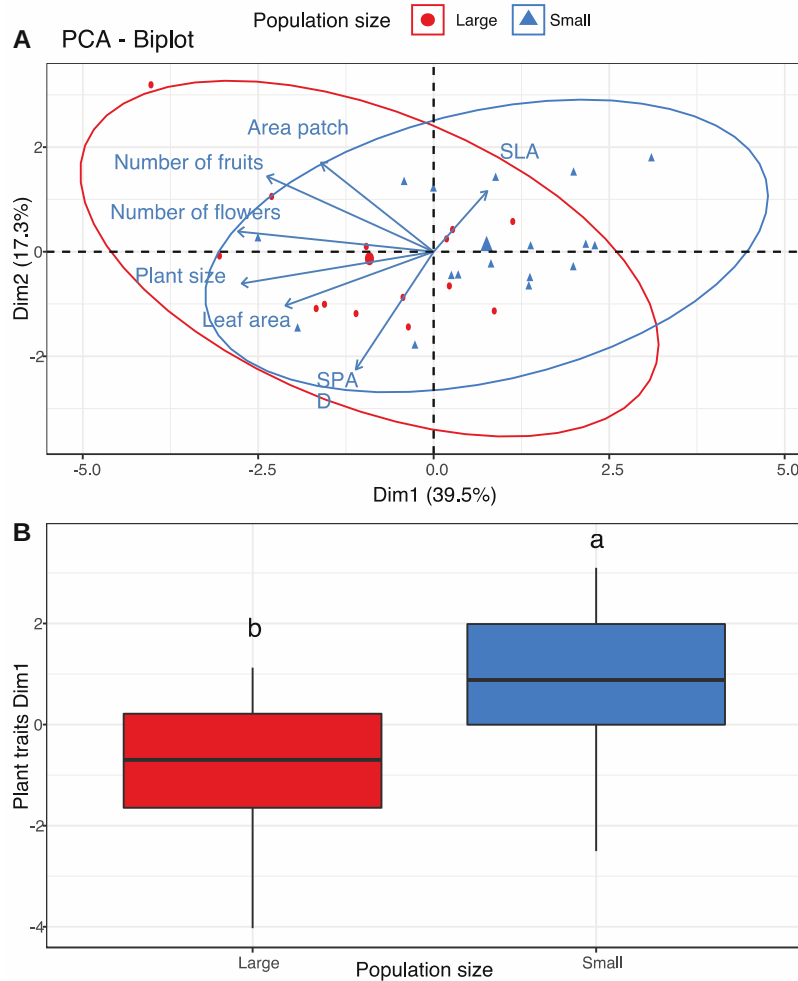


Figure 2. Functional traits characterizing *C. calceolus* population size. A. Principal component analysis (PCA) of seven plant traits describing the functional form of the different *C. calceolus* populations (see methods for details on the traits). Ellipses show the 95% confidence intervals characterizing small (blue area; < 10 individuals), and large (red areas, > 20 individuals) populations. B. Boxplots describing average values of small (blue) and large (red) based on the first axis of the PCA as shown above. Letters above boxplots show significant differences among populations (TukeyHSD, $p < 0.05$).

2.4.3. Soil properties across site and relationship with *C. calceolus* population size and functional traits

First, we classified soil profiles following two nomenclatures, the Référentiel pédologique classification (Baize and Girard 2009), and the world soil reference database WRB (IUSS Working Group 2015). Humus forms were classified according to Zanella et al. (2018). Next, we performed a principal component analysis (PCA) to visualize the multivariate space of soil properties for all *C. calceolus* populations (*dudi.pca* function in the package *ade4* (Dray and Dufour 2007)). We performed a Regularized Discriminant Analysis (RDA) with the *rda* function in the package *vegan* (Oksanen et al. 2013), and a multivariate analysis of variance (MANOVA) to assess the effect of population size (two levels; small and large) on the soil properties of *C. calceolus* populations. Site was included as a blocking factor in the analyses. Second, we assessed the potential correlation between individual soil properties

and the *C. calceolus* population functional trait matrix using redundancy analysis (*rda* function) and the *ordistep* function (package *vegan*) to perform a backward selection of variables in the model.

3. Results

3.1. *C. calceolus* population functional traits

We found that large populations of *C. calceolus* contained on average 72% bigger patches with 2.5 times more stems, 18% bigger plants, 5.5% more leaves, and 20% bigger and 7% harder leaves, and produced 83% more flower per individual than small populations (Figure S2, Table S1). Overall, multivariate trait space was significantly separated by the categorical variable of population size (Figure 2A, RDA with 999; $F_{1,221} = 4.39$, $p = 0.001$), and accordingly, along the first axis of the PCA (Figure 2B, population size treatment effect; $F_{1,221} = 24.62$, $p < 0.001$, and site effect; $F_{32,221} = 4.65$, $p < 0.001$).

3.2. Vegetation community relationships with *C. calceolus* population size and functional traits

Based on the vegetation surveys, we found that *C. calceolus* populations were associated with 349 vascular plant species in total and found in 10 different vegetation alliances (Table 1), with surrounding plant species richness ranging from 17 species to a maximum of 60 species per site (Table 1). The dominant species varied strongly between sites, with a total of 34 different plant species being dominant when considering all sites (Table 1). Overall, we found that the best clustering model identified 12 groups, suggesting a high variability in vegetation types across sites (Figure 3). Accordingly, we did not find a correlation between the population functional trait matrix and the vegetation community structure (Mantel test on 10000 permutations, $r = 0.14$, $z = 53.95$, $p = 0.14$, Figure 3). Similarly, the population size had no effect on the surrounding vegetation community structure (PERMANOVA, $F_{1,33} = 0.97$, $p = 0.48$). These results were also supported by a generally weak dendrogram distance-based signal (k and λ values) for all traits (see k and λ values in Table S2 indicating a weak correlation between the trait values and the branch length of the dendrogram; i.e., more similar vegetation types do not correspond to more similar traits values; $p > 0.05$).

The indicator species analysis highlighted that the most discriminant species based on *C. Calceolus* population size were *Ajuga reptans* ($r = 0.64$, $p = 0.014$), *Juniperus communis* ($r = 0.56$, $p = 0.019$), *Equisetum telmateia* ($r = 0.50$, $p = 0.037$), and *Gymnadenia conopsea* ($r = 0.50$, $p = 0.037$). Indeed, *A. reptans* was 19 times more abundant (glm with quasipoisson distribution with Type II analysis of deviance, Chi-sq = 9.41, $p = 0.002$), *J. communis* was 1.7 times more abundant (Chi-sq = 12.56, $p = 0.003$), *E. telmateia* was in average present with a relative abundance of 2.11% in large populations, while virtually absent in small populations (Chi-sq = 7.83, $p = 0.005$), and *G. conopsea* was 12 times more abundant (Chi-sq = 9.02, $p = 0.003$) in large *C. calceolus* populations than in small populations. We also found that small population of *C. calceolus* were significantly associated to two species; *Sesleria caerulea* (Chi-sq = 0.68, $p = 0.031$), and *Carex sempervirens* (Chi-sq = 0.53, $p = 0.044$).

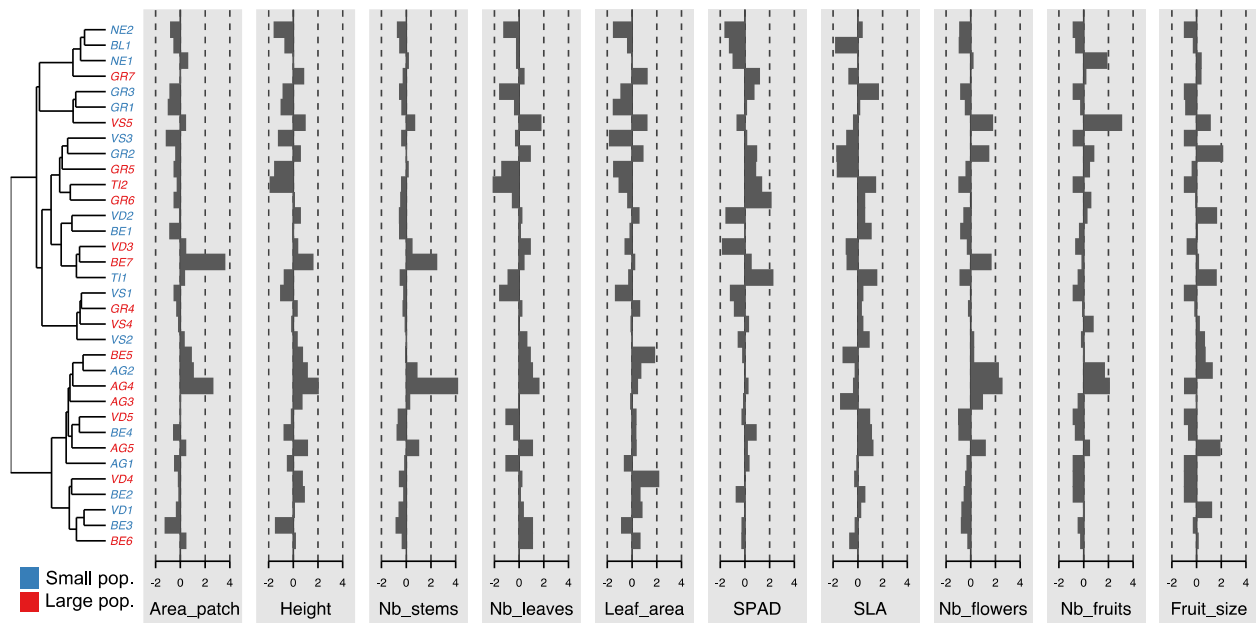


Figure 3. Similarities across *C. calceolus*-associated vegetation communities. Shown is a dendrogram based on Bray-Curtis dissimilarities values of plant species occurrences across 34 sites. Each site is coloured based on the size of *C. calceolus* population (small = blue colour; < 10 individuals, and large = red colour, > 20 individuals). Adjacent to the dendrogram are shown the scaled and centered values for all functional vegetation traits in each population, as shown in Figure S2.

3.3. Soil properties across site and relationship with *C. calceolus* population size and functional traits

Overall, we identified three major soil classes according to Baize and Girard (2009), all being calcareous or dolomitic soils: 23 Calcosols (Calcaric Cambisol (IUSS Working Group 2015)), four Rendosols (Calcaric Leptosols, (IUSS Working Group 2015)), and seven Dolomitosols (Dolomitic Cambisols, (IUSS Working Group 2015) (Table 1 and Supplementary data D1). Humus forms ranged between Mull and Moder (Supplementary data D1). We found that small and large populations significantly differed in soil physicochemical properties, with small populations inhabiting a wider range of variance than large populations (Figure S3; RDA with 999; $F_{33,69} = 6.53$, $p = 0.001$). Considering all traits together also revealed clear differences among sites (Figure 4, MANOVA, population size effect; Pillai = 0.68, approximation_{10,60} = 12.98, $p < 0.001$, site effect; Pillai = 6.3, approximation_{320,6900} = 3.67, $p < 0.001$). Specifically, we found that sites occupied by small populations had soil containing 21% more SOM, 26% more Corg, and 40% more CaCO_3 , but 16% less CEC than soil beneath large populations of *C. calceolus* (Figure 4 and Table S3).

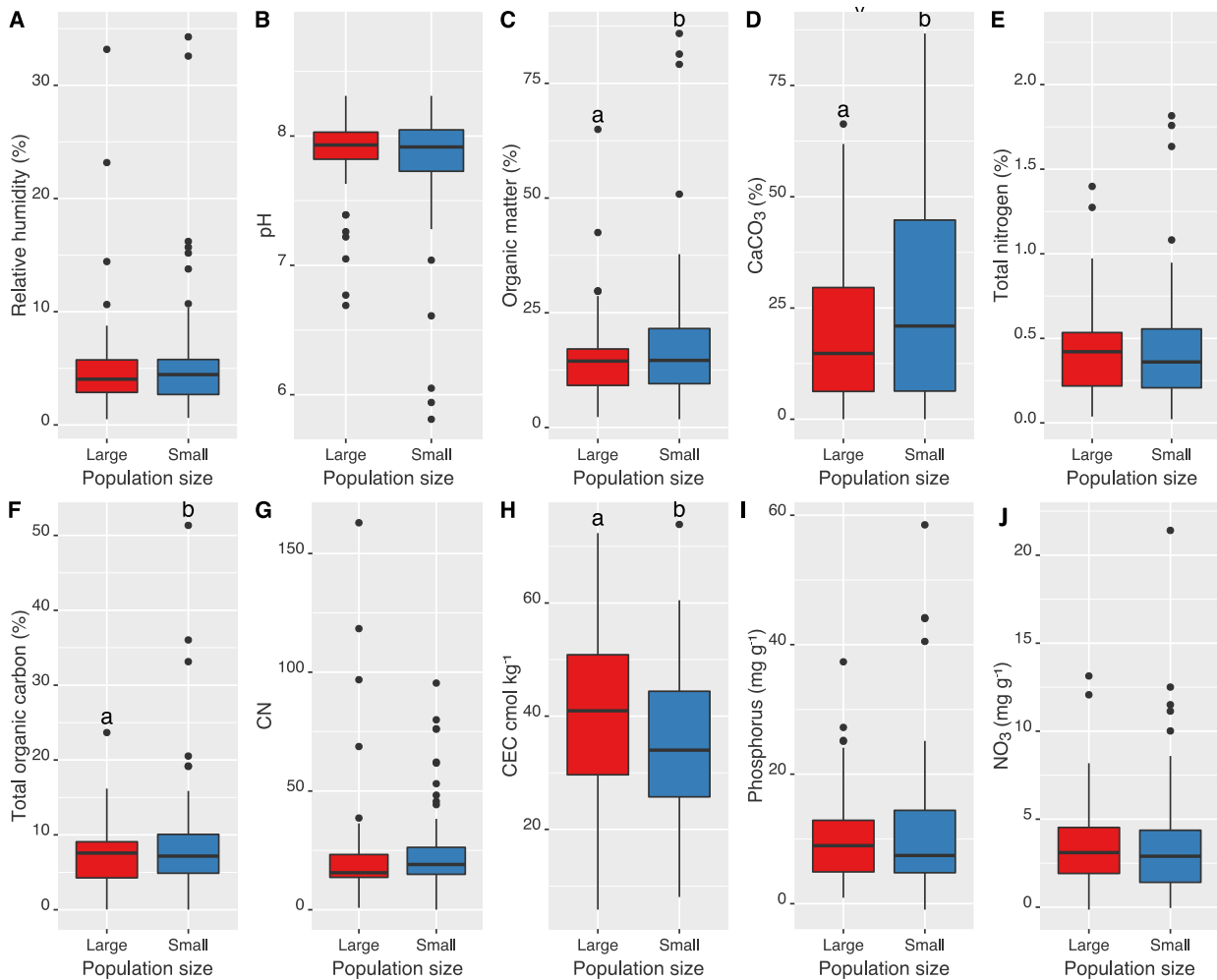


Figure 4. Soil physico-chemical parameters of *C. calceolus* populations. Shown are average trait values distributions for A. soil relative humidity (HR) B. pH, C. total soil organic matter content (SOM), D. CaCO_3 , E. Total N, F. total C, G. carbon-to-nitrogen ratio (CN), H. cation exchange capacity (CEC), I. total phosphorous, and J. total nitrates (NO_3) for small (blue boxplots; < 10 individuals), and large (red boxplots, > 20 individuals) populations. Different letters above boxplots show significant differences ($p < 0.05$) based on the multivariate analysis of variance as shown in Table S3.

Through the RDA analysis and stepwise model selection, we found that the soil characteristics that best explain *C. calceolus* population functional traits were pH ($F_{1,60} = 2.54$, $p = 0.05$), HR ($F_{1,60} = 2.61$, $p = 0.06^*$), P ($F_{1,60} = 3.37$, $p = 0.03$), SOM ($F_{1,60} = 3.78$, $p = 0.02$), and Corg ($F_{1,60} = 4.25$, $p = 0.02$) (Figure 5). In other words, we observed that high values of pH (alkaline soils) correlated positively with leaf area and SLA. In addition, P, SOM and Corg were positively correlated with the patch area and the number of flowers, while high HR values were negatively correlated with population fitness traits (Figure 5).

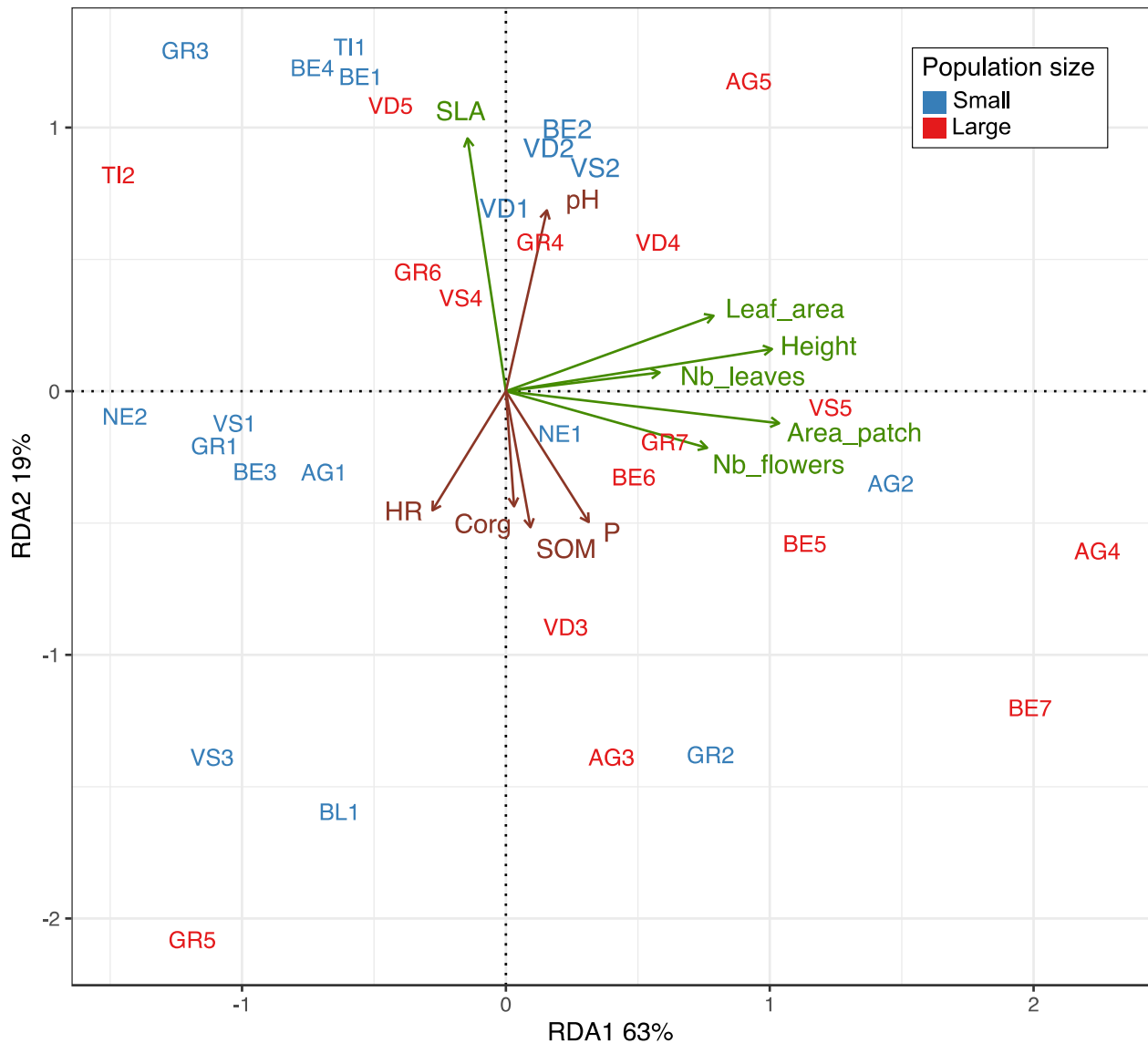


Figure 5. Correlation between plant functional traits and soil physico-chemical parameters. Shown is a Regularized Discriminant Analysis (RDA) biplot for highlighting the relative importance of soil physicochemical parameters (brown arrows) on the functional traits (green arrows) of *C. calceolus* populations. Site names are coloured based on the categorical variable population size (small = blue colour; < 10 individuals, and large = red colour, > 20 individuals). Only those soil variables that are significantly correlated with the vegetation functional trait matrix are shown with blue arrows ($p < 0.05$).

4. Discussion

Through extensive and fine-grained field work we explored the links between functional traits related to population health status and vegetation and soil parameters. We found that large (>20 individuals) populations of *C. calceolus* display a specific assemblage of measurable characteristics that discriminate them from small (<10 individuals) populations, indicating that it is possible to assess the health of a population of a rare plant species by measuring a specific set of functional traits. While we could not highlight a direct link between vegetation alliances and soil types and population health status, we show that the unique combination of companion plants, and several edaphic parameters, such as SOM, CaCO₃, pH, and P, could be used to potentially assess the optimal sites to implement (re)-introduction actions for this emblematic and patrimonial orchid species.

4.1. Functional trait variation across small and large populations

We found that, based on our classification, small and large populations display different functional signatures, in which, large populations of *C. calceolus* displayed significantly higher values for most measured plant traits, compared to small populations. This indicates that classic plant functional traits (Díaz et al. 2016; Wright et al. 2004) could be used to characterize the quality of *C. calceolus* populations (Adler et al. 2014). In plant evolutionary ecology and conservation, the predicted relationship between plant population size and fitness has been amply observed (Reed 2005). For instance, Leimu et al. (2006) performed a meta-analysis on 105 studies focusing on correlations between plant population size, fitness and genetic variation. They highlighted that all these correlations were significantly positive. Moreover, rather interestingly for our study, the same research also showed that these positive relationships were more pronounced for rare than for common species, an effect thus likely heightened when large population size differences exist in nature. Relationships between populations size and fitness may happen for two reasons: (i) an extinction vortex causing a decrease in genetic variation leading to inbreeding depression (Ruegg and Turbek 2022), a reduced mate availability or random genetic drift that consequently reduce populations fitness; or (ii) a difference in habitat quality (Ellstrand and Elam 1993; Fischer and Matthies 1998; Leimu et al. 2006). From a conservation point of view, these hypotheses are even more central in the case of an endangered species such as *C. Calceolus*. Indeed, small populations, which can be crucial for a species survival, are more vulnerable to stochastic events and fluctuations (Honnay and Jacquemyn 2007; Reed 2005). The recovery time after a perturbation is lengthened by a reduced fitness and will make the population more prone to extinction when supplementary perturbations happen (Reed 2005). In fact, smaller populations appear to be less able to adapt to new environmental changes because of the loss of adaptative genetic variation through genetic drift (Reed et al. 2003; Willi et al. 2007). In addition, reduced pollinator activity in small populations of rare species generally contributes to reduced plant fitness (Leimu et al. 2006). For the *C. calceolus* populations studied here, further investigations should be made to understand whether the relationship between population size and fitness is due to inbreeding depression or to habitat quality. Genetic studies performed in Europe on *C. calceolus* populations showed that this species has a relatively high genetic diversity compared with

rare taxa and taxa with the same life history (Brzosko 2002; Brzosko et al. 2002). On the other hand, signatures of a bottleneck effect and recent founder events were identified in Estonia, and genetic and genotypic diversity variables were significantly correlated with population size in Poland (Brzosko et al. 2011; Gargiulo et al. 2018). Finally, in addition to genetic studies and for conservation purposes, it would be necessary to understand the minimum size of *C. calceolus* populations so as not to be threatened by the extinction vortex and then ensure that populations stay under this threshold.

4.2. Vegetation communities associated with *C. calceolus*

Across our sampling, we found that *C. calceolus* grows on 11 vegetation alliances, with those where *C. calceolus* mostly occurred being xerothermophilic beech forests (*Cephalanthero-Fagenion*) and the low elevation mesophyll beech forest (*Galio-Fagenion*) – as observed by Käsermann and Moser (1999), but we couldn't find a recurrent pattern between vegetation composition and *C. calceolus* population size. It is essential to highlight that vegetation alliances were difficult to assess because *C. calceolus* often grows in somewhat hybrid environments (i.e., transition zones) that do not always fit traditional classification methods (Delarze et al. 2015). Consequently, we were only able to associate our vegetation inventories with the closest vegetation alliances found in the literature (see Table 1). All identified vegetation alliances were forests, except for the pre-forest shrub stage (*Sambuco-Salicion*). Together, these findings indicate that *C. calceolus* prefer to grow at the forest edges, or in the ecotones, of mixed-stands forest type, therefore preferring intermediate levels of direct solar radiations (i.e., not in full light, but never in full understory shading) (Rusconi et al. 2022). Along these lines, Hurskainen et al. (2017) highlighted that removing trees to create forest gaps favoured *C. calceolus* populations significantly. These observations point to the very complex ecology of this orchid and the delicate balance of light parameters it requires to grow (Kirillova and Kirillov 2019). Accordingly, because open forests are disappearing in Switzerland, recovery of *C. calceolus* populations might be impacted. In this regard, specific forest management plans should be implemented to favour this species optimal light requirements (Bornand et al. 2016). However, based on our findings, vegetation composition *per se* is not a sufficiently strong marker for the identification of suitable (re)introduction sites, and other parameters should be accounted for.

4.3. The use of indicator species for finding suitable habitats

Through species indicator analyses, we found that large and small *C. calceolus* populations were best discriminated by four species, positively by *Ajuga reptans*, *Juniperus communis*, *Equisetum telmateia*, and *Gymnadenia conopsea*, while negatively by *Sesleria caerulea* and *Carex sempervirens*. Interestingly, the ecological characteristics of *A. reptans* (the most discriminant species for large populations of *C. calceolus*) reflect the ecological needs of *C. calceolus*: clear forests with average humidity and average soil nutrients (Landolt et al. 2010). However, *A. reptans* would not be a good indicator species for finding *C. calceolus* suitable habitats, as it is even more broadly distributed than *C. calceolus* in Switzerland, growing in the sub-alpine regions (Lauber et al. 2018). The three other species positively discriminating large populations also shared similar or close ecological requirements to *C. calceolus* and the same elevation optimum (Landolt et al. 2010). The two species discriminating

small populations had the same soil nutrient requirements than *C. calceolus*, but their ecological optima are at higher elevations (Landolt et al. 2010). Therefore, a combination of some of the observed positively and negatively discriminating species could be used to find suitable habitats for *C. calceolus*. Such an approach could be confirmed using a combination of fieldwork for assessing population fitness (as was done here) and species distribution modelling (Guisan et al. 2006).

4.4. Soil characteristics associated with *C. calceolus* population health status

Based on the soil horizon profile analysis, we observed that *C. calceolus* grows on three, or two depending on the soil nomenclature, soil types, which corresponds to the observations made by Käsermann and Moser (1999). In opposition with the results of the vegetation types, and based on the total number of existing soil references (110 in Baize (2009) and 32 soil groups in IUSS (IUSS Working Group 2015)), we thus found that *C. calceolus* grows on a very limited range of soil types, attesting that *C. calceolus* preferentially grows on calcareous or dolomitic substrate. Moreover, the physicochemical characteristics results corroborate what is generally found in the literature: *C. calceolus* grows in soil with neutral to alkaline pH (Rusconi et al. 2022) with the presence of calcium carbonates (in the form of CaCO_3 or $\text{CaMg}(\text{CO}_3)_2$) (Käsermann and Moser 1999) and with on average a high concentration of soil organic matter (Klaviņa and Osvalde 2017). The observations regarding the humus forms, Mull-to-Moder, also support the notion that the rooting of this plant is where biological activity is relatively intense and where the organic matter is well and rapidly integrated into the soil matrix (Zanella et al. 2018). Moreover, through multivariate comparative analysis, we found that soil parameters that most strongly influenced *C. calceolus* functional traits were pH, HR, P, Corg and SOM. In this regard, our results contradict those found by Klaviņa and Osvalde (2017), in which pH did not affect population vitality. A precise characterization of the edaphic niche of endangered species (as we did in this study) is crucial for implementing conservation plans and identifying suitable (re)introduction sites. Specifically for *C. calceolus*, we encourage to perform soil physicochemical analysis to verify that the preselected zone has the following edaphic properties: presence of CaCO_3 or $\text{CaMg}(\text{CO}_3)_2$, neutral to alkaline pH, about 15% of organic matter and a CEC of about 40 cmol/kg.

In conclusion, with this work, we provide an additional step toward a better understanding how the selection of (re)introduction sites for the conservation of endangered plant species can be resolved. Specifically, we argue that beyond the use of classic approaches for site selection, such as building species distribution models (Guisan et al. 2013; Pecchi et al. 2019; Prasad et al. 2016), or, on the opposite, using only practitioner-informed current or past occurrence knowledge (Rusconi et al. 2022), might not provide the most accurate predictions for finding optimal sites to enhance or protect the target species. Instead, a fine-grained analysis of multiple targeted ecosystem variables is generally needed (Prasad et al. 2016; Richardson et al. 2009). Moreover, the analyses derived from our fieldwork also highlighted a direct link between plant and population life history traits and in situ measurable functional traits. In return, we then highlighted that narrow ranges of edaphic factors best correlated with unique sets of the measured plant traits, ultimately corroborating the importance of above-belowground links in plant ecology (Wardle 2002), and conservation biology.

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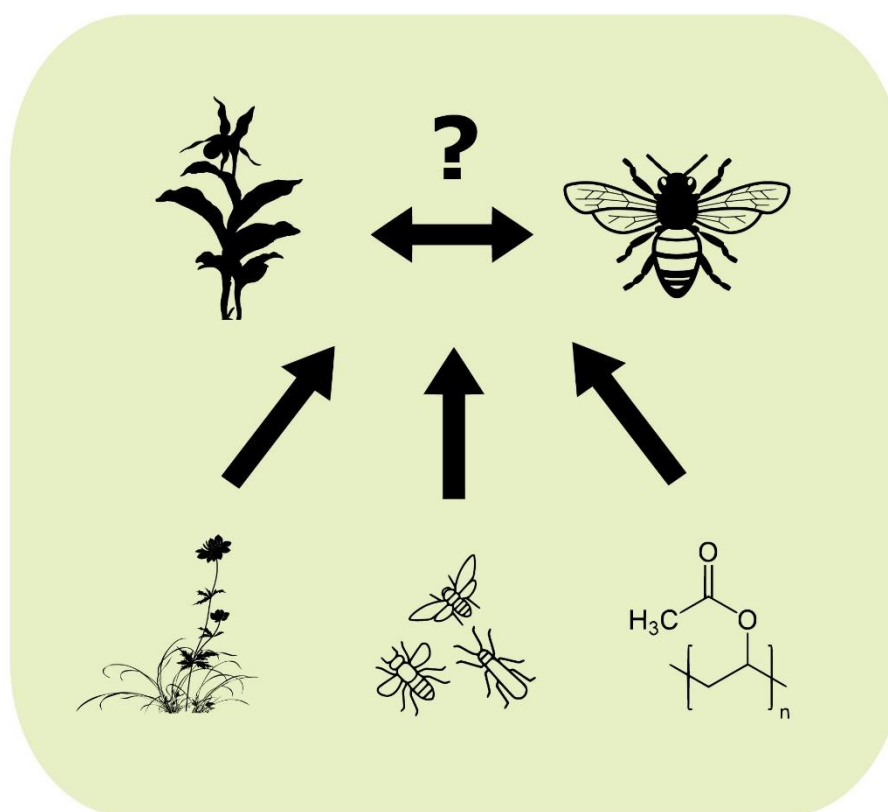
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CHAPTER III : RARE ORCHID SPECIES COMPENSATE FOR SURROUNDING LOW INSECT AND PLANT DIVERSITY BY PRODUCING MORE ODOUR BLENDS FOR POLLINATOR ATTRACTION



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AUTHOR CONTRIBUTIONS

OR and SR developed the ideas and the sampling design. OR, RB, and TS collected the data. CP identified insects and provide information about pollinators biology. OR and SR analysed the data. OR and SR wrote the first draft of the manuscript, and all co-authors contributed to editing

Abstract

The fate of flowering plants is tightly linked to the diversity and activity of their pollinators. Within the context of current worldwide biodiversity decline, plant reproductive fitness can thus be impacted if insects are also declining. This pattern should be even more accentuated for rare plant species that principally rely on attracting pollinators for their reproduction. To attract pollinators, flowering plants produce both visual and odorant cues (volatile organic compounds – VOCs). Therefore, if small populations of one plant species live in habitats where pollinator diversity is limited, those plants should be destined to extinction if they cannot overcompensate by producing more attractive cues than in large populations. We tested this hypothesis using a threatened orchid, *Cypripedium calceolus* (L.), as a model species. We selected 30 populations of which half were categorized as small (<10 individuals), and the other half as large (>20 individuals). In each population, we sampled site-level vegetation and insect diversity, flower visitor diversity, flower VOCs, plant growth traits, and fruit production. We found that even if small *C. calceolus* populations occur in less diverse sites, both in terms of surrounding plants and insects, and were physically smaller and generated less flowers, they produced the same number of seeds per capita as large populations. We also observed that flowers in small populations produced in average 97% more VOCs than flowers in large populations. We thus suggest that plants from small populations compensate the lack of pollinator diversity by providing stronger attractive cues in form of flower VOCs. These findings not only provide novel evidence for how changes in population structure can impact functional traits and species interactions, but they can inform conservation practices in terms of prioritizing the selection of genotypes depending on the need of creating novel founder populations or incrementing existing ones.

Keywords - Conservation biology, deceptive pollination, red-list species, linalool, octyl-acetate, solitary bees

1. Introduction

The fate of flowering plants and their pollinators are often intrinsically coupled (e.g. Traveset et al. 2017; van der Kooi et al. 2021), since flowering plants depend on pollinators to maintain reproductive fitness (Gómez et al. 2011; Wei et al. 2021). As such, factors that affect one are likely to affect the other (Chomicki et al. 2019; Christopher et al. 2019; van der Kooi et al. 2021). Today, the unprecedented biodiversity loss occurring worldwide is not only causing losses of plants and insects individually, but also driving changes to the processes that underpin community interaction networks, such as predation, parasitism and pollination (Albert et al. 2021; Ceballos et al. 2017; Gérard et al. 2020; Linders et al. 2019; MEA 2005; Ripple et al. 2019). In turn, alterations to interaction networks can alter the functioning, resilience and stability of entire ecosystems (Cardinale et al. 2012; Díaz et al. 2019; Tylianakis et al. 2008; Weiskopf et al. 2020). While biodiversity loss is therefore expected to have widespread impacts on plant fitness and reproductive success (Krushelnysky 2014), such impacts are likely to be particularly severe for rare plant species. This is because rare species are typically found in fragmented habitats that can be disconnected from pollinators (Lennartsson 2002; Xiao et al. 2016) and population fitness can be strongly affected by small changes in pollinator biodiversity (Parreño et al. 2022). Despite this, studies on how rare plants maintain fitness despite small, variable, pollinator communities are rare (Burkle and Runyon 2019; Wei et al. 2021).

Flowering plants have evolved a wide range of characteristics that serve as visual and olfactory cues to attract pollinators to reproductive organs (Chittka and Raine 2006; Lucas-Barbosa et al. 2016). Visual cues include the shape and colour of flowers, with different colours being especially important for triggering insect attraction from relatively long distances (Renoult et al. 2014; van der Kooi et al. 2018). Olfactory cues are also crucial for attracting pollinators, especially for plants pollinated at night or with highly specialized pollination systems (Barragán-Fonseca et al. 2020; Braunschmid et al. 2017). Plants give olfactory signals to pollinators in the form of floral volatile organic compounds (VOCs) (Burkle and Runyon 2019). More than 1700 individual VOCs have been found in the plant kingdom and countless combinations of them are emitted by flowers (Knudsen et al. 2006). Such odour blends have been shown to vary across and within species (Braunschmid et al. 2021; Braunschmid et al. 2017; Mant et al. 2005; Stökl et al. 2009; Sun et al. 2013), a variability most likely driven by both genetic and environmental variation (Zu et al. 2016). The crucial role played by floral scents in the attraction of pollinators is increasingly appreciated even if their role in reproductive fitness remain largely unknown (Burkle and Runyon 2019; Schiestl 2015). Moreover, knowledge about the importance and the abundance of floral VOCs production in rare plant are still missing.

The rare Lady's slipper orchid *Cypripedium calceolus* L. (Orchidaceae) is an iconic species of high relevance to this issue. While the species is broadly distributed from the United Kingdom to the Pacific Ocean (Kull 1999), the number and size of its populations have drastically declined over the last decades, and is now listed in the ICUN red list as threatened or critically endangered in multiple countries (Devillers-Terschuren 1999; Rankou and Bilz 2014). In addition to overharvesting and habitat destruction, its disappearance can be linked to its biology and ecological interactions (Swarts and Dixon

2009). *Cypripedium calceolus* plants have a very slow development time and a long life expectancy, potentially living more than 30 years. The first green leaves appear between 1 and 4 years after germination and flowering occurs 6 to 16 years after germination (Rasmussen 1995). *Cypripedium calceolus* can either reproduce vegetatively or sexually, through pollination. Vegetative reproduction occurs through the production of tufts of clones from rhizome ramifications. Regarding sexual reproduction, its pollination system is no exception: insects are attracted in the *C. calceolus* labellum by odour and by visual cues (Antonelli et al. 2009; Bergstrom et al. 1992; Nilsson 1979). Once in the labellum, the pollinators will immediately try to leave the flower because this orchid does not provide reward (such as nectar, deceptive pollination). To leave the flower, insects have to climb through the posterior exit where pollinia are deposited on their body (Antonelli et al. 2009). *Cypripedium calceolus*, as rare species is particularly sensitive to interaction with the surrounding pollinator guild and to their diversity (Krushelnicky 2014). Moreover, as this species uses olfactory cues in a deceptive pollination system, they should play a key role in maintaining plant reproductive fitness. However, the degree of importance represented by floral VOCs in such a scenario is unknown.

Here, we use *C. calceolus* as a model species to understand the role of olfactory signals in maintaining fitness in a rare orchid species. We focused on multiple *C. calceolus* individuals from 30 populations (15 small, 15 large) across Switzerland, for which we combined vegetation and insect diversity surveys, measurements of population-level plant functional traits related to growth and reproduction as indicators of fitness (Adler et al. 2014) and analysis of floral VOC quantity and composition. We specifically asked: (i) do more diverse sites also have higher pollinator diversity; (ii) do differences in pollinator diversity among sites lead to differences in orchid (reproductive) fitness; and (iii) what role do floral VOCs play in mediating this response.

2. Material and methods

2.1. Site selection and vegetation monitoring

To address the role of olfactory signals in maintaining fitness in small or large populations of plants, we randomly selected *C. calceolus* populations throughout Switzerland based on known occurrences, provided by the national data and information centre on the Swiss flora (www.infoflora.ch) (Figure 1A). Additional selection criteria included site accessibility, possibility of obtaining a visitation permit by the cantonal offices and population size. For this latter criterion, we categorized populations as being *small* (i.e., less than 10 inventoried individuals per population), or *large* (i.e., more than 20 individuals per population). Each stem or group of stems (patch), separated by a minimum of 70 cm from each other, is considered as an individual in *C. calceolus* (Kull 1999) (Figure 1B, average for small populations = 5.94 individuals, average for large populations = 78.12 individuals, generalized linear model with quasibinomial distribution, $\text{Chisq} = 47.99$, $p < 0.001$). Ultimately, we selected 30 populations (15 *small* and 15 *large*) across Switzerland (Table S1).

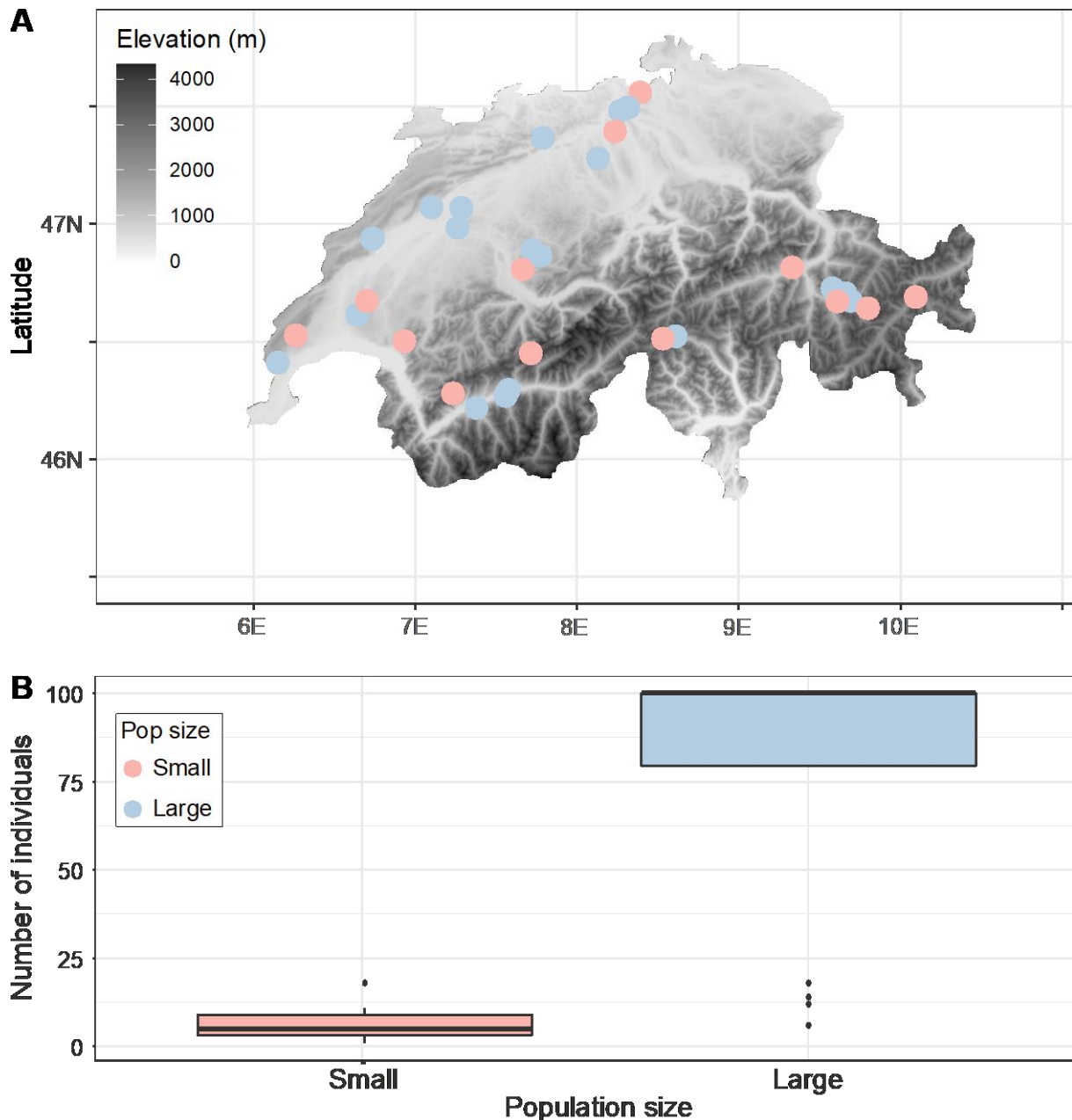


Figure 1. Experimental design of the study. Shown are A. an elevation map of Switzerland with the 30 different populations of *Cypripedium calceolus* sampled, and B. boxplots showing the average number individuals found in small (< 10 individuals, red boxplots), and large (> 20 individuals, blue boxplots) populations.

Each population was visited between 2017 and 2021; once during the peak flowering period, between April and June, and one after the production of fruits (i.e., seed pods), between July and August. At each site, we performed an exhaustive vegetation survey on a 10x10 m² plot placed in the centre of the *C. calceolus* population to measure local vegetation biodiversity, and to detect potential co-flowering species that produced yellow flowers. Vegetation surveys were done in the most homogenous vegetation type following the Braun-Blanquet method (Braun-Blanquet 1964). Based on the vegetation community structure (characteristic and most abundant species), we assigned to each

vegetation type an alliance name based on phytosociological nomenclature (Barkman et al. 1986), and according to Delarze et al. (2015).

2.2. Insect and pollinator survey

2.2.1. Local insect biodiversity survey

To obtain a snapshot of the entire diversity of flying insects visiting *C. calceolus* populations during flowering, we installed four yellow plates, filled with water and a drop of soap around *C. calceolus* individuals chosen randomly during their blooming period (Figures 2 and S1) (Prado et al. 2017). The plates were separated from each other by a minimum of 20 meters. Insect sampling was done on sunny days and, and for each population, plates were installed at 9 am and removed at 4 pm. Trapped insects were washed from the soapy solution and stored in 70% EtOH. Potential pollinators present in each population, specifically bees and syrphid flies (Antonelli et al. 2009; Braunschmid et al. 2021; Erneberg and Holm 1999) were determined to the species level. In addition, their gender was identified. All other insects were categorized using the morphospecies classification technique under a stereo microscope (Oliver and Beattie 1993, 1996).

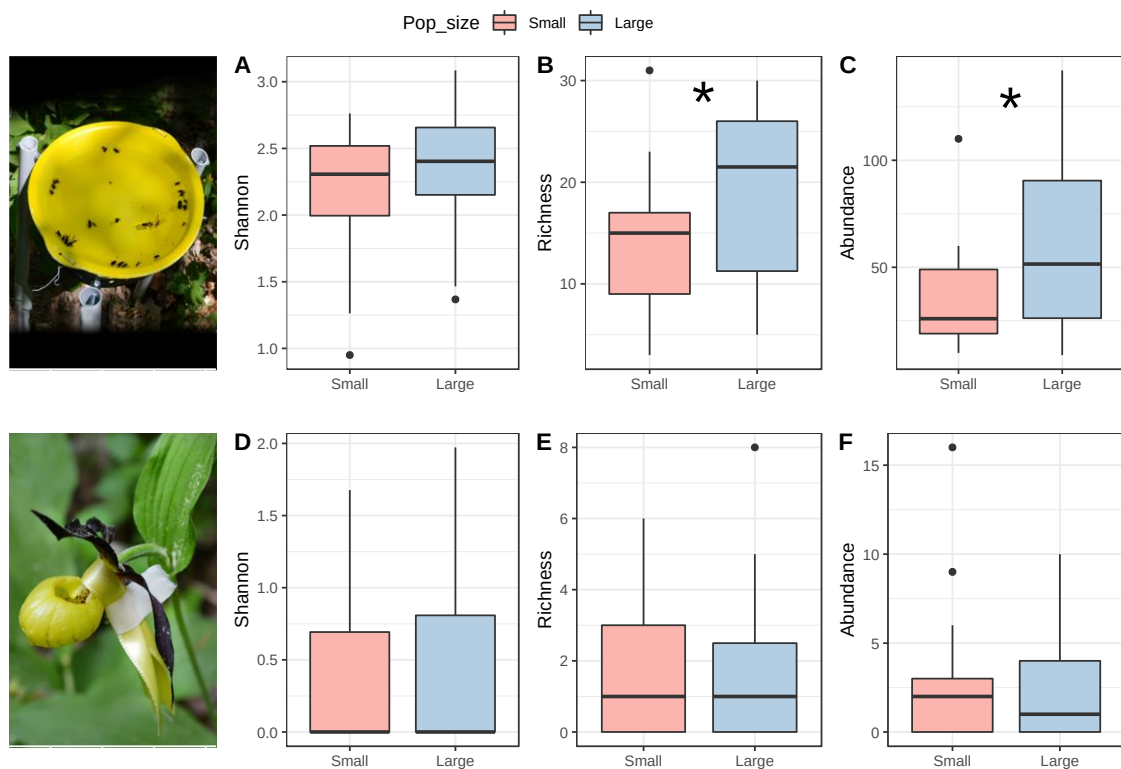


Figure 2. Insect biodiversity survey. Shown are the different biodiversity indices (Shannon diversity, species richness and total abundance) for insect collected using yellow plate traps (A, B, C, respectively), and insect trapped in *Cypripedium calceolus* labella (D, E, F, respectively) in small (< 10 individuals, red boxplots), and large (> 20 individuals, blue boxplots) populations. Asterisks indicate significant differences across populations sizes ($p < 0.05$).

2.2.2. Flower-visiting insect survey

To determine the insects visiting the labellum of *C. calceolus* during the blooming period, we performed an experiment as described by Case (2009) on ten (or less depending on population size) individuals per population. We used the natural trapping system of the lady's slipper orchid and blocked its exit with a yellow ribbon (Figure 2). By doing so, we were able to catch the insect trapped in the flower by taking off the ribbon, positioning a net around the flower and by waiting until the insect emerged from the labellum into the net. We attributed each insect to one of the two following categories: i) without pollinia, or ii) with pollinia, predominantly placed on their thorax (Case and Bradford 2009). All bees and syrphid flies were determined to the species level and their gender was identified. All other insects caught in the labellum were determined to the most accurate possible taxonomic classification and according to a morphospecies system (as described above).

2.3. *Life history traits*

For all sites, on each individual of *C. calceolus* ($n = 2-10$ depending on population size) we measured several functional traits directly related to fitness and reproduction (Adler et al. 2014; Díaz et al. 2016). We randomly choose individuals within a 100 m² area, and separated by a minimum of 2 m from each other. We measured i) clonal patch area, measured as an ellipse circumscribing the individual patch, ii) the number of flowers per patch (measured during the first visit), iii) number of fruits (pods) per plant (during the second visit), and iv) reproductive success for each plant patch, estimated as the total number of fruits per patch divided by the total number of flowers per patch.

2.4. *Collection of flower VOCs*

For flower volatile organic compounds (VOCs) collection, we followed a similar protocol as described by Braunschmid et al. (2017), and applied a dynamic headspace collection and analysis method as described in Dötterl and Jürgens (2005), with some modifications. Specifically, we enclosed individual flowers in a nalophan oven bag (Tangan N°34, Migros-Genossenschafts-Bund, Switzerland), fixed to a bamboo stick and closed with a rope around the stalk (Figure S2). After 15 minutes, we punched a hole in the plastic bag and fixed a plastic pipe (Tecnotubi Picena Srl, 4 mm inner diameter, 40 cm long) connected to an adsorbent glass tube filled with Tenax-TA (GERSTEL GmbH & Co., Buchem B.V., The Netherlands), itself plugged using a Teflon glass connector (Figure S2). The headspace around the flower was then adsorbed on the Tenax tube for 15 minutes (200 ml/min) with an AirChek® 52 Sample Pump (SKC Company, Chino Hills, CA, USA). In each population, we also included a “blank” by collecting air contained inside an empty nalophan oven bag fixed to a bamboo stick. Tenax tubes were then stored in coolers at 4 °C and at -80 °C until analysis. Next, when available, we also sampled VOCs from the co-flowering species *Hieracium murorum* aggr. flowers. As shown in Table S2, we found several co-flowering species across the field campaign. From those we selected *H. murorum* because it was the most common co-flowering species that produces yellow-coloured flowers across all sites, and that can be visited by short-tongued bees (see discussion section on co-flowering plants).

2.5. Analysis of flower VOCs

Identification and relative quantification of the odorant substances contained in each Tenax adsorbent tube were carried out using an automatic thermal desorption system coupled to a gas chromatographer and a mass spectrometer (GC-MS) (GC: Agilent 7890A, MS: Agilent 5975C, Agilent, Santa Clara, CA, USA). As internal standard, 1 μL of a solution of naphthalene in dichloromethane was added right before analysis (concentration = 20 ng/ μL) to each sample. The Tenax tube were desorbed during 3 minutes (initial temperature: 50°C, hold time 0.25 min, ramp 60°C / min until 250°C), then cryofocused in the CIS at -80°C (PTV solvent vent mode, 250°C, 14 psi pressure, 50 mL/min purge and vent flows, helium as carrier gas). After desorption, temperature in CIS was rapidly increased to 260°C (rate 12°C/s) and compounds were transferred into a polar column (HP-5MS, 5% Phenyl Methyl Silox, 30 meter long, 250 μm diameter, 0.25 μm film thickness) for separation. The carrier gas was helium (constant flow mode at 0.9 mL/min). The GC program started at a temperature of 50°C for 30 seconds and increased 5°C/min until it reached 250°C; it was stabilized at this temperature for 1 minute, with a post-run at 260°C for 1 min. The transfer line temperature to the mass spectrometer was set at 280°. The mass detector in EI mode at 70 eV was used to scan over the mass range from m/z 33 to 350. The chemical compounds were aligned based on retention time and ion spectra using the MZmine ver. 2.53 software (Katajamaa et al. 2006). Identification of individual compounds was done by comparison with pure standards and with the NIST05 database, and their relative abundance was calculated as a function of the internal standard (naphthalene). For statistical analyses, because samples were collected across three seasons, and across a wide variety of habitats, we choose the conservative approach of only retaining VOCs that were present in at least 3 individuals across all years, and that were clearly not present in the blank.

2.6. Data analysis

2.6.1. Diversity of plants and insects

For both vegetation and insect dataset, we calculated three indices of community diversity: i) the species richness, ii) the Shannon diversity index, and iii) the total abundance of individuals using the *diversity* function in the *vegan* package (Oksanen et al. 2013) in R (R Core Development Team 2021). Next, we assessed the effect of population size (two levels; small and large) for each index individually using one-way ANOVAs. Insect total abundance was log-transformed to meet homoscedasticity assumptions. For the diversity of the insects trapped in the labellum, we performed generalised linear models (glm) with population size as fixed factor and with a quasipoisson distribution.

2.6.2. Life history traits

We assessed the effect of population size (two levels) on patch size and number of flowers using one-way ANOVAs. In this case, as we sampled multiple individuals per site, we included site as blocking factor in the model. Patch size was log-transformed to meet homoscedasticity assumptions. The effect of population size on the number of fruits and the reproductive success was assessed using glm with

quasipoisson and quasibinomial distributions, respectively. Population was also included as blocking factor.

2.6.3. Flowers VOCs

We first assessed the effect of *C. calceolus* population size (two levels) and site on the entire volatile matrix with a permutational ANOVA (PERMANOVA) using the *Adonis* function in the *vegan* package in R (R Core Development Team 2021). The Bray–Curtis metric was used to calculate the dissimilarity matrix of all compounds across species. We next assessed the effect of population size, the identity of the volatile compounds and their interaction on the amount of the compounds using a two-way ANOVA on log-transformed data. Population was included as blocking factor on all analyses. Finally, we compared the scents emitted by *H. murorum* to the scents emitted by all *C. calceolus* flowers sampled using a non-metric multidimensional scaling (NMDS with Bray-Curtis distance) ordination.

3. Results

3.1. Diversity of plants and insects

Based on the vegetation surveys, we found that *C. calceolus* populations were, overall, associated with 349 vascular plant species and found in 10 different vegetation alliances, with plant species richness ranging from 17 species to a maximum of 60 species per site (Table S1). We found that sites with large populations of *C. calceolus* have 10% higher Shannon diversity than small populations (Figure S3A; $F_{1,32} = 4.29$, $p = 0.047$), while we found no effect of population size for either species richness (Figure S3B; $F_{1,32} = 1.03$, $p = 0.319$), nor for total vegetation abundance (Figure S3C; $F_{1,32} = 0.71$, $p = 0.407$). For the total community of insects within each site, we found that large populations of *C. calceolus* had on average the same Shannon diversity (Figure 2A; $F_{1,31} = 1.00$, $p = 0.325$), but they bore 36% more species (Figure 2B, $F_{1,31} = 3.46$, $p = 0.072$), and 70% more individual insects (Figure 2C, $F_{1,31} = 4.22$, $p = 0.048$) than small populations. Regarding insects found trapped in the labellum, we found no differences between small and large populations, either for the Shannon diversity (Figure 2D, $\text{Chisq}_{1,31} = 0.02$, $p = 0.897$), species richness (Figure 2E, $\text{Chisq}_{1,31} = 0.10$, $p = 0.751$), nor for total abundance (Figure 2F, $\text{Chisq}_{1,31} = 0.09$, $p = 0.762$).

In total, we found 32 species of Hymenoptera and Diptera in *C. calceolus* labella (Table 1). Most insect species were polylectic short-tongued solitary bees and syrphid flies. Overall, we found many more females than males visiting the flowers (Figure S4; 6 females /6 dipteran (Chi-squared = 4.17, $df = 1$, $p\text{-value} = 0.041$), and 54 females /62 hymenopteran (Chi-squared = 32.66, $df = 1$, $p\text{-value} < 0.001$)). Fifteen species (from 25 individuals) were identified as pollinators, meaning that they exited *C. calceolus* labellum carrying pollinia on their torso. Accordingly, we found four new species of Hymenoptera pollinating *C. calceolus*: *Andrena fulvata*, *Andrena strohmei*, *Lasioglossum nitidulum* and *Lasioglossum pygmaeum* and one species of Diptera: *Melanostoma scalare* (Table 1). We also identified five new species of solitary bees and four species of syrphid flies that have never been

previously reported to visit (i.e., existing the labellum by the posterior exit but without pollinia, Table 1) *C. calceolus* flowers.

Table 1. Insect pollinators of *Cypripedium calceolus*. The table describes all species of insects found in *C. calceolus* labellum across 34 populations in Switzerland. Shown are the number of observations per species (Nb), whether the insect carried pollinia or not on their torso (P), whether this was a first record of such an event (in bold ^N) and if they belong to the order of Hymenoptera (H) or Diptera (D).

Species	Nb	P	O	Species	Nb	P	O
<i>Andrena bicolor</i>	3	1	H	<i>Lasioglossum c.f albipes</i>	1	0	H
<i>Andrena bucephala</i> ^N	1	0	H	<i>Lasioglossum c.f calceatum</i>	1	1	H
<i>Andrena fulvata</i> ^N	4	2	H	<i>Lasioglossum calceatum</i>	4	3	H
<i>Andrena haemorrhoa</i>	2	1	H	<i>Lasioglossum cf. albipes</i>	2	0	H
<i>Andrena nigroaenea</i>	1	1	H	<i>Lasioglossum cf. calceatum</i>	1	1	H
<i>Andrena rogenhoferi</i>	4	0	H	<i>Lasioglossum fulvata</i> ^N	1	0	H
<i>Andrena scotica</i>	5	0	H	<i>Lasioglossum lativentre</i> ^N	1	1	H
<i>Andrena cf. scotica</i>	1	0	H	<i>Lasioglossum minutulum</i> ^N	1	0	H
<i>Andrena strohmeilla</i> ^N	1	1	H	<i>Lasioglossum morio</i>	1	1	H
<i>Cheilosia flavipes</i> ^N	1	0		<i>Lasioglossum nitidulum</i> ^N	15	6	H
<i>Cheilosia proxima</i> ^N	1	0		<i>Lasioglossum parvulum</i> ^N	1	0	H
<i>Cheilosia rhynchops</i> ^N	1	0		<i>Lasioglossum pygmaeum</i> ^N	2	1	H
<i>Dasysyrphus lenensis</i> ^N	2	0		<i>Melanostoma mellinum</i>	2	0	H
<i>Halictus tumulorum</i>	2	1	H	<i>Melanostoma scalare</i> ^N	3	3	H
<i>Lasioglossum albipes</i>	4	0	H	<i>Platycheirus parmatus</i>	1	0	
<i>Lasioglossum bluethgeni</i> ^N	4	0	H	<i>Symphita</i>	1	1	H

3.2. Life history traits

We found that large populations of *C. calceolus* have in average 70% bigger individuals (patch size) (Figure 3A; patch size effect: $F_{1,221} = 28.31$, $p = < 0.001$, site effect: $F_{32,221} = 3.34$, $p = < 0.001$), and produced 83% more flowers (Figure 3B; patch size effect: $F_{1,221} = 28.45$, $p = < 0.001$, population effect: $F_{32,221} = 5.40$, $p = < 0.001$) than small populations. We also found 49% more fruits in large population than in small populations (Figure 3C, patch size effect: $\text{Chisq}_{1,253} = 8.07$, $p = 0.036$, population effect: $\text{Chisq}_{32,253} = 231.68$, $p < 0.001$). On the other hand, we found no difference between large and small populations in term reproductive success (Figure 3D, patch size effect: $\text{Chisq}_{1,253} = 0.21$, $p = 0.518$, population effect: $\text{Chisq}_{32,253} = 47.01$, $p < 0.001$).

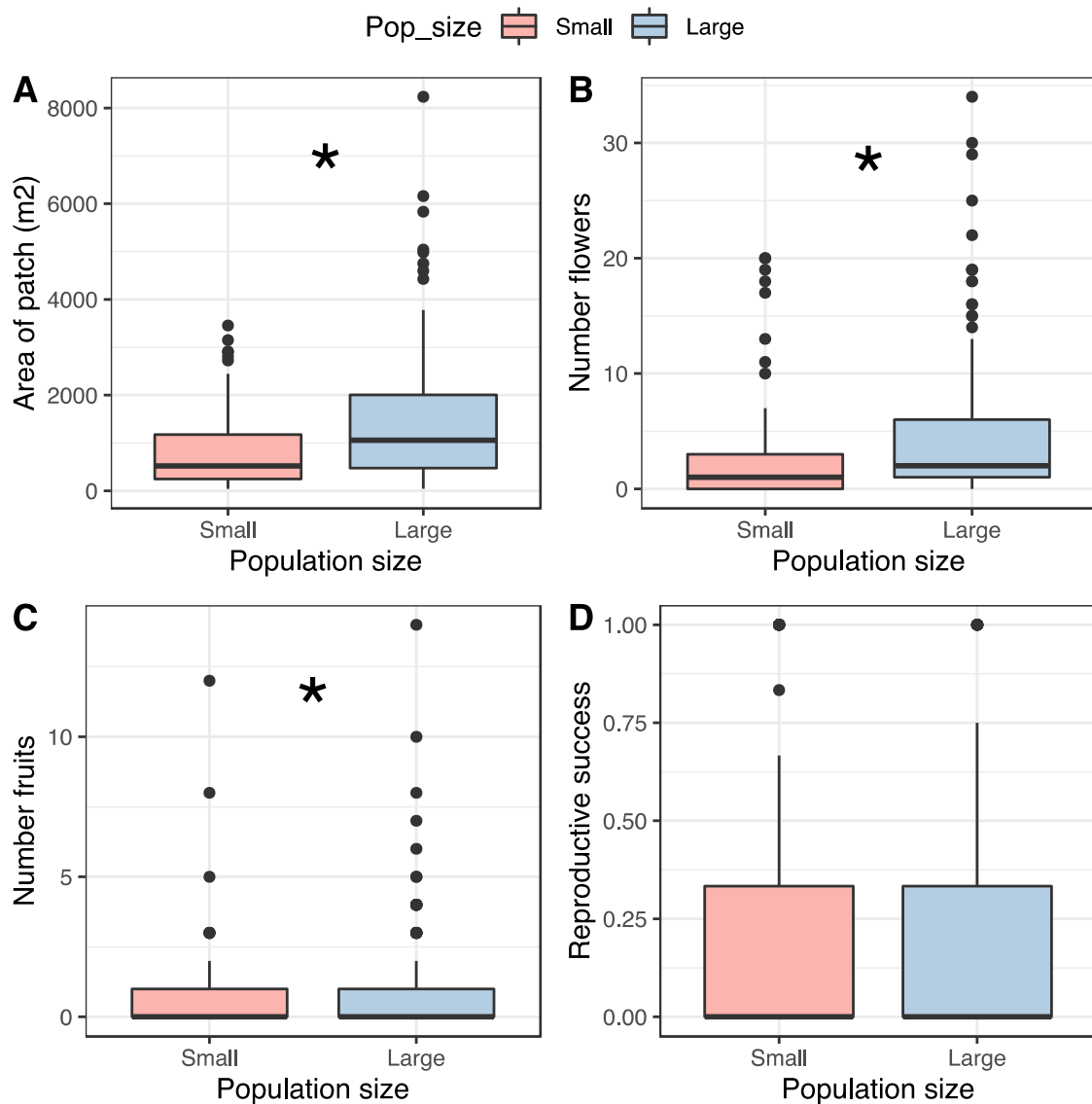


Figure 3. Plant fitness and reproduction traits. Shown are the average A. *Cyripedium calceolus* individual patch size, B. number of flowers per plant, C. number of flowers per plant, and D. reproductive success (fruits per flowers) in small (< 10 individuals, red boxplots), and large (> 20 individuals, blue boxplots) populations. Asterisks indicate significant differences across populations sizes ($p < 0.05$).

3.3. Flower VOCs

Across the three years of sampling and 95 *C. calceolus* individuals studied, we found a total of more than 80 compounds that were likely produced by *C. calceolus* flowers. To avoid biases due to interannual variation, out of the total number of VOCs observed, we selected 20 VOCs that were represented across all years (Figure 4). Of those, 11 VOCs belonged to the aliphatic hydrocarbons, six to the terpenoid and three to aromatic hydrocarbon class. We found a significant effect of population size and population on the entire volatile blend, which fits with what maintain deceptive pollination (PERMANOVA: $F_{1,93} = 0.79$, $p < 0.001$, and population effect: $F_{26,93} = 11.83$, $p < 0.001$). We also found

that, on average, small populations produced 97% more volatiles than large populations, but such effect varied across compounds (Figure 4; population size effect: $F_{1,1906} = 12.2$, $p < 0.001$, compound effect: $F_{20,1906} = 437.7$, $p < 0.001$, population effect: $F_{26,1906} = 109.3$, $p < 0.001$, and compound by population size interaction: $F_{20,1906} = 26.9$, $p < 0.001$). Specifically, we found that 8 out of 21 compounds were more abundant in small populations, including the characteristics *C. calceolus* flower VOCs (e.g. linalool and octyl-acetate) (Braunschmid et al. 2021; Braunschmid et al. 2017) and one (benzyl acetate) that was more abundant in large populations. We also found that *H. murorum* flowerheads produced a wide range of VOCs (Figure S5A), but with almost no overlap with *C. calceolus* VOCs profiles (Figure S5B), such as the complete absence of linalool or octyl-acetate.

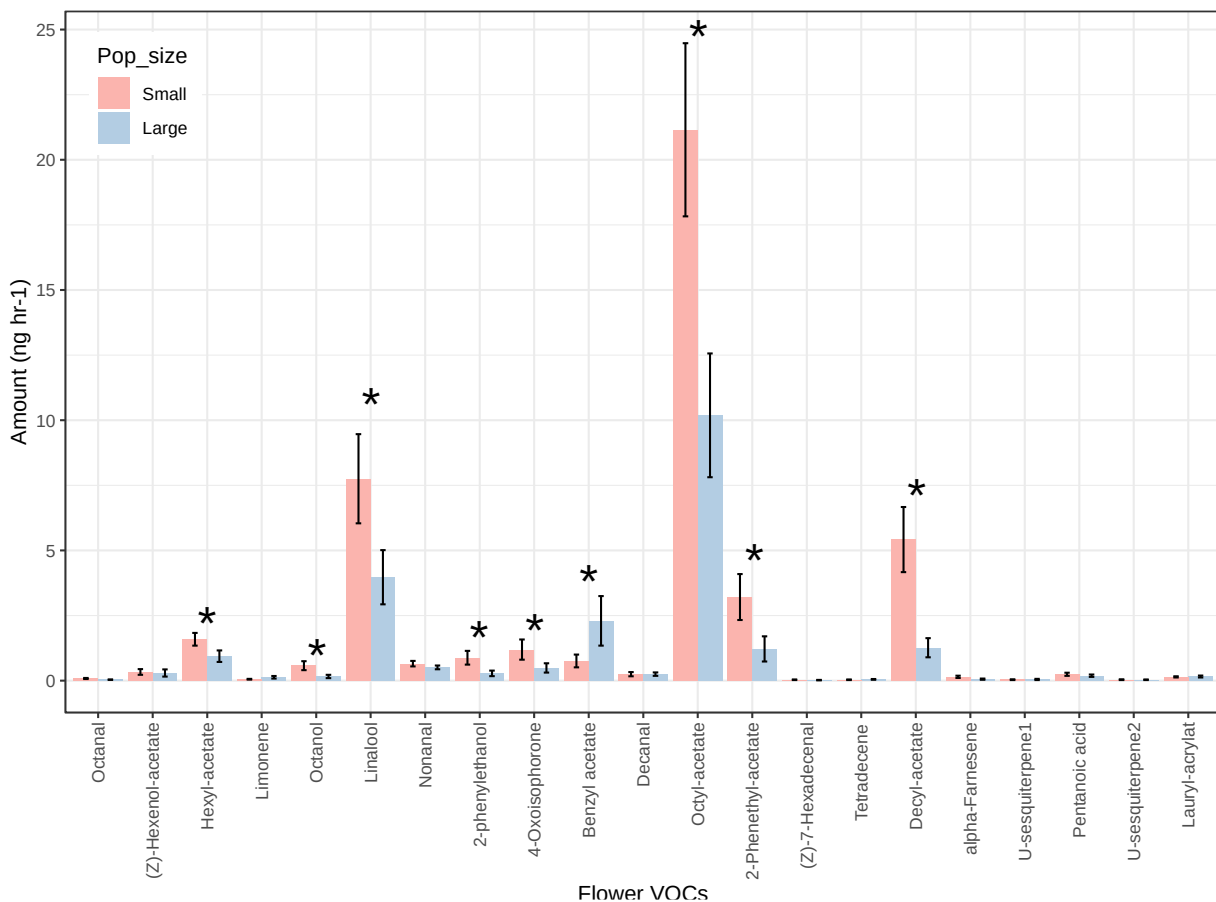


Figure 4. *Cyripedium calceolus* flower VOCs. Barplots show means $\pm$ S.E. for each individual VOC found across small (< 10 individuals, red bars), and large (> 20 individuals, blue bars) populations. Asterisks indicate significant differences across populations sizes ($p < 0.05$).

4. Discussion

We here aimed to understand the role of olfactory signals in maintaining fitness in a rare orchid species. We found that small *C. calceolus* populations occur in less diverse sites, both in terms of surrounding plants and insects. However, while plants in larger populations were physically larger and produce more flowers per capita, they produced the same number of seeds per capita (i.e. seed set) as small populations. This occurred despite the lower pollinator diversity present at the small population sites, meaning that plants from small populations must compensate by providing a stronger attraction to pollinators. Indeed, we discovered that floral VOCs are produced in higher quantity from the flowers of plants from small populations, suggesting that small populations compensate for low pollinator diversity by producing a stronger floral scent.

It is assumed and generally observed, when performing common garden experimental manipulations for example (Barnes et al. 2020; Cook-Patton et al. 2011; Ebeling et al. 2018; Johnson et al. 2006), that insect diversity positively correlates with vegetation diversity at the plot level. By providing a wider range of habitats and other niche axes, more diverse vegetation communities directly favour the presence of a wider range of insect species and trophic groups (Ebeling et al. 2018; Wan et al. 2020). Accordingly, through our survey, we also highlighted a positive link between vegetation diversity and insect diversity, at least for some diversity metrics studied. Interestingly, more diverse sites also corresponded to places where the populations of *C. calceolus* were large (i.e., containing more than 20 individuals). In other words, we found that more diverse sites also harbour more fit *C. calceolus* populations (since large populations of *C. calceolus* also include bigger individuals, with more stems, a large patch size, more vegetative growth, and so on (Rusconi et al. 2022)). Two non-exclusive explanations might drive such patterns. First, large populations of *C. calceolus* grow in sites that are more suitable for vegetation growth, such as having richer soils, and milder climatic conditions, in turn favouring both vegetation and insect diversity (Kull 1999). Second, larger populations grow in places that underwent less habitat destruction and fragmentation than smaller populations, again benefiting other facets of the local biodiversity (Wiegand et al. 2005; Xiao et al. 2016). At this stage, we don't dispose of the necessary means to address both hypotheses, but we advocate for future work in this direction, to further address the interrelated links between the surrounding vegetation diversity, insect diversity and rare species population performance across a broad range of habitats. Looking at our insect surveys from natural history point of view, we found four unreported species of solitary bees and one species of syrphid fly also carrying pollinia on their thorax, and six new *C. calceolus* visitors. Indeed, syrphid flies have also been previously shown to pollinate *C. calceolus* (Braunschmid et al. 2021; Braunschmid et al. 2017), or at least to act as flower visitors on this species (Antonelli et al. 2009; Blinova 2002; Ishmuratova et al. 2006). Our results thus confirm that the range of species pollinating this orchid is wide and composed of generalist pollinators (Braunschmid et al. 2021; Braunschmid et al. 2017), in majority consisting of polylectic short-tongued solitary bees (Antonelli et al. 2009; Braunschmid et al. 2021).

Despite the higher insect pollinator diversity in large *C. calceolus* populations, the reproductive success (i.e. the total number of fruits per patch divided by the total number of flowers per patch) was the same between small and large populations. This occurred even though plants from small populations were smaller in size and produced fewer flowers. Therefore, while individuals produced more flowers and more fruits in absolute terms in larger populations of *C. calceolus*, the probability for a flower to become a fruit is the same in a small or in a large population. This indicates that despite growing in sites with a higher biodiversity, plants from small populations, per capita, can attract as many pollinators as plants in large populations where pollinators are more abundant (see discussion paragraph above). This finding is however in contrast with the hypothesis that pollination efficiency and fruit set depend on pollinator population size and density (Loy and Brosi 2022). For instance, Albrecht et al. (2012) reported enhanced pollination services with more diverse pollinator communities at the plant population level in a study focusing on *Raphanus sativus*. The authors of this study suggested that such a positive relationship is driven both by the presence of specific taxa and the niche complementarity (i.e., species of pollinators that are active at different daytime and that select plant of different height) in the pollinator community and, therefore more pollinator diversity equals more fertilization events (Albrecht et al. 2012). Along these lines, Agren (1996) reported a positive correlation between *Lythrum salicaria* population size and seed set, showing that this occurs due to insufficient pollen transfer when plant populations are small. Similarly, the reproductive success of the orchid *Listera ovata* has been shown to increase with increasing *L. ovata* population size – at least up to 30-40 flowering plants (Brys et al. 2008), and a few studies have shown that *C. calceolus* population size and pollination rate are positively correlated (Brzosko et al. 2017; Nilsson 1979). That said, our results are not unique. Other studies on *C. calceolus* have shown a negative correlation between population size and reproductive success (Internicola et al. 2006). These results were explained based on the fact that *C. calceolus* is a rewardless species and so reproductive success is reduced if pollinators learn to avoid it – which is more likely in larger plant populations. However, we would argue that this explanation is not well supported. Indeed, even if pollinators learn to avoid *C. calceolus* flowers (Aguiar et al. 2020), this would not explain why per flower pollination rate does not vary among populations regardless of fewer pollinators being present in small population sites. We thus suggest that other mechanisms could be involved in generating a negative correlation between population size and pollination rate.

Focusing on the causes of the convergence of the reproductive success between small and large populations, two main hypotheses are emerging. First, it might be that in large populations, *C. calceolus*, pollination success is reduced because it competes with co-flowering species for generalist pollinator (i.e. *C. calceolus* pollinators, see above) visits (Vamosi et al. 2006). Yet, a preliminary analysis of the flower VOCs of *Hieracium murorum* agg., the most common co-flowering species with yellow flowers in our system, showed practically no overlap, in terms of VOCs composition, with *C. calceolus*, suggesting no competition at this level, although not excluding it completely with other untested species. The second hypothesis is that small *C. calceolus* plants can disproportionately attract pollinators by producing more attractive cues. Accordingly, we found that small populations of *C.*

calceolus emitted significantly more floral scents than large populations. Based on our analyses of the 20 most common VOCs, linalool, octyl-acetate, and a series of aliphatic, terpenoid and aromatic compounds (Braunschmid et al. 2021; Braunschmid et al. 2017), a large fraction of those were more abundantly emitted by plants growing in small populations. Emission of floral scents are known to be sensitive to numerous environmental factors (Zu et al. 2016) such as difference between day and night (Balao et al. 2011; Hoballah et al. 2005; Okamoto et al. 2008), air moisture (Jakobsen and Olsen 1994), solar radiation (Jakobsen and Olsen 1994) and temperature (Jakobsen and Olsen 1994; Nielsen et al. 1995). Moreover, biotic processes can also modify scent emission such as pollination (Barragán-Fonseca et al. 2020; Negre et al. 2003; Schiestl and Ayasse 2001; Theis and Raguso 2005) and herbivory (Kessler and Halitschke 2009; Schiestl et al. 2014; Zangerl and Berenbaum 2009). In our case, we can exclude that the observed differences were due to non-random effects of abiotic or biotic factors across populations for three reasons. First, we collected *C. calceolus* VOCs during three consecutive years using a fully randomized sampling design, both for selecting populations and plants within populations. Second, sites were always sampled at the peak of the flowering period, and at the same time of the day when the weather was warm and sunny. Third, we could not visually detect differences in herbivory across large and small populations (OR, personal observations), thus largely excluding the effect of abiotic and biotic factors on the differences in scent emissions between small and large populations. Therefore, to sum up, independently of the proximate causes, all evidence points to the fact that by producing higher levels of floral VOCs, individuals living in small populations compensate the lack of pollinator diversity, and ultimately, per capita, produce the same number of fruits.

In summary, our findings that floral VOC production is higher in small population individuals despite similar pollinator visitation of small and large population individuals together suggest that floral VOCs are crucial for attracting pollinators and thus in maintaining plant reproductive fitness (Burkle and Runyon 2019). Variation in floral VOCs has already been demonstrated at the individual level (Kuppler et al. 2016) and at the population level (Braunschmid et al. 2021). However, our findings provide the first empirical evidence that floral scents may play a role in compensating for the small size of a plant population or reduced local pollinator diversity. Indeed, intraspecific variation in species interaction patterns, in particular floral VOCs, has not been given its due importance during decades (Dicke 2006; Kuppler et al. 2016; Raguso 2008a, b; Whitehead and Peakall 2009). We thus speculate that VOC production is one part of such variation and thus contributes to the maintenance of plant reproductive fitness *via* plant-pollinator interactions (Wei et al. 2021). We further suggest that VOC production may be particularly important in maintaining reproductive fitness in rare plant species. We emphasise that our findings are the first demonstration that VOC production is linked to reproductive fitness and come from an observational study on one species in a small portion of its geographical range. Nevertheless, our findings advocate for explicit consideration of such a coupling in future plant-pollinator research. Such consideration is particularly true for rare plant species that grow in fragmented and pollinator-poor habitats, where reproductive fitness is likely to be low (Frankham et al. 2017). Moreover, these findings could have conservation implications. Indeed, one could argue that it might be worth maintaining and using the genotypes from small populations for future (re)introduction campaigns in

novel habitats, since these genotypes should be better at establishing novel plant-pollinator networks. Second, generating seeds through hand pollination should be made by mating individuals from large populations, which are generally more vigorous, with individuals from small populations, which should produce more flowers.

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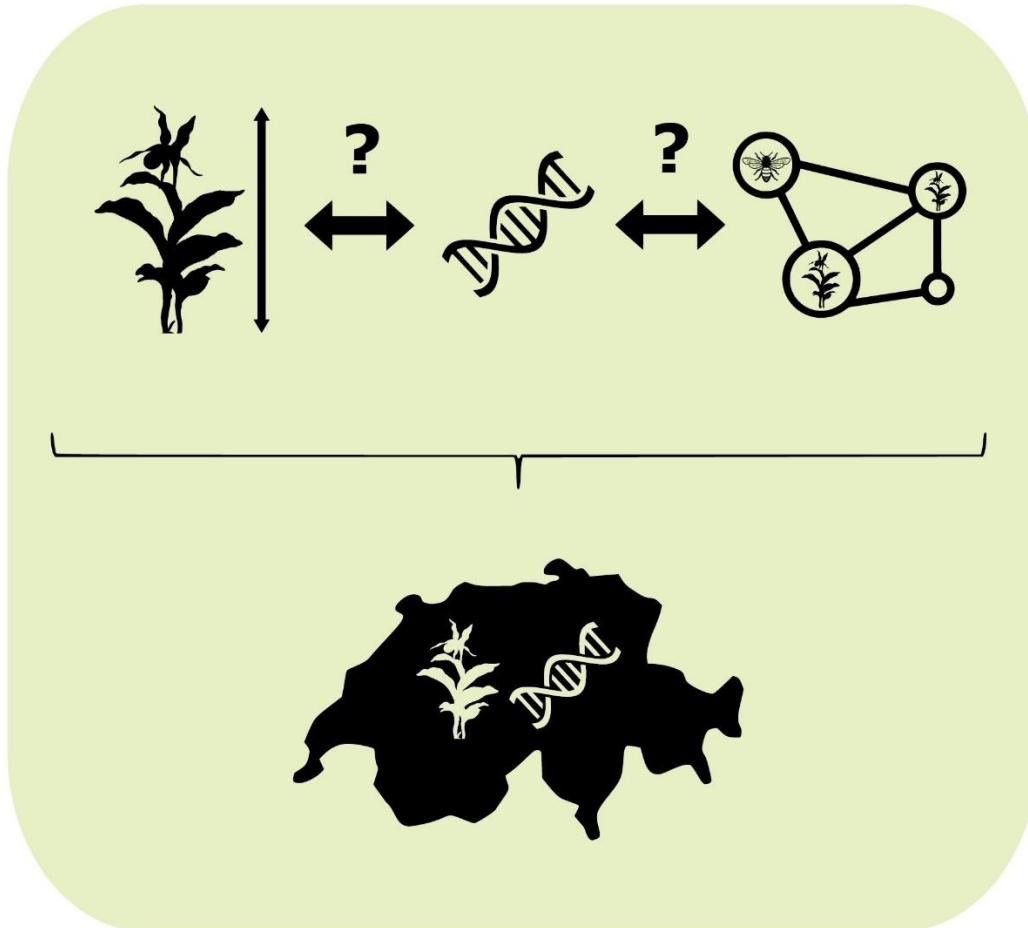
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PERSPECTIVE CHAPTER I: GENETICS AND DEMOGRAPHY OF *CYPRIPEDIUM CALCEOLUS* IN SWITZERLAND



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OR and SR developed the ideas and the sampling design. OR and TS collected the data. FG and RG provided the know-how for genetic analyses. OR, FG, RG and SR analysed the data. OR, TS and SR wrote the first draft of the manuscript.

1. Introduction

In the current times, the viability of plant populations is gravely endangered by habitat loss and fragmentation caused by human activities (Aguilar et al. 2006; Aguilar et al. 2019; Aguilar et al. 2008; Minasiewicz et al. 2018; Vranckx et al. 2012). As a result, the disappearance of large and non-fragmented natural habitats is driving the reduction of natural plant populations' size and number (Browne and Karubian 2018). As a consequence, a decline in genetic variability can be generally observed in natural plant populations, likely caused by random genetic drift and inbreeding (Reed 2004). Such a loss of diversity may diminish the ability of plant populations to respond to environmental changes and increases extinction risks (Minasiewicz et al. 2018; Reed 2004). While such effects are highly visible for annual or ephemeral plants, species capable of clonal reproduction are supposed to be less exposed to the negative effects of habitat fragmentation due to their long life span (e.g., Bossuyt and Honnay 2006; Minasiewicz et al. 2018; Van Geert et al. 2008). On the other hand, long-lived clonal plant species tend to display a lower potential to colonize new habitat patches and to respond to environmental changes, which also may cause local extinctions if their local patches have undergone destruction and fragmentation (Buckley and Freckleton 2010; Wiberg et al. 2016; Zobel et al. 2010). Therefore, from a conservation biology perspective, to better understand the effect of fragmentation on plant population genetics and demography, investigations encompassing both local and regional scales are necessary in order to evaluate these effects across scales.

With this work, we addressed the population genetics of an endangered clonal and outcrossing orchid species: *Cypripedium calceolus* L. (Orchidaceae). This species has suffered a widespread decline throughout its Eurasian range (Devillers-Terschuren 1999). While according to the International Union for Conservation of Nature (IUCN), fragmentation among populations is not considered as a main threat to this orchid (Rankou and Bilz 2014), several studies, on the contrary, are showing that habitat fragmentation might bear negative effects on *C. calceolus* survival, likely reflecting on genetic diversity erosion (e.g., Brzosko et al. 2017; Kull 1997; Minasiewicz et al. 2018). Therefore, in order to improve conservation measures for this species, more fine-grained studies on the population genetic of this species are needed.

The population genetic structure of *C. calceolus* has been studied in Poland (Brzosko 2002; Brzosko et al. 2002; Brzosko et al. 2011), Estonia (Gargiulo et al. 2018), Italy (Gargiulo et al. 2021) and at a broader scale across Europe (Fay et al. 2009). Even though different techniques to characterize genetic diversity of populations were used (e.g., analyses of allozymes, chloroplast DNA and total genomic DNA), the main results concur. First, the genetic diversity within *C. calceolus* is relatively high compared with rare taxa and taxa with the same life history (Brzosko 2002; Brzosko et al. 2002). Second, genetic diversity within populations is much larger than genetic diversity among populations at both national and international scales (Brzosko 2002; Brzosko et al. 2002; Fay et al. 2009). Third, Brzosko et al. (2002) and Gargiulo et al. (2018) did not find a strict correlation between genetic diversity and geographical factors, while Brzosko et al. (2011) found a significant pattern of isolation by distance. Fourth, signatures of a bottleneck effect and recent founder events were identified in Estonia, and genetic and

genotypic diversity variables were significantly correlated with population size in Poland (Brzosko et al. 2011; Gargiulo et al. 2018). Fifth, Brzosko et al. (2002) suggested that the main factors maintaining genetic diversity in *C. calceolus* populations are the longevity of genets, the out-crossing breeding system and the presence of recruitment of seeds. Finally, it has been shown that *C. calceolus* actual genetic differentiation preserves the mark of climatic fluctuations in the historical range of its distribution (Gargiulo et al. 2019).

In this study, we addressed the link between *C. calceolus* population fitness and genetic diversity. Specifically, we aimed (i) to compare the functional traits of *C. calceolus* related to fitness with the genetic diversity of this species (Adler et al. 2014; Díaz et al. 2016; Pérez-Harguindeguy et al. 2016), and (ii) to assess the effect of habitat fragmentation and connectivity of the landscape on genetic differentiation of the species. To this end, we genotyped at 17 microsatellite loci *C. calceolus* individuals and measured functional traits related to plant growth and reproduction in 35 native populations (18 small and 17 large populations) in Switzerland. In addition, we genotyped plants from 4 Swiss populations where *C. calceolus* were reintroduced. Specifically, we predicted that (i) Swiss metapopulations of *C. calceolus* are no more genetically connected, (ii) smaller populations of *C. calceolus* have a smaller genetic pool and (iii) *C. calceolus* populations with a reduced genetic pool have a lower fitness.

2. Material and methods

2.1. Population selection criteria

We chose *C. calceolus* populations of varying sizes from throughout Switzerland based on known occurrences of viable populations (data provided by www.infoflora.ch). We selected a total of 35 native populations, 18 small (i.e., less than 10 individuals per population) and 17 large (i.e., more than 20 individuals per population), encompassing the six major biogeographic regions of Switzerland (Fig 1a, Table 1). In addition, we sampled *C. calceolus* individuals in 4 sites where they were reintroduced (Fig 1a, Table 1).

2.2. Genetic sample collection and plant traits

In each of the 39 studied *C. calceolus* populations (native and reintroduced), we sampled the leaves for genetic analyses ($n = 20$ individuals, or less if there were fewer plants) and we measured ten functional traits with known relationships with plant life history parameters (Adler et al. 2014; Díaz et al. 2016; Pérez-Harguindeguy et al. 2016) that could be quantified using non-intrusive methods. At each of the 34 native site, we sampled traits on a maximum of 10 randomly chosen individuals growing within a 100 m² area and separated by a minimum of 2 m from each other. These 10 randomly chosen individuals were a subset of the 20 individuals we sampled for genetic analysis. Each stem or group of stems (patch), separated by a minimum of 70 cm from each other, was considered as an individual (i.e., a clonal patch) (Kull 1999).

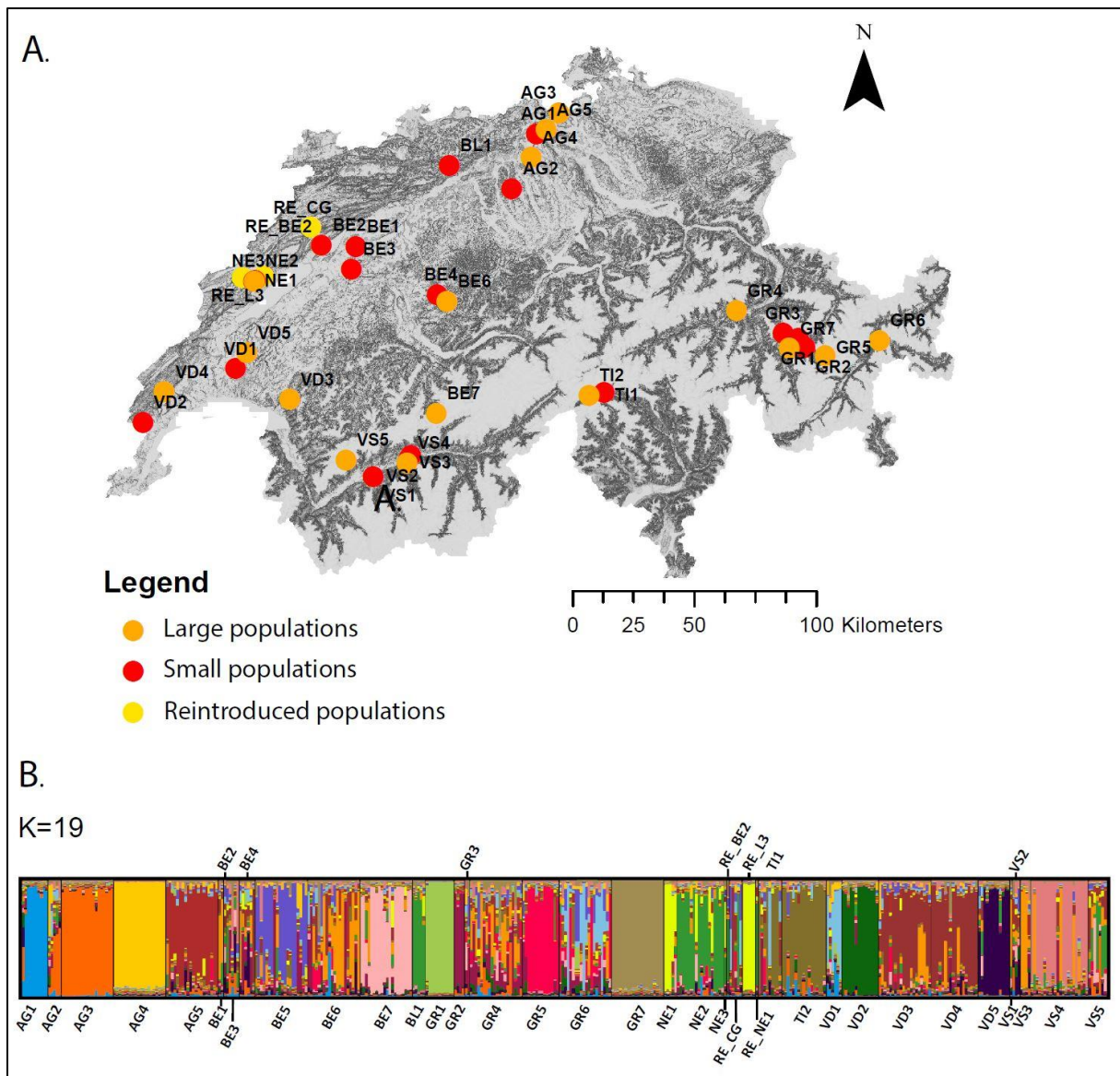


Figure 1. Population genetic sampling for Swiss populations of *C. calceolus*. A. Map of Switzerland showing the 39 populations of *Cypripedium calceolus* visited in the scope of this study. We categorized native populations as being small (i.e., less than 10 inventoried individuals per population; red dots), or large (i.e., more than 20 individuals per population; orange dots). In addition, we studied 4 reintroduced populations (yellow dots). The complete name of each population is given in Table 1. On the map, shades of grey indicate the habitat suitability for *Cypripedium calceolus* based on a climatic niche model with light grey representing low suitability and dark grey high suitability (Rusconi et al. 2022). B. Barplots drawn from CLUMPP average for each of K=19.

Table 1: The 35 Swiss native and the 4 reintroduced *Cypripedium calceolus* populations visited in the scope of this study. Site names are abbreviations of the canton where the population stands and the site number (Aargau: AG, Bern: BE, BL: Basel-Landschaft, GR: Graubünden, NE: Neuchâtel, VD: Vaud, VS: Valais and TI: Ticino). Populations were categorized as being small (i.e., less than 10 inventoried individuals per population), or large (i.e., more than 20 individuals per population).

Site name	Canton	Municipality	Small or large population	Native (N) or reintroduced population (R)
AG1	Aargau	Turgi	Small	N
AG2	Aargau	Gontenschwil	Small	N
AG3	Aargau	Rümikon	Large	N
AG4	Aargau	Dottikon	Large	N
AG5	Aargau	Freienwil	Large	N
BE1	Bern	Kappelen	Small	N
BE2	Bern	La Neuveville	Small	N
BE3	Bern	Radelfingen	Small	N
BE4	Bern	Bowil	Small	N
BE5	Bern	Fahrni	Large	N
BE6	Bern	Röthenbach	Large	N
BE7	Bern	Kandersteg	Large	N
BL1	Basel-Landschaft	Langenbruck	Small	N
GR1	Graubünden	Albula/Alvra	Small	N
GR2	Graubünden	Bergün Filisur	Small	N
GR3	Graubünden	Vaz/Obervaz	Small	N
GR4	Graubünden	Trin	Large	N
GR5	Graubünden	Bergün Filisur	Large	N
GR6	Graubünden	Zernez	Large	N
GR7	Graubünden	Albula/Alvra	Large	N
NE1	Neuchâtel	La Grande Béroche	Small	N
NE2	Neuchâtel	La Grande Béroche	Large	N
NE3	Neuchâtel	La Grande Béroche	Small	N
VD1	Vaud	Bretigny-sur-Morrens	Small	N
VD2	Vaud	Chésereux	Small	N
VD3	Vaud	Saint-Légier-La Chiésaz	Large	N
VD4	Vaud	Saint-George	Large	N
VD5	Vaud	Fey	Large	N
VS1	Valais	Sion	Small	N
VS2	Valais	Salgesch	Small	N
VS3	Valais	Sierre	Small	N
VS4	Valais	Anniviers	Large	N
VS5	Valais	Conthey	Large	N
TI1	Ticino	Airolo	Small	N
TI2	Ticino	Bedretto	Large	N
RE_CG	Bern	Villeret	Large	R
RE_L3	Neuchâtel	La Grande Béroche	Small	R
RE_BE2	Bern	La Neuveville	Large	R
RE_NE1	Neuchâtel	La Grande Béroche	Small	R

For growth-related traits, we measured clonal patch area (cm²), the vegetative plant height (cm), leaf number and median leaf area (cm², ellipse area formula) of the highest stem in a clonal patch and the median leaf photosynthetic activity (SPAD chlorophyllometer, Konica Minolta, Osaka Japan) and specific leaf area (SLA, mm²/mg) of 1 leaf per patch. For reproductive traits, we counted the number of stems and the number of flowers per patch on the first visit, and on the second visit we counted, per patch, the number fruits (pods) and estimated median fruit volume (approximated as a cylinder).

2.3. Genetic analyses

DNA was extracted from dry leaf tissue (427 individuals from 39 populations) using a KingFisher Cell and Tissue DNA Kit (ThermoFisher Scientific, Waltham, Massachusetts, United States), following the manufacturer's protocol. All individuals were genotyped at 17 microsatellite loci (Ccal_5, Ccal_7, Ccal_9, Ccal_19, Ccal_24, Ccal_25, Ccal_34, Ccal_39, Ccal_47, Ccal_48, Ccal_49, Ccal_50, Ccal_51, Ccal_53, IKA8, IKA9, and IM30B)(Gargiulo et al. 2018; Leitch et al. 2009; Minasiewicz and Znaniecka 2014); see Table 2 for specifics of all loci.

Microsatellite loci were PCR-amplified together in multiplex reactions (Table 3 and Table 4). Each of the four multiplexes were amplified in 10 µL final volumes, containing 1.5 µL genomic DNA, 0.3 µL of Bovine serum albumin (BSA), between 0.27 and 0.56 µL each of 10 µM dye-tagged forward primer and un-tagged reverse primer, 4.5 µL of GoTaq G2 Hot Start Taq Polymerase and between 0.08 and 1.75 µL of H2O-Milli Q (Tables 3 and 4). Then, PCR amplifications were carried out with different conditions depending on the multiplexes (Tables 3 and 4).

PCR products of each multiplex were diluted in H2O-Milli Q (10 µL of PCR product/ 20 µL of water). Diluted PCR products (1 µL of multiplex 1.1 and 1 µL of multiplex 1.2 forming Mix1 and respectively 1 µL of multiplex 2.1 and 1 µL of multiplex 2.2 forming Mix2) were added to 15 µL HiDi Liz500 (1000 µL HiDi/12 µL Liz500, Applied Biosystems, Foster City, California, United States) and run on an ABI3730-4 DNA Analyzer (Applied Biosystems, Foster City, California, United States). Afterwards, fragment sizing was realized with GeneMapper v5 (Applied Biosystems, Foster City, California, United States). Chromatograms were manually scored, checked and recorded.

Table 2 : Nuclear microsatellite loci used to in this study. Forward and reverse primer sequences, including fluorescent dye, repeat motifs, fragment size ranges, optimal annealing temperatures (TA), and references for each locus are shown above.

Locus	Primer Sequence (5'-3')	Repeat Motif	Size Range (bp)	T _A (°C)	Reference
Ccal_5	(FAM) F: CCACAAAGCCACACTACATAACA R: TGTAAGGGTGATCTTGGAAGC	(AG) ₉	141–143	62	Minasiewicz and Znaniecka (2014)
Ccal_7	(A532) F: TAAGCACTTCTTGGGAGGCA R: GAGGTTGAGCACAAGAAAGAAA	(AGT) ₁₂	195–207	60	Minasiewicz and Znaniecka (2014)
Ccal_9	(FAM) F: AGAAGAGATGTGGAAGCCC R: CTTCCAAGCTCCAGCTACCA	(AT) ₁₀	122–124	59	Minasiewicz and Znaniecka (2014)
Ccal_19	(FAM) F: CGAGCCTCTCGACAACATCT R: TGGTGGTTGTTTGGTGTGAA	(AT) ₁₀	291–293	60	Minasiewicz and Znaniecka (2014)
Ccal_24	(A532) F: GGCTTATAGAGAGAGAGGCTATGG R: CATGGGAGCTGACTCATCAT	(ATC) ₁₃	118–123	59	Minasiewicz and Znaniecka (2014)
Ccal_25	(A532) F: CAGCATTTTGTCTAATGTCTTT R: CAGATAATGGCCCTTGGTC	(ATC) ₁₆	162–180	60	Minasiewicz and Znaniecka (2014)
Ccal_34	(A565) F: CATGGAAGGGAATAACATCCT R: TGCAATTCCATGTACTTGTTCTTA	(AT) ₁₀	128–136	58	Minasiewicz and Znaniecka (2014)
Ccal_39	(FAM) F: TTCTCCTCAAAGAATGATTCCA R: CCATTGGGCAATTCATCAT	(AC) ₁₅	154–164	59	Minasiewicz and Znaniecka (2014)
Ccal_47	(A532) F: AAGGCTCAAGATCCCAAGGA R: ATCATTATGGTTGTCTCTTTATCGTT	(AAC) ₁₁	127–136	60	Minasiewicz and Znaniecka (2014)
Ccal_48	(A550) F: CAATAAGCTAAGTGAGTAGCAGGTTG R: AGGTTCTTCTTTCATTCCTACTACC	(AAC) ₁₂	141–150	61	Minasiewicz and Znaniecka (2014)
Ccal_49	(A565) F: TGAAGGGTCATGTTACTAGCAG R: TGGTGATGACACAATACTCCA	(AAG) ₁₃	115–143	58	Minasiewicz and Znaniecka (2014)
Ccal_50	(A550) F: GAGAAGGGATTCAATAGGTTTGG R: AAGTTCCTTCTCATTTCTAGCTCTC	(AAG) ₁₀	122–134	60	Minasiewicz and Znaniecka (2014)
Ccal_51	(A565) F: TTCTCCTCAAAGAATGATTCCA R: CCATTGGGCAATTCATCAT	(AAG) ₈	174–177	60	Minasiewicz and Znaniecka (2014)
Ccal_53	(FAM) F: CCTACCTCCACCTGACACA R: TGAGGCCTAGGCTAGCAAGT	(AAG) ₁₃	164–185	60	Minasiewicz and Znaniecka (2014)
IK8	(A565) F: GTGATGGGGTTAATGACAAAGG R: GGCAGCTATGATGATGATGATG	(CTT) _n or (CAT) _n	200-206	58	Leitch et al. (2009)
IK9	(A550) F: GCTGATGGTGATGATGCTTAG R: CCTTTATAGTTGTGAAAGAGGG	(GAT) _n	167-185	56	Leitch et al. (2009)
IM30B	(FAM) F: TGAGTCGATGTGTTGAGGTGA R: GAGTGTGCATGAGCATCGAA	(AAG) ₈	227	60	Gargiulo et al. (2018)

Table 3 : DNA microsatellite loci and other substances used to in Mix1 for PCR reactions.

Mix1					
PCR Multiplex 1.1			PCR Multiplex 1.2		
Substance	Concentration	Volume [μ l]	Substance	Concentration	Volume [μ l]
GoTaq G2 HotStart		4.5	GoTaq G2 HotStart		4.5
Ccal_5_F_FAM	10 μ M	0.28	Ccal_34_F_A565	10 μ M	0.4
Ccal_5_R	10 μ M	0.28	Ccal_34_R	10 μ M	0.4
Ccal_7_F_A532	10 μ M	0.3	Ccal_47_F_A532	10 μ M	0.37
Ccal_7_R	10 μ M	0.3	Ccal_47_R	10 μ M	0.37
Ccal_9_F_FAM	10 μ M	0.3	Ccal_53_F_FAM	10 μ M	0.42
Ccal_9_F_R	10 μ M	0.3	Ccal_53_R	10 μ M	0.42
Ccal_19_F_FAM	10 μ M	0.28	BSA	10 μ M	0.3
Ccal_19_R	10 μ M	0.28	H ₂ O		1.32
Ccal_48_F_A500	10 μ M	0.35	DNA	10 ng/ μ l	1.5
Ccal_48_R	10 μ M	0.35	Total		10
Ccal_51_F_A565	10 μ M	0.3			
Ccal_51_R	10 μ M	0.3			
BSA	10 μ M	0.3			
H ₂ O		0.08			
DNA	10 ng/ μ l	1.5			
Total		10			
PCR cycling profile Multiplex 1.1			PCR cycling profile Multiplex 1.2		
95 °C	5 min		95 °C	5 min	
95 °C	30 s		95 °C	30 s	
60 °C	30 s	31 cycles	60 °C	30 s	34 cycles
72 °C	45 s		72 °C	45 s	
70° C	30 min		70° C	30 min	
10 °C	∞		10 °C	∞	

Table 4 : DNA microsatellite loci and other substances used to in Mix2 for PCR reactions.

Mix2					
PCR Multiplex 2.1			PCR Multiplex 2.2		
Substance	Concentration	Volume [μ l]	Substance	Concentration	Volume [μ l]
GoTaq G2 HotStart		4.5	GoTaq G2 HotStart		4.5
Ccal_39_F_FAM	10 μ M	0.4	Ccal_24_F_A532	10 μ M	0.27
Ccal_39_R	10 μ M	0.4	Ccal_24_R	10 μ M	0.27
IKA8_F_A565	10 μ M	0.3	Ccal_25_F_A532	10 μ M	0.35
IKA8_R	10 μ M	0.3	Ccal_25_R	10 μ M	0.35
IKA9_F_A550	10 μ M	0.125	Ccal_49_F_A565	10 μ M	0.56
IKA9_R	10 μ M	0.125	Ccal_49_R	10 μ M	0.56
IM30B_F_FAM	10 μ M	0.15	Ccal_50_A550	10 μ M	0.4
IM30B_R	10 μ M	0.15	Ccal_50_R	10 μ M	0.4
BSA	10 μ M	0.3	BSA	10 μ M	0.3
H ₂ O		1.75	H ₂ O		0.54
DNA	10 ng/ μ l	1.5	DNA	10 ng/ μ l	1.5
Total		10	Total		10
PCR cycling profile Multiplex 2.1			PCR cycling profile Multiplex 2.2		
95 °C	5 min		95 °C	5 min	
95 °C	30 s		95 °C	30 s	
59 °C	30 s	38 cycles	60 °C	30 s	30 cycles
72 °C	45 s		72 °C	45 s	
72° C	30 min		70° C	30 min	
10 °C	∞		10 °C	∞	

2.4. Statistical analysis

2.4.1. Data preparation

After having obtained sequences for microsatellites loci, 13 individuals presented missing data and were removed from the dataset (initial number of individuals: 427, removed: 13, kept: 414). Moreover, one nuclear microsatellite locus (IKA8) produced unexploitable data and was excluded for further statistical analysis.

2.4.2. Null Alleles and Hardy-Weinberg Equilibrium

First, we used MICRO-CHECKER 4.05 (Van Oosterhout et al. 2004) to test for homozygote excess at each locus, which may suggest the presence of null alleles. To ensure that our microsatellite data meets assumptions of Hardy-Weinberg (HW) equilibrium and independence of loci, we tested for any departures from HW equilibrium in R (R Core Development Team 2021) using the *pegas* package (Paradis 2010).

2.4.3. Genetic clustering, Discriminant analysis of principal components (DAPC) and Pairwise F_{ST}

To test for the presence of genetic clusters across the sampled range, we first used a model-based clustering method using STRUCTURE 2.3.4 (Pritchard et al. 2000). We employed the Bayesian maximum likelihood approach that does not explicitly require data on sampling locations as prior information. After a burn-in period of 100,000 replicates, we ran 500,000 Markov chain Monte Carlo (MCMC) iterations in each run. We initially tested all values of K from 1 to 39 across the entire range (i.e. the number of sampled sites in this study), with 20 replicates for each K value. We then uploaded the results to Structure Harvester (Earl and von Holdt 2012) to determine the log probability of the generated data, $\ln P(K)$, and ΔK , a measure of the rate of change in $\ln P(K)$ over the range of K values tested. A spike in the ΔK graph indicates the most probable K value (Evanno et al. 2005), and after identifying this K value and running an additional 20 replicates for it, we averaged all 20 runs in CLUMPP 1.1 (Jakobsson and Rosenberg 2007) and visualized the results in DISTRUCT (Rosenberg 2004), as implemented in the CLUMPAK web pipeline (Kopelman et al. 2015).

Secondly, in order to visualise the optimal number of clusters found in the previous step, we produced a dendrogram plot using the function *hclust* in the R package *stats* (R Core Development Team 2021). We performed a DAPC which particularly relevant to infer clusters for genetically related individuals (Jombart et al. 2010). Moreover, we calculated pairwise F_{ST} between populations in R using the *HierFstat* package (Goudet 2005; Nei 1987).

3. First results

3.1. Null Alleles and Hardy-Weinberg Equilibrium

The probability of having null alleles in our dataset was negligible. We observed no major deviation from Hardy-Weinberg Equilibrium.

3.2. Genetic clustering, DAPC and Pairwise F_{ST}

The ΔK table for STRUCTURE runs from $K=1$ to $K=39$ indicated that $K=19$ was the most likely number of clusters ($\Delta K=8.8$). Barplots drawn from CLUMPP averages for each of $K=19$ are presented in Figure 1b. Most of the populations had a distinct genetic signature (e.g., AG1, AG4 and AG5; Figure 1b). We observed that genetic clustering was not always related to geographical distribution. On the other hand, some populations were composed with individuals with a heterogeneous genetic composition (e.g., BE3, GR4 and GR6; Figure 1b). The hierarchical clustering shows the same tendencies (Figures 2a and 2b). When visualizing genetic differentiation using DAPC, we found that 2 clusters were substantially differentiated from the rest, these were populations GR1 and GR7 (Figure 3). Pairwise F_{ST} values are shown in Table 4.

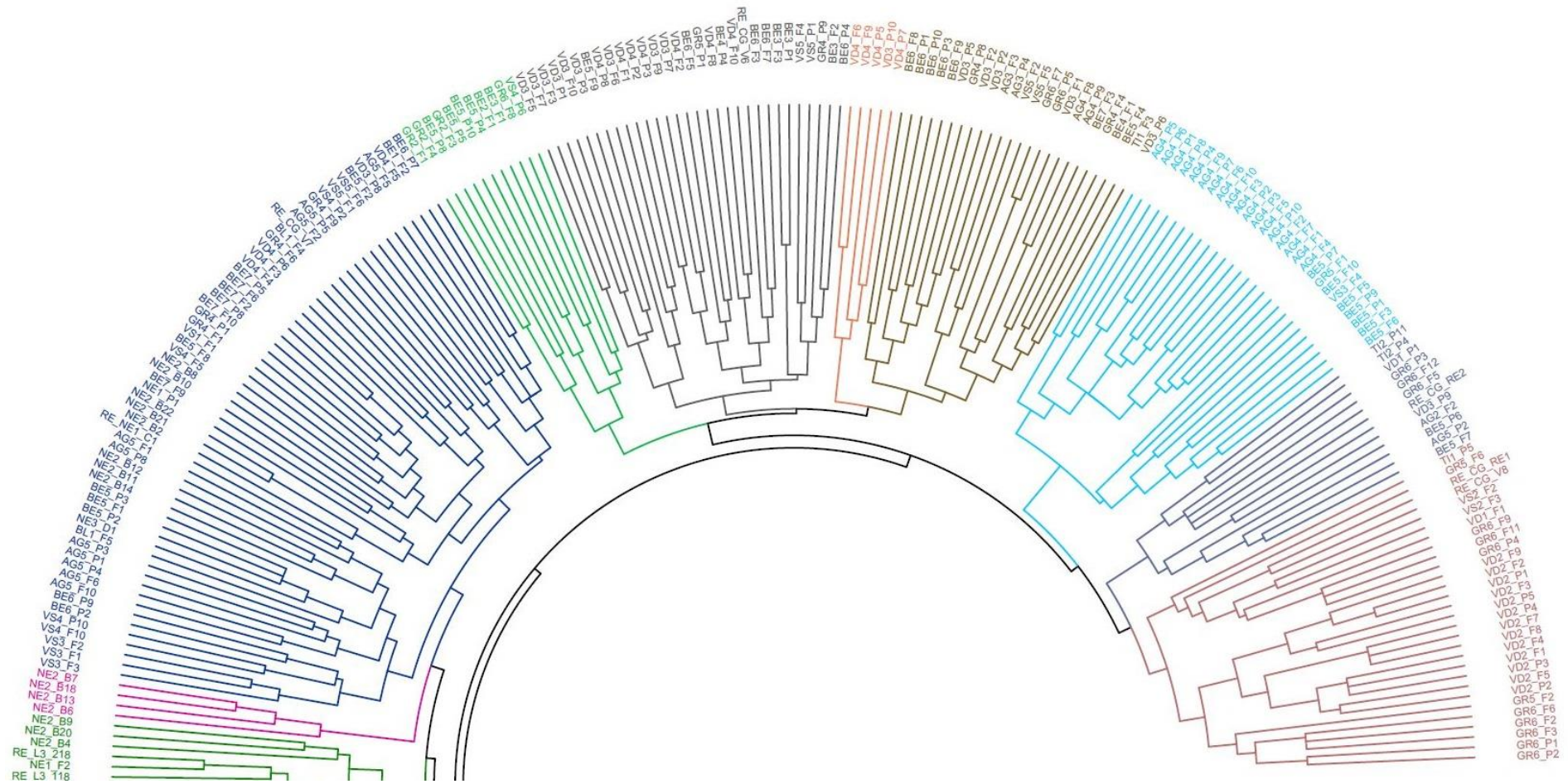


Figure 2a. Hierarchical clustering of *Cypripedium calceolus* displayed as a radial dendrogram. Each genetic cluster (K=19) is represented in a different colour. The dendrogram is split in two parts for readability purposes. The second half of the graph is shown in Figure 2b.

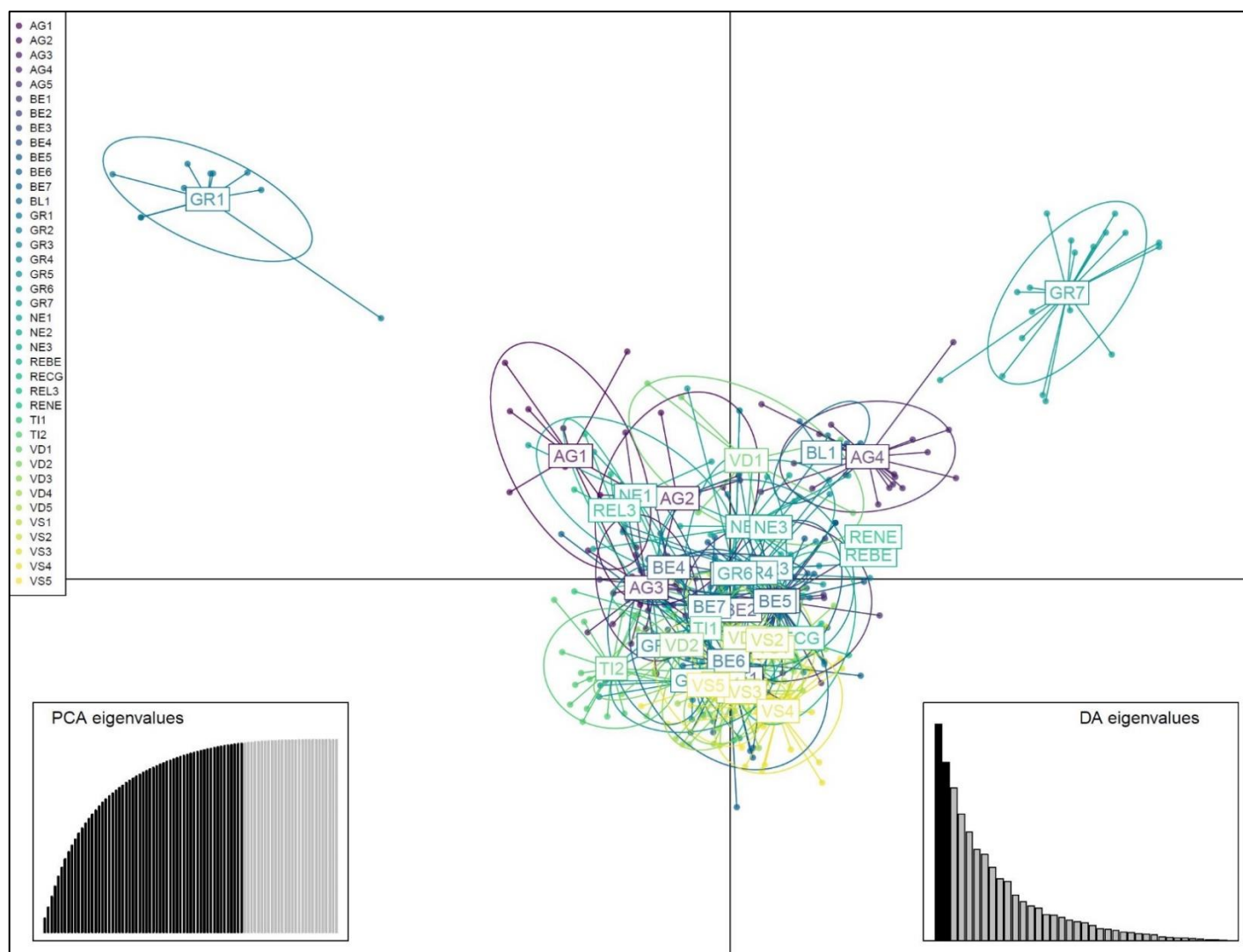


Figure 3. Discriminant analysis of principal components (DAPC) plots of the 39 *Cypripedium calceolus* sampled populations. Inset plots: The darkened portion represents the retained principal components, and the proportion of the variance they explain.

Table 5. Pairwise F_{st} values among all studied populations. Population pairs with F_{st} values <0.15 (red) are generally considered to be moderately differentiated, and population pairs with F_{st} values >0.20 (green) are highly differentiated.

VS5-	0.218	0.086	0.155	0.212	0.093	0.035	0.035	0.071	0.079	0.116	0.04	0.066	0.129	0.33	0.142	0.061	0.081	0.139	0.11	0.212	0.108	0.038	0	0.048	0.09	0.143	0.039	0.162	0.181	0.251	0.223	0.054	0.064	0.124	0	0.026	0.126	0.108		
VS4-	0.286	0.136	0.238	0.22	0.12	0.041	0.114	0.131	0.125	0.152	0.083	0.158	0.201	0.401	0.238	0.099	0.087	0.147	0.108	0.217	0.2	0.112	0.06	0.074	0.101	0.256	0.12	0.17	0.204	0.272	0.193	0.127	0.137	0.152	0.026	0.07	0.127	0.108		
VS3-	0.369	0.202	0.259	0.242	0.158	0.068	0.3	0.218	0.17	0.159	0.113	0.195	0.267	0.422	0.322	0.195	0.137	0.263	0.16	0.304	0.261	0.155	0.144	0.195	0.189	0.334	0.287	0.278	0.302	0.339	0.322	0.146	0.168	0.18	0.148	0.115		0.127	0.126	
VS2-	0.198	0.04	0.163	0.144	0.038	0	0	0.049	0.015	0.06	0	0.072	0.126	0.333	0.178	0.039	0.037	0.111	0.042	0.141	0.106	0.103	0	0	0.052	0.187	0	0.109	0.109	0.147	0.123	0.044	0.077	0.013	0		0.115	0.07	0.026	
VS1-	0.27	0.086	0.25	0.195	0.074	0		0	0.062	0.014	0	0.067	0.119	0.45	0.246	0.053	0.034	0.059	0.021	0.183	0.12	0.001			0	0.238		0.029	0.089	0.233	0.216	0.026	0.037	0.128		0	0.148	0.026	0	
VD5-	0.265	0.118	0.202	0.191	0.129	0.158	0.125	0.151	0.086	0.15	0.092	0.16	0.251	0.388	0.247	0.118	0.103	0.211	0.132	0.217	0.192	0.188	0.12	0.237	0.167	0.256	0.216	0.19	0.167	0.186	0.175	0.149	0.152		0.128	0.013	0.18	0.152	0.124	
VD4-	0.241	0.138	0.17	0.196	0.151	0.059	0.113	0.091	0.071	0.126	0.074	0.135	0.215	0.351	0.165	0.098	0.119	0.136	0.094	0.207	0.196	0.12	0.02	0.143	0.063	0.243	0.116	0.159	0.221	0.214	0.198	0.058		0.152	0.037	0.077	0.168	0.137	0.064	
VD3-	0.249	0.138	0.194	0.166	0.115	0.058	0.057	0.088	0.067	0.073	0.047	0.13	0.167	0.35	0.173	0.125	0.113	0.136	0.104	0.206	0.171	0.116	0.013	0.096	0.069	0.24	0.129	0.149	0.172	0.248	0.246		0.058	0.149	0.026	0.044	0.146	0.127	0.054	
VD2-	0.294	0.171	0.296	0.289	0.244	0.215	0.289	0.218	0.194	0.232	0.187	0.227	0.375	0.434	0.285	0.188	0.178	0.217	0.152	0.278	0.278	0.276	0.256	0.394	0.221	0.307	0.362	0.228	0.236	0.272		0.246	0.198	0.175	0.216	0.123	0.322	0.193	0.223	
VD1-	0.284	0.176	0.329	0.213	0.249	0.258	0.179	0.23	0.222	0.229	0.194	0.282	0.372	0.419	0.309	0.112	0.213	0.273	0.136	0.284	0.325	0.289	0.25	0.363	0.188	0.38	0.366	0.207	0.27		0.272	0.248	0.214	0.186	0.233	0.147	0.339	0.272	0.251	
TI2-	0.295	0.122	0.235	0.219	0.187	0.216	0.104	0.151	0.156	0.129	0.147	0.233	0.287	0.416	0.211	0.143	0.156	0.178	0.156	0.242	0.201	0.237	0.063	0.259	0.166	0.256	0.217	0.097		0.27	0.236	0.172	0.221	0.167	0.089	0.109	0.302	0.204	0.181	
TI1-	0.3	0.103	0.25	0.19	0.175	0.164	0.001	0.154	0.142	0.128	0.105	0.228	0.276	0.39	0.171	0.074	0.125	0.128	0.087	0.214	0.189	0.198	0	0.178	0.099	0.218	0.133	0	0.097	0.207	0.228	0.149	0.159	0.19	0.029	0.109	0.278	0.17	0.162	
RENE-	0.353	0.134	0.313	0.254	0.013	0.103		0.075	0.097	0.152	0.02	0.135	0.144	0.489	0.247	0.158	0.087	0.082	0.103	0.249	0.06	0			0.069	0.18		0.133	0.217	0.366	0.362	0.129	0.116	0.216		0	0.287	0.12	0.039	
REL3-	0.332	0.164	0.3	0.268	0.198	0.248	0.273	0.199	0.193	0.25	0.194	0.19	0.281	0.401	0.289	0.267	0.2	0.248	0.211	0.339	0.001	0.132	0.068	0.346	0.28		0.18	0.218	0.256	0.38	0.307	0.24	0.243	0.256	0.238	0.187	0.334	0.256	0.143	
REC3-	0.246	0.136	0.215	0.186	0.152	0.026	0.024	0.082	0.126	0.098	0.067	0.159	0.165	0.387	0.183	0.024	0.124	0.083	0.07	0.188	0.21	0.121	0	0.035		0.28	0.069	0.099	0.166	0.188	0.221	0.069	0.063	0.167	0	0.052	0.189	0.101	0.09	
REBE-	0.321	0.142	0.271	0.281	0.095	0.015		0.055	0.1	0.121	0.004	0.152	0	0.458	0.224	0.095	0.062	0.17	0.13	0.186	0.146	0.045			0.035	0.346		0.178	0.259	0.363	0.394	0.096	0.143	0.237		0	0.195	0.074	0.048	
NE3-	0.208	0	0.159	0.111	0	0.013		0	0	0	0	0.082	0.042	0.384	0.131	0.076	0	0.073	0	0.13	0	0			0	0.068		0	0.063	0.25	0.256	0.013	0.02	0.12		0	0.144	0.06	0	
NE2-	0.259	0.14	0.22	0.217	0.126	0.071	0.098	0.087	0.114	0.174	0.095	0.107	0.11	0.347	0.206	0.123	0.114	0.157	0.132	0.227	0.093		0	0.045	0.121	0.132	0	0.198	0.237	0.289	0.276	0.116	0.12	0.188	0.001	0.103	0.155	0.112	0.038	
NE1-	0.277	0.149	0.262	0.196	0.152	0.154	0.151	0.151	0.121	0.196	0.148	0.132	0.154	0.331	0.253	0.201	0.16	0.212	0.182	0.274		0.093	0	0.146	0.21	0.001	0.06	0.189	0.201	0.325	0.278	0.171	0.196	0.192	0.12	0.106	0.261	0.2	0.108	
GR7-	0.29	0.144	0.243	0.251	0.175	0.205	0.206	0.178	0.166	0.142	0.126	0.236	0.221	0.431	0.233	0.156	0.117	0.217	0.165		0.274	0.227	0.13	0.186	0.188	0.339	0.249	0.214	0.242	0.284	0.278	0.206	0.207	0.217	0.183	0.141	0.304	0.217	0.212	
GR6-	0.223	0.077	0.194	0.133	0.116	0.105	0	0.1	0.078	0.08	0.061	0.153	0.211	0.304	0.15	0.037	0.083	0.089		0.165	0.182	0.132	0	0.13	0.07	0.211	0.103	0.087	0.156	0.136	0.152	0.104	0.094	0.132	0.021	0.042	0.16	0.108	0.11	
GR5-	0.28	0.142	0.246	0.214	0.163	0.108	0.12	0.159	0.135	0.151	0.11	0.157	0.237	0.372	0.179	0.09	0.121		0.089	0.217	0.212	0.157	0.073	0.17	0.083	0.248	0.082	0.128	0.178	0.273	0.217	0.136	0.136	0.211	0.059	0.111	0.263	0.147	0.139	
GR4-	0.223	0.048	0.13	0.199	0.072	0.059	0.045	0.121	0.096	0.105	0.031	0.108	0.177	0.338	0.164	0.032		0.121	0.083	0.117	0.16	0.114	0	0.062	0.124	0.2	0.087	0.125	0.156	0.213	0.178	0.113	0.119	0.103	0.034	0.037	0.137	0.087	0.081	
GR3-	0.224	0.045	0.149	0.205	0.106	0.078	0	0.068	0.142	0.122	0.039	0.108	0.192	0.347	0.127		0.032	0.09	0.037	0.156	0.201	0.123	0.076	0.095	0.024	0.267	0.158	0.074	0.143	0.112	0.188	0.125	0.098	0.118	0.053	0.039	0.195	0.099	0.061	
GR2-	0.3	0.155	0.224	0.295	0.197	0.229	0.088	0.159	0.163	0.155	0.131	0.256	0.293	0.361		0.127	0.164	0.179	0.15	0.233	0.253	0.206	0.131	0.224	0.183	0.289	0.247	0.171	0.211	0.309	0.285	0.173	0.165	0.247	0.246	0.178	0.322	0.238	0.142	
GR1-	0.402	0.299	0.415	0.404	0.369	0.364	0.448	0.363	0.284	0.348	0.329	0.377	0.403		0.361	0.347	0.338	0.372	0.304	0.431	0.331	0.347	0.384	0.458	0.387	0.401	0.489	0.39	0.416	0.419	0.434	0.35	0.351	0.388	0.45	0.333	0.422	0.401	0.33	
BL1-	0.31	0.219	0.284	0.259	0.195	0.133	0.189	0.15	0.184	0.188	0.144	0.147		0.403	0.293	0.192	0.177	0.237	0.211	0.221	0.154	0.11	0.042	0	0.165	0.281	0.144	0.276	0.287	0.372	0.375	0.167	0.215	0.251	0.119	0.126	0.267	0.201	0.129	
BE7-	0.248	0.138	0.178	0.218	0.156	0.074	0.137	0.15	0.108	0.177	0.108		0.147	0.377	0.256	0.108	0.108	0.157	0.153	0.236	0.132	0.107	0.082	0.152	0.159	0.19	0.135	0.228	0.233	0.282	0.227	0.13	0.135	0.16	0.067	0.072	0.195	0.158	0.066	
BE6-	0.219	0.07	0.159	0.177	0.074	0.022	0	0.079	0.059	0.067		0.108	0.144	0.329	0.131	0.039	0.031	0.11	0.061	0.126	0.148	0.095	0	0.004	0.067	0.194	0.02	0.105	0.147	0.194	0.187	0.047	0.074	0.092	0	0	0.113	0.083	0.04	
BE5-	0.267	0.115	0.198	0.146	0.12	0.116	0.05	0.116	0.096		0.067	0.177	0.188	0.348	0.155	0.122	0.105	0.151	0.08	0.142	0.196	0.174	0	0.121	0.098	0.25	0.152	0.128	0.129	0.229	0.232	0.073	0.126	0.15	0.014	0.06	0.159	0.152	0.116	
BE4-	0.214	0.087	0.157	0.16	0.118	0.081		0.046	0.083		0.096	0.059	0.108	0.184	0.284	0.163	0.142	0.096	0.135	0.078	0.166	0.121	0.114	0	0.1	0.126	0.193	0.097	0.142	0.156	0.222	0.194	0.067	0.071	0.086	0.062	0.015	0.17	0.125	0.079
BE3-	0.192	0.106	0.176	0.213	0.148	0.125	0		0.083	0.116	0.079	0.15	0.15	0.363	0.159	0.068	0.121	0.159	0.1	0.178	0.151	0.087	0	0.055	0.082	0.199	0.075	0.154	0.151	0.23	0.218	0.088	0.091	0.151	0	0.049				

4. Summary of this study and further perspectives

From our first preliminary analyses performed on 35 Swiss populations of *C. calceolus*, we were able to highlight several (19) different genetic clusters spread across Switzerland. What we observed, and according to the predictions, most of Swiss populations were genetically distinct (e.g., AG1, AG4 and AG5), however, at the population level, genetic similarities were not related to population location. For example, populations from the Canton Aargau (AG) are geographically close to one another but exhibit highly distinct genetic patterns. Moreover, according to pairwise F_{ST} values and the DAPC analysis, populations such as GR1 and GR7, which are relatively disconnected geographically to the rest of Switzerland, are indeed highly genetically differentiated from the others.

While these preliminary observations are encouraging in terms of better understanding the genetic underlying the populations structure across the regions of Switzerland, future work needs to be devoted to (i) compare genetic patterns of small, large and reintroduced *C. calceolus*, (ii) link between genetic patterns to plant functional traits and (iii) integrate connectivity analysis that were performed in the framework of a Msc thesis included in this PhD.

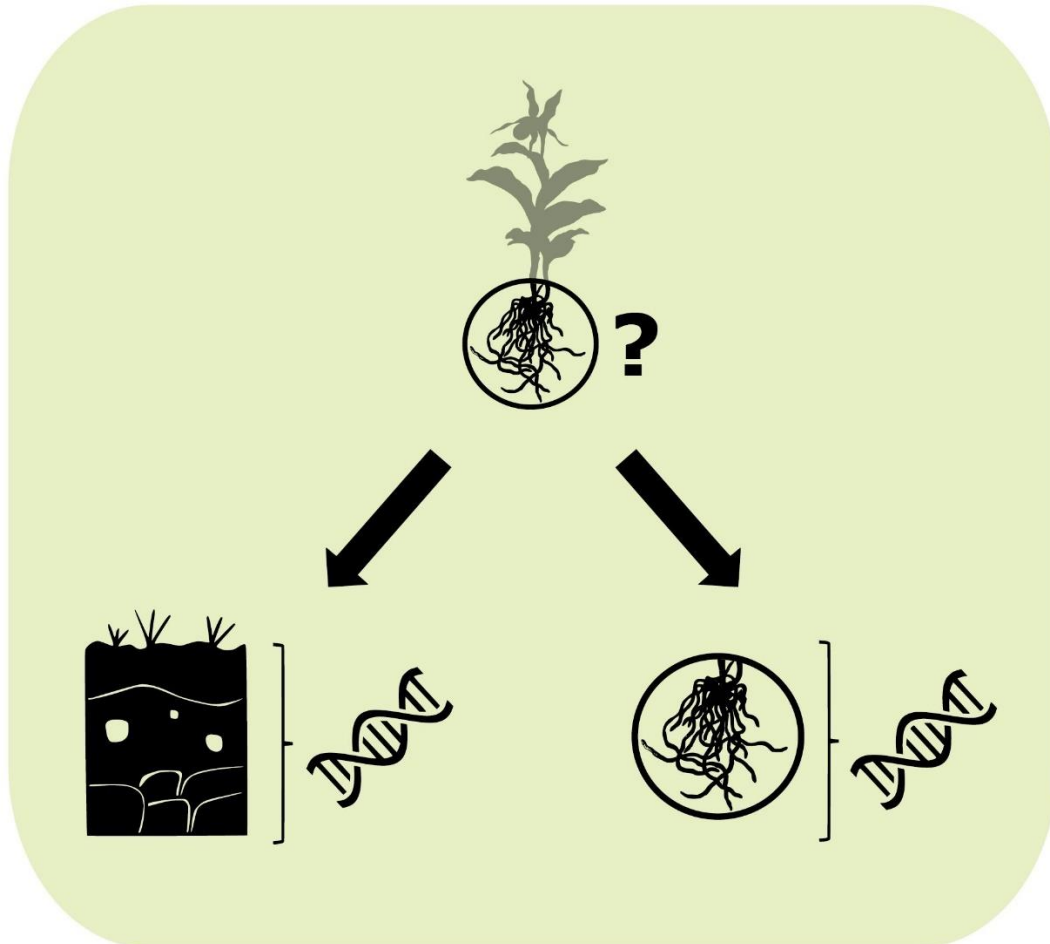
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PERSPECTIVE CHAPTER II: *CYPRIPEDIUM CALCEOLUS* - MYCORRHIZAL INTERACTIONS



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AUTHOR CONTRIBUTIONS

OR and SR developed the ideas and the sampling design. OR and BC collected the data. BC did the DNA extraction. GL analysed the data. OR, GL and SR wrote the first draft of the manuscript.

1. Introduction

Orchidaceae, as 80% of the plant families in the world, are partially mycoheterotrophic (Bidartondo et al. 2004; Gebauer and Meyer 2003; Smith and Read 2010). While most plant species are associated with arbuscular or ectomycorrhiza, orchids seems to be associated with mycorrhizae that are sufficiently distinct from the rest that mycologist have grouped them into a distinct guild of fungi called “orchids type mycorrhizae” (OM) (Smith and Read 2010). The general dogma is that because orchids have dust-like seeds (0.3-14 µg), with minimal nutrients reserves, their interaction with OM is vital for the plant to overcome the first steps of germination and development (e.g., Sathiyadash et al. 2012; Smith and Read 2010). On the other hand, there is also the common assumption that during the adult phase, full photosynthetic capacity relieves the orchids from their fungal dependence (Sathiyadash et al. 2012; Smith and Read 2010). However, numerous studies observed the presence of OM also during the adult phase, but with still little knowledge of the physiological interactions between the two (e.g., Fay et al. 2018; Sathiyadash et al. 2012; Shefferson et al. 2007). For instance, Fay et al. (2018), by analysing the enrichment of isotopes of carbon (C), nitrogen (N) and hydrogen (H), which is indicative for exchange of matter between fungi and orchids in plant leaves, confirmed healthy mycorrhizal activity on all adult individuals (native and introduced plants) studied (Fay et al. 2018). In addition, Roy et al. (2009) showed that the relationships with mycorrhizae can change during an orchid’s lifetime with different fungal species or genotypes playing different roles at different life stages (Fay et al. 2015).

Such complex ecological interactions between orchids and mycorrhizal fungi (Swarts and Dixon 2017) is believed to drive the precariousness of several orchid species’ survival (Phillips et al. 2011). Indeed, as mentioned above, the initial dependence to OM can be thought to dramatically influence the capability of most orchids to germinate and colonize new areas (De Long et al. 2012; Fay et al. 2015). Accordingly, the rarity of some orchid species could be linked to the sparse distribution or narrow ecological requirements of their OM partners (e.g., Fay et al. 2015; Nurfadilah et al. 2013; Phillips et al. 2011). Consequently, a better understanding of the association, distribution and ecological requirements of different OM within the distribution of their plant host is needed to improve conservation strategies for orchid species that are threatened to disappear in the near future.

The rare Lady’s slipper orchid *Cypripedium calceolus* L. (Orchidaceae) is an iconic species of high relevance for understanding orchid-OM interaction within the context of conservation biology. While the species is broadly distributed from the United Kingdom to the Pacific Ocean (Kull 1999), the number and size of its populations have drastically declined over the last decades and is now listed in the IUCN red list as threatened or critically endangered in multiple countries (Devillers-Terschuren 1999; Rankou and Bilz 2014). In addition to overharvesting and habitat destruction, its disappearance can be linked to its biology and ecological interactions (Swarts and Dixon 2009), including the lack of favourable conditions to form the symbioses with different OM species. However, this broad-level knowledge is yet lacking for this species. Indeed, while *C. calceolus* plants have been reported to be almost exclusively associated with fungi from the Tulasnellaceae family (Shefferson et al. 2007;

Shefferson et al. 2005), the entire community of mycorrhizal species interacting with *C. calceolus* has not been clearly identified yet (Fay et al. 2018). Moreover, to date, it is still not known whether the fungi involved in the mycorrhizal relationships with adult plants (Fay et al. 2018) are a mere reflection of what is found in the surrounding soil fungal community or if adult plants carry imprints from the seedling stage, independently of the local conditions. Interestingly, Shefferson et al. (2007) observed a lack of morphological evidence of mycorrhizal colonization in some *C. calceolus* seedlings, whereas adult plants always exhibited pelotons. While these results counter the initial hypothesis that seedlings should be more dependent to OM than adult plants, they again raise the question of whether young seedlings are actually mycorrhized, and if yes, how different this fungal community is from the one found in adult plants (Shefferson et al. 2007).

In this chapter, we pursued these broad questions in order to better characterize the community of OM interaction with *C. calceolus* in relation to local soil conditions, ultimately to better inform the causes potentially explaining this species' fast population disappearance. Specifically, we aimed to explore the link between the OM community within roots and the surrounding soil fungal community, and also to explore the link between *C. calceolus* population fitness and mycorrhizal diversity in *C. calceolus* roots by asking the following questions: (i) Is the diversity of soil fungi reflected in the diversity of OM in *C. calceolus* roots? (ii) Are similar edaphic and climatic conditions resulting in similar OM communities? (iii) Are healthy *C. calceolus* populations characterized by a unique combination of OM species? To this end, we sampled local habitat (soil and vegetation) parameters and *C. calceolus* life-history traits parameters across 34 Swiss populations of *C. calceolus* (17 small and 17 large populations). From soils and root samples, we extracted and amplified DNA using first a general primer for fungi and second, with a primer designed for Tulasnellaceae. Specifically, we predicted that (i) *C. calceolus* is associated with one or more species of OM throughout its life-cycle which is the reflection of the local fungal community, (ii) similar habitat conditions would favour convergence in the fungal community across the landscape, and (iii) larger (more fit) *C. calceolus* populations should host a more diverse or more unique diversity of OM in their roots.

2. Material and methods

2.1. Population selection criteria

We chose *C. calceolus* populations of varying sizes throughout Switzerland based on known occurrences of viable populations (data provided by www.infoflora.ch). We selected a total of 34 native populations, 17 small (i.e., less than 10 individuals per population) and 17 large (i.e., more than 20 individuals per population), encompassing the six major biogeographic regions of Switzerland (Figure 1, Table 1). Each population was visited between 2017 and 2021, once during the peak flowering period, between April and June, and once after the production of fruits (i.e., seed pods), between July and August.

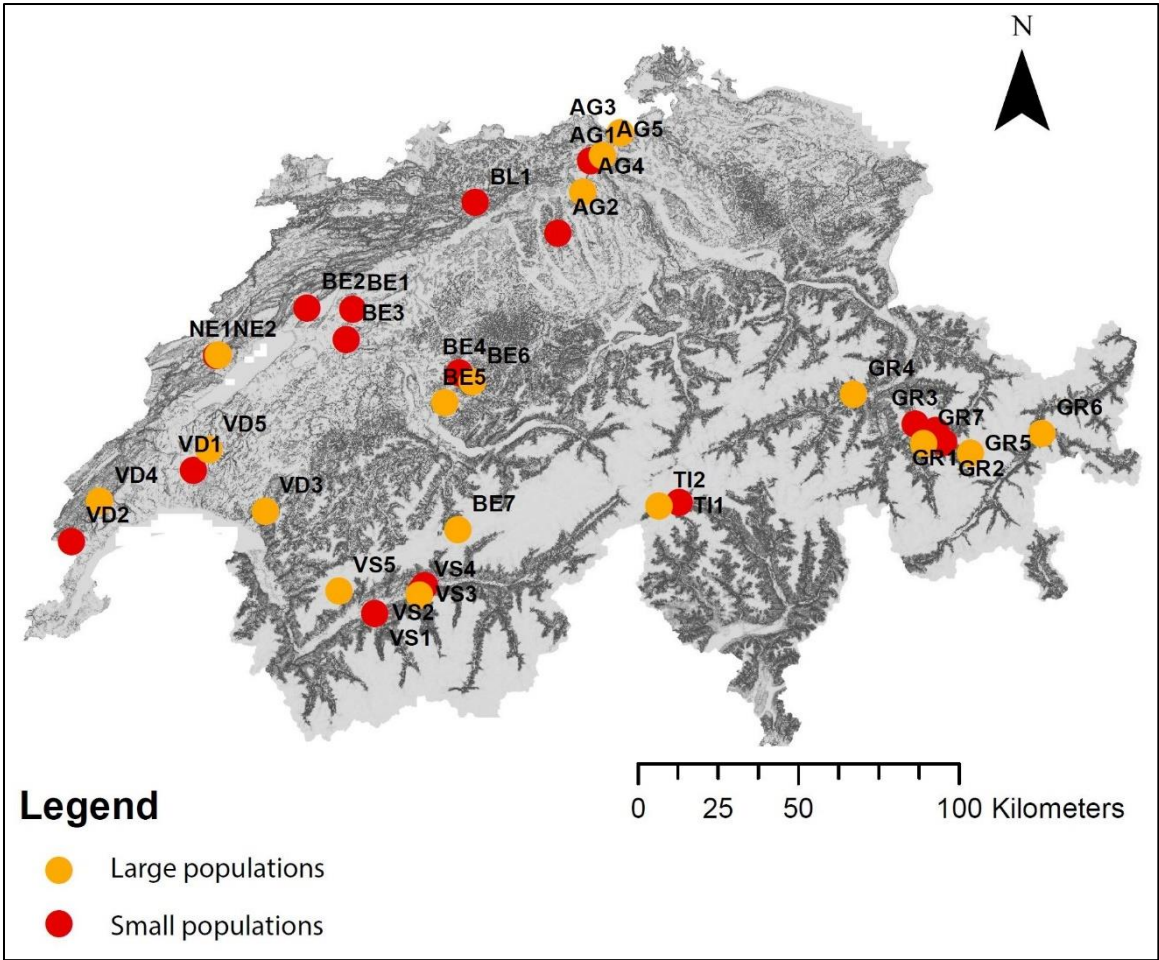


Figure 1. The 34 populations of *Cypripedium calceolus* visited in the scope of this study. We categorized populations as being small (i.e., less than 10 inventoried individuals per population, red dots), or large (i.e., more than 20 individuals per population, orange dots). The complete name of each population is given in Table 1. On the background map, shades of grey indicate the habitat suitability for *C. calceolus* based on a climatic niche model with light grey representing low suitability and dark grey high suitability (Rusconi et al. 2022a).

Table 1: The 34 Swiss *Cypripedium calceolus* populations visited in the scope of this study. Site names are abbreviations of the canton where the population stands and the site number (Aargau: AG, Bern: BE, BL: Basel-Landschaft, GR: Graubünden, NE: Neuchâtel, VD: Vaud, VS: Valais and TI: Ticino). Populations were categorized as being small (i.e., less than 10 inventoried individuals per population), or large (i.e., more than 20 individuals per population).

Site name	Canton	Municipality	Small or large population
AG1	Aargau	Turgi	Small
AG2	Aargau	Gontenschwil	Small
AG3	Aargau	Rümikon	Large
AG4	Aargau	Dottikon	Large
AG5	Aargau	Freienwil	Large
BE1	Bern	Kappelen	Small
BE2	Bern	La Neuveville	Small
BE3	Bern	Radelfingen	Small
BE4	Bern	Bowil	Small
BE5	Bern	Fahrni	Large
BE6	Bern	Röthenbach	Large
BE7	Bern	Kandersteg	Large
BL1	Basel-Landschaft	Langenbruck	Small
GR1	Graubünden	Albula/Alvra	Small
GR2	Graubünden	Bergün Filisur	Small
GR3	Graubünden	Vaz/Obervaz	Small
GR4	Graubünden	Trin	Large
GR5	Graubünden	Bergün Filisur	Large
GR6	Graubünden	Zernez	Large
GR7	Graubünden	Albula/Alvra	Large
NE1	Neuchâtel	La Grande Béroche	Small
NE2	Neuchâtel	La Grande Béroche	Large
NE3	Neuchâtel	La Grande Béroche	Small
VD1	Vaud	Bretigny-sur-Morrens	Small
VD2	Vaud	Chésereux	Small
VD3	Vaud	Saint-Légier-La Chiésaz	Large
VD4	Vaud	Saint-George	Large
VD5	Vaud	Fey	Large
VS1	Valais	Sion	Small
VS2	Valais	Salgesch	Small
VS3	Valais	Sierre	Small
VS4	Valais	Anniviers	Large
VS5	Valais	Conthey	Large
TI1	Ticino	Airolo	Small
TI2	Ticino	Bedretto	Large

2.2. *Vegetation and C. calceolus functional traits*

During the first visit to each *C. calceolus* population, we performed a comprehensive vegetation survey on a 10 x 10 m² plot comprehending the most homogenous vegetation type of each site following the Braun-Blanquet method (Braun-Blanquet 1964). We used the measured vegetation community structure (characteristic and most abundant species) to assign an alliance name to each vegetation type based on phytosociological nomenclature (Barkman et al. 1986), and according to Delarze et al. (2015). We next measured ten functional traits with known relationships with plant life history parameters (Adler et al. 2014; Díaz et al. 2016; Pérez-Harguindeguy et al. 2016) that could be quantified using non-intrusive methods. At each of the 34 native site, we sampled traits on a maximum of 10 randomly chosen individuals growing within a 100 m² area and separated by a minimum of 2 m from each other. Each stem or group of stems (patch), separated by a minimum of 70 cm from each other, was considered as an individual (i.e., a clonal patch) (Kull 1999). For growth-related traits, we measured clonal patch area (cm²), the vegetative plant height (cm), leaf number and median leaf area (cm², ellipse area formula) of the highest stem in a clonal patch and the median leaf photosynthetic activity (SPAD chlorophyllometer, Konica Minolta, Osaka Japan) and specific leaf area (SLA, mm²/mg) of 1 leaf per patch. For reproductive traits, we counted the number of stems and the number of flowers per patch on the first visit, and on the second visit we counted, per patch, the number fruits (pods) and estimated median fruit volume (approximated as a cylinder).

2.3. *Soil parameters (for edaphic properties)*

In each site, we dug a soil profile from the surface to the bedrock or parental material at a minimum distance of 40 cm from the nearest plant. We identified organic, organo-mineral and mineral horizons to characterise the soil type (Baize and Girard 2009; IUSS Working Group 2015) and corresponding humus forms (Zanella et al. 2018). Then, within the 100 m² area of each site, we sampled approximately 300 g of the organo-mineral horizon (A) three times, corresponding to a depth of 20.9 +/- 8.5 cm – i.e., the rooting zone of *C. calceolus*. From the A horizons, we measured eight soil physicochemical parameters, including 1) relative humidity (HR), obtained by subsequent desiccation at 105 °C and weighing. 2) Total organic carbon (Corg) to total nitrogen (Ntot), and subsequent carbon-to-nitrogen ratio (CN), which were measured using an elemental analyzer (Flash 2000, CHN-O Analyzer, Thermo Scientific, Waltham, Massachusetts, United States). 3) Total soil organic matter content (SOM), measured via the loss of ignition (LOI) method, by heating the samples at 450°C for two hours. 4) pH, measured in distilled water with a Metrohm 827 pH meter (Metrohm AG, Herisa, Switzerland). 5) Soil total cationic exchange capacity (CEC), using the “cobalt hexamine trichloride” method (Ciesielski and Sterckeman 1997). 6) Total carbonates, estimated by CaCO₃ dissolution after HCl 6M addition, and according to the Calcimeter Bernard method (Dreimanis 1962). 7) Bioavailable phosphorus (P) estimated by extraction with sodium bicarbonate (Olsen 1954). 8) Nitrate (NO₃⁻) quantified after an extraction with potassium sulphate (Bremner and Shaw 1955). In addition, on soils that developed on a dolomitic substrate (geological information obtained from www.mapgeo.admin.ch), we performed dolomite quantification using the Calcimeter Bernard method (Dreimanis 1962) and

gypsum quantification using a method with successive weighing after a passage in the desiccator and in the oven (105 °C) (Lebron et al. 2009).

2.4. Soil and *C. calceolus* root collection for mycorrhizal analysis, DNA extraction, amplification and Illumina sequencing

To assess mycorrhizal diversity in *C. calceolus* roots and in the soil, we collected 3 samples of roots (1 cm) on 3 *C. calceolus* adult individuals in each *C. calceolus* population. In addition, we randomly collected 10 soil samples of approximately 300 g of the organo-mineral horizon (A), corresponding to a depth of 20.9 +/- 8.5 cm – i.e., the rooting zone of *C. calceolus*. Soil and root samples were stored at -80 °C until analysis. Before DNA extraction, root and soil samples were pooled by population. DNA extraction was performed using DNeasy PowerSoil Kit (Qiagen) following the manufacturer's protocol. The same extraction kit was used for root and soil samples in order to ensure the comparability of results. We used DNA extracted from samples containing only Milli-Q water as a negative control for the rest of the analysis process. For DNA amplification, we selected two pairs of primers. The first pair of primers ITS86F/ITS4 targets the general fungal diversity that is present in samples (Turenne et al. 1999). The second one 5.8S-Tulngs/ITS4-Tul2 targets specifically *Tulasnella* species (Oja et al. 2015). To control for potential tag jump, we used as a positive control the DNA extracted from *Serpula lacrymans*, a fungus species that is never found in soil. DNA amplification and sequencing using Illumina techniques were performed by Fasteris SA (Fasteris SA, CH 1228 Plan-les-Ouattes).

2.5. Bioinformatic processing of Illumina sequencing results

The full bioinformatic analyses were automatically processed using DeltaMP v. 0.6 (Lentendu 2022) on a HPC ("Centre de Calcul de la Faculté des Sciences", UniNe). Raw Illumina reads were demultiplexed using cutadapt v. 2.10 (Martin 2011) allowing until 2 mismatches on the barcode sequences. The barcode sequence was strip off the 5'-end of both R1 and R2 libraries. In the first sequencing run (Illumina MiSeq 2x300bp), 37.65 % of the 1.3M reads were assigned to the 11 samples of fungal ITS, 44.88 % to the 11 samples of Tulasnellaceae ITS. In the second run, 58.31 % of the 23.3M reads were assigned to the 72 samples of fungal ITS, 26.15 % to the 72 samples of Tulasnellaceae ITS. Primer sequences were then removed from both ends in both libraries using the linked adapter strategy of cutadapt. For that, the full primer at 5'-end was requested to be anchored at sequence 5'-end (after stripping the barcode) and at least 8 nucleotides of the second primer should be found toward the 3'-end of the sequence, with allowing until 20 % mismatches over the matching length. This step produced four sets of sequences per sample, including sequences from both orientation (i.e. originally starting with the forward or the reverse primer) and in both R1 and R2 libraries. After stripping the primer sequences, only the amplified biological sequences were further analysed. In order to avoid low quality end of reads, only the 210 to 215 first nucleotides of each sequence were kept, and sequences with a maximum expected error above 3.0 for fungal ITS and above 0.5 for Tulasnellaceae dataset were removed using VSEARCH v. 2.13.6 (Rognes et al. 2016). Amplicon sequence variants (ASV) were then created using the DADA2 R package v. 1.14.1 (Callahan et al. 2016). For that, one error model was built for each R1 and R2 libraries separately and then applied to call ASVs for each

orientation in each library, turning the pool option on. Paired reads in R1 and R2 libraries were assembled with the dada2 mergePairs function, with a minimum overlap length of 10 nucleotide and a maximum of 2 mismatches in the overlap region. ASV originally with the reverse primer in the R1 library were reverse complemented using the seqinr package v. 3.6-1 (Charif and Lobry 2007). Presence of chimera was double checked first with the dada2 function removeBimeraDenovo and with the UCHIME de-novo algorithm as implemented in VSEARCH (Edgar et al. 2011). Taxonomy was assigned to each ASV using the VSEARCH option `-usearch_global` without allowing any end-gap against reference sequences of the UNITE v9.0 database (Abarenkov et al. 2022). When multiple best matches were found in the database, a consensus taxonomy was resolved at a threshold of 60%.

3. First results

3.1. Taxonomic composition

In root samples, ITS dataset was dominated by fungi regarding species richness and by Tulasnellaceae regarding relative abundance (Figure 2). In soil samples, fungal ITS dataset was dominated by fungi (richness and relative abundance, Figure 2). Tulasnellaceae-specific ITS marker dataset was dominated by unknown sequences (i.e., sequences with no match in database, Figure 3).

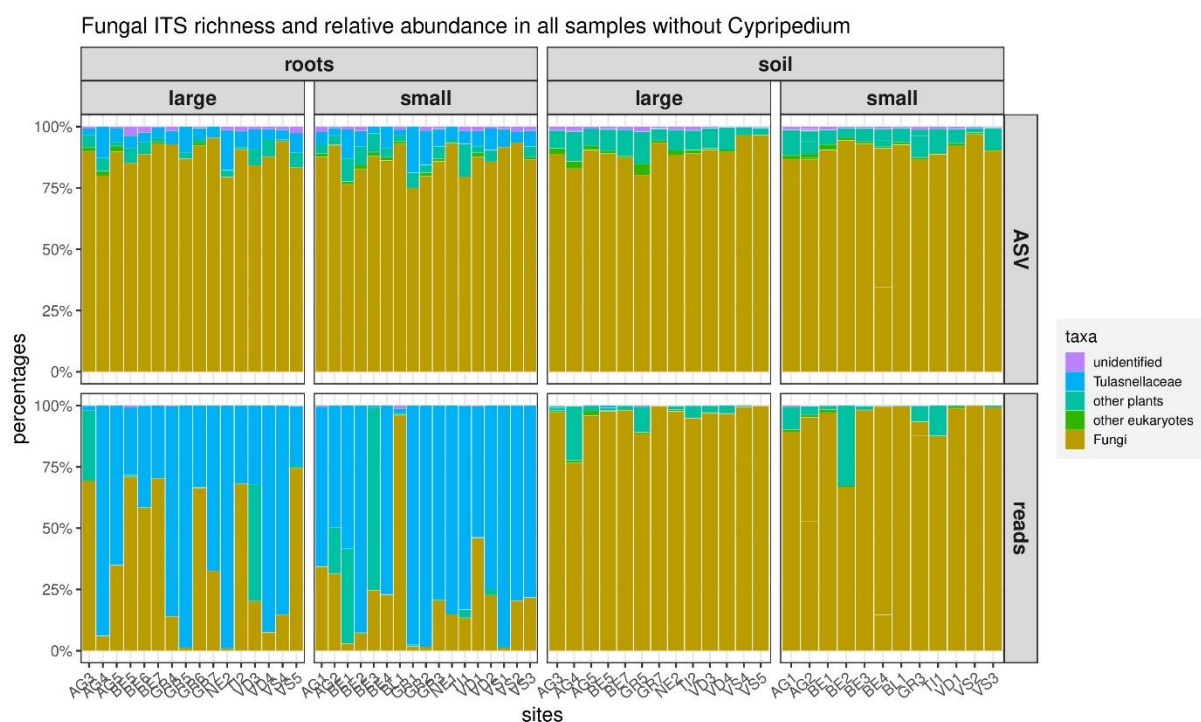


Figure 2. Barplots representing fungal ITS richness (ASV) and relative abundance (reads) in all samples after removing *Cyripedium calceolus* sequences of all organisms. Results for root and soil samples are shown for large and small populations of *C. calceolus*. The different colours refer to the taxa present in samples

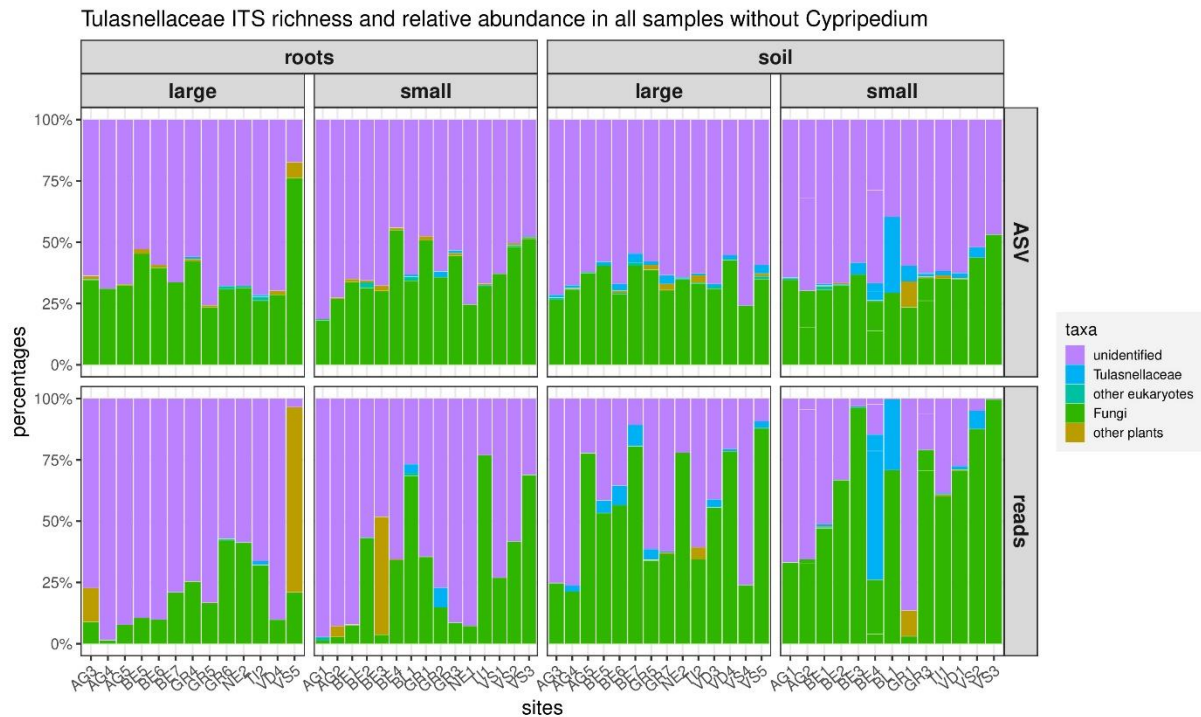


Figure 3. Barplots representing Tulasnellaceae ITS richness (ASV) and relative abundance (reads) in all samples after removing *Cypripedium calceolus* sequences of all organisms. Results for root and soil samples are shown for large and small populations of *C. calceolus*. The different colours refer to the taxa present in samples.

3.2. Taxonomic composition of Fungi

After removing all ASV that were not fungi or were unidentified and ASV occurring only in one sample, in root samples, the fungal ITS dataset was dominated by Tulasnellaceae (relative abundance, Figure 4). On the other hand, Tulasnellaceae ITS dataset was dominated by the group of Saccharomycetes in the roots, and the group of Orbiliaceae in the soil (Figure 5). As most of the diversity covered by the “Tulasnellaceae” specific ITS dataset did not include the Tulasnellaceae, we decided to not analyse such diversity patterns further, as the fungal community covered with this dataset is most probably skewed due to primer bias.

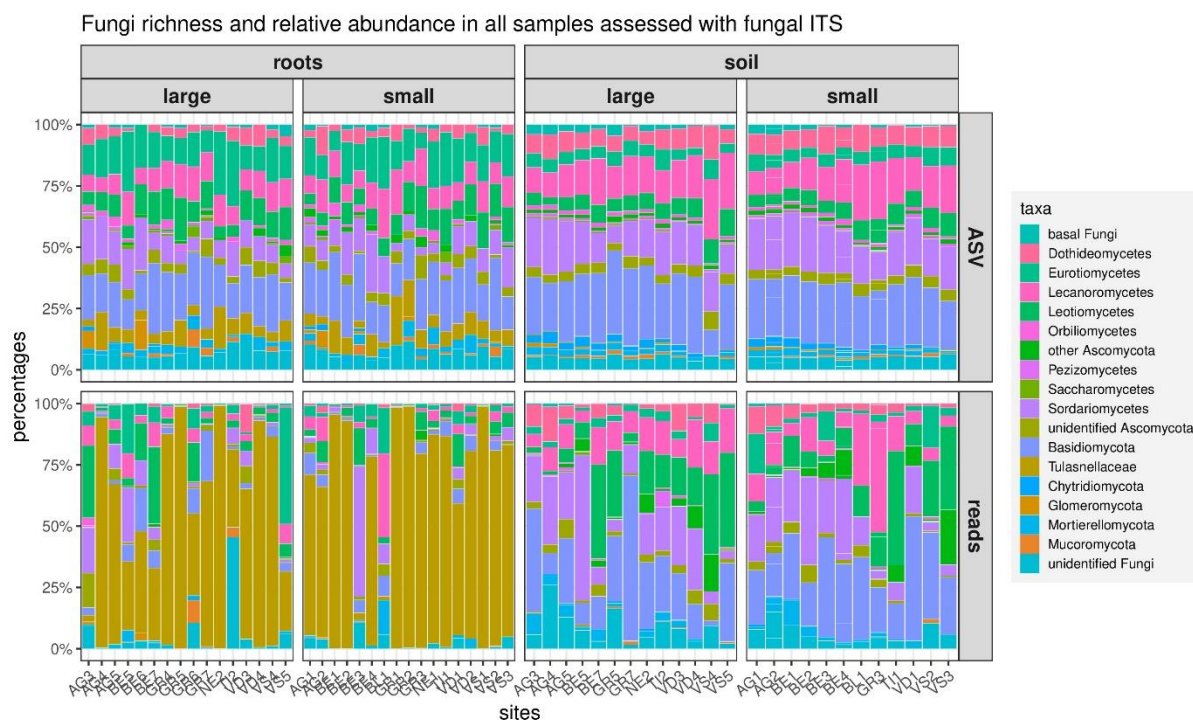


Figure 4. Barplots representing fungal ITS richness (ASV) and relative abundance (reads) of fungi in all samples. Results for root and soil samples are shown for large and small populations of *C. calceolus*. The different colours refer to the taxa of fungi.

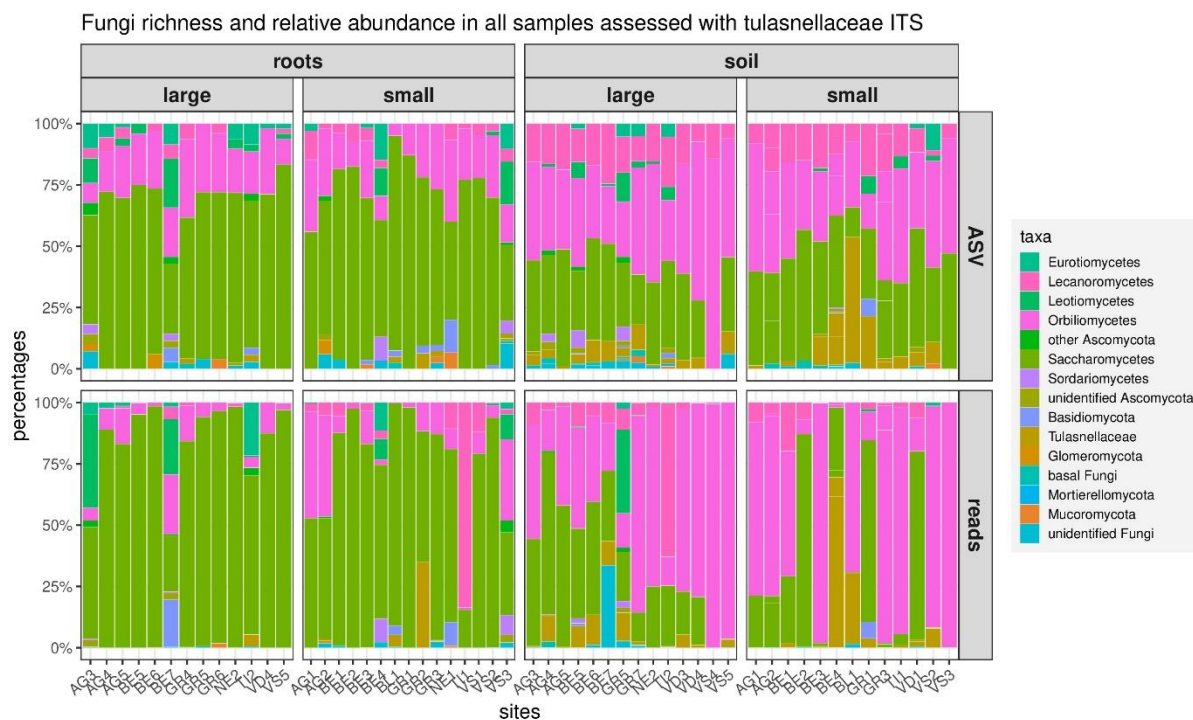


Figure 5. Barplots representing Tulasnellaceae ITS richness (ASV) and relative abundance (reads) of fungi in all samples. Results for root and soil samples are shown for large and small populations of *C. calceolus*. The different colours refer to the taxa of fungi.

3.3. Effect of population size on the alpha diversity (fungal ITS dataset only)

Focusing on alpha diversity (Species richness, Shannon index and Simpson index), we found no significant differences between small and large populations of *C. calceolus* (Figure 6). The same tendencies were observed when looking at the Tulasnellaceae present in the fungal ITS dataset.

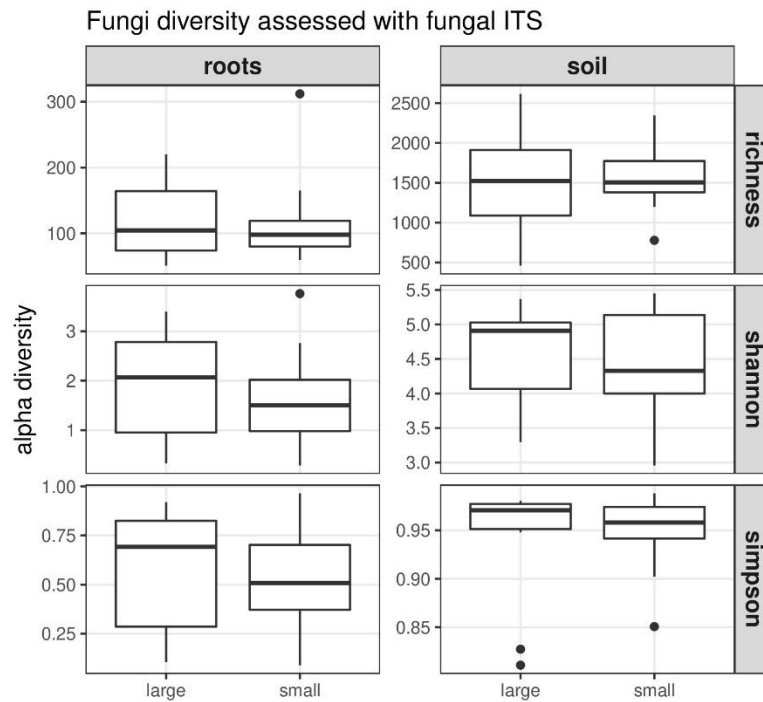


Figure 6. Boxplots illustrating alpha diversity (species richness, Shannon and Simpson index) assessed with fungal ITS in small and large populations of *Cypripedium calceolus*.

3.4. Beta diversity (fungal ITS dataset only) -variation across habitats

Difference of beta diversity in the fungal community composition was explained by the geographical location of the populations (i.e., the canton in which they are located, PERMANOVA, $R^2 = 0.181$, p-value < 0.001) and the type of samples (i.e., roots or soil, PERMANOVA, $R^2 = 0.12$, p-value < 0.001 , Figure 7). We observed no effect of the *C. calceolus* population size (PERMANOVA, $R^2 = 0.015$, p-value < 0.163). Difference of beta diversity of Tulasnellaceae community composition was explained by the geographical location of the populations (i.e., the canton in which they are located, PERMANOVA, $R^2 = 0.424$, p-value < 0.001 , Figure 8). We observed no effect of the *C. calceolus* population size (PERMANOVA, $R^2 = 0.041$, p-value < 0.102).

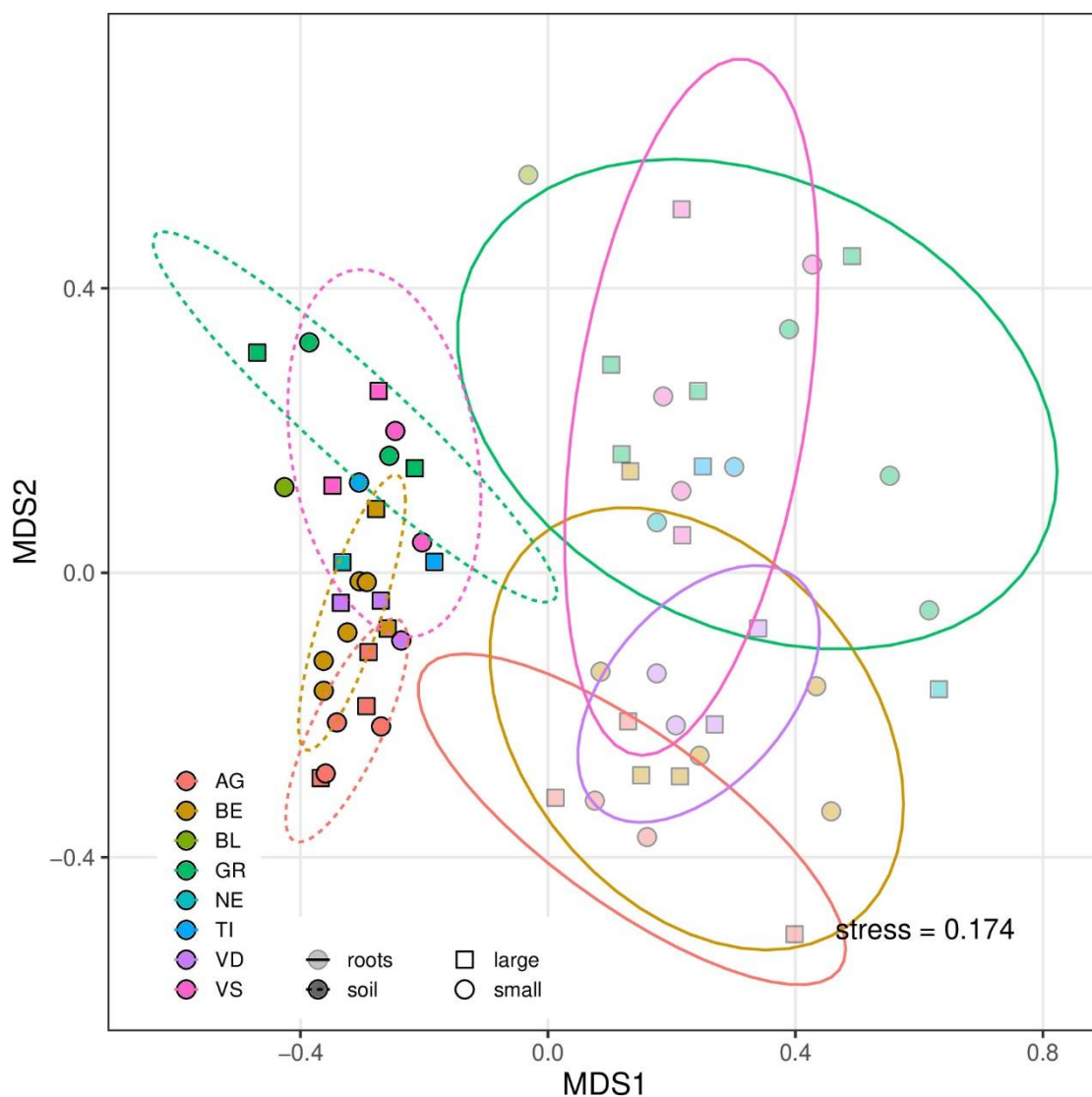


Figure 7. NMDS visualization of dissimilarity in Fungi community composition as computed with Bray-Curtis index on Hellinger transformed matrix (square-root of relative abundance). Different colours refer to *Cypripedium calceolus* populations (Table 1). Continuous line refers to root samples and dotted line refers to soil samples. Square refers to large and circle to small *C. calceolus* populations.

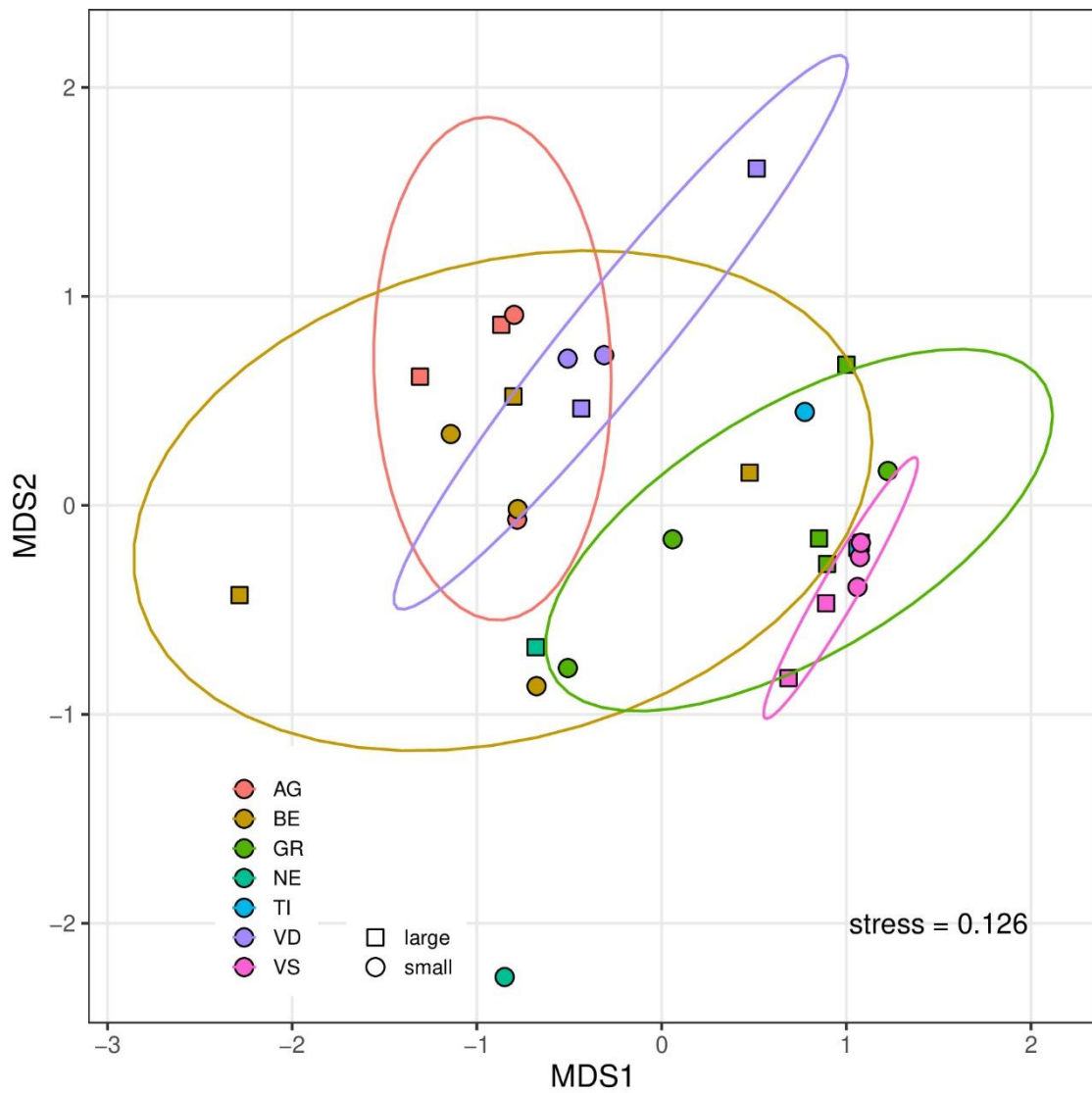


Figure 8. NMDS visualization of dissimilarity in Tulasnellaceae community composition as computed with Bray-Curtis index on Hellinger transformed matrix (square-root of relative abundance). Different colours refer to *Cyripedium calceolus* populations (Table 1). Square refers to large and circle to small *C. calceolus* populations.

4. Summary of this study and further perspectives

From our analyses performed on 34 Swiss populations of *C. calceolus*, we highlighted, looking at fungal ITS dataset, that Tulasnellaceae are present and dominant in *C. calceolus* roots. On the other hand, results from Tulasnellaceae ITS do not allow to further identify the specific member of this family in *C. calceolus* roots. This could be explained by a unique (not yet characterized) diversity of OM that is present in the *Cypripedium* roots, whereas in soils, broad fungal diversity has been better characterized. Therefore, the lack of correct (in-depth) amplification of the Tulasnellaceae-specific ITS could be explained by the presence of a clade of Tulasnellaceae that is not yet characterized and that the Tulasnellaceae marker was unable to detect. Nonetheless, if the lack of amplification success is the reflection of a unique, relatively rare, set of closely related species, such result could help to partially explain the rarity of *C. calceolus* (e.g., Fay et al. 2015; Nurfadilah et al. 2013; Phillips et al. 2011). On the other hand, we cannot exclude that these unidentified sequences are artefacts or chimeras but it is more probable that they are Fungi missed by the standard ITS fungi primers and so lacking from the databases.

Regarding small and large populations of *C. calceolus*, we found no difference be it in alpha or in beta diversity of fungi, suggesting that broad diversity metrics of soil fungal communities as analysed here are poor predictors of *C. calceolus* population health status, as it was observed for specific combinations of soil physicochemical parameters (Rusconi et al. 2022b). That said, future multivariate-based analyses will be needed to explore the relative linkage of the different plant life-history traits measured and the local functional community matrix.

On the other hand, we found geographical differentiation of the fungal community mediated by the geographic borders of the Swiss cantons. This result suggests that geographically closer sites are more similar in terms of fungal communities than more distantly related sites. These results also suggest, surprisingly, that soil physicochemical parameters or vegetation types do not contribute in structuring the fungal communities in soils or in the roots of these plants, since, within the same canton soils and vegetation can differ from each other by several orders of magnitude (Rusconi et al. 2022b). Future analyses by specifically comparing soil and vegetation trait distance matrices with fungal distance matrices will allow confirming these observations.

To our knowledge, the approach presented here is among the first attempts to link the local fungal diversity with the diversity of fungi present in *C. calceolus* plants. By including more in-depth statistical analyses, we hope to be able to pinpoint to fungal indicator species of healthy and viable *C. calceolus* populations.

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General discussion

In this PhD thesis, we aimed to develop a comprehensive conservation method for endangered plant species based on fundamental knowledge about plant natural history, biology, demography and genetics. To this end, we used the iconic and patrimonial orchid *Cypripedium calceolus* as a model species. We studied (i) the climatic and topographic niche of *C. calceolus* by applying climate-niche modelling techniques (**Chapter 1**) and (ii) its habitat requirements (soil and vegetation type and structure) through observational and experimental approaches (**Chapter 2**). In addition, we explored its (iii) pollination biology and underlying chemical ecology (**Chapter 3**), (iv) population genetics and demography and (v) relationship with mycorrhiza (**Chapters 4 & 5**, presented as Perspective Chapters I and II).

In the **first chapter**, we developed a two-step approach to identify suitable sites for the (re)introduction of *C. calceolus* populations. First, we found that predictions of occurrence probability based on climate alone, although not very precise, were nevertheless sufficient to indicate a decline of the species range during the next 50 years in response to predicted climate warming. Next, by incorporating topographical layers and vegetation structure for the modelling approach at the regional scale, we revealed that *C. calceolus* was more likely to occur near forest edges. Finally, by analysing in situ soil factors, we found that soil organic matter, cation-exchange capacity and pH explained best *C. calceolus* populations fitness variables as described by multivariate function trait space. We thus advocate for the combination of modelling tools with fine scale ecological surveys to identify suitable reintroduction sites for this, and potentially other, endangered plant species (Figure 1).

In the **second chapter**, we explored the links between functional traits related to population health status and vegetation and soil parameters through extensive and fine-grained field work. We found that large (>20 individuals) populations of *C. calceolus* displayed a specific assemblage of measurable characteristics that discriminate them from small (<10 individuals) populations, indicating that it is possible to assess the health of a population of a rare plant species by measuring a specific set of functional traits. While we could not predict population health directly from vegetation (phytosociological alliances) and soil types, we identified a unique combination of companion plants, and edaphic parameters, such as SOM, CaCO₃, pH, and P, that could be used to select potentially optimal sites to implement (re)-introduction actions for this emblematic and patrimonial orchid species (Figure 1).

In the **third chapter**, we aimed to understand the role of olfactory signals in maintaining fitness in a rare orchid species. We found that small *C. calceolus* populations occur in less diverse sites, both in terms of surrounding plants and insects. However, while individuals in larger populations were physically larger and produced more flowers per capita, they produced the same number of seeds per capita (i.e., seed set) as small populations. This occurred despite the lower pollinator diversity present at the small population sites, suggesting that plants from small populations must compensate by providing a stronger attraction to pollinators. Indeed, we discovered that floral volatile organic

compounds (VOCs) were produced in higher quantity in individuals from small populations, suggesting that small populations compensate for low pollinator diversity by producing a stronger floral scent (**Figure 1**).

The **two additional perspective chapters (I and II)** focused on the genetic structure of the Swiss *C. calceolus* populations and their association with mycorrhizal fungi. While these data opened new interesting venues, these topics merits further investigations in the future.

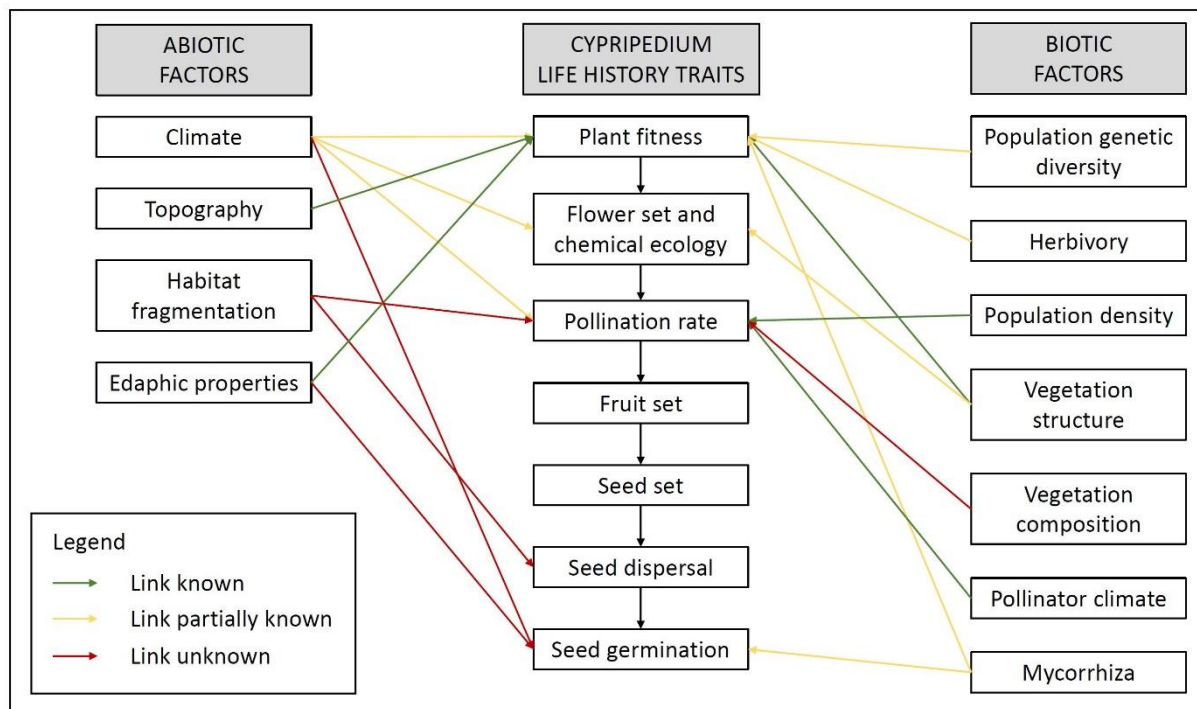


Figure 1 : Links between *Cypripedium calceolus* life history traits and biotic and abiotic factors. Green, yellow and red arrows highlight state of knowledge according to the current understanding. This PhD thesis contributed to better understand the links between (i) edaphic properties and *C. calceolus* plant fitness traits, (ii) *C. calceolus* population density and pollination rate, (iii) vegetation structure and *C. calceolus* fitness traits and (iv) pollinator climate (composition, abundance, and efficiency of pollinators, (Grant and Grant 1965)) and pollination rate.

The present study contributes to a better understanding of the fundamental knowledge about *C. calceolus* regarding its biotic and abiotic habitat preferences as well as its pollination system and its interactions with pollinators, filling existing theoretical gaps (Devillers-Terschuren 1999; Fay and Taylor 2015; Kull 1999). Concretely, in the framework of *C. calceolus* conservation, this study makes links between fundamental research and practical actions allowing to convert theoretical knowledge into specific conservation measures. For example, we advocate that the modelling of this species niche and the identification of its edaphic requirements will improve the selection of future translocation sites. In addition, the description of the vegetation type (phytosociological alliances) and structure (tree strata) will contribute to help forest managers contribute to maintenance of areas favourable for the growth of *C. calceolus*. In addition, the identification of some pollinators of *C. calceolus* in Switzerland can help maintaining gene flow through pollination if in turn favourable conditions for these insect

species can be identified and maintained. The improvement of knowledge and integrated research about the biology and the ecology of a plant species is crucial to develop efficient conservation measures and actions (Heywood and Iriondo 2003). This is particularly important for species such as *C. calceolus*, characterized by complex ecological interactions with pollinators and fungi (Fay 2016; Fay and Chase 2009; Swarts and Dixon 2017; Swarts and Dixon 2009). The conservation of *C. calceolus* individuals and populations will allow to preserve genetic variations within this species and therefore its ability to adapt to environmental changes which is essential for plants that are sensitive to global warming (Kolanowska and Jakubska-Busse 2020; Reed et al. 2003, Rusconi et al. 2022; Rusconi 2017). Moreover, with the pace and scale of current global changes (e.g.: www.ipcc.ch), conservation translocations of plants tend to increase. To be successful, these actions however, require excellent understanding of species ecology (Silcock et al. 2019). Since the selection of translocation sites is a particularly complex task and the success of plant translocation can vary at a scale of less than 1 m² (Godefroid et al. 2011; Maschinski and Haskins 2012), extensive biological and ecological knowledge as we proposed in this study are necessary.

In the framework of orchid and plant conservation, this thesis gives an example of an integrated study allowing to link the different aspects of a species biology and ecology leading to potential specific protection actions for plant individuals and populations (Swarts and Dixon 2017). In this special case, we focused on *C. calceolus* but the methodology we used can be transposed to other plant species (orchid or plant from another family). In concrete terms, to adapt our approach to another threatened species, it requires: (i) research in the literature and information gathering from practitioners to understand the causes of decline and the key aspects of the species' ecology; (ii) research combining field and laboratory work on keystone ecological factors of the focal species and (iii) transfer of theoretical results to specific conservation actions and measures. In addition, as mentioned above, studies such as this thesis will diminish the rate of plant translocation failures through a more informed choice of suitable planting sites (Godefroid et al. 2011; Guerrant Jr and Kaye 2007). In turn higher success rates of translocation will likely feedback positively to restoration efforts as the means invested will be matched with success, making it easier to advocate for further investment in conservation. Moreover, this PhD thesis perfectly fits with the third objective of conservation biology, namely to develop practical approaches to prevent species extinction (Primack 2014).

Our methodology that aimed to develop conservation actions for endangered plant species using a comprehensive approach present theoretical and practical limitations. First, the huge complexity of nature and its interaction network makes it impossible to take in account and to understand all the relevant factors on which the survival of a plant species depends. Because of this, choices have to be made on which aspects of the species ecology will be targeted in a particular study, leading to trade-offs. These risks overlook crucial parameters for this species survival (Hirsch et al. 2011). Moreover, studying the ecology of species in order to develop targeted conservation actions is time-consuming for different reasons such as: (i) studies on the field have to been done to cover the relevant life stages of the targeted species (i.e.: when the species is not in a dormant form such as the flowering period), which represent often several years of data collection (Heywood and Iriondo 2003); (ii)

multidimensional aspects of the ecology of the species need to be investigated (Heywood and Iriondo 2003) and (iii) once a conservational measure is taken for a species, the monitoring of the efficiency of this action takes years (Godefroid et al. 2011). The length of such projects can be complicated at different levels. First, long studies are expensive and budgets allocated to this kind of conservation projects are often insufficient (Naidoo et al. 2006). Second, due to the academic research system in place, researchers often do not work long enough on the same subject and thirdly, conservation actions are sometimes urgently needed if species are under severe threat. Indeed, as highlighted by Soulé (1985), conservation biology is sometimes a crisis discipline, meaning that rapid actions are necessary to assure the persistence of species and populations. One more limitation of this study is the complexity of the interactions between all stakeholders (researchers, practitioners, actors from private foundations, NGO, etc.) (Groce et al. 2019; Van Dyke and Lamb 2020). The different points of view of these actors, even though they have the same common goal: the protection of a species, can hinder the success of a project (Van Dyke and Lamb 2020).

Focusing on the limitations of this PhD thesis in the particular case of *C. calceolus*, the question of the duration of studies is particularly relevant. Indeed, this species, such as numerous other perennial plants, has a very long and slow lifecycle making its study especially complicated (Kull 1999). In addition, monitoring of taken conservation measures or of *C. calceolus* translocations would be needed over a period of several decades, which is unfortunately often technically not possible. Moreover, in the framework of this PhD, final results about *C. calceolus* mycorrhizal associations and population genetics are still missing, which constitutes an important gap to set up conservation measures. Indeed, the identification of mycorrhizal symbionts and the possibility of determining their presence in a candidate site would be very informative to assess the potential of reintroduction success. In addition, information about population genetics in Switzerland will inform for example about the pertinence of population reinforcements with *C. calceolus* specimen from other Swiss cantons or about the need of cross-pollination action by hand (Gargiulo et al. 2021). These kinds of actions would favour gene flow between populations that are not connected anymore leading to more genetic variability, adaptability and resilience to environmental changes in focal populations (Garland and Curry 2022).

Concentrating more in details about possible improvements, outlook and future directives for *C. calceolus* conservation, despite decades of research, the complex ecology of this species is still not completely understood to improve its conservation (Rusconi et al. 2022), as is the case for many other plant species. This is particularly important because *C. calceolus* is very sensitive to global warming and suitable growing areas will decrease in coming decades (Kolanowska and Jakubska-Busse 2020; Rusconi et al. 2022). Furthermore, population genetic studies are needed over a larger geographical scale to evaluate the risk of phenomenon such as bottleneck effect, founder effect or genetic drift (Gargiulo et al. 2021). This would be essential to evaluate the ability of this species to adapt to current global warming (Reed et al. 2003; Willi et al. 2007).

Based on the case study of *C. calceolus* and in order to improve the conservation methodology for threatened plant species more generally, the following suggestions can be made. First, the

development of conservation methods that are less time-consuming and therefore less expensive would allow to speed-up concrete conservation actions in the field. On the other hand, the complexity of the ecology of plants, and especially orchids, requires integrated conservation research and measures (Gale et al. 2018). Compromises need to be found between financial and temporal costs, the quality of information emerging from research and the efficacy of concrete conservation measures. Moreover, close collaboration between conservation stakeholders, such as researchers, practitioners and state environmental services, is key (Mascia et al. 2003). Indeed, in the context of current global warming and the urgency to protect plant species, these actions could save precious time by avoiding miscommunication or conflicting opinions between different actors. In addition, international collaboration between universities and research centres needs to be strengthened to combine forces and avoid duplicating studies. To this end, the initiation of an official, international and up-to-date database about research and findings on plant conservation would facilitate the flow of information and avoid duplication of identical and redundant projects, as it is aimed by the *Global Strategy for Plant Conservation* (Convention on Biological Diversity 2011; Fay 2016; Godefroid and Vanderborght 2011). Global climate change makes the conservation of endangered plant species even more challenging and measures which seem relevant today may soon be obsolete due to rapid and unpredictable climate warming (Prober et al. 2019). In such a context, the development of integrated conservation measures for endangered plants species and plant populations that include climate are more necessary and relevant than ever before (Swarts and Dixon 2017).

Beyond the target species itself, even if conservation at the species level is relevant, as the results of this study show, conservation efforts also need to focus on other organisation levels of the ecosystems (e.g., communities, ecological networks, etc.) (Pickett et al. 1992). Indeed, despite the best efforts of conservationists, with the scale and pace of current climatic global warming, our capacity to develop solution at the species-level seems outstripped (Gale et al. 2018). This assumption is true for plant species such as orchids but also for the rest of the living world (Hanson et al. 2020). In order to protect and conserve species at another level than the species level, alternatives exist. First, Fay et al. (2018), in the special framework of orchid conservation, advised to protect more processes than species and to conserve species that grow sympatrically. Another option is to focus on ecological niche protection. With environmental changes, species will need to adapt to persist (e.g.: Garland and Curry 2022) and ensuring that protected areas cover a broad range of environmental conditions will safeguard this adaptation process (Hanson et al. 2020). Indeed, expanding nature reserve areas without ensuring that the various niches needed by the different species are available is not an effective conservation measure (Hanson et al. 2020). It is also possible to concentrate on the conservation of ecological networks and more precisely food webs in order to link species-specific variability to the overall network persistence (Stouffer et al. 2012). Another approach focusing on species networks proposes to explicitly link multiple species and habitat resources in order to estimate the importance of a specific species or habitat in a given landscape and to quantify the emerging properties of the complete habitat network (Marini et al. 2019). On the other hand, and as mentioned above, conservation at the species-level is still necessary even in a context of global changes. Indeed, it is still relevant for some species at

great risks and biodiversity has to be protected for its intrinsic value (Fay 2018; Van Dyke and Lamb 2020). In addition, analyses of interaction networks such as the one proposed by Stouffer et al. (2012) can highlight a species whose role is crucial in the community persistence and should receive priority in conservation efforts. Moreover, emblematic and patrimonial species (i.e.: species with a great symbolic value (Cortés-Avizanda et al. 2022)) such as *C. calceolus* have a strong emotional impact on people and drive general interest for nature conservation. This allows to raise awareness among the general public on the importance of preserving diversity which is essential for the future of conservation biology and for the fate of biodiversity (Van Dyke and Lamb 2020).

A more personal note to finish

Overall, this thesis shows that working on conservation of endangered species is fascinating, motivating and challenging. However, with the pace and the scale of global changes and the evidence of their consequences on biodiversity, it is sometimes difficult to see a happy ending for the species to which conservationists devote time and energy. Nowadays, conservation biologists suffers from a wave of hopelessness and this affects the ability of scientists to mobilize actions among the public at large and to draw others into the conservation field (Swaigood and Sheppard 2010; Van Dyke and Lamb 2020). Some scientists even argued that because extinction is part of evolution, we don't need to do anything to protect threatened species (Pyron 2017; Varzinczak 2018). On the other hand, it is obvious that conservation biologists can play a crucial role in facing and overcoming hopelessness. As Van Dyke and Lamb (2020) wrote, "there is joy in conservation success, and it is in hope that long-term solutions to these and other current problems are possible that conservation biologists work so tirelessly to achieve it". In addition, conservation efforts depend on how they are perceived by politicians and by the general public (Beever 2000). Although conservation biologists must warn these groups of ecological challenges, they also have the duty to give them hope (Beever 2000). In these dark times for biodiversity, I strongly believe that conservation biologists must keep hope alive and as scientist we have the ethical and moral responsibility to act.

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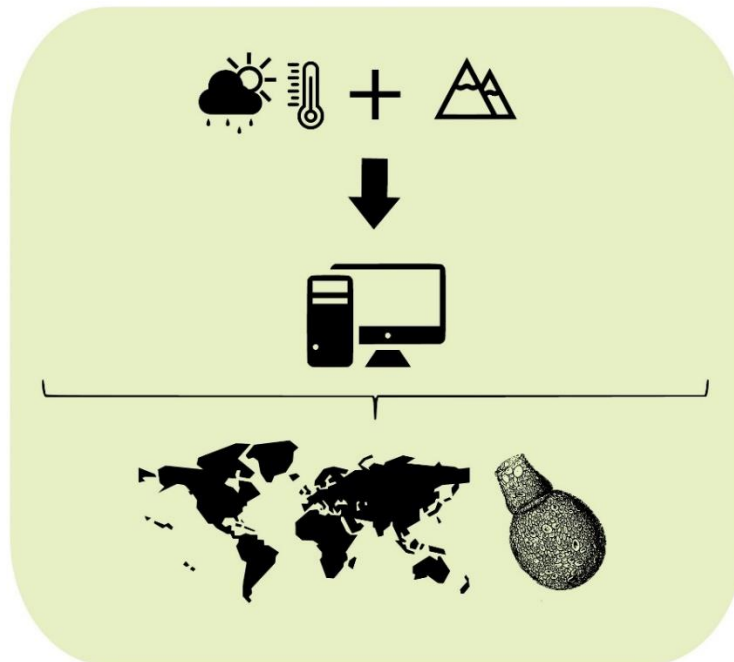
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APPENDIX I : DISTRIBUTION MODELLING OF SOIL PROTIST BIOGEOGRAPHY REVEALS DISPERSAL LIMITATION AND THREAT TO MICROBIAL DIVERSITY FROM CLIMATE WARMING



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E.A.D.M., O.R. and O.B. conceptualized the project, VK, AK, EA, Lbe, QB, AB, Lbu, CD, LDF, TJH, IK, DJGL, MM, RM, DS, EV, DMW, JW, SW, & EADM collected field samples and generated the data, VK, AK, EA, Lbe, QB, AB, Lbu, CD, LDF, AG, TJH, IK, DJGL, MM, RM, DS, EV, DMW, JW & EADM compiled published literature, OR, OB, EPB, RJ, & EADM analysed the data, OR, EPB & EADM led the writing with input from all co-authors.

Abstract

Aim

The goal of this study was to predict the present, past (Last Glacial Maximum - LGM) and future (2061-80) potential distribution of the testate amoeba *Apodera vas*, a morphologically conspicuous and model soil microbe in microbial biogeography. We especially aimed to assess if the well-documented distribution of *A. vas* across the Southern Hemisphere and inter-tropical zone could be explained by its climatic niche rather than palaeogeography and its potential vulnerability in the face of ongoing climate change due to potential dispersal limitation.

Location

Documented records: Southern Hemisphere and inter-tropical zone; modelling: Global.

Methods

We validated 391 georeferenced records for *A. vas* and developed a climatic niche model to predict its present, past, and future potential distributions in responses to climate by relating to climatic and topographic predictors.

Results

Potential distribution maps for past, current and future climates suggest that favourable climatic conditions existed across mountains of East Africa, the Atlantic coast of Brazil, and Southern Australia during the Last Glacial Maximum. The potential distribution range contracted strongly during the current interglacial. Further range contraction is predicted in response to global warming (IPSL-CM5A-MR scenario for 2061-80) with only marginal favourable areas remaining in Africa mainland Australia and Eastern South America. Our models show that favourable climatic conditions for *A. vas* currently exist in the British Isles, an especially well-studied region for testate amoebae where this unmistakable species has never been found.

Main conclusions

This study illustrates the role played by historical factors in shaping microbial biogeography. Our models suggest that the effect of climate change on the potential geographical distribution of terrestrial microscopic organisms at broad spatial scales could possibly lead to regional extinction. Such loss of microbial biodiversity, may in turn affect ecosystem function, thus illustrating the pertinence of including microorganisms in biodiversity conservation research and actions.

Keywords

Climate change; Conservation biogeography; Cosmopolitanism; Endemism; Free-living protists; Gondwana; Microbial biogeography; Palaeogeography; Soil biodiversity; Species distribution modelling; Testate amoebae

1. Introduction

1.1 *Soil microbial diversity patterns and conservation*

Biodiversity conservation requires an up-to-date knowledge on the diversity of organisms and their distribution patterns to prioritize areas for protection and/or restoration (Bragazza 2009; Tittensor et al. 2010; Whittaker et al. 2005). This information is primarily based on empirical species distribution and biodiversity data, or on model predictions derived from them (Rondinini et al. 2006). Climate-based species distribution models (SDM) are thus useful tools to potentially support conservation (Araújo et al. 2011; Araújo et al. 2019; Franklin 2013; Guisan et al. 2013). These models have often been based on current climate data, but palaeoecological data (Nogué et al. 2017) have been shown to be equally good complements for understanding the temporal dynamics of species responses to past environmental changes (Maiorano et al. 2013), including past human influence (Phelps et al. 2020). Thus, conservation biogeography can build on past climate data and models to provide guidance for present and future conservation (Barnosky et al. 2017), illustrating the value of biogeography in applied ecology (Yannic et al. 2014).

The functional importance of soil microorganisms has long been recognised (Sandon 1927), but their diversity remains largely unknown (Decaëns 2010; Eisenhauer et al. 2017; Geisen et al. 2018). As the long-term maintenance and restoration of soil fertility ultimately depends on soil biodiversity (Birkhofer et al. 2008; Decaëns et al. 2006; Morriën et al. 2017), its knowledge and conservation are of vital importance. In line with this, soil microbial biogeography is now a dynamic field of research (Chu et al. 2020), increasingly making use of compiled morphological data sets (Fernández et al. 2016) and high throughput sequencing data to assess diversity patterns at regional to global scales for different groups including bacteria (Ramirez et al. 2018; Yashiro et al. 2016), fungi (Pellissier et al. 2014; Tedersoo et al. 2014), nematodes (van den Hoogen et al. 2019) and protists (Bates et al. 2013; Fernández et al. 2022; Heger et al. 2018; Oliverio et al. 2020; Seppey et al. 2020; Singer et al. 2019a; Singer et al. 2021). The amount and quality of data generated allows for a better assessment of microbial biodiversity distribution patterns, potential threats such as changes in climate or soil conditions (Mod et al. 2021), and cascading impacts on ecosystem functioning (Heleno et al. 2020).

The microbial world constitutes the life support of the biosphere. Changes in microbial biodiversity and associated ecosystem functioning will affect the resilience of other organisms and potentially their ability to respond to climate change. Concerns about biodiversity loss, as well as its possible impact on ecosystem functioning (Heleno et al. 2020) therefore require that microorganisms be considered in conservation strategies, but, like biogeography in general, conservation biogeography is still largely focused on macroscopic plants and animals (Ladle et al. 2011). A further paradox is that microorganisms are crucial in regulating climate change, yet they are rarely the focus of climate change studies. Their diversity and response to environmental change and fluctuation in climate make determining their role in ecosystems challenging. Thus, we need an improved understanding of microbial processes and their response to climate change to ensure an environmentally secure future. Finally, in addition to being essential for soil functioning and natural soil fertility and hence to plant

health and agricultural production, soil microorganisms also have intrinsic value as elements of biodiversity worthy of preservation (Cotterill et al. 2009). We therefore have moral as well as practical and economic reasons to better document soil microbial diversity as a basis for its conservation and understanding its functions. Such moral arguments are, however, currently applied mainly to a small number of plants and animals (O'Malley 2007).

1.2 Species distribution data and models

While studies on diversity patterns are valuable, they do not inform on individual species and hence to what extent global warming (Seppey et al. 2020) and other impacts of human activities may threaten specific microbial species. For this, data at the species resolution are needed, but although regional climate change assessments exist (e.g., Mod et al. 2020), similar assessments are rare for soil microbial taxa at the global scale (Fontaneto et al. 2007). Nevertheless, new molecular methods now make it possible to obtain reliable data at large scales (Robeson et al. 2011), but the exact distribution of soil microbial taxa remains generally unknown. Indeed, existing records are patchy due to highly uneven and non-systematic sampling, with regions such as Europe and North-America typically better covered than other parts of the world (Burdman et al. 2021; Geisen et al. 2018). Such geographical sampling bias is a known problem in biogeography (Meyer et al. 2015; Troudet et al. 2017), and it is no surprise that the less studied microbes suffer from it as much if not more than macroscopic organisms (Yang et al. 2013).

The testate amoeba *Apodera vas* (Certes, 1889) (Amoebozoa: Arcellinida: Hyalospheniformes) is a notable exception to the general scarcity of spatial data for soil microorganisms (Figure 1). Originally described by the late nineteenth century French naturalist Adrien Certes as *Nebela vas* (Certes 1889) based on material collected on Hoste Island (Tierra del Fuego, Chile), *A. vas* is an iconic and highly conspicuous taxon in the debate over microbial biogeography (Finlay et al. 2004; Foissner 1999; Heger et al. 2011b; Mitchell and Meisterfeld 2005; Smith et al. 2009). Indeed, it is one of the few microbial examples to be found in biogeography textbooks (Cox et al. 2016).

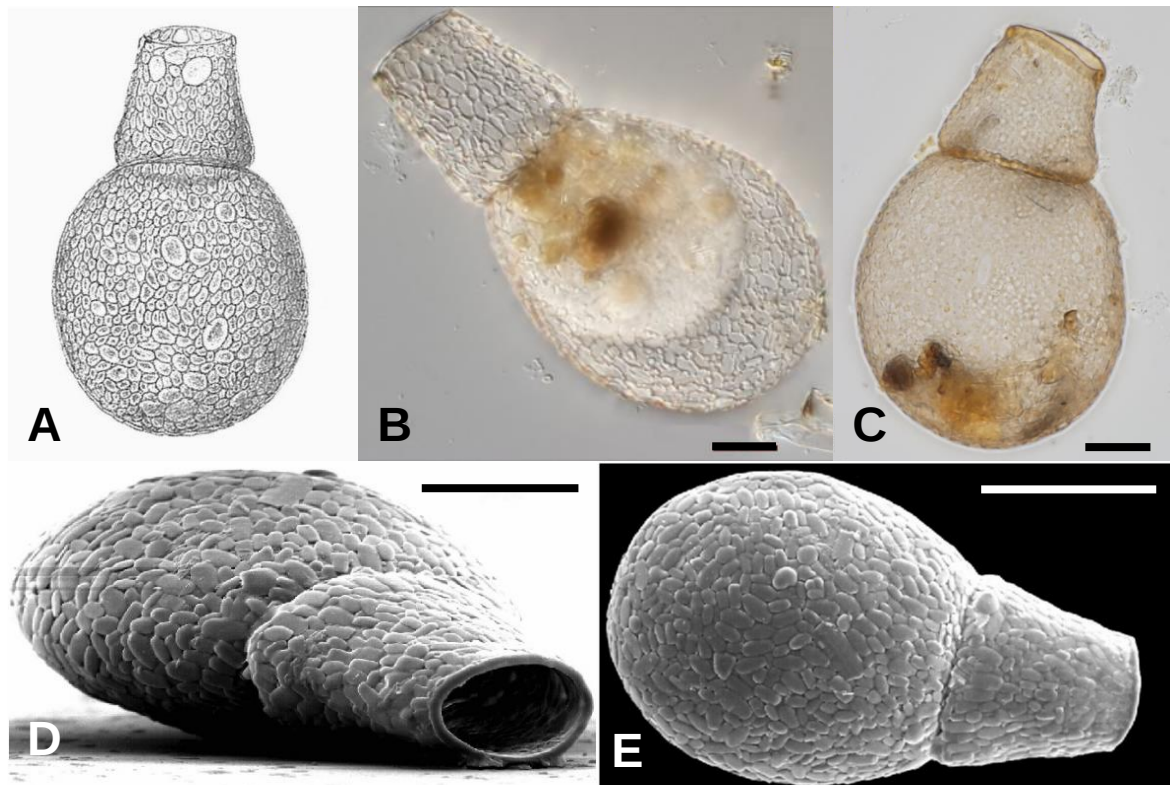


Figure 1. Pictures and microphotographs of *Apodera vas*: A. drawing by Adrian Certes in the original description of *Nebela vas* in 1889 (Certes 1889), B. light microscopy of *A. vas* from Chile (DIC), C. light microscopy of *A. vas* from New Zealand (DIC, extended depth focus image), D. and E. scanning electron microscopy of *A. vas* from Tanzania (Mitchell and Meisterfeld 2005). Scale bars: B & C = 20 μ m, D & E: 50 μ m. Images B-E by E. Mitchell. Images B and C unpublished, Images D & E reproduced from Mitchell & Meisterfeld (Mitchell and Meisterfeld 2005).

Its known distribution is mostly restricted to regions located South of a desert barrier that follows the Cancer Tropic (Figure 2) (Smith et al. 2009; Smith and Wilkinson 2007), suggesting an origin within former super-continent Gondwana, with only limited subsequent dispersal into central America (Bobrov et al. 2013; Golemansky 1967; Heger et al. 2011b; Laminger 1973) and South-East Asia (Bobrov et al. 2013; Bonnet 1980; van Oye 1949). *Apodera vas* is common in wet to humid forest humus and mosses, including *Sphagnum* (Smith et al. 2009). Such environments are common in northern temperate regions, but protistologists have so far failed to find it there, in clear contradiction with the once popular idea that all free-living microorganisms should potentially be cosmopolitan (“everything is everywhere, ...”), their occurrence in a given place being determined only by the local environmental conditions (“... but, the environment selects”) (Baas Becking 1934; Beijerinck 1913; De Wit and Bouvier 2006; Sherwood et al. 2015).

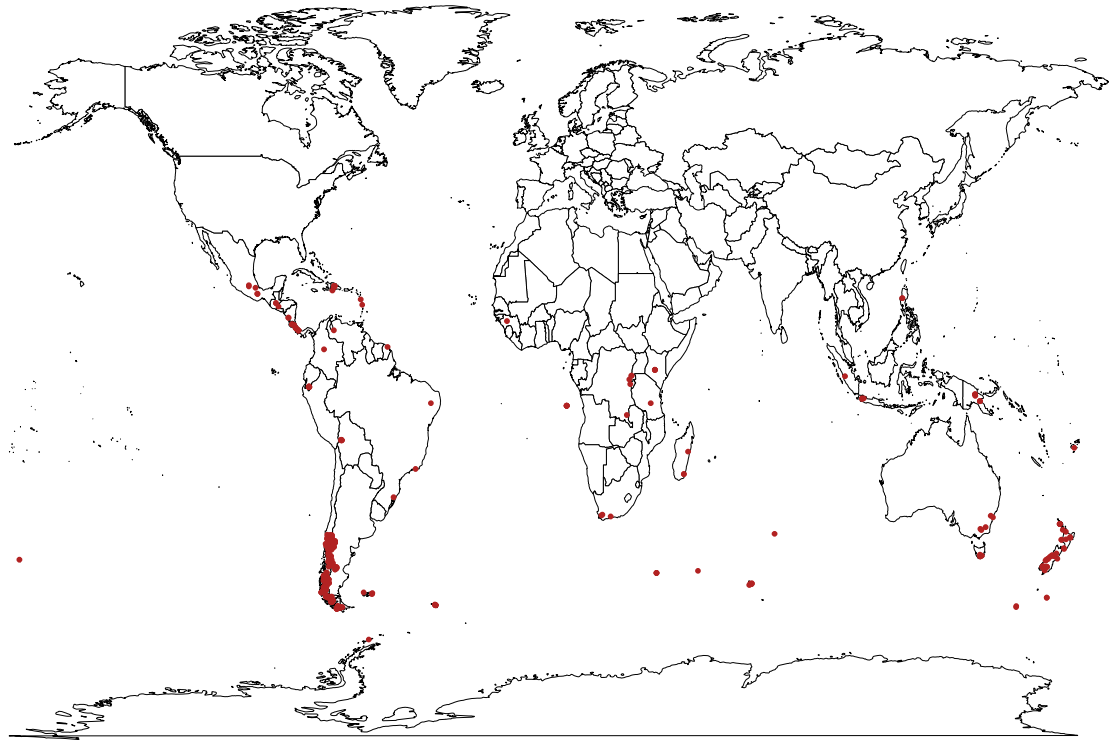


Figure 2. Geographical position of 391 validated occurrences of *Apodera vas*. Occurrences (red points) compiled from the literature and personal observations. Grey areas correspond to the potential distribution in which background points were randomly selected for the modelling.

Although the potential distribution of *A. vas* had not yet been modelled, the abundance of records in the literature has now made this possible, thus providing a unique test case to use a predictive bioclimatic niche-based species distribution models (SDM) in soil microbial biogeography. Indeed, although this approach has been widely used in studies of multicellular taxa, there are only a few SDM microbial studies (e.g., Mod et al. 2021; Mod et al. 2020). However, studies on larger organisms have indicated that the performance of such models is independent of the trophic level (Huntley et al. 2004), suggesting they are also applicable to soil microorganisms (Schröder 2008).

1.3 Conservation of microbial diversity

Due to the large census population sizes that can be seen in microorganisms, it used to be considered unlikely that any may be endangered (Finlay et al. 2004), but this view has been challenged (Cotterill et al. 2009). Extinction threat increases with decreasing population size and geographic range (Wilson and MacArthur 1967) and it is now demonstrated that at least some soil microbes also have limited geographical ranges (Beyens and Bobrov 2016; Boenigk et al. 2006; Chao et al. 2006; Foissner 2006, 2009; Geisen and Bonkowski 2018; Heger et al. 2011a). However, it remains difficult to demonstrate that a microbial species may indeed be threatened and to forecast the effect of its potential loss on ecosystem functioning. Climate-niche models are useful to compare the predicted and realised distribution of taxa, and thus to assess the possible role of historical factors such as palaeogeography

or glaciations in shaping current patterns. For example, if these models are used to predict the potential distribution under different climatic scenarios, in the past (e.g., Schorr et al. 2013) and in the future (e.g., Mod et al. 2021), it is then possible to infer the magnitude of potential range expansions or contractions over glaciation cycles and the likely impact of future climate warming. Finally, SDM are also potentially useful to guide the sampling in regions suitable for the species but where it was not yet observed.

Based on the geographical coordinates of 391 curated *Apodera vas* occurrences (Figure 2), we built a bioclimatic niche-based species distribution model and determined its potential distribution according to current climate, future climate scenarios (Coupled Model Intercomparison Project - CMIP5 predictions for 2061-2080, representative concentration pathways 4.5 and 8.5) and past climatic conditions during the last glacial maximum (LGM, i.e., 21 ± 3 ka BP; Palaeoclimate Modelling Intercomparison Project, hereafter PMIP3). Our objectives were to 1) compile an updated distribution map of *Apodera vas*, 2) build a climate niche model and predict the current, past, and future potential distribution of *Apodera vas* in the absence of any dispersal barriers and evaluate the magnitude of potential range expansions or contractions in response to past and on-going climatic changes, 3) use these models to predict phylogeographic patterns and the likelihood of cryptic diversity and possible threats to this yet undescribed diversity from climate and land-use changes, and 4) assess the implications of climate niche models for research on microbial biodiversity and biogeography (e.g. identifying priorities for future sampling), ecosystem functioning and applications in conservation.

2. Methods

2.1 Study area and species data

We compiled 391 occurrence points from all known published records of *A. vas* worldwide (Smith and Wilkinson 2007) and from our own unpublished data (Figure 2, Table S1). When no precise location was given, we estimated the most likely location given the available information in the text, especially considering the specified or likely (i.e., forests or peatlands) habitat-type and elevation, resulting in an estimated geographical accuracy of less than 2 km from the original sampling location for most samples. A dubious unconfirmed record from Antarctica (an apparent albeit surprising confusion with *Diffflugia vas*, now *Lagenodiffflugia vas* (Murray 1910; Penard 1911) was excluded, but the record of Zapata & Matamala (Zapata and Matamala 1987) from King George Island, 120 km off the coast of the Antarctic Peninsula is validated despite being from lake sediments, which is not the typical habitat of *A. vas*. Records from Vancouver, Canada (Penard 1911), Iceland (Decloître 1965), Nepal (Bonnet 1977), Hawaii and Japan (Richters 1908), likely corresponding to mis-identifications, and records with insufficient information to infer a location with the required degree of certainty for this were also excluded (see supporting information for a more detailed discussion of critical points).

To estimate the past, current and future global bioclimatic niche of *Apodera vas*, we first calibrated models with all available *A. vas* occurrences at a 2.5 arcmin resolution (~5 km). Then, we disaggregated

A. vas occurrences to avoid spatial auto-correlation (grid resolution = 2.5 arcmin * 10, occurrences kept: n = 136).

2.2 Current bioclimatic variables and topographic data

We used the high-resolution (30 arcsec, ~1km) climate database CHELSA Bioclim v1.2 (Karger et al. 2017; Nikolaus Karger et al. 2018) to retrieve bioclimatic variables. We selected five variables to describe the current climatic niche of *A. vas*: (i) bioclim4: temperature seasonality, (ii) bioclim9: mean temperature of driest quarter, (iii) bioclim10: mean temperature of warmest quarter, (iv) bioclim15: precipitation seasonality, and (v) bioclim18: precipitation of warmest quarter (Table S2; Figures S2 and S3). These descriptors were selected as complementary based on their position in a PCA of all variables and a low pair-wise correlation (computation matrix of Person and Spearman rank-correlation < 0.7; (Dormann et al. 2013)) while being consistent with the ecology of this microorganism and the general observation that terrestrial testate amoeba communities respond to soil moisture (Bonnet 1990; Lousier 1974b). Bioclimatic data were upscaled at a 2.5 arcmin resolution. The Topographic Position Index (TPI) was used to retrieve information on the elevation in each grid cell (Karger et al. 2017; Nikolaus Karger et al. 2018).

2.3 Spatial modelling

All statistical analyses and modelling processes were performed in the R environment version 4.1.1 (R Core Development Team 2021) using package *biomod2* (v. 3.5.1, Georges and Thuiller 2013; Thuiller et al. 2016). The full code is provided in the supporting information section.

To build the bioclimatic niche-based SDM, we first randomly selected 10 sets of 1000 background points (Barbet-Massin et al. 2012; Barve et al. 2011). Each set of background points was randomly selected in the area accessible to the species *A. vas* (Figure 2) (sensu Barve et al. 2011). Then, the background points datasets and occurrences were processed with three algorithms included in *biomod2*: (i) Generalized Linear Model (GLM – (Guisan et al. 2002)), (ii) Boosted Regression Trees (GBM = Boosted Regression Trees - BRT- (Elith et al. 2008)) and (iii) Maximum Entropy Method (MAXENT; (Phillips and Dudík 2008)). For each set of background points, each modelling technique (i.e., the three algorithms) was run three times. All models were validated using a 80/20% split sample cross-validation where 80% of the points were used to build the model and the remaining 20% were used to validate it. All models were evaluated based on the maximization of the true skill statistics (TSS, Allouche et al. 2006), see (Guisan et al. 2017) for the maximization) and the area under the receiver operating characteristic curve (AUC, Fielding and Bell 1997). We draw response curves for each variable and for each model to show how predicted values changed along each variable gradient while keeping all other variable at their mean value (Elith et al. 2005).

2.4 Current, past and future predictions

Finally, we used the resulting models to determine the global potential distribution of *A. vas* according to three climatic conditions at a 2.5 arcmin resolution: (i) current climate, (ii) past climate during the last glacial maximum (LGM), and (iii) future climate scenario for 2061-2080. For the future scenario,

we used the IPSL-CM5A-MR scenario for 2061-80 using the representative concentration pathways (RCP) 4.5 and 8.5 from the CMIP5 project (<https://pcmdi.llnl.gov/mips/cmip5/>) and current TPI. For LGM we used the scenario IPSL-CM5A-LR from the PMIP3 project (<https://pmip3.lscce.ipsl.fr/>) and LGM topographic position index. All these data were downloaded from the CHELSA database v1.2 (Karger et al. 2017; Nikolaus Karger et al. 2018). We used Ensemble Forecasting to create global final prediction maps. Two maps were produced for each time period: (i) a map showing mean prediction weighted by model quality (TSS and AUC), and (ii) a map illustrating coefficients of variation between different models. Then, Ensemble Forecasting was also evaluated with True Skill Statistic (TSS) and the Area Under the Receiver Operating Characteristics Curve (hereafter AUC).

To downweight predictions in non-analogous climatic areas, we used a Multivariate Environmental Similarity Surfaces (MESS) analysis that measures the climatic differences between the calibration and projection areas for each time scenario. Negative MESS values indicate localities that are climatically non-analogous to the calibration area (Elith et al. 2005). Final SDM prediction maps were adjusted using the MESS results in the following way: (i) areas with MESS values > 0 were not modified and (ii) areas with MESS values < 0 were weighted with MESS values (i.e., multiplied by MESS values normalized between 0 and 1). As bioclim10 (i.e., mean temperature of warmest quarter) was by far the variable that had the strongest influence in the SDM modelling process, final results presented here are based on a MESS calibrated only on bioclim10.

3. Results

3.1 Updated distribution map of *Apodera vas*

The 391 validated occurrences of *Apodera vas* (Figure 2) show that this species has a broad distribution in the Southern Hemisphere and inter-tropical zone. It confirms its absence from mid- to high-latitudes of the Northern Hemisphere above 20°N. *Apodera vas* is found at higher elevation at low latitudes (20°N-20°S; average 2369m, SE 129m, median 2145m) than at high latitude (>35°S; average 542m, SE 30m, median 414) (Figure 3)

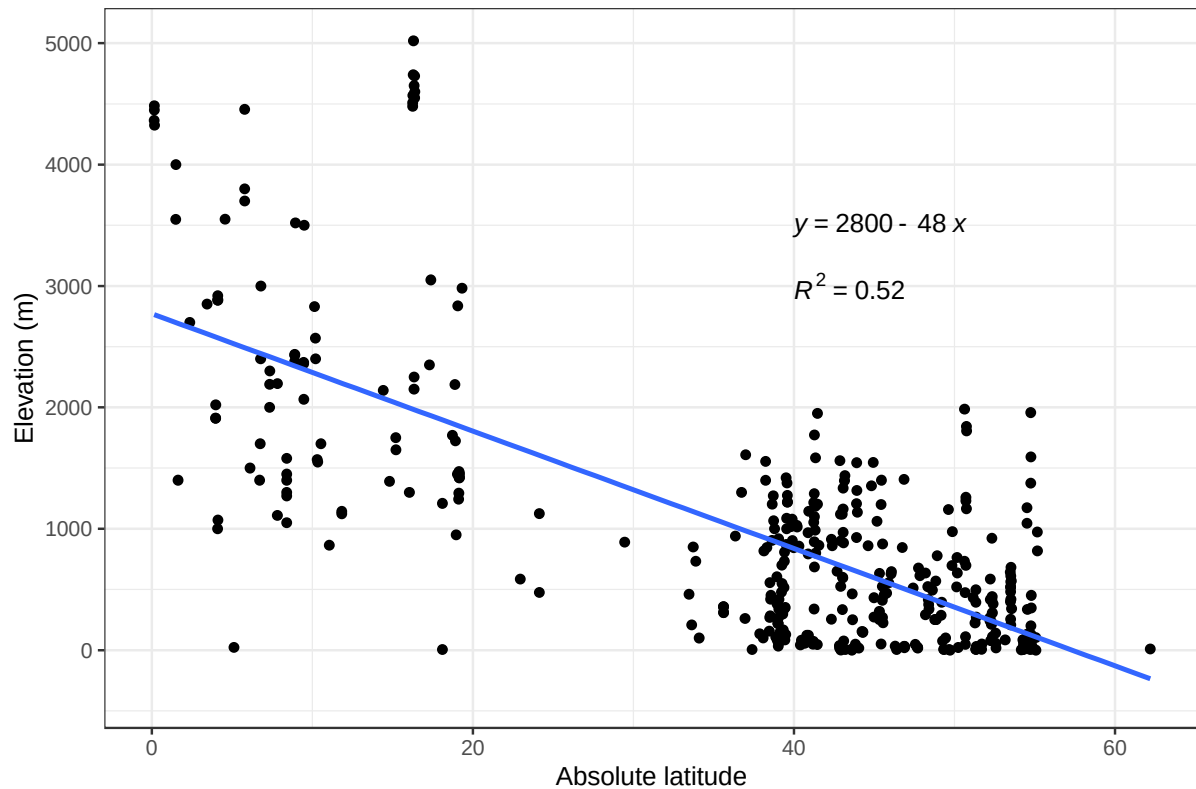


Figure 3. Biplot of the absolute value of latitude vs. elevation of 391 *Apodera vas* geographical records. The line shows a linear regression and confidence intervals.

3.2 Model performance, variable importance and probability of occurrences maps

We successfully obtained species distribution models for *A. vas* and generated a map showing probability of occurrences for this species weighted with MESS of bioclim10 (Figure S3). Model performances were based on TSS (mean: 0.77, sd: 0.11) and AUC (mean: 0.92, sd: 0.06) (Table S3). The importance of variables highlighted the central role of the mean temperature of the warmest quarter (bioclim 10). This descriptor was the most determinant one for each algorithm (GLM: 0.98, sd: 0.02; GBM: 0.70, sd: 0.04; MAXENT: 0.73, sd: 0.20; Table S4). Looking at response curves (Figure S4), this taxon is mainly found in temperate to cold climate. Indeed, *A. vas* is likely to be found in regions where the maximal temperature during the warmest quarter (bioclim10) is below ca. 20°C. This is also evident from the relationship between latitude and elevation in our records (Figure 3). Finally, we merged all the best performing models together to obtain final maps of probability of occurrences for each climatic scenarios (Figure 3; evaluation with TSS: 0.84 and AUC: 0.97; Table S3).

3.3 Current, past, and future potential distributions of *Apodera vas*

Our prediction maps show that *Apodera vas* could, with the present climatic conditions, potentially occur on all continents with highest predicted probability of occurrence in temperate oceanic regions such as the Pacific coast forests of Canada, Southern Chile and Tierra del Fuego, South-East Australia, Tasmania and New Zealand as well as tropical mountain areas of South and Central America, the

Caribbean, Sub-Saharan Africa, North-West Europe and South-East Asia (Figure 4). The validity of these models was tested by observing (in the field) moss samples from rocks and trees in the mountains of SE Madagascar by one of our team members (Sebastien Wohlhauser) (Figure S5).

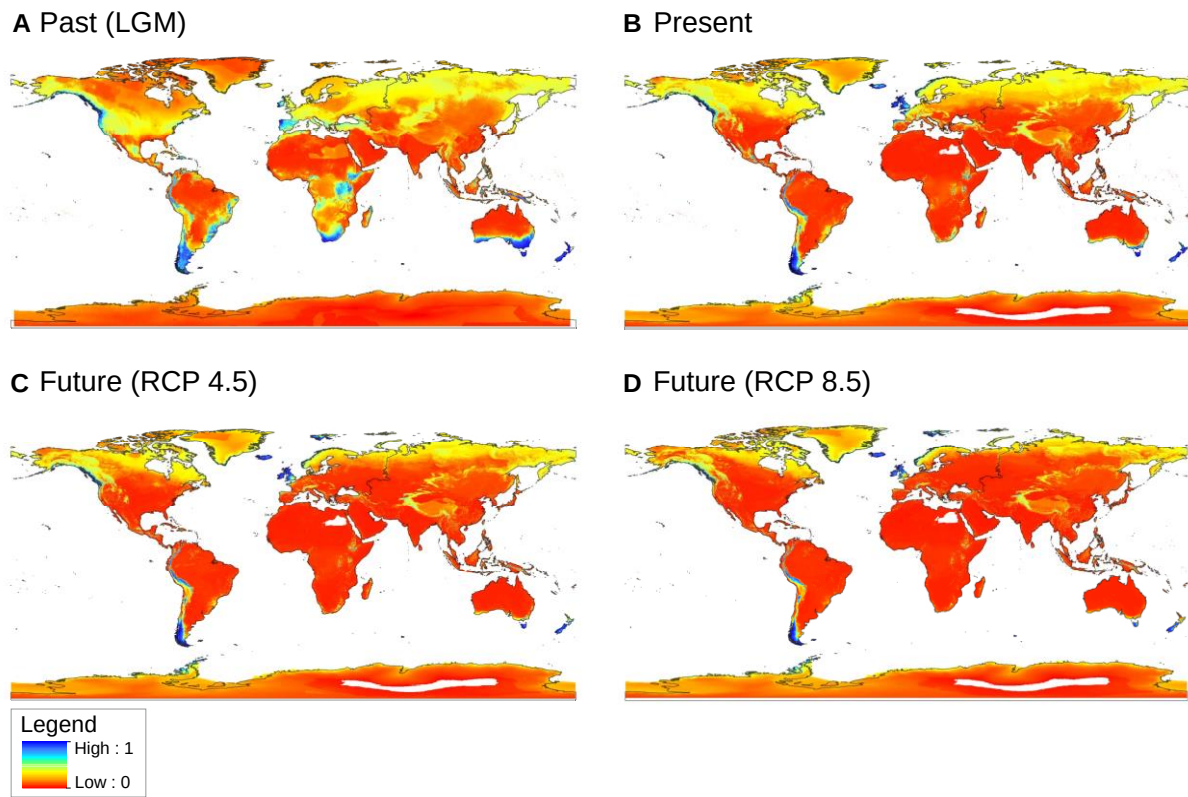


Figure 4. Modelled potential distribution of *Apodera vas*. Distribution for A. Late Glacial Maximum climatic conditions (LGM-PMIP3), B. current climate, C. future climate (CMIP5 prediction for 2016-80, RCP 4.5) and D. future climate (CMIP5 prediction for 2016-80, RCP 8.5). Note the high probability of occurrence in some temperate regions in the Northern Hemisphere especially the British Isles, and the reduction in overall potential favourable habitat and the loss of connectivity between remaining favourable habitats (e.g., Southern Australia, Southern and Eastern Africa).

The comparison of current and past (LGM) models (Figures 4 and S6-8) reveals strong range contractions of *A. vas*, which was particularly evident in Africa where an almost continuum between mountains of Angola and Ethiopia shrinks to isolated patches corresponding to the highest elevation mountain ranges. Similarly, the large band of moderately favourable habitat along the Atlantic coast of south America and the even more favourable band along the southern coast of Australia both shrink.

The comparison of current and future climate scenarios models predicts further range reduction in these same regions with some contrast between RCP 4.5 and 8.5 projections (Figures 4 and S6-8). The potential distribution in RCP 8.5 represents an extreme among the four projections with several areas such as SE England, SE Australia and the North Island of New Zealand still reasonably favourable (light blue) in the RCP 4.5 scenario becoming clearly unfavourable (orange and red) under RCP 8.5. Furthermore, in the RCP 4.5 and 8.5 projections some isolated potentially favourable areas such as the African mountains, Madagascar and the Atlantic Forest of Brazil, which had already seen their surfaces shrink considerably between LGM and current conditions, may move outside of the climatic niche space of *A. vas*. The latter two regions have been subject to intense deforestation in the past decades, which added to climate change certainly contributes to a strong reduction in potential habitat and population sizes.

4. Discussion

4.1 Updated distribution map of *Apodera vas* - Taxonomy as a possible caveat

The 391 validated occurrences of *Apodera vas* (Figure 2) represent a substantial increase in comparison to the 46 sites included in the analysis of Smith and Wilkinson (2007), which also included several errors. The most critical points are discussed in the supporting information. Two possible caveats should be mentioned here: The first is that some published records may be wrong due to misidentification. For example, *A. vas* resembles *Lagenodiffugia vas* (Finlay et al. 2004), an unrelated species (Mitchell and Meisterfeld 2005) in general shape, and we suspect this confusion could explain the unconfirmed record of *A. vas* from mainland Antarctica (Murray 1910; Penard 1911). A second caveat is that it is impossible to account for cryptic or pseudo-cryptic diversity when using morphology-based records. Cryptic or pseudo-cryptic diversity is commonly reported within broadly defined morphological species of protists and other micro-metazoa (de Vargas et al. 1999; Foissner et al. 2001; Fučíková and Lahr 2016; Heger et al. 2009; Kosakyan et al. 2012; Leasi et al. 2013; Singer et al. 2015). It has long been suspected that *A. vas* is a species complex (Penard 1911; Zapata and Fernandez 2008) and this is supported by the recent finding of a long-forgotten yet highly conspicuous species within genus *Apodera* in New Zealand and the associated high genetic variability within morphotypes identified as *A. vas* in New Zealand and Macquarie Island (Duckert et al. 2021). Thus, relying on morphological identification and partly unverifiable sources carries the risk of overestimating the ecological or climatic niche of a species. This caveat suggests that, if our predicted distributions for *A. vas* were biased, this bias would be towards an overestimation of its distribution. We therefore regard our results as being valid for *A. vas* as a species complex.

4.2 Model parameters and performance

The performance of our models was good (Table S3), but an unexpected result was that temperature was the most important variable rather than moisture (Table S4). Indeed, as soil moisture is a key factor controlling the productivity (Lousier 1974a, b) and community structure (Koenig et al. 2018) of soil protists, we expected bioclimatic variables related to moisture (e.g., precipitation) to significantly explain a high fraction of the distribution of *A. vas*. However, the clear relationship between latitude and elevation in the compiled records of *A. vas* (Figure 3) confirms the importance of temperature as a factor determining the presence of this species. This apparent paradox may also be due to the fact that precipitation only partly predicts soil moisture (Mod et al. 2016; Piedallu et al. 2013; Scherrer and Guisan 2019). Soil moisture is indeed partly determined by local factors such as microtopography (Lembrechts et al. 2020; Lembrechts et al. 2019), soil texture, shading, or interception by tree foliage, all of which vary substantially at the local scale, decreasing the pertinence – and hence predictive power of precipitation for soil biotic communities (le Roux et al. 2013; Lembrechts et al. 2020). The lower importance of precipitation variables in the models could also be due to the fact that temperature data are more accurate than precipitation data in the CHELSA Bioclim data base. This potential bias is illustrated by a comparative study of temperature and precipitation data inferred from vascular plant data recorded in the Swiss biodiversity monitoring network (BDM) using expert-based plant ecological indicator values (EIVs) (Ellenberg et al. 1992). The comparison of this well validated method with climatic extrapolations from meteorological stations showed that while the model-inferred temperature data was quite good, the inference was poorer for other variables, and hence model performance doubled when variables such as soil properties were included (Scherrer and Guisan 2019). Indeed, model performance only improved marginally when EVIs were included instead of mapped temperature data. Despite these limitations, the model performance was sufficient for answering the hypotheses of this specific study (Table S3).

4.3 Current, past, and future potential distribution of *Apodera vas* and implications for microbial phylogeography

The potential current distribution of *A. vas* logically matches well the known occurrences of *A. vas* in the Southern Hemisphere and part of the tropics (Figure 2) but the absence of documented localities in well studied regions of the Holarctic with a high probability of occurrence such as Britain, Ireland, Iceland and the Pacific Coast of Canada is remarkable (Figures 4 and S7). Favourable habitat such as *Sphagnum* peatlands and forests with well-developed humus are indeed widespread in these regions (Figure S8). Its absence from these regions can therefore be interpreted as evidence for limited dispersal. The British Isles are arguably the most intensively studied region for the current and past (palaeoecology) diversity of testate amoebae, but *A. vas* has never been reported there (Amesbury et al. 2016; Mitchell et al. 2008), except from horticultural *Sphagnum* mosses imported from New Zealand to be used in decorative hanging baskets (Wilkinson 2010). This absence was interpreted as a sign of limited dispersal (Foissner 2006, 2009; Smith et al. 2009) and our modelling results bring further support to this hypothesis through clear and quantitative predictive data. To our knowledge this is the first documented case for a microbial species.

Our modelling results also have implications for the possible invasion of free-living microbial species. Indeed, no living specimen of *A. vas* has yet been observed in the British Isles, but should some specimen survive the transport and conditioning of the peat, *A. vas* could likely colonise the British Isles and other places such as Japan which also imports *Sphagnum* from New Zealand (Wilkinson 2010). Indeed, our models clearly show that the climate would be favourable for its development (e.g., maximal temperature during the warmest quarter below ca. 20°). This suggests that importation of mosses and soil, including through horticultural products, should be more strongly regulated to prevent the spread of potentially invasive microorganisms.

Our models predict substantial range contraction and expansion over glacial-interglacial cycles (Figures 4 and S6-8). Such changes likely result in complex phylogeographical patterns, as reported for *Hyalosphenia papilio*, a common *Sphagnum*-dwelling hyalospheniid testate amoeba taxon found in Northern Hemisphere peatlands (Heger et al. 2013; Singer et al. 2019b). Further marked range contraction is predicted as a response to on-going climate warming (Figures 4 and S6-8). Although the contrast is not as strong as between current and LGM models, which is similar to findings for soil microorganisms at the more regional scale (Mod et al. 2021; Mod et al. 2020), this predicted contraction suggests that *A. vas* may become rare or may even go extinct in some regions for both future scenarios (i.e., RCP 4.5 and RCP 8.5; Fig. S6-8). Assessing the likelihood of such a scenario would require ground truthing by conducting surveys in regions where models predict that *A. vas* was likely abundant but has become rare.

A somewhat surprising result is that some regions that were glaciated during the LGM, such as the British Isles and the Alps, do not appear as absolutely unfavourable (i.e., yellow shading). By contrast the region covered by the larger North American ice sheet is clearly mapped as unfavourable (i.e., red). Such patterns suggest that only the dark and light blue areas should be considered as climatically potentially favourable for *A. vas*.

Our results are valuable to target potential sampling sites for *A. vas*, especially the Sub-Saharan African mountain ranges (i.e., from the highlands of Ethiopia to the mountains of Southern Africa and Cape region, Mt. Cameroon and the mountains of Angola; Figure S9), the Atlantic Forest in Brazil, Andes and Central America and SE Asia (Figure 4). Sampling in these places would allow us first test the validity of our model and, if the species is found, to isolate specimens for DNA barcoding and phylogeographical analysis. As other microbial taxa may exhibit a distribution similar to *A. vas*, a sampling targeting this taxon would allow us to assess phylogeographical patterns in several microbial groups, such as other hyalospheniidae (e.g., genera *Nebela*, *Padaungiella*, *Alocodera*, and *Certesella*), ciliates (Kumar and Foissner 2016), or bdelloid rotifers (Fontaneto and Ricci 2006). Such a multi-group approach would be useful to clarify the extent of microbial diversity and biogeography more generally.

4.4 Linnean and Wallacean shortfalls in the study of soil microbial biodiversity

The study of soil biodiversity in general suffers from two main curses: the Linnean shortfall (most species remain undescribed) and the Wallacean shortfall (the geographic distribution of known species is incomplete) (Hortal et al. 2015). The Linnean shortfall is illustrated by general estimates of the

“known and unknown” diversity (Chao et al. 2006; Decaëns 2010; Foissner 1999), as well as by studies reporting new protist species, often from specific habitats (Dunthorn et al. 2012; Foissner and Stoeck 2011). Clearly, much remains to be done to clarify the taxonomy of most microbial groups (Eisenhauer et al. 2017; Geisen and Bonkowski 2018). Taxonomic uncertainty in turn hinders biogeographical inference (Caron 2009; Heger et al. 2009) and, together with very patchy sampling, contributes to the Wallacean shortfall, the scarcity of geographic distribution data for most taxa (Hortal et al. 2015). Many species are known from only a small number of localities, often within a given region (Heger et al. 2011a), but sometimes there is a puzzling disjunct distribution with reports from distant regions (Bourland 2017; Nicholls 2015). As most of these records are based on morphological data only, strange patterns may hide cryptic or pseudo-cryptic diversity, and hence emphasizing the need for more taxonomic studies combining morphological and molecular approaches (Foissner 2009; Foissner et al. 2001; Heger et al. 2014).

4.5 Species distribution models to predict cryptic diversity, distribution and threat

As was shown for *Hyalosphenia papilio*, a ubiquitous hyalospheniid testate amoeba common in northern Hemisphere *Sphagnum*-dominated peatlands (Heger et al. 2013; Singer et al. 2019b), a single morphospecies may correspond to numerous distinct species. As each of these may occur only in a fraction of the whole range of the morphospecies, some of this diversity may indeed be threatened and, as such, worthy of concern to the same degree as macroscopic plants or animals. There is no reason to believe that the pattern of geographically structured cryptic diversity observed for *H. papilio* would not also be found in *A. vas*. Quite the contrary: the distribution of *A. vas* is disjunct due to the distribution of land masses in the Southern Hemisphere, and, within continents, it is much patchier than that of *H. papilio* due to the ecological preference of this taxon. The predicted distribution of this morphospecies therefore suggests the existence of a diverse species complex. As our models show that many currently favourable locations will become unsuitable for *A. vas* in a warmer future, *A. vas* should be considered at risk of becoming locally extinct, and if these local populations correspond to genetically distinct species this would mean a net loss of species diversity (Bickford et al. 2007). If we are to understand the true morphological and molecular diversity within this taxon, it is therefore urgent to study such relict isolated populations. This illustrates how useful species distribution models are to identify areas where taxa are potentially threatened due to direct impact (e.g., land-use changes) and climate change.

4.6 Implications for biodiversity conservation and ecosystem functioning

Global warming is a recognised major threat to plant (Harter et al. 2015) and animal (Díaz et al. 2019) biodiversity, especially in places such as oceanic islands from where migration to more suitable habitats is limited. Our data suggest that it may also cause a loss of diversity in free-living protists. Considering other components of global change, it is likely that many microbial species have already disappeared and many more are currently threatened. Such a loss has only very rarely been documented for protists. One such example is *Nebela carinatella* a highly conspicuous species described as subfossil from Subatlantic peat deposits in Belgium but absent from the communities

living at the surface (Beyens and Chardez 1982). A record from China for an aquatic habitat and which we could not find any illustration is not considered here (Yang et al. 2004). The loss of microbial diversity represents a potential threat to ecosystem functioning through disruptions of food webs (Heleno et al. 2020). It is therefore now urgent to invest in taxonomy to better document this mostly unknown diversity, evaluate how much of it is threatened and what would be the ecological consequences of their disappearance.

Protists and microbes in general are rarely considered to be of any concern for biodiversity conservation despite their major ecological roles and huge diversity (O'Malley 2014; Wilkinson 2007). The almost complete exclusion of microbes in conservation efforts is partly due to a historical focus on macroscopic organisms, which ignores large parts of biodiversity resulting in a general lack of expertise on microbial biodiversity (Wilkinson 1998). But is also likely due to the belief that owing to their high abundance and dispersal potential it is all but impossible for any microbial species to go extinct (Fenchel and Finlay 2003). However, even within the microbial world, most attention has been recently on bacteria and fungi, compared with the relatively under-studied protists (Caron 2009; Geisen et al. 2017). Reaching a better balance in the study of different groups of organisms is a clear challenge, as is bringing microbes and especially protists to the attention of conservationists. *Apodera vas* is not just a scientific curiosity; the contrast between its current and projected future distribution tells us a lot about the impact of human activities on the biosphere and suggests that, just as for macroscopic organisms, many microbes may currently be threatened with unknown consequences on ecosystems.

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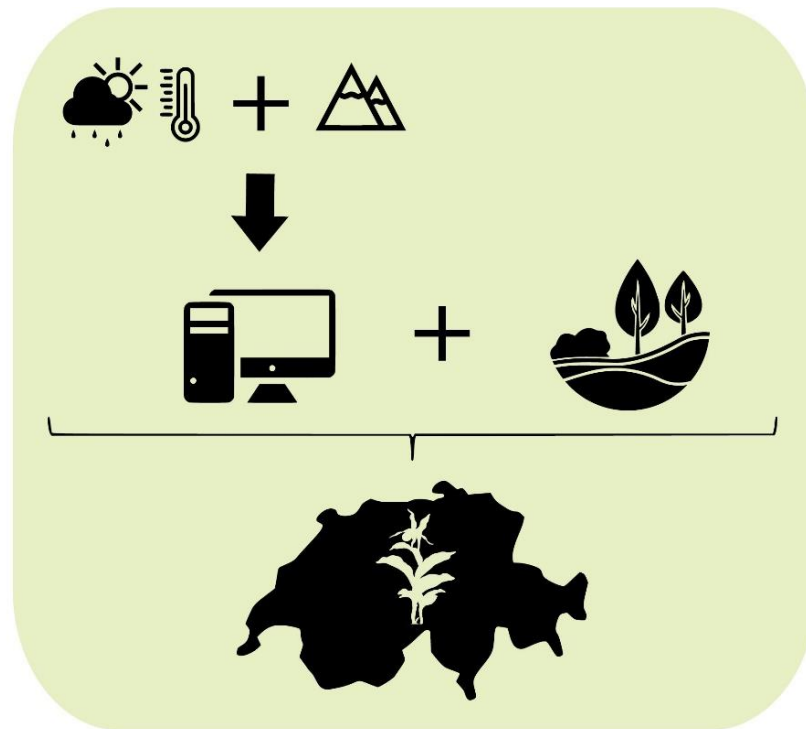
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APPENDIX II : SUPPLEMENTARY MATERIAL FOR CHAPTER I: DETECTING PRESERVATION AND REINTRODUCTION SITES FOR ENDANGERED PLANT SPECIES USING A TWO-STEP MODELLING AND FIELD APPROACH



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Supplementary data D1. Soil profiles of 16 *Cypripedium calceolus* populations. In each *C. calceolus* population, we dug a soil profile until bedrock or parental material, and we separated soil profiles in organic, organo-mineral and mineral horizons according to each homogenous zone observed. We identified each horizon and classified both the soils and humus forms (Baize and Girard 2009; IUSS Working Group 2015; Zanella et al. 2018). The humus and soil horizons were named according to the Référentiel pédologique (Baize and Girard 2009), and the World Reference Base for Soil Resources (WRB) (IUSS Working Group 2015) (see glossary of soil terms at the end of the document. Finally, vegetation types were also identified and classified according to Delarze et al. (2015).

Glossary for humus forms and soil horizons according to (Zanella et al. 2018) with (IUSS Working Group 2015) equivalent.

Horizon name	Definition	WRB
nOL and vOL	The OL horizon is characterized by the accumulation of mainly leaves/needles, twigs and woody materials, most of the original plant organs being easily discernible to the naked eye. The humic component amounts to less than 10% by volume; recognizable remains 90% and more, up to 100% in non-decomposed litter. Suffixes characterize neither fragmented nor transformed/discolored leaves and/or needles (nOL), and slightly altered, discolored, bleached, softened up, glued, matted, skeletonized, sometimes only slightly fragmented leaves and/or needles (vOL)	O
BryoOL	OL horizon with Bryo characteristics defined as an horizon with “byophytes or arbuscular lichens or small stonecrop plants totally covering the soil and forming a stratified carpet or cushion with living (green) parts overgrowing a layer of dead stems and leaves	O
OF	The OF horizon is characterized by the accumulation of partly decomposed litter, mainly from transformed leaves/needles, twigs and woody materials, but without any entire plant organ. The proportion of humic component is 10% to 70% by volume. Depending on humus form, decomposition is mainly accomplished by soil fauna (OFzo) or cellulose–lignin decomposing fungi (OFnoz). Slow decomposition is revealed by a partly decomposed matted layer, permeated by hyphae	O
OH	The OH horizon is characterized by an accumulation of zoogenically transformed material, i.e. black, gray-brown, brown, reddish-brown well-decomposed litter, mainly comprised of aged animal droppings. A large part of the original structures and materials are not discernible, the humic component amounting to more than 70% by volume. The OH horizon differs from the OF horizon by a more advanced transformation (fragmentation, humification, ...) due to the action of soil organisms	O

Soil horizons and layers according to (Baize and Girard 2009) with (IUSS Working Group 2015) equivalent.

Horizon name	Definition	WRB
A	A horizon presents a mix of organic and mineral material and is situated under holorganic horizons.	A
Aca	Aca is an A horizon with cold HCl effervescence.	A
Acah	Acah is an Aca horizon that is humus-rich.	A
Aci	Aci is an Aca horizon which has been decarbonated. Aci is thus not carbonated in fine soil or or only occasionally or locally.	A
Acih	Acih is an Aci horizon that is humus-rich.	A
Cca	C horizon is a depth mineral horizon. Its components have undergone, throughout their mass, significant fragmentation and/or some geochemical alteration. Cca is calcareous C horizon.	C
Dca	D layer is formed with hard materials fragmented, then moved or transported, not consolidated, forming a pseudo-fixed set where the coarse elements dominate (scree, moraines, etc.). This not very coherent layer is often easy to work in the absence of blocks. Dca is calcareous M layer.	-
Mca	M layer is formed by continuous, loose or soft rocks, not or slightly fragmented, with possibly locally or partially altered micro-cracks. This layer are coherent, but easy to work with tools. Mca is calcareous M layer.	-
Rca	R layer is formed with hard, continuous, massive or slightly fragmented rocks, generally with cracks. These cracks can trap material from the upper horizons. Localized dissolution, disintegration and alteration occur in the rock seals. This layer is very difficult to break up and the soil cannot be deepened by the usual tillage tools. Rca is calcareous R layer.	R
S	S horizon is a structural horizon. It is an alteration horizon. Process such as alteration of primary minerals, release of iron oxyhydroxides, decarbonation take place in that horizon.	B
Sca	Sca is calcareous S horizon.	B

Soil name (Baize and Girard, 2009)	CALCOSOL
Soil name (IUSS Working Group, 2022)	Calcaric Cambisol
Humus form (Zanella et al., 2018)	Oligomull
Site name	NAT-GE1

Station parameters		
Location	Blécheins	
Date	12.08.2016	
Altitude	1122 m	
Slope	5°	
Aspect	342°	
Topography	Slight slope	
Geology	NA	
Vegetation alliance	<i>Molinio-Pinion</i>	

Soil profile (horizon names from Baize and Girard, 2009)		
BryoOL-nOL-vOL +1 0 Aca-Sca-[OF] 3 Sca 14 Sca-Mca		

Horizon depth (cm)	Horizon name	Coarse fraction (%)				Acidity, presence of calcium carbonates		Texture	Soil structure					Roots (index from 1 to 5)			Soil color (Munsell)
		2-20 mm	2-5 cm	5-20 cm	> 20 cm	pH (Hellige)	HCl		single-grain	crumb	sub-blocky	blocky	fibrous	0-2 mm	2-5 mm	> 5 mm	
+1 - 0	BryoOL-nOL-vOL	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
0 - 3	Aca-Sca-[OF]	3	-	-	-	-	4/4	silty-clayey	-	X	X	-	-	1	-	-	10YR 4/1
3 - 14	Sca	3	-	-	-	7.51	4/4	silty-clayey	-	-	-	X	-	1	-	-	2.5Y 5/2
14 - 26+	Sca-Mca	3	-	-	-	7.08	4/4	silty-clayey	-	-	-	X	-	1	-	-	2.5Y 5/3
Comments		-															

Soil name (Baize and Girard, 2009)	RENDOSOL pachique, issu de molasse
Soil name (IUSS Working Group, 2022)	Rendzic Calcaric Leptosol
Humus form (Zanella et al., 2018)	Hemimoder
Site name	NAT-VD1

Station parameters		
Location	Fey	
Date	21.07.2016	
Altitude	621 m	
Slope	5°	
Aspect	136°	
Topography	Slight slope below a molasse cliff	
Geology	Molasse	
Vegetation alliance	<i>Galio-Fagenion</i>	

Soil profile (horizon names from Baize and Girard, 2009)		
BryoOL-vOL vOL-[BryoOL] OF-[OH]-[vOL] Aca +2 +1 0 3		

Horizon depth (cm)	Horizon name	Coarse fraction (%)				Acidity, presence of calcium carbonates		Texture	Soil structure					Roots (index from 1 to 5)			Soil color (Munsell)
		2-20 mm	2-5 cm	5-20 cm	> 20 cm	pH (Hellige)	HCl		single-grain	crumb	sub-blocky	blocky	fibrous	0-2 mm	2-5 mm	> 5 mm	
+2 - +1	BryoOL-vOL	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
+1 - 0	vOL-[BryoOL]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
0 - 3	OF-[OH]-[vOL]	-	-	-	-	7.58	1/4	-	-	-	-	-	-	-	-	-	10YR 3/2
3 - 30	Aca	-	-	-	-	8.52	2/4	silty	-	X	-	-	-	3	3	-	2.5Y 6/4
30 - 30+	Mcag	-	-	-	-	8.6		sandy	-	-	-	X	-	-	-	-	2.5Y 6/4
Comments						Oxidoreduction stains in the Mcag horizon											

Soil name (Baize and Girard, 2009)	CALCOSOL
Soil name (IUSS Working Group, 2022)	Calcaric Cambisol
Humus form (Zanella et al., 2018)	Dysmull
Site name	NAT-VD2

Station parameters		
Location	Chésereux	
Date	03.08.2016	
Altitude	666 m	
Slope	3°	
Aspect	80°	
Topography	Slight slope until a river 200 m below	
Geology	NA	
Vegetation alliance	<i>Molinio-Pinion</i>	

Soil profile (horizon names from Baize and Girard, 2009)		
1 0 3 10 vOL-nOL OF-Aca Aca Aca-Sca		

Horizon depth (cm)	Horizon name	Coarse fraction (%)				Acidity, presence of calcium carbonates		Texture	Soil structure					Roots (index from 1 to 5)			Soil color (Munsell)
		2-20 mm	2-5 cm	5-20 cm	> 20 cm	pH (Hellige)	HCl		single-grain	crumb	sub-bblocky	blocky	fibrous	0-2 mm	2-5 mm	> 5 mm	
+1 - 0	nOL-vOL	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
0 - 3	OF-Aca	-	-	-	-	-	4/4	-	-	-	-	-	-	3	-	-	-
3 - 10	Aca	5	-	-	-	7.71	4/4	clayey	-	X	X	-	-	3	3	3	10YR 3/1
10 - 21	Aca-Sca	-	-	-	-	7.85	4/4	clayey	-	X	X	-	-	-	-	-	10YR 4/2
21 - 50	Mca1	-	-	-	-	8.46	4/4	sandy (thin)	-	-	-	X	-	-	-	-	10YR 5/3
50 - 50+	Mca2	-	-	-	-	8.62	4/4	sandy (coarse)	-	-	-	X	-	-	-	-	10YR 6/4
Comments		-															

Soil name (Baize and Girard, 2009)	CALCOSOL décarbonaté en surface
Soil name (IUSS Working Group, 2022)	Calcaric Cambisol
Humus form (Zanella et al., 2018)	Dysmull
Site name	NAT-VD3

Station parameters		
Location	Saint-George	
Date	03.08.2016	
Altitude	1122 m	
Slope	15°	
Aspect	15°	
Topography	Slope	
Geology	NA	
Vegetation alliance	<i>Abieti-Fagenion</i>	

Soil profile (horizon names from Baize and Girard, 2009)		
nOL-vOL OF-Aci Aca 13 Aca-Sca		

Horizon depth (cm)	Horizon name	Coarse fraction (%)				Acidity, presence of calcium carbonates		Texture	Soil structure					Roots (index from 1 to 5)			Soil color (Munsell)
		2-20 mm	2-5 cm	5-20 cm	> 20 cm	pH (Hellige)	HCl		single-grain	crumb	sub-blocky	blocky	fibrous	0-2 mm	2-5 mm	> 5 mm	
+1 - 0	nOL-vOL	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
0 - 1	OF-Aci	-	-	-	-	-	0/4	-	-	-	-	-	-	3	-	-	-
1 - 13	Aca	-	5	-	-	7.59	1/4	silty-clayey	-	X	-	-	-	3	3	-	10YR 3/1
13 - 23	Aca-Sca	-	5	-	-	7.79	3/4	clayey-silty	-	-	-	X	-	1	1	-	10YR 3/2
23 - 23+	Mca	-	-	-	-	8.28	4/4	sandy (thin)	-	-	-	-	-	-	-	-	10YR 6/3
Comments		-															

Soil name (Baize and Girard, 2009)	CALCOSOL colluvial, leptique issu d'éboulis calcaires
Soil name (IUSS Working Group, 2022)	Calcaric Cambisol (Litholinic)
Humus form (Zanella et al., 2018)	Dysmoder
Site name	NAT-NE1

Station parameters		
Location	Creux-du-Van	
Date	11.07.2016	
Altitude	1166 m	
Slope	45°	
Aspect	295°	
Topography	Steep slope	
Geology	Calcareous scree	
Vegetation alliance	Lonicero-Fagenion	

Soil profile (horizon names from Baize and Girard, 2009)		
+4 0 10 15 20 [BryoOL]-[vOL]-[vOL] OF-OH Aca Cca-[Sca]		

Horizon depth (cm)	Horizon name	Coarse fraction (%)				Acidity, presence of calcium carbonates		Texture	Soil structure					Roots (index from 1 to 5)			Soil color (Munsell)
		2-20 mm	2-5 cm	5-20 cm	> 20 cm	pH (Hellige)	HCl		single-grain	crumb	sub-blocky	blocky	fibrous	0-2 mm	2-5 mm	> 5 mm	
+1 - 0	[BryoOL]-[vOL]-[nOL]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
0 - 10	OF-OH	-	-	-	-	-	-	-	X	-	-	-	-	5	1	-	-
10 - 15	Aca	25	5	-	-	7.48	4/4	silty-clayey	-	X	X	-	-	2	5	-	10YR 3/1
15 - 20+	Cca-[Sca]	35	5	-	-	7.84	4/4	silty-clayey	-	-	-	X	-	1	4	-	10YR 3/2
Comments		-															

Soil name (Baize and Girard, 2009)	RENDOSOL colluvial issu d'éboulis calcaires
Soil name (IUSS Working Group, 2022)	Rendzic Calcaric Leptosol (Solimovic)
Humus form (Zanella et al., 2018)	Dysmoder
Site name	NAT-NE2

Station parameters		
Location	Creux-du-Van	
Date	23.08.2016	
Altitude	1187 m	
Slope	55°	
Aspect	336°	
Topography	Steep slope with a rocky break	
Geology	Calcareous scree	
Vegetation alliance	Abieti-Fagenion	

Soil profile (horizon names from Baize and Girard, 2009)		
+4 vOL-BryoOL 0 OF-OH 5 Aca 20 Dca-[Aca]		

Horizon depth (cm)	Horizon name	Coarse fraction (%)				Acidity, presence of calcium carbonates		Texture	Soil structure					Roots (index from 1 to 5)			Soil color (Munsell)
		2-20 mm	2-5 cm	5-20 cm	> 20 cm	pH (Hellige)	HCl		Single-grain	crumb	sub-blocky	blocky	fibrous	0-2 mm	2-5 mm	> 5 mm	
+4 - 0	vOL-BryoOL	-	-	-	-	-	-	-	-	-	-	-	-	1	1	-	-
0 - 5	OF-OH	10	-	-	15	-	-	-	-	-	-	-	-	5	5	-	-
5 - 20	Aca	15	-	-	15	7.51	1/4	silty-clayey	-	X	-	-	-	5	5	-	7.5YR 3/2
20 - 20+	Dca-[Aca]	25	-	-	30	7.08	2/4	silty-clayey	-	X	-	-	-	5	5	-	10YR 3/2
Comments		-															

Appendix II

Soil name (Baize and Girard, 2009)	CALCOSOL issu de molasse
Soil name (IUSS Working Group, 2022)	Calcaric Cambisol
Humus form (Zanella et al., 2018)	Oligomull
Site name	NAT-BE1

Station parameters		
Location	Oltigen	
Date	30.07.2016	
Altitude	491 m	
Slope	35°	
Aspect	225°	
Topography	Slope below a molasse cliff	
Geology	Molasse	
Vegetation alliance	<i>Cephalanthero-Fagenion</i>	

Soil profile (horizon names from Baize and Girard, 2009)		
nOL-vOL-[BryoOL]-[OF] +1 0 1 Aca1 Aca2 16 Sca1		

Horizon depth (cm)	Horizon name	Coarse fraction (%)				Acidity, presence of calcium carbonates		Texture	Soil structure					Roots (index from 1 to 5)			Soil color (Munsell)
		2-20 mm	2-5 cm	5-20 cm	> 20 cm	pH (Hellige)	HCl		single-grain	crumb	sub-blocky	blocky	fibrous	0-2 mm	2-5 mm	> 5 mm	
+1 - 0	nOL-vOL-[BryoOL]-[OF]	-	5	-	-	-	-	-	-	-	-	-	-	-	-	-	-
0 - 1	Aca1	3	2	-	-	7.66	3/4	silty-clayey	-	-	X	-	-	1	-	-	10YR 3/2
1 - 16	Aca2	3	-	-	-	7.83	4/4	silty-clayey	-	X	-	-	-	3	3	-	7.5Y 3/2
16 - 32	Sca1	-	-	-	-	8.03	4/4	silty-clayey	-	-	-	X	-	3	3	-	2.5Y 4/2
32 - 48	Sca2	-	2	-	-	8.04	4/4	silty-clayey	-	-	-	X	-	-	-	-	2.5Y 4/2
48 - 63+	Mca	-	2	-	-	8.48	4/4	silty-clayey	-	-	-	X	-	-	-	-	2.5Y 5/4
Comments																	

Soil name (Baize and Girard, 2009)	CALCOSOL issu de calcaires du Portlandien
Soil name (IUSS Working Group, 2022)	Calcaric Cambisol
Humus form (Zanella et al., 2018)	Dysmull
Site name	NAT-BE2

Station parameters		
Location	Neuveville	
Date	29.07.2016	
Altitude	606 m	
Slope	5°	
Aspect	68°	
Topography	Lower slope	
Geology	Portlandien limestone	
Vegetation alliance	<i>Lonicero-Fagenion</i>	

Soil profile (horizon names from Baize and Girard, 2009)		
+2 vOL-nOL 0 OF-[Aca] 2 Aca-[OF] 4 Aca-Sca		

Horizon depth (cm)	Horizon name	Coarse fraction (%)				Acidity, presence of calcium carbonates		Texture	Soil structure					Roots (index from 1 to 5)			Soil color (Munsell)
		2-20 mm	2-5 cm	5-20 cm	> 20 cm	pH (Hellige)	HCl		single-grain	crumb	sub-blocky	blocky	fibrous	0-2 mm	2-5 mm	> 5 mm	
+2 - 0	vOL-nOL	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
0 - 2	OF-[Aca]	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-
2 - 4	Aca-[OF]	8	8	-	-	7.38	3/4	silty	-	X	-	-	-	5	-	-	10YR 3/1
4 - 30	Aca-Sca	25	-	-	30	7.62	4/4	silty	-	-	X	-	-	3	3	-	10YR 3/1
30 - 30+	Cca	40	55	-	-	7.72	4/4	silty	-	-	X	-	-	1	1	-	2.5Y 2.5/1
Comments						-											

Appendix II

Soil name (Baize and Girard, 2009)	RENDOSOL colluvial issu d'éboulis calcaires
Soil name (IUSS Working Group, 2022)	Rendzic Calcaric Leptosol (Solimovic)
Humus form (Zanella et al., 2018)	Hemimoder
Site name	NAT-BL1

Station parameters		
Location	Langenbruck	
Date	25.08.2016	
Altitude	933 m	
Slope	35°	
Aspect	156°	
Topography	Steep slope	
Geology	Calcareous scree	
Vegetation alliance	Abieti-Fagenion	

Soil profile (horizon names from Baize and Girard, 2009)		
+1 0 vOL-nOL-[BryoOL] OF-[OH] 5 Acah-Cca 10		

Horizon depth (cm)	Horizon name	Coarse fraction (%)				Acidity, presence of calcium carbonates		Texture	Soil structure					Roots (index from 1 to 5)			Soil color (Munsell)
		2-20 mm	2-5 cm	5-20 cm	> 20 cm	pH (Hellige)	HCl		single-grain	crumb	sub-blocky	blocky	fibrous	0-2 mm	2-5 mm	> 5 mm	
+1 - 0	vOL-nOL-[BryoOL]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
0 - 5	OF-[OH]	-	-	-	-	-	-	-	-	-	-	-	-	5	-	-	-
5 - 10+	Acah-Cca	25	25	-	-	7.40	2/4	silty-clayey	-	X	-	-	-	5	5	-	10YR 2/1
Comments																	

Soil name (Baize and Girard, 2009)	CALCOSOL issu de molasse
Soil name (IUSS Working Group, 2022)	Calcaric Cambisol
Humus form (Zanella et al., 2018)	Dysmull
Site name	NAT-AG1

Station parameters		
Location	Freienwil	
Date	02.08.2016	
Altitude	566 m	
Slope	20°	
Aspect	90°	
Topography	Slope below a molasse cliff	
Geology	Molasse	
Vegetation alliance	<i>Lonicero-Fagenion</i>	

Soil profile (horizon names from Baize and Girard, 2009)		
BryoOL nOL-vOL-OF-BryoOL +2 0 1 Aca-Sca 20 Sca-Dca		

Horizon depth (cm)	Horizon name	Coarse fraction (%)				Acidity, presence of calcium carbonates		Texture	Soil structure					Roots (index from 1 to 5)			Soil color (Munsell)
		2-20 mm	2-5 cm	5-20 cm	> 20 cm	pH (Hellige)	HCl		single-grain	crumb	sub-blocky	blocky	fibrous	0-2 mm	2-5 mm	> 5 mm	
+2 - 0	BryoOL	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
0 - 1	nOL-vOL-OF-BryoOL	5	-	-	-	-	-	-	-	-	-	-	-	2	-	-	-
1 - 20	Aca-Sca	5	15	-	-	7.84	4/4	clayey-silty	-	X	-	-	-	2	2	-	10YR 2/2
20-20+	Sca-Dca	-	10	70	-	8.18	4/4	clayey	-	-	-	X	-	1	1	-	10YR 4/3
Comments		-															

Soil name (Baize and Girard, 2009)	CALCOSOL issu de molasse
Soil name (IUSS Working Group, 2022)	Calcaric Cambisol
Humus form (Zanella et al., 2018)	Dysmull
Site name	NAT-ZH1

Station parameters		
Location	Bühl	
Date	04.08.2016	
Altitude	619 m	
Slope	5°	
Aspect	15°	
Topography	Light slope	
Geology	Molasse	
Vegetation alliance	Transition <i>Atropion</i> and <i>Abieti-Fagenion</i>	

Soil profile (horizon names from Baize and Girard, 2009)		
+1.5 BryoOL-nOL-vOL 0 OF 3 Aca 6 Aca-Sca 21		

Horizon depth (cm)	Horizon name	Coarse fraction (%)				Acidity, presence of calcium carbonates		Texture	Soil structure					Roots (index from 1 to 5)			Soil color (Munsell)
		2-20 mm	2-5 cm	5-20 cm	> 20 cm	pH (Hellige)	HCl		single-grain	crumb	sub-blocky	blocky	fibrous	0-2 mm	2-5 mm	> 5 mm	
+1.5 - 0	bryoOL-nOL-vOL	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
0 - 3	OF	-	-	-	-	-	1/4	-	-	-	-	-	-	1	-	-	-
3 - 6	Aca	-	15	-	-	7.45	2/4	clayey-silty	-	X	X	-	-	4	4	-	10YR 3/2
6 - 21	Aca-Sca	-	10	-	-	7.68	3/4	silty-clayey	-	-	-	X	-	4	4	-	10YR 4/3
21-26+	Sca	-	-	-	-	8.15	4/4	sandy-clayey	-	-	-	X	-	-	1	-	10YR 5/6
Comments						Hard and very sandy horizon below the Sca horizon											

Soil name (Baize and Girard, 2009)	CALCOSOL leptique, issu d'éboulis calcaires
Soil name (IUSS Working Group, 2022)	Leptic Calcaric Cambisol
Humus form (Zanella et al., 2018)	Dysmull
Site name	REINTRO-NE1

Station parameters		
Location	Creux-du-Van	
Date	23.08.2016	
Altitude	1055 m	
Slope	50°	
Aspect	320°	
Topography	Steep slope with limestone blocks	
Geology	Calcareous scree	
Vegetation alliance	Abieti-Fagenion	

Soil profile (horizon names from Baize and Girard, 2009)		
nOL-vOL-BryoOL OF-Aca Cca-Aca Cca-Aca-Sca +1 0 5 15		

Horizon depth (cm)	Horizon name	Coarse fraction (%)				Acidity, presence of calcium carbonates		Texture	Soil structure					Roots (index from 1 to 5)			Soil color (Munsell)
		2-20 mm	2-5 cm	5-20 cm	> 20 cm	pH (Hellige)	HCl		single-grain	crumb	sub-blocky	blocky	fibrous	0-2 mm	2-5 mm	> 5 mm	
+2 - 0	nOL-vOL-BryoOL	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
0 - 5	OF-Aca	-	15	-	-	6.49	1/4	silty-clayey	-	X	-	-	-	4	4	-	10YR 2/2
5 - 15	Cca-Aca	-	20	-	40	6.79	2/4	clayey-silty	-	X	-	-	-	4	4	-	10YR 2/1
15 - 30+	Cca-Aca-Sca	-	75	-	5	7.03	3/4	clayey-silty	-	-	X	-	-	4	4	-	10YR 2/2
Comments																	

Soil name (Baize and Girard, 2009)	CALCOSOL présentant des cutanes, issu d'éboulis calcaires
Soil name (IUSS Working Group, 2022)	Calcaric Cambisol (Clayic, Litholinic)
Humus form (Zanella et al., 2018)	Dysmull
Site name	REINTRO-NE2

Station parameters	
Location	Creux-du-Van
Date	06.08.2016
Altitude	1079 m
Slope	45°
Aspect	15°
Topography	Steep slope
Geology	Calcareous scree
Vegetation alliance	<i>Abieti-Fagenion</i>



Soil profile (horizon names from Baize and Girard, 2009)

Soil profile (horizon names from Baize and Girard, 2009)

Horizon names and depths (cm):

- vOL-nOL-BryoOL: 0 to 1
- OF: 1 to 2
- Aca-Sca: 2 to 10
- Sca: 10 to 20
- Dca: 20 to 30

The photograph shows a soil profile with a yellow measuring tape placed vertically for scale. The soil is dark and moist, with some organic matter visible at the surface. The horizons are labeled on the left side of the image.

[illegible]

Soil name (Baize and Girard, 2009)	CALCOSOL présentant des cutanes, issu d'éboulis calcaires
Soil name (IUSS Working Group, 2022)	Calcaric Cambisol (Clayic, Litholinic)
Humus form (Zanella et al., 2018)	Dysmull
Site name	REINTRO-NE3

Station parameters	
Location	Creux-du-Van (Site G)
Date	07.08.2016
Altitude	750 m
Slope	50°
Aspect	10°
Topography	Steep slope
Geology	Calcareous scree
Vegetation alliance	<i>Abieti-Fagenion</i>



Soil profile (horizon names from Baize and Girard, 2009)

Soil profile (horizon names from Baize and Girard, 2009)

Horizon names and depths (cm):

- vOL-nOL-BryoOL: 0 to +1
- OF-BryoOL: +1 to 8
- Sca1: 8 to 15
- Sca2: 15 to 20
- Dca-Sca: 20 to 25

[illegible]

Soil name (Baize and Girard, 2009)	RENDOSOL décarbonaté en surface issu de dalle calcaire
Soil name (IUSS Working Group, 2022)	Rendzic Calcaric Leptosol
Humus form (Zanella et al., 2018)	Oligomull
Site name	REINTRO-BE1

Station parameters		
Location	Saint-Imier	
Date	01.08.2016	
Altitude	1114 m	
Slope	35°	
Aspect	180°	
Topography	Slope under a pasture	
Geology	Limestone slab	
Vegetation alliance	<i>Abieti-Fagenion</i>	

Soil profile (horizon names from Baize and Girard, 2009)		
+4 0 2 6 20 nOL-vOL Acih-[OF] Acih Acah Rca		

Horizon depth (cm)	Horizon name	Coarse fraction (%)				Acidity, presence of calcium carbonates		Texture	Soil structure					Roots (index from 1 to 5)			Soil color (Munsell)
		2-20 mm	2-5 cm	5-20 cm	> 20 cm	pH (Hellige)	HCl		single-grain	crumb	sub-blocky	blocky	fibrous	0-2 mm	2-5 mm	> 5 mm	
+4 - 0	nOL-vOL	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
0 - 2	Acih-[OF]	-	10	-	-	6.48	0/4	silty-clayey	-	X	-	-	-	5	-	-	10YR 3/2
2 - 6	Acih	-	20	20	-	6.94	0/4	silty-clayey	-	X	-	-	-	5	-	-	10YR 3/1
6 - 20	Acah	-	-	60	-	7.21	1/4	silty-clayey	-	-	-	X	-	3	3	-	10YR 3/1
20-20+	Rca	-	-	-	-	-	4/4	-	-	-	-	-	-	-	-	-	-
Comments		-															

Soil name (Baize and Girard, 2009)	BRUNISOL EUTRIQUE issu de loess
Soil name (IUSS Working Group, 2022)	Eutric Cambisol (Siltic)
Humus form (Zanella et al., 2018)	Dysmull
Site name	REINTRO-BE2

Station parameters		
Location	Neuveville	
Date	29.07.2016	
Altitude	616	
Slope	5°	
Aspect	140°	
Topography	Just under a break in the slope	
Geology	Loess	
Vegetation alliance	<i>Cephalanthero-Fagenion</i>	

Soil profile (horizon names from Baize and Girard, 2009)		
+3 vOL-nOL 0 OF-[A] 2 S		

Horizon depth (cm)	Horizon name	Coarse fraction (%)				Acidity, presence of calcium carbonates		Texture	Soil structure					Roots (index from 1 to 5)			Soil color (Munsell)
		2-20 mm	2-5 cm	5-20 cm	> 20 cm	pH (Hellige)	HCl		single-grain	crumb	sub-blocky	blocky	fibrous	0-2 mm	2-5 mm	> 5 mm	
+3 – 0	vOL-nOL	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
0 – 2	OF-[A]	10	-	-	-	5.77	0/4	silty	-	X	-	-	-	3	-	-	10YR 4/3
2 – 10+	S	5	5	-	-	5.78	0/4	silty	-	-	-	X	-	-	-	1	10YR 4/3
Comments		-															

Table S1. Workflow of the spatial modelling approach. The approach is divided in a first modelling step, a second projection step, and a third forecasting step. Analyses integrate two spatial layers, first an Eurasian layer, and a second more precise Swiss layer of occurrence data and top-climatic variables. See details of the workflow in the Material and Methods section.

Modelling process for <i>C. calceolus</i> distribution		
	Eurasian scale	Swiss scale
1) Modelling step		
Geographic range (variables and background points)	Eurasia	Switzerland
Occurrences	GBIF Gargiulo (2019) Averyanov (1999)	Info Flora AGEO Canton of Fribourg
Number of occurrences	2'854	1'796
Variables	Bioclim 1 (Chelsa, 1km2) Bioclim 2 (Chelsa, 1 km2) Bioclim 5 (Chelsa, 1 km2) Bioclim 12 (Chelsa, 1 km2) Bioclim 15 (Chelsa, 1 km2)	EU climatic niche (results from modelling at Eurasian scale, 25 m2) Max canopy height (25 m2) Forest distance (25 m2) Aspect (25 m2) Slope (25 m2) Topographic position (25 m2)
Background points	1 set of 100'000 points	1 set of 100'000 points
Number of runs	30	30
Algorithms (in biomod2)	GLM GBM GAM	GLM GBM GAM
Model evaluation	POD ROC TSS	POD ROC TSS
2) Projection step		
Projection area	Switzerland	Switzerland
Variables	Bioclim 1 (MeteoSuisse, 25m2) Bioclim 2 (MeteoSuisse, 25m2) Bioclim 5 (MeteoSuisse, 25m2) Bioclim 12 (MeteoSuisse, 25m2) Bioclim 15 (MeteoSuisse, 25m2)	EU climatic niche (results from modelling at Eurasian scale, 25 m2) Max canopy height (25 m2) Forest distance (25 m2) Aspect (25 m2) Slope (25 m2) Topographic position (25 m2)
3) Ensemble forecasting		
Model evaluation	TSS	TSS

Table S2. Mean values for *C. calceolus* vegetation parameters per population. In each populations, we measured plant size from the ground to the top of the highest leaf, number of stems per plant, number of flowers per plant, number of leaves per plant, number of pods (fruits) per plant, leaf area (measured as the median leaf area of the highest stem, calculated using the ellipse area formula), and photosynthetic activity (measured with a SPAD chlorophyllometer).

Population name	Population size	Status	Plant size [cm]	Nb stems	Nb flowers	Nb leaves	Nb fruits	Leaf area [cm ²]	Chlorophyll
AG1	large	native	44.35	12.3	5.6	5.2	1.2	91.26	32.44
BE1	small	native	26.58	1	0.83	5.5	0.5	58.87	24.27
BE2	small	native	41	3	3	5	0	98.01	31.6
BL1	small	native	23.64	7.14	0.42	4	0.14	53.01	21.34
GE1	small	native	43.71	3.14	2	5.43	0.07	85.78	26.74
NE1	small	native	46.62	9.25	4.25	5	0.5	122.57	30.62
NE2	large	native	27.18	1.87	0.25	4.25	0	60.16	27.26
VD1	large	native	36.97	3.1	0.4	4.2	0.1	78.40	30.41
VD2	small	native	37.05	2.6	0.8	4.6	0.2	100.57	27.9
VD3	large	native	35.8	3	2.6	4.7	0.1	80.66	27.27
ZH1	large	native	42.8	13.1	10	5	4.7	76.07	28.21

Table S3. Soil physico-chemical parameters in the first organo-mineral horizon across 16 *C. calceolus* populations. Populations are sorted per status (native or reintroduced). For each soil, we quantified water content, organic mater (OM), cationic exchange capacity (CEC), water pH (pHH₂O), total carbonates and organic carbon relative to total nitrogen (CN).

Population name	Status	Population size	Horizon name	Water content [%]	OM [%]	CEC [cmol/kg]	pHH ₂ O	Total carbonates [%]	CN
AG1	Native	Large	Aca-Sca	27.45	7.39	23.92	7.84	7.78	12.5
BE1	Native	Small	Aca	15.57	9.95	24.62	7.83	7.19	15.92
BE2	Native	Small	Aca-Sca	17.42	13.07	36.45	7.62	16.86	14.64
BL1	Native	Small	Ahca-Cca	31.08	45.62	70.79	7.4	25.84	29.57
GE1	Native	Small	Aca-Sca-[OF]	30.94	16.64	33.86	7.71	25.24	14.84
NE1	Native	Small	Aca	34.76	24.95	51.45	7.48	57.81	20.47
NE2	Native	Large	Aci	46.29	24.77	63.84	7.51	9.33	10.43
VD1	Native	Large	Aca	21.9	9.51	26.87	7.58	6.11	16.17
VD2	Native	Small	Aca	42.43	23.11	47.35	7.71	63.45	14.49
VD3	Native	Large	Aci	40.48	22.17	51.11	7.59	33.59	12.39
ZH1	Native	Large	AcaSca	31.41	11.57	38.79	7.68	4.43	14.1
R-BE1	Reintroduced	-	Aci1	33.21	30.6	63.24	6.94	0.34	19.05
R-BE2	Reintroduced	-	OF-[A]	12.39	13.52	18.45	5.77	0	33.64
R-NE1	Reintroduced	-	OF-Aca	52.71	65.81	70.51	6.49	2.16	26.61
R-NE2	Reintroduced	-	Aca-Sca	33.95	13.68	26.98	7.72	57.85	16.07
RNE3	Reintroduced	-	Sca1	23.28	6.79	11.39	8.18	67.74	2.32

Table S4. Envift analysis result table on the multivariate correlation between soil physico-chemical properties and *C. calceolus* growth- and reproduction-related plant traits.

	NMDS1	NMDS2	r2	Pr(>r)
OM	0.090392	0.99591	0.5028	0.071 °
CEC	0.138965	0.9903	0.5581	0.042 *
pH	-0.090154	-0.99593	0.8428	0.002 **
CACO3	-0.092979	0.99567	0.1368	0.583
CN	-0.038634	0.99925	0.2896	0.255

Signif. Codes: 0.001 '***' 0.01 '**' 0.05 '*' 0.1 '.' 1

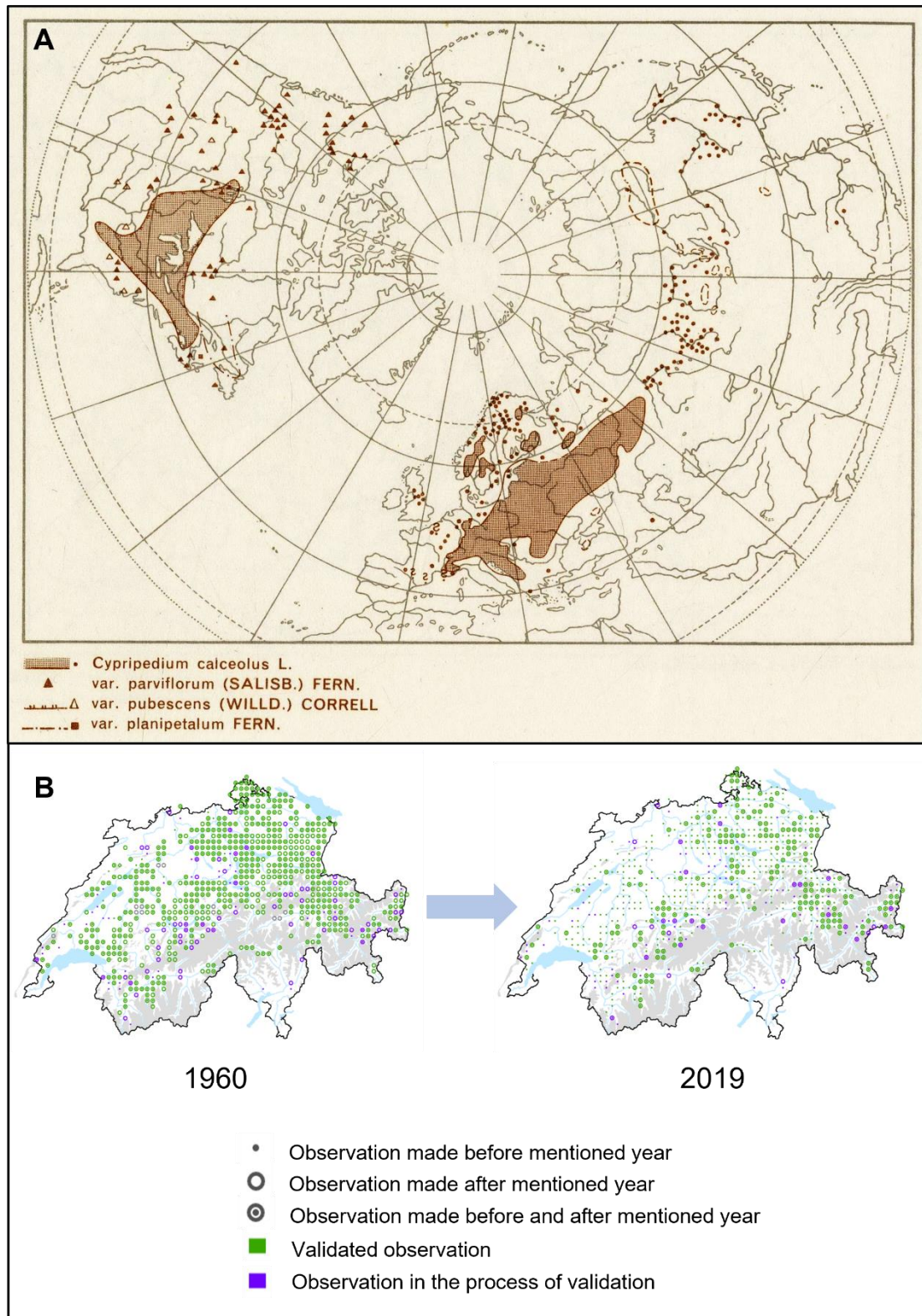


Figure S1. Eurasian distribution of *C. calceolus*. A) Map showing *C. calceolus* Eurasian distribution (Source: (Meusel et al. 1978)), and B) *C. calceolus* Swiss population evolution and decline between the years 1960 and 2019. These maps don't show the actual populations of *C. calceolus* for a given year but the observations that were made before and after the mentioned the focus year (Source: www.infoflora.ch.)

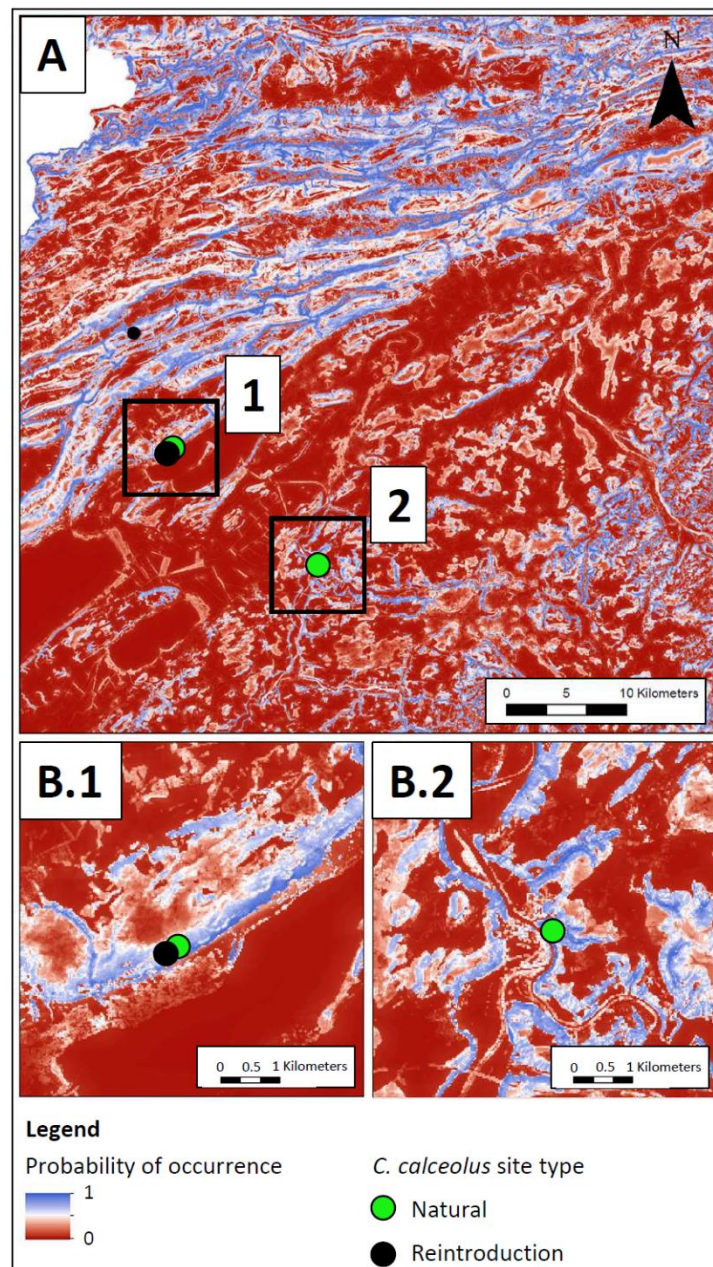


Figure S2. Probability of occurrences of *Cypripedium calceolus* in Switzerland. The map is coloured based on the spatial modelling integrating Swiss and Eurasian populations, whereby red indicates low probability of occurrence and blue indicates high probability of occurrence based on the climatic and topographic variables at 25 m resolution scale. Green dots represent natural populations of *C. calceolus* while black dot represents a reintroduced population. A. Probability of occurrences of *C. calceolus* in the north-west of Switzerland. B.1. Zoom on a natural and a reintroduced population in the lake Neuchâtel region. B.2. Zoom on a natural population by a riverside.

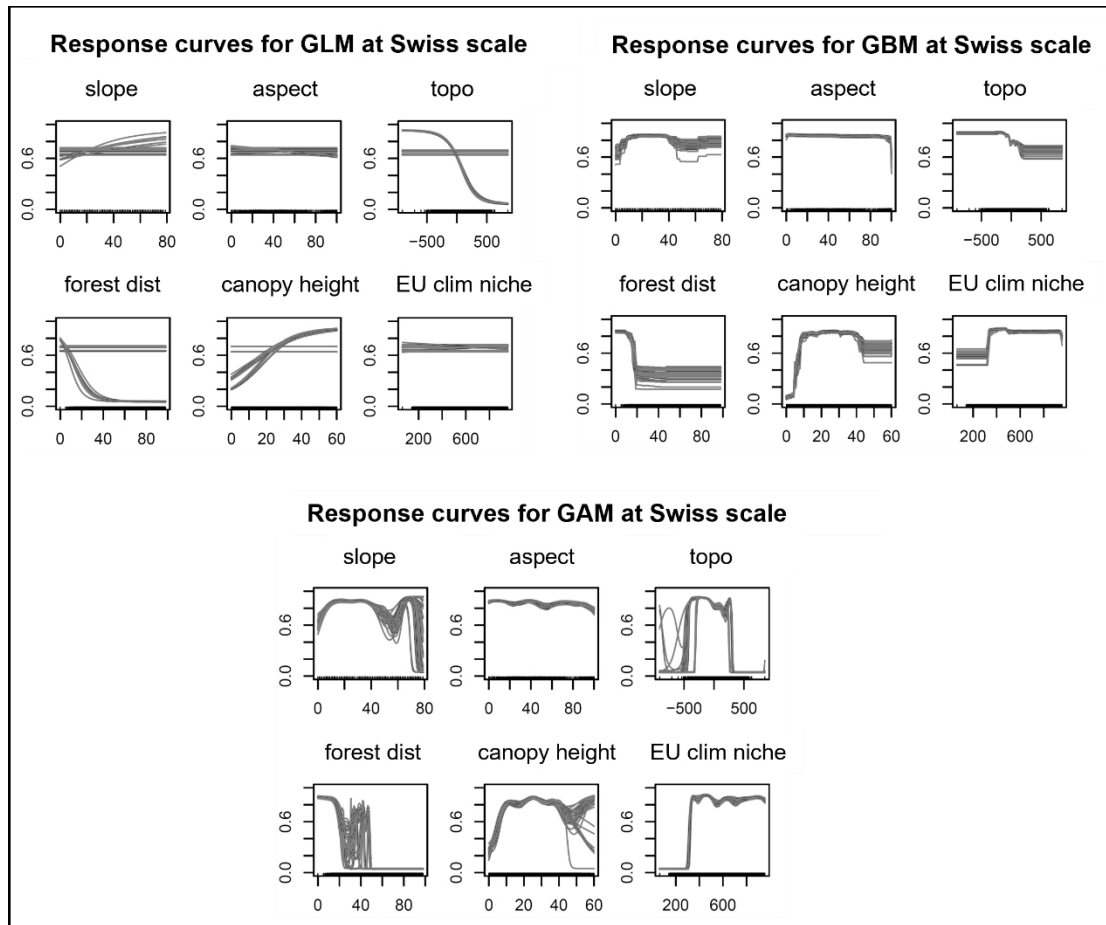


Figure S3. Response curves for *Cyripedium calceolus* modelling process at Swiss scales. Response curves are presented by model (GLM, GBM and GAM) for each variable: slope, aspect, topographic position (topo), forest distance (forest dist), maximum canopy height (canopy height) and Eurasian climatic niche (EU clim niche).

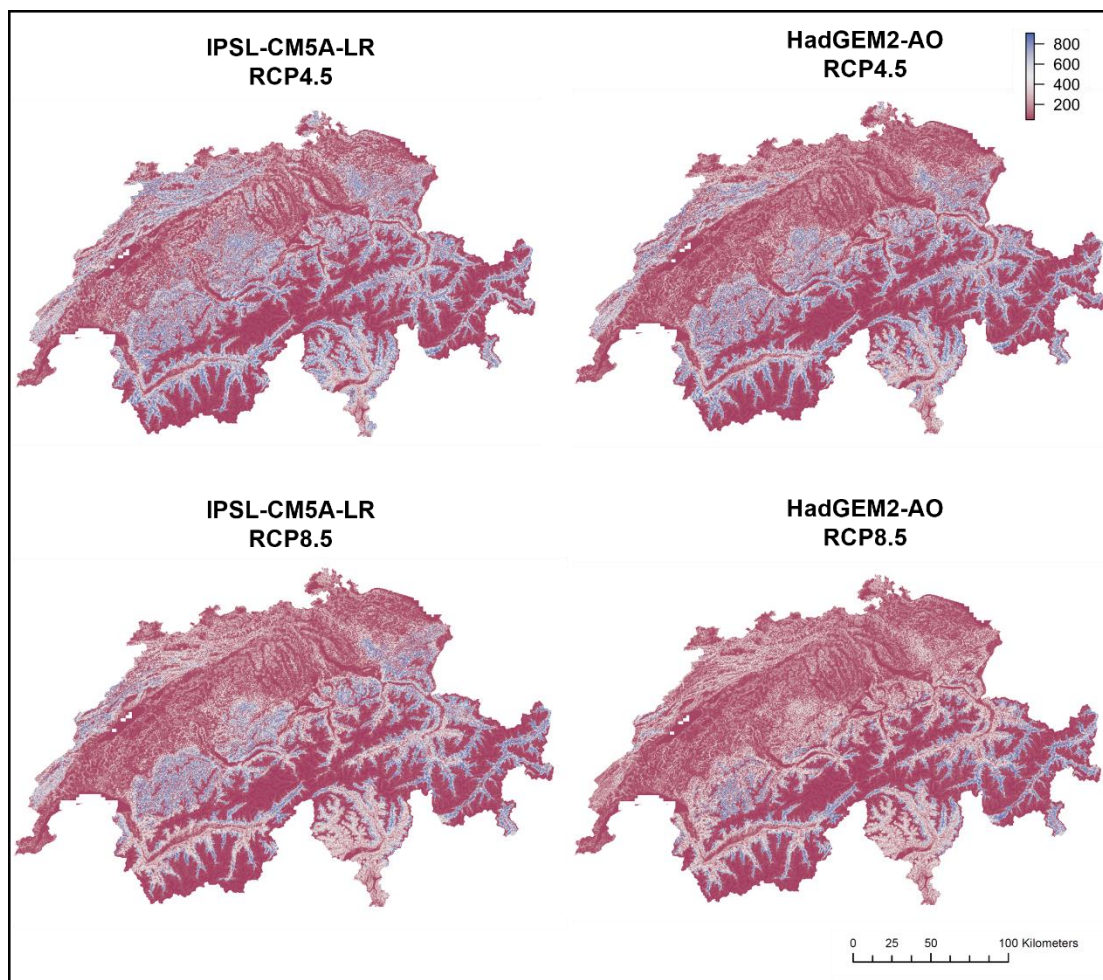


Figure S4. Future climatic scenarios (2061-2080) forecasting *Cypripedium calceolus* populations probability of occurrence in Switzerland. The map is coloured based on the spatial modelling integrating Swiss and Eurasian populations; warm red colouring indicates low, while cold blue colouring indicates high probability of occurrence based on the climatic and topographic variables at 25 m resolution scale. We used two Global Circulation Models (GCMs): IPSL-CM5A-LR (Dufresne et al. 2013) and HadGEM2-AO (Collins et al. 2008) together with two Representative Concentration Pathway (RCP): RCP4.5 and RCP8.5 (Van Vuuren et al. 2011).

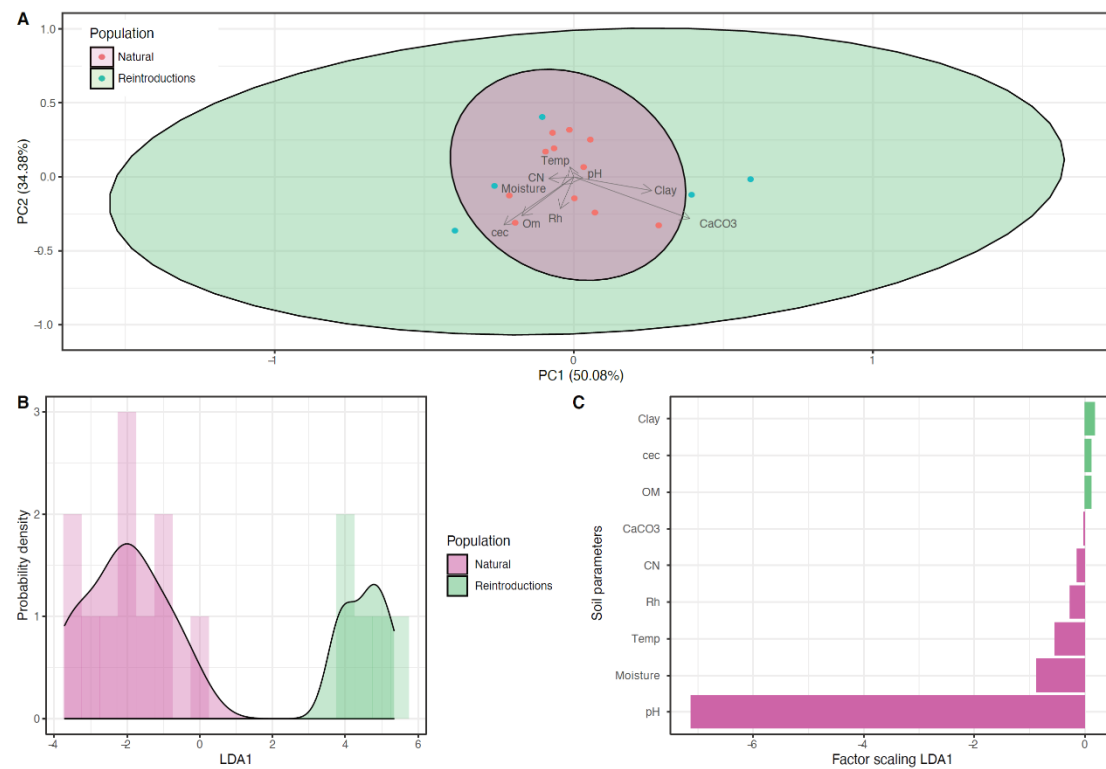
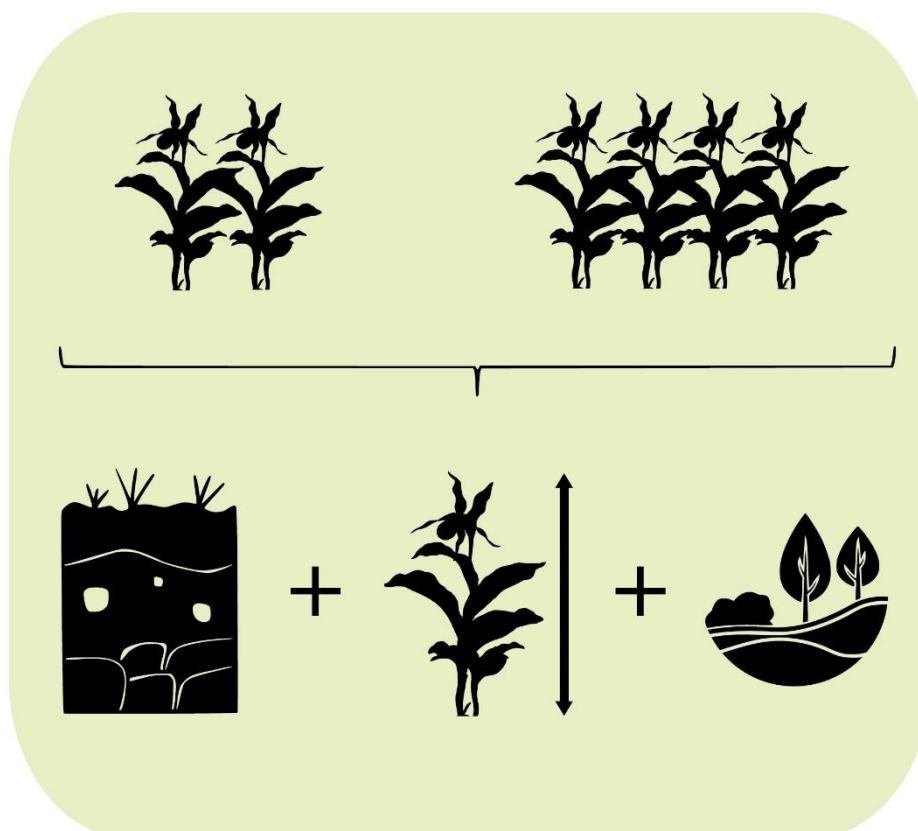


Figure S5. Soil properties across *Cypripedium calceolus* populations. Shown are A) the principal component analysis (PCA) of all soil properties: soil temperature (Temp), pH, total organic carbon to total nitrogen ratio (CN), water content (Moisture), total organic matter content (OM), soil total cationic exchange capacity (CEC), total carbonates (CaCO₃), residual humidity (Rh) and clay content (Clay), Natural: pink shading, Reintroductions: green shading B) Histogram showing the distribution of discriminant scores of climatic profiles originating from the two categories of *C. calceolus* populations (Natural: pink shading, Reintroductions: green shading) C) Discriminant coefficients for soil variable. Variables with negative coefficients (in pink) reflect negative discriminant scores of the soil niche (natural populations), whereas variables with positive coefficient (in green) reflect positive discriminant scores (reintroductions).

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APPENDIX III : SUPPLEMENTARY MATERIAL FOR CHAPTER II: SOIL PROPERTIES AND PLANT SPECIES CAN PREDICT POPULATION HEALTH AND POTENTIAL INTRODUCTION SITES OF THE ENDANGERED ORCHID *CYPRIPEDIUM CALCEOLUS*



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Table S1. Multivariate analysis of variance (MANOVA) results table for all plant functional traits measured across 34 *Cypripedium calceolus* populations.

Response variable	Factor	Df	Pillai	Fnum/Fden	Pr(>F)	
All_traits	Pop_size	1	0.3066	10/212	<0.001	***
	Site	32	3.2981	320/2210	<0.001	***
	Residuals	221				
		Df	SumSq	F	Pr(>F)	
Plant size	Pop_size	1	2079.2	27.22	<0.001	***
	Site	32	10833.7	4.43	<0.001	***
	Residuals	221	16880.2			
Nb of Flower	Pop_size	1	303.6	12.72	<0.001	***
	Site	32	2728.9	3.57	<0.001	***
	Residuals	221	5275.4			
Nb of fruits	Pop_size	1	7.06	2.45	0.12	
	Site	32	213.13	2.31	0.00	***
	Residuals	221	637.55			
Fruit volume	Pop_size	1	462777	1.38	0.24	
	Site	32	35980689	3.36	<0.001	***
	Residuals	221	74011715			
Nb of stems	Pop_size	1	1951.9	28.15	<0.001	***
	Site	32	14595.7	6.58	<0.001	***
	Residuals	221	15323.2			
Nb of leaves	Pop_size	1	4.202	5.93	0.02	*
	Site	32	79.452	3.50	<0.001	***
	Residuals	221	156.683			
Leaf area	Pop_size	1	11048	18.80	<0.001	***
	Site	32	86449	4.60	<0.001	***
	Residuals	221	129845			
SPAD	Pop_size	1	40.9	1.87	0.17	
	Site	32	2356.8	3.36	<0.001	***
	Residuals	221	4848			
SLA	Pop_size	1	0.04262	19.04	<0.001	***
	Site	32	0.78676	10.98	<0.001	***
	Residuals	221	0.4948			
Area patch	Pop_size	1	21941923	22.38	<0.001	***
	Site	32	132072274	4.21	<0.001	***
	Residuals	221	216707442			

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Table S2. Phylogenetic signal type of analyses to detect potential association between dendrogram branch length (as shown in Figure 3) and trait values across 34 populations of *Cypripedium calceolus*.

Signals are calculated both with K and lambda statistics. The associated p-values indicate potential deviation from null values, if $p < 0.05$.

Variable	K	P (K)	Lambda (I)	P (I)
Patch area	0.59	0.11	0.00	1.00
Plant height	0.56	0.20	0.05	0.71
Nb stems	0.56	0.25	0.00	1.00
Nb leaves	0.55	0.22	0.04	0.75
Leaf area	0.56	0.20	0.14	0.27
SPAD	0.58	0.13	0.00	1.00
SLA	0.56	0.19	0.00	1.00
Nb flowers	0.56	0.19	0.00	1.00
Nb fruits	0.48	0.70	0.00	1.00
Fruit size	0.49	0.62	0.00	1.00

Table S3. Multivariate analysis of variance (MANOVA) results table for all soil physico-chemical properties measured across 34 *Cypripedium calceolus* populations.

Response variable	Factor	Df	Pillai	Fnum/Fden	Pr(>F)	
All_traits	Pop_size	1	0.68	10/60	<0.001	***
	Site	32	3.67	320/690	<0.001	***
	Residuals	69				
		Df	SumSq	F-value	Pf(>F)	
HR	Pop_size	1	10.7	0.55	0.462	
	Site	32	2370.3	3.79	<0.001	***
	Residuals	69	1349.8			
SOM	Pop_size	1	294.4	6.17	0.015	*
	Site	32	19554.6	12.81	<0.001	***
	Residuals	69	3292			
CaCO3	Pop_size	1	1722	21.76	<0.001	***
	Site	32	45210	17.85	<0.001	***
	Residuals	69	5461			
pH	Pop_size	1	0.1631	2.40	0.126	
	Site	32	16.3437	7.52	<0.001	***
	Residuals	69	4.6879			
P	Pop_size	1	16.5	0.59	0.444	
	Site	32	8121.7	9.14	<0.001	***
	Residuals	69	1916.4			
CEC	Pop_size	1	1024.4	13.19	0.001	***
	Site	32	15773.2	6.34	<0.001	***
	Residuals	69	5360.7			
Ntot	Pop_size	1	0.0133	0.40	0.531	
	Site	32	10.5278	9.84	<0.001	***
	Residuals	69	2.306			
CN	Pop_size	1	78	0.22	0.642	
	Site	32	37225	3.27	<0.001	***
	Residuals	69	24526			
NO3	Pop_size	1	1.88	0.34	0.560	
	Site	32	719.12	4.10	<0.001	***
	Residuals	69	378.13			
Corg	Pop_size	1	96.846	7.5121	0.008	**
	Site	32	128.293	9.9514	<0.001	***
	Residuals	69	12.892			

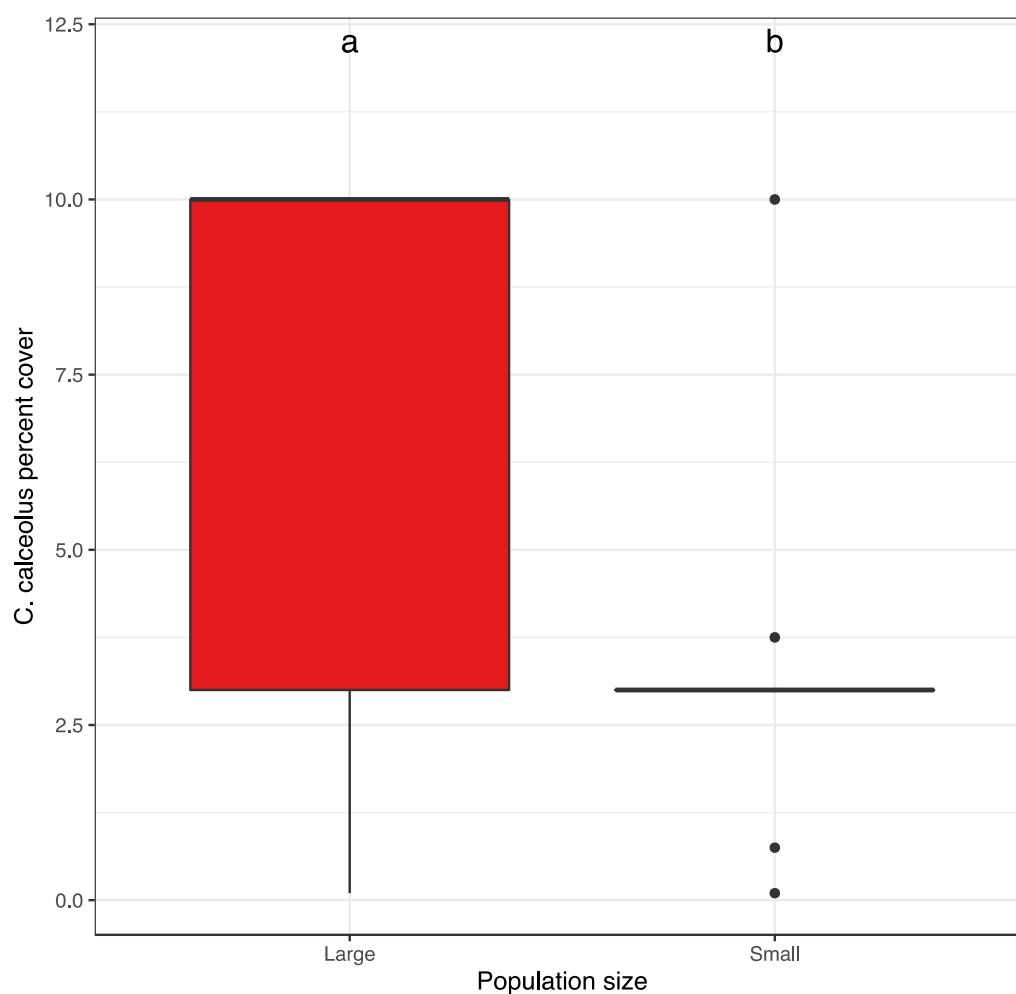


Figure S1. Average percent cover of *Cypripedium calceolus* populations in the surveyed vegetation patches according to the factorial discrimination of population size (Small = blue boxplots, and Large = red boxplots). Letters above boxplots indicate significant differences (glm with quasipoisson distribution, $t_{1,29} = -3.202$, $p = 0.003$).

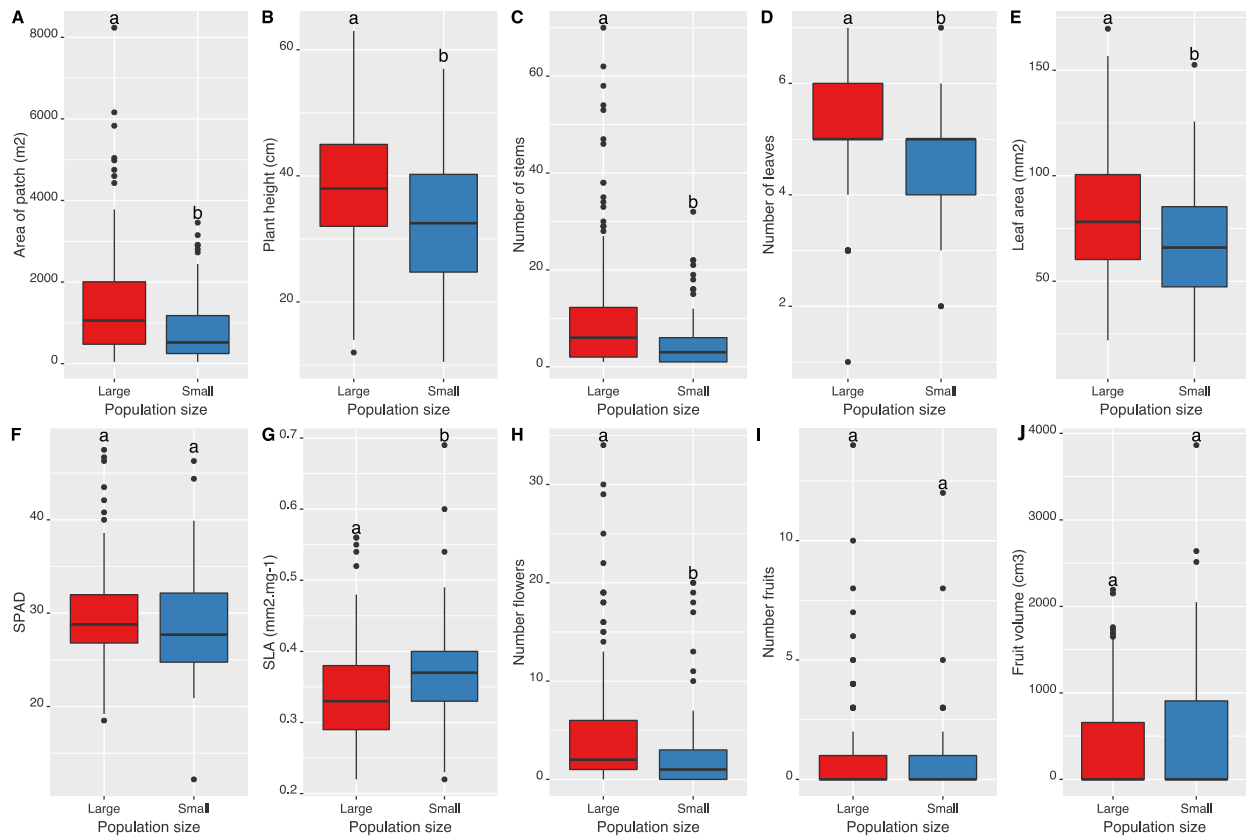


Figure S2. *Cypripedium calceolus* population functional traits. Shown are average trait values distributions for A. individual patch area (approximated to a ellipse) B. plant height, C. Number of stems per patch, D. Number of leaves per plant, E. Leaf area, F. Chlorophyll content (SPAD), G. Specific leaf area (SLA), H. Number of flowers per plant, I. Number of fruits per plant, J. Fruit volume (approximated to a cylinder) for small (blue boxplots; < 10 individuals), and large (red boxplots, > 100 individuals) populations. Different letters above boxplots show significant differences ($p < 0.05$) based on the multivariate analysis of variance as shown in Table S1.

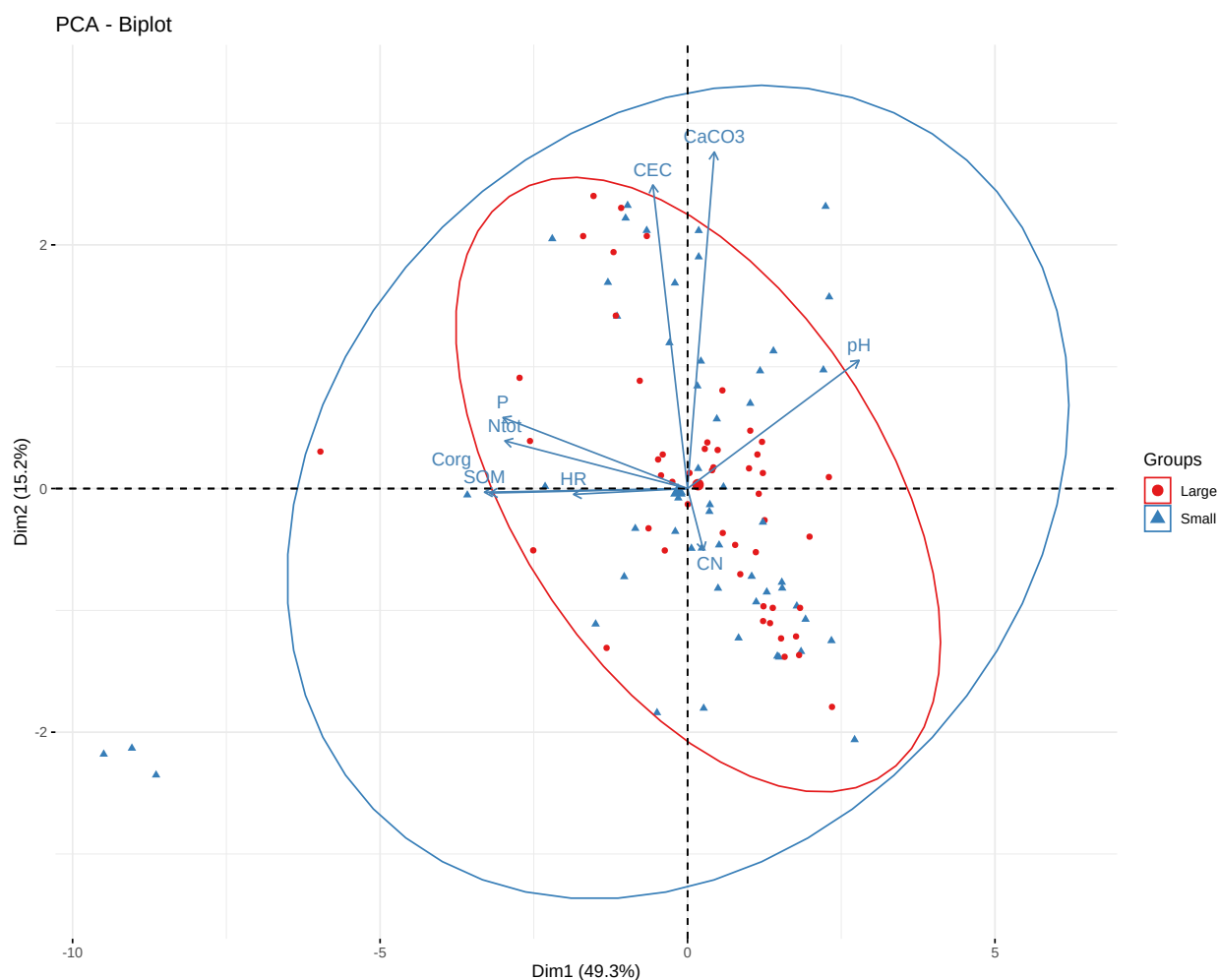


Figure S3. Principal component analysis (PCA) of ten soil physicochemical traits describing the soils form of the 34 different *Cypripedium calceolus* populations. Ellipses show the 95% confidence intervals characterizing small (blue area; < 10 individuals), and large (red areas, > 20 individuals) populations. Soil traits included: soil relative humidity, pH, total soil organic matter content, CaCO₃, Total N, total C, G) carbon-to-nitrogen ratio (CN), cation exchange capacity (CEC), total phosphorous (P), and total nitrates (NO₃).

Supplementary data D1. Soil profiles of 34 *Cypripedium calceolus* populations. In each *C. calceolus* population, we dug a soil profile until bedrock or parental material, and we separated soil profiles in organic, organo-mineral and mineral horizons according to each homogenous zone observed. We identified each horizon and classified both the soils and humus forms (Baize and Girard 2009; IUSS Working Group 2015; Zanella et al. 2018). The humus and soil horizons were named according to the Référentiel pédologique (Baize and Girard 2009), and the World Reference Base for Soil Resources (WRB) (IUSS Working Group 2015) (see glossary of soil terms at the end of the document. Finally, vegetation types were also identified and classified according to Delarze et al. (2015).

Glossary for humus forms and soil horizons according to (Zanella et al. 2018) with (IUSS Working Group 2015) equivalent.

Horizon name	Definition	WRB
nOL and vOL	The OL horizon is characterized by the accumulation of mainly leaves/needles, twigs and woody materials, most of the original plant organs being easily discernible to the naked eye. The humic component amounts to less than 10% by volume; recognizable remains 90% and more, up to 100% in non-decomposed litter. Suffixes characterize neither fragmented nor transformed/discolored leaves and/or needles (nOL), and slightly altered, discolored, bleached, softened up, glued, matted, skeletonized, sometimes only slightly fragmented leaves and/or needles (vOL)	O
BryoOL	OL horizon with Bryo characteristics defined as an horizon with “byophytes or arbuscular lichens or small stonecrop plants totally covering the soil and forming a stratified carpet or cushion with living (green) parts overgrowing a layer of dead stems and leaves	O
OF	The OF horizon is characterized by the accumulation of partly decomposed litter, mainly from transformed leaves/needles, twigs and woody materials, but without any entire plant organ. The proportion of humic component is 10% to 70% by volume. Depending on humus form, decomposition is mainly accomplished by soil fauna (OFzo) or cellulose–lignin decomposing fungi (OFnoz). Slow decomposition is revealed by a partly decomposed matted layer, permeated by hyphae	O
OH	The OH horizon is characterized by an accumulation of zoogenically transformed material, i.e. black, gray-brown, brown, reddish-brown well-decomposed litter, mainly comprised of aged animal droppings. A large part of the original structures and materials are not discernible, the humic component amounting to more than 70% by volume. The OH horizon differs from the OF horizon by a more advanced transformation (fragmentation, humification, ...) due to the action of soil organisms	O

Soil horizons and layers according to (Baize and Girard 2009) with (IUSS Working Group 2015) equivalent.

Horizon name	Definition	WRB
A	A horizon presents a mix of organic and mineral material and is situated under holorganic horizons.	A
Aca	Aca is an A horizon with cold HCl effervescence.	A
Acaho	Acaho is an Aca horizon that is humus-rich.	A
Aci	Aci is an Aca horizon which has been decarbonated. Aci is thus not carbonated in fine soil or only occasionally or locally.	A
Ado	Ado is an A horizon which is made up of hard dolomitic rocks.	A
Adoy	Adoy is an A horizon which is made up of hard dolomitic rocks and contains gypsum.	A
Agca	Agca is an Aca horizon with cold HCl effervescence and with redox properties.	
Cca	C horizon is a depth mineral horizon. Its components have undergone, throughout their mass, significant fragmentation and/or some geochemical alteration. Cca is calcareous C horizon.	C
Cdo	Cdo is an C horizon which is made up of hard dolomitic rocks.	C
Mca	M layer is formed by continuous, loose or soft rocks, not or slightly fragmented, with possibly locally or partially altered micro-cracks. This layer is coherent, but easy to work with tools. Mca is calcareous M layer.	-
S	S horizon is a structural horizon. It is an alteration horizon. Process such as alteration of primary minerals, release of iron oxyhydroxides, decarbonation take place in that horizon.	B
Sca	Sca is calcareous S horizon.	B
Sdo	Sdo is an S horizon which is made up of hard dolomitic rocks.	B
Sdoy	Sdoy is an S horizon which is made up of hard dolomitic rocks and contains gypsum.	B
Sgca	Sgca is an Sca horizon with cold HCl effervescence and with redox properties.	B

Soil classification	
Soil name (Baize and Girard, 2009)	CALCOSOL colluvial, issu d'éboulis calcaires
Soil name (WRB, 2022)	Calcaric Cambisol (Litholinic)
Humus form (Zanella et al., 2018)	Eumull
Horizon sequence (Référentiel pédologique, 2009)	[OL]-OF-BryoOL / Aca / Sca-Aca / Sca-[Aca]
Site name	AG1
Station parameters	
Location	Ramsel Hang
Year of sampling	2018
Altitude	501 m
Slope	40°
Aspect	330°
Topography	Steep slope
Geology	Calcareous scree
Vegetation type	<i>Galio-Fagenion</i>
	
Soil profile	
	

Soil classification		
Soil name (Baize and Girard, 2009)		CALCOSOL issu de molasse
Soil name (WRB, 2022)		Calcaric Cambisol
Humus form (Zanella et al., 2018)		Eumull
Horizon sequence (Référentiel pédologique, 2009)		[OL]-BryoOL / Aca / Aca-Sca / Sca
Site name		AG2
Station parameters		
Location	Erli/Bööndler	
Year of sampling	2018	
Altitude	600 m	
Slope	8°	
Aspect	0°	
Topography	Light north-facing slope	
Geology	Molasse	
Vegetation type	<i>Galio-Fagenion</i>	
Soil profile		
		

Soil classification		
Soil name (Baize and Girard, 2009)		CALCOSOL issu de molasse
Soil name (WRB, 2022)		Calcaric Cambisol
Humus form (Zanella et al., 2018)		Eumull
Horizon sequence (Référentiel pédologique, 2009)		[OL] / BryoOL-OF-Aca / Aca / Sca1 / Sca2
Site name		AG3
Station parameters		
Location	Grashalde	
Year of sampling	2018	
Altitude	450 m	
Slope	10°	
Aspect	47°	
Topography	Light slope	
Geology	Molasse	
Vegetation type	<i>Cephalanthero-Fagenion</i>	
Soil profile		
		

Soil classification		
Soil name (Baize and Girard, 2009)		CALCOSOL issu de molasse
Soil name (WRB, 2022)		Calcaric Cambisol
Humus form (Zanella et al., 2018)		Eumull
Horizon sequence (Référentiel pédologique, 2009)		[nOL-vOL] / Aca-Sca / Aca-Sca&Cca
Site name		AG4
Station parameters		
Location	Rotebüel	
Year of sampling	2018	
Altitude	467 m	
Slope	10°	
Aspect	180°	
Topography	Light slope	
Geology	Molasse	
Vegetation type	Galio-Fagenion	
Soil profile		
		

Soil classification		
Soil name (Baize and Girard, 2009)		CALCOSOL colluvial, issu d'éboulis calcaires
Soil name (WRB, 2022)		Calcaric Cambisol (Litholinic)
Humus form (Zanella et al., 2018)		Eumull
Horizon sequence (Référentiel pédologique, 2009)		[OL]-OF-BryoOL / Aca-Sca / Mca-[Aca-Sca]
Site name		AG5
Station parameters		
Location	Risi	
Years of sampling	2016 and 2018	
Altitude	564 m	
Slope	40°	
Aspect	90°	
Topography	Steep slope	
Geology	Calcareous scree	
Vegetation type	<i>Galio-Fagenion</i>	
Soil profile		
		

Soil classification		
Soil name (Baize and Girard, 2009)		CALCOSOL issu de molasse
Soil name (WRB, 2022)		Calcaric Cambisol
Humus form (Zanella et al., 2018)		Mesomull
Horizon sequence (Référentiel pédologique, 2009)		nOL-BryoOL / Aca / Sca / Sca-Mca / Mca
Site name		BE1
Station parameters		
Location	Chatzestiel	
Year of sampling	2018	
Altitude	440 m	
Slope	0°	
Aspect	0°	
Topography	Edge of a forest road, flat	
Geology	Molasse	
Vegetation type	<i>Cephalanthero-Fagenion</i>	
Soil profile		
		

Soil classification		
Soil name (Baize and Girard, 2009)		CALCOSOL issu de calcaires du Portlandien
Soil name (WRB, 2022)		Calcaric Cambisol
Humus form (Zanella et al., 2018)		Dysmull
Horizon sequence (Référentiel pédologique, 2009)		nOL-vOL / OF-[Aca] / Aca-[OF] / Aca-Sca / Cca
Site name		BE2
Station parameters		
Location	La Neuveville	
Year of sampling	2016 and 2021	
Altitude	606 m	
Slope	5°	
Aspect	68°	
Topography	Concave bottom of slope	
Geology	Portlandian limestone	
Vegetation type	Cephalanthero-Fagenion	
Soil profile		
		

Soil classification		
Soil name (Baize and Girard, 2009)	CALCOSOL colluvial, issu d'éboulis calcaires	
Soil name (WRB, 2022)	Calcaric Cambisol (Litholinic)	
Humus form (Zanella et al., 2018)	Mesomull	
Horizon sequence (Référentiel pédologique, 2009)	nOL-OF / Aca / Sca / Sca-Cca	
Site name	BE3	
Station parameters		
Location	Schlosshubel	
Year of sampling	2016 and 2018	
Altitude	500 m	
Slope	45°	
Aspect	220°	
Topography	Steep slope under a molasse cliff	
Geology	Molasse and calcareous scree	
Vegetation type	<i>Cephalanthero-Fagenion</i>	
Soil profile		
		

Soil classification		
Soil name (Baize and Girard, 2009)		CALCOSOL colluvial, issu d'éboulis calcaires
Soil name (WRB, 2022)		Calcaric Cambisol (Litholinic)
Humus form (Zanella et al., 2018)		Oligomull
Horizon sequence (Référentiel pédologique, 2009)		OL / OF-Aca / Aca-Sca / Sca1 / Sca2
Site name		BE4
Station parameters		
Location	Schwändiwald	
Year of sampling	2018	
Altitude	870 m	
Slope	35°	
Aspect	220°	
Topography	Steep slope	
Geology	Calcareous scree	
Vegetation type	<i>Lonicero-Fagenion</i>	
Soil profile		
		

Soil classification		
Soil name (Baize and Girard, 2009)		CALCOSOL issu de molasse
Soil name (WRB, 2022)		Calcaric Cambisol
Humus form (Zanella et al., 2018)		Oligomull
Horizon sequence (Référentiel pédologique, 2009)		OL / BryoOL-OF-Aca / Aca / Aca-Sca-Cca
Site name		BE5
Station parameters		
Location	Zapfenmoos	
Year of sampling	2018	
Altitude	780 m	
Slope	15°	
Aspect	0°	
Topography	Light slope	
Geology	Molasse	
Vegetation type	<i>Lonicero-Fagenion</i>	
Soil profile		
		

Soil classification		
Soil name (Baize and Girard, 2009)		CALCOSOL colluvial, issu d'éboulis calcaires
Soil name (WRB, 2022)		Calcaric Cambisol (Litholinic)
Humus form (Zanella et al., 2018)		Oligomull
Horizon sequence (Référentiel pédologique, 2009)		OL / OF-Aca / Aca-Sca / Cca
Site name		BE6
Station parameters		
Location	Sahlenweidli	
Year of sampling	2018	
Altitude	915 m	
Slope	40°	
Aspect	160°	
Topography	Steep slope	
Geology	Calcareous scree	
Vegetation type	<i>Lonicero-Fagenion</i>	
Soil profile		
		

Soil classification	
Soil name (Baize and Girard, 2009)	CALCOSOL à horizon A hémiorganique, colluvial, issu d'éboulis calcaires
Soil name (WRB, 2022)	Calcaric Cambisol (Litholinic, Humic)
Humus form (Zanella et al., 2018)	Mesomull
Horizon sequence (Référentiel pédologique, 2009)	OL / Acaho / Acaho-Sca-Cca / Sca-Cca
Site name	BE7
Station parameters	
Location	Gasterntal
Year of sampling	2018
Altitude	1540 m
Slope	17°
Aspect	230°
Topography	Slope with flat areas
Geology	Calcareous scree
Vegetation type	<i>Vaccinio-Piceion</i>
	
Soil profile	
	

Soil classification		
Soil name (Baize and Girard, 2009)		RENDOSOL colluvial, issu d'éboulis calcaires
Soil name (WRB, 2022)		Rendzic Calcaric Leptosol (Litholinic)
Humus form (Zanella et al., 2018)		Hemimoder
Horizon sequence (Référentiel pédologique, 2009)		vOL-nOL-BryoOL / OF-[OH] / Acaho-Cca
Site name		BL1
Station parameters		
Location	Langenbruck	
Year of sampling	2016 and 2021	
Altitude	933 m	
Slope	35°	
Aspect	156°	
Topography	Steep slope	
Geology	Calcareous scree	
Vegetation type	<i>Abieti-Fagenion</i>	
Soil profile		
		

Soil classification		
Soil name (Baize and Girard, 2009)	DOLOMITOSOL	
Soil name (WRB, 2022)	Dolomitic Cambisol	
Humus form (Zanella et al., 2018)	Oligomull	
Horizon sequence (Référentiel pédologique, 2009)	nOL-vOL / Ado1 / Ado2 / Sdo	
Site name	GR1	
Station parameters		
Location	Schmitten	
Year of sampling	2019 and 2021	
Altitude	1927 m	
Slope	10°	
Aspect	88°	
Topography	Light slope, edge of a forest road	
Geology	Dolomite	
Vegetation type	<i>Erico-Pinion uncinatae</i>	
Soil profile		
		

Soil classification		
Soil name (Baize and Girard, 2009)		DOLOMITOSOL à horizons gypsiques
Soil name (WRB, 2022)		Gypsic Dolomitic Cambisol
Humus form (Zanella et al., 2018)		Dysmull
Horizon sequence (Référentiel pédologique, 2009)		[nOL]-[vOL]-OF / Adoy / Cdo1 / Cdo2 / Cdo3
Site name		GR2
Station parameters		
Location	Filisur (Bim Schloss)	
Year of sampling	2019	
Altitude	1128 m	
Slope	32°	
Aspect	253°	
Topography	Steep slope between a forest road and railroad	
Geology	Dolomite	
Vegetation type	<i>Cephalanthero-Fagenion</i>	
Soil profile		
		

Soil classification	
Soil name (Baize and Girard, 2009)	DOLOMITOSOL à horizons gypsiques
Soil name (WRB, 2022)	Gypsic Dolomitic Cambisol
Humus form (Zanella et al., 2018)	Eumull
Horizon sequence (Référentiel pédologique, 2009)	nOL / Adoy / SdoY
Site name	GR3
Station parameters	
Location	Lenzerheide
Year of sampling	2019 and 2021
Altitude	1922 m
Slope	35°
Aspect	218°
Topography	Steep slope, scree
Geology	Dolomite
Vegetation type	<i>Erico-Pinion sylvestris</i>
	
Soil profile	
	

Soil classification		
Soil name (Baize and Girard, 2009)		CALCOSOL à horizon A hémiorganique
Soil name (WRB, 2022)		Calcaric Cambisol (Humic)
Humus form (Zanella et al., 2018)		Pachyamphi
Horizon sequence (Référentiel pédologique, 2009)		vOL-nOL / BryoOL-OF-OH / Acaho / Sca
Site name		GR4
Station parameters		
Location	Bonaduz	
Year of sampling	2019	
Altitude	880 m	
Slope	30°	
Aspect	94°	
Topography	Slopes and flat areas	
Geology	Mixed moraine	
Vegetation type	<i>Cephalanthero-Fagenion</i>	
Soil profile		
		

Soil classification		
Soil name (Baize and Girard, 2009)	DOLOMITOSOL à horizons gypsiques	
Soil name (WRB, 2022)	Gypsic Dolomitic Cambisol	
Humus form (Zanella et al., 2018)	Rhizoamphi	
Horizon sequence (Référentiel pédologique, 2009)	nOL-[vOL] / OF-[OH] / Adoy / Adoy-Sdoy	
Site name	GR5	
Station parameters		
Location	Bergün	
Year of sampling	2019	
Altitude	1700 m	
Slope	38°	
Aspect	350°	
Topography	Steep slope and edge of a road	
Geology	NA	
Vegetation type	<i>Vaccinio-Piceion</i>	
Soil profile		
		

Soil classification		
Soil name (Baize and Girard, 2009)		DOLOMITOSOL
Soil name (WRB, 2022)		Dolomitic Cambisol
Humus form (Zanella et al., 2018)		Mesomull
Horizon sequence (Référentiel pédologique, 2009)		OF / Ado / Ado-Sdo / Sdo
Site name		GR6
Station parameters		
Location	Zernez	
Year of sampling	2019 and 2021	
Altitude	1526 m	
Slope	15°	
Aspect	340°	
Topography	Light slope, edge of a forest road	
Geology	NA	
Vegetation type	<i>Vaccinio-Piceion</i>	
Soil profile		
		

Soil classification		
Soil name (Baize and Girard, 2009)		CALCOSOL hypocalcaire, colluvial, issu d'éboulis calcaires
Soil name (WRB, 2022)		Calcaric Cambisol (Litholinic)
Humus form (Zanella et al., 2018)		Oligomull
Horizon sequence (Référentiel pédologique, 2009)		[nOL]-[vOL] / S*-OF / [OF]-Aca / Aca-Sca / Dca
Site name		GR7
Station parameters		
Location	Ruina Belfort	
Year of sampling	2019 and 2021	
Altitude	1153 m	
Slope	35°	
Aspect	205°	
Topography	Very steep slope	
Geology	Calcareous scree	
Vegetation type	<i>Erico-Pinion sylvestris</i>	
Soil profile		
		

Soil classification		
Soil name (Baize and Girard, 2009)		CALCOSOL colluvial, leptique, issu d'éboulis calcaires
Soil name (WRB, 2022)		Calcaric Cambisol (Litholinic)
Humus form (Zanella et al., 2018)		Dysmoder
Horizon sequence (Référentiel pédologique, 2009)		[BryoOL]-[vOL]-[nOL] / OF-OH / Aca / Cca-[Sca]
Site name		NE1
Station parameters		
Location	Creux-du-Van	
Year of sampling	2016 and 2018	
Altitude	1166 m	
Slope	45°	
Aspect	295°	
Topography	Steep slope	
Geology	Calcareous scree	
Vegetation type	<i>Lonicero-Fagenion</i>	
Soil profile		
		

Soil classification		
Soil name (Baize and Girard, 2009)		RENDOSOL colluvial, issu d'éboulis calcaires
Soil name (WRB, 2022)		Rendzic Calcaric Leptosol (Litholinic)
Humus form (Zanella et al., 2018)		Dysmoder
Horizon sequence (Référentiel pédologique, 2009)		vOL-BryOL / OF-OH / Aca / Dca-[Aca]
Site name		NE2
Station parameters		
Location	Creux-du-Van	
Year of sampling	2016 and 2021	
Altitude	1187 m	
Slope	55°	
Aspect	336°	
Topography	Steep slope with a rocky break	
Geology	Calcareous scree	
Vegetation type	Abieti-Fagenion	
Soil profile		
		

Soil classification		
Soil name (Baize and Girard, 2009)	DOLOMITOSOL	
Soil name (WRB, 2022)	Dolomitic Cambisol	
Humus form (Zanella et al., 2018)	Oligomull	
Horizon sequence (Référentiel pédologique, 2009)	nOL / OF / Ado	
Site name	T11	
Station parameters		
Location	Airolo	
Year of sampling	2019 and 2021	
Altitude	1276 m	
Slope	20°	
Aspect	326°	
Topography	North-facing slope	
Geology	NA	
Vegetation type	<i>Sambuco-Salicion</i>	
Soil profile		
		

Soil classification		
Soil name (Baize and Girard, 2009)	DOLOMITOSOL	
Soil name (WRB, 2022)	Dolomitic Cambisol	
Humus form (Zanella et al., 2018)	Mesomull	
Horizon sequence (Référentiel pédologique, 2009)	vOL / Ado / Ado-Sdo / Sdo1 / Sdo2	
Site name	TI2	
Station parameters		
Location	Bedretto	
Year of sampling	2019 and 2021	
Altitude	1495 m	
Slope	45°	
Aspect	80°	
Topography	Landslide cone	
Geology	Dolomite	
Vegetation type	<i>Vaccinio-Piceion</i>	
Soil profile		
		

Soil classification		
Soil name (Baize and Girard, 2009)		CALCOSOL colluvial, issu d'éboulis calcaires
Soil name (WRB, 2022)		Calcaric Cambisol (Litholinic)
Humus form (Zanella et al., 2018)		Oligomull
Horizon sequence (Référentiel pédologique, 2009)		OL / OF-Aca / Aca-OF / Sca
Site name		VD1
Station parameters		
Location	Moulin d'Assens	
Year of sampling	2018	
Altitude	658 m	
Slope	40°	
Aspect	290°	
Topography	Steep slope above a forest path	
Geology	Calcareous scree	
Vegetation type	<i>Galio-Fagenion</i>	
Soil profile		
		

Soil classification		
Soil name (Baize and Girard, 2009)		RENDOSOL issu de molasse
Soil name (WRB, 2022)		Calcaric Cambisol (Humic)
Humus form (Zanella et al., 2018)		Mesomull
Horizon sequence (Référentiel pédologique, 2009)		OL / Acaho1 / Acaho2 / Mca
Site name		VD2
Station parameters		
Location	Grande Côte de Baumont	
Year of sampling	2016 and 2018	
Altitude	669 m	
Slope	7°	
Aspect	110°	
Topography	Slight slope	
Geology	Molasse	
Vegetation type	<i>Molinio-Pinion</i>	
Soil profile		
		

Soil classification		
Soil name (Baize and Girard, 2009)		CALCOSOL issu de Molasse
Soil name (WRB, 2022)		Calcaric Cambisol
Humus form (Zanella et al., 2018)		Mesomull
Horizon sequence (Référentiel pédologique, 2009)		OL / Aca-Sca / Sca-Aca
Site name		VD3
Station parameters		
Location	Vallon de la Veveyse	
Year of sampling	2018	
Altitude	994 m	
Slope	50°	
Aspect	90°	
Topography	Slope up a river	
Geology	Molasse	
Vegetation type	<i>Abieti-Fagenion</i>	
Soil profile		
		

Soil classification		
Soil name (Baize and Girard, 2009)		CALCOSOL décarbonaté en surface
Soil name (WRB, 2022)		Calcaric Cambisol
Humus form (Zanella et al., 2018)		Oligomull
Horizon sequence (Référentiel pédologique, 2009)		OL / OF-Aci / Aci / Aca-Cca / Sca
Site name		VD4
Station parameters		
Location	Saint-George	
Year of sampling	2016 and 2018	
Altitude	1122 m	
Slope	15°	
Aspect	15°	
Topography	Slope	
Geology	NA	
Vegetation type	<i>Abieti-Fagenion</i>	
Soil profile		
		

Soil classification		
Soil name (Baize and Girard, 2009)		RENDOSOL pachique issu de molasse
Soil name (WRB, 2022)		Rendzic Calcaric Leptosol
Humus form (Zanella et al., 2018)		Hemimoder
Horizon sequence (Référentiel pédologique, 2009)		BryoOL-vOL / OL-[BryoOL] / OF-[OH]-[vOL]-[BryoOL] / Aca
Site name		VD5
Station parameters		
Location	Fey	
Year of sampling	2016 and 2021	
Altitude	621 m	
Slope	5°	
Aspect	136°	
Topography	Slight slope below a molasse cliff	
Geology	Molasse	
Vegetation type	<i>Galio-Fagenion</i>	
Soil profile		
		

Soil classification		
Soil name (Baize and Girard, 2009)		CALCOSOL leptique
Soil name (WRB, 2022)		Leptic Calcaric Cambisol
Humus form (Zanella et al., 2018)		Dysmull
Horizon sequence (Référentiel pédologique, 2009)		nOL-[vOL] / OF / [OF]-Aca / Sca1 / Sca2
Site name		VS1
Station parameters		
Location	Sion	
Year of sampling	2019	
Altitude	731 m	
Slope	28°	
Aspect	14°	
Topography	Edge of a forest path	
Geology	NA	
Vegetation type	<i>Cephalanthero-Fagenion</i>	
Soil profile		
		

Soil classification	
Soil name (Baize and Girard, 2009)	CALCOSOL rédoxique
Soil name (WRB, 2022)	Stagnic Calcaric Cambisol
Humus form (Zanella et al., 2018)	Dysmoder
Horizon sequence (Référentiel pédologique, 2009)	vOL / OF-OH / Agca / Agca-Sgca
Site name	VS2
Station parameters	
Location	L'Ermitage
Year of sampling	2019
Altitude	571 m
Slope	17°
Aspect	217°
Topography	Next to a water chanel (bisse)
Geology	NA
Vegetation type	Cephalanthero-Fagenion
	
Soil profile	
	

Soil classification		
Soil name (Baize and Girard, 2009)	CALCOSOL	
Soil name (WRB, 2022)	Calcaric Cambisol	
Humus form (Zanella et al., 2018)	Hemimoder	
Horizon sequence (Référentiel pédologique, 2009)	[nOL]-vOL / S*-OF-[OH] / Aca-Sca / Dca	
Site name	VS3	
Station parameters		
Location	Forêt de Sierre	
Year of sampling	2019	
Altitude	1075 m	
Slope	31°	
Aspect	354°	
Topography	Edge of a forest road	
Geology	NA	
Vegetation type	<i>Abieti-Piceion</i>	
Soil profile		
		

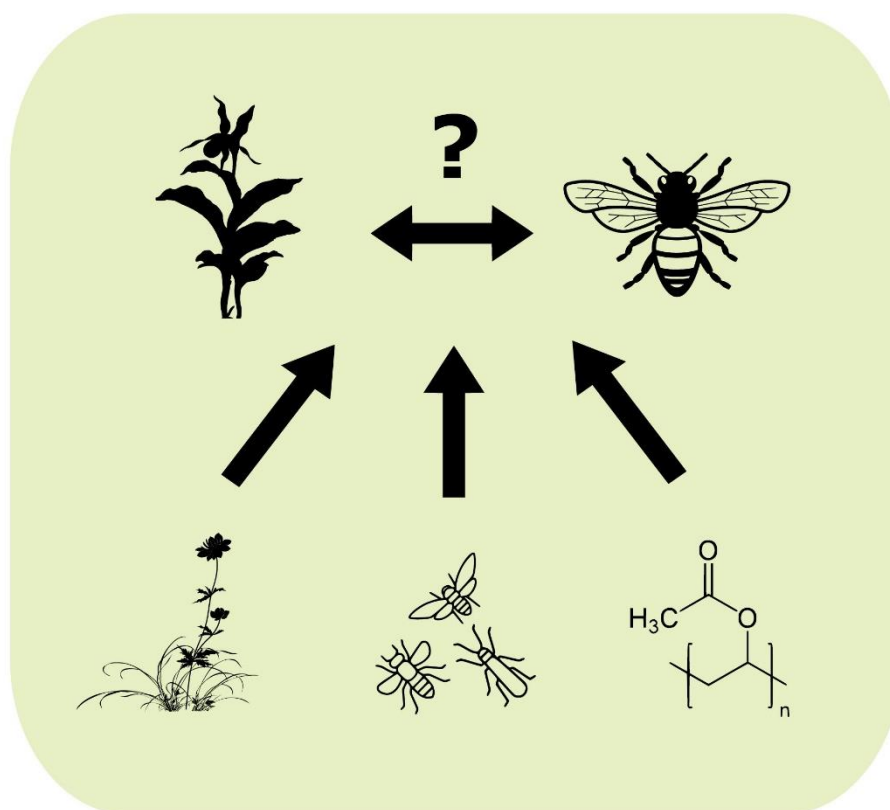
Soil classification		
Soil name (Baize and Girard, 2009)		RENDOSOL colluvial, issu d'éboulis calcaires
Soil name (WRB, 2022)		Calcaric Leptosol (Solimovic)
Humus form (Zanella et al., 2018)		Dysmull
Horizon sequence (Référentiel pédologique, 2009)		[nOL]-vOL / OF / Aca1 / Aca2 / Dca
Site name		VS4
Station parameters		
Location	Niouc	
Year of sampling	2019	
Altitude	1040 m	
Slope	30°	
Aspect	278°	
Topography	Bottom of a steep slope	
Geology	Calcareous scree	
Vegetation type	<i>Cephalanthero-Fagenion</i>	
Soil profile		
		

Soil classification		
Soil name (Baize and Girard, 2009)		CALCOSOL
Soil name (WRB, 2022)		Calcaric Cambisol
Humus form (Zanella et al., 2018)		Oligomull
Horizon sequence (Référentiel pédologique, 2009)		nOL-[vOL] / S*-OF / Aca / Sca
Site name		VS5
Station parameters		
Location	Les Diablerets	
Year of sampling	2019	
Altitude	1450 m	
Slope	6°	
Aspect	190°	
Topography	Flat area	
Geology	NA	
Vegetation type	<i>Erico-Pinion uncinatae</i>	
Soil profile		
		

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APPENDIX IV : SUPPLEMENTARY MATERIAL FOR CHAPTER III: RARE ORCHID SPECIES COMPENSATE FOR SURROUNDING LOW INSECT AND PLANT DIVERSITY BY PRODUCING MORE ODOUR BLENDS FOR POLLINATOR ATTRACTION



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Table S1. Sampling sites of *Cypripedium calceolus*. Shown are the sites names (Abbreviations refer to the different Swiss cantons visited: AG = Aargau, BE = Bern, GR = Graubünden, NE = Neuchâtel, VD = Vaud, VS = Valais, TI = Ticino). Habitat type refers to the vegetation alliance of each *C. calceolus* populations. Size refers to *C. calceolus* population size (Small means < 10 individuals, Large means > 20 individuals). Plant richness refers to the number of plant species at each site. Insect local richness refers to the number of insect morphospecies trapped in the yellow plants, and the flower insect richness refers to the number of insects species found visiting *C. calceolus* flowers. The number of fruits (pods) produced at the end of the season is also displayed. NA means that data were not collected due to logistical reasons.

Site	Habitat type	Size	Plant richness	Insect local richness	Flower insects richness	Nb fruits
AG1	<i>Galio-Fagenion</i>	Small	39	13	5	0
AG2	<i>Galio-Fagenion</i>	Small	44	44	2	2.6
AG3	<i>Cephalanthero-Fagenion</i>	Large	47	62	4	0.4
AG4	<i>Galio-Fagenion</i>	Large	48	89	2	3
AG5	<i>Galio-Fagenion</i>	Large	60	60	6	1.4
BE1	<i>Cephalanthero-Fagenion</i>	Small	34	60	1	0.5
BE2	<i>Cephalanthero-Fagenion</i>	Small	16	24	3	0
BE3	<i>Cephalanthero-Fagenion</i>	Small	37	19	0	0.4
BE4	<i>Lonicero-Fagenion</i>	Small	36	19	0	0.17
BE5	<i>Lonicero-Fagenion</i>	Large	41	95	3	1
BE6	<i>Lonicero-Fagenion</i>	Large	39	34	1	0.6
BE7	<i>Vaccinio-Piceion</i>	Large	49	111	4	0.7
GR1	<i>Erico-Pinion uncinatae</i>	Small	35	10	0	0.6
GR2	<i>Cephalanthero-Fagenion</i>	Small	44	120	2	1.75
GR3	<i>Erico-Pinion sylvestris</i>	Small	37	26	0	0
GR4	<i>Cephalanthero-Fagenion</i>	Large	31	45	0	0.8
GR5	<i>Vaccinio-Piceion</i>	Large	40	128	5	1.4
GR6	<i>Vaccinio-Piceion</i>	Large	41	NA	NA	1.5
GR7	<i>Erico-Pinion sylvestris</i>	Large	26	14	0	1.1
NE1	<i>Lonicero-Fagenion</i>	Small	32	39	6	2.8
NE2	<i>Abieti-Fagenion</i>	Large	29	27	0	0
VD1	<i>Galio-Fagenion</i>	Small	29	49	2	1
VD2	<i>Molinio-Pinion</i>	Small	43	10	9	1.2
VD3	<i>Abieti-Fagenion</i>	Large	35	48	0	0.2
VD4	<i>Abieti-Fagenion</i>	Large	48	17	0	0
VD5	<i>Galio-Fagenion</i>	Large	34	9	1	0
VS1	<i>Cephalanthero-Fagenion</i>	Small	34	11	0	0
VS2	<i>Cephalanthero-Fagenion</i>	Small	29	21	2	0.67
VS3	<i>Abieti-Piceion</i>	Small	47	59	16	NA
VS4	<i>Cephalanthero-Fagenion</i>	Large	25	55	10	1.7
VS5	<i>Erico-Pinion uncinatae</i>	Large	46	28	2	4
TI1	<i>Sambuco-Salicion</i>	Small	51	NA	NA	0.4
TI2	<i>Vaccinio-Piceion</i>	Large	52	NA	NA	0

Table S2. Results from the coflowering plant survey. This table presents co-flowering plants of *Cypripedium calceolus* (Species), in how many sites we collected samples of each species (Nb of sites) and the colour of the corolla of co-flowering plants (Corolla colour, data from www.infoflora.ch). It is shown if the co-flowering species are potential food sources for short-tongued solitary bees and for syrphid flies as well as their potential pollinators species. Species in bold are the species we found in this study acting as pollinators or visitors in *C. calceolus* flowers.

Species	Nb of sites	Corolla colour (www.infoflora.ch)	Potential food source for short-tongued solitary bees	Potential pollinators – Solitary bee species (www.swisswildbees and Westrich 2019)	Potential food source for syrphid flies	Potential pollinators – Syrphid fly species (Speight 2020)
<i>Ajuga genevensis</i>	1	blue	no	Long-tongued bees (<i>Osmia</i> , <i>Anthophora</i> , <i>Xylocopa</i>)	yes	<i>Cheilosia albitarsis</i> , <i>Merodon equestris</i> and <i>Neoascia annexa</i>
<i>Allium ursinum</i>	1	white	yes	Short-tongued bees (<i>Anrdena helvola</i> and <i>Andrena nitida</i>)	yes	<i>Brachyopa pilosa</i> , <i>Cheilosia albitarsis</i> , <i>Cheilosia caerulescens</i> , <i>Cheilosia carbonaria</i> , <i>Cheilosia chloris</i> , <i>Cheilosia fasciata</i> , <i>Cheilosia lenis</i> , <i>Cheilosia pagana</i> , <i>Cheilosia semifasciata</i> , <i>Chrysotoxum cautum</i> , <i>Criorhina asilica</i> , <i>Criorhina berberina</i> , <i>Dasysyrphus venustus</i> , <i>Eumerus strigatus</i> , <i>Heringia heringi</i> , <i>Melanostoma mellinum</i> , <i>Melanostoma scalare</i> , <i>Merodon ruficornis</i> , <i>Neoascia podagrica</i> , <i>Pipiza quadrimaculata</i> , <i>Platycheirus complicatus</i> , <i>Platycheirus parmatus</i> , <i>Platycheirus peltatus</i> , <i>Portevinia maculata</i> , <i>Syrphus torvus</i> and <i>Xanthogramma laetum</i>
<i>Aquilegia vulgaris</i>	1	purple-blue	no	-	no	-
<i>Aster bellidiastrum</i>	2	white and yellow	yes	NA	yes	<i>Eristalinus aeneus</i> , <i>Helophilus trivittatus</i> , <i>Merodon equestris</i> , <i>Platycheirus scutatus</i> , <i>Sphaerophoria rueppelli</i> , <i>Spilomyia longicornis</i> , <i>Syrphid pipiens</i> and <i>Syrphus torvus</i>
<i>Berberis vulgaris</i>	1	yellow	yes	Short-tongued bees (<i>Andrena bicolor</i> , <i>Andrena fulva</i> and <i>Andrena varians</i>)	yes	<i>Blera fallax</i> , <i>Brachypalpus laphriformis</i> , <i>Criorhina asilica</i> , <i>Criorhina brevipila</i> , <i>Dasysyrphus venustus</i> , <i>Epistrophe flava</i> , <i>Helophilus pendulus</i> , <i>Mallota eurasatica</i> , <i>Platycheirus scutatus</i> , <i>Sphecomyia vespiformis</i> and <i>Xanthogramma pedissequum</i>
<i>Cardamine heptaphylla</i>	1	white or lilac	yes	NA	yes	<i>Brachyopa pilosa</i> , <i>Brachyopa scutellaris</i> , <i>Cheilosia albipila</i> , <i>Cheilosia antiqua</i> , <i>Cheilosia frontalis</i> , <i>Cheilosia lenis</i> , <i>Cheilosia pubera</i> , <i>Criorhina asilica</i> , <i>Criorhina ranunculi</i> , <i>Eristalis anthophorina</i> , <i>Eristalis cryptarum</i> , <i>Eristalis horticola</i> , <i>Eristalis nemorum</i> , <i>Eristalis oestracea</i> , <i>Eristalis picea</i> , <i>Eristalis rupium</i> , <i>Eurimyia lineata</i> , <i>Megasyrphus erraticus</i> , <i>Neoascia meticulosa</i> ,

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						<i>Orthonevra geniculata</i> , <i>Parasyrphus annulatus</i> , <i>Parasyrphus malinellus</i> , <i>Parasyrphus punctulatus</i> , <i>Parhelophilus versicolor</i> , <i>Pipiza quadrimaculata</i> , <i>Platycheirus europaeus</i> , <i>Platycheirus manicatus</i> , <i>Platycheirus sticticus</i> , <i>Sericomyia lappona</i> , <i>Sphegina clunipes</i> , <i>Syrretta pipiens</i> , <i>Syrphocheilosia claviventris</i> and <i>Tropidia scita</i>
<i>Centaurea sp.</i>	1	blue	yes	NA	yes	<i>Brachypalpus chrysites</i> , <i>Cheilosia nebulosa</i> , <i>Epistrophe grossulariae</i> , <i>Eriozona syrphoides</i> , <i>Helophilus trivittatus</i> , <i>Leucozona lucorum</i> , <i>Paragus quadrfasciatus</i> , <i>Rhingia rostrata</i> , <i>Sericomyia superbiens</i> and <i>Volucella zonaria</i>
<i>Crataegus monogyna</i>	2	white	yes	Short-tongued bees (<i>Andrena barbilabris</i> , <i>Andrena labiata</i> , <i>Andrena scotica</i> , <i>Andrena synadelpha</i> and <i>Andrena varians</i>) and long-tongued bees (<i>Osmia cornuta</i>)	yes	More than 100 species whose <i>Cheilosia proxima</i>
<i>Euphorbia cyparissias</i>	2	yellow	no	NA	yes	More than 100 species whose <i>Cheilosia flavipes</i> , <i>Cheilosia proxima</i> , <i>Cheilosia rhynchops</i> , <i>Melanostoma mellinum</i> and <i>Melanostoma scalare</i>
<i>Fragaria vesca</i>	2	white and yellow	yes	Short-tongued bees (<i>Andrena alfkenella</i> , <i>Andrena dorsata</i> , <i>Andrena gravida</i> , <i>Andrena minutula</i> , <i>Andrena minutuloides</i> , <i>Andrena nana</i> , <i>Andrena pilipes</i> , <i>Andrena subopaca</i> , <i>Lasioglossum laevigatum</i> , <i>Lasioglossum laticeps</i> , <i>Lasioglossum malachurum</i> , <i>Lasioglossum subfasciatum</i>) and long-tongued bees (<i>Osmia bicolor</i>)	yes	<i>Cheilosia antiqua</i> , <i>Cheilosia lasiopa</i> , <i>Cheilosia pagana</i> , <i>Dasysyrphus pinastri</i> , <i>Eumerus funeralis</i> , <i>Eumerus strigatus</i> , <i>Merodon alexandri</i> , <i>Orthonevra nobilis</i> , <i>Pipiza quadrimaculata</i> , <i>Sphaerophoria virgata</i> and <i>Sphegina montana</i>
<i>Frangula alnus</i>	1	white	yes	Short-tongued bees (e.g.: <i>Andrena synadelpha</i>)	yes	<i>Caliprobola speciosa</i> , <i>Cheilosia chloris</i> , <i>Chrysotoxum cautum</i> , <i>Chrysotoxum fasciatum</i> , <i>Criorhina asilica</i> , <i>Criorhina berberina</i> , <i>Dasysyrphus pinastri</i> , <i>Dasysyrphus venustus</i> , <i>Meligramma guttata</i> , <i>Meligramma triangulifera</i> , <i>Orthonevra intermedia</i> , <i>Orthonevra stackelbergi</i> , <i>Parhelophilus frutetorum</i> , <i>Platycheirus tarsalis</i> , <i>Psilota anthracina</i> , <i>Syrphus torvus</i> , <i>Trichopsomyia flavitarsis</i> and <i>Volucella inflata</i>
<i>Galium odoratum</i>	2	white	yes	NA	yes	More than 40 species but no <i>C. calceolus</i> visitor or pollinator identified in this study
<i>Geranium robertianum</i>	1	pink	yes	Short-tongued bees (<i>Andrena bicolor</i> , <i>Lasioglossum calceatum</i> , <i>Lasioglossum leucozonium</i> , <i>Lasioglossum malachurum</i>)	yes	<i>Eumerus ornatus</i> , <i>Platycheirus scutatus</i> , <i>Rhingia borealis</i> and <i>Rhingia rostrata</i>
<i>Hieracium murorum aggr.</i>	6	yellow	yes	Short-tongued bees (<i>Andrena nigriceps</i> , <i>Dufourea alpina</i> , <i>Halictus subauratus</i> , <i>Lasioglossum albipes</i> , <i>Lasioglossum limbellum</i>)	yes	<i>Cheilosia ahenea</i> , <i>Cheilosia bergenstammi</i> , <i>Cheilosia scutellata</i> , <i>Cheilosia mutabilis</i> , <i>Cheilosia scutellata</i> , <i>Chrysotoxum festivum</i> , <i>Criorhina asilica</i> , <i>Dasysyrphus pinastri</i> ,

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				and <i>Lasioglossum punctatissimum</i>) and long-tongued bees (<i>Osmia fulviventris</i> , <i>Osmia leaiana</i> and <i>Megachile alpicola</i>)		<i>Ferdinandea cuprea</i> , <i>Melanogaster aerea</i> , <i>Merodon equestris</i> , <i>Pelecocera lusitanica</i> , <i>Pelecocera tricineta</i> and <i>Syrphus torvus</i>
<i>Lamium galeobdolon</i>	3	yellow	no	-	yes	<i>Xanthogramma pedissequum</i>
<i>Listera ovata</i>	1	yellow-greenish	no	-	yes	<i>Cheilosia uviformis</i>
<i>Lonicera xylosteum</i>	1	white-yellowish	no	-	yes	<i>Criorhina asilica</i> , <i>Criorhina berberina</i> , <i>Dasysyrphus albostratus</i> , <i>Dasysyrphus hilaris</i> , <i>Dasysyrphus pinastri</i> , <i>Dasysyrphus venustus</i> , <i>Epistrophe melanostoma</i> , <i>Melangyna lasiophthalma</i> , <i>Melangyna quadrimaculata</i> , <i>Rhingia borealis</i> and <i>Temnostoma vespiforme</i>
<i>Melittis melysophyllum</i>	2	white or pink	no	-	no	-
<i>Phyteuma spicatum</i>	3	white-yellowish	yes	NA	yes	<i>Xanthogramma laetum</i>
<i>Platanthera bifolia</i>	1	white-greenish	no	-	no	-
<i>Polygonatum multiflorum</i>	3	white and green	no	Long-tongued bees (<i>Osmia pilicornis</i>)	no	-
<i>Polygonatum verticillatum</i>	1	white and green	no	Long-tongued bees	no	-
<i>Potentilla erecta</i>	1	yellow	yes	Short-tongued bees (<i>Andrena tarsata</i> , <i>Andrena coitana</i> , <i>Andrena dorsata</i> , <i>Andrena minutula</i> , <i>Halictus leucaneus</i> , <i>Halictus maculatus</i> and <i>Lasioglossum lucidulum</i>)	yes	More than 60 species whose <i>Melanostoma mellinum</i>
<i>Ranunculus sp.</i>	1	yellow	yes	Short-tongued bees (<i>Andrena</i> and <i>Lasioglossum</i>) and long-tongued bees (<i>Osmia</i>)	yes	More than 250 species whose <i>Cheilosia flavipes</i>, <i>Cheilosia proxima</i>, <i>Cheilosia rhynchops</i>, <i>Melanostoma mellinum</i>, <i>Melanostoma scalare</i> and <i>Platycheirus parvatus</i>
<i>Tamus communis</i>	1	yellow-greenish	no	-	no	-
<i>Valeriana montana</i>	2	pink to white	yes	NA	no	-
<i>Viburnum sp.</i>	1	white	yes	Short-tongued bees (<i>Andrena fulva</i>)	yes	More than 40 species but no <i>C. calceolus</i> visitor or pollinator identified in this study

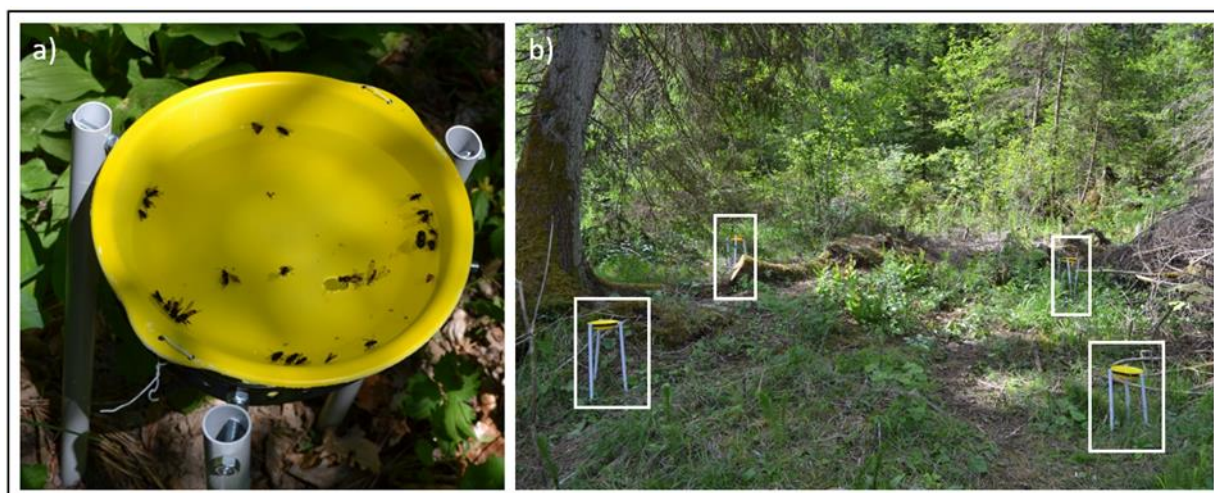


Figure S1. Yellow plates insect sampling methods. a) Shows a close-up of yellow plate, and b) shows an example of how the four yellow plates were spaced in the environment. Each plate was filled with water and soap and left for about 8hrs to catch flying insects.

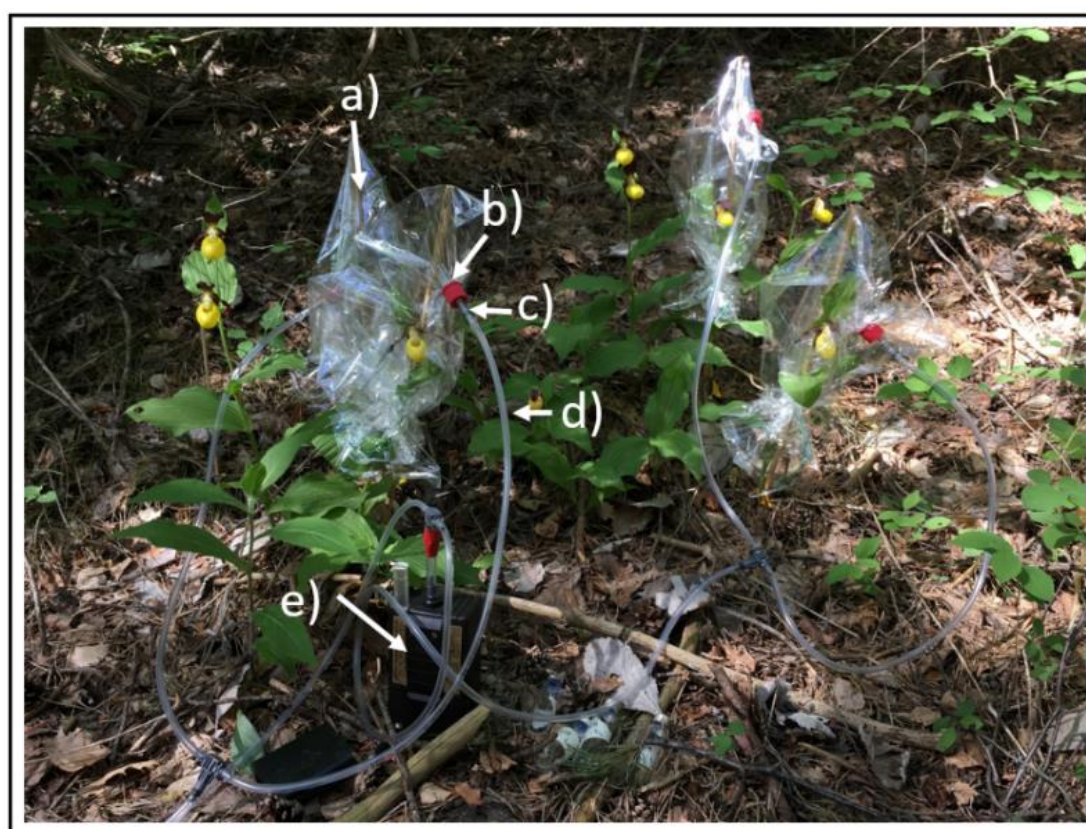


Figure S2. Volatile organic compounds sampling methods. In order to catch *Cypripedium calceolus* scents, flowers were enclosed in a nallophan oven bag (a). A red socket was fixed on the plastic bag (b). An adsorbent tube filled with Tenax (c) was then plugged with the socket and a plastic pipe (d) in order to pump (e) the air trapped in the oven bag.

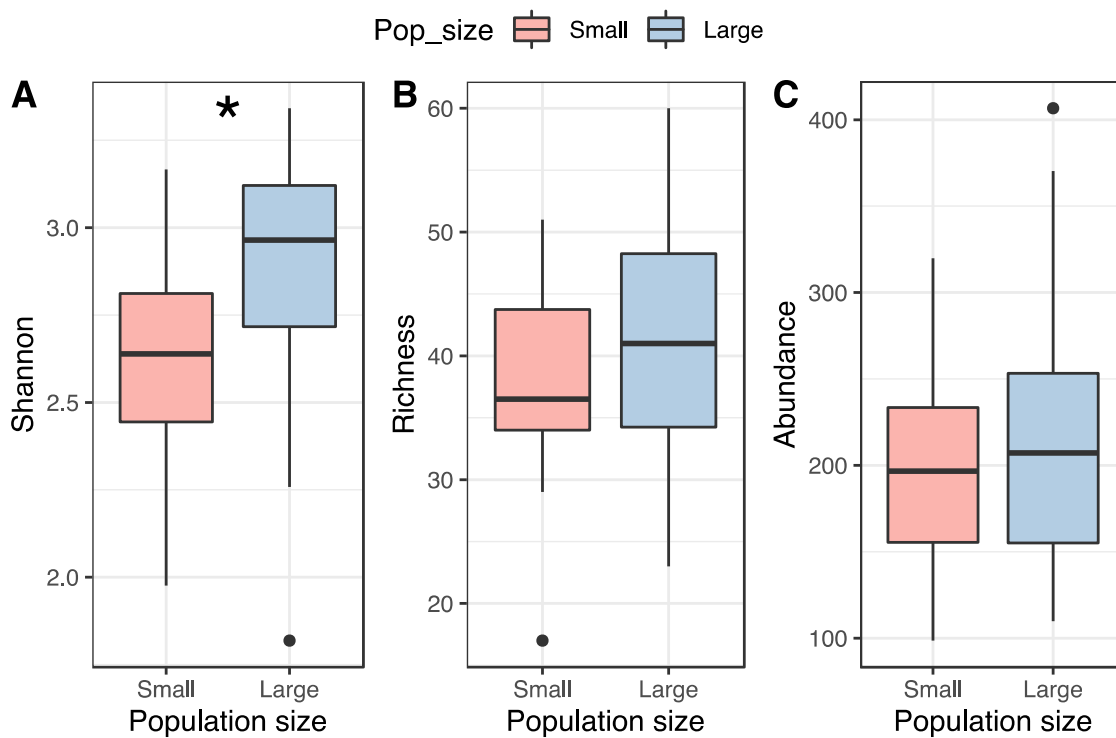


Figure S3. Vegetation biodiversity survey. Shown are the different biodiversity indices; A. Shannon diversity, B. species richness and C. total abundance for plants sampled around each *C. calceolus* site in small (< 10 individuals, red boxplots), and large (> 20 individuals, blue boxplots) populations. Asterisks indicate significant differences across populations sizes ($p < 0.05$).

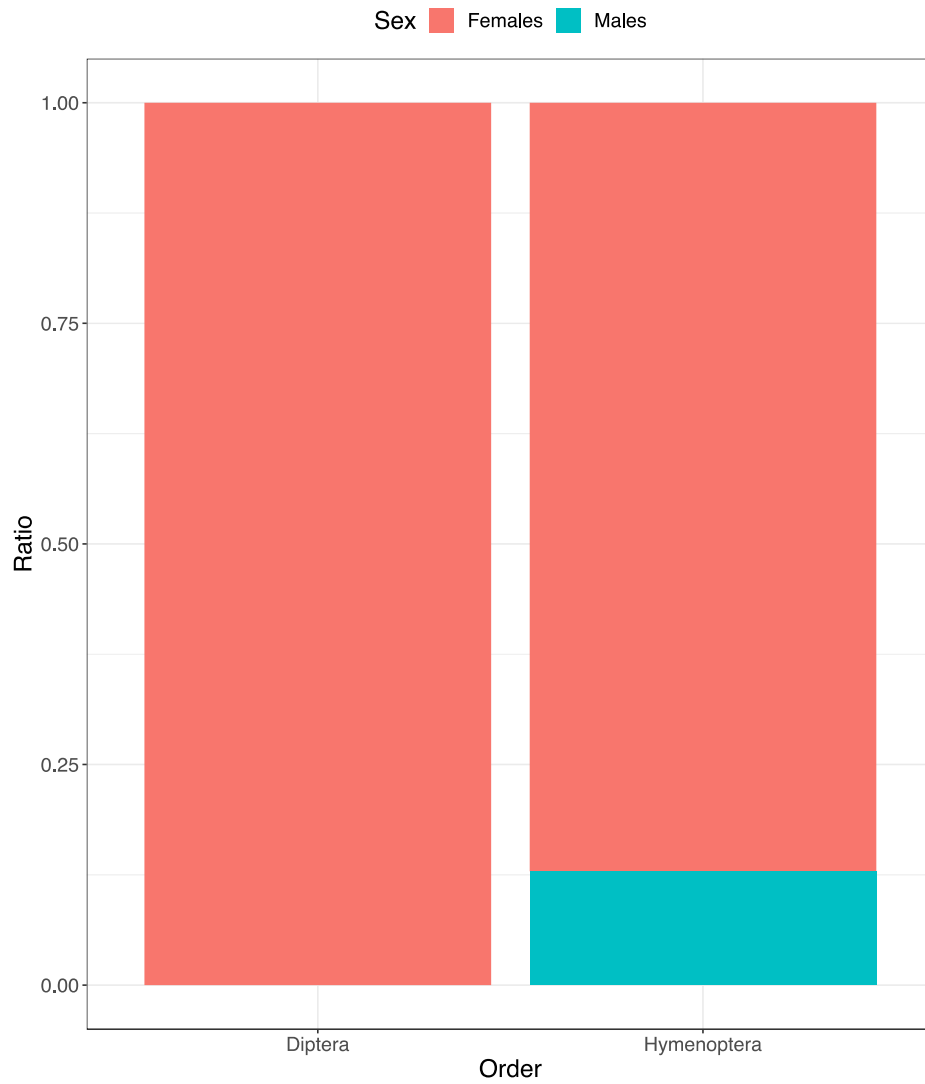


Figure S4. Sex ratio of *Cyripedium calceolus* flower visitors. Shown are the proportion of females (red bars) and males (green bars) of Diptera and Hymenoptera species found visiting the labella of *C. calceolus* flowers. Overall, we found many more females than males visiting the flowers (6/6 dipteran (Chi-squared = 4.17, df = 1, p-value = 0.041), and 54/62 hymenopteran (Chi-squared = 32.66, df = 1, p-value < 0.001)).

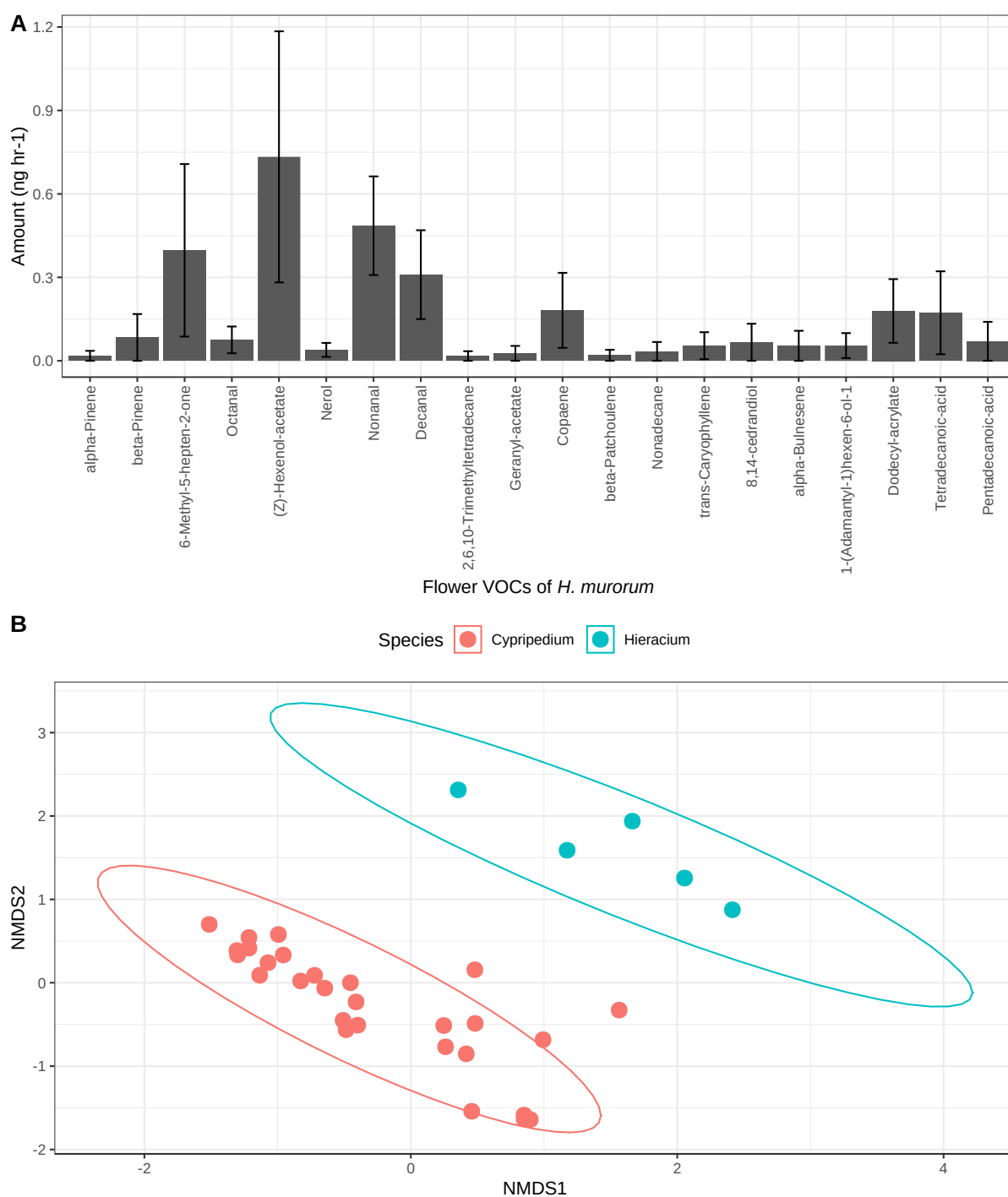
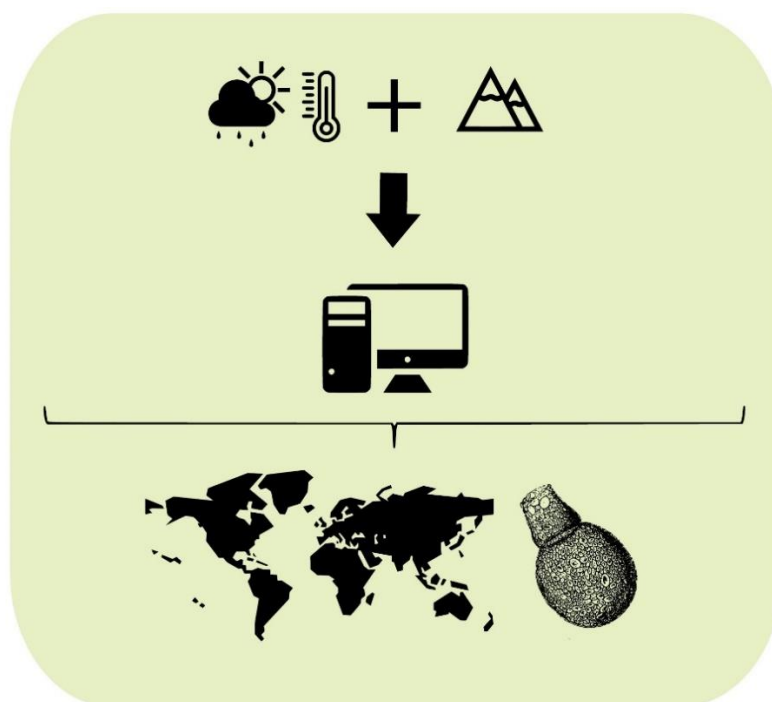


Figure S5. Flower Volatile organic compounds (VOCs) profiles of *Hieracium murorum* aggr. A. VOCs found across 16 sites, B. non-metric multidimensional scaling (NMDS) of flower VOCs from *C. calceolus* (red color) and *H. murorum* (blue color) (stress = 0.1). Overall, we found that *Hieracium* flowerheads produced a wide range of VOCs, but with almost no overlap with *C. calceolus* VOCs profiles, such as the complete absence of linalool or octyl-acetate

References

- Speight M. (2020) StN key for the identification of the genera of European Syrphidae 2020. *Syrph the Net*, the database of European Syrphidae (Diptera) 105, 1-46.
- Westrich P. (2019) Die Wildbienen Deutschlands, 2. ed. Verlag Eugen Ulmer Stuttgart, Deutschland.

APPENDIX V : SUPPLEMENTARY MATERIAL FOR DISTRIBUTION MODELLING OF SOIL PROTIST BIOGEOGRAPHY REVEALS DISPERSAL LIMITATION AND THREAT TO MICROBIAL DIVERSITY FROM CLIMATE WARMING



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Discussion on some excluded geographical records of *Apodera vas*.

The most extreme record in terms of climate, geography, and ecology was from mainland Antarctica (Penard 1911). This occurrence was reported by James Murray as “*Diffflugia vas*” (Murray 1910). However, Eugène Penard did not observe the species *Nebela (Apodera) vas* in the samples he analysed (Penard 1911). Given the extreme climate of the sampling location and the extreme paucity of testate amoebae in the samples, it is very unlikely that this identification is correct. We presume that the observation rather corresponds to *Lagenodiffflugia vas* (originally indeed *Diffflugia vas*), as mentioned by Murray. This would imply that Penard surprisingly erroneously believed it was *Nebela (Apodera) vas*. Indeed, Murray wrote the following: “The paucity of Rhizopods at Cape Royds was surprising, after it became known that so many other kinds of microscopic life abounded in the lakes. In the lakes only two testaceous species were observed: the well-known *Diffflugia vas*, and a very small kind which appears to be a *Quadrula*. Among the moss there was another species, not identified.” Penard (1911) reported finding *Quadrula irregularis* (current name: *Paraquadrula irregularis*) but not *Apodera (Nebela) vas*. He did not either find any heliozoan in a sample fixed with formalin, although Murray found these in high abundance, albeit in a sample collected in April 1908 while the sample Penard observed was from January 1909. Murray did not specify in which habitat “*Diffflugia vas*” was observed. Penard assumed it was in Blue Lake. Despite his extensive knowledge on protists, Penard was most likely misled in thinking the taxon mentioned by Murray under the name *Diffflugia vas* should correspond to *Nebela (Apodera) vas*. Interestingly, Penard did not either find *Diffflugia (Lagenodiffflugia) vas*. It may be that the shell of this species built from mineral grains (xenosome) broke in the formalin-preserved sample and was no-longer visible by the time Penard could access the samples. Another possible explanation is that he observed a different set of samples. Unfortunately, *Diffflugia vas* was not included in the microphotographs and drawing of the plates illustrating Murray’s report. As Murray specified that this species was “well-known” he might have considered it unnecessary to illustrate it; little did he realise that this puzzle would only be clarified more than one century later! The absence of any favourable habitat on mainland Antarctica in our modelling data further confirms that Penard’s report was due to a taxonomic confusion as this record is clearly well beyond the climate niche of *Apodera vas*.

The record from Nepal (Bonnet 1977) is also considered dubious. Louis Bonnet (pers. comm. to Sebastien Wohlhauser) mentioned that “the morphology was close to *Padaungiella lageniformis* and thus that the observed specimen may not correspond to *A. vas*”. Given that no permanent slide is available in the collection Louis Bonnet deposited at the Natural History Museum of Geneva, that no illustration exists for this record and that this was the only record from the Indian subcontinent, we excluded it. This means that to our knowledge there is currently no validated record of *A. vas* from the Indian subcontinent.

Richters (1908) mentioned that *Apodera (Nebela) vas* was partly represented as intermediate forms with a shell covered by mineral grains (in German “Steinchen”), which clearly does not correspond to the diagnosis of the genus *Apodera* but more likely *Lagenodiffflugia*, as discussed previously (Mitchell

and Meisterfeld 2005). As the detail of which records correspond to which morphotype is not given, none of his records can be accepted with certainty. Only the highly likely records are thus included here. Given this taxonomic uncertainty, the records that have not been confirmed by later studies are excluded, and most significantly those from Hawaii and Japan. We explored the diversity of testate amoebae in Hawaii (Big Island, Maui and Kauai) along elevation gradients from the sea level to the alpine level in the summer of 2022. We did not find *Apodera vas* in over 50 samples analysed, although other large Hyalospheniidae were observed in several bog and wet forest samples.

The record for Vancouver, Canada (Penard 1911) having never been confirmed despite recent sampling in the area by one of us (Thierry J. Heger), is also considered dubious.

Decloître (1965) reported *Nebela strangularia* from a pond on the island of Grímsey off the northern coast of Iceland. The description is very superficial and is only illustrated by a simple line drawing that resembles *Apodera vas*. As this species has never been observed since its description and this location would be extremely distant from the next nearest record, we exclude it.

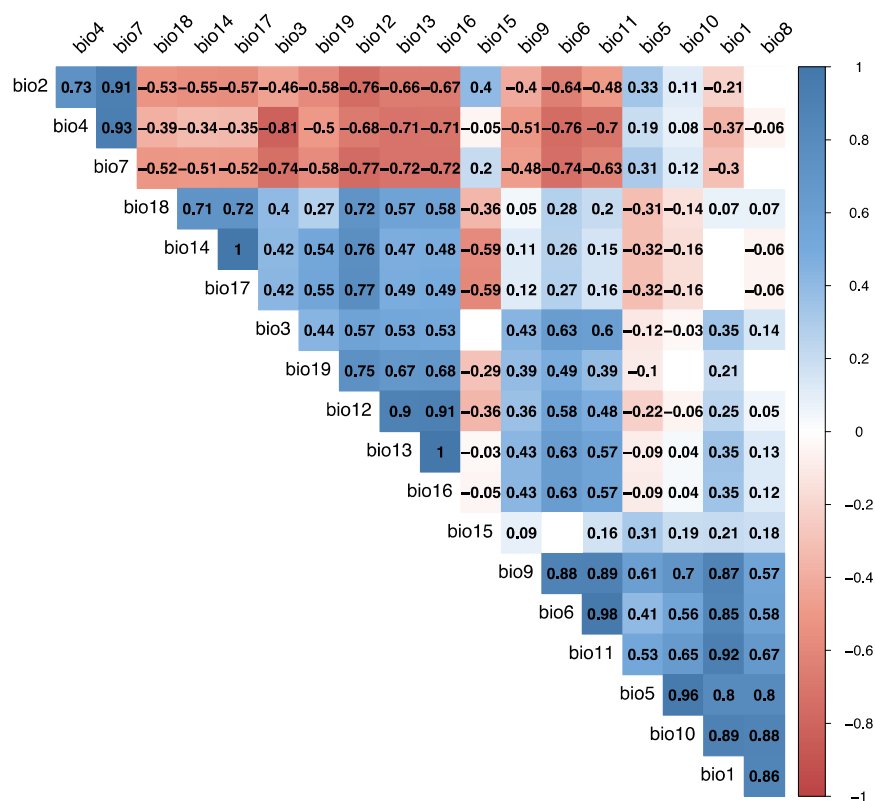


Figure S1. Spearman correlation among aggregated current climate CHELSA bioclim variables. The variables are listed in Table S1.

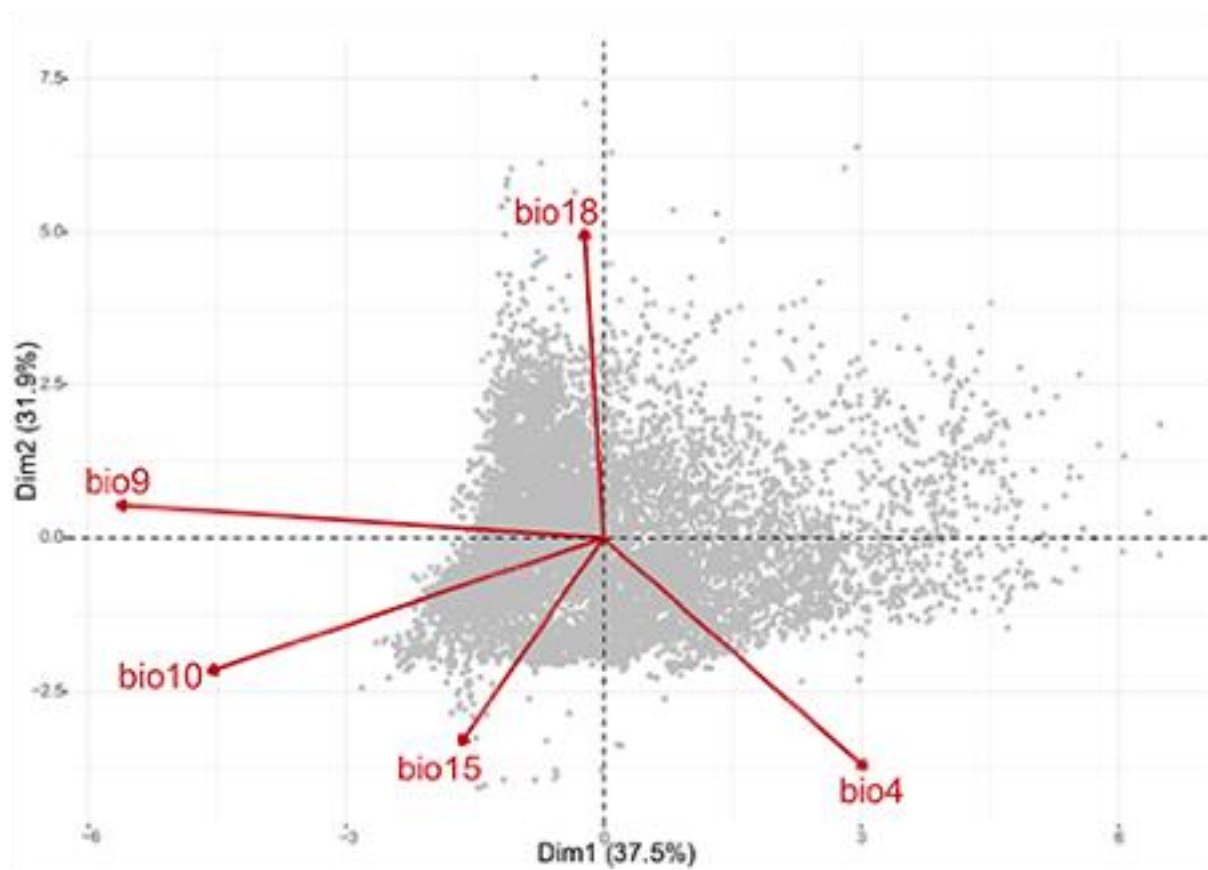


Figure S2. PCA selection of CHELSA bioclim variables. The grey dots correspond to the validated occurrences. The variables are listed in Table S1.

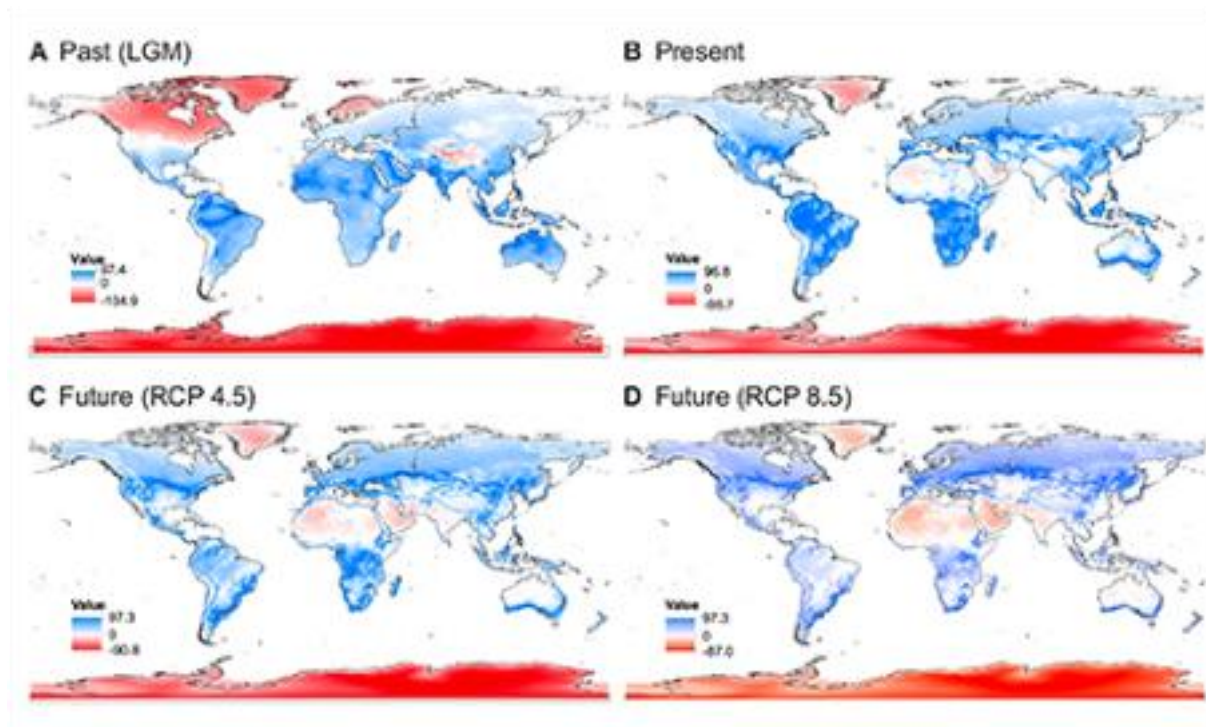


Figure S3. Analogous and non-analogous climatic areas weighted with Multivariate Environmental Similarity Surfaces (MESS) of CHELSA variable bioclim10 (mean temperature of warmest quarter) for A. past, B. current, C. future RCP 4.5, and D. future RCP 8.5 scenarios.

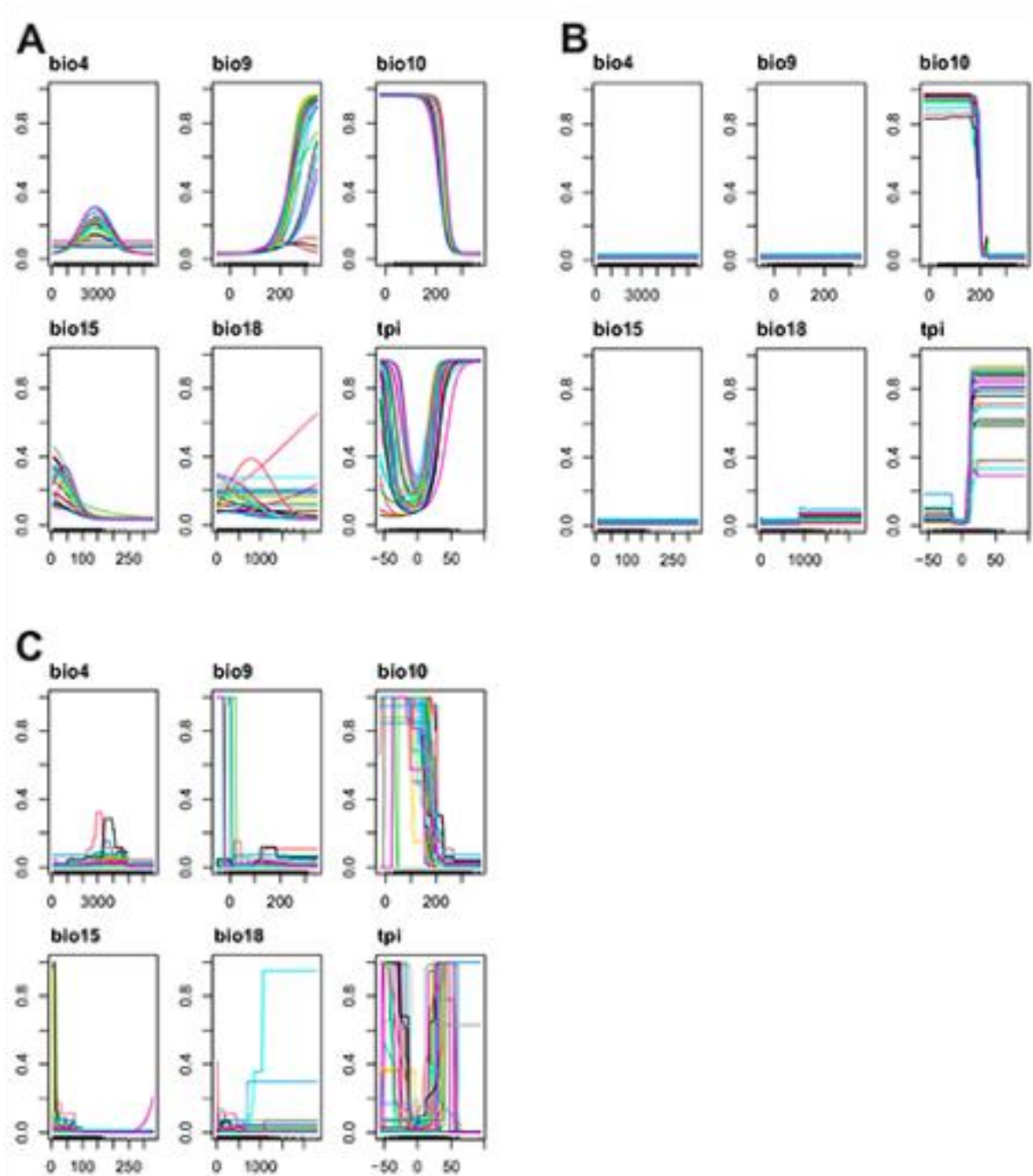


Figure S4. Response curves of the CHELSA bioclim variables used in the models: A. Generalized Linear Model (GLM), B. Boosted Regression Trees (GBM), and C. the Maximum Entropy Method (MAXENT). The x-axes show the bioclim variable or TPI, the y-axes show the probability of occurrence of *Apodera vas* in relation to the selected variable.

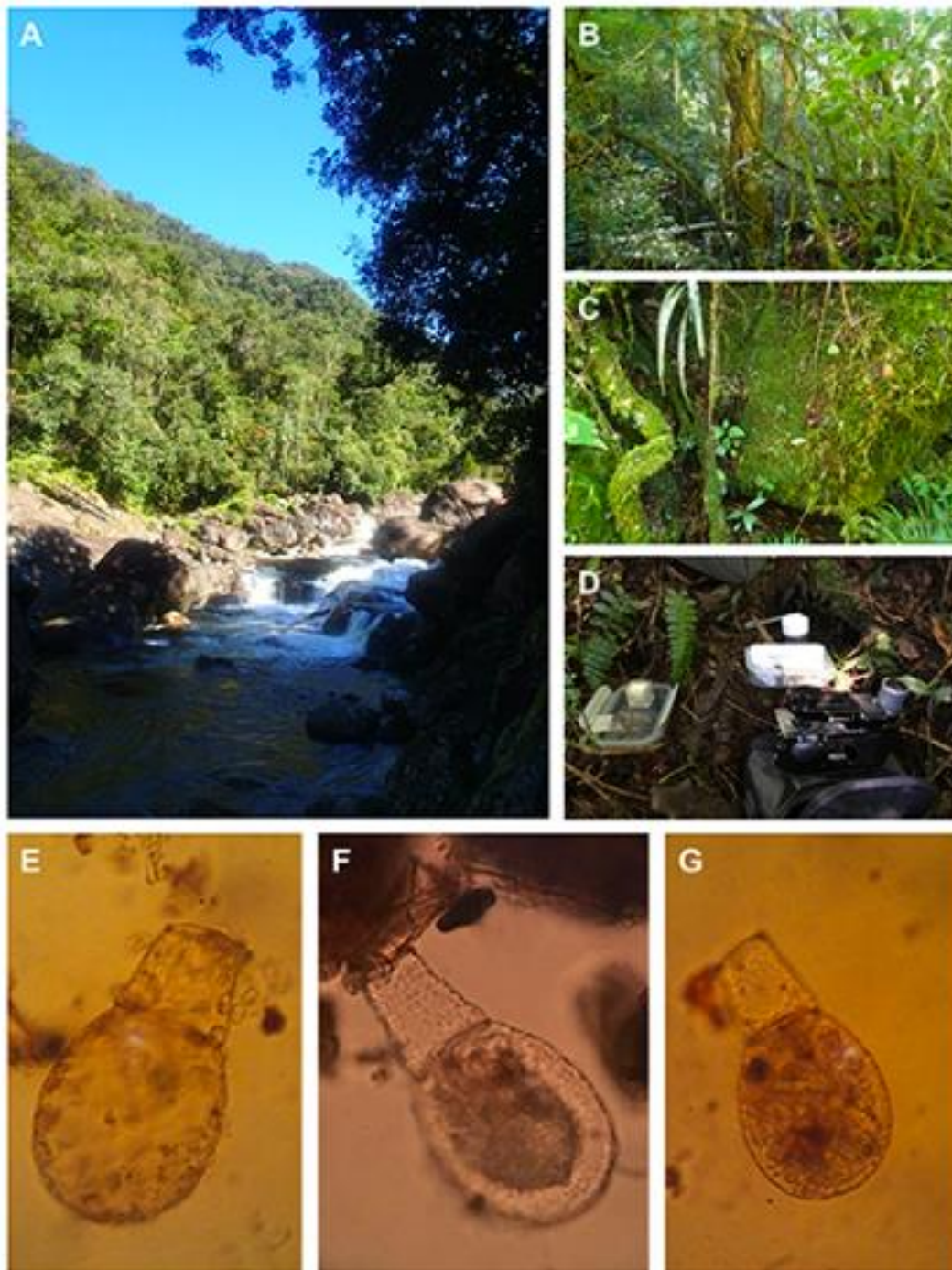


Figure S5. During an expedition in the Beampingaratsy Mountains, Anosyenne Mountain Range, Municipality of Analamary, SE Madagascar led by Janet Wilmshurst, testate amoebae were observed and photographs taken in the field by Sebastien Wohlhauser using a Nikon H portable inverted microscope (picture D). As predicted by the model, *Apodera vas* was found in this region along the Mananjary River, at 475m a.s.l. (WGS84 coordinates: -24.137895; 47.08264, image A, specimen F & G) and at 1125m a.s.l. (WGS84 coordinates: -24.13494; 47.05677, images B & C, specimen E) half way up to the Vohibanga summit.

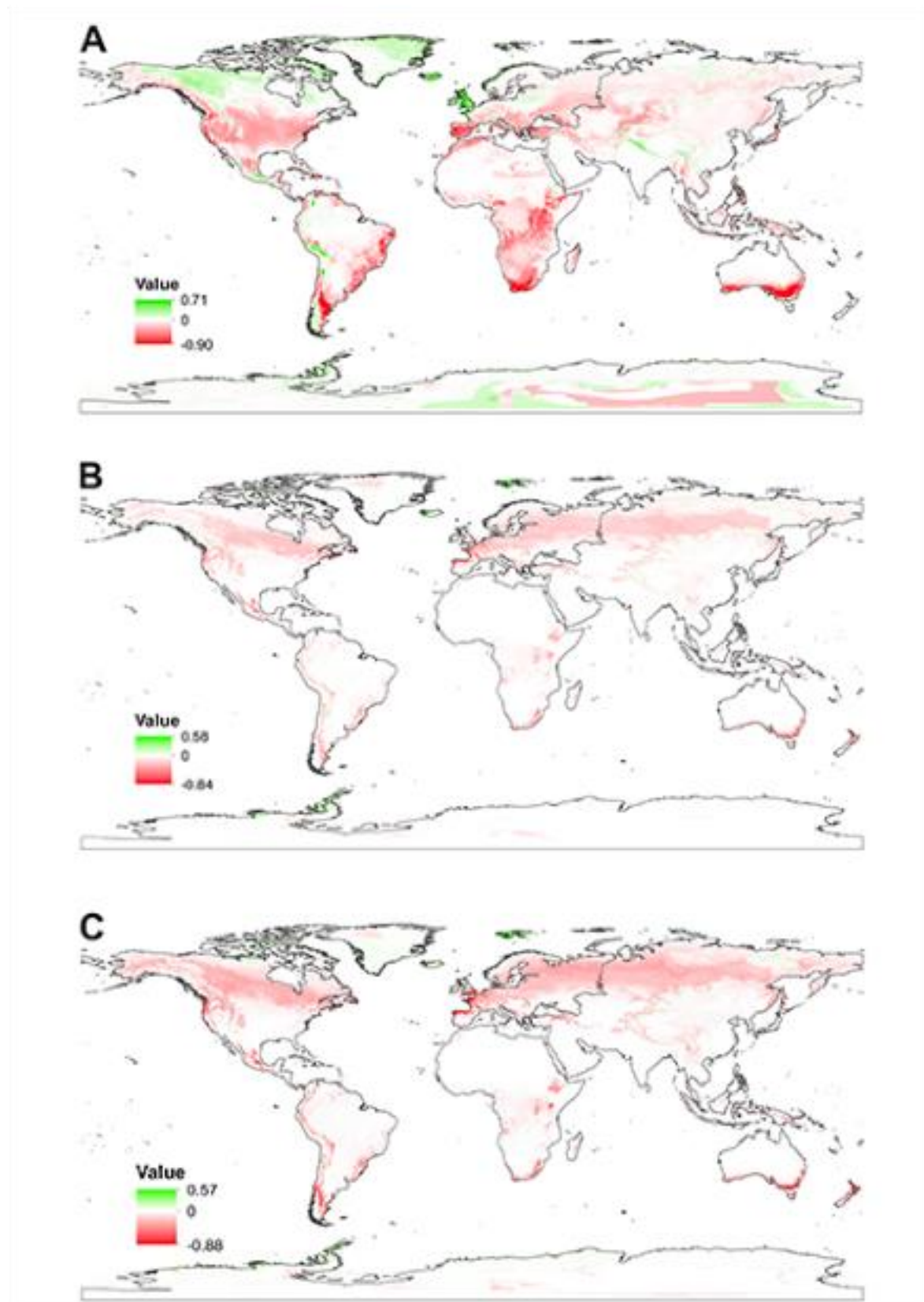


Figure S6. Comparisons of loss (red) and gain (green) of suitable habitats (climatic conditions) for *Apodera vas* between distribution models corresponding to different climatic conditions: A. past (LGM) vs present, B. present vs future (RCP 4.5), and C. present vs future (RCP 8.5).

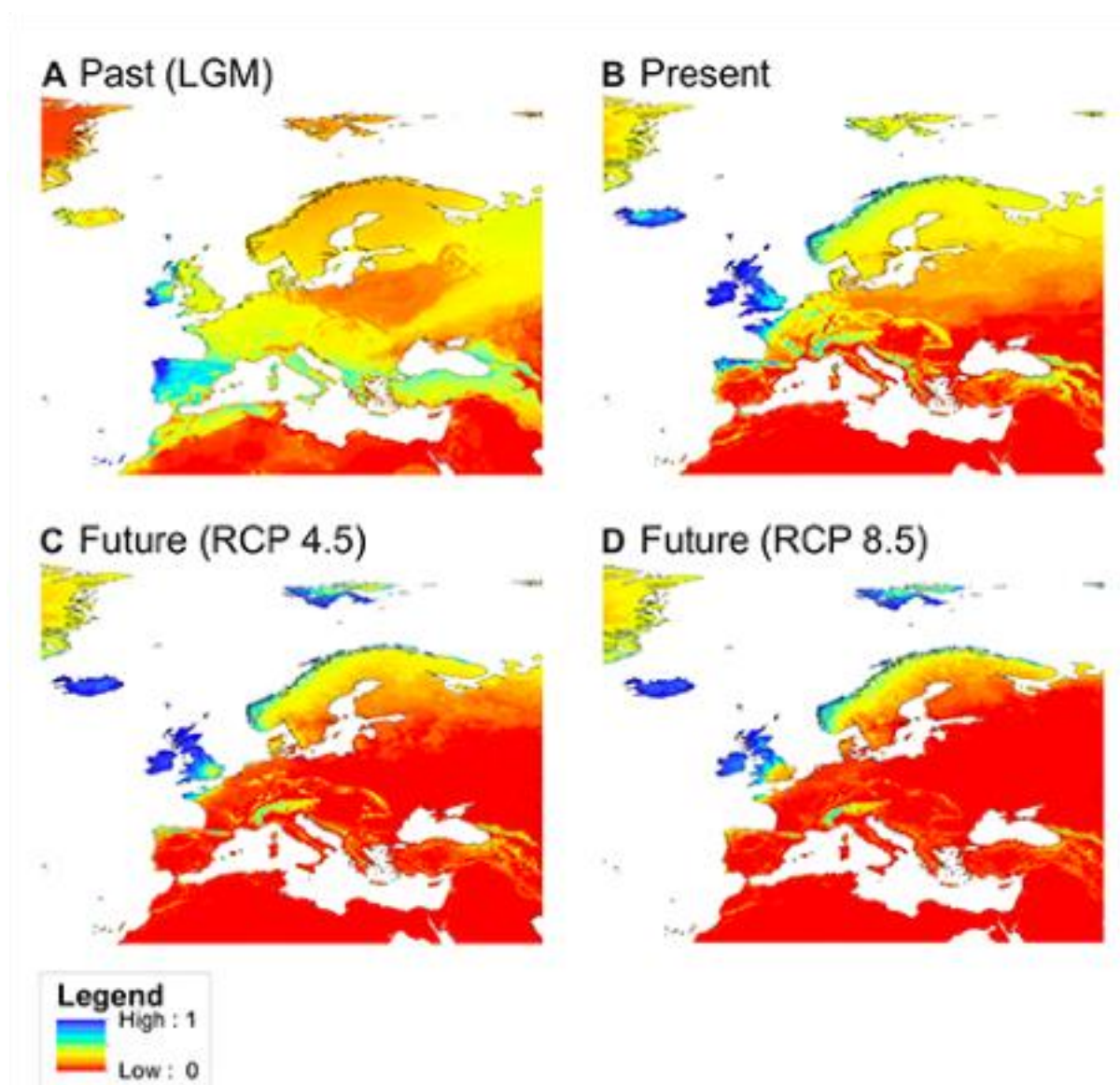


Figure S7. Modelled potential distribution of *Apodera vas* in Europe and Northern Africa for A. Late Glacial Maximum climatic conditions (LGM-PMIP3), B. current climatic conditions, and C. and D. future climatic conditions (CMIP5 prediction for 2016-80, RCP 4.5 and RCP 8.5). “High” and “Low” indicates high and low probability of occurrence, respectively.

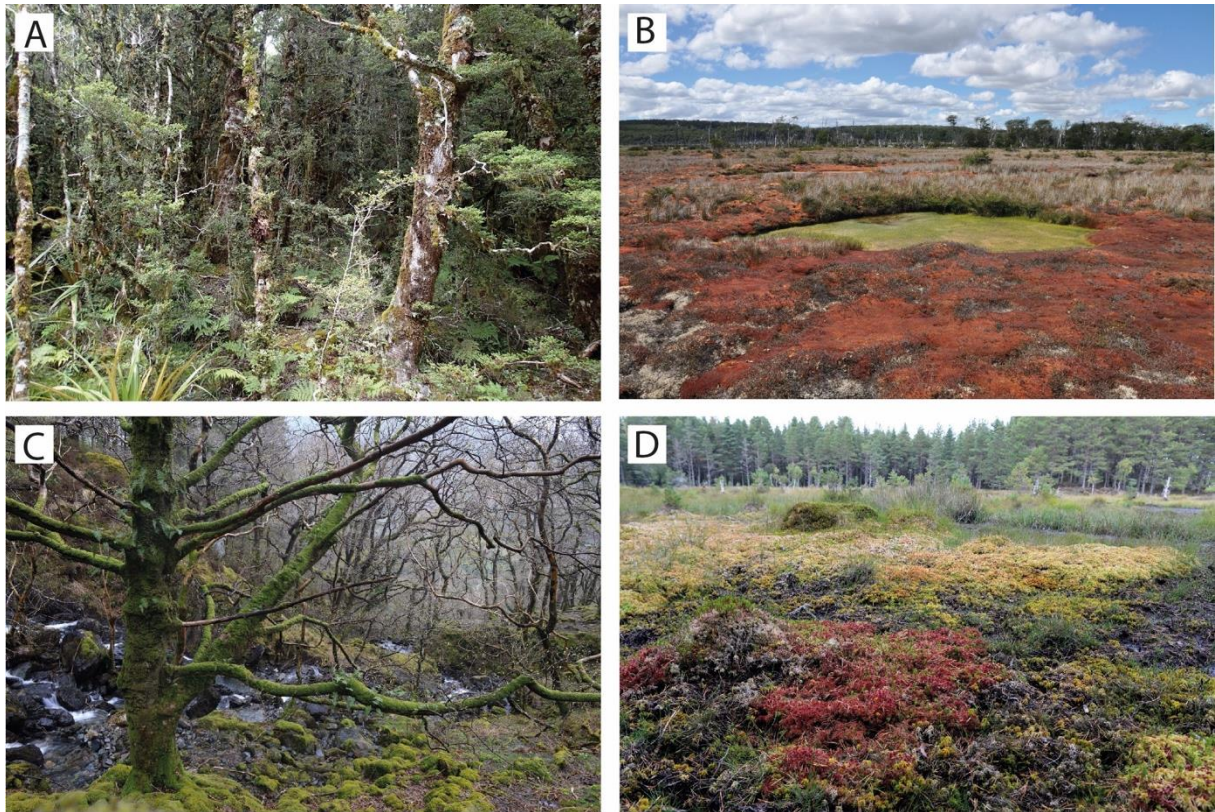


Figure S8. Examples of potentially favourable habitats. A. Temperate rain forest on the South Island of New Zealand along the Old Ghost Road, B. *Sphagnum magellanicum*-dominated peatland in Tierra-del-Fuego, Chile, C. temperate rain forest in Wales, D. *Sphagnum*-dominated peatland in England. *Apodera vas* was present in A & B but absent from C & D. Pictures A & B by Edward A. D. Mitchell, C & D by David M. Wilkinson.

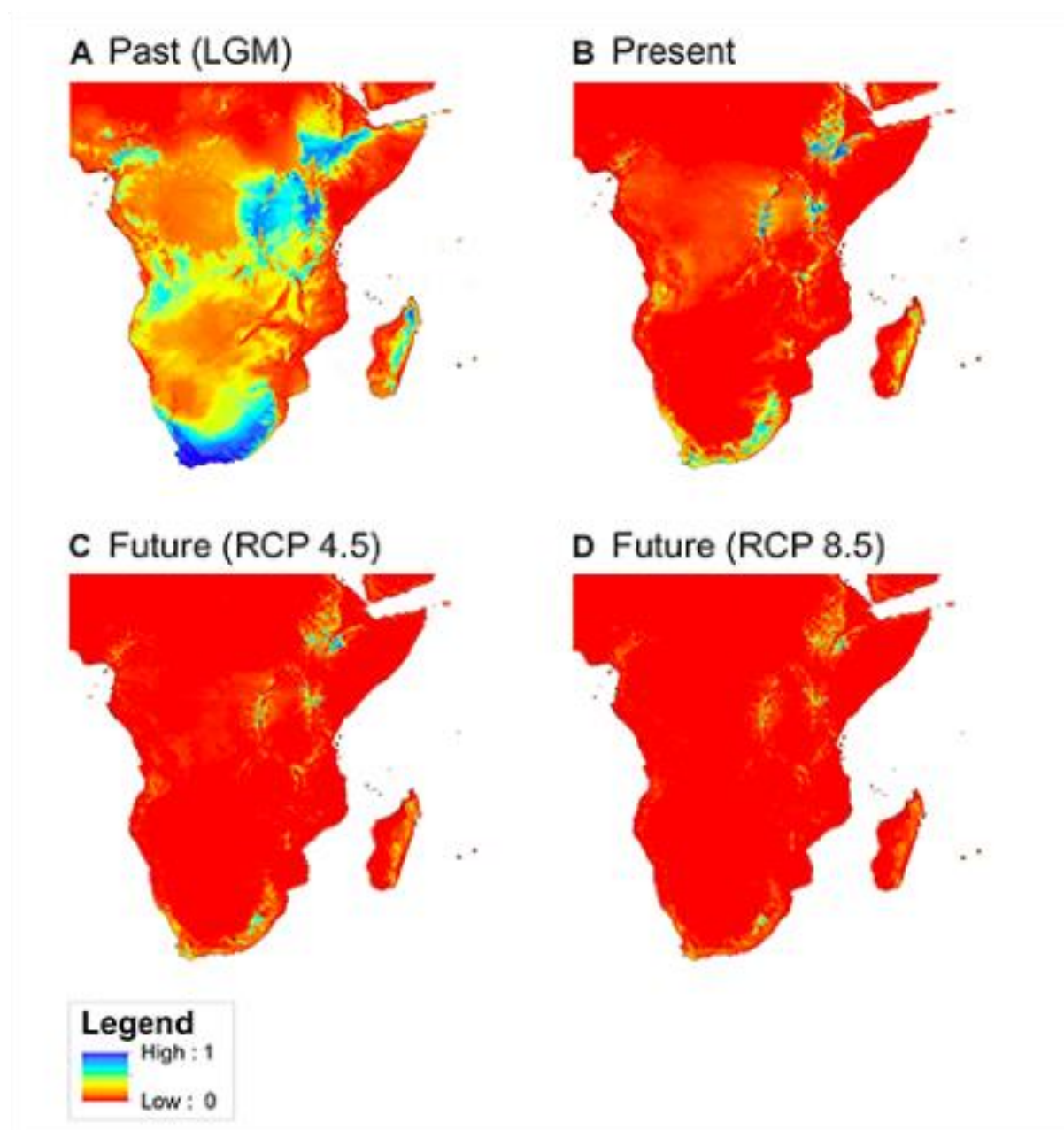


Figure S9. Modelled potential distribution of *Apodera vas* in most of sub-Saharan Africa for A. Late Glacial Maximum climatic conditions (LGM-PMIP3), B. current climatic conditions, and C. and D. future climatic conditions (CMIP5 prediction for 2016-80, RCP 4.5 and RCP 8.5). High and Low indicates high and low probability of occurrence, respectively.

Table S1. List of occurrences of *Apodera vas* used in the model

Country	Latitude (dec. deg.)	Longitude (dec. deg.)	Elevation (m a.s.l.)
Antarctica (South Shetland)	-62.1942	-58.9640	10
Argentina	-54.8330	-68.5666	9
Argentina	-54.8109	-68.3246	38
Argentina	-54.8336	-68.4646	99
Argentina	-54.7463	-67.8258	120
Argentina	-54.7580	-68.1958	131
Argentina	-54.7554	-68.3381	200
Argentina	-43.8978	-71.4237	928
Argentina	-39.5094	-70.3136	870
Argentina	-39.5386	-70.3986	1000
Argentina	-39.5956	-71.1003	1221
Argentina	-39.5058	-71.2558	1420
Argentina	-39.9828	-70.4017	844
Argentina	-39.8056	-70.2694	1018
Argentina	-39.8836	-70.4386	1046
Argentina	-39.8883	-70.4486	1079
Argentina	-39.9008	-70.3844	903
Argentina	-39.9364	-70.3703	855
Argentina	-40.8778	-70.7092	968
Argentina	-40.8836	-70.6769	792
Argentina	-40.1981	-70.9750	1015
Argentina	-40.3047	-70.9261	857
Argentina	-41.2258	-71.7164	1216
Argentina	-41.2342	-71.6497	1053
Argentina	-41.2761	-71.4067	1773
Argentina	-41.2778	-71.4908	1099
Argentina	-41.3275	-71.4869	1185
Argentina	-41.3422	-71.3478	1584
Argentina	-41.5508	-71.5461	863
Argentina	-41.3181	-71.7369	988
Argentina	-42.8581	-71.8961	1561
Argentina	-43.0639	-71.9100	970
Argentina	-43.0653	-71.8994	1335
Argentina	-43.0425	-71.8294	596
Argentina	-43.0008	-71.6794	1120
Argentina	-43.0858	-71.7314	884
Argentina	-44.9347	-71.8581	1546
Argentina	-44.8275	-71.4992	1354
Argentina	-44.6197	-71.0372	860
Argentina	-45.1706	-71.2494	1062

Argentina	-46.0678	-70.3289	647
Argentina	-46.0475	-70.2794	628
Argentina	-45.3261	-70.6575	632
Argentina	-45.5061	-70.2231	525
Argentina	-45.5064	-69.8328	411
Argentina	-45.6231	-69.7711	458
Argentina	-45.7006	-69.8264	468
Argentina	-45.7664	-69.8789	470
Argentina	-45.8972	-69.8767	554
Argentina	-48.8600	-72.6036	252
Argentina	-49.1675	-72.3597	287
Argentina	-49.2022	-72.3281	395
Argentina	-49.8678	-73.0831	976
Argentina	-49.8469	-73.1978	697
Australia (Macquarie Island)	-54.7111	158.8532	5
Australia (Macquarie Island)	-54.4993	158.9051	19
Australia (Tasmania)	-43.0996	146.7155	76
Australia	-34.0924	151.0540	100
Australia (Tasmania)	-42.9114	147.2532	525
Australia (Tasmania)	-42.6811	146.6747	650
Australia	-33.7224	150.3224	850
Australia	-36.3485	148.6012	940
Australia (Tasmania)	-42.8902	147.2313	1120
Australia	-36.7325	146.8103	1300
Australia	-36.9921	147.1614	1609
Bolivia	-16.2514	-68.2432	4480
Bolivia	-16.2494	-68.2309	4510
Bolivia	-16.3739	-68.1354	4550
Bolivia	-16.2598	-68.2163	4570
Bolivia	-16.3816	-68.1344	4600
Bolivia	-16.3408	-68.0394	4650
Bolivia	-16.3649	-68.1345	4730
Bolivia	-16.2869	-68.1255	4740
Bolivia	-16.2982	-68.1714	5020
Brazil	-22.9512	-43.2139	585
Brazil	-29.4528	-50.6047	890
Brazil	-7.8203	-38.0561	1110
Chile	-41.4536	-72.7272	46
Chile	-53.1548	-70.9522	84
Chile	-44.2858	-72.5331	146
Chile	-44.2896	-72.5290	150
Chile	-44.2384	-72.5038	154
Chile	-39.0050	-72.2310	220
Chile	-40.1720	-73.4918	1028
Chile	-39.0050	-72.2310	219

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Chile	-38.2336	-71.3833	1399
Chile	-38.2322	-71.3411	1555
Chile	-38.1294	-71.9683	817
Chile	-38.0828	-72.9281	101
Chile	-38.4961	-72.6364	556
Chile	-39.4875	-72.7372	129
Chile	-38.5231	-72.6594	283
Chile	-38.4625	-72.7953	156
Chile	-38.4850	-72.4017	269
Chile	-38.5631	-72.6106	452
Chile	-38.9375	-71.8933	604
Chile	-38.7317	-71.8453	1066
Chile	-38.9506	-71.9500	380
Chile	-38.9572	-72.7722	82
Chile	-38.8675	-72.9756	108
Chile	-38.5403	-72.6153	420
Chile	-38.6411	-71.7939	1202
Chile	-38.7147	-71.3117	1272
Chile	-38.3106	-71.9206	845
Chile	-39.0386	-72.9108	57
Chile	-39.0211	-72.9714	33
Chile	-39.1100	-72.6322	102
Chile	-39.1575	-72.6242	106
Chile	-39.3303	-72.5514	166
Chile	-39.0614	-72.7419	418
Chile	-39.1156	-72.7642	103
Chile	-39.0403	-71.9542	917
Chile	-39.3731	-72.2544	338
Chile	-39.4097	-72.1408	808
Chile	-39.3989	-72.1625	733
Chile	-39.3778	-72.1381	516
Chile	-39.5867	-71.5867	1218
Chile	-39.5397	-71.6042	1086
Chile	-39.5694	-71.5550	1377
Chile	-39.5917	-71.4783	1275
Chile	-39.4353	-72.7408	84
Chile	-39.4575	-72.5717	351
Chile	-39.0283	-72.3681	267
Chile	-39.0608	-72.3378	339
Chile	-39.2547	-71.9106	549
Chile	-39.2844	-71.8622	306
Chile	-39.3022	-72.2331	297
Chile	-40.4003	-73.1264	43
Chile	-40.4614	-73.0889	81
Chile	-40.8019	-72.4944	123

Chile	-40.5900	-73.2911	82
Chile	-40.6731	-73.1803	57
Chile	-40.8928	-72.2853	123
Chile	-40.9186	-72.2078	1144
Chile	-41.4606	-71.9750	1203
Chile	-41.4661	-71.8436	1951
Chile	-41.1925	-72.6800	53
Chile	-41.2100	-72.5158	73
Chile	-41.2578	-72.1486	339
Chile	-41.2650	-72.0925	1289
Chile	-41.2664	-72.0511	893
Chile	-41.2739	-71.9900	685
Chile	-42.3314	-72.5164	255
Chile	-42.3319	-72.5847	913
Chile	-43.1728	-72.3456	1438
Chile	-43.1458	-72.2742	1397
Chile	-43.0569	-71.9678	1163
Chile	-43.0169	-72.5533	334
Chile	-43.6458	-71.8769	251
Chile	-43.6267	-71.8400	464
Chile	-43.9500	-72.1667	1136
Chile	-43.9133	-72.0483	1315
Chile	-43.9039	-71.9642	1544
Chile	-43.8964	-71.8642	1207
Chile	-44.9839	-72.0889	432
Chile	-44.9874	-72.1370	273
Chile	-45.4289	-72.4382	51
Chile	-46.7467	-73.2175	845
Chile	-46.8536	-73.0772	1407
Chile	-47.4189	-74.0989	512
Chile	-47.5542	-74.1853	47
Chile	-47.6943	-73.1335	21
Chile	-47.7121	-73.1643	17
Chile	-47.7012	-73.0761	24
Chile	-47.7533	-73.7914	676
Chile	-47.8431	-74.1067	614
Chile	-48.6208	-74.2283	495
Chile	-48.1853	-72.7589	292
Chile	-48.1928	-74.0844	635
Chile	-48.3359	-72.4524	523
Chile	-48.3701	-72.4955	416
Chile	-48.3895	-72.5113	376
Chile	-48.4104	-72.5283	338
Chile	-48.9208	-73.0072	778
Chile	-48.8308	-72.8208	569

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Chile	-48.8161	-72.9536	252
Chile	-48.7628	-72.7611	252
Chile	-49.6211	-73.6928	1158
Chile	-49.7211	-73.6575	0
Chile	-50.2318	-74.1790	22
Chile	-50.1747	-74.1050	636
Chile	-50.1592	-74.1003	763
Chile	-50.1275	-74.0164	521
Chile	-50.6858	-73.7967	111
Chile	-50.7039	-73.6525	1230
Chile	-50.7092	-73.6542	1259
Chile	-50.7372	-73.4789	1165
Chile	-50.7497	-73.4244	1806
Chile	-50.7031	-73.5772	698
Chile	-50.6766	-73.4429	48
Chile	-50.6611	-73.6147	474
Chile	-50.6228	-73.4617	732
Chile	-50.6253	-73.2800	1985
Chile	-50.7414	-73.4053	1843
Chile	-51.3488	-74.8227	11
Chile	-51.3489	-74.8206	7
Chile	-51.3531	-74.7881	272
Chile	-51.3244	-74.9231	497
Chile	-51.3294	-74.1592	394
Chile	-51.3214	-74.0905	6
Chile	-51.1611	-73.7781	432
Chile	-51.2789	-74.7886	224
Chile	-51.3442	-74.9731	9
Chile	-52.2200	-73.2294	252
Chile	-52.2089	-73.1917	268
Chile	-52.2058	-73.1733	242
Chile	-52.2303	-73.1894	417
Chile	-52.2281	-73.1628	586
Chile	-52.2519	-73.2733	79
Chile	-52.3264	-73.3564	922
Chile	-52.3569	-73.4089	307
Chile	-52.3003	-72.3814	212
Chile	-52.3294	-72.3897	225
Chile	-52.3542	-72.4356	77
Chile	-52.3572	-72.4075	111
Chile	-52.4042	-72.3531	381
Chile	-52.5825	-72.6775	18
Chile	-52.3331	-71.5197	416
Chile	-52.3439	-71.4619	441
Chile	-53.4975	-71.2572	518

Chile	-53.4914	-71.2644	481
Chile	-53.4897	-71.2819	255
Chile	-53.4989	-71.1861	208
Chile	-53.5017	-71.3831	563
Chile	-53.5108	-71.4572	419
Chile	-53.5025	-71.4911	611
Chile	-53.4958	-71.5072	522
Chile	-53.4750	-71.5014	643
Chile	-53.4800	-71.5658	399
Chile	-53.5422	-71.5828	571
Chile	-53.5547	-71.6222	342
Chile	-53.5197	-71.5958	682
Chile	-54.5108	-69.3942	1173
Chile	-54.5156	-69.4042	1045
Chile	-54.5419	-69.4011	336
Chile	-54.5444	-69.3653	133
Chile	-54.7839	-69.5556	348
Chile	-54.7853	-69.5375	451
Chile	-54.7450	-69.5103	1957
Chile	-54.7492	-69.4564	1376
Chile	-54.7569	-69.4214	1592
Chile	-55.0708	-69.8150	0
Chile	-55.0625	-69.7853	103
Chile	-55.1542	-69.6867	973
Chile	-55.1633	-69.7072	818
Columbia	4.5675	-74.0314	3550
Costa Rica	10.3333	-84.7833	1550
Costa Rica	10.3000	-84.7833	1570
Costa Rica	9.4647	-83.5675	2066
Costa Rica	9.4598	-83.5607	2360
Costa Rica	9.4543	-83.5486	2370
Costa Rica	10.1997	-84.2249	2400
Costa Rica	10.1908	-84.2329	2570
Costa Rica	10.1333	-84.1000	2830
Costa Rica	9.4931	-83.4861	3500
Domimican_Republic	18.0984	-71.1894	1209
Domimican_Republic	19.1092	-70.5242	1244
Domimican_Republic	19.1194	-70.5234	1293
Domimican_Republic	19.1533	-70.5186	1419
Domimican_Republic	19.1419	-70.5193	1423
Domimican_Republic	19.1436	-70.5192	1423
Domimican_Republic	19.1508	-70.5181	1447
Domimican_Republic	19.0400	-70.5181	1450
Domimican_Republic	19.1514	-70.5177	1456
Domimican_Republic	19.1361	-70.5178	1472

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Domimican_Republic	18.9100	-70.6962	1724
Domimican_Republic	18.8786	-70.5912	2188
Democratic Republic of Congo	-10.5349	27.8470	1700
Democratic Republic of Congo	-2.3621	28.6893	2700
Democratic Republic of Congo	-3.4390	29.0088	2851
Democratic Republic of Congo	-1.4911	29.4380	3549
Democratic Republic of Congo	-1.4987	29.4468	4000
El Salvador	14.4145	-89.3512	2140
Ecuador	-4.1000	-78.9667	1000
Ecuador	-4.1160	-78.9686	1072
Ecuador	-3.9733	-79.0764	1911
Ecuador	-3.9733	-79.0764	1911
Ecuador	-3.9803	-79.0838	2021
Ecuador	-4.1000	-79.1667	2884
Ecuador	-4.1160	-78.9686	2884
Ecuador	-4.1097	-79.1798	2920
Fiji	-18.0986	178.4311	5
France (Ile de la Possession)	-46.3925	51.8060	5
France (Kerguelen)	-49.6587	69.0832	5
France (Kerguelen)	-49.3503	69.9038	5
France (Kerguelen)	-49.3198	69.4121	7
France (French Guiana)	5.1153	-52.6498	23
France (Kerguelen)	-49.2552	69.9518	75
France (Kerguelen)	-49.4472	69.8303	100
France (Amsterdam Island)	-37.8651	77.5758	135
France (Guadelupe)	16.0448	-61.6667	1300
France (Martinique)	14.8097	-61.1667	1390
Guatemala	15.2097	-90.2060	1650
Guatemala	15.2000	-90.2167	1750
Guinea	11.0564	-12.4544	864
Indonesia (Sumatra)	-1.6363	101.2639	1400
Indonesia (Java)	-6.7153	107.5830	1400
Indonesia (Java)	-6.7535	106.9862	1700
Indonesia (Java)	-6.7773	106.9745	2400
Indonesia (Java)	-6.7899	106.9833	3000
Ivory_Coast	-8.4004	7.6169	1050
Ivory_Coast	-8.4033	7.6216	1270
Ivory_Coast	-8.3986	7.6240	1300
Ivory_Coast	-8.4007	7.6237	1400
Ivory_Coast	-8.4072	7.6308	1450
Ivory_Coast	-8.4038	7.6254	1580
Kenya	-0.1681	37.3205	4324
Kenya	-0.1443	37.3010	4364
Kenya	-0.1551	37.3327	4450
Kenya	-0.1549	37.2968	4485

Madagascar	-24.1379	47.0826	475
Madagascar	-18.9586	48.4652	950
Madagascar	-24.1349	47.0568	1125
Mexico	18.7256	-97.0769	1770
Mexico	17.2958	-96.3981	2350
Mexico	19.3192	-99.3134	2982
Mexico	17.3803	-96.4475	3050
Mexico	19.0559	-99.3201	2836
New_Zealand (Noth Island)	-35.6097	173.5346	358
New_Zealand (Noth Island)	-35.6097	173.5346	358
New_Zealand (Noth Island)	-35.6151	173.5320	310
New_Zealand (Noth Island)	-35.6151	173.5320	310
New_Zealand (Noth Island)	-36.9528	174.5632	261
New_Zealand (Noth Island)	-37.3900	175.5800	5
New_Zealand (Noth Island)	-38.6298	177.0308	904
New_Zealand (Noth Island)	-38.7929	177.0957	1000
New_Zealand (Noth Island)	-39.1405	175.6333	150
New_Zealand (Noth Island)	-39.2251	174.1228	478
New_Zealand (Noth Island)	-39.2431	174.0877	700
New_Zealand (Noth Island)	-41.1808	174.9627	50
New_Zealand (Noth Island)	-41.3563	174.9852	800
New_Zealand (South Island)	-42.3800	172.4000	860
New_Zealand (South Island)	-42.8286	170.9378	37
New_Zealand (South Island)	-42.8367	171.0047	19
New_Zealand (South Island)	-42.9140	171.5591	900
New_Zealand (South Island)	-42.9213	170.7506	0.4
New_Zealand (South Island)	-43.0275	171.6872	600
New_Zealand (South Island)	-43.1998	170.1911	5
New_Zealand (South Island)	-43.5900	169.6200	6
New_Zealand (South Island)	-43.6033	172.7193	0
New_Zealand (South Island)	-43.6111	169.6154	8
New_Zealand (South Island)	-43.8017	169.1019	23
New_Zealand (Chatham Island)	-43.8233	-176.5284	49
New_Zealand (South Island)	-43.9102	168.9809	27
New_Zealand (South Island)	-44.0335	168.7507	17
New_Zealand (South Island)	-45.3197	167.8202	252
New_Zealand (South Island)	-45.3200	167.8100	320
New_Zealand (South Island)	-45.4300	169.1400	1200
New_Zealand (South Island)	-45.4500	169.2000	1400
New_Zealand (South Island)	-45.5100	167.7100	270
New_Zealand (South Island)	-45.5200	169.2500	875
New_Zealand (South Island)	-45.5502	167.6836	225
New_Zealand (South Island)	-46.3035	168.4417	33
New_Zealand (Campbell Isand)	-52.5597	169.1623	140
New_Zealand (Campbell Isand)	-52.5680	169.1399	56

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Nicaragua	11.8353	-85.9794	1123
Nicaragua	11.8342	-85.9800	1143
Panama	8.9014	-82.6181	2380
Panama	8.9014	-82.6203	2430
Panama	8.8996	-82.6197	2436
Papua New Guinea	-6.1199	145.1266	1500
Papua New Guinea	-7.3341	146.6813	2000
Papua New Guinea	-7.3425	146.6687	2190
Papua New Guinea	-7.3486	146.6743	2300
Papua New Guinea	-5.7881	145.0507	3700
Papua New Guinea	-5.7856	145.0488	3800
Papua New Guinea	-5.7802	145.0298	4455
Philippines	16.3333	120.5500	2150
Philippines	16.3356	120.5609	2250
South_Africa (Marion Island)	-46.8768	37.8572	20
South_Africa (Marion Island)	-46.8833	37.8500	27
South_Africa (Marion Island)	-46.8776	37.8492	30.35
South_Africa (Marion Island)	-46.8766	37.8572	31
South_Africa	-33.6320	19.3117	208
South_Africa	-33.4671	19.6442	460
South_Africa	-33.8835	22.4310	732
Tanzania	-7.8262	35.9261	2196
UK (South Georgia)	-54.3039	-36.4980	5
UK (South Georgia)	-54.2993	-36.4982	50
UK (South Georgia)	-54.2959	-36.4948	30
UK (South Georgia)	-54.2944	-36.5023	75
UK (South Georgia)	-54.2934	-36.4940	10
UK (South Georgia)	-54.2906	-36.5146	85
UK (South Georgia)	-54.2556	-36.4958	85
UK (South Georgia)	-54.2522	-36.4912	15
UK (South Georgia)	-54.1575	-36.7161	3
UK (Falklands)	-51.7007	-57.8515	50
UK (Falklands)	-51.6855	-57.9245	7
UK (Falklands)	-51.5064	-57.7602	15
UK (Falklands)	-51.3511	-60.6894	30
Venezuela	8.9437	-70.7176	3520

Table S2. CHELSA bioclim variables (Karger et al. 2017).

Code	Variable	Unit	Resolution	Database	Reference
bio1	Annual Mean Temperature	°C *10	30 arc sec	CHELSA V1.2	Karger et al., 2017
bio2	Mean Diurnal Range	°C *10	30 arc sec	CHELSA V1.2	Karger et al., 2017
bio3	Isothermality	°C *10	30 arc sec	CHELSA V1.2	Karger et al., 2017
bio4	Temperature Seasonality	°C *10	30 arc sec	CHELSA V1.2	Karger et al., 2017
bio5	Max Temperature of Warmest Month	°C *10	30 arc sec	CHELSA V1.2	Karger et al., 2017
bio6	Min Temperature of Coldest Month	°C *10	30 arc sec	CHELSA V1.2	Karger et al., 2017
bio7	Temperature Annual Range	°C *10	30 arc sec	CHELSA V1.2	Karger et al., 2017
bio8	Mean Temperature of Wettest Quarter	°C *10	30 arc sec	CHELSA V1.2	Karger et al., 2017
bio9	Mean Temperature of Driest Quarter	°C *10	30 arc sec	CHELSA V1.2	Karger et al., 2017
bio10	Mean Temperature of Warmest Quarter	°C *10	30 arc sec	CHELSA V1.2	Karger et al., 2017
bio11	Mean Temperature of Coldest Quarter	°C *10	30 arc sec	CHELSA V1.2	Karger et al., 2017
bio12	Annual Precipitation	kg m ⁻²	30 arc sec	CHELSA V1.2	Karger et al., 2017
bio13	Precipitation of Wettest Month	kg m ⁻²	30 arc sec	CHELSA V1.2	Karger et al., 2017
bio14	Precipitation of Driest Month	kg m ⁻²	30 arc sec	CHELSA V1.2	Karger et al., 2017
bio15	Precipitation Seasonality	kg m ⁻²	30 arc sec	CHELSA V1.2	Karger et al., 2017
bio16	Precipitation of Wettest Quarter	kg m ⁻²	30 arc sec	CHELSA V1.2	Karger et al., 2017
bio17	Precipitation of Driest Quarter	kg m ⁻²	30 arc sec	CHELSA V1.2	Karger et al., 2017
bio18	Precipitation of Warmest Quarter	kg m ⁻²	30 arc sec	CHELSA V1.2	Karger et al., 2017
bio19	Precipitation of Coldest Quarter	kg m ⁻²	30 arc sec	CHELSA V1.2	Karger et al., 2017

Table S3. Evaluation values of modeling and Ensemble modeling steps based on the maximization of the true skill statistics (TSS) and the area under the receiver operating characteristic curve (AUC).

Model evaluation						
	TSS			AUC		
Modeling	Mean TSS for all models			Mean AUC for all models		
	0.77 (sd : 0.11)			0.92 (sd : 0.06)		
	Mean TSS per model			Mean AUC per model		
	GLM	GMB	MAX	GLM	GMB	MAX
	0.79 (sd : 0.05)	0.81 (sd : 0.04)	0.70 (sd : 0.16)	0.94 (sd : 0.02)	0.94 (sd : 0.02)	0.87 (sd : 0.09)
	TSS			AUC		
Ensemble modeling	0.84			0.97		

Table S4. Importance of the five CHELSA bioclim variables selected by PCA and TPI used to model *A. vas climatic niche*. The importance of each variable is given for each of the modelling algorithms GLM (Generalized Linear Model, GBM and MAXENT (Maximum Entropy Method).

Variable importance			
	GLM	GBM	MAX
Bioclim 4 <i>Temperature seasonality</i>	0.09 (sd : 0.06)	0.03 (sd : 0.02)	0.07 (sd : 0.18)
Bioclim 9 <i>Mean temperature of the driest quarter</i>	0.11 (sd : 0.03)	0.006 (sd : 0.003)	0.04 (sd : 0.04)
Bioclim 10 <i>Mean temperature of the warmest quarter</i>	0.98 (sd : 0.02)	0.70 (sd : 0.04)	0.73 (sd : 0.20)
Bioclim 15 <i>Precipitation seasonality</i>	0.05 (sd : 0.02)	0.1 (sd : 0.04)	0.12 (sd : 0.08)
Bioclim 18 <i>Precipitation of the warmest quarter</i>	0.002 (sd : 0.007)	0.006 (sd : 0.003)	0.03 (sd : 0.04)
TPI	0.06 (sd : 0.01)	0.14 (sd : 0.02)	0.15 (sd : 0.21)

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