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COI gene and ecological data suggest size-dependent high dispersal and low intra-specific diversity in free-living terrestrial protists (Euglyphida: *Assulina*)

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ABSTRACT

Aim Propagule size and ecological requirements are believed to be major factors influencing the passive dispersal of free-living terrestrial protists. We compared the colonization potential of three closely related testate amoeba species (*Assulina muscorum*, *A. seminulum*, *A. scandinavica*, ranging from 40 to 100 μm in length).

Location Europe.

Methods We collected individual *Assulina* species cells from *Sphagnum* peatlands across Europe. We sequenced a 550-bp fragment of the mitochondrial cytochrome *c* oxidase subunit I gene (COI) to estimate the within-species variability, as a proxy for gene flow. We reviewed existing ecological and palaeoecological data to assess the ecological tolerance of *Assulina* species and how rapidly they colonized developing peatlands.

Results We obtained COI sequences for 30 individuals of *A. seminulum* from eleven sites, for 39 of *A. muscorum* from six sites, and for six of *A. scandinavica* from two sites. We observed three haplotypes for *A. seminulum* and two for *A. muscorum*, often co-existing in the same sites. The sequences of *A. scandinavica* from the two sites were identical. *Assulina muscorum* and *A. seminulum* haplotypes varied by only 1–2 nucleotides, resulting in >99.5% similarity. Genetic diversity within *A. seminulum* was higher than that within *A. muscorum*. Ecological and palaeoecological records showed that *A. muscorum* was much more frequent and abundant than *A. seminulum*, and had a somewhat broader ecological tolerance for pH, moisture and water-table depth. *Assulina muscorum* always appeared early during the developmental history of peatlands, either before or simultaneously with *A. seminulum.*

Main conclusions The lack of genetic structure observed with a variable marker such as COI suggests high gene flow between the sites and thus rapid transport (at an evolutionary scale) over large distances, in agreement with the palaeoecological records. Thus, geographical distance alone does not seem to prevent the dispersal of testate amoebae, at least within Europe. Nevertheless, genetic diversity was significantly lower within *A. muscorum* than within *A. seminulum*, suggesting that its smaller size and abundance and/or broader ecological tolerance influence its effective dispersal capacity. These results are in agreement with the often earlier colonization of peatlands by *A. muscorum* and its broader ecological tolerance.

Keywords ***Assulina*, COI gene, dispersal, Europe, palaeoecological records, peat bog, *Sphagnum*, terrestrial, testate amoebae, Western Palaearctic.**

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INTRODUCTION

The existence or absence of biogeographical patterns in the distribution of microorganisms is a long-standing yet unresolved debate. Defenders of the 'ubiquity theory' claim that all microorganisms can be found anywhere on Earth as long as suitable conditions are met (Finlay & Clarke, 1999; Finlay *et al.*, 1999, 2001; Finlay & Fenchel, 2004), while proponents of the 'moderate endemicity theory' claim that at least some species have limits to their distribution ranges (Foissner, 2006; Smith & Wilkinson, 2007; Foissner *et al.*, 2008; Smith *et al.*, 2008; Van de Vijver & Mataloni, 2008; Vanormelingen *et al.*, 2008). Considerable efforts have been invested over the last decade to acquire the new data needed to inform this debate (Caron, 2009). Several terrestrial testate amoeba species are increasingly cited as examples corroborating the moderate endemicity theory, because some morphologically very distinctive species, such as *Apodera vas* (=*Nebela vas*), have been shown to present truly geographically limited ranges (Smith & Wilkinson, 2007; Smith *et al.*, 2008). In most other cases, however, morphology alone cannot be used to delimit reliable taxonomic units (Heger *et al.*, 2009), and studies based on intra-morphospecies genetic diversity are urgently needed (Heger *et al.*, 2010a).

The main dispersal agent of testate amoebae is thought to be the wind (Wilkinson, 2001), but other vectors such as birds, mammals, insects or human activities have been reported for other soil protists (Revill *et al.*, 1967; Schlichting & Sides, 1969; Charalambidou & Santamaría, 2002; Wilkinson, 2010). Several factors have been proposed in the literature to explain the distribution range of testate amoebae: test (shell) size (Wilkinson, 2001; Yang *et al.*, 2010), population size (Finlay & Fenchel, 2004) and the ability to form drought-resistant propagules (cysts) (Corliss & Esser, 1974; Foissner, 1987). The ecological tolerance spectrum (Mitchell & Meisterfeld, 2005) and the spatial distribution of favourable habitats in the landscape are also potential, but less often discussed, factors. The potential habitat of a species with a narrow ecological spectrum can be hypothesized to be patchier than the generalists, and these species will therefore be less likely to reach a suitable habitat by random dispersal.

The distribution of species observed today can be considered as a snap-shot of a pattern changing with time (Lara *et al.*, 2008; Smith *et al.*, 2008). Factors controlling this pattern therefore also influence the pace at which new environments are colonized as well as the genetic patterns at different spatial scales. A propagule from a large, rare, drought-sensitive species that is restricted to a very particular environment will theoretically have few chances to be transported to a new suitable habitat and to form a new population. These events will thus happen rarely. Conversely, a small, abundant, cyst-forming species with a broad ecological spectrum will have many chances to form new populations. Thus, we can expect that the first species will build new populations at a slower rate than the second; in other words, it will disperse more slowly. A second corollary of that example is that, as propagules move more slowly in the first case than in the second, mutations will also spread more slowly, and we can thus expect a higher degree of genetic differentiation among populations than in the second case (assuming that selective pressure is homogeneous in all cases and that generation times are similar).

In this study we aimed to bring new elements to the cosmopolitanism versus endemism debate by estimating the colonization potential of three closely related species of euglyphid terrestrial testate amoebae (Lara *et al.*, 2007): (1) *Assulina scandinavica* Penard, 1890, a large (*c.* 100 μm long and 1.9×10^5 μm^3), rare species restricted to *Sphagnum* habitat; (2) *A. seminulum* (Ehrenberg, 1848) Leidy, 1879, a medium-sized (*c.* 85 μm long and 0.55×10^5 μm^3), more frequent but also ecologically restricted species; and (3) *A. muscorum* Greeff, 1888, a small (*c.* 45 μm long and 0.12×10^5 μm^3), frequent and tolerant species (Fig. 1). We compared the genetic differentiation among populations at the European scale and reviewed ecological and palaeoecological data to determine the colonization pace of these species during peatland development and the breadth of their respective ecological niches.

MATERIALS AND METHODS

Sampling

Sphagnum mosses potentially containing *Assulina* species were collected from 13 locations in northern and central Europe (Fig. 2; Table 1). Cells were then extracted from wet *Sphagnum* mosses and isolated using inverted microscopy. Only living

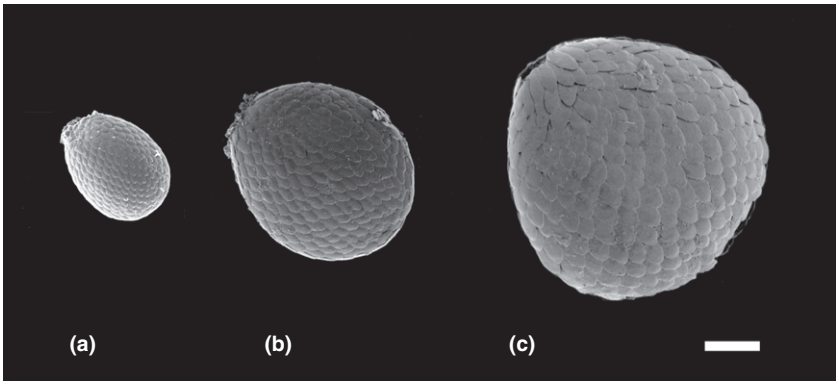

Figure 1 Scanning electron microscope images of (a) *Assulina muscorum*, (b) *A. seminulum* and (c) *A. scandinavica*. The three cells are represented at the same scale. Scale bar = 20 μm.

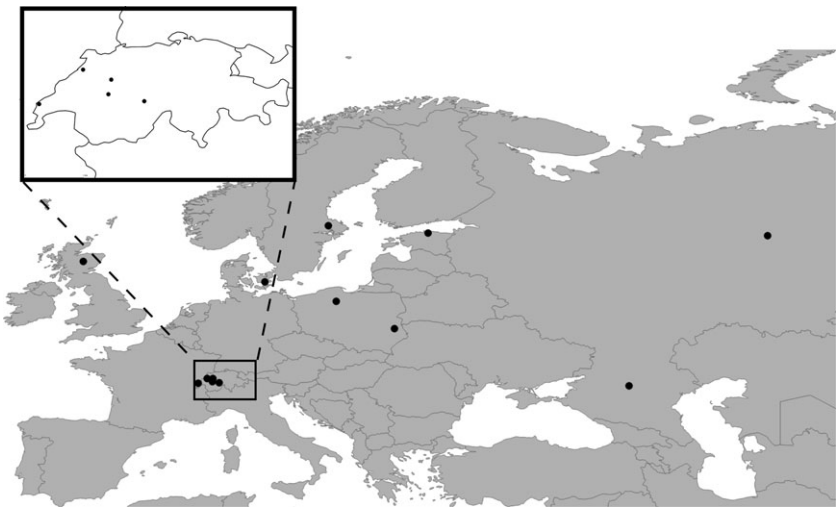

Figure 2 Map of the study area showing the locations of our 13 sampling sites of *Assulina* species in Denmark, Estonia, Poland, Russia, Switzerland (inset), Sweden and the United Kingdom.

Table 1 List of sites from which *Assulina* taxa were sampled for COI gene sequencing.

				Haplotypes					
				A. muscorum		*A. seminulum*			
Site	Country	Latitude	Longitude	AM1	AM2	AS1	AS2	AS3	*A. scandinavica*
Praz Rodet	Switzerland	46°34′ N	6°10′ E	3	0	2	1	0	0
Sortel	Switzerland	46°44′ N	7°22′ E	0	0	0	1	0	0
Lörmoos	Switzerland	46°59′ N	7°25′ E	18	0	3	2	0	0
Grindelwald	Switzerland	46°37′ N	7°59′ E	0	0	0	2	0	0
Chaux d'Abel	Switzerland	47°59′ N	6°55′ E	2	0	0	0	0	3
Glenn Dee	UK	56°59′ N	3°39′ W	0	0	0	0	0	3
Holmegard Moose	Denmark	55°16′ N	11°52′ E	0	0	1	0	0	0
Ryggmossen	Sweden	60°02′ N	17°19′ E	0	0	0	1	0	0
Bory Tucholskie	Poland	53°36′ N	18°00′ E	3	2	3	0	0	0
Moszne	Poland	51°16′ N	22°60′ E	0	0	1	1	2	0
Verkhozimskie	Russia	43°04′ N	46°23′ E	4	0	5	1	0	0
Zuratkul	Russia	54°53′ N	59°13′ E	7	0	0	0	0	0
Pikassaare	Estonia	59°25′ N	25°51′ E	0	0	0	4	0	0

cells were taken: these could easily be recognized by the presence of a nucleus surrounded by small vesicles of granular appearance. Each cell was isolated with a narrow-diameter pipette under an inverted microscope, washed in distilled water, deposited individually (one cell per tube) inside a polymerase chain reaction (PCR) tube containing 10 μL of PCR reaction buffer with concentrations of 1.5 mM $MgCl_2$ and 10 nmol of each dNTP (deoxynucleotide triphosphate) (Promega), and stored at −20 °C or processed immediately for the PCR reaction. Scanning electron microscopy (SEM) of *Assulina* species was performed as described in Heger *et al.* (2009).

PCR and sequencing

Polymerase and primers were added subsequently to the tubes containing the cells to perform the PCR reaction (15 μL containing 1.5 mM $MgCl_2$, 10 nmol of each dNTP, 20 pmol of each primer and 1 U Taq DNA polymerase; GoTaq, Promega Madison, WI, USA). A first PCR was performed using Eucox1F and Euglycox1R, a pair of euglyphid-specific cytochrome *c* oxidase subunit I (COI) primers designed in a previous study (Heger *et al.*, 2010b). The cycling profile consisted of a 5-min initial denaturation step (95°); followed by 40 cycles of 95 °C for 15 s, 40 °C for 30 s, and 72 °C for 90 s; and a final extension at 72 °C for 10 min. *Assulina*-specific primers were designed for a second PCR (Assucox 1F: 5′-AAYATGAGRGCYAGRGG-3′ and Assucox 1R: 5′-CGTAATGAAARTGWCCYACC-3′). A nested PCR protocol was applied using these last two primers for both *Assulina muscorum* and *A. seminulum*. The cycling profile was a 2-min initial denaturation step (95 °C); followed by 40 cycles of 95 °C for 15 s, 52 °C for 30 s, and 72 °C for 90 s; and a final extension at 72 °C for 10 min. In the case of *A. scandinavica*, a combination of Assucox 1F and Euglycox 1R was used for the second step of the nested protocol; the cycling profile was the same, except for the annealing temperature (45 °C). The PCR

products were purified using either a High Pure PCR Purification Kit (Roche, Basel, Switzerland) or a Wizard® SV Gel and PCR Clean-Up System (Promega), and directly sequenced without a cloning step. Sequencing was carried out using a BigDye197 Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Carlsbad, CA, USA) and analysed with an ABI-3130xl DNA Sequencer (Applied Biosystems). COI sequences were deposited in GenBank with the accession numbers HM641248–HM641252. Sequences were manually aligned and compared, and sequence identity values were obtained using the software BioEdit 7.0.9.0 (Hall, 1999). The COI sequences were easily aligned, as no insertions or deletions were detected.

Genetic diversity patterns of *A. muscorum* and *A. seminulum* were evaluated using the software DnaSP (Rozas *et al.*, 2003). The following standard genetic diversity indices were calculated: number of segregating sites (*S*); number of haplotypes (*h*); haplotype diversity (*Hd*); average number of differences (Π) and nucleotide diversity (π). Standard diversity indices are measures of the frequency of each haplotype in the sample (Nei & Jin, 1989). Haplotype diversity *Hd* is defined as the probability that two randomly chosen haplotypes are different in the sample. The mean number of pairwise differences (Π) is defined as the mean number of differences between all pairs of haplotypes in a sample. Nucleotide diversity (π) is defined as the mean number of nucleotide differences per site between two sequences. In addition, in order to test if there is a correlation between the number of haplotypes observed and the number of sampling sites, we performed a Kendall test on the data (i.e. a nonparametric rank test) using the R software package (R Development Core Team, 2008).

Review of present-day and palaeoecological data

We reviewed 767 published present-day (surface samples) and palaeoecological samples from Europe and Alaska taken from the following publications (original sources for these data): Mitchell *et al.*, 1999, 2001; Lamentowicz & Mitchell, 2005; Payne *et al.*, 2006; Payne & Mitchell, 2007; Lamentowicz *et al.*, 2008, 2010a,b. In addition, we analysed unpublished data from Karelia and Penza regions (kindly provided by Yuri Mazei, Department of Zoology and Ecology, Penza VG Belinsky State Pedagogical University). We used the present-day and palaeoecological data to evaluate: (1) frequency of presence, (2) relative dominance, (3) relative abundance, and (4) density of *A. muscorum* and *A. seminulum*. We used the present-day data to evaluate the ecology [optimum and tolerance for depth to water table (DWT), percentage moisture, and pH] of *A. muscorum* and *A. seminulum* in peatlands. We used the palaeoecological data to assess: (1) how rapidly *Assulina* species colonized peatlands during their developmental history, (2) which species appeared first out of *A. muscorum* and *A. seminulum* (*A. scandinavica* is too rarely reported to allow any meaningful interpretation), and (3) in cases where several species co-occurred, how many years separated their respective first records.

RESULTS

Molecular data

Our PCR protocol allowed us to amplify sequences of *c.* 550 bp. Sequences from *A. muscorum* showed the existence of two haplotypes (AM1 and AM2), which differed by a single position, representing 0.2% of the sequence divergence. In total, 37 (95%) sequences belonged to haplotype AM1, and two (5%) to haplotype AM2. In the case of *A. seminulum*, we discovered three haplotypes (AS1, AS2 and AS3), which differed by 1–2 nucleotides (see Fig. 3), and two sites exhibiting single nucleotide polymorphism (SNP); this represents between 0.2 and 0.4% of the sequence divergence. The haplotypes were distributed in the following manner: 15 belonged to AS1 (50%), 13 to AS2 (43%) and 2 to AS3 (7%). For *A. scandinavica*, we found only one haplotype (i.e. all six sequences were identical). There was no obvious geographical pattern for the distribution of the various haplotypes encountered: AS1 was found in six sites located in four countries, and AS2 in eight sites in five countries. In addition, some sites hosted several haplotypes: for *A. seminulum* this was the case for Lörmoos (AS1 and AS2), Praz Rodet (AS1 and AS2), Moszne (AS1, AS2 and AS3) and Verkhozimskie (AS1 and AS2). For *A. muscorum*, AM1 and AM2 were found in Bory Tucholskie (Table 1, Figs 1 & 2). The diversity indices show that *A. seminulum* was genetically more diverse than

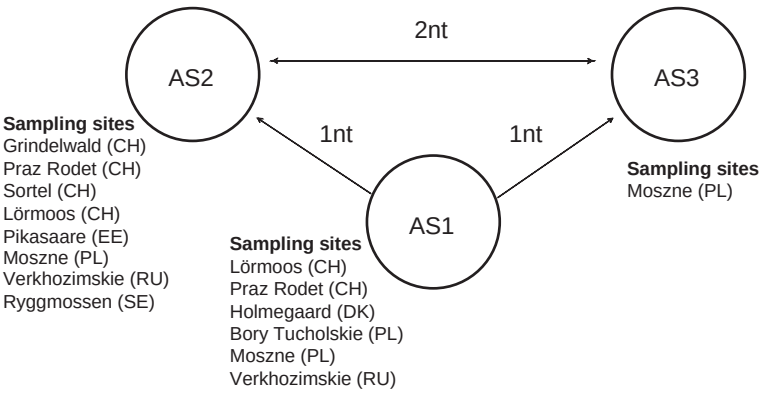

Figure 3 Representation of the three haplotypes found in *Assulina seminulum* (AS1, 2 and 3) and the differences observed between sequences. The sampling sites at which they were found are also indicated. nt denotes a single nucleotide difference.

Table 2 Values obtained for the various genetic diversity estimators within *Assulina muscorum* and *A. seminulum* using DnaSP (Rozas *et al.*, 2003), with the number of segregating sites (*S*), the number of haplotypes (*h*), haplotype diversity (*Hd*), the average number of differences (Π) and nucleotide diversity (π).

Species	Individuals	*S*	*h*	*Hd*	Π	π
Assulina seminulum	30	2	3	0.577	0.637	0.00115
Assulina muscorum	40	1	2	0.097	0.097	0.00018

A. muscorum (Table 2). All observed SNPs concerned only silent mutations (i.e. they occurred in the third position of the codon and therefore did not change the amino acid sequence), except for the mutation that differentiated AS1 from AS2, which was responsible for a change between a valine and an alanine (two hydrophobic amino acids). The Kendall test showed that there was no significant correlation between the number of sampling sites and the number of haplotypes in the case of *A. muscorum* ($P = 0.720$, $n = 40$ for the global analysis; if we exclude the Lörmoos site from which we retrieved 18 sequences, $P = 1.000$). However, this relationship proved to be almost significant for *A. seminulum* ($P = 0.057$, $n = 30$).

In contrast to the low level of within-taxon genetic diversity, the three species were clearly distinct from each other. The percentage of similarity of the nucleotide sequences (calculated by direct comparison of the sequences excluding the gaps and non-alignable parts if encountered) was 84% between *A. seminulum* and *A. muscorum*, 82% between *A. seminulum* and *A. scandinavica*, and 79% between *A. muscorum* and *A. scandinavica*. When translated into amino acid sequences, the percentages of similarity are, respectively, 95% between *A. seminulum* and *A. muscorum*, 88% between *A. seminulum* and *A. scandinavica*, and 87% between *A. muscorum* and *A. scandinavica*.

Ecological data

Assulina species are very common in surface moss samples as well as in subfossil samples from peatlands in Europe and Alaska. Indeed, *A. muscorum* was the most frequent testate amoeba species listed in a compilation of European and North American data, while *A. seminulum* ranked fourth (Gilbert & Mitchell, 2006). Of the 767 peat samples analysed in this study, at least one *Assulina* species was observed in 650 samples (84.7%). Furthermore, *A. muscorum* and *A. seminulum* co-occurred in 52.5% of samples, whereas *A. muscorum* was found alone in 30.2%. *Assulina seminulum* was found alone in only 2.0% of the samples. Furthermore, *A. muscorum* was more abundant than *A. seminulum* in 86.9% of the samples in which both species were observed, whereas *A. seminulum* was more abundant than *A. muscorum* in only 10.6% of these cases (Table 3). *Assulina scandinavica* was not found in these samples and is in fact only rarely recorded in ecological studies.

Assulina muscorum was also systematically more abundant than *A. seminulum* in all samples for which abundance was recorded. This was the case in terms of both relative abundance (percentage of total number of testate amoebae) and density (cells per gram of dried material; Table 4). When present, *A. scandinavica* was much less abundant than *A. seminulum* (data not shown).

Table 3 Summary statistics of presence and relative dominance of *Assulina muscorum* versus *A. seminulum* in surface and palaeoecological peat samples from Europe and Alaska ($n = 767$). See text for the list of corresponding references.

Samples with:	Total *n*	%
Assulina spp.	650	84.7
A. muscorum only	223	30.2
A. seminulum only	13	2.0
A. muscorum and *A. seminulum*	403	52.5*
A. muscorum > *A. seminulum*	565	86.9*
A. seminulum > *A. muscorum*	69	10.6*
A. muscorum = *A. seminulum*	16	2.5*

*Percentage of samples containing both *Assulina* species.

Present-day ecological studies also reveal that *A. muscorum* generally occurs in slightly drier and less acidic microsites than *A. seminulum* and has a broader tolerance with respect to the ecological gradients to which testate amoebae have been shown to respond most strongly: moisture content of mosses, water-table depth and pH. However, the differences in optima were not systematic, and differences in tolerance were mostly small: in some cases *A. seminulum* had an optimum in drier microsites and a slightly larger tolerance than *A. muscorum* (Table 5).

Palaeoecological data

We analysed 13 published palaeoecological studies of peat bogs (Table 3). In 38% of cases, *A. muscorum* and *A. seminulum* were observed for the first time in the same sample. They are thus recorded in these cases as having arrived 'at the same time', although it is clearly impossible to ascertain this, given the relatively low temporal resolution of the records, especially in the basal part of the peat deposits. In 62% of cases, *A. muscorum* appeared before *A. seminulum*, and we did not find a single study in which *A. seminulum* appeared first. Based on the age–depth chronologies of the studies, we estimated the time lag between the appearance of *A. muscorum* and *A. seminulum* to be between 30–50 years and 7500 years. In the latter case, unfavourable ecological conditions over much of the developmental history of the peatland are suspected to be the main explanation for the absence of *A. seminulum* (Table 6).

DISCUSSION

The genetic diversity observed within the three species examined in this study can be considered as very low. The

Table 4 Relative abundance and density of *Assulina muscorum* and *A. seminulum* in surveys of present-day communities.

Location	*A. muscorum*	*A. seminulum*	Reference
Toolik Lake (Alaska, USA), poor fen, control plots of nutrient enrichment experiment	9337 (1634)	1118 (1118)	Mitchell, 2004
Alps of northern Italy, *Hylocomium splendens* along elevational transects	4299 (608)	47 (33)	Mitchell *et al.*, 2004
Swiss Jura Mountains, Le Cachot bog	12.9% (2.3)	1.2% (0.5)	Mitchell & Gilbert, 2004
North-west Poland, peatlands	19.1% (6.1)	2.2% (1.6)	Lamentowicz & Mitchell, 2005
Swiss Jura Mountains, Le Cachot bog	11.8% (2.4)	3.8% (1.3)	Kishaba & Mitchell, 2005
Swiss Jura Mountains, Chaux d'Abel bog	3146 (954)	459 (231)	Laggoun-Défarge *et al.*, 2008
Swiss Jura Mountains, Chaux d'Abel bog	18.7% (4.6)	1.6% (1.0)	Laggoun-Défarge *et al.*, 2008
Swiss Eastern Alps, Subalpine peatlands	14.4% (2.0)	2.6% (0.7)	Lamentowicz *et al.*, 2010b
European & North American peatlands	8.5% (0.5)	2.3% (0.3)	Gilbert & Mitchell, 2006

Data are either densities (expressed as individuals per gram dry weight of moss) or relative abundances (expressed as percentage of total testate amoeba community; indicated by %); values in brackets are standard errors of the means.

Table 5 Optimum and tolerance values from ecological studies of *Assulina muscorum* and *A. seminulum* for percentage moisture, depth to water table (DWT) and pH.

		A. muscorum		*A. seminulum*		
Location	Variable	Optimum	Tolerance	Optimum	Tolerance	Reference
Jura (Switzerland and France)	Percentage moisture	34	19	38	18	Mitchell *et al.*, 1999
Europe	Percentage moisture	*c.* 89	+	*c.* 90.5	−	Charman *et al.*, 2007
Canada (NE Ontario)	Percentage moisture	*c.* 68	(−)	*c.* 70	(+)	Charman & Warner, 1992
Canada (Lake Superior)	Percentage moisture	*c.* 72	+	*c.* 71	−	Booth, 2001
Jura (Switzerland and France)	DWT (cm)	59	49	38	33	Mitchell *et al.*, 1999
Finland	DWT (cm)	16.6	11.6	10.9	7.57	Tolonen *et al.*,1992
Europe	DWT (cm)	*c.* 18	+	*c.* 18.5	−	Charman *et al.*, 2007
Canada (Lake Superior)	DWT (cm)	*c.* 15	+	*c.* 17	−	Booth, 2001
Canada (Newfoundland)	DWT (cm)	17.44	11	13.95	9.68	Charman & Warner, 1997
Canada (NW Ontario-Minnesota)	DWT (cm)	42.53	25.59	39.29	17.84	Charman & Warner, 1997
Jura (Switzerland and France)	pH	4.3	0.3	4	0.1	Mitchell *et al.*, 1999
Finland	pH	4	0.5	4	0.48	Tolonen *et al.*, 1992
Canada (Lake Superior)	pH	4.6	+	4.2	−	Booth, 2001

Optima are calculated by the weighted average of species' relative abundance (see Mitchell *et al.*, 1999 for the formula). Signs '+' and '−' indicate which species had the highest tolerance; when differences are minimal, these signs are in brackets.

0.2 and 0.4% of intraspecific COI sequence divergence observed for *Assulina* species is far below the threshold of 2–3% generally accepted for discriminating species of Metazoa (Hebert *et al.*, 2003). Our values of intraspecific genetic divergence are comparable to those observed in scleractinian corals, which are well known for the conservation of their mitochondrial genes (Shearer *et al.*, 2002). However, in contrast to these cnidarians, interspecific variation is important in the genus *Assulina*, reaching about 20% between the three species that constitute this genus. COI is indeed quite variable in euglyphids: a recent study of another euglyphid genus, *Cyphoderia*, showed that this gene was three times more variable than small subunit ribosomal RNA (SSU rRNA; Heger *et al.*, 2010b). Within the genus *Assulina*, COI might be relatively even more variable than in *Cyphoderia*: we observe as much as 97.2% sequence identity between *A. muscorum* (AJ418791) and *A. seminulum* (EF456749) at the SSU rRNA gene level and only 84% at the COI level (in other words, COI appears more than five times more variable than SSU rRNA).

In addition, it appears that some haplotypes are 100% identical, even though they originated from sites as far from each other as Praz Rodet (Switzerland), Pikassaare (Estonia), Ryggmossen (Central Sweden) and Verkhozimskie (Russia), as in the case of AS2 for *A. seminulum*, or Glenn Dee (Scotland) and Chaux d'Abel (Switzerland) for *A. scandinavica*. These locations represent separations of thousands of kilometres, mostly over apparently unsuitable habitat (i.e. not *Sphagnum* peatlands), especially for *A. seminulum* and *A. scandinavica*. Most interestingly, several cells collected from the same site belonged to different haplotypes. Assuming that COI is sensitive enough to reveal the genetic structure of *Assulina* populations, our data strongly suggest that cells can move

Table 6 Palaeoecological records of *Assulina muscorum* and *A. seminulum*: position of the first record of *A. muscorum* from the base of the record, number of samples between the first records of *A. muscorum* and *A. seminulum*, and estimated corresponding time lag.

Country	Location	Context	*A. muscorum* present in *X*th oldest sample	Lag between first records of *A. muscorum* and *A. seminulum* (number of samples)	Lag between first records of *A. muscorum* and *A. seminulum* (years)	Reference
Belgium	Ipenrooi, Valley of River Mark	Holocene sequence	2	4	380–480	Beyens, 1985
USA	Upper Michigan	Holocene sequence	1	9	300	Booth *et al.*, 2004
Scotland	Traligill Basin, blanket peat – UAM1	Last *c.* 2700 years	1	3	30–50	Charman *et al.*, 2001
Scotland	Traligill Basin, peat above cave entrance – UAM4	Last *c.* 2200 years	8	0	0	Charman *et al.*, 2001
Estonia	Männikjärve bog	Last 60 years	1	0	0	Charman *et al.*, 2004
Switzerland	Praz-Rodet	Holocene sequence	6	2	100–150	Mitchell *et al.*, 2001
Poland	Tuchola	Holocene sequence	2	0	0	Lamentowicz *et al.*, 2008
Scotland	Temple Hill Moss	Last *c.* 7000 years	1	1	100–200	Langdon *et al.*, 2003
Québec, Canada	Tourbière du lac Malbaie – Core Mal-1	Holocene sequence	2	26	7500*	Lavoie & Richard, 2000
Québec, Canada	Tourbière du lac Malbaie – Core Mal-2	Holocene sequence	26	0	0	Lavoie & Richard, 2000
Québec, Canada	Tourbière du lac Malbaie – Core Mal-3	Holocene sequence	9	10	4500*	Lavoie & Richard, 2000
Northern Ireland	Glen West, County Fermanagh	Last *c.* 2500 years	2	25	2300*	Swindles *et al.*, 2007
New Zealand	Eweburn Bog	Holocene sequence	2	0	0	Wilmshurst *et al.*, 2003

*Habitat not suitable for *A. seminulum* over much of this period (probably too minerotrophic).

quite rapidly across Europe. Another explanation would be recent selective sweeps (Hurst & Jiggins, 2005) that might have occurred in all three *Assulina* species and eliminated or strongly reduced genetic diversity.

Several other protists also present low genetic variability of the COI gene across large geographical distances. In ciliates, it has been observed that isolates of *Tetrahymena thermophila* from relatively distant geographic origins within North America [Woods Hole (MA) and Allegheny National Forest (PA)] have COI sequences that diverge by less than 1% (Lynn & Strüder-Kypke, 2006). Strains of *Paramecium multimicronucleatum* originating from Italy, Germany and Australia presented identical COI sequences (Barth *et al.*, 2006), suggesting that these species frequently cross major biogeographical barriers. *Carchesium polypinum*, another ciliate, did not show patterns of COI genetic diversity associated with geographical distance at the Grand River basin scale, in North America (Gentekaki & Lynn, 2009). The marine prasinophyte *Micromonas pusilla* also presented cosmopolitan COI haplotypes (Šlapeta *et al.*, 2006), and identical COI sequences of the freshwater diatom *Sellaphora capitata* have been found in Belgium and Scotland (Evans *et al.*, 2007). Thus, the reduced intraspecific genetic diversity found in other protists is consistent with our observations in *Assulina* species, whose ability to travel rapidly across large distances hence appears unsurprising. Such results can be seen as corroborating the 'ubiquity theory', providing evidence for the more or less instantaneous dispersal of microbial propagules across the globe. The conclusions drawn from all these aquatic species forming small propagules can thus be extended to the larger terrestrial testate amoebae. However, it is possible that genetic markers that are even more sensitive than COI, such as microsatellites, could reveal more about the biogeography of *Assulina* species, as shown for freshwater diatoms (Evans *et al.*, 2009).

In our study, all diversity estimators revealed a higher genetic diversity in *A. seminulum* than in *A. muscorum* (Table 2). *Assulina scandinavica*, the largest, rarest species and the only one whose test length is around the 100-μm threshold proposed for the cosmopolitan distribution of testate amoebae (Wilkinson, 2001), did not present any SNPs in the six sequences we analysed. This might suggest a rapid migration capacity in this species also. However, we were able to retrieve only a limited number of cells, and it would be necessary to sample more sites and retrieve more individuals to assess the genetic diversity in this species at the COI level. This is consistent with our hypothesis that a rarer, larger, more specialized species that forms smaller populations is expected to spread more slowly than a more frequent, smaller, more tolerant species that forms larger populations.

These results are partly corroborated by our observations of ecological and palaeoecological data, which show that: (1) *A. muscorum* is more frequent than *A. seminulum* in a large data set from European and North American peatlands, and, where it co-occurs with *A. seminulum*, it is in most cases the more abundant species (see Tables 3 & 4); and (2) *A. seminulum* became established at the same time as or later than *A. muscorum* during the developmental history of the studied peatlands (see Table 6).

The higher abundance of *A. muscorum* could be attributed to its smaller size. Within closely related taxa, smaller organisms can be expected to reach higher densities than larger ones. The higher frequency of *A. muscorum* could, on the other hand, also be explained by its broader ecological tolerance and preference for more common microenvironmental conditions (e.g. drier microsites). Although there is indeed a trend for an optimum (in relative abundance) in drier, less acidic microsites and for a higher tolerance at least with respect to the two most commonly measured variables, pH and water table depth, these differences in ecological preferences were not as clear as for population size and abundance (Table 5). Furthermore, when other ecological variables are compared, the pattern is even more pronounced (e.g. see Tolonen *et al.*, 1992).

In some cases (Tourbière du Lac Malbaie cores 1 and 3, Lavoie & Richard, 2000; and Glenn West, Swindles *et al.*, 2007), changes in the ecology of these peatlands could be held responsible for the absence of *A. seminulum* for a long time. Indeed, other indicators have shown that these sites were relatively minerotrophic. This may have prevented the establishment of this species. However, as existing ecological data did not reveal any marked difference in ecological optima between the two species it is not clear if these palaeoecological patterns can be held responsible for the absence of *A. seminulum*. In all other records, a lag of between 0 and 480 years has been observed between the appearance of *A. muscorum* and of *A. seminulum*. We can thus reasonably consider that *A. muscorum* spreads more rapidly than *A. seminulum* (and probably even more rapidly than *A. scandinavica*, although there are not enough data for this species to draw any firm conclusion).

These data suggest that new suitable environments can be colonized within at most a couple of hundred years, depending probably on factors such as distance to the closest population nucleus, competition of migrants with locally established populations (although this effect seems to be marginal, if it exists at all; Wanner & Xylander, 2005; Wanner *et al.*, 2008), direction of dominant winds, migratory patterns of birds, and, of course, 'chance events', as attested by the critical importance of the tail-end of propagule dispersal distribution functions for explaining the rapid post-glacial colonization patterns of trees (Clark, 1998).

The available data may not allow us to draw definitive conclusions about the causes of the observed patterns. However, (1) the clear differences in size (and hence even more in biovolume and biomass) and abundance (either relative or density) observed between *A muscorum* and *A. seminulum*; and (2) the comparatively smaller differences in optima and tolerance for the main ecological gradients suggest that size (and related population size) matters more than the (relatively small and partly inconsistent) differences in ecological requirements in determining the dispersal potential of *Assulina* species.

CONCLUSIONS

Molecular and palaeoecological data suggest that the terrestrial euglyphid testate amoebae of the genus *Assulina* are capable of rapid colonization of new habitats. However, both lines of evidence also suggest that the colonization pace was slower in *A. seminulum*, a larger, less abundant, and ecologically slightly more specialized species than *A. muscorum*. Propagule size and to a lesser extent biotic constraints of species therefore influence the dispersal capability of *Assulina* species. Further studies combining molecular and ecological data are required to determine the degree to which this may also hold true for testate amoebae in general and for other microbial species.

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