Published in Journal of Biogeography 38, issue 10, 1897-1906, 2011
which should be used for any reference to this work

Rediscovery of *Nebela ansata* (Amoebozoa: Arcellinida) in eastern North America: biogeographical implications

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ABSTRACT

Aim The question whether free-living protists are generally cosmopolitan is currently a matter of debate. In this study we investigate the geographical distribution of a distinctive testate amoeba species, *Nebela ansata*, and use our data to assess the potential for highly restricted distribution patterns in some protist species.

Location Global.

Methods We analysed (1) 3400 testate amoeba publications from North America and other continents, (2) unpublished slides of the Penard Collection of the Natural History Museum, London, UK, and (3) 104 *Sphagnum* samples from eastern North America. Non-metric multidimensional scaling (NMDS) was used to visualize the similarities in testate amoeba community composition among 1012 North American samples, including two communities that contained *N. ansata*.

Results We rediscovered *N. ansata* at a site in New Jersey located close to its type locality, and in Nova Scotia. We also report the existence of an apparently unpublished museum specimen originally collected from New Jersey. Our extensive literature survey confirmed the presence of this species only in the temperate part of eastern North America. The NMDS revealed that communities with *N. ansata* were less similar to each other than to communities from other parts of North America, suggesting that favourable habitats for *N. ansata* occur in other *Sphagnum*-dominated peatlands, a habitat type that has been extensively sampled in North America and elsewhere.

Main conclusions These data provide an unusually convincing case of a free-living microorganism with a very limited distribution range in the temperate part of eastern North America. The remarkably restricted distribution of *N. ansata* highlights the extent of our ignorance about the natural history of free-living microorganisms, and raises questions about the lack of attention to microbial diversity in conservation biology.

Keywords

Biodiversity, biogeography, conservation biogeography, eastern North America, endemism, microbial diversity, *Nebela ansata*, protist, testate amoebae.

INTRODUCTION

In contrast to macroscopic organisms, for which limited dispersal is common and therefore local endemism frequent, microorganisms are often considered to be mostly cosmopolitan, and therefore the presence of a given species at a given location is controlled only by its tolerance to local environmental conditions (Sandon, 1927; Finlay, 2002;

Fenchel & Finlay, 2004). However, the degree of cosmopolitanism in microorganisms has been the subject of considerable debate in recent years (e.g. (Finlay, 2002; Foissner, 2006; Smith & Wilkinson, 2007; Evans *et al.*, 2009; Boo *et al.*, 2010). Based on morphologically defined taxa, there is now substantial evidence that at least some microorganisms have restricted distributions unrelated to differences in ecological conditions, leading to the concept of regional endemism for at least some free-living microorganisms (Foissner, 2006; Smith & Wilkinson, 2007; Stoeck *et al.*, 2007; Smith *et al.*, 2008; Vanormelingen *et al.*, 2008). The often cited examples of the latter case are terrestrial testate amoebae of the Arcellinida group, *Apodera* (*Nebela*) *vas* or *Certesella* spp., which have been found only in the Southern Hemisphere and the tropics (Smith *et al.*, 2008; Heger *et al.*, 2011). Indeed, *A. vas* is now used as a 'textbook' example of a free-living microorganism of limited distribution (Cox & Moore, 2010), even though its Southern Hemisphere + tropical distribution pattern is still quite widespread in comparison to many macroscopic organisms. It remains unclear whether some protists have highly restricted distributions comparable in scale to the regional endemism observed in some macroscopic organisms such as plants.

Arcellinida are protozoa in which the single cell is enclosed within a shell – usually referred to as a 'test'. The tests of these amoebae are morphologically distinctive, allowing the identification of morphospecies even in fossils (Foissner & Schiller, 2001; Mitchell *et al.*, 2008). Some taxa, such as *Nebela ansata*, have particularly characteristic shells. Joseph Leidy (b. 1823, d. 1891), one of the great American naturalists of the 19th century (Warren, 1998; Corliss, 2001), first described *N. ansata*, writing that it was 'distinguished by the spur-shaped appendages projecting from the sides of the neck' (Leidy, 1879a; p. 158). Such appendages are very unusual in testate amoebae, making *N. ansata* easily identifiable even by non-specialists. Joseph Leidy found this 'remarkable'-looking shelled protozoan while making microscopical investigations of samples from bog moss (*Sphagnum*) collected from cedar swamps near Absecom (Leidy, 1874, 1876, 1879a) and Malaga, Gloucester County (Leidy, 1879b), in New Jersey, USA. Since these initial investigations, only three publications have reported finding the species, at a site near the original Leidy locality (Stokes, 1882; Hoogenraad & de Groot, 1952) and at a site near Lanoraie, in southern Quebec (Fantham & Porter, 1945).

In this study we assessed whether the Arcellinida testate amoeba *N. ansata* has a restricted or widespread distribution by examining (1) previous publications, (2) slides from a specialized museum archive, and (3) new samples from North America. In addition, we provide the first modern description of this taxon, including light and scanning electron microscopy images and a molecular phylogenetic analysis. We discuss the factors that may have contributed to its distribution pattern and we argue that the discovery and rediscovery of *N. ansata*, and its localized distribution, has wide implication for conservation biogeography.

MATERIALS AND METHODS

Literature survey and analysis of unpublished slides

To assess the geographical distribution of *N. ansata*, we first conducted an extensive literature survey of 3400 publications that contain testate amoeba records from North America and other continents. In addition to Leidy's publications, we found original *N. ansata* records in three papers (Stokes, 1882; Fantham & Porter, 1945; Hoogenraad & de Groot, 1952). The '*Nebela* sp. *ansata*?' record reported by Stenroos (1898) was excluded from our *N. ansata* biogeographical analysis because the morphological characteristic of the briefly described specimen did not match with the original *N. ansata* description. In addition to this literature survey, we analysed unpublished slides of the Penard Collection in the Natural History Museum, London, UK, and 104 *Sphagnum* samples from sites in eastern North America.

Sampling sites in eastern North America

Sixty-five samples from *Sphagnum*-dominated peatlands near the original type locality in the Pine Barrens, New Jersey, were collected for this study, as well as 39 samples in Nova Scotia, an area of similar habitat *c.* 1100 km north along the Atlantic coast of North America (Fig. 1).

The Pine Barrens is a region characterized by pine (*Pinus*)- and oak (*Quercus*)-dominated upland vegetation, and is a mosaic of sandy, infertile upland soils, slow-moving streams, and numerous acidic wetlands and peatlands (Collins & Anderson, 1994; Forman, 1998). Cedar (*Chamaecyparis thyoides*) swamps are particularly common in the lowland areas, and, given that Leidy's habitat description for *N. ansata* was a cedar swamp, our sampling was designed to include several of these habitats. The *N. ansata* sampling site was located in a relatively open wetland with scattered small cedar trees (Webb's Mill Bog). The water-table depth and pH at the sampling site were 3 cm and 4.14, respectively. Temperature records from nearby Tom's River (39°96′ N, 74°21′ W) are characterized by an annual mean of 11.7 °C, with average January and July temperatures of −0.44 and 23.8 °C, respectively. The total annual precipitation is *c.* 124 cm, and this is relatively evenly distributed across the course of the year (http://climate.rutgers.edu/stateclim_v1/norms/monthly/index.html, last accessed 1 July 2010).

Peggy's Cove is located on the Atlantic coast *c.* 30 km southwest of Halifax, Nova Scotia (Table 1 and Fig. 1b). The landscape consists of a glaciated coastline of granite shores and numerous glacier erratics on the gently rolling topography. The bedrock in this area is monzo-granite plutonic igneous rocks (Keppie, 1979). Soils are typically shallow and predominantly podzolic, with significant stony and lithic phases (Clayton *et al.*, 1977). The climate in the region is oceanic and relatively mild. It is characterized by warm summers and cold winters with some snow. Climate data from 1975 to 2000 at the nearby Sandy Cove NRC station (44°28′ N, 63°34′ W) show

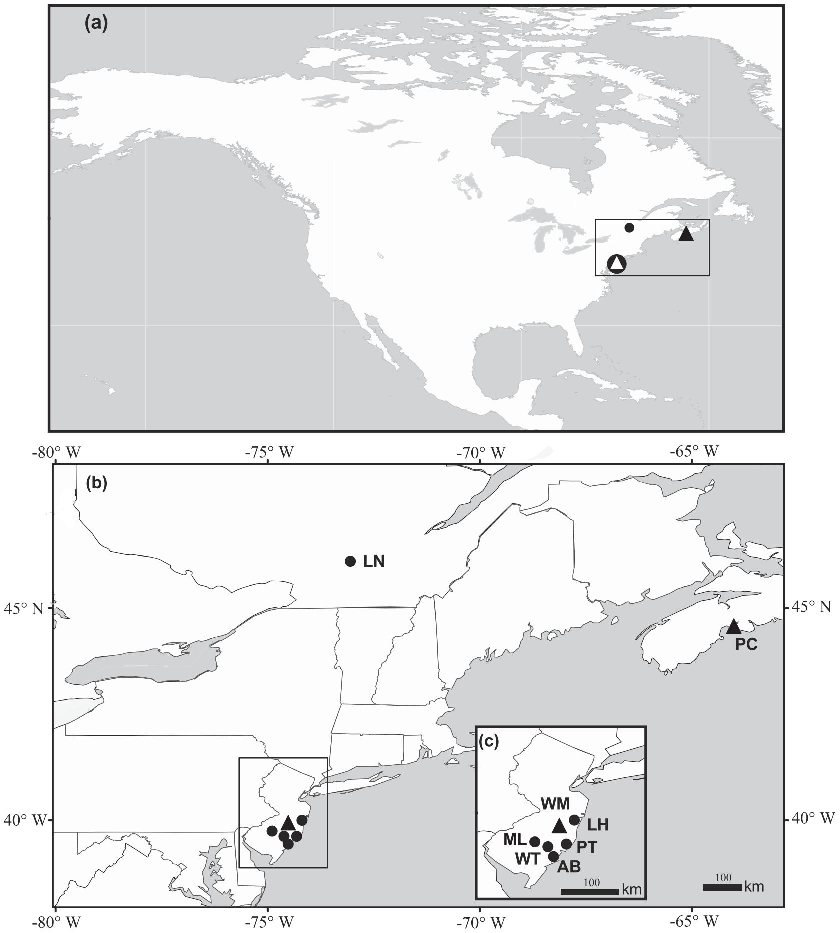

Figure 1 Sketch maps of North America showing (a) the general study area, followed by detailed maps showing (b) *Nebela ansata* records in eastern North America and (c) *N. ansata* records in New Jersey. ML, Malaga (NJ); WT, Weekstown (NJ); AB, Absecon (NJ); PT, Parkertown (NJ); WM, Webb's Mill (NJ); LH, Lakehurst (NJ); LN, Lanoraie (Quebec); PC, Peggy's Cove (Nova Scotia). Circles and triangles represent published and new observations, respectively. Details of sites (including references) are given in Table 1.

that the annual average of daily average temperature is 6.2 °C, with a highest daily average of 16.7 °C in August and a lowest of −3.9 °C in January. Mean annual precipitation is 136 cm, which is relatively evenly distributed throughout the year. The highest recorded snowfall is 28.4 cm in January (Environment Canada, 2010). The sampling site is a few hundred metres inland from the shoreline, on a rolling landscape with prominent outcrops, scattered boulders, and small open ponds in depressions (Fig. 2). Vegetation is mostly dominated by ericaceous and other low shrubs, including *Gaylussacia baccata*, *Nemopanthus mucronata*, *Arctostaphylos uva-ursi*, *Myrica gale*, *Rhododendron groenlandicum*, *Vaccinium oxycoccus*, *Juniperus communis*, and scattered *Alnus* spp. and black spruce (*Picea mariana*). *Pteridium aquilinum* is common in relatively dry areas, and *Sphagnum* spp. are common on wet, peaty soils.

Testate amoeba analyses and ecological context

Water-table depth and pH were measured at each sampling point in New Jersey. To extract testate amoebae, moss samples were shaken energetically in distilled water (Nova Scotia samples) or boiled (New Jersey samples) and then sieved and back-sieved using appropriate mesh sizes (250 or 300 and 10 or 15 μm). The occurrence of *N. ansata* was checked using a light microscope under 200× magnification. In the samples in which *N. ansata* was observed, a total of 100 testate amoebae were then identified to morphospecies at 400× magnification in order to characterize the community context of this species. Hoogenraad & de Groot (1952) also analysed the testate amoeba communities in two samples in which *N. ansata* was recorded, but Leidy (1874, 1876, 1879a,b) did not provide this information. A semi-quantitative assessment of the hydrological conditions of the site where *N. ansata* was found was made by examining the preferences of the recorded species of four samples harbouring *N. ansata* (Hoogenraad & de Groot, 1952; present study). Taxa were assigned to three categories when unambiguous ecological requirements were available (i.e. wet, moist or dry) (see Appendix S1 in Supporting Information) (Charman & Warner, 1992; Tolonen *et al.*, 1994; Mitchell *et al.*, 1999; Booth, 2001, 2008; Booth & Zygmunt, 2005), and a mean value of this index was calculated.

In order to determine whether the communities from the *N. ansata* samples were more similar in composition to each other than to other North American testate amoeba communities, we conducted non-metric multidimensional scaling (NMDS) ordination of 1012 North American samples, including the two *N. ansata* samples from Webb's Mill and Peggy's Cove as well as 1010 North American *Sphagnum*-dominated peatland samples from the mid-continent and eastern North America (Booth, 2002, 2008), the Central Rocky

Table 1 Characteristics of the *Nebela ansata* sampling sites.

Site	Country	Habitat	Coordinates		Number of samples analysed	Number of samples with *N. ansata*	Sampling year	References
Absecon (Absecom), New Jersey	USA	*Sphagnum* mosses	*c.* 39°25′ N	74°30′ W	n.a.	n.a.	≤ 1874	Leidy (1874)
Absecon (Absecom), New Jersey	USA	*Sphagnum* mosses	*c.* 39°25′ N	74°30′ W	n.a.	n.a.	≤ 1879	Leidy (1879a)
Near Malaga, Gloucester County, New Jersey	USA	*Sphagnum* mosses	*c.* 39°34′ N	75°04′ W	n.a.	n.a.	≤ 1879	Leidy (1879b)
Southern part of New Jersey	USA	*Sphagnum* mosses	n.a.	n.a.	n.a.	n.a.	≤ 1882	Stokes (1882)
Lakehurst town, New Jersey	USA	n.a.	*c.* 40°00′ N	74°18′ W	n.a.	n.a.	≤ 1920	Present study
Parkertown (Parkerton), New Jersey	USA	*Sphagnum pylaesii*	*c.* 39°37′ N	74°18′ W	6	2	1916	Hoogenraad & de Groot (1952)
Weekstown, New Jersey	USA	*Sphagnum torreyanum*	39°35′ N	74°36′ W			1933	
Lanoraie, Quebec	Canada	*Sphagnum wulfianum*	*c.* 45°58′ N	73°16′ W	4 (from the Lanoraie region)	1	≤ 1945	Fantham & Porter (1945)
Webb's Mill, Pine Barrens, New Jersey	USA	*Sphagnum* mosses	39°53′ N	74°22′ W	65	1	2008	Present study
Peggy's Cove, Nova Scotia	Canada	*Sphagnum* mosses	44°29′ N	63°53′ W	39	1	2008	Present study

Figure 2 Freshwater wetland near Peggy's Cove, Nova Scotia (Canada).

Mountains (Booth & Zygmunt, 2005), the Southeast (Pocosin) (Booth *et al.*, 2008) and Alaska (Markel *et al.*, 2010). The two *N. ansata* samples described by Hoogenraad & de Groot (1952) were not included in this analysis because quantitative data on testate amoeba communities were unavailable. The analysis was performed using the PC-ORD statistical software package and Sørensen's distance measure (McCune & Grace, 2002). Prior to ordination, percentage data were square-root-transformed to reduce the effects of the most abundant taxa. The abundance of *N. ansata* was not included in the ordination. Proximity of samples in the NMDS ordination space indicates similarity of peatland testate amoeba communities.

Morphometry

To determine the morphometric variability within and among populations of *N. ansata*, specimens from Peggy's Cove and Webb's Mill were analysed by light microscopy and scanning electron microscopy. Scanning electron microscopy (SEM) was performed as described in Heger *et al.* (2009). Five morphological characteristics were measured: shell length (L), pseudostome width (P), length of the horn (H) and shell widths (W1 and W2). W1 corresponds to the width between the ends of the horns and W2 to the width of the *fundus* (width of the terminal body shell). A total of 20 *N. ansata* individuals were analysed for the population from Peggy's Cove, but only eight from Webb's Mill, owing to the low number of specimens available from this site.

DNA extraction, polymerase chain reaction amplification, sequence alignment and phylogenetic analyses

In order to determine the phylogenetic position of *N. ansata*, we prepared four different DNA extractions. Only individuals from Peggy's Cove were used in this analysis. The relatively low number of *N. ansata* individuals present in the Webb's Mill samples was not sufficient for molecular investigation. For each of the four extractions, between 1 and 11 individuals were isolated under a light microscope using fine-diameter glass pipettes. The amoebae were subsequently washed three times in distilled water, and a guanidine thiocyanate protocol was used to extract DNA (Chomczynski & Sacchi, 1987). Three 1142-bp portions of the small subunit ribosomal RNA (SSU rRNA) gene were amplified by nested polymerase chain reaction (PCR) with the universal eukaryotic primers in the first PCR and then using a specific and a universal eukaryotic primer (Appendix S2). The PCR cycling profile was the same for all PCRs: a 30-s initial denaturation step (95 °C), followed by 40 cycles of 95 °C for 30 s, 50 °C for 30 s and 72 °C for 90 s, and a final extension at 72 °C for 10 min. The PCR products were purified using the High Pure PCR Purification Kit (Roche, Basel, Switzerland). 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). The three portions of the *N. ansata* SSU rRNA gene sequence were identical. Sequences were deposited in GenBank with the accession numbers HM050411–HM050413. The three *N. ansata*, 16 Hyalospheniidae and three environmental sequences were manually fitted to a general alignment of eukaryotic SSU rRNA gene sequences (Berney & Pawlowski, 2004) using the BioEdit 7.0.9 sequence alignment editor (Hall, 1999). This last alignment was based on a universal model of eukaryotic SSU rRNA secondary structure (Van de Peer *et al.*, 2000). Ambiguously aligned regions and gaps were excluded from the analyses. Our tree was rooted with the sequence of *Argynnia dentistoma* (EU392158), *Heleopera rosea* (EU392157) and one environmental sequence (GQ330615). The MrAIC 1.4.3 program (Nylander, 2004) identified the Hasegawa–Kishino–Yano with invariable sites and gamma distribution (HKYIG) as the most appropriate model of sequence evolution. Maximum likelihood (ML) analyses were performed using treefinder (Jobb *et al.*, 2004). The bootstrap analysis option was used to assess nodal support on the ML tree (1000 replicates, consensus level 50). Bayesian analysis was performed using the software MrBayes 3.1.2 (Huelsenbeck & Ronquist, 2001). Three simultaneous Markov chains were run for 10,000,000 generations with trees sampled every 10 generations, 250,000 initial trees discarded as 'burn-in'. ML and Bayesian analyses were run through the Bioportal web-based service platform for phylogenomic analysis at the University of Oslo (http://www.bioportal.uio.no, last accessed 1 November 2010).

RESULTS

Occurrence of *Nebela ansata* in eastern North America

We recorded *N. ansata* in the two sampled areas, namely New Jersey and Nova Scotia. The sample containing *N. ansata* from New Jersey was collected from Webb's Mill Bog located *c.* 60 km north of Absecon and Malaga, where Leidy's collections were made (Fig. 1b,c). The second sample containing *N. ansata* was from Peggy's Cove (Nova Scotia) (Fig. 1b). These two newly recorded *N. ansata* populations, as well as the other populations already mentioned in the literature (i.e. Leidy's publications; Stokes, 1882; Fantham & Porter, 1945; Hoogenraad & de Groot, 1952), were all isolated from *Sphagnum* samples collected in *Sphagnum*-dominated peatlands, at low elevation (< 50 m) (Table 1).

A previously unpublished historical record of *Nebela ansata* from New Jersey

There is a slide containing examples of *N. ansata* in the Penard Collection of the Natural History Museum, London. This material was collected by George Herbert Wailes from a site near Lakehurst, New Jersey, and sent to Eugène Penard in Geneva – presumably around the early 20th century. The slide was accessioned into the museum's collection in 1920 but the date of its preparation is unknown. The material on the slide is in outstanding condition considering its age [this is common for Penard's preserved slides (Corliss, 2001)]; however, as the site is near the original localities described by Leidy (1879a) it adds little to our knowledge of the biogeography of this taxon.

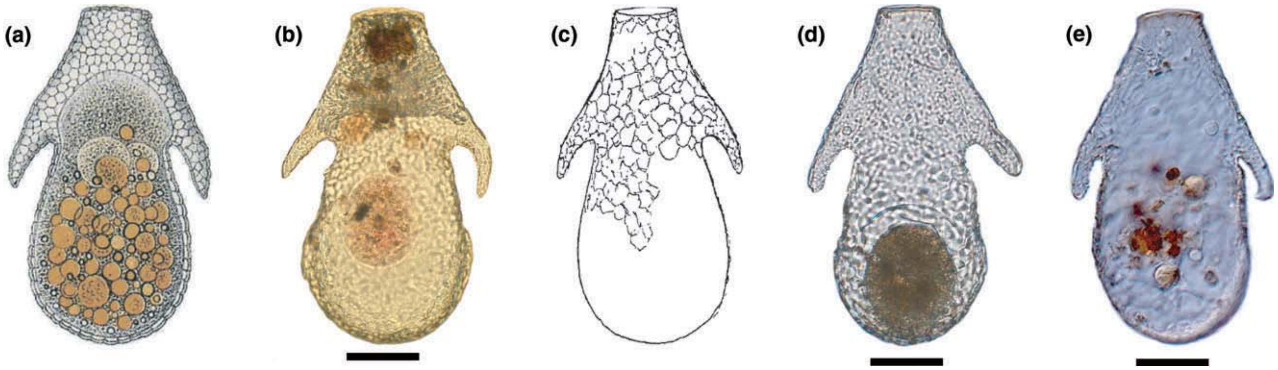

Figure 3 *Nebela ansata*: (a) Absecon, New Jersey (Leidy, 1879a), (b) Lakehurst town, New Jersey (previously unpublished record of the Penard Collection), (c) Weekstown, New Jersey (Hoogenraad & de Groot, 1952), (d) Webb's Mill, New Jersey (present study), and (e) Peggy's Cove, Nova Scotia (present study). Scale bars = 50 μm.

Nebela ansata morphology

Our results demonstrate that *N. ansata* is morphologically well characterized, with the populations from Absecon, Lakehurst town, Webb's Mill, Weekstown, Parkertown and Peggy's Cove being similar based on shape, composition of the shell or morphometric measurements (Figs 3 & 4, Appendix S3). The ranges of lengths (L) of individuals in these six populations were respectively: 220–264, 240 ($n = 1$), 213–253, 233–250, 225–270 and 195–235 μm. Leidy's (1879a) original length measurements ranged from 216 to 260 μm. Further biometric measurements (i.e. pseudostome width, length of the horn and shell widths) are reported in Appendix S3.

Testate amoeba communities of four *Sphagnum* samples harbouring *Nebela ansata*

A total of 62 testate amoeba taxa (including species, infraspecific taxa and morphotypes of uncertain taxonomic affiliation) were recorded in the two samples analysed in this study and the two samples from Hoogenraad & de Groot (1952) (Appendix S1). Of the 62 taxa recorded, four taxa could not be assigned a precise name based on their morphology: *Arcella* sp., *Difflugia* sp., *Euglypha* sp. and *Pseudodifflugia* sp. Besides *Nebela ansata*, only one taxon, *Assulina muscorum*, was found in all four samples. Nine taxa were recorded in three samples, 10 taxa in two samples and 41 taxa in only a single sample. The species richnesses of the Peggy's Cove, Webb's Mill, Weekstown and Parkertown samples were 25, 18, 31 and 25, respectively. Most recorded species were characteristic of wet or moist habitats. However, a small number of species characteristic of dry habitats were also encountered in the samples (Appendix S1).

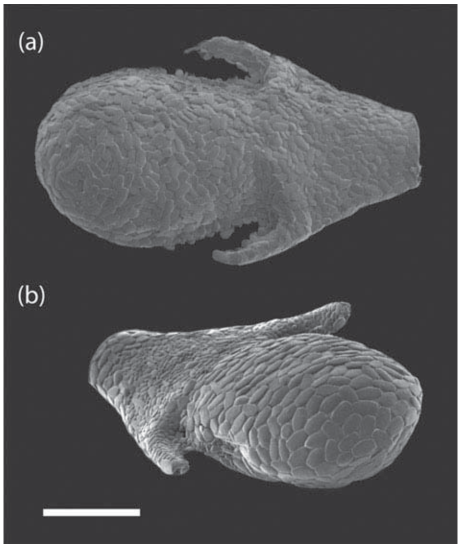

Figure 4 Scanning electron microscopy pictures illustrating *Nebela ansata* specimens from (a) Peggy's Cove, Nova Scotia, and (b) Webb's Mill, New Jersey. Scale bar = 50 μm.

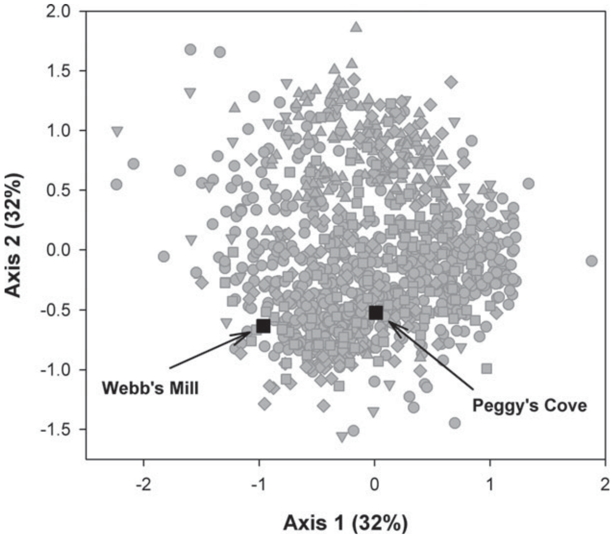

Figure 5 Non-metric multidimensional scaling ordination of 1012 North American samples, including the *Nebela ansata* samples from Webb's Mill (New Jersey) and Peggy's Cove (Nova Scotia). The abundance of *N. ansata* was not included for the ordination. The percentage variance represented by each axis is indicated. Symbols are as follows: circles, mid-continent; point-up triangles, Central Rocky Mountains; point-down triangles, Southeast (Pocosin); squares, Northeast; diamonds, Alaska. Stress = 39.5.

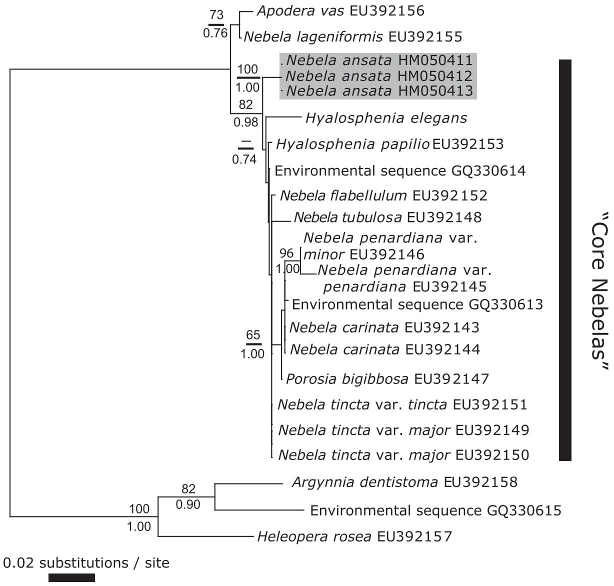

Figure 6 Maximum likelihood phylogeny of 22 small subunit ribosomal DNA Hyalospheniidae sequences based on 775 nucleotide positions. The best-fit model selected in MrAIC (Nylander, 2004) was the Hasegawa–Kishino–Yano with invariable sites and gamma distribution (HKYIG). Numbers represent values of posterior probabilities as calculated with Bayesian analyses and the bootstraps obtained by the maximum likelihood method. A dash indicates that the bootstrap value is lower than 50.

Ordination results

The NMDS analysis of 1012 North American samples showed that the two *N. ansata* samples from Webb's Mill and Peggy's Cove were not very closely related in terms of community structure (Fig. 5). In fact, these two samples were less similar in composition to each other than to many North American samples lacking *N. ansata*, suggesting that apparently suitable habitat for *N. ansata* exists elsewhere in North America.

Molecular phylogeny of *Nebela ansata* as inferred from SSU rDNA

Phylogenetic analyses of the data set containing 22 Hyalospheniidae taxa with three new *N. ansata* sequences demonstrated that *N. ansata* is truly distinct from other species in the *Nebela* genus. The new *N. ansata* sequences branched within a clade composed of two *Hyalosphenia*, one *Porosia (Nebela) bigibbosa*, nine *Nebela* and two environmental sequences (Fig. 6). Within this clade, which corresponds to the 'Core Nebelas' described by Lara *et al.* (2008), the phylogenetic relationships among sequences were not or were only weakly supported. This Core Nebelas clade branched as a sister group to a group containing *Apodera (Nebela) vas* and *Nebela lageniformis*.

DISCUSSION

Despite the unambiguous morphology of *N. ansata* and its occurrence in a well-studied habitat type (*Sphagnum* peatlands), this species had been recorded only three times in temperate eastern North America (Stokes, 1882; Fantham & Porter, 1945; Hoogenraad & de Groot, 1952) since its description by Leidy (if we exclude Leidy's own later observations). However, when we specifically looked for this species in eastern North America, near the place where it was originally described and in comparable habitats further north, we were immediately successful in finding it. Although this testate amoeba was never a dominant taxon in our samples, its comparatively large size and unambiguous morphology allowed us to easily rediscover this species in two samples among a total of 104 eastern North American *Sphagnum* samples. *Nebela ansata* has not been reported between Nova Scotia and New Jersey, although *Sphagnum*-dominated peatlands exist. The absence of *N. ansata* in this area may be a result of undersampling.

In addition to the rediscovery of *N. ansata* in New Jersey and Nova Scotia, our extensive literature survey based on 3400 publications confirmed the absence of this species from the rest of North America as well as from other continents, although suitable habitat probably exists elsewhere in North America (Fig. 5). It is highly unlikely that the testate amoeba taxonomists, palaeoecologists and ecologists who have intensively studied the peatlands and mineral soils of Europe and North America and to a lesser extent of the Southern Hemisphere for over 100 years would have overlooked it because of the characteristic morphology and the relatively large size of this species.

Together, these data provide an unusually convincing case of a free-living microorganism with a very limited distribution range in the temperate part of eastern North America, representing a clear exception to the view that microorganisms have cosmopolitan distributions (i.e. the 'everything is everywhere' hypothesis) and hence low overall diversity (Finlay, 2002). Several other testate amoeba taxa, as well as other microorganisms, have been shown to have limited distribution ranges (Smith & Wilkinson, 2007; Smith *et al.*, 2008; Vanormelingen *et al.*, 2008; Heger *et al.*, 2011), but, to our knowledge, convincing examples of heterotrophic microeukaryotes with the highly restricted endemism exhibited by *N. ansata* are absent from the literature.

Could the current distribution of *Nebela ansata* be a legacy of the Ice Age?

A possible explanation for the distribution of *N. ansata* lies in the unusual history of North American peatlands. The North American east coast represents one of the rare regions in the continent where peatlands are likely to have occurred during the Last Glacial Maximum (Halsey *et al.*, 2000) and probably during much of the Pleistocene. By contrast, the vast expanses of peatlands that currently characterize the northern part of the North American continent – and where *N. ansata* has never been recorded – only developed during the Holocene. Although the factors that govern the distribution of *N. ansata* remain poorly understood, we hypothesize that during the Last Glacial Maximum *N. ansata* occurred in glacial refugia peatlands of the American east coast and has not yet been successful in colonizing other favourable habitats that developed during post-glacial peatland development.

Nebela ansata may be a particularly good candidate for a free-living microbe with significant limitations in its ability to disperse, particularly if it was unable to recolonize much of North America during the Holocene following glacial retreat. The limited passive dispersal capability of *N. ansata* may be explained by its comparatively large size, and perhaps by its unique morphology. Indeed, Wilkinson (2001) and Yang *et al.* (2010) recently observed that, for testate amoebae, limited geographical distribution becomes more common above sizes of 100–150 μm. Furthermore, Lara *et al.* (2011a) suggested a relationship between testate amoeba shell size and both genetic diversity and colonization of developing peatlands for the genus *Assulina* (Euglyphida). In addition, an obvious speculation is that the characteristic 'horns' of *N. ansata* may help anchor the test amongst vegetation in very wet sites, and, if this is the case, they may also limit dispersal. Alternatively, *N. ansata* may represent a recently diverged species that has not yet colonized favourable habitats at a large scale. Palaeoecological and molecular data could be used in tandem to test this hypothesis. However, it remains difficult to assess whether *N. ansata* had a more widespread distribution in former times because of the paucity of older testate amoeba fossils.

This forgotten microbe illustrates the knowledge gap between microbial and visible biodiversity

Beyond the simple fact that we have rediscovered a forgotten but highly conspicuous (by microbial standards) taxon, our results illustrate how little is known about microbial biogeography and diversity and raise the following questions. How many protists and other microbes also have geographically restricted distributions? How many endemic species remain undescribed? How many microbial species are currently threatened by direct habitat destruction or other global changes? If the distribution and diversity of free-living microbes is so poorly known that a distinctive species such as *N. ansata* can vanish from the scientific record for over 50 years, in countries as well studied as the USA and Canada, then the potential threats to microbial biodiversity are likely to be greatly underestimated.

The year 2010 was declared the International Year of Biodiversity by the United Nations, with the aim of increasing public awareness of the value of and threats to biodiversity (Williams, 2010). As the world's attention is focused on biodiversity and the alarming rate of its current destruction we should not forget the mostly unknown and under-appreciated diversity of free-living microorganisms. The protection of biodiversity can be viewed as a moral question, and it can be argued that every life-form equally deserves to be protected from extinction (e.g. Midgley, 2001). However, in the case of free-living microorganisms this is clearly not the only reason to worry about their diversity and conservation. Microorganisms are generally recognized as essential for the functioning of all ecosystems. They play fundamental roles in food-web processes and in nutrient cycling, much more so than the larger organisms that are generally the object of conservation measures. Further studies are needed to improve our understanding of how particular microbial species such as *N. ansata* influence ecosystem functioning.

In order to assess microbial biogeography and biodiversity, reliable taxonomy is an essential prerequisite. Although classical taxonomy allows the identification of some species such as *N. ansata* or other charismatic microorganisms, it does not allow precise identification of all microorganisms. This, however, should not be used as an excuse for not assessing microbial biogeography and biodiversity. Indeed, recent studies have shown that the use of modern molecular approaches can be extremely powerful and effective methods to recognize and quantify microbial biodiversity (López-García & Moreira, 2008; Caron *et al.*, 2009; Lara *et al.*, 2011b). Although small organisms are generally ignored in conservation management, a few pioneering projects are beginning to include such groups of organisms into biodiversity surveys (Esteban & Finlay, 2010). Similar and expanded efforts will certainly lead to better and more comprehensive conservation strategies, and to a better understanding of microbially driven ecosystem processes (Lewis, 2010).

ACKNOWLEDGEMENTS

This work was funded by Swiss NSF projects nos 205321–109709/1 and 205321–109709/2 to E.A.D.M., Swiss NSF PBELP2–122999 to T.J.H., the National Science and Engineering Research Council of Canada no. 283091–09 to B. Leander (Laboratory of Protozoan Diversity and Evolution at the University of British Columbia), Scientific & Technological Cooperation Programme Switzerland-Russia faculty exchange grant to E.A.D.M. and Y.M., and an award from the Royal Society International Travel Grant Scheme (2009R1) to Y.M. Work in the Pine Barrens of New Jersey was supported by Lehigh University. The authors wish to thank Brian Leander and Enrique Lara for fruitful discussions, Humphrey Smith and two anonymous referees for helpful suggestions on the manuscript, and Chitchai Chantangsi for his help with the first SEM imaging tests. SEM at the EPFL was possible through the Interdisciplinary Center for Electron Microscopy (CIME).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Testate amoeba species of four *Sphagnum* samples containing *Nebela ansata* specimens.
Appendix S2 Sequences of the primers used in this study.
Appendix S3 Comparative morphometrical characters of the five *Nebela ansata* populations recorded.

BIOSKETCH

Thierry J. Heger is currently a postdoctoral researcher at the University of British Columbia. His research interests include protist diversity assessment, protist biogeography and microbial ecology.

Author contributions: T.J.H., R.K.B., M.E.S., D.M.W. and E.A.D.M. conceived the idea; T.J.H., R.K.B., M.E.S., B.G.W., T.A., Y.M., R.M. and E.A.D. collected the data; T.J.H., R.K.B., M.E.S., R.M. and E.A.D.M. analysed the data; and T.J.H., R.K.B., D.M.W., M.E.S., T.A. and E.A.D.M. wrote the paper.

Editor: John Lambshead