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Speciation in protists: Spatial and ecological divergence processes cause rapid species diversification in a freshwater chrysophyte

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Abstract

Although eukaryotic microorganisms are extremely numerous, diverse and essential to global ecosystem functioning, they are largely understudied by evolutionary biologists compared to multicellular macroscopic organisms. In particular, very little is known about the speciation mechanisms which may give rise to the diversity of microscopic eukaryotes. It was postulated that the enormous population sizes and ubiquitous distribution of these organisms could lead to a lack of population differentiation and therefore very low speciation rates. However, such assumptions have traditionally been based on morphospecies, which may not accurately reflect the true diversity, missing cryptic taxa. In this study, we aim to articulate the major diversification mechanisms leading to the contemporary molecular diversity by using a colonial freshwater flagellate, *Synura sphagnicola*, as an example. Phylogenetic analysis of five sequenced loci showed that *S. sphagnicola* differentiated into two morphologically distinct lineages approximately 15.4 million years ago, which further diverged into several evolutionarily recent haplotypes during the late Pleistocene. The most recent haplotypes are ecologically and biogeographically much more differentiated than the old lineages, presumably because of their persistent differentiation after the allopatric speciation events. Our study shows that in microbial eukaryotes, species diversification via the colonization of new geographical regions or ecological resources occurs much more readily than was previously thought. Consequently, divergence times of microorganisms in some lineages may be equivalent to the estimated times of speciation in plants and animals.

KEYWORDS
biogeography, chrysophyceae, pleistocene, protists, speciation, taxonomy

1 | INTRODUCTION

While eukaryotic microorganisms (protists) are extremely numerous, diverse and essential to global ecosystem functioning, due to their small size and difficulty with culturing, our knowledge of their diversity and evolutionary processes is considerably limited. Advances in molecular techniques have brought new insights into global protist diversity and revealed a high degree of cryptic diversification (Boenigk, Ereshefsky, Hoef-Emden, Mallet, & Bass, 2012). Molecular markers have been used in species delineation, and revealed that classical, morphological approaches drastically underestimated diversity (Boenigk et al., 2012). Several large-scale studies based on

numerous isolates from a wide range of habitats have revealed that actual protist diversity is much higher than previously assumed (Howe, Bass, Vickerman, Chao, & Cavalier-Smith, 2009; Kooistra et al., 2008; Sáez et al., 2003). More recently, the growing number of next generation sequencing (NGS) studies employing amplicon sequencing of microbial eukaryotes has confirmed the vast genetic diversity and abundance of protists (Behnke et al., 2011; Gimmler, Korn, De Vargas, Audic, & Stoeck, 2016; Pawlowski et al., 2012). Moreover, several studies have shown that both terrestrial and freshwater protist communities are strongly shaped by the combination of geographical distance and environmental factors (Bates et al., 2013; Boenigk et al., 2018; Ragon, Fontaine, Moreira, & López-García, 2012).

However, little is known about the mechanisms generating and maintaining the protist diversity. The uncertainty of these processes, coupled with fluid or inconsistent species concepts, has led some authors to the contrary opinion that protist diversity is actually quite low, perhaps less so than even that of metazoans (Fenchel & Finlay, 2006; Mora, Tittensor, Adl, Simpson, & Worm, 2011). According to these authors, the enormous population sizes and unlimited dispersal characteristics of protists should lead to the lack of population differentiation with no barriers to gene flow, leading to very low speciation rates (Fenchel & Finlay, 2006). They further postulate that the use of genetic data has brought confusion into the estimates of real diversity in protists, because the variation in molecular markers reflected the accumulation of neutral mutations over historical times rather than the existence of large numbers of cryptic taxa. Therefore, it is essential to understand the nature of genetic variability in protists, in particular, to disentangle the drivers of population differentiation that lead to speciation processes.

Several examples of population differentiation in protists have been described, along with the geographical, ecological or temporal patterns. Geographical speciation seems to represent the major isolating force, especially in diatoms (Casteleyn et al., 2010; Evans et al., 2009; Souffreau, Vanormelingen, Verleyen, Sabbe, & Vyverman, 2010). Population differentiation has been reported among both distantly and closely related localities, indicating that gene flow may be limited to a few kilometres (Rengefors, Logares, & Laybourn-Parry, 2012). A remarkable example of recent ecological speciation has been reported by Logares et al. (2007), who identified the post-glacial marine–freshwater transition of a dinoflagellate connected with the speciation event. Host specialization has been shown to be the major diversification force in symbiotic algae (LaJeunesse & Thornhill, 2011; Thornhill, Lewis, Wham, & Lajeunesse, 2014). Finally, temporal differentiation of phytoplankton has been reported in the marine diatom *Ditylum brightwellii* (Rynearson, Newton, & Armbrust, 2006) and freshwater raphidophyte *Gonyostomum semen* (Lebret, Kritzberg, Figueroa, & Rengefors, 2012).

Silica-scaled chrysophytes are an interesting group to study speciation mechanisms in protists because of their potential to improve our knowledge gathered primarily from diatoms and dinoflagellates. First, recently published phylogenetic investigations demonstrate the existence of evolutionarily recent radiations within several traditionally characterized species (Jo, Kim, Škaloud, Siver, & Shin, 2016; Scoble & Cavalier-Smith, 2014; Škaloud, Škaloudová, Procházková, & Němcová, 2014). Second, the biogenesis of species-specific siliceous scales allows us to morphologically differentiate most of these extant taxa (Siver et al., 2015). Finally, deposition of silica scales in sediments of ponds and lakes leaves a recognizable fossil record, which enables the calibration of divergence times (Siver et al., 2015; Siver, Lott, & Wolfe, 2013).

In this study, we aim to articulate the major diversification mechanisms that generated the contemporary molecular diversity within the silica-scaled chrysophyte *Synura sphagnicola* (Chrysophyceae, Stramenopiles), a colonial, cosmopolitan freshwater flagellate that often dominates in slightly acidic waters. To study both molecular and morphological diversity in detail, we cultured more than 70 newly established strains isolated from water bodies differing in climatic and chemical characteristics. We used a time-calibrated phylogenetic analysis of five molecular loci to infer patterns of diversity and to estimate divergence times among the lineages. We then assessed the relative contributions of geography, climate and habitat in promoting the diversification of lineages at different times of their evolutionary history. We recovered rapid diversification that occurred during the late Pleistocene, with the most recent haplotypes being ecologically and biogeographically much more differentiated than the old, morphologically distinguishable lineages which diverged during the Miocene. Accordingly, the colonization of new geographical regions or ecological resources seems to represent an important diversification force in protists, despite their enormous population sizes and cosmopolitan dispersal.

2 | MATERIALS AND METHODS

2.1 | Sampling, isolation and cultivation

The strains used in this study were obtained during an extensive investigation of the genus *Synura* in Europe, Newfoundland and Korea (2007–2017). The material was sampled using a plankton net with 20-μm mesh. Abiotic factors including water pH, temperature and specific conductivity were measured using a combined pH/conductometer (WTW 340i; WTW GmbH). Collected samples were kept in a polystyrene box equipped with a cooling gel pad for a few hours until they were processed at the research base. To establish uni-algal cultures, the individual colonies were isolated from the natural samples by micropipetting, and transferred into separate wells of a 96-well polypropylene plate filled with MES-buffered DY IV liquid medium (pH ≈ 6; Andersen, Berges, Harirson, & Watanabe, 2005). All cultures were then grown in 50-ml Erlenmeyer flasks at 15°C, under the permanent illumination of 7–40 μmol m^{-2} s^{-1} (various light sources, including fluorescent and LED lamps). From a total of 2,450 isolated cultures, 71 strains corresponded morphologically well with the description of *Synura sphagnicola*. These strains were further analysed in this study (Supporting Information Table S1).

2.2 | DNA sequencing and phylogenetic analyses

DNA was isolated as described by Škaloud et al. (2014). Five molecular loci were amplified by PCR (polymerase chain recation): the hypervariable nuclear internal transcribed spacer (ITS) rDNA, plastid *psa*A and mitochondrial *coxI* were obtained from the 69, 45 and 39 strains, respectively. Two additional molecular markers (nuclear small subunit [SSU] rDNA and plastid *rbcL*) were obtained from the five selected strains to assess the phylogenetic position and estimate the ages of *S. sphagnicola* lineages. The amplification of ITS rDNA was performed as described by Kynčlová, Škaloud, and Škaloudová (2010). Amplification of the *psaA* and *coxI* markers were performed according to Boo et al. (2010), with the newly designed forward *cox* primer Synura-cox1-F1 (5′-TTTGATCCTGCAGGNGGDGG-3′). Amplification of the SSU rDNA and *rbcL* was performed as described by Škaloud, Kristiansen, and Škaloudová (2013). The PCR products were purified and sequenced at Macrogen Inc., using an ABI 3730 DNA analyzer (Applied Biosystems).

Multiple alignments of the ITS rDNA, *psaA*, *coxI*, *rbcL* and SSU rDNA loci sequences were either manually built in MEGA6 (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013) or constructed using MAFFT version 6, applying the Q-INS-i strategy (Katoh, Misawa, Kuma, & Miyata, 2002). Positions with deletions prevailing in a majority of sequences were removed from the alignment. The resulting data sets comprised 447 nucleotide sites of the ITS rDNA, 958 sites of the *psaA*, 958 sites of the *psaA*, 542 sites of the *coxI*, 1,663 sites of the SSU rDNA and 1,236 sites of the *rbcL* gene.

Bayesian evolutionary analysis was performed to infer the phylogenetic position and estimate the age of splits of *S. sphagnicola* lineages, using the program BEAST version 1.8.2. The analysis was performed on the concatenated and partitioned SSU rDNA and *rbcL* data set (Supporting Information Table S2), using the lognormal relaxed clock model. The most appropriate partition-specific substitution models were recognized by the Bayesian information criterion (BIC)-based model in jModelTest 2.1.4 as follows: (i) TIM2 + I + Γ for SSU rDNA, (ii) TIM1 + I + Γ for the 1st *rbcL* codon partition, (iii) TPM3 + I + Γ for the 2nd *rbcL* codon partition and (iv) TIM3 + I + Γ for the 3rd *rbcL* codon partition. However, the GTR + I + Γ substitution model was applied to all partitions, as the most similar applicable option. A birth–death diversification process was used as a prior on the distribution of node heights. Eleven secondary calibration constraints were used, following the inferred molecular divergence times published by Siver et al. (2015). These includes the *Synura* calibration points 3, 4, 5, 6, 7, 9, 14 and 15, the ages of the genera *Mallomonas* (124 Ma, *SD* 15) and *Synura* (100 Ma, *SD* 15), and the estimated divergence between *Mallomonas* and *Synura* (130 Ma, *SD* 15). Five Markov chain Monte Carlo (MCMC) analyses were run for 100 million generations (burn-in 20 million generations). The convergence diagnostics was performed in Tracer 1.6. To check for the reliability of using secondary calibration constraints, we further performed the molecular clock analysis based on fewer but primary calibrations. We analysed the concatenated SSU rDNA and *rbcL* data set of Chrysophyceae published by Škaloud et al. (2013), applying seven calibration points as follows: (i) the split between *Mallomonas cratis* and *M. pseudocratis*, (ii) the node including all species from the section Mallomonas, (iii) the node including *M. insignis* and *M. bangladeshica*, (iv) the node including *M. matvienkoae*, *M. caudata*, *M. oviformis* and *M. heterospina*, (v) the parent node of *M. insignis*, (iv) the parent node of *Synura uvella*, and (vii) the node including *S. spinosa*, *S. curtispina* and *S. mollispina*. For all calibration points, we used an offset of 40 Ma, a mean of 15, and a standard deviation of 0.6, based on the observation of the above-mentioned *Mallomonas* and *Synura* scales in the laminated sediments of the Giraffe Pipe core (Siver, Lott, & Wolfe, 2009; Siver & Wolfe, 2005), estimated to have a minimum age of approximately 40 Ma (Siver & Wolfe, 2009). All the other parameters were the same as described above. The estimated node ages were highly congruent in both analyses (e.g., the split of *S. sphagnicola* was estimated to 15.4 ± 8.4 and 16.2 ± 10.3 Ma, respectively).

Genealogical relationships of ITS rDNA, *psaA* and *coxI* loci were investigated by constructing the maximum parsimony haplotype networks using the Haplotype Viewer (Ewing, available at www.cibiv.at/~greg/haploviewer). Because the loci were incongruent in haplotype composition and their relationships, the species tree was inferred by the StarBEAST analysis using the two-step approach. First, the concatenated alignment of SSU rDNA, *rbcL*, ITS rDNA, *psaA* and *coxI* sequences of 14 Petersenianae taxa (Supporting Information Table S3) was built to estimate the substitution rates of ITS rDNA, *psaA* and *coxI* molecular loci. The following partition-specific substitution models were selected according to the BIC-based model in jModelTest 2.1.4: (i) GTR + I + Γ for SSU rDNA, (ii) GTR + I + Γ for the *rbcL* partition, (iii) JC for the ITS rDNA partition, (iv) HKY + I for the *coxI* partition and (iv) HKY + I for the *psaA* partition. The alignment was analysed by BEAST version 1.8.2 (Suchard et al., 2018), with substitution and clock models unlinked among the partitions. Lognormal relaxed clock models were selected for the partitions, and a birth–death diversification process was used as a prior on the distribution of node heights. Four temporal constraints were used to calibrate the phylogeny, following the inferred molecular divergence times published by Siver et al. (2015). These constrains include nodes 5, 9, 14 and 15 in Siver et al. (2015). Five MCMC analyses were run for 50 million generations, sampling every 10,000 generations. After the diagnosis for convergence using Tracer 1.6, the log files were merged using the burn-in set to 10 million generations. Accordingly, the mean rates and coefficients of variation were obtained for ITS rDNA (9.569×10^{-3}, 1.414), *psaA* (2.571×10^{-3}, 0.417) and *coxI* (4.962×10^{-2}, 0.222) loci, respectively. The estimated rates were then applied to infer the phylogenetic position and node ages of *S. sphagnicola* lineages, using the StarBEAST as implemented in BEAST2 version 2.4.7 (Bouckaert et al., 2014). The analysis was performed on the concatenated and partitioned ITS rDNA, *psaA* and *coxI* data set, using only those strains for which all three partitions were successfully sequenced (Supporting Information Table S1). Seven haplotypes were defined in StarBEAST taxon sets (1A, 1B, 1C, 1D, 2A, 2B, 2C), according to the results of haplotype networks. MCMC analyses were

run for 100 million generations, using the same setting as specified above. The convergence diagnostics was performed in Tracer 1.6.

2.3 | Statistical analyses

Although we measured the temperature, pH and conductivity for the most of studied sites, these values are probably influenced by short-term variations occurring as a result of biological activity and climatic deviations. Therefore, more biologically meaningful variables were calculated for each sampling site, using several preprocessed environmental data layers. First, altitude and 19 bioclimatic variables were obtained from the WorldClim database (Hijmans, Cameron, Parra, Jones, & Jarvis, 2005) at resolution of 2.5 arc minutes to characterize the general climatic conditions. Because the studied organisms naturally exhibit temporal changes in their composition and abundance, we additionally retrieved average, minimum and maximum temperatures for the period of 1 month preceding the sampling date, using the timeseries data sets in the Chelsa database (Karger et al., 2017). Finally, seven physical and chemical soil properties were obtained from the SoilGrids database (soilgrids.org): pH, cation exchange capacity, clay, silt and sand content, and organic carbon stock and content. At every sampling site, climatic and habitat data were obtained by applying either a 1-km or 5-km buffer to limit the effects of spatial bias. No sampling site was located on a hillside, to prevent inaccurate data buffering. Geographical distance (latitude and longitude) was transformed to the principal coordinates of neighbour matrices (PCNM; Dray, Legendre, & Peres-Neto, 2006) via the distGPS and pcnm functions in BoSSA (Lefeuvre, 2018) and vegan (Oksanen et al., 2016) packages in R (version 3.3.3; R Development Core Team), respectively.

The relative effects of climate, habitat and geographical distance on the variance in *Synura* diversity were analysed using variation partitioning in redundancy analyses, with the varpart function in vegan package in R. The effects of environmental and spatial factors were analysed at three levels of diversity, using the affiliation of samples into (i) two main *S. sphagnicola* lineages, (ii) four sp1 haplotypes 1A–D and (iii) three sp2 haplotypes 2A–C, as a response variable. The analyses were run on the subset of 48 samples for which the affiliation into one of the seven sp1 and sp2 haplotypes was recovered (Supporting Information Table S1). To identify significant explanatory variables associated with the tested levels of *Synura* diversity, forward selection by redundancy analysis was used prior to variation partitioning, using the ordiR2step function in vegan package in R.

The morphological analyses were based on 15 cultures randomly selected in lineage sp1 (seven cultures) and lineage sp2 (eight cultures). The cultures were inoculated into fresh media, and after 4–5 weeks of cultivation they were examined with a JEOL 1011 transmission electron microscope. For each of these cultures, the five characters were measured in at least 40 randomly selected scales, as follows: (i) scale length, (ii) scale width, (ii) spine length, (iv) spine width and (v) rim width. The morphological differentiation of *S. sphagnicola* lineages was evaluated using principal component analyses (PCOs). Linear discriminant analysis (LDA) was used to test for the morphological distinction of the lineages. We further evaluated the LDA by randomly selecting three-quarters of scales, estimating the parameters on the training data, and classifying the remaining quarter scales of the hold-out sample. We repeated this 100 times. All the analyses were performed in R, using the packages scales (Wickham, 2017), VEGAN, MASS (Ripley, Venables, Hornik, & Ripley, 2015), and DISCRIMINER (Gaston & Sanchez, 2013).

3 | RESULTS

3.1 | Phylogenetic relationships and timing of divergence

A total of 71 strains were isolated from various water bodies in Newfoundland, Korea, the Czech Republic, Norway and British Isles

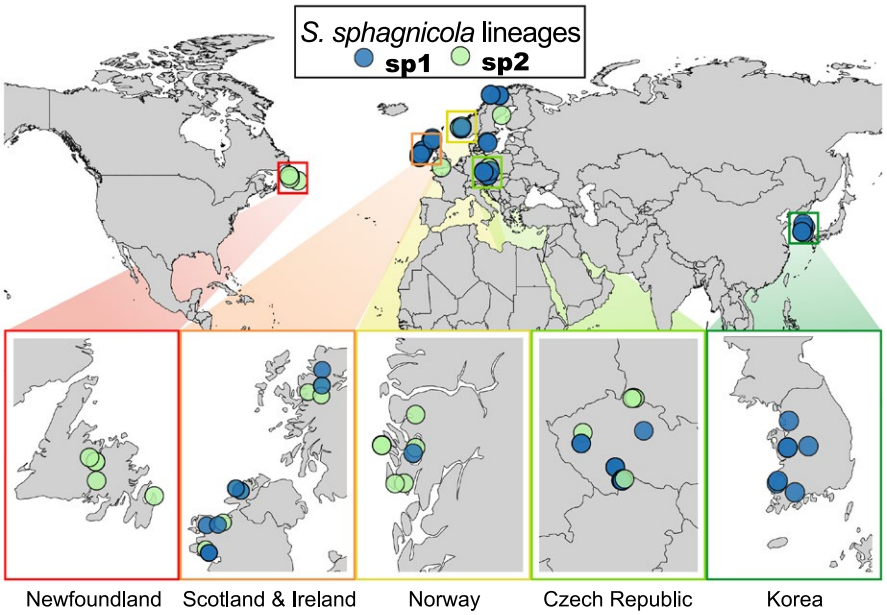

FIGURE 1 Distribution of the two main *Synura sphagnicola* lineages. Five regions are enlarged in inset figures for better clarity [Colour figure can be viewed at wileyonlinelibrary.com]

(Figure 1). All strains were inferred to be closely related, forming a single, monophyletic group (Figure 2a). The clade was split into two distinct lineages at about 15.4 (± 8.4) Ma, here referred to as sp1 and sp2. These lineages frequently co-occurred in European areas, but were not found together in Newfoundland and Korea (Figure 1). Additional molecular diversity was revealed within both of these lineages, by sequencing fast evolving ITS rDNA, *psaA* and *coxI* loci (Figure 2b,c). Four haplotypes were identified within lineage sp1. Haplotypes 1A and 1B were closely related, differentiated only by the *psaA* locus. Whereas 1A occurred only in the Czech Republic, the latter haplotype was isolated from the Czech Republic, Sweden, Scotland and Ireland (Figure 3). Haplotypes 1C and 1D were recognized by ITS rDNA and *psaA* loci, and both were endemic to Korea. Molecular diversity within lineage sp2 was recovered only by the *coxI* locus, identifying three haplotypes. Haplotypes 2A and 2B were closely related, the former sampled in the Czech Republic, Scotland and England, and the latter distributed in the Czech Republic, Norway and Newfoundland (Figure 3). Haplotype 2C, occurring in northern Europe (Scotland, Ireland, Sweden and Norway), was rather distantly related to the other haplotypes.

Because ITS rDNA, *psaA* and *coxI* loci fragment the molecular diversity within the sp1 and sp2 lineages differently, we inferred the putative, time-calibrated species tree of *Synura sphagnicola* haplotypes based on all haplotype data. The resulting species tree strongly supported the differentiation of all haplotypes, providing evidence for their recent radiation in the Pleistocene (Figure 2c). Using the haplotype-specific mean rates, we estimate sp1 and sp2 diverged 17.7 (± 12.1) Ma, which corresponds closely to the 15.4 (± 8.4) Ma estimate based on the fossil-calibrated phylogeny of Synurales (Figure 2a).

3.2 | Explaining patterns in molecular diversity

To assess the role of environmental and spatial processes on the pattern of observed genetic diversity, we performed variation

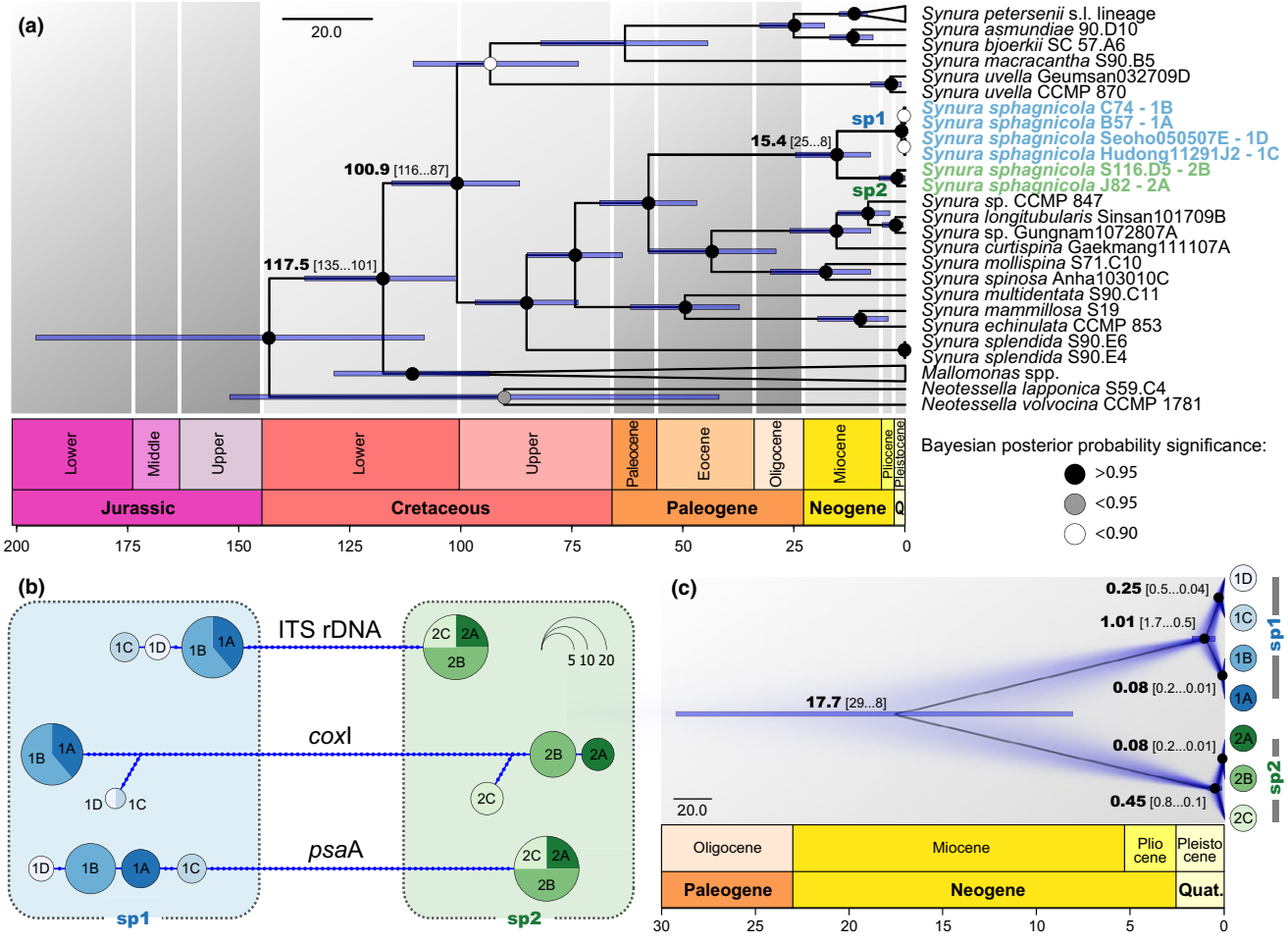

FIGURE 2 Phylogenetic relationships and timing of divergence. (a) Time-calibrated phylogeny for the Synurales based on concatenated SSU rDNA and *rbcL* sequences. Lineages sp1 and sp2 are given in blue and green, respectively. (b) Statistical parsimony networks of four sp1 and three sp2 haplotypes based on the analysis of three loci sequences. Circle sizes are proportional to haplotype frequency. Lines between haplotypes are single mutational steps; small dots indicate missing haplotypes. (c) Time-calibrated coalescence species tree with visualization of the most probable topologies. In (a) and (c), mean divergence times are given for selected nodes, along with 95% highest posterior density (HPD) values in square brackets. HPD values are illustrated for each node by blue bars. Time axis is million years ago, along with chronological dating of geological intervals [Colour figure can be viewed at wileyonlinelibrary.com]

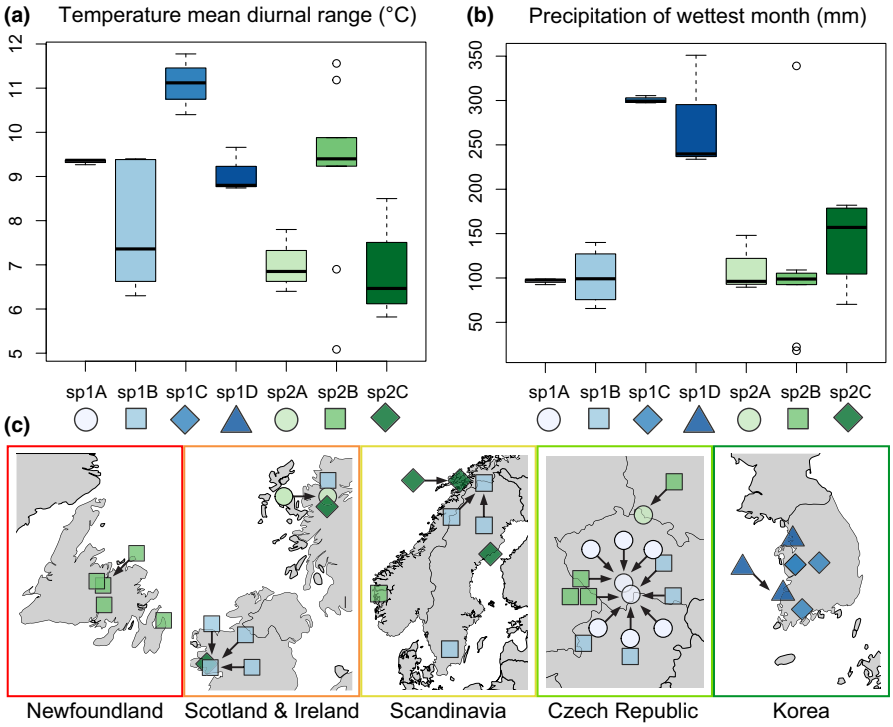

FIGURE 3 Ecology and distribution of seven sp1 and sp2 haplotypes. Differences in the distribution of seven *Synura sphagnicola* haplotypes along the gradient of (a) mean diurnal temperature range (BIO2) and (b) precipitation of wettest month (BIO13). (c) Distribution patterns in five investigated regions. Multiple samples at the same location are presented by arrows [Colour figure can be viewed at wileyonlinelibrary.com]

partitioning analyses on three levels of diversity (Figure 4). At the level of lineages sp1 and sp2, only the net effect of climate made a significant contribution to explaining the overall variation ($p = 0.029$; Figure 4a). Season precipitation (BIO15) and wettest month precipitation (BIO13) were the main factors explaining the divergence of the lineages. The spatial and habitat differences were nested within the effect of climate. Most of the variation (64%) remained unexplained, suggesting that the lineages are highly correlated to their environmental preferences and spatial distribution.

In contrast, when analysing the diversity within the recently diverging haplotypes there was much less unexplained variance (29% and 22% for sp1 or sp2, respectively). The haplotypes were well differentiated by their geography and climate (Figures 3 and 4b,c). Interestingly, the patterns of variation in partitioning were very similar, irrespective of explaining the variability within lineage sp1 or sp2. The largest part of the variation was explained by the spatial pattern (sp1 haplotypes: 32% net effect, $p = 0.001$; sp2 haplotypes: 41% net effect, $p = 0.010$), followed by the climatic factors (sp1 haplotypes: 16% net effect, $p = 0.004$; sp2 haplotypes: 18% net effect, $p = 0.043$). Mean diurnal temperature range (BIO2) and wettest month precipitation (BIO13) were the most informative bioclimatic variables in the model.

3.3 | Morphological analyses

All strains morphologically well fit the circumscription of *S. sphagnicola*: they formed multicellular colonies composed of two-flagellated cells up to 11(–16) μm in diameter, often filled with bright red granules (Figure 5a). The cells were covered by a layer of imbricate scales composed of a perforated basal plate with an upturned rim and an apical spine (Figure 5b). PCO clearly separated lineages sp1 and sp2, based on the morphometric characteristics of silica scales analysed in 15 cultures (Figure 5c). LDA revealed significant morphological differentiation of the lineages, indicating that all but one morphological feature (spine width) was significant for lineage recognition ($p < 0.00001$), with spine length being the most discriminant character (Wilk's $\lambda = 0.43$, $F = 1063.4$). Indeed, the average spine length was 1.58 ± 0.31 in sp1 and 2.64 ± 0.56 in sp2 (Figure 5d). The predictive discrimination of individual scales on the basis of their morphology reached 94.1%. In addition to the distinction between lineages sp1 and sp2, we also detected apparent differences among the cultures belonging to the same lineages (Figure 5c). This variation cannot be explained by the origin of analysed strains, as the morphologically most similar strains were isolated from geographically distantly related and ecologically distinct localities. It appears that the morphological variation could rather be attributed to the distinction between the recently diverging haplotypes, although additional measurements based on much larger samples are required to support this explanation.

4 | DISCUSSION

4.1 | Protist speciation

In general, speciation models assume that species may diverge either with or without gene flow, or by secondary contact between the diverging species (Richards, Servedio, & Martin, 2018). Gene flow restriction is the most commonly documented mechanism (Sobel, Chen, Watt, & Schemske, 2010), occurring in both spatially

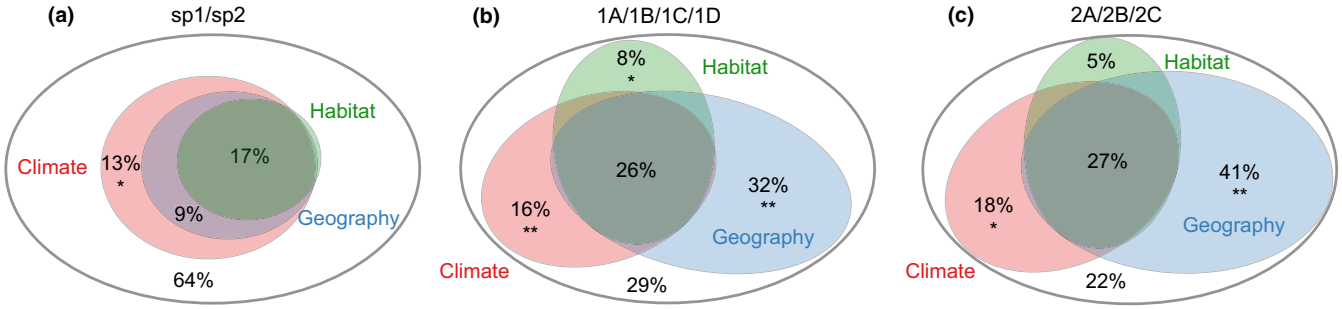

FIGURE 4 venn diagrams showing the relative effects of climate, habitat and geographical distance on the variance in *Synura* diversity. Areas not covered by coloured ellipses represent the portion of unexplained variability. (a) Differences between lineages sp1 and sp2. (b) Differences among the sp1 haplotypes. (c) Differences among the sp2 haplotypes. Significant net effects are indicated by asterisks (*$p < 0.05$, **$p < 0.01$). Values lower than 1% are not shown [Colour figure can be viewed at wileyonlinelibrary.com]

structured (allopatry) and well-mixed, initially randomly mating populations (classical sympatry). However, species may also emerge in sympatry when gene flow is present (Richards et al., 2018). Interestingly, genomic studies based primarily on animal models show that high levels of genome-wide divergence can be established along with ongoing gene flow (Martin et al., 2013; Ravinet et al., 2018). Unfortunately, no such genomic studies have been performed in protists so far, with the exception of investigations based on yeasts (Leducq et al., 2016). From a genetic point of view, isolating barriers may be based on the divergent ecological selection of alleles (e.g., habitat and temporal isolation), cytological divergence (e.g., polyploidization, chromosome rearrangements) or species hybridization (Futuyma, 2013).

Herein, we had an excellent opportunity to document the patterns in evolutionary divergence in eukaryotic microorganisms (protists), based on our investigations of spatial and ecological structuring of genetic diversity in *S. sphagnicola*. We were able to overcome several obstacles that have limited research in this field so far. First, it is usually very difficult to trace recently derived protist lineages in nature due to their small size and paucity of differentiating morphological features. Second, detailed comparative research requires labour-intensive cultivation practices. We were able to circumvent these issues due to the pre-existing, well-characterized phylogeny of the genus *Synura* (Jo et al., 2016; Škaloud et al., 2014) with a concurrent well-established fossil record (Siver et al., 2015; Siver, Wolfe, Rohlf, Shin, & Jo, 2013). This enabled us to set up reliable calibration points on several diversification events. Although logistical constraints prevent investigation of hundreds of strains, the observed differentiation of lineages in organisms studied herein is so strong that it cannot be considered to be the result of random processes.

The results of our variation partitioning analyses clearly show that the most recently diverged haplotypes differ significantly by their distribution and ecological preferences. Because the distribution patterns of recently derived haplotypes probably reflect the possible causes of speciation (Barraclough & Nee, 2001), allopatric and divergent ecological speciation seems to represent a major speciation force in *S. sphagnicola* despite the assumed enormous population sizes and unlimited dispersal of this protist taxon. Specifically, we showed that clear restriction in gene flow was maintained between geographically differentiated populations, despite only recently diverging. However, how can populations be resistant to gene flow under the continuous influx of immigrant cells? One possible explanation could lie in the unfavourable conditions to which the protists are exposed during their long-distance transport. Drought, freezing and ultraviolet radiation may be lethal for protist propagules, making it impossible for them to colonize geographically remote new habitats. Indeed, freshwater diatoms were recognized to be extremely sensitive to desiccation stress (Souffreau et al., 2010). On the other hand, ~200 viable airborne protist taxa were recorded around the world (Sharma, Rai, Singh, & Brown, 2007), indicating that under some microclimatic and physicochemical conditions microorganisms may be able to successfully colonize remote habitats.

However, high migration rates do not necessarily prevent speciation processes. In plants and animals, several examples of species divergence have been reported for populations occurring over local distance scales, even under a high migration frequency. For example, sexual selection (Elmer, Lehtonen, & Meyer, 2009; Higashi, Takimoto, & Yamamura, 1999) or disjunction in flowering time (Savolainen et al., 2006), which are usually tightly coupled with ecological specialization (Feder et al., 1994; Mallet, Meyer, Nosil, & Feder, 2009), are among the best-documented examples of speciation maintained in the face of gene flow. In protists, which are characterized by rather rare sexual reproduction, population differentiation may be related to mechanisms that effectively prevent immigrant genotypes from being incorporated into local populations, such as the founder effect of resident species that have monopolized the resources (De Meester, Gómez, Okamura, & Schwenk, 2002). Indeed, numerous studies have determined the importance of the founder effect in structuring bacterial (Svoboda, Lindström, Ahmed Osman, & Langenheder, 2018), fungal (James & Vilgalys, 2001) and even protist (Weisse et al., 2011) communities. Protists are generally characterized not only by high dispersal rates but also by rapid growth rates and short generation times. Rapid population growth combined with the formation of seed banks may result in a strong numerical

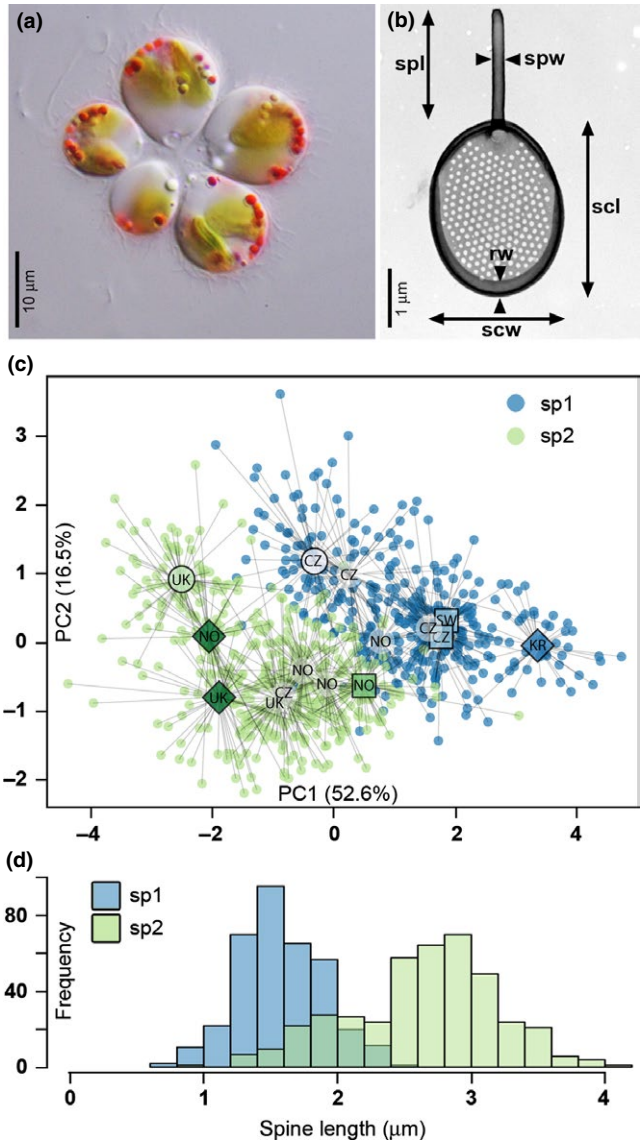

FIGURE 5 Morphological analyses. (a) Light micrograph of a colony of *Synura sphagnicola*. Note the layer of scales encircling the cells. (b) Transmission electron micrograph of a silica scale (scl – scale length, scw – scale width, spl – spine length, spw – spine width, rw – rim width). (c) Principal component analysis ordination of 810 silica scales showing the morphological distinction of lineages sp1 and sp2. The scales are clustered based on their affiliation to 15 investigated strains, and their haplotype identity (if known) and geographical origin are provided by symbols (see Figure 4) and country acronyms (CZ – Czech Republic, KR – Korea, NO – Norway, SW – Sweden, UK – United Kingdom). (d) The distribution of spine lengths in lineages sp1 and sp2 [Colour figure can be viewed at wileyonlinelibrary.com]

advantage for the first colonizers. In addition, rapid adaptation of populations to local environments may strongly enhance the priority effect that hinders immigration (Urban & De Meester, 2009). The combination of frequent clonal and rare sexual reproduction, typical for the majority of protist species, represents a powerful tool for promoting local adaptation (De Meester et al., 2002).

In addition to the dominant spatial pattern of recent *S. sphagnicola* haplotypes, a significant part of the overall genetic variability has been explained by the net effect of climatic and habitat factors. This may represent one of the consequences of speciation following founder effect; in other words, the ecological differentiation of closely related species to local conditions. However, this pattern may also relate to the divergent ecological selection that occurs during sympatry. Indeed, closely related *S. sphagnicola* genotypes were sometimes found to occur in sympatry (Figure 3c). In protists, the above-mentioned combination of frequent clonal and rare sexual reproduction may primarily facilitate a specific mechanism of ecological speciation referred to as "periodic selection" (Atwood, Schneider, & Ryan, 1951; Cohan, 2001; Maynard Smith & Haigh, 2008). This is characterized by periods of relaxed selection followed by bouts of pronounced selection, yielding a vast reduction of genetic diversity (Cohan, 2001). However, if a new mutant differs in its ecological niche, it could escape the diversity-purging effects of periodic selection, giving rise to the new, ecologically defined species (Škaloud & Rindi, 2013).

4.2 | Revisiting the species concept of protists

The proper delimitation of species is an essential requirement for both biodiversity assessments and a correct understanding of their ecology, biogeography and evolutionary history. As is the case for almost every group of organisms, the species concept in protists has been discussed in many publications yet remains highly controversial (Boenigk et al., 2012; Leliaert et al., 2014; Malavasi et al., 2016). The great majority of protist species are still delimited morphologically, although morphology alone is generally insufficient to distinguish natural species units (Hoef-Emden, 2007; Škaloud & Rindi, 2013). However, this concept has frequently been applied in proposing and testing hypotheses of distribution patterns and speciation mechanisms in protists (Fenchel, 2005; Finlay & Fenchel, 1999), which can lead to confusing inferences.

Importantly, the lack of clear morphological delineation between recently derived taxa of protists may obscure evolutionary relationships and tempos of change. This bias, based on the morphological perception of species, is well demonstrated when comparing macro- and microorganisms. Estimated divergence times of plant or animal sister species generally range from tens of thousands to several million years, with a considerable proportion of species originating during the Pleistocene glaciations (Johnson & Cicero, 2004; Wallis, Waters, Upton, & Craw, 2016). For example, estimated speciation times span 0.03–4.1 Ma in birds (Johnson & Cicero, 2004), 0.002–4.6 Ma in cichlids (Genner et al., 2007; McCune, 2001), 0.5–1.5 Ma in sea stars (Foltz, Nguyen, Kiger, & Mah, 2008), ~1.5 Ma in frogs (Sasa, Chippindale, & Johnson, 1998), and <1 Ma in endemic cyprinodontid and semionotid fishes, fruit flies and *Tetragnatha* spiders (McCune, 2001). In addition, extremely fast speciation times, evolving within thousands of generations, have been observed in numerous plant and animal taxa (Hendry, Nosil, & Rieseberg, 2007).

In contrast to macroorganisms, the estimated divergence times of sister protist species are usually much longer. Dinoflagellate

species divergence varies from 5.5 to 32 Ma (Leaw et al., 2016; Lowe, Martin, Montagnes, & Watts, 2012), diatom divergence times then generally span 2.9–11.7 Ma (Brown & Sorhannus, 2010; Sörhannus, Fenster, Hoffman, & Burckle, 1991; Whittaker, Rignanese, Olson, & Rynearson, 2012). Of note, more recent divergence times were estimated for those protist groups producing a variety of beautifully ornamented shells or scales, such as Radiolaria (~1.7 Ma; Ishitani, Ujiié, de Vargas, Not, & Takahashi, 2012) and Coccolithophores (0.3–5.6 Ma; Sáez et al., 2003). Clearly, the scarcity of morphological features in protists lead to ignoring recently diverged lineages and recognizing rather evolutionary old lineages as species units, often artificially lumping together ecologically well-differentiated young lineages. In addition, because increasing phylogenetic resolution correlates well with detecting geographically restricted lineages (Bass & Boenigk, 2011), such taxonomic bias can fundamentally influence our estimates of the principal mechanisms of speciation towards the higher probability of allopatric speciation.

In addition, because morphological change often lags behind ecological change, morphologically defined taxa may mask cryptic, ecologically distinct lineages, leading to erroneous estimations of their ecological niches (Malavasi et al., 2016; Škaloud & Rindi, 2013). Considering our model, *S. sphagnicola* is currently regarded as a broadly distributed species occurring in a wide variety of mainly acidic water bodies (Siver, 1989). However, our data indicate it may consist of several both geographically and ecologically distinct entities. Although additional sampling is needed to support our observations, the majority of haplotypes exhibit nonrandom distribution patterns (Figure 3c). For example, it appears that haplotype 1A is restricted to central Europe, whereas haplotypes 1C and 1D are distributed in more humid and warmer Korean environments. Similarly, haplotypes 2A and 2C appear to be restricted in their distribution to colder regions of Europe.

We feel that our perception of protist species should be revised considerably in terms of distinguishing between much more recent and morphologically similar, but genetically, spatially and ecologically isolated lineages. It is highly probable that numerous protist species originated during the Quaternary, as a consequence of a fragmentation of previously contiguous populations by the advances of glaciers and associated sea-level variations. Indeed, some striking examples of Pleistocene protist speciation have been documented, for example in the marine foraminifer *Globigerinoides ruber* (Aurahs, Grimm, Hemleben, Hemleben, & Kucera, 2009), marine diatom *Pseudo-nitzschia pungens* (Casteleyn et al., 2010), freshwater diatom *Stephanodiscus yellowstonensis* (Theriot, Fritz, Whitlock, & Conley, 2006), and brackish–freshwater dinoflagellates *Peridinium aciculiferum* and *Scrippsiella hangoei* (Logares et al., 2007).

5 | CONCLUSIONS

We report rapid diversification of a freshwater microbial eukaryote that occurred during the late Pleistocene, resulting in several geographically and ecologically diverse populations. Our study shows that ecological and allopatric speciation seem to occur much more readily in microbial eukaryotes than was previously thought. The colonization of new geographical regions or ecological resources seems to promote protist speciation, despite their enormous population sizes and cosmopolitan dispersal. Although it is generally difficult to compare speciation times of microorganisms and macroorganisms due to their different generation times, these findings imply they may be equivalent, contrary to hypotheses that suggest the slow evolution of protist species (Fenchel & Finlay, 2003, 2006). Future studies using detailed, genome-wide population analyses, such as restriction-site associated DNA sequencing, will help us to better understand the ecology of evolutionarily young haplotypes and their roles in ecosystem functioning, as well as to establish a solid taxonomy based on the identification of ecologically and evolutionarily meaningful units.

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AUTHOR CONTRIBUTIONS

Conceptualization, P.Š. and P.Dv.; methodology, P.Š.; formal analysis, P.Š. and P.Do; investigation, M.Š., P.Do., J.I.K. and W.S.; resources, P.Š., M.Š., P.Do., J.I.K. and W.S.; writing – original draft, P.Š.; writing – review and editing, P.Š., M.Š. and P.Dv.; visualization, P.Š.; funding acquisition, P.Š. and W.S.

DATA ACCESSIBILITY

DNA sequence data have been submitted to the DDBJ/EMBL/GenBank databases under accession numbers MK322768–MK322930. DNA alignments are freely available on Mendeley Data: http://dx.doi.org/10.17632/83dcg6ypp4.1.

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