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Dispersal limitations and historical factors determine the biogeography of specialized terrestrial protists

David Singer[1,2] | Edward A. D. Mitchell[1,3] | Richard J. Payne[4] | Quentin Blandenier[1,5] | Clément Duckert[1] | Leonardo D. Fernández[1,6] | Bertrand Fournier[7] | Cristián E. Hernández[8] | Gustaf Granath[9] | Håkan Rydin[9] | Luca Bragazza[10,11,12] | Natalia G. Koronatova[13] | Irina Goia[14] | Lorna I. Harris[15] | Katarzyna Kajukało[16] | Anush Kosakyan[17] | Mariusz Lamentowicz[16] | Natalia P. Kosykh[13] | Kai Vellak[18] | Enrique Lara[1,5]

[1]Laboratory of Soil Biodiversity, Institute of Biology, University of Neuchâtel, Neuchâtel, Switzerland

[2]Department of Zoology, Institute of Biosciences, University of São Paulo, São Paulo, Brazil

[3]Jardin Botanique de Neuchâtel, Neuchâtel, Switzerland

[4]Environment, University of York, York, UK

[5]Real Jardín Botánico, CSIC, Madrid, Spain

[6]Centro de Investigación en Recursos Naturales y Sustentabilidad (CIRENYS), Universidad Bernardo O'Higgins, Santiago, Chile

[7]Community and Quantitative Ecology Laboratory, Department of Biology, Concordia University, Montreal, QC, Canada

[8]Facultad de Ciencias Naturales y Oceanográficas, Departamento de Zoología, Universidad de Concepción, Barrio Universitario de Concepción, Chile

[9]Department of Ecology and Genetics, Evolutionary Biology Centre, Uppsala University, Uppsala, Sweden

[10]WSL Swiss Federal Institute for Forest, Snow and Landscape Research, Lausanne, Switzerland

[11]Laboratory of Ecological Systems (ECOS), Ecole Polytechnique Féderale de Lausanne (EPFL), School of Architecture, Civil and Environmental Engineering (ENAC), Lausanne, Switzerland

[12]Department of Life Science and Biotechnologies, University of Ferrara, Ferrara, Italy

[13]Laboratory of Biogeocenology, Institute of Soil Science and Agrochemistry, Siberian Branch of Russian Academy of Sciences, Novosibirsk, Russia

[14]Department of Taxonomy and Ecology, Faculty of Biology and Geology, Babeș-Bolyai University, Cluj-Napoca, Romania

[15]School of Geography and Earth Sciences, McMaster University, Hamilton, ON, Canada

[16]Laboratory of Wetland Ecology and Monitoring, Faculty of Geographical and Geological Sciences and Department of Biogeography and Paleoecology, Adam Mickiewicz University, Poznań, Poland

[17]Institute of Parasitology, Biology Center, Czech Academy of Sciences, České Budějovice, Czech Republic

[18]Institute of Ecology and Earth Sciences, Natural History Museum, University of Tartu, Tartu, Estonia

Correspondence
David Singer, Department of Zoology, Institute of Biosciences, São Paulo, Brazil.
Email: david.singer.bio@outlook.com

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Abstract

Recent studies show that soil eukaryotic diversity is immense and dominated by micro-organisms. However, it is unclear to what extent the processes that shape the distribution of diversity in plants and animals also apply to micro-organisms. Major diversification events in multicellular organisms have often been attributed to long-term climatic and geological processes, but the impact of such processes on protist diversity has received much less attention as their distribution has often been

We would like to dedicate this paper to co-author Richard Payne. Richard was a member of a group of 8 climbers caught in an avalanche in the Himalayas at the end of May.

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believed to be largely cosmopolitan. Here, we quantified phylogeographical patterns in *Hyalosphenia papilio*, a large testate amoeba restricted to Holarctic *Sphagnum*-dominated peatlands, to test if the current distribution of its genetic diversity can be explained by historical factors or by the current distribution of suitable habitats. Phylogenetic diversity was higher in Western North America, corresponding to the inferred geographical origin of the *H. papilio* complex, and was lower in Eurasia despite extensive suitable habitats. These results suggest that patterns of phylogenetic diversity and distribution can be explained by the history of Holarctic *Sphagnum* peatland range expansions and contractions in response to Quaternary glaciations that promoted cladogenetic range evolution, rather than the contemporary distribution of suitable habitats. Species distributions were positively correlated with climatic niche breadth, suggesting that climatic tolerance is key to dispersal ability in *H. papilio*. This implies that, at least for large and specialized terrestrial micro-organisms, propagule dispersal is slow enough that historical processes may contribute to their diversification and phylogeographical patterns and may partly explain their very high overall diversity.

KEYWORDS
distribution, Holarctic, *Hyalosphenia papilio*, phylogeography, protists, *Sphagnum* peatland

1 | INTRODUCTION

The question of whether the same rules structure the diversity of all eukaryotes, micro- and macroscopic alike, has been the subject of heated debate since the early 2000s. The classical paradigm is that "everything is everywhere, but, the environment selects" (Baas-Becking, 1934). Defenders of this paradigm have argued that geographical barriers are ineffective in preventing the dispersal of microbes (Fenchel, 2005; Finlay, 1998). Other researchers, while accepting that some microbes do indeed occur worldwide, have argued that others are clearly restricted to certain regions: the "moderate endemicity" model (Foissner, 1999). This argument is based particularly on a limited number of so-called "biogeographical flagship species," with conspicuous morphology.

The application of barcoding to protists has brought new nuance to the debate (Pawlowski et al., 2012). Single-cell DNA barcoding studies (Pawlowski et al., 2012) of individual "morphospecies" are now revealing the existence of numerous "cryptic" biological species (Singer et al., 2018). Barcoding studies have recently demonstrated geographically limited distributions in soil (Ryšánek, Hrčková, & Škaloud, 2015), freshwater (Škaloud et al., 2019) and marine organisms (Santoferrara, Rubin, & Mcmanus, 2018), although cases of cosmopolitan distribution have also been reported (Geisen, Fiore-Donno, Walochnik, & Bonkowski, 2014; Šlapeta, López-García, & Moreira, 2005). The development of microbial phylogeography, combining biogeography and molecular phylogeny, in turn has allowed the evaluation of possible drivers of diversity patterns, and comparison with those known to drive plant and animal diversity (Martiny et al., 2006).

Among terrestrial micro-organisms, testate amoebae are particularly useful models for phylogeographical studies. Testate amoebae are conspicuous and relatively easy to identify and are also large enough to be isolated individually for DNA barcoding. Many species have narrow ecological tolerances and thus can only colonize specific, often geographically discontinuous habitats (Singer et al., 2018). Furthermore, some species have well-documented distributions and ecology. A good example is *Hyalosphenia papilio*, a widely recorded and morphologically distinctive testate amoeba taxon (Figure 1). Of particular interest for phylogeographical studies is that, based on single-cell barcoding and the variable molecular marker cytochrome *c* oxidase subunit I (*COI*), *H. papilio* is known to represent a species complex of at least 12 lineages (Heger, Mitchell, & Leander, 2013).

Hyalosphenia papilio is found exclusively in Holarctic *Sphagnum*-dominated peatlands (Amesbury et al., 2018, 2016) and it is known to be absent from similar southern hemisphere sites despite extensive study (Fernández, Lara, & Mitchell, 2015; Smith, Bobrov, & Lara, 2007). *Sphagnum*-dominated peatlands are comparatively young ecosystems, dating back to the expansion of boreal and subarctic environments near the Pliocene (Shaw et al., 2010). *Sphagnum* is an ecosystem engineer that modifies habitats by increasing soil wetness and decreasing pH and available nutrient content, producing decay-resistant litter rich in phenols and sphagnan (van Breemen, 1995) and hosting very distinctive prokaryotic, algal and fungal communities (Kostka et al., 2016; Mutinová, Neustupa, Bevilacqua, & Terlizzi, 2016). Thus, *Sphagnum* represents a highly selective habitat for macro- and micro-organisms. This explains why *Sphagnum*-dominated ecosystems are species-poor and these same factors are likely to also drive evolutionary adaptations in testate amoebae (Kosakyan

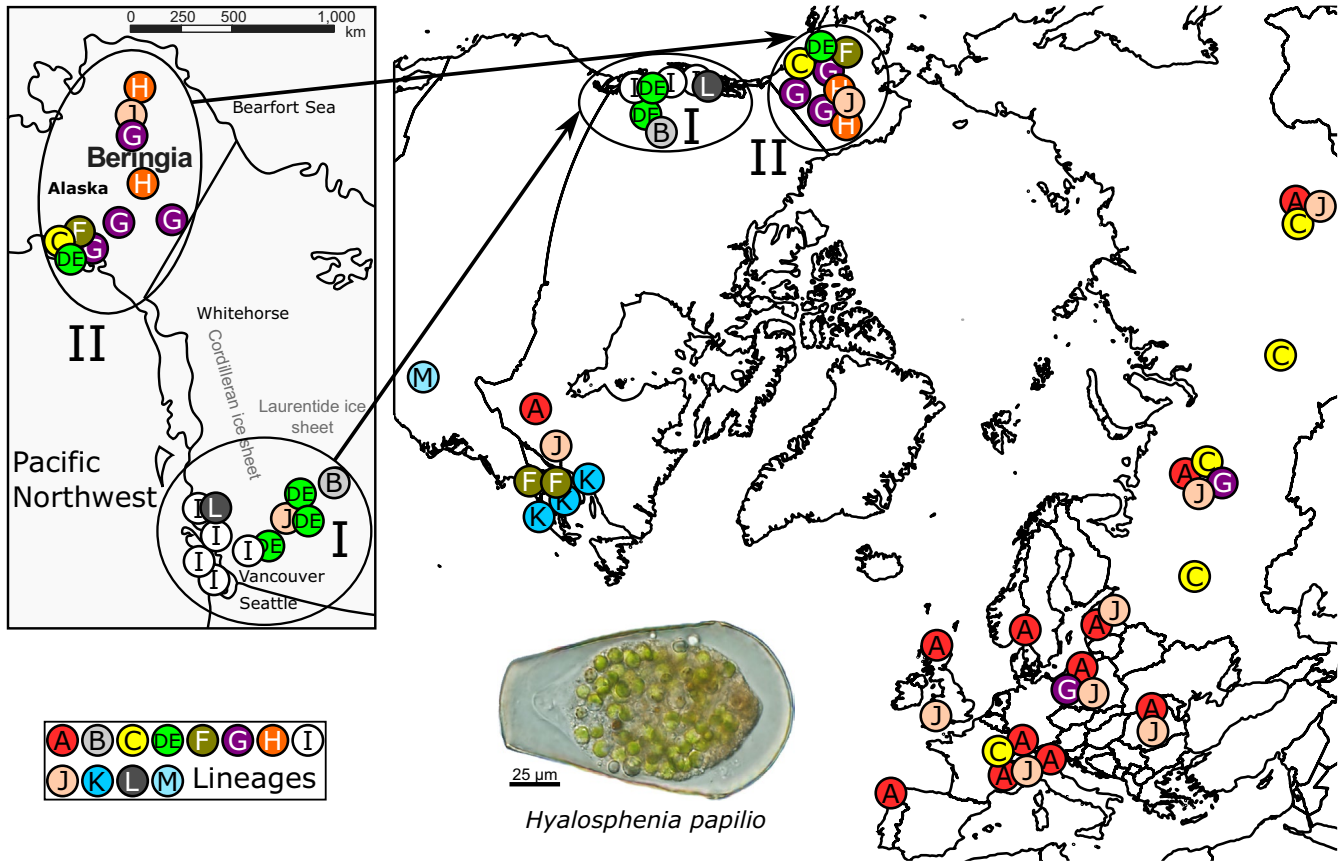

FIGURE 1 Holarctic distribution of *Hyalosphenia papilio* lineages. Each circle corresponds to a sampling site where the lineage has been detected. Lineage codes correspond to phylogenetic groups, as identified in Heger et al. (2013). I and II provide a detailed representation of the Beringia area. Inset: light micrograph of *H. papilio*. The pyriform outline corresponds to the shell that protects the single-cell body of the organism and its endosymbiotic microalgae (green dots)

et al., 2016; Singer et al., 2018). Hence, it is likely that this taxon does not pre-date the radiation of peat-forming *Sphagnum* species between 17 and 7 Ma (Shaw et al., 2010). Large extents of *Sphagnum* comparable to modern *Sphagnum*-dominated peatlands probably appeared during the late Miocene/early Pliocene, concomitantly with global cooling, that is, between 7 and 5.5 Ma (Herbert et al., 2016). While *Sphagnum* occurs at low as well as high latitudes it is only a dominant component of peatlands at higher latitudes (e.g., Tierra-del-Fuego and the boreal zone of the Holarctic). The taxonomic richness of the genus is low in the Southern Hemisphere high latitudes and high in the Northern Hemisphere high latitudes which correspond to its inferred origin (Shaw, Carter, Aguero, da Costa, & Crowl, 2019).

Holarctic *Sphagnum*-dominated peatlands have experienced considerable changes in their extent due to the repeated advances and retreats of ice sheets during the Quaternary. Many of the largest areas covered by peatlands today were under ice during the Last Glacial Maximum (e.g., Fennoscandia, boreal Canada), while peatlands may have persisted in others (e.g., Pacific coast of Canada) (Treat et al., 2019). These successive glacial expansions and contractions are known to have shaped genetic diversity in multicellular taxa (Schönswetter, Stehlik, Holderegger, & Tribsch, 2005), whose dispersal is assumed to be slow in contrast to eukaryotic micro-organisms (Bahram et al., 2016). If, like plants and animals, protist dispersal is relatively slow, the genetic structure of their populations will bear traces of such range expansions and contractions, and the origin of taxa can potentially be inferred and the timing of phylogenetic events estimated based on molecular clocks (Arbogast et al., 2006; Arbogast, Edwards, Wakeley, Beerli, & Slowinski, 2002). By contrast, fast dispersal in protists would blur any such signature, and taxonomic or phylogenetic diversity would tend to be distributed randomly and peak in areas with the largest extent of favourable habitats (Forest, Colville, & Cowling, 2018).

It follows that the phylogeographical pattern of a given taxon, here *H. papilio*, can be used to test two alternative hypotheses. (a) Dispersal is low and/or slow enough so that traces of glacial cycles are reflected in its extant diversity. The highest diversity, and the probable geographical origin of *H. papilio* would be expected to occur in refugia corresponding to the margins of ice sheets during the Last Glacial Maximum where *Sphagnum* peatlands could survive. (b) Dispersal is high and/or fast, and diversity would be expected to be maximal where the largest expanses of *Sphagnum* peatlands are found today (e.g., western Siberia). Empirical evidence demonstrates a relationship between testate amoeba shell size and geographical range (Wilkinson, 2001). Population genetics analyses

(Lara, Heger, Scheihing, & Mitchell, 2011) and modelling (Wilkinson, Koumoutsaris, Mitchell, & Bey, 2011) show a decline in dispersal potential for testate amoebae and theoretical organisms of smaller sizes (*c*. 60 μm) than that reported for *H. papilio* (size range 90–175 μm). The ability of entering a dormant stage (cysts) which can withstand desiccation and other stresses is considered to be a key dispersal trait in protists (Geisen et al., 2018); however, such structures have never been reported in *H. papilio*. We therefore predict that the first hypothesis is more likely to be supported.

2 | MATERIAL AND METHODS

2.1 | Data set preparation

We retrieved all 360 existing *COI* gene sequences of *Hyalosphenia papilio* from GenBank, together with information on the origin of the cells from four studies (Gomaa et al., 2014; Heger et al., 2013; Kosakyan et al., 2012; Oliverio, Lahr, Grant, & Katz, 2015). In addition, we isolated 57 single cells of *H. papilio* from *Sphagnum* samples collected at 13 new sites, targeting under-sampled regions to compile a global data set (Table S1). Briefly, the cells were washed three times in autoclaved distilled water before DNA extraction, which was performed using the guanidine thiocyanate-base protocol (Duckert et al., 2018). Amplifications of *COI* gene fragments were performed in two steps: a first polymerase chain reaction (PCR) was undertaken with the general *COI* primers LCO1490 and HCO2198 (Folmer, Black, Hoeh, Lutz, & Vrijenhoek, 1994), which was followed by a nested PCR, using *H. papilio*-specific primers HPcoiF and HPcoiR (Gomaa et al., 2014). The first DNA amplification profiles consisted of an initial denaturation step for 3 min at 95°C, followed by 39 cycles of 15 s of denaturation at 95°C, 15 s of annealing at 43°C and 1 min of elongation at 72°C with an additional final elongation step at 72°C for 10 min. The procedure for the second PCR profile was the same except that the annealing temperature was increased to 55°C. Sequencing was carried out using a BigDye197 Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) and analysed with an ABI-3130XL DNA sequencer (Applied Biosystems). Sequences were deposited in GenBank with the following accession numbers: MK823130–MK823186. *COI* sequences were edited and aligned (CLUSTALW algorithm; Thompson, Gibson, & Higgins, 2003) using BIOEDIT (version 7.2.3; Hall, 1999). The final data set including published and new sequences consisted of 418 sequences from 61 sites (Table S1).

2.2 | Lineage delineation

We delimited genetic lineages following the approach described by Heger et al. (2013). Briefly, to obtain a general overview of the existing lineages, we first constructed the phylogenetic tree based on the matrix of the unique sequences (haplotypes) among the 418 considered in this study. The sequence lengths of the data set vary from 430 to 620 bp (depending on the primers used to barcode the isolated cells). We constructed both a maximum likelihood (ML) and a Bayesian tree, with the RAxML algorithm (Stamatakis, Hoover, Rougemont, & Renner, 2008) and MRBAYES version 3.2.6 (Ronquist & Huelsenbeck, 2003), using in both cases a GTR + Γ model. We then tested if the haplotypes were distributed randomly by comparing our observed distribution with a null model obtained by haplotypes randomly attributed to lineages (10,000 replicates). The tree root was placed between two major clades (clade I contains lineages L, K, M and J and clade II contains lineages C, DE, F, B, A, G, H and I) that appeared well supported in previous studies (Heger et al., 2013; Kosakyan et al., 2012). Bipartition support values were evaluated with 1,000 bootstrap replicates. Bayesian Markov chain Monte Carlo analysis was carried out with two simultaneous chains and 50,000,000 generations. Trees were sampled every 1,000th generation and the burn-in was set at 25%. The trees were rooted internally based on the topology of trees obtained in previous studies (Heger et al., 2013; Kosakyan et al., 2016), which showed two major clades with maximum support; we rooted the tree between these two clades. As both trees were congruent, we presented only the ML tree and used the Bayesian analysis to evaluate each node's posterior probability (pp). We used three independent methods of lineage delimitation to compare our assignments with those of Heger et al. (2013): (a) Automatic Barcode Gap Discovery (ABGD) (Fontaneto, Flot, & Tang, 2015; Puillandre, Lambert, Brouillet, & Achaz, 2011) using the ABGD web-server http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html; (b) sequence divergences using the Kimura 2-parameter method (Kimura, 1980; Nassonova, Smirnov, Fahrni, & Pawlowski, 2010) using the "APE" package (version 3.2; Paradis, Claude, & Strimmer, 2004) in R (version 3.0.1; R Core Team, 2014); and (c) generalized mixed yule coalescent (GMYC) analysis performed with the SPLITS package, version 1.0-19 (Fujisawa & Barraclough, 2013) coded in R, version 3.1.2 (R Core Team, 2014).

2.3 | Haplotype and lineage network

Haplotypes (defined as genetic units separated by at least a single mutation) were assigned to the previously determined lineages. Haplotype networks were constructed using minimum spanning network analysis as implemented in the software POPART (version 1.7; Leigh & Bryant, 2015 [Table S2]). Four main geographical zones (Eastern North America, Western North America, Europe and Asia) were defined to highlight the distribution of the haplotypes.

2.4 | Historical biogeography

We first tested whether the observed patterns could be due to chance or to a sampling bias by calculating the observed beta diversity (following Legendre & De Cáceres, 2013). We then tested if the observed beta diversity was higher than expected by chance. We simulated beta diversity values under null expectations and compared them to observed beta diversity values to obtain *p*-values and standardized effect sizes (SESs). Simulated beta diversity values were calculated using the same approach (Legendre & De Cáceres, 2013) on a permuted site by species matrix. Permutations were conducted using the permatswap algorithm of the R-package "VEGAN" (Oksanen, Blanchet, & Kindt, 2015),

which preserves column sums. This allows us to randomly attribute species to station while preserving species total abundance.

To determinate whether species distribution areas were correlated with ecological tolerance, we determined the climatic niche breadth for each species using the tolerance index (Dolédec, Chessel, & Gimaret-Carpentier, 2000), with the R package "ADE4" (Dray & Dufour, 2007). This index estimates niche breadth based on environmental tolerance (i.e., climate) (Hurlbert, 1978; Thuiller, 2004) using the dispersion of geographical cells that contain the target species in the climatic multivariate space. Low values of the index suggest narrow tolerance while high values correspond to generalists. These indices were inferred based on geographical coordinates for each occurrence (Table S1) and interpolated climate data sets (Bioclim, 19 variables) that were generated at 2.5 arcmin resolution from meteorological data (Hijmans, Cameron, Parra, Jones, & Jarvis, 2005). We then estimated distribution areas of the species based on the number of plots in which a given species was observed (i.e., a rough estimate of the spatial range) and plotted it against the estimated climatic niche breadths.

To evaluate the evolutionary events that may explain the current distribution of *H. papilio* (e.g., dispersal, extinction, range-switching, sympatry, vicariance and founder effect), we estimated the ancestral distribution and the frequency of event counts in each of 1,000 biogeographic stochastic mapping (BSM) analyses, using the BIOGEOBEARS package (Matzke, 2013) in R (version 3.0.1; R Core Team, 2014). BIOGEOBEARS allows for the estimation of ancestral geographical ranges on dated phylogeny, comparing several models of range evolution. We used the DEC model (Ree, Smith, & Baker, 2008) with two free parameters: "d" (dispersal rate) and "e" (extinction rate), and a fixed cladogenetic model (cladogenetic event allowed: vicariance, sympatric-subset speciation and sympatric range-copying). We also used a DEC model with an extra parameter, "j," which represents the founder-event speciation, where the new species "jumps" to a range outside of the ancestral range (DEC + j model). The comparison of these two models was performed using Akaike's information criterion (AIC). The age of the nodes of the rescaled BEAST tree of *H. papilio* was estimated by constraining its root to 7 Ma, which corresponds to the documented origin of *Sphagnum* peatlands (Stenøien, Shaw, Shaw, Hassel, & Gunnarsson, 2010), as all known lineages of *H. papilio* are restricted to these environments. This type of approach has been used to identify the geographical origin of multicellular taxa of different ages, dispersion strategies and lifestyles, including hyacinthoid monocots (Ali, Yu, Pfosser, & Wetschnig, 2012), chameleons (Tolley, Townsend, & Vences, 2013) and bees (Trunz, Packer, Vieu, Arrigo, & Praz, 2016), and is used here, to our knowledge, for the first time in micro-organisms. This approach allowed us to test the two hypotheses: if the frequency of event counts in each of 1,000 BSM sustains a low frequency of dispersal events, related to other biogeographical events, the first hypothesis is supported ([a] dispersal is low and/or slow enough so that traces of glacial cycles are reflected in its extant diversity), and otherwise, the second hypothesis is supported ([b] dispersal is high and/or fast, and diversity would be expected to be maximal where the largest expanses of *Sphagnum* peatlands are found today).

3 | RESULTS

3.1 | Lineage delineation and diversity

The 418 mitochondrial *COI* sequences of *Hyalosphenia papilio* revealed the existence of 13 or 14 distinct lineages (Figure 2). The Kimura 2-parameter test suggested the existence of 14 lineages based on a threshold of ≥1% sequence divergence. The GMYC method yielded 13 lineages (lower and upper confidence intervals: 10 and 29 lineages, respectively; $p = 0.046$) based on single threshold methods. Finally, the ABGD method identified 13 lineages, using a distinctive barcoding gap of 7%. One of these lineages, called here "M," has not been previously recorded. This lineage was recovered from localities not included in previous studies (Gomaa et al., 2014; Heger et al., 2013; Kosakyan et al., 2012; Oliverio et al., 2015). It was supported by all analyses, although the Kimura 2-parameter test suggested dividing it into two (Figure 2).

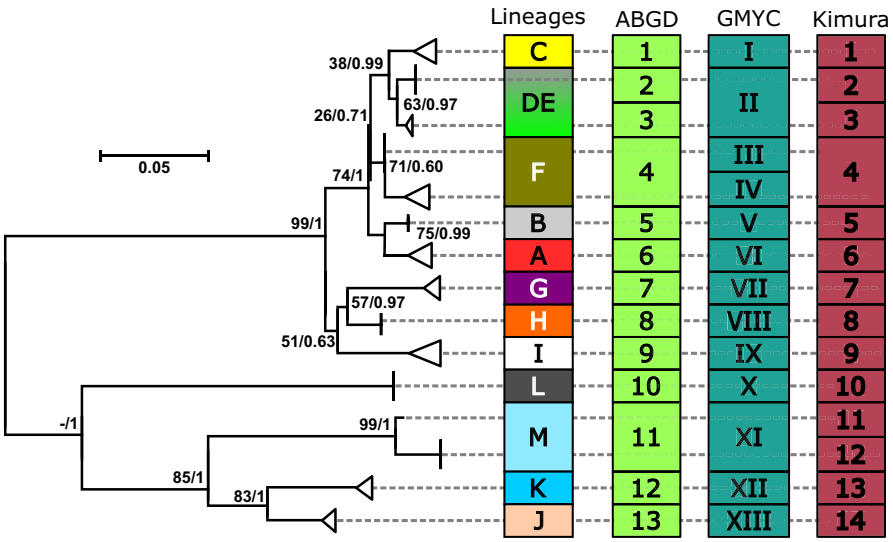

FIGURE 2 Maximum likelihood (ML) and Bayesian concatenated phylogenetic tree from 76 unique sequences of *Hyalosphenia papilio* isolated from *Sphagnum* peatlands across the Holarctic realm. Numbers along branches represent, respectively, bootstrap support values obtained by ML and posterior probabilities as calculated with Bayesian analyses. Trees were rooted internally based on the topology of trees obtained in previous studies (Heger et al., 2013; Kosakyan et al., 2016), which showed two major clades with maximum support; we rooted the tree between these two clades. The tree also represents the different lineages obtained with the ABGD and GMYC analyses and the Kimura 2-parameter test

3.2 | Phylogenetic reconstruction

Sequences from the previously overlooked lineage M diverged from all others (pp = 1) and branched as a sister group to lineages J and K (Figure 2). Only a single haplotype was retrieved from lineage E, and five from lineage D (sensu Heger et al., 2013). Here again the genetic divergence was low (i.e., at most six nucleotides difference between the sequence of lineage E, and the five sequences of lineage D, all of which were separated by a single nucleotide [Figure 3]).

3.3 | Haplotype network

The haplotype network (Figure 3) showed that some lineages (B, H and L) were composed of only a single haplotype, whereas others included several haplotypes, independently of the number of individual cells barcoded. Some lineages were relatively rare (e.g., B, L and M with seven, two and seven individuals, respectively) whilst others were extensively recorded (e.g., lineage A was identified more than 100 times). Null model analyses show that such a pattern is not expected under random assembly of lineages (Figure S1).

3.4 | Spatial patterns of phylogenetic richness

We found that the observed beta diversity was significantly higher than expected by chance (SES = 1.71; p = 0.99), showing a strong spatial structuring of diversity. We also found a strong positive correlation between niche breadth as estimated using Dolédec tolerance indices and distribution areas (R^2 = 0.75; p = 0.001). We also found that lineages differed in their climatic niches with some lineages preferring colder and drier conditions (Figure S2, lineage H) and others preferring warmer conditions with abundant precipitation (Figure S2, lineage I).

The geographical distribution of phylogenetic richness showed a clear contrast (Figures 1 and 3). Only four lineages (A, C, J and G) were recovered from all of Eurasia, five from Eastern North America (A, F, K, J and M), and nine from Western North America (six in Alaska and five in the Pacific Northwest, only two being shared between these two regions). Thus, regional as well as overall diversity and diversity turnover were all higher in North America than in Eurasia.

The distribution of the different lineages (Figure 3) suggests that several haplotypes are specific to certain geographical areas (B, DE, H, I and L occur only in Western North America, while K and M occurred only in Eastern North America), whereas others were geographically widespread (e.g., J is found throughout the Holarctic realm). Null model analyses show that such a pattern is not expected under random assembly of lineages (Figure S1). This structure in lineage distribution suggests that geographical dispersal has occurred comparatively slowly, allowing it to be recovered with a genetic marker such as mitochondrial *COI* used for species-level delineation in this group of organisms (Kosakyan et al., 2012).

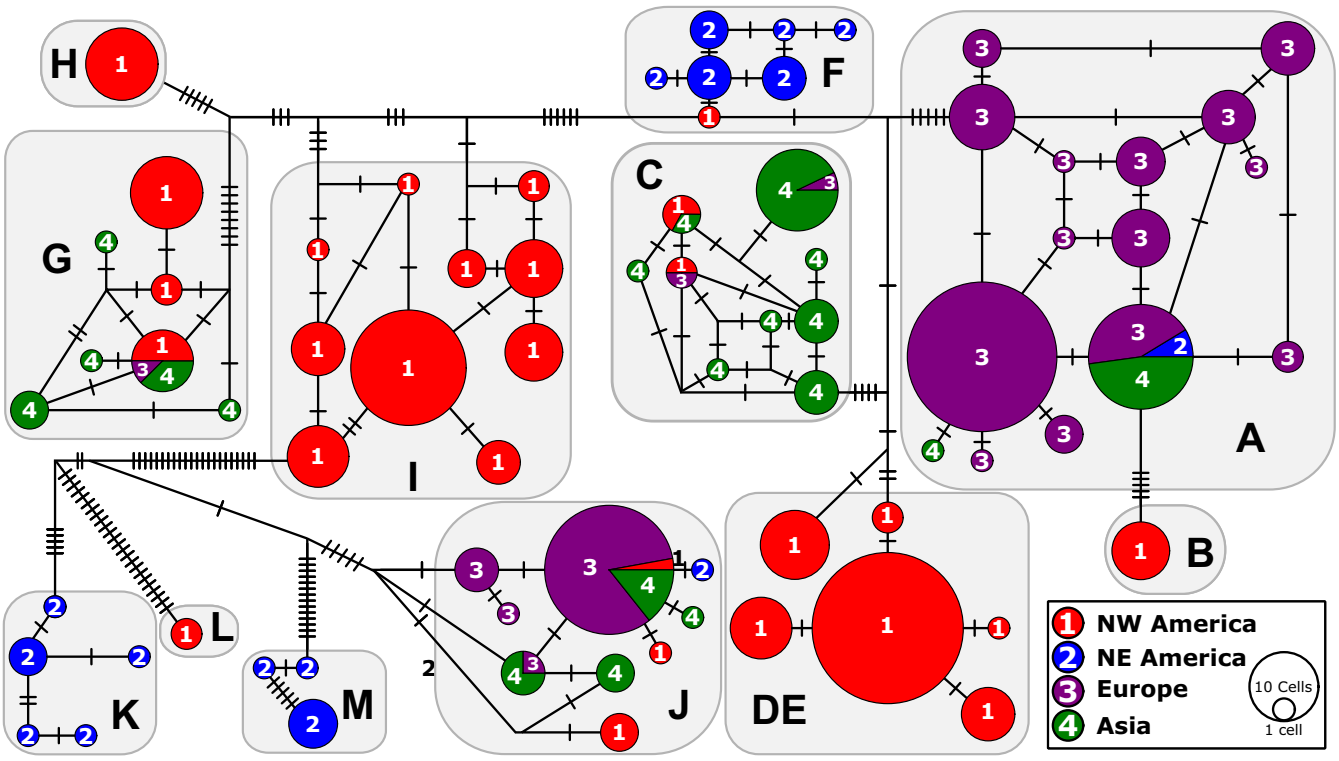

FIGURE 3 Median joining haplotype network of the cytochrome oxidase subunit 1 (*COI*) gene of *Hyalosphenia papilio* from *Sphagnum* peatlands in the Holarctic realm. Grey boxes and letters represent the different lineages identified in the present study. Colours indicate geographical regions (legend: bottom right inset). Circle sizes are proportional to the number of sequenced single cells of *H. papilio* within each haplotype. Cross lines show the number of mutational steps between haplotypes

3.5 | Origin of lineages and evaluation of the diversification processes

The AIC selection of biogeographical models implemented in BIOGEOBEARS indicated that a DEC model was the best supported (Table S3). Based on this model, the most likely ancestral areas for *H. papilio* are in Western North America (Figure 4). The dispersal summary extracted from the 1,000 BSM maps showed that most of the dispersal events occurred from Western North America and Asia to the other biogeographical areas, and from Asia to Europe (Table S4). The results of the ancestral area estimation and number of dispersal events analyses showed that the most frequent process during the historical biogeography of *H. papilio* was narrow sympatry (i.e., when the ancestral range contains one area, and both daughter lineages inherit that area), followed by a low frequency of dispersal events (range expansion) (Figure S1). The importance of vicariance and founder events was comparatively limited (Figure S1).

4 | DISCUSSION

4.1 | Diversity and geographical distribution of the lineages and haplotypes

The *Hyalosphenia papilio* species complex is represented by at least 13 lineages in the Holarctic region, one of which had not been previously detected. Although it is possible that some lineages remain to be discovered, our globally extensive sampling retrieved only one additional lineage (M), suggesting that we have now captured most of the group's diversity. The genetic distances determined by our taxon delineating approaches are consistent with the barcoding gap (<4%) used to discriminate species in other related testate amoebae lineages (e.g., genus *Nebela*, Hyalospheniidae). The above-mentioned lineages were defined as species under multiple and independent concepts, including ecological, morphological and evolutionary (Singer et al., 2018). This might imply that the lineages retrieved in the present study can all be considered as separate species (Kosakyan, Gomaa, Mitchell, Heger, & Lara, 2013; Kosakyan et al., 2012; Singer, Kosakyan, Pillonel, Mitchell, & Lara, 2015; Singer et al., 2018). The accuracy of a species tree built on a single locus may be still questioned, especially in the case of recent radiations, as the existence of several caveats (e.g., sequencing pseudogenes, ongoing hybridization processes) cannot be ruled out and may distort the tree's topology. In Amoebozoa, *COI* has been chosen as the most accurate marker notably because of its sensitivity and lack of intra-individual variability (Nassonova et al., 2010) and we therefore consider it reliable.

Lineages of *H. papilio* show different distribution patterns over the Holarctic realm. Four lineages (J, A, C, G) were found in several regions with contrasting climates (Figure 1) suggesting that they have a greater ecological tolerance. This is corroborated by the strong correlation between climatic niche breadth and estimated distribution ranges (Figure 5), suggesting that colonization capacity is constrained by specific tolerance to climates. If these distributional patterns reflect evolutionary adaptation to long-distance

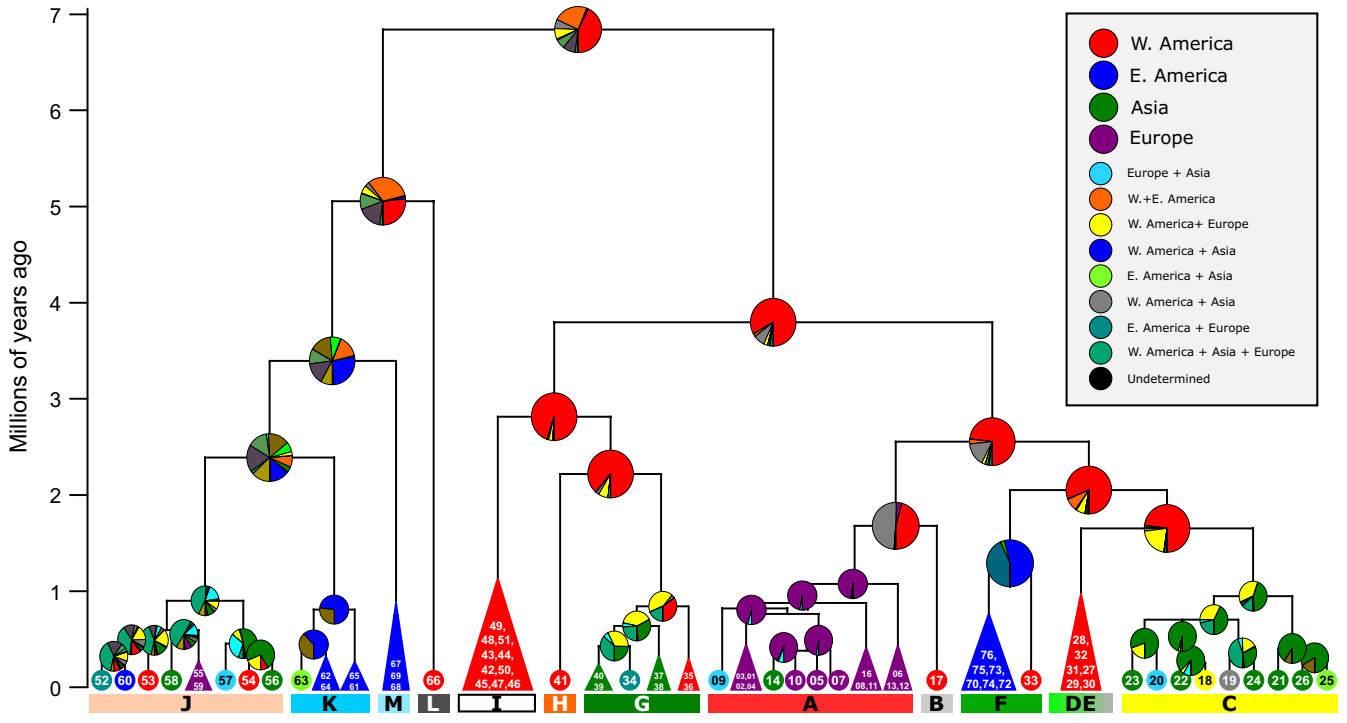

FIGURE 4 Biogeographical analysis of *Hyalosphenia papilio* from *Sphagnum* peatlands across the Holarctic realm using BIOGEOBEARS. The four biogeographical areas are: Eastern North America (in blue), Western North America (in red), Europe (in purple) and Asia (in green). Pie charts at nodes indicate support for each area. The tips are labelled with present-day distributions. The secondary colours indicate range combinations of the tip ranges

dispersal, it would then imply that the required physiological/lifestyle adaptations to long-range migration have appeared independently at least four times in the history of the *H. papilio* species complex (Figure 4).

The existence of restricted distributions is even clearer at the haplotype level. Of the 74 total *H. papilio* haplotypes, only seven (9.2%) were present in two zones, two in three zones (2.6%) and no single haplotype was found in all four zones. This indicates that even widespread lineages (e.g., lineage J) show high infraspecific genetic structuring, which suggests limited gene flow among sites, and thus geographical isolation (Fernández, Hernández, Schiaffino, Izaguirre, & Lara, 2017; Lara et al., 2011).

Hypothesis 2, that diversity is maximal where the largest expanses of *Sphagnum* peatlands are found today, cannot be supported by our data and analyses. Under this hypothesis, highest diversity would be expected in regions such as Western Siberia where peatlands are at their most extensive and cover more than 20% of the landscape (Peregon, Maksyutov, Kosykh, & Mironycheva-Tokareva, 2008). However, only four "far travelled" lineages were found in all of Eurasia, as compared to 13 in North America. This is despite a larger overall area, greater extent of *Sphagnum* peatland and an extensive range of climatic conditions. Six lineages, 50% more than the entire of Eurasia, were found only in Alaska. Moreover, most genetic diversity seems to be located along the Western North American coast, a region where peatlands are typically small and scattered today. This fact, together with the strong spatial patterns in lineage distribution observed (Figures 1 and 4), advocates against our hypothesis 2 (fast dispersal).

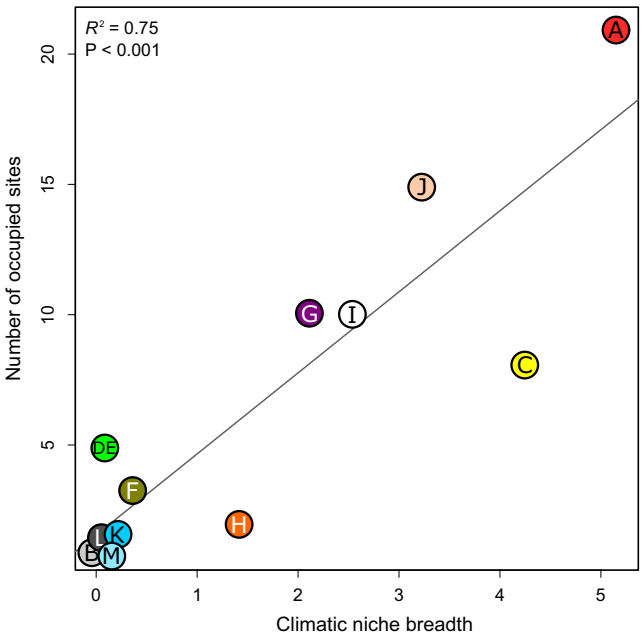

FIGURE 5 Relationship of lineage climatic niche breadth to lineage range size (estimated as the number of locations where a given lineage is present) of *Hyalosphenia papilio*. Climatic niche breadth was estimating from Bioclim variables using the tolerance index of Dolédec et al. (2000)

4.2 | Geographical origin and influence of historical events

All Eurasian lineages identified were also present in North America, while several lineages were restricted to North America. This observation alone suggests an American origin for *H. papilio*. Our ancestral range reconstruction corroborates this inference, placing the most probable origin of the *H. papilio* complex in Western North America (Figure 4).

Dating speciation events is difficult in testate amoebae as their lineages cannot be morphologically distinguished (Mulot et al., 2017); testate amoeba shell records in peat are rare before the Holocene. Nevertheless, it is still possible to infer a time window for the radiation of the lineages indirectly based on the very strict habitat specificity of this taxon. All lineages of *H. papilio* thus far identified are restricted to *Sphagnum* peatlands. It is therefore reasonable to assume that this highly adapted taxon evolved within these ecosystems. The oldest fossils of the genus *Hyalosphenia* were described from the Triassic (*H. baueri*, 220 Ma) (Schönborn, Dörfelt, Foissner, Krienitz, & Schäffer, 1999). *H. baueri* shares some traits like an "indistinctly vase shape" and the presence of an organic lip surrounding the aperture with *H. papilio*. However, it is far from clear that the two taxa are directly related. First, it has been shown that the genus *Hyalosphenia* is paraphyletic, as *H. papilio* and *H. elegans* are only distantly related (Lahr et al., 2019; Lara, Heger, Ekelund, Lamentowicz, & Mitchell, 2008). Furthermore, a rough calculation can rule out the possibility of a very old age for *H. papilio*. "Standard" *COI* (estimated for animals) mutation rates are typically in the range of a few per cent per million years (Ho & Lo, 2013; Papadopoulou, Anastasiou, & Vogler, 2010), sometimes much higher (Ney, Frederick, & Schul, 2018). The most divergent *H. papilio* sequences are separated by roughly 10%, which implies that, in order for the deepest branching in the complex (see Figure 4) to be 100 million years old, the mutation rate would need to be of 0.01% Ma^{-1}. This is far below all rates known to date, and even lower than the mutation rate of cnidarians, which are known for their extremely slow evolving mitochondria (Park et al., 2012). By contrast, to obtain an age of 7 million years the mutation rate would need to be 1.3% Ma^{-1}, which is similar to the mutation rate of many arthropods and thus more parsimonious than the alternative.

During the Pleistocene, large areas of North America were intermittently covered by ice although ice-free refugia remained. The area of *Sphagnum* peatlands probably repeatedly expanded during interglacial periods and contracted in response to glacial periods when ice masses covered most of the landscape (Shaw et al., 2014). This period is also contemporary with most cladogenesis in the *H. papilio* phylogenetic history, which suggests a series of speciation events by cladogenetic range evolution that may have occurred during interglacial periods (Figure 4). Indeed, our analyses show that at least eight out of 12 cladogenesis events occurred during the Pleistocene, immediately after the 2.5 Ma boundary (Figure 4).

This hypothesis is also in line with the fact that the BIOGEOBEARS analyses designated narrow sympatry or the inheritance of the

ancestral area of a range by both daughter lineages, as a key process explaining the distribution of *H. papilio* lineages. At the onset of Quaternary glaciations (2.58 Ma), one lineage probably existed in Eastern and two in Western North America (Figure 4). While the first lineage probably survived south of the ice sheet, where conditions were wet enough to allow the development of peatlands (Shaw et al., 2010), the two others were probably confined to refugia in Western North America.

The location of these refugia is known to have shaped the distribution of plants (Eidesen et al., 2013) and animals (Klütsch, Manseau, Anderson, Sinkins, & Wilson, 2017). In particular, Eastern Beringia (today Alaska and Yukon Territory) was wet enough to support the growth of *Sphagnum* mosses and *Sphagnum* peatlands (Shaw et al., 2013, 2014). These peatlands allowed the survival of associated organisms, probably including the lineages of *H. papilio*. In contrast, Western Beringia (today far eastern Russia) was too dry to support large expanses of *Sphagnum* peatlands (Shaw et al., 2013, 2014) and probably constituted a barrier for the migration of *H. papilio* westwards. Our data suggest that colonization of the Palaearctic region occurred recently, possibly after the last glaciation (Figure 1). Western Siberia, which was a cold desert during the Last Glacial Maximum, became covered with peatlands after 11,000 years ago (Velichko, Timireva, Kremenetski, MacDonald, & Smith, 2011) and could have constituted a bridge that facilitated the invasion of the Western Palaearctic by "far travelled" lineages of *H. papilio*. Interestingly, a similar pattern has been suggested for *Sphagnum angermanicum* (Stenøien et al., 2010).

The present-day distribution of lineages and the local palaeogeographical context designates Eastern Beringia or the Pacific Coast as the most probable origin for all extant *H. papilio* lineages. The higher diversity of *H. papilio* haplotypes in North America as compared to Europe mirrors the higher diversity of vascular plants (Earl Latham & Ricklefs, 1993; Svenning, 2003), and both were probably similarly driven by glaciations. The phylogeographical history of *H. papilio*, used here as a convenient model taxon for protists lacking specialized morphological adaptation for dispersal, thus highlights the importance of historical processes in explaining the distribution of extant microbial diversity.

Therefore, following a dispersal event, sympatric diversification could indeed have played a major role in shaping the current phylogeography of *H. papilio* (Figure S1). It remains to be determined if *H. papilio* is representative of free-living micro-organisms in general. *H. papilio* is large by microbial standards; testate amoebae mostly range between 20 and 200 µm and many other protists and most fungi and prokaryotes are smaller. *H. papilio* is also restricted to *Sphagnum* mosses, which, although widespread across the Holarctic, nevertheless constitute a very specific habitat. More generalist, smaller species and/or species possessing structures adapted to dispersal (e.g., fruiting bodies as in many other Amoebozoa; Shadwick, Spiegel, Shadwick, Brown, & Silberman, 2009) may show patterns which agree better with the second hypothesis. Elucidating the historical processes shaping the diversity of protists with different dispersal strategies, and comparing patterns with better known macroscopic organisms, will open the way to understanding the processes of diversification that produced the immense diversity existing today.

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CONFLICT OF INTEREST

The material in this manuscript is original research, has not been previously published and has not been submitted for publication elsewhere while under consideration for *Molecular Ecology*. The authors have no conflicts of interest to declare.

AUTHOR CONTRIBUTION

D.S., E.A.D.M. and E.L. designed the experiments; D.S., E.A.D.M., G.G., H.R., L.B., N.G.K., I.G., L.I.H., K.K., M.L., N.P.K., R.J.P. and K.V. collected the samples; D.S., Q.B., C.D., L.D.F., B.F., C.E.H. and E.L. analysed the data; D.S., E.A.D.M., R.J.P. and E.L. wrote the first version of the manuscript, which was then edited by all co-authors.

DATA AVAILABILITY STATEMENT

The 57 DNA sequences of the *COI* gene of *Hyalophenia papilio* are available in GenBank with the following accession numbers: MK823130–MK823186.

ORCID

David Singer https://orcid.org/0000-0002-4116-033X
Edward A. D. Mitchell https://orcid.org/0000-0003-0358-506X
Quentin Blandenier https://orcid.org/0000-0002-4297-0262
Gustaf Granath https://orcid.org/0000-0002-3632-9102
Håkan Rydin https://orcid.org/0000-0002-7582-3998
Enrique Lara https://orcid.org/0000-0001-8500-522X

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