

One Alga to Rule them All: Unrelated Mixotrophic Testate Amoebae (Amoebozoa, Rhizaria and Stramenopiles) Share the Same Symbiont (Trebouxiophyceae)

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Endosymbiosis is a central and much studied process in the evolution of eukaryotes. While plastid evolution in eukaryotic algae has been extensively studied, much less is known about the evolution of mixotrophy in amoeboid protists, which has been found in three of the five super groups of Eukaryotes. We identified the green endosymbionts in four obligate mixotrophic testate amoeba species belonging to three major eukaryotic clades, *Hyalosphenia papilio* and *Heleopera sphagni* (Amoebozoa: Arcellinida), *Placocista spinosa* (Rhizaria: Euglyphida), and *Archerella flavum* (Stramenopiles: Labyrinthulomycetes) based on *rbcL* (ribulose-1,5-diphosphate carboxylase/oxygenase large subunit) gene sequences. We further investigated whether there were different phylotypes of algal endosymbionts within single *H. papilio* cells and the degree of host-symbiont specificity by amplifying two genes: COI (mitochondrial cytochrome oxidase subunit 1) from the testate amoeba host, and *rbcL* from the endosymbiont. Results show that all studied endosymbionts belong to genus *Chlorella* sensu stricto, closely related to *Paramecium bursaria* *Chlorella* symbionts, some lichen symbionts and also several free-living algae. Most *rbcL* gene sequences derived from symbionts from all testate amoeba species were almost identical (at most 3 silent nucleotides difference out of 780 bp) and were assigned to a new Trebouxiophyceae taxon we named TACS (Testate Amoeba *Chlorella* Symbionts). This “one alga fits all mixotrophic testate amoeba” pattern suggests that photosynthetic symbionts have pre-adaptations to endosymbiosis and colonise diverse hosts from a free-living stage.

Key words: Secondary endosymbiosis; peatlands; *Hyalosphenia papilio*; *rbcL* gene; COI gene; co-evolution; *Chlorella*.

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Introduction

Endosymbiosis with photosynthetic organisms has played a major role in the evolution of life and represents an evolutionary strategy for many eukaryotic organisms to acquire new biochemical and metabolic functions (Nowack and Melkonian 2010; Raven et al. 2009). The most conspicuous endosymbiotic relationships encountered in the domain Eukaryota is the primary endosymbiosis that happened between a phototrophic cyanobacterium and a heterotrophic organism, giving rise to plants, green, red and glaucophyte algae in the eukaryotic supergroup Archaeplastida, and the unusual testate amoeba *Paulinella chromatophora* in the Rhizaria (Keeling 2010; Sagan 1967; Yoon et al. 2006). Further associations between some of these organisms and phagotrophic protists eventually gave rise to secondary and tertiary plastid acquisition in all major eukaryotic supergroups (Archibald 2009; Keeling 2009). These photo-heterotrophic consortia appeared several times independently in the course of evolution of eukaryotes, giving rise the rest of the diverse array of algae found in the modern ecosystems (Johnson 2011). The degree of metabolic integration and dependency of the symbiont within the host that acquired phototrophs varies widely, from the facultative mid-term storage of algae within vacuoles prior to later digestion (Esteban et al. 2010; Johnson 2011) to obligatory symbiosis, that can be sometimes combined with gene transfers from the endosymbiont to the heterotrophic partner's nucleus (Figueroa et al. 2009; Nowack and Melkonian 2010).

A first step in this hetero-phototrophic association (usually referred as mixotrophy) can be found in some species of marine dinoflagellates such as *Noctiluca scintillans* and freshwater ciliates such as *Paramecium bursaria* where the host can switch to heterotrophic nutrition if exposed to prolonged darkness or if the environment lacks symbionts. This association, albeit facultative, increases the growth rate of the host (Stoecker et al. 2009). Furthermore, host selectivity and specificity to symbiont species have been demonstrated experimentally in such facultative symbiosis, for example in *P.bursaria* (Summerer et al. 2007, 2008).

In a further step, these consortia can become obligatory for the host at least, which can no longer survive in the long run without its symbionts; this type of association is commonly encountered in diverse groups of organisms

including corals, lichens, ciliates, planktonic and benthic foraminifera, polycystines and acantharea (Esteban et al. 2006; Stoecker et al. 2009). Overall, these organisms harbour a broad range of algae such as dinoflagellates, diatoms, chlorophytes, chrysophytes, and haptophytes.

Host cells are supposed to acquire their endosymbionts by two different mechanisms 1) vertical transmission (i.e. inherited) when cells divide as observed in ciliates, lichens and some foraminifers which have a predominantly asexual life cycle (Garcia-Cuetos et al. 2005) or 2) acquisition from the surrounding environments with a highly selective recognition mechanism such as in most foraminiferans, Radiozoa and Hexacoralia (Gast and Caron 1996, 2001; Gast et al. 2000; Santos et al. 2004; Zoller and Lutzoni 2003). The difference between these two levels of integration in the host-symbiont relationship is not always clear-cut. Soritid foraminifera can acquire symbionts both maternally and horizontally from the environment (Garcia-Cuetos et al. 2005). When the katablepharid cryptophyte *Hatena arenicola* divides, only one of the resulting cells inherits maternally the single symbiont, while the other one has to acquire it from the environment through a highly selective recognition mechanism (Okamoto and Inouye 2006). Although the host cell still retains the ability of heterotrophic nutrition mode, it was reported that most of these mixotrophic organisms cannot survive for long time if the endosymbiont has been lost or could not be acquired at a certain life stage (Caron et al. 1982; Stoecker et al. 2009).

The most stable type of endosymbiotic association occurs in some species of dinoflagellates that host diatom endosymbiont such as *Kryptoperidinium foliaceum*, and the marine ciliates *Myrionecta rubra* (= *Mesodinium rubrum*), which host cryptomonads. Here both host cell and endosymbiont are highly integrated, with a synchronized cell cycle, and endosymbiotic gene transfer was even reported from the symbiont genome to their host (dinoflagellate) nucleus (Figueroa et al. 2009; Nowack and Melkonian 2010; Stoecker et al. 2009).

Despite the fact that many studies have succeeded in identifying the symbionts and documented the modalities of endosymbiotic associations in protists, the nature of this symbiosis remains unknown for certain groups including testate amoebae. Testate amoebae are a polyphyletic group of free-living unicellular eukaryotes, characterized by the presence of shell that can be agglutinated, proteinaceous, siliceous, or

calcareous (Meisterfeld 2002a, b). They are distributed in three major groups of eukaryotes, Amoebozoa (Arcellinida: Nikolaev et al. 2005), Rhizaria (Euglyphida: Bhattacharya et al. 1995; Cavalier-Smith 1997; Lara et al. 2007) and, as discovered recently, Stramenopiles (Amphitremida: Gomaa et al. 2013). As with other groups of protists, some species do harbour green-colored symbionts, especially those that live in nutrient-poor environments (with the exception of *Diffugia pyriformis* var. *venusta*, a limnetic form that has been found in mesotrophic habitats (Glime 2013; Penard 1902)). Because many testate amoebae are impossible to maintain in culture, there is very little information on the modalities of the symbiosis that they have established with their phototrophic partners.

In this study, we have identified the in hospite symbionts in four mixotrophic testate amoeba species belonging to three different major eukaryotic clades; *Hyalosphenia papilio*, *Heleopera sphagni* (Amoebozoa: Arcellinida) (Nikolaev et al. 2005), *Placocista spinosa* (Rhizaria: Euglyphida) (Bhattacharya et al. 1995; Cavalier-Smith 1997; Cavalier-Smith 1998), and *Archerella flavum* (Labyrinthulomycetes: Stramenopiles) (Gomaa et al. 2013) (Fig. 1) collected from different geographical locations (Table 1). In addition, we focused on the particular case of *H. papilio* to explore more in-depth the modalities of the association by 1) assessing whether there are different phylotypes of algal endosymbionts within single amoeba cells, and 2) assessing whether there was a correlation between host and symbiont phylotypes that would suggest co-evolutionary patterns. For this purpose, we used two molecular markers to resolve the phylogenetic affiliation to a fine taxonomic resolution, the chloroplastic *rbcl* (ribulose-1,5-diphosphate carboxylase/oxygenase large subunit) marker for the endosymbionts and the mitochondrial COI (cytochrome oxidase subunit 1) marker for *H. papilio* (host). The chloroplast-encoded *rbcl* marker has been used to assess the diversity among green algae (i.e. chlorophytes) (Ghosh and Love 2011; Novis et al. 2009) and, more specifically, Trebouxiophyceae (Rindi et al. 2007; Sherwood et al. 2000). This marker was shown to have high universality as well as high genetic variation at the species level (Fucikova et al. 2011). The COI marker is a commonly used DNA barcode for species identification in eukaryotes including arcellinids and euglyphid testate amoebae (Heger et al. 2011, 2013; Kosakyan et al. 2012, 2013).

Results

Phylogenetic Affinity and Molecular Identification of the Endosymbionts

Our phylogenetic data analysis based on *rbcl* gene sequences identified all examined endosymbionts as members of the genus *Chlorella* sensu stricto (class Trebouxiophyceae, family Chlorellaceae) (Fig. 2). Forty-six out of 49 sequences (all the *rbcl* gene sequences obtained from *Heleopera sphagni*, *Placocista spinosa*, *Archerella flavum* and 37 out of 40 *Hyalosphenia papilio*) clustered together in a single clade with a maximum support values including 100% Bootstrap values (BS) and 1.00 Bayesian inference posterior probability (PP). This clade is closely related to other symbiotic Chlorellaceae, such as *Paramecium bursaria* *Chlorella* symbionts, lichen associated-*Trebouxia*, as well as related free-living strains such as *Chlorella variabilis* (also from *P.bursaria*), *C. vulgaris* and *C. sorokiniana*, *C. pyrenoidosa*, and *Auxenochlorella protothecoides* (for the corresponding GenBank accession numbers see Fig. 2). Our newly identified clade comprised, to our knowledge, only testate amoeba endobiotic *Chlorella*; we refer here-after to this clade as the TACS clade for “Testate amoeba *Chlorella* Symbionts” (Fig. 2). All *rbcl* gene sequences from *Heleopera sphagni* (Amoebozoa) and *Placocista spinosa* (Rhizaria) symbionts were strictly identical to each other and to 25 *rbcl* sequences obtained from symbionts of single cells of *Hyalosphenia papilio* (Amoebozoa) (Fig. 2). The three *rbcl* gene sequences of *Archerella flavum* (Stramenopile) symbionts were identical to each other and branch off first from the TACS clade. The genetic diversity among the remaining sequences in this clade (i.e. 12 sequences of *Hyalosphenia papilio* symbionts) was low <0.3% (i.e. 1 to 3 nucleotides out of 780 bp) and mutations were exclusively observed at the third position of the codon (i.e. silent mutations).

The three remaining sequences did not belong to the TACS clade; *H. papilio* symbionts 10/BD-A/6 and 12/BD-B/6 branched with *Paramecium bursaria* symbionts, and diverged from the TACS clade by 2.7% (Fig. 2). The *H. papilio* symbiont 18/LC/10 was more distantly related to the rest of testate amoeba associated *Chlorella* spp., and had 7.3% genetic divergence with TACS (including mutations across all codon positions).

We also determined the number of endosymbiont phylotypes within 24 individual cells of *H. papilio* (from the 40 above-mentioned cells) by cloning the amplified *rbcl* fragment of the algae. The recovered

Table 1. List of sequenced taxa and sampling locations.

Taxa	Sampling site	Country	Coordinates	No. of <i>rbcL</i> clones	GenBank accession number <i>rbcL</i>/COI
1- <i>Hyalosphenia papilio</i> (PR)	Poor fen, small pool, submerged <i>Sphagnum</i> , Praz-Rodet bog	Switzerland	46°34'N 06°10'E	–	(KJ446794/KJ446843)
2- <i>Hyalosphenia papilio</i> (PR)	Poor fen, small pool, submerged <i>Sphagnum</i> , Praz-Rodet bog	Switzerland	46°34'N 06°10'E	6	(KJ446795/KJ446844)
3- <i>Hyalosphenia papilio</i> (PR)	Poor fen, small pool, submerged <i>Sphagnum</i> , Praz-Rodet bog	Switzerland	46°34'N 06°10'E	8	(KJ446796/KJ446845)
4- <i>Hyalosphenia papilio</i> (PR)	Poor fen, small pool, submerged <i>Sphagnum</i> , Praz-Rodet bog	Switzerland	46°34'N 06°10'E	5	(KJ446797/KJ446846)
5- <i>Hyalosphenia papilio</i> (PR)	Poor fen, small pool, submerged <i>Sphagnum</i> , Praz-Rodet bog	Switzerland	46°34'N 06°10'E	–	(KJ446798/KJ446847)
6- <i>Hyalosphenia papilio</i> (PR)	Poor fen, small pool, submerged <i>Sphagnum</i> , Praz-Rodet bog	Switzerland	46°34'N 06°10'E	5	(KJ446799/KJ446848)
7- <i>Hyalosphenia papilio</i> (PR)	Poor fen, small pool, submerged <i>Sphagnum</i> , Praz-Rodet bog	Switzerland	46°34'N 06°10'E	–	(KJ446800/KJ446849)
8- <i>Hyalosphenia papilio</i> (BD-A)	Submerged <i>Sphagnum</i> , Bois-des-lattes bog	Switzerland	46°58'N 06°42'E	8	(KJ446801/KJ446850)
9- <i>Hyalosphenia papilio</i> (BD-A)	Submerged <i>Sphagnum</i> , Bois-des-Lattes bog	Switzerland	46°58'N 06°42'E	–	(KJ446802/KJ446851)
10- <i>Hyalosphenia papilio</i> (BD-A)	Submerged <i>Sphagnum</i> , Bois-des-Lattes bog	Switzerland	46°58'N 06°42'E	6	(KJ446803/KJ446852)

11- <i>Hyalosphenia papilio</i> (BD-A)	Submerged <i>Sphagnum</i> , Bois-des-Lattes bog	Switzerland	46°58'N 06°42'E	5	(KJ446804/KJ446853)
12- <i>Hyalosphenia papilio</i> (BD-B)	Submerged <i>Sphagnum</i> , Bois-des-Lattes bog	Switzerland	46°58'N 06°42'E	6	(KJ446805/KJ446854)
13- <i>Hyalosphenia papilio</i> (BD-B)	Submerged <i>Sphagnum</i> , Bois-des-Lattes bog	Switzerland	46°58'N 06°42'E	5	(KJ446806/KJ446855)
14- <i>Hyalosphenia papilio</i> (BD-B)	Submerged <i>Sphagnum</i> , Bois-des-Lattes bog	Switzerland	46°58'N 06°42'E	—	(KJ446807/KJ446856)
15- <i>Hyalosphenia papilio</i> (BD-B)	Submerged <i>Sphagnum</i> , Bois-des-Lattes bog	Switzerland	46°58'N 06°42'E	6	(KJ446808/KJ446857)
16- <i>Hyalosphenia papilio</i> (BD-B)	Submerged <i>Sphagnum</i> , Bois-des-Lattes bog	Switzerland	46°58'N 06°42'E	—	(KJ446809/KJ446858)
17- <i>Hyalosphenia papilio</i> (LC)	<i>Sphagnum</i> mosses, Le Cachot bog, Jura Mountains	Switzerland	47°05'N 06°04'E	10	(KJ446810/KJ446859)
18- <i>Hyalosphenia papilio</i> (LC)	<i>Sphagnum</i> mosses, Le Cachot bog, Jura Mountains	Switzerland	47°05'N 06°04'E	10	(KJ446811/KJ446860)
19- <i>Hyalosphenia papilio</i> (PZ-A)	Wet <i>Sphagnum</i> , Bezimyanoë bog, 15 Km North- East Penza city	Russia	53°37'N 45°24'E	5	(KJ446812/KJ446861)
20- <i>Hyalosphenia papilio</i> (PZ-A)	Wet <i>Sphagnum</i> , Bezimyanoë bog, 15 Km North- East Penza city	Russia	53°37'N 45°24'E	—	(KJ446813/KJ446862)
21- <i>Hyalosphenia papilio</i> (PZ-A)	Wet <i>Sphagnum</i> , Bezimyanoë bog, 15 Km North- East Penza city	Russia	53°37'N; 45°24'E	5	(KJ446814/KJ446863)

Table 1 (Continued)

Taxa	Sampling site	Country	Coordinates	No. of <i>rbcL</i> clones	GenBank accession number <i>rbcL</i>/COI
22- <i>Hyalosphenia papilio</i> (PZ-A)	Wet <i>Sphagnum</i> , Bezimyanoë bog, 15 Km North- East Penza city	Russia	53°37'N 45°24'E	7	(KJ446815/KJ446864)
23- <i>Hyalosphenia papilio</i> (PZ-B)	Wet <i>Sphagnum</i> , Bezimyanoë bog, 15 Km North- East Penza city	Russia	53°37'N 45°24'E	—	(KJ446816/KJ446865)
24- <i>Hyalosphenia papilio</i> (PZ-B)	Wet <i>Sphagnum</i> , Bezimyanoë bog, 15 Km North- East Penza city	Russia	53°37'N 45°24'E	—	(KJ446817/KJ446866)
25- <i>Hyalosphenia papilio</i> (PZ-B)	Wet <i>Sphagnum</i> , Bezimyanoë bog, 15 Km North- East Penza city	Russia	53°37'N 45°24'E	—	(KJ446818/KJ446867)
26- <i>Hyalosphenia papilio</i> (PZ-B)	Wet <i>Sphagnum</i> , Bezimyanoë bog, 15 Km North- East Penza city	Russia	53°37'N 45°24'E	7	(KJ446819/KJ446868)
27- <i>Hyalosphenia papilio</i> (PZ-B)	Wet <i>Sphagnum</i> , Bezimyanoë bog, 15 Km North- East Penza city	Russia	53°37'N 45°24'E	6	(KJ446820/KJ446869)
28- <i>Hyalosphenia papilio</i> (PZ-B)	Wet <i>Sphagnum</i> , Bezimyanoë bog, 15 Km North- East Penza city	Russia	53°37'N 45°24'E	6	(KJ446821/KJ446870)
29- <i>Hyalosphenia papilio</i> (PZ-B)	Wet <i>Sphagnum</i> , Bezimyanoë bog, 15 Km North- East Penza city	Russia	53°37'N 45°24'E	—	(KJ446822/KJ446871)
30- <i>Hyalosphenia papilio</i> (PZ-B)	Wet <i>Sphagnum</i> , Bezimyanoë bog, 15 Km North- East Penza city	Russia	53°37'N 45°24'E	—	(KJ446823/KJ446872)

31- <i>Hyalosphenia papilio</i> (PZ-B)	Wet <i>Sphagnum</i> , Bezimyanae bog, 15 Km North- East Penza city	Russia	53°37'N 45°24'E	—	(KJ446824/KJ446873)
32- <i>Hyalosphenia papilio</i> (AK-A)	<i>Sphagnum</i> , rich fen near Chena river on side of road, higher topographic position, near fen-bog transition, Alaska	USA	64°51'N 147°24'W	7	(KJ446825/KJ446874)
33- <i>Hyalosphenia papilio</i> (AK-B)	Brown mosses, rich fen on side of road - short vegetation, small hummock, Alaska	USA	61°24'N 143°03'W	—	(KJ446826/KJ446875)
34- <i>Hyalosphenia papilio</i> (AK-C)	<i>Sphagnum</i> , rich fen on side of road, <i>Sphagnum</i> hummock, Alaska	USA	61°24'N 143°03'W	—	(KJ446827/KJ446876)
35- <i>Hyalosphenia papilio</i> (AK-C)	<i>Sphagnum</i> , rich fen on side of road, <i>Sphagnum</i> hummock, Alaska	USA	61°24'N 143°03'W	6	(KJ446828/KJ446877)
36- <i>Hyalosphenia papilio</i> (AK-C)	<i>Sphagnum</i> , rich fen on side of road, <i>Sphagnum</i> hummock, Alaska	USA	61°24'N 143°03'W	—	(KJ446829/KJ446878)
37- <i>Hyalosphenia papilio</i> (AK-C)	<i>Sphagnum</i> , rich fen on side of road, <i>Sphagnum</i> hummock, Alaska	USA	61°24'N 143°03'W	8	(KJ446830/KJ446879)
38- <i>Hyalosphenia papilio</i> (AK-C)	<i>Sphagnum</i> , rich fen on side of road, <i>Sphagnum</i> hummock, Alaska	USA	61°24'N 143°03'W	6	(KJ446831/KJ446880)
39- <i>Hyalosphenia papilio</i> (BC)	Wet <i>Sphagnum</i> , Allan Creek, South central British Columbia	Canada	52°32'N 119°06'W	6	(KJ446832/KJ446881)

Table 1 (Continued)

Taxa	Sampling site	Country	Coordinates	No. of <i>rbcL</i> clones	GenBank accession number <i>rbcL</i> /COI
40- <i>Hyalosphenia papilio</i> (BC)	Wet <i>Sphagnum</i> , Allan Creek, South central British Columbia	Canada	52°32'N 119°06'W	5	(KJ446833/KJ446882)
41- <i>Heleopera sphagni</i> (PR)	Small pool, submerged <i>Sphagnum</i> , Praz-Rodet bog	Switzerland	46°34'N 6°10'E	6	(KJ446834/—)
42- <i>Heleopera sphagni</i> (PR)	Small pool, submerged <i>Sphagnum</i> , Praz-Rodet bog	Switzerland	46°34'N 6°10'E	5	(KJ446835/—)
43- <i>Heleopera sphagni</i> (PR)	Small pool, submerged <i>Sphagnum</i> , Praz-Rodet bog	Switzerland	46°34'N 6°10'E	—	(KJ446836/—)
44- <i>Heleopera sphagni</i> (PR)	Small pool, submerged <i>Sphagnum</i> , Praz-Rodet bog	Switzerland	46°34'N 6°10'E	—	(KJ446837/—)
45- <i>Placocista spinosa</i> (PT)	<i>Sphagnum</i> mosses, poor fen on the side of Pechora River	Russia	61°55'N 57°54'E	7	(KJ446838/—)

46- <i>Placocista spinosa</i> (PT)	<i>Sphagnum</i> mosses, poor fen on the side of Pechora River	Russia	61°55'N 57°54'E	5	(KJ446839/—)
47- <i>Archerella flavum</i> (BC)	Brown submerged mosses, West of Duffey Lake, South central British Columbia	Canada	50°23'N 122°27'W	4	(KJ446840/—)
48- <i>Archerella flavum</i> (BC)	Brown submerged mosses, West of Duffey Lake, South central British Columbia	Canada	50°23'N 122°27'W	7	(KJ446841/—)
49- <i>Archerella flavum</i> (BC)	Brown submerged mosses, West of Duffey Lake, South central British Columbia	Canada	50°23'N, 122°27'W	—	(KJ446842/—)

“—” Indicates that sequences have been obtained from direct PCR.

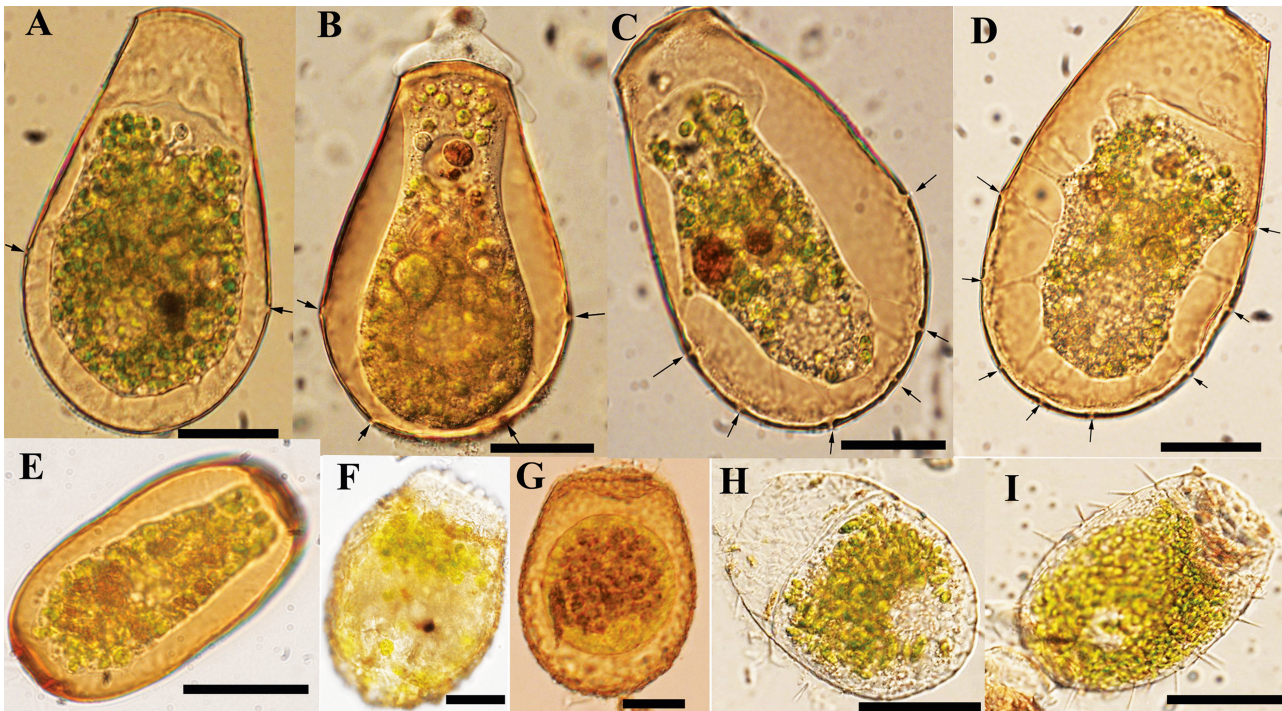


Figure 1. Light microscopy photographs of (A-D) *Hyalosphenia papilio*, arrows indicate the number of pores present at the shell edge, (E) *Archerella flavum*, (F, G) *Heleopera sphagni* and (H, I) *Placcocista spinosa*. For all photographs scale bar is 20 μm .

sequences of the clones (5-10 in each cell) were strictly identical within each host cell.

Genetic Characterization of One Host: *Hyalosphenia papilio*

In contrast to the algal symbiont, we observed clear genetic diversity in the studied host (*Hyalosphenia papilio* morphospecies): the 40 analysed cells formed five different lineages (here designated as A, C, D, G, and J) as described in Heger et al. (2013) (Fig. 3). Inter-clade genetic variability ranged between 2.5 and 7%, and the intra-clade variability was below 1%. We did not detect any morphological feature concomitant with assignment to one or another clade. For example, the number of pores along the shell margin (Fig. 1, varied independently from genetic affiliation. However, the phylogenetic tree suggested a possible pattern of geographical origin (Fig. 3).

Discussion

Our results based on the chloroplast-encoded *rbcL* gene sequence identified the in hospite symbionts of four taxonomically distantly related mixotrophic

testate amoeba taxa (*Hyalosphenia papilio*, *Heleopera sphagni*, *Placcocista spinosa* and *Archerella flavum*) (Fig. 1) as members of one single genus, *Chlorella* sensu stricto. Our sequences are therefore included within a larger clade containing many endosymbiotic forms such as *Paramecium bur-saria* and lichen symbionts, as well as free-living forms, such as *Chlorella variabilis*, *C. vulgaris*, *C. pyrenoidosa* and *Auxenochlorella protothecoides* (Fig. 2). *Chlorella* belongs to class Trebouxiophyceae, which contains most known green algal endosymbionts, living in lichens, unicellular eukaryotes (e.g. ciliates, foraminifera etc.), plants (e.g. *Ginkgo*), animals (e.g. cnidarians, mussels, flat-worms, etc.), and even parasites such as some *Coccomyxa* species (Lewis and Muller-Parker 2004; Pröschold et al. 2011; Rodríguez et al. 2008; Trémouillaux-Guiller and Huss 2007).

Almost all symbiont's *rbcL* sequences fall within the TACS (Testate Amoeba *Chlorella* Symbionts) clade (Fig. 2). Diversity within TACS (0.3%) is far below the level of genetic divergence usually observed between plant species (i.e. a 1-2% threshold is generally accepted for specific discrimination in higher plants (CBOL Plant Working Group 2009; Burgess et al. 2011)). This suggests that TACS actually correspond to a single

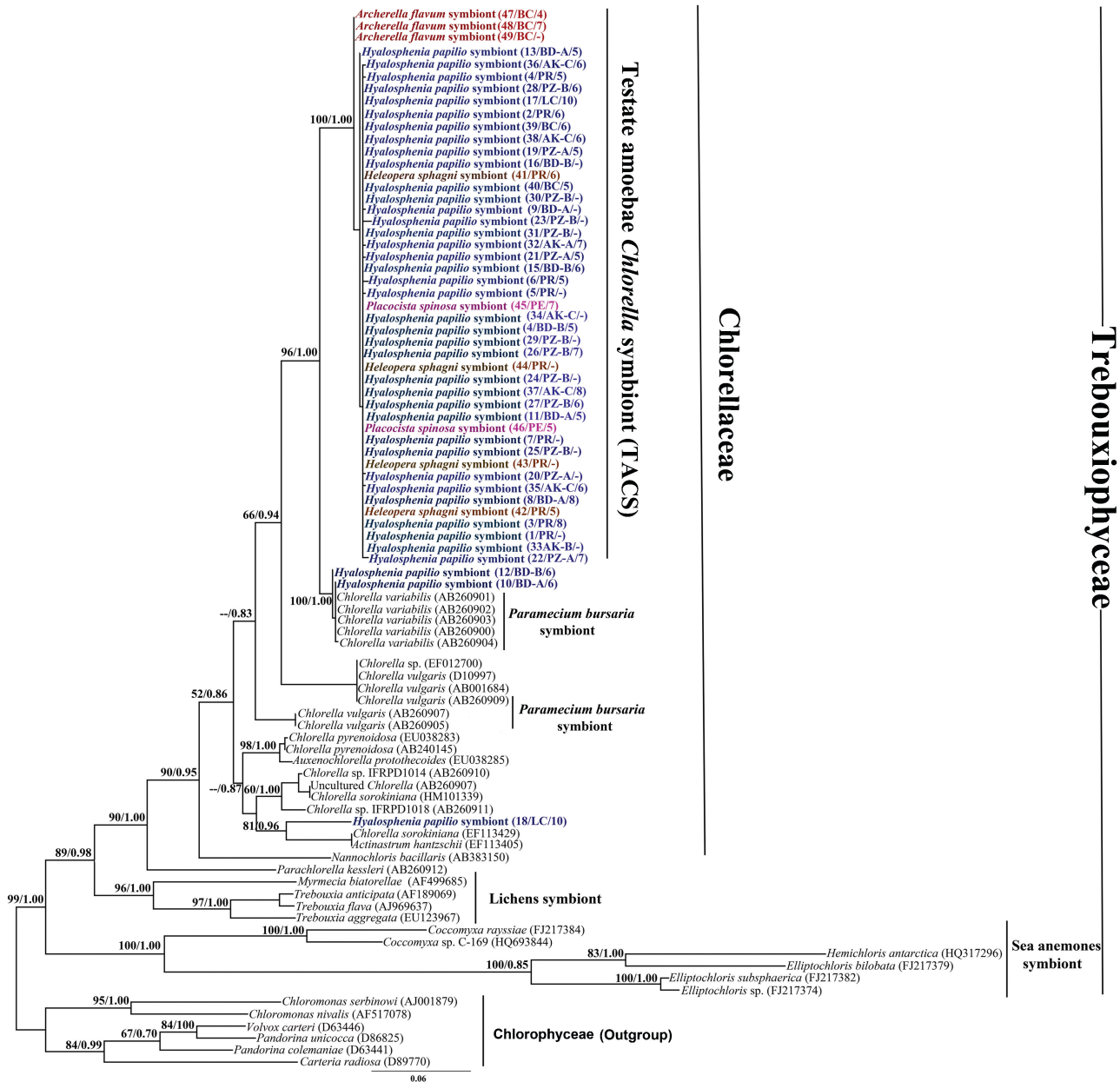


Figure 2. Molecular phylogenetic tree inferred from both maximum likelihood and Bayesian analysis based on the large subunit of the ribulose-bisphosphate carboxylase (*rbcL*) gene sequences obtained from the endosymbionts of four mixotrophic testate amoebae species *Hyalosphenia papilio* (blue), *Archerella flavum* (red), *Heleopera sphagni* (brown) and *Placocista spinosa* (violet). For each sequence we indicated the (number of sample / geographical origin - code of the *Sphagnum* samples where applicable / number of *rbcL* clones). The tree illustrating the phylogenetic position of the Testate Amoeba *Chlorella* Symbionts (TACS) belong to class Trebouxiophyceae. Numbers at nodes indicate the bootstrap values / posterior probabilities. Only values above 50/0.50 are shown. The tree was rooted with the group of Ulvophyceae. The scale bar indicates 0.06% sequence divergence.

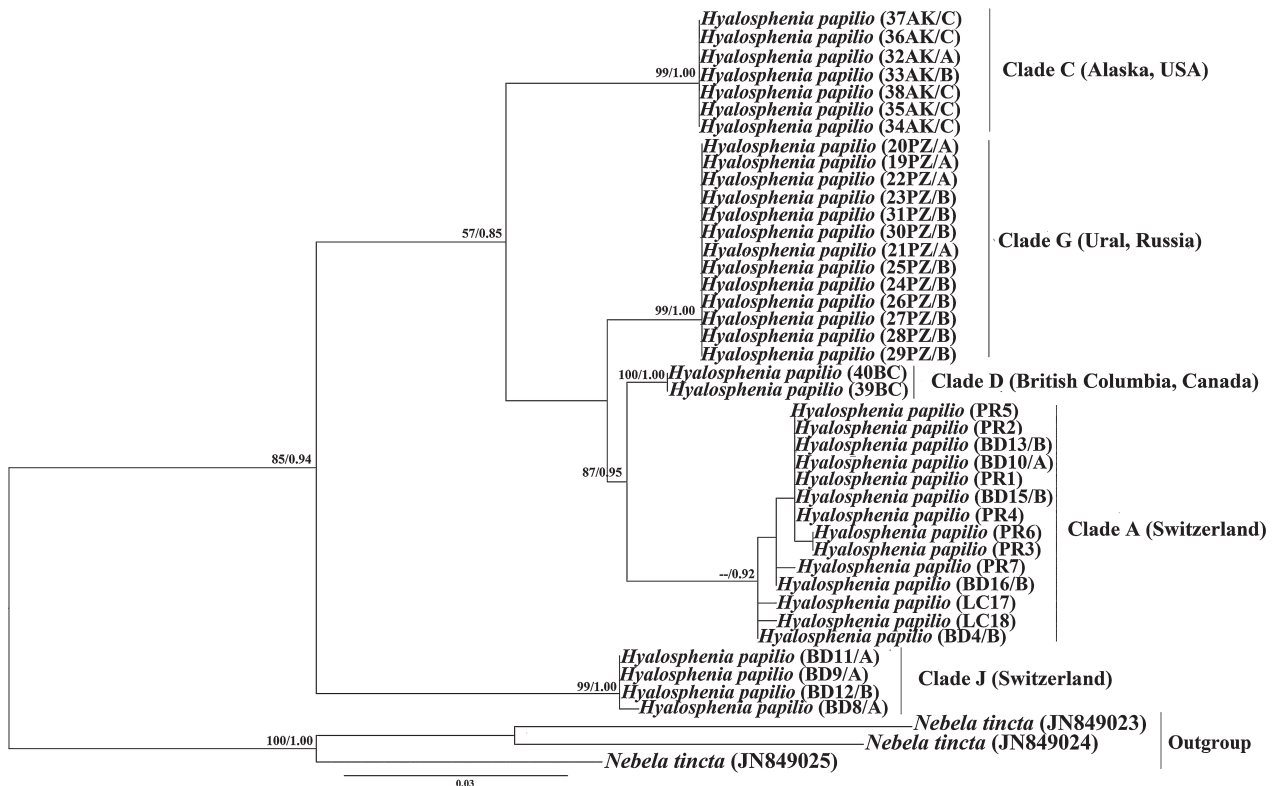


Figure 3. Molecular phylogenetic tree inferred from both maximum likelihood and Bayesian analysis based on mitochondrial cytochrome oxidase first subunit (COI) gene sequences obtained from 40 individual cells of *Hyalosphenia papilio*. The numbers along the branches represent respectively the bootstraps and the posterior probabilities. Only values above 50/0.50 are shown. The tree was rooted with the outgroup *Nebela tinctoria*. The scale bar indicates 0.03% sequence divergence.

species. In contrast, testate amoebae hosts belong to three different major eukaryotic clades (Amoebozoa, Rhizaria and Stramenopiles) (Bhattacharya et al. 1995; Gomaa et al. 2013; Nikolaev et al. 2005) whose roots dive deeply into the origins of the domain. The likelihood that this pattern results from a single endosymbiosis which would have taken place in the common ancestor of these three clades, close to the base of the eukaryotic tree, is highly unlikely because traces of this event would have been detectable in most present day eukaryotic taxa. The most likely explanation that is suggested by our data is that symbiosis occurred relatively recently and independently in the different lineages in species sharing the same lifestyle and evolutionary pressures. Indeed, mixotrophy is favoured in nutrient-depleted environments such as peat bogs (Gerea et al., 2013; Johnson 2011). Not only does it constitute a “starvation insurance” for the host, allowing it to survive periods of low food availability in light conditions and outcompete

heterotrophs (Rothhaupt 1996), it also allows it to exploit prey sources beyond thresholds allowing sustainable growth of potential competitor (Tittel et al. 2003). However, to clearly determine whether or not the TACS clade is exclusive to mixotrophic testate amoebae, further research ideally targeting multiple genetic markers and a broader taxonomic sampling particularly from different mixotrophic organisms would be required.

Furthermore, in contrast to the low diversity observed in the algal symbionts derived from different geographical locations, we found a large genetic diversity within the amoeba *H. papilio* at the COI level. This is consistent with previous results on *H. papilio* and other Hyalospheniidae (Heger et al. 2013), and has been interpreted as cryptic and/or intra-species genetic diversity. Indeed, the *H. papilio* sequences branch in five different groups that can be discriminated by their geographic origin, suggesting possible allopatric speciation events. This pattern clearly differs from the *rbcl* data; most

rbcL sequences from the symbionts belong to the same (TACS) group, regardless of their geographic origin. Some *rbcL* sequences have been obtained from cells collected in sites as distant as British Columbia, Switzerland, Alaska and the Ural, and are indeed exactly identical. Although COI and *rbcL* have different evolutionary histories (and possible different evolutionary rates), the almost total lack of diversity within the TACS group corroborates further the hypothesis that these organisms evolved independently from their hosts.

The lack of evidence for co-evolutionary patterns in turn suggests the existence of a pool of symbionts in the bog environment that are used by the amoeba hosts from all phylogenetic origins. This implies that the algal symbionts probably have an independent life-stage, as in the case of *Chlorella variabilis*, which can live independently from *Paramecium bursaria* (Blanc et al. 2010; Summerer et al. 2007). Thus, the symbionts do not appear to be strictly “maternally” transferred along the amoeba lineages, even within individual amoeba species. Symbionts, in turn, belong to a limited number of species, which suggests that only those photosynthetic organisms that have pre-adaptations to intra-vacuolar life will be kept. Indeed, the nuclear genome of *C. variabilis* contains genes coding for many protein involved in protein-protein interaction and adhesion domains, a trait that appears commonly in endosymbiotic eukaryotes and bacteria (Blanc et al. 2010) We show here that *C. variabilis* can also live within *H. papilio* (as exemplified by the sequences 10/BD-A/6 and 12/BD-B/6, Table 1). In addition, a recent experimental study showed that the digestive vacuoles of *Paramecium bursaria* have the ability to recognize and differentiate symbiotic algae from other ingested food particles based on their size and shape, allowing the *Chlorella* symbiont to escape from the digestive vacuole by budding from the vacuole membrane (Kodama and Fujishima 2012). The ability of *Chlorohydra* and some marine invertebrates to recognize symbiotic algae is believed to depend on algal cell wall associated proteins / proteo-glycans (Huss et al. 1993; Robert 1989). It is therefore most likely that the TACS clade members also have pre-adaptations for endosymbiotic life; a pre-adaptation of testate amoebae symbionts would agree with Law’s (1985) hypothesis that “*through natural selection genotypes of symbionts are produced that are so accommodating they could be transferred even among unrelated hosts*” (Piercey-Normore and Depriest 2001). It is probable that TACS have similar adaptations to

intracellular life as *C. variabilis*. Furthermore, the existence of the distantly related strain 18/LC/10 (Table 1) suggests that other Trebouxiophyceae strains can bear similar adaptations. It cannot be ruled out however that these rare cases correspond to undigested prey and not to genuine symbionts.

In contrast to *Paramecium bursaria*, *H. papilio* has never been observed alive without endosymbionts since its description over 130 years ago (Leidy 1879). Although *H. papilio* was shown to prey on various microorganisms (microalgae, protozoa, micro-metazoa and fungi) (Jassey et al. 2012), it appears not to survive without its algal symbionts. The situation is likely to be similar with some other mixotrophic testate amoebae, such as *Archerella flavum*. Although stable isotopic signals suggest that *A. flavum* feeds on bacteria (Jassey et al. 2013), it seems that it is not able to survive for long periods (i.e. three months in Schönborn’s in-situ shading experiment) in the absence of light (Schönborn 1965). Half-digested *Chlorella* cells were observed in the cytoplasm of *A. flavum*, suggesting that it uses its algae both as food and as photobionts (Bonnet et al. 1981). In the process of association between a phototrophic symbiont and its heterotrophic host, the mixotrophic testate amoebae have reached the stage where they rely on their symbionts for survival. This close and obligate association contrasts with the symbionts of *Paramecium bursaria*. Such associations can be considered as an intermediate step of integration possibly announcing a specialization of the symbiont for intracellular life, gene transfers to the host and further processes of “algal enslavement” as observed in chlorarachniophytes and cryptophytes (Keeling 2009; Lane and Archibald 2008).

Methods

Samples collections and documentation: Four species of mixotrophic testate amoebae (*Hyalosphenia papilio*, *Heleopera sphagni*, *Placocista spinosa* and *Archerella flavum*) (Fig. 1) were collected from their natural environments in *Sphagnum* peatlands (Table 1). Testate amoebae were isolated from *Sphagnum* mosses through serial filtrations, then washed several times with ddH₂O and sorted using a fine capillary pipette under an inverted microscope. We prepared independent extractions; two from *Placocista spinosa*, four from *Heleopera sphagni* and three from *Archerella flavum*, each extractions of *Heleopera sphagni* and *Placocista spinosa* contained from 4 to 10 different cells, and for *Archerella flavum* from 50 to 70 cells. We used single cells for *Hyalosphenia papilio* in forty different extractions.

DNA extraction, amplification, cloning and sequencing: DNA was extracted using guanidine thiocyanate protocol (Chomczynski and Sacchi 1987). The extracted DNA was

pelleted, and re-suspended in 15 μ L of Trisbuffer (pH 8.5). The *rbcL* gene was amplified in two steps, using general green algae primers that were designed based on sequences retrieved from GenBank. A first amplification was conducted with primers RBF1 (CGGGCAGAKTGCA) and RB800r (TGTRAAACCACCWGTTAAG), and the second amplification with RBF2 (CTCCACAACTGAACTARAG) and RB800r. The temperature profile for both reactions consisted of 5 minutes at 95 °C, followed by 45 cycles of 1 min at 94 °C, 1 min at 55 °C and 2 min at 72 °C, with a final elongation of 10 min at 72 °C. For *Hyalosphenia papilio*, we used half of the re-suspended DNA from the single cell for *rbcL* amplification (symbionts) as detailed previously and half for mitochondrial cytochrome oxidase sub-unit 1 (COI) of the host. The COI fragments were amplified in two steps. The first amplification was performed using uni-versal COI primers LCO and HCO designed by Folmer et al. (1994) following the original protocol. The obtained product served as template for the second amplification using *H. papilio*-specific primers HPCOIF (GTTATTGTTACTGCTCATGCC) and HPCOIR (ATACAAAATAGGATCACCTCCACC) in a total volume of 30 μ L with amplification profile consisting of (5 minutes at 95 °C followed by 40 cycles 15 sec at 94 °C, 15 sec at 55 °C and 1 min 30 sec at 72 °C with a final elongation of 10 min at 72 °C). Nineteen out of 49 *rbcL* products were sequenced directly, while the remaining 30 *rbcL* products (24 out of 40 from *H. papilio*, 2 from *Placocista spinosa*, 2 out of 4 from *Heleopera sphagni* and 2 out of 3 from *Archerella flavum*) (Table 1) were cloned into pCR2.1 Topo TA cloning vector (Invitrogen) and transformed into *E. coli* TOP10' One Shot cells (Invitro-gen) according to the manufacturer's instructions. Clone inserts were amplified with vector T7 (5'-TAATACGACTCACTATAGG-3'), SP6 (5'-GATTTAGGTGACACTATAG-3') Primers (Promega, Switzerland). From five to ten clones were purified with the NucleoFasts 96 PCR Clean Up kit from Macherey-Nagel (Düren, Germany) and sequenced with an ABI PRISM 3700 DNA Analyzer (PE Biosystems, Genève, Switzerland) using a BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (PE Biosystems).

Alignment and phylogenetic analysis: Both COI and *rbcL* sequences were aligned manually using the BioEdit software (Hall 1999) together with sequences retrieved from GenBank that are highly similar to our obtained sequences. Our alignments had respectively 480 bp for COI and 780 by for *rbcL*, and we used respectively members of the *Nebela tincta-collaris-bohemica* complex (Kosakyan et al. 2012) as the outgroup for the COI alignment and members of Ulvophyceae as the outgroup for the *rbcL* gene alignment.

The maximum likelihood tree was built using RAxML version 7.2.8 algorithm (Stamatakis et al. 2005) as proposed on the Black Box portal (<http://phylobench.vital-it.ch/raxml-bb/>) using the GTR+ Γ +I model. The reliability of internal nodes was estimated by bootstrapping (1000 replicates).

The obtained tree was compared to a phylogeny generated through Bayesian analysis using the software MrBayes v. 3.1.2 (Huelsenbeck and Ronquist 2001). We performed two simultaneous MCMC chains, and 500,000 generations. The generations were added until standard deviation of split frequencies fell below 0.01 according to the manual of MrBayes 3.1. For every 100th generation, the tree with the best likelihood score was saved, resulting in 5000 trees. The burn in value was set to 25%. Trees were viewed using FigTree (a program distributed as part of the BEAST package <http://tree.bio.ed.ac.uk/software/figtree/>). The divergences between sequences were calculated using the program BioEdit software (Hall 1999).

Acknowledgement

This work was funded by Science and Technology Cooperation Program Switzerland – Russia grant IZLR Z3_128338, Swiss NSF grants n° PZ00P2_122042 (Ambizione fellowship to E. Lara), IB73A0-111064/1 (SCOPES), 205321-109709/1 and 205321-109709/2 and PA00P3 145374. We also would like to thank Professor Jan Pawlowski for his insightful discussion during this project.

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