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Reframing the 'Everything is everywhere' debate: evidence for high gene flow and diversity in ciliate morphospecies

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ABSTRACT: Current debate on microbial diversity contrasts the 'cosmopolitan' hypothesis, which argues for high gene flow and low diversity, with the 'endemism' hypothesis, which argues for high diversity and geographically restricted gene flow. Our analyses of genetic variation in ciliate morphospecies isolated from ephemeral environments (freshwater ponds and tide pools) redefine this debate. In 2 different clades of oligotrich ciliates (in the genera *Halteria/Meseres* and *Strombidium*), we found both high levels of diversity *and* evidence of high gene flow as indicated by the presence of identical haplotypes across broad geographic ranges. Five recognizable morphospecies of *Halteria/Meseres* were found to be composed of 7 different clades, differing by as much as 7.6% sequence divergence at the ITS locus (ITS1, ITS2 and 5.8S rDNA). Two recognizable morphospecies of *Strombidium* (*S. oculatum* and *S. stylifer*) resolved into 10 distinct clades, differing by as much as 15.7% at the same locus. For both groups of ciliates, the genetic divergence underlying these morphospecies may be related to cycles of isolation in their ephemeral habitats (freshwater lakes and ponds for *Halteria/Meseres* and tide pools for *Strombidium*). By comparison, there is both low diversity and high gene flow in published data on ciliates from open coastal water (*Laboea strobila* and several species of tintinnids), a more stable environment over evolutionary time-scales. Our analyses indicate that models of microbial diversity must test for ecologically driven patterns in the interactions of gene flow and species richness to account for observed patterns of high dispersal and high gene flow.

KEY WORDS: Ciliate phylogeography · Endemism · Cosmopolitanism · Gene flow · Cryptic species

INTRODUCTION

Recent discussions on ciliate biogeography reflect 2 schools of thought in the broader debate on the phylogeography of eukaryotic microbes (protists). The first maintains that all microbes, including ciliates, have cosmopolitan distributions, with a low degree of endemism and low species numbers (Finlay et al. 1996a,b, Fenchel et al. 1997). Proponents of this view argue that microbial species are characterized by large population sizes and, hence, high dispersal probability, which prevents isolation and allopatric speciation. In support of this view, Fenchel et al. (1997) found that approximately 8% of all described, free-living ciliate morphospecies were present in core samples representing less than 50 cm^2 total of sediment from a single small lake and a shallow marine bay. They concluded that 'in the case of microorganisms… global species diversity is relatively limited' (Fenchel et al. 1997). An opposing school of thought holds that fewer than half of the exist-

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ing species of ciliates have been described and posits a greater degree of endemism in ciliate species (Foissner 1998, 1999). Evidence in support of this includes the observation of apparently geographically limited 'flagship species' and the continued high rate of new morphospecies descriptions (Foissner 1997, in press).

To evaluate these hypotheses, we characterized DNA polymorphisms at the internally transcribed spacer regions (ITS) of the rDNA locus (ITS1, 5.8S rDNA, ITS2) in 2 clusters of morphospecies isolated from ephemeral environments: the freshwater species *Halteria grandinella* and several close relatives (Spirotrichea: Oligotrichia), and the marine tide pool species *Strombidium oculatum* and *S. stylifer* (Spirotrichea: Oligotrichia). We also compared patterns of variation from these ciliates to those of related planktonic morphospecies, including previously published sequences of *Eutintinnus pectinis*, *Tintinnopsis* sp. and *Favella ehrenbergii*, and *Laboea strobila* (Spirotrichea: Choreotrichia) (Snoeyenbos-West et al. 2002, Agatha et al. 2004). Our collection sites included places in North America, South America, Africa, and Europe.

MATERIALS AND METHODS

Ciliates were either picked directly from natural populations (all of the *Strombidium oculatum* samples) or cultured in the laboratory before characterization of individual clones (most *S. stylifer* and *Halteria* spp. samples; Tables 1 & 2). Samples of *H. grandinella*, 3 distinct undescribed *Halteria* spp. and the closely related *Meseres corlissi* were collected from 13 sites, and in many cases clonal lines were generated by passing single cells through several washes (Table 1). No substantial differences were seen in genetic variation (ITS locus) in *Halteria* spp. samples from clonal lines compared to those from natural populations (data not shown). We also include previously published sequences from Massachusetts population 1 and Colorado in our analyses (Table 1). Most *Halteria* populations were characterized morphologically and morphometrically using protargol impregnation and scanning electron microscopy (Foissner 1991).

We collected 25 samples of *Strombidium oculatum* and *S. stylifer* from populations located along the

Table 1. Populations of *Halteria grandinella* and its relatives. Clade numbers refer to Fig. 1

Morphospecies	Sample location	Latitude	Longitude	Date isolated	Clonal line	No. of clones	Clade
H. grandinella	Freshwater pond off I-90 near Missoula, MT	47° 09′ N	114° 50′ W	Jun 02	Yes	6	1
H. grandinella	Freshwater pond off Willcutt Road in Chesterfield, MA; (MA 2)	42° 1′ N	72° 50′ W	Jul 04	No	14	1
H. grandinella	Freshwater pond (Meekin Brook) rte-143W Chesterfield, MA; (MA 3)	42° 23′ N	72° 46″ W	Jul 04	No	8	1
H. grandinella	Freshwater pond (Meekin Brook) rte-143W Chesterfield, MA; (MA 4)	42° 23′ N	72° 46′ W	Jul 04	No	6	1
H. grandinella	Freshwater pond in Lory State Park, Bellevue, CO	40° 34′ N	105° 10′ W	Jun 04	No	1	2
H. grandinella	Freshwater pond, Botanical Gardens, Univ Puerto Rico, Rio Piedras	18° 23′ N	66° 2′ W	Jan 03	Yes	8	2
H. grandinella	Freshwater pond, Harbour Branch Ocean Station, Ft. Pierce, FL	27° 22′ N	80° 19′ W	Jul 02	Yes	4	2
H. grandinella	Freshwater pond, San Francisco Botanical Gardens, San Francisco, CA	37° 46′ N	112° 24′ W	Jul 03	Yes	6	3
H. grandinella	Simmelried bog near Constance, Germany	47° 40′ N	9° 05′ E	Aug 04	Yes	2	3
H. grandinella	Zicklacke (alkaline lake) near Vienna, Austria	47° 45′ N	16° 50′ E	Aug 04	Yes	3	3
H. grandinella	Tank bromeliad in the Tiputini Biodiversity Station, Coca, Ecuador	0° 27′ S	76° 59′ W	Jun 04	No	14	2
Halteria sp.	Mangrove swamp (slightly saline), Dominican Republic	19° N	70° W	Jun 02	No	2	7
Halteria sp.	Floodplain soil from the Chobe River, Botswana, Africa	23° S	21° E	Aug 04	No	5	5
Halteria sp.	Floodplain soil from the Panará River near town of Maringa, Brazil	22° 40′ S	53° 15′ W	Jun 02	No	3	6
Meseres corlissi	Floodplain soil from the Panara River near town of Maringa, Brazil	22° 40′ S	53° 15′ W	Jun 02	No	2	4
H. grandinella	Pond University of Colorado Campus, Boulder, CO, GB#AF508759	From GenBank				1	1
H. grandinella	Smith College Greenhouse Pond, Northampton, MA, GB#AY007444	From GenBank				6	3

coasts of New England, Ireland, the Isle of Man (British Isles), and Brazil (Table 2). The *Strombidium* samples were collected over short temporal and spatial scales (consecutive low tides and adjacent pools) in order to assess intrapopulation diversity. With the exception of populations of *S. stylifer* from Connecticut, no clonal lines were generated for these taxa. In fact, none of the *S. oculatum* could be maintained in cultures and hence all data on this taxon are from field collections.

Nucleic acid extraction and amplification were performed according to the protocol described in Snoeyenbos-West et al. (2002). PCR reactions were run in the Peltier Thermal Cycler (PTC-200) under the same conditions as those described in Snoeyenbos-West et al. (2002). Cloning was performed using the TOPO TA Cloning Kit (Invitrogen #45-0641). Direct sequencing of PCR products from cloned plasmid DNA was accomplished in the forward direction using gene-

Table 2. *Strombidium oculatum* and *S. stylifer* populations. Clade numbers refer to Fig. 3. All *S. oculatum* samples were picked from natural populations; all *S. stylifer* populations were from clonal cultures, except Cove Island Park, Connecticut, 27 September 2002, and Brazil. ANP = Acadia National Park in Bar Harbor, ME; SML = Shoals Marine Lab, Appledore Island, ME

Morpho-species	Sample location (~distance from Pool 1)	Date isolated	Total no. of clones	Clade	Clones per clade
S. oculatum	Dublin Bay, Pool 1[a] (0 m), AM low tide	14 May 02	3	II	2
				III	1
S. oculatum	Dublin Bay, Pool 2 (5 m), AM low tide	14 May 02	4	IV	4
S. oculatum	Dublin Bay, Pool 4 (5 m), AM low tide	14 May 02	58	VIII	19
				V	1
				IV	34
				X	2
				IX	2
S. oculatum	Dublin Bay, Pool 6 (5 m), AM low tide	14 May 02	3	IV	3
S. oculatum	Dublin Bay, Pool T (10 m), AM low tide	14 May 02	2	II	2
S. oculatum	Dublin Bay, Pool 1 (0 m), PM low tide	14 May 02	2	IV	1
				I	1
S. oculatum	Dublin Bay, Pool 4 (5 m), PM low tide	14 May 02	66	VIII	60
				IV	3
				II	2
				IX	1
S. oculatum	Dublin Bay, Pool T (10 m), PM low tide	14 May 02	4	VIII	2
				II	2
S. oculatum	Dublin Bay, Pool 13 (3.7 km), AM low tide	11 Jun 02	3	IV	3
S. oculatum	Dublin Bay, Pool 13 (3.7 km), PM low tide	10 Jun 02	6	IV	2
				II	4
S. oculatum	Dublin Bay, Pool 1 (0 m)	6 Jun 02	8	II	8
S. oculatum	Dublin Bay, Pool 4 (5 m)	9 Apr 02	2	IV	2
S. oculatum	Dublin Bay, Pool 9 (1 km)	23 Apr 02	9	IV	9
S. oculatum	Galway Bay (220 km)	25 Jun 02	44	II	44
S. oculatum	Isle of Man, Pool A (125 km)	5 May 02	8	VIII	6
				II	2
S. oculatum	Isle of Man, Pool B (125 km)	5 May 02	12	VIII	5
				IV	2
				II	5
S. oculatum	Maine: ANP (4500 km)	Aug 04	28	II	28
S. oculatum	Maine: SML (4750 km)	15 Aug 02	33	II	33
S. oculatum	Maine: SML (4750 km)	18 Aug 03	7	II	7
S. stylifer	Maine: SML (4750 km)	18 Aug 03	5	VII	5
S. stylifer	Maine: SML (4750 km)	18 Aug 03	6	VII	6
S. stylifer	Connecticut (5070 km)	27 Sep 02	8	VI	1
				VII	7
S. stylifer	Maine: SML (4750 km)	18 Aug 03	6	VII	6
S. stylifer	Maine: SML (4750 km)	18 Aug 03	5	VII	5
S. stylifer	Brazil (9375 km)	16 Dec 04	3	VII	3

[a]Dublin Bay Pool 1 location 53° 17.5′ N, 06° 7.5′ W

specific primers and the BigDye terminator kit (Perkin Elmer). Sequences were run on an ABI 3100 automated sequencer.

Sequences generated using a single primer were compared in SeqMan (DNAStar) and ambiguous sites were checked by eye. An alignment of data was performed using the Clustal W algorithms (Thompson et al. 1994) as implemented in Megalign (DNAStar). The ITS alignment yielded 512 characters in total and 468 after excluding ambiguously aligned regions.

Genealogical analysis of ITS nucleotide data was performed using the maximum likelihood (ML) and maximum parsimony (MP) algorithms of PAUP* version 4.0b10 (Swofford 2002). For MP, the most parsimonious consensus tree was generated in an exhaustive search with gaps treated as a fifth character or removed. For ML, the most likely consensus tree was built in an heuristic search with gaps removed and with gaps treated as missing data. Modeltest Version 3.06 (Posada & Crandall 1998) was used to determine an appropriate model for ML analysis as well as to estimate the parameters for the data set. Bootstrap values were calculated using 100 replicates under all models. Percent uncorrected nucleotide divergence was calculated using PAUP* version 4.0b10.

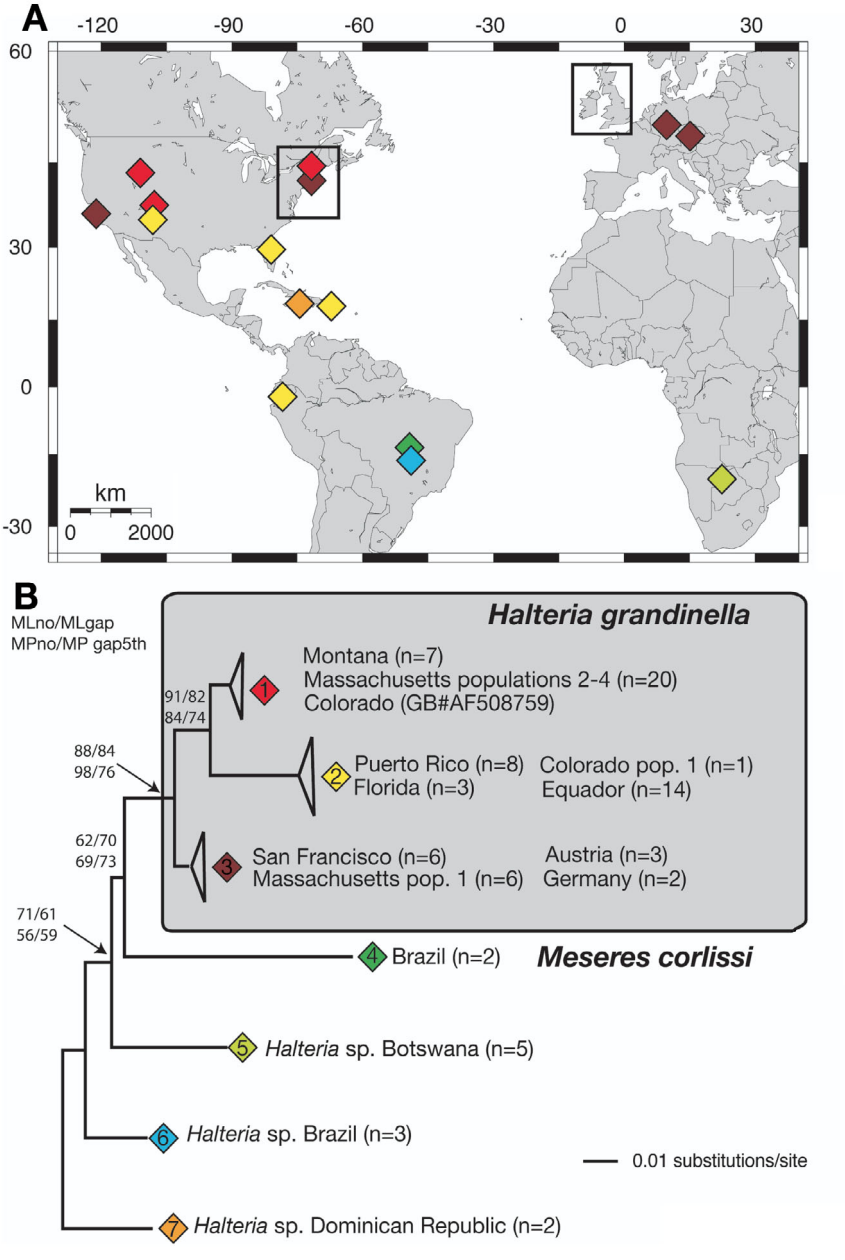

Fig. 1. (A) Locations of *Halteria grandinella* and its relatives (see Table 1 for description of locations). Colors correspond to the different clades in the *H. grandinella* genealogy; black insets refer to regions in Fig. 3. (B) Genealogy based on maximum-likelihood analysis of ITS data. Bootstrap values are shown at nodes: upper values are maximum-likelihood with gaps removed (MLno), and maximum-likelihood with gaps included (MLgap); lower values are maximum parsimony with gaps excluded (MPno), and maximum parsimony gaps treated as 5th character (MPgap5th)

RESULTS

We analyzed variation in the ITS locus in clones from 15 populations of the morphospecies *Halteria grandinella* and its relatives *Halteria* spp. (3 distinct morphospecies) and *Meseres corlissi* from North America, South America, Europe and Africa (Figs. 1 & 2, Table 1). Genealogical analyses of the resulting sequences reveal 7 distinct clades of sequences (<2% divergence within clades). We chose a 2% cutoff as a starting point, as some authors have suggested that 1 to 2% rDNA divergence is equivalent to morphospecies designations (Jerome et al. 1996, Shin et al. 2000). In fact, intraclade divergences based on this cutoff are all ≤0.5%, while interclade divergences are as high as 7.64% (Table 3). Hence, in contrast to a continuous distribution of diversity among sequences, as would be expected if we had sampled a genetically cohesive cluster of lineages, we find ciliates with identical or nearly identical ITS sequences in disparate locations. For example, Clade 3 contains 17 sequences that diverge on average by 0.25% and represent populations from both sides of the United States (San Francisco and Massachusetts) and from Europe (Austria and Germany). In

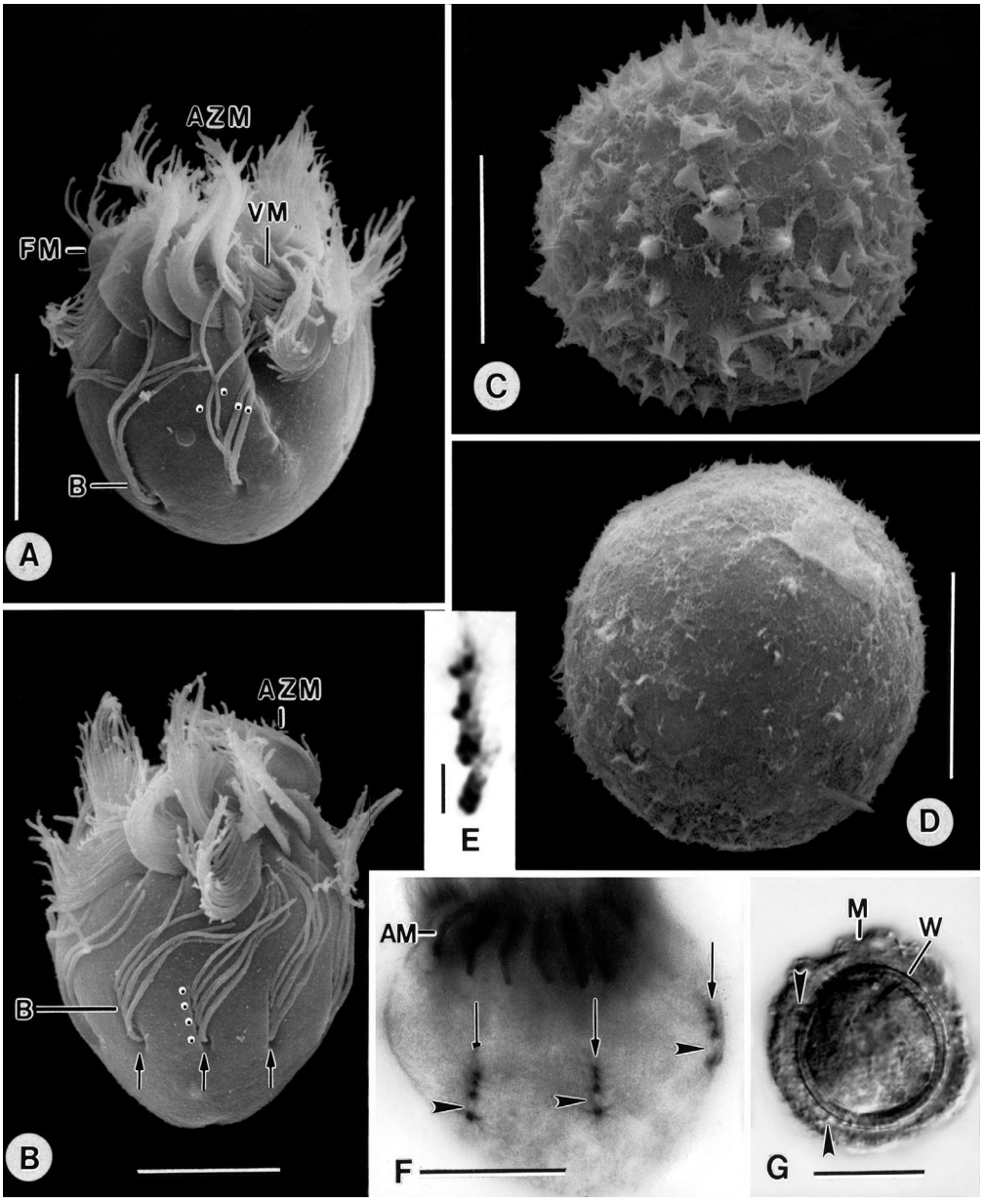

Fig. 2. *Halteria* populations from (A–C) the Dominican Republic, (D) Austria, and (E–G) Botswana in the scanning electron microscope (A–D), after protargol impregnation (E,F), and from life (G). (A,B) Ventral and dorsal overviews, respectively. The general appearance and the basic structure of the bristle rows (arrows) are the same in all populations investigated (Table 1). The bristle rows each consist of 2 single cilia anteriorly and 2 cilia pairs posteriorly (dots). (C,D,G) The 3 populations shown can be distinguished by the resting cysts. The cyst of the Dominican population (C) has conical scales attached to a fibrous mucus layer, while the Austrian specimens (D) have a smooth wall. The Botswanan population has conical scales (G, arrowheads) embedded in a thick, mucus envelope. (E,F) Typically, the jumping bristle rows of *Halteria* each consist of 4 equidistantly spaced complexes (B,F, arrows), while the distance is increased between complexes 3 and 4 in the Botswanan specimens (F, arrowheads). AM: adoral membranelles; AZM: adoral zone of membranelles; B: jumping bristles; FM: frontal (collar) membranelles; M: mucus envelope; VM: ventral membranelles; W: cyst wall. Scale bars = 10 µm (A– D), 2 µm (E), and 20 µm (F,G)

contrast, Clade 3 is 2.0 and 3.7 % divergent from Clades 1 and 2 (its closest relatives), respectively.

These analyses demonstrate that at least some *Halteria* spp. experience high gene flow, as some clades (e.g. 1 to 3) are found across broad geographic areas. For example, sequences obtained from populations in Northampton, Massachusetts (Clade 3) are more similar to sequences from Germany than they are to those

Table 3. Percent divergence within and between clades of *Halteria grandinella* and its relatives, calculated using uncorrected distances

Clade[a]	1	2	3	4	5	6	7
1	0.13						
2	2.93	0.25					
3	1.95	3.71	0.25				
4	6.69	7.31	6.49	0.39			
5	6.05	7.64	4.88	8.22	0.20		
6	5.07	5.88	4.10	7.22	5.23	0.41	
7	5.61	7.41	5.20	7.80	6.15	4.55	0.20

[a]Descriptions of populations/clades refer to Table 1 and Fig. 1

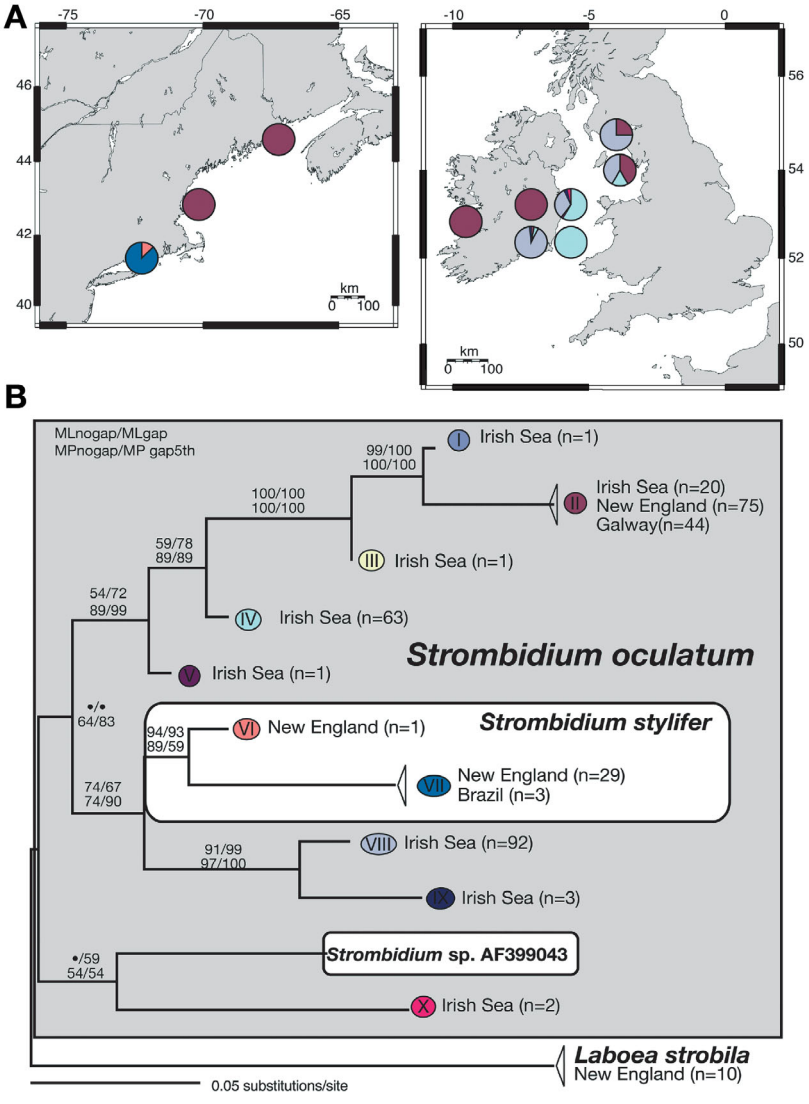

Fig. 3. (A) Map indicating the locations of *Strombidium oculatum* and *S. stylifer* sampling sites, from which at least 8 clones were sequenced (see Table 2). Only a few clones were sequenced from many pools and although they are included in the genealogy, these populations are not displayed on the map. Colors correspond to the different clades in the *S. oculatum* genealogy. (B) Genealogy based on maximum-likelihood analysis of ITS data (see 'Materials and methods'). The genealogy is rooted with previously published sequences of *Laboea strobila.* Bootstrap values as in Fig. 1

from nearby Chesterfield, Massachusetts (Clade 1; Fig. 1). While other clades may be geographically isolated (e.g. Clades 4 and 6 were only found in South America and Clade 5 in Africa), substantially more data are required to determine the extent to which gene flow occurs or is limited among locations.

To further assess morphospecies descriptions, protargol staining was carried out on representative *Halteria* populations from Clades 2, 3, 4, 5 and 7. Consistent with our genealogy, Clades 5 to 7 are morphologically distinct at this more detailed level of microscopy (Table 4). Intriguingly, the Southern Hemisphere clades (4 to 7) are present in morphospecies that have spiny cysts, while *H. grandinella* from Europe and North America (Clades 1 to 3) make smooth cysts (Fig. 2C,D, Table 4).

Analysis of the second species cluster, the tide pool morphospecies *Strombidium oculatum* and *S. stylifer*, collected over short temporal (tidal) and both small and large spatial scales reveals similar patterns of high genetic diversity, except that considerable variation also exists within populations of these taxa (Figs. 3 & 4, Tables 2 & 5). These species can be distinguished by the habit of *S. oculatum* to encyst with a tidal periodicity, and by a posterior thorn-like projection that is usually present on *S. stylifer*. In *S. stylifer*, the adoral zone extends further down the ventral side than that of *S. oculatum*, and the girdle kinety of *S. oculatum* is closer to the mouth. Both species inhabit similar environments, contain a prominent eyespot, and are mixotrophs, harboring 'slave' chloroplasts derived from green macroalgae (McManus et al. 2004).

Genealogical analyses of 336 clones from *Strombidium oculatum* and *S. stylifer* isolated from both sides of the North Atlantic and from the western South Atlantic (coastal Brazil) reveal not 2 but 10 distinct clades (>2% sequence divergence; Fig. 3). As with *Halteria* spp., there is evidence of both genetic diversity and high levels of gene flow in *S. oculatum* despite the seemingly restricted habitat of this tide pool ciliate (Fig. 3, Table 2). For example, individuals represented in Clade II are found in both the Irish Sea and on the East Coast of North America. To our

Table 4. Morphometric and morphological characteristics and classification of 7 *Halteria* populations from Eurasia, Africa, South Central, and North America. NA: not available

Morphological classification	Clade (Fig. 1)	Sample location	Body length (µm)[a,b]	No. of frontal membranelles[a,c]	No. ventral membranelles[a,c]	No. bristle rows[a,c]	No. bristle groups in the first row right of buccal cavity[a,c]	Posterior group of bristle rows separated[a,d]	Cyst wall
H. grandinella	3	Austria, Europe	20.1 (±3.3)	16–17 (16)	7–8 (7)	7 (7)	4 (4)	No	Smooth
H. grandinella	3	Germany, Europe	23.9 (±4.0)	16 (16)	7 (7)	7–8 (7)	4 (4)	No	Smooth
H. grandinella	2	Colorado (Lory State Park), USA	24.3 (±3.1)	16 (16)	7 (7)	7 (7)	4 (4)	No	Smooth
H. grandinella	3	Massachusetts (Northampton), USA	26.9 (±2.3)	16–17 (16)	7–8 (7)	7 (7)	4 (4)	No	Smooth
Distinct species 1[f]	5	Botswana, Africa	40.1 (±7.7)	15–16 (16)	9–11 (10)	8–9 (8)	4 (4)	Yes	Spiny[e]
Distinct species 2	6	Brazil, South America	23.8 (±1.9)	16–18 (16)	7–8 (7)	7 (7)	5 (5)	No	NA
Distinct species 3	7	Dominican Rep., Central America	31.0 (±3.2)	16–17 (16)	6–8 (7)	8–9 (8)	4 (4)	No	Spiny

[a]Based on protargol silver preparations
[b]Arithmetic mean and ±SD in brackets. Width very similar and thus not shown
[c]Extremes and median in brackets
[d]A bristle (ciliary) row consists of 4 or 5 groups of cilia. Usually, these groups are equidistantly spaced (Fig. 2B,E), but in the Botswanan specimens the posterior group is separated from the anterior groups by an increased distance (Fig. 2F)
[e]Surrounded by a conspicuous, dense mucus coat (Fig. 2G)
[f]It is not *H. geleiana* Szabó (1935) because this has 6 (vs. 2) paroral cilia

knowledge, *S. oculatum* has never been observed in open waters outside of tide pools, yet identical haplotypes of this morphospecies can be found thousands of kilometers apart on both sides of the North Atlantic Ocean. Moreover, we again find low levels of diversity within clades and high divergence (up to 15.7%) between clades (Table 5). Because these ciliates are capable of sexual recombination, the low divergence within clades and high divergence between them is evidence that they are reproductively isolated. Moreover, we find no footprint of recent recombination in the ITS locus (i.e. exchange of linked polymorphisms), indicating that haplotypes are not the result of recent hybridization.

The greatest genetic diversity in *Strombidium oculatum* is present within the Irish Sea, suggesting that this region may have served as a refugium for *S. oculatum* during the last glaciation. For example, individuals in Clades I to V, and VIII to X all coexist in pools along the Irish Sea, where we sampled most intensively (Table 2). However, our largest sample is from this area: more samples from across the North Atlantic and across different time periods are needed. We found no evidence that cyclical encystment behavior of *S. oculatum* (Montagnes et al. 2002) results in genetically isolated populations. For example, Clades IV, VIII, and IX were all found in a single pool on 2 consecutive low tides in Dublin Bay (on 14 May 2002; Pool 4, Table 2).

Morphospecies designations in *Strombidium oculatum* and *S. stylifer* are not consistent with genetic haplotypes. The clades of *S. oculatum* are paraphyletic with respect to *S. stylifer* and a previously characterized *Strombidium* sp. (GenBank AF399043) isolated from open coastal waters in the western North Atlantic (Fig. 3). We speculate that detailed microscopy will show that this high genetic divergence is matched by fine-scale morphological and/or behavioral variations within *S. oculatum* and *S. stylifer.*

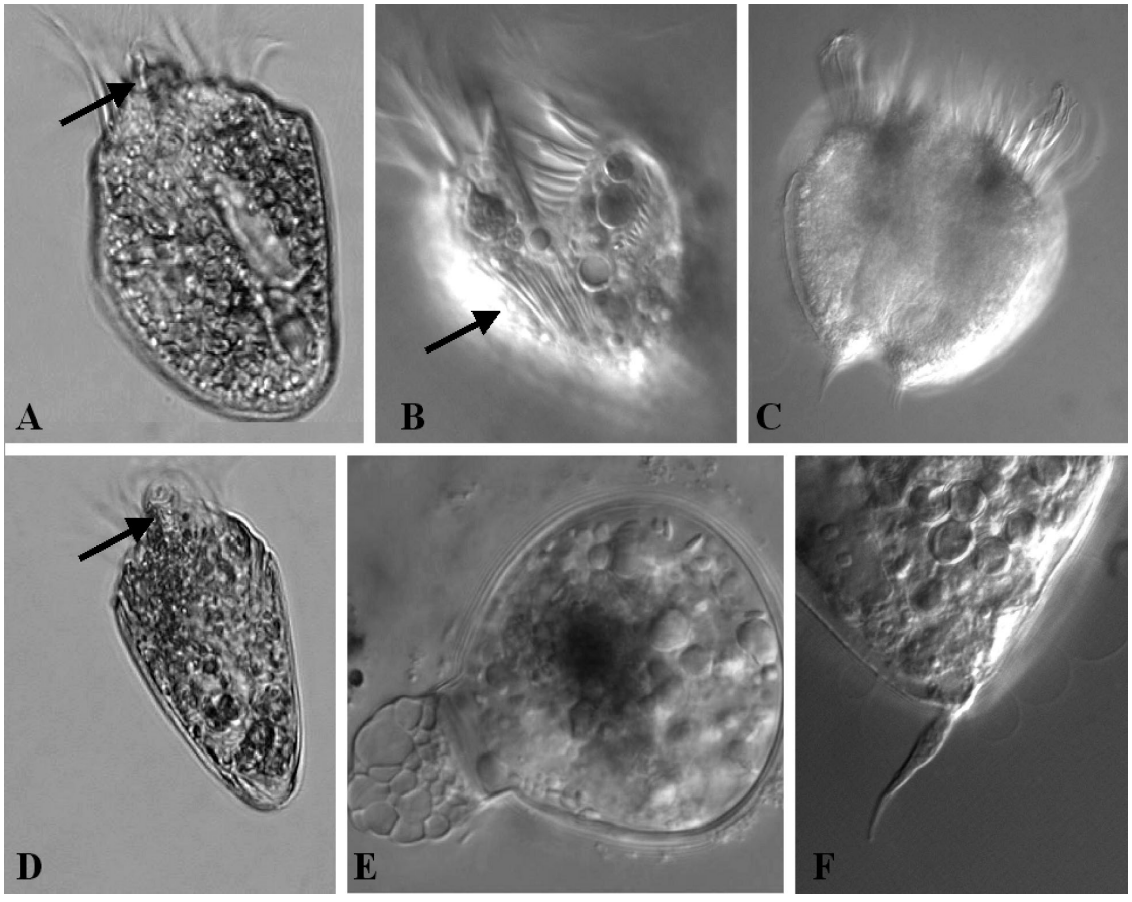

Fig. 4. Morphology of *Strombidium oculatum* and *S. stylifer*. (A,D) *S. oculatum* from live populations sampled in Dublin Bay, Ireland, with arrows showing eyespots. (B) *S. stylifer* from a culture isolated from Scotland, with arrow showing extrusomes. (C) Conjugating pair of *S. stylifer* from a culture isolated from a tide pool in Scotland. (E) Cyst from a New England *S. stylifer* culture. (F) Closeup of the 'stachel', a posterior thorn-like projection from a culture of *S. stylifer* isolated from Brazil. The ciliates are approximately 65 µm in length; the stachel in this case is ca. 10 µm, but the size and morphology of this structure varies widely

Table 5. Percent divergence within and between *Strombidium* spp. clades. –: containing only 1 clone. S: *Strombidium*; L: *Laboea*. Sampling information from Table 2

	Clade I	Clade II	Clade III	Clade IV	Clade V	Clade VI	Clade VII	Clade VIII	Clade IX	*S.* sp.	Clade X	*L. strobila*
Clade I	–											
Clade II	4.48	0.18										
Clade III	2.20	5.92	–									
Clade IV	6.78	10.59	4.59	0.07								
Clade V	8.02	12.59	6.03	2.90	–							
Clade VI	12.75	13.27	11.33	9.01	7.80	–						
Clade VII	13.66	14.20	13.06	10.75	11.85	6.31	0.23					
Clade VIII	15.03	14.06	13.61	11.00	8.28	7.49	11.18	0.16				
Clade IX	13.40	12.01	11.15	6.35	9.21	9.72	11.48	4.55	1.00			
Strombidium sp. AF399043	16.07	14.90	14.67	12.38	11.37	12.31	13.22	12.24	13.57	–		
Clade X	15.72	15.48	14.15	10.57	11.33	12.75	14.68	12.07	11.63	11.39	0	
Laboea strobila AF399079	18.72	19.01	18.13	15.57	15.39	14.89	16.39	16.22	16.87	16.14	16.26	–

DISCUSSION

We propose that the ephemeral nature of tide pools and freshwater bodies, at least over evolutionary scales, drives the diversification of these morphospecies due to past periods of isolation (Fig. 5). In the case of *Strombidium oculatum* and *S. stylifer*, for example, we estimate the divergences in these populations to have been initiated during the Miocene, ~6 to 20 million yr ago (mya), based on a rate of mutation in the ITS of roughly 0.5 to 1%/million yr (Bargues et al. 2000, LaJeunesse 2005). Intriguingly, similar divergence times have been estimated for the early diversification of species of symbiotic marine dinoflagellates (LaJeunesse 2005). Our data suggest that tide pool populations became isolated during periods such as the sharp drop in eustatic sea level during the upper Miocene about 10 mya (Haq et al. 1987), which corresponds to the estimate of earliest divergence among our clades. Such an isolation could have occurred due to loss of appropriate habitats and/or extinction of intervening populations. Furthermore, at the last glacial maximum ca. 20 000 yr BP, probably all of the rocky shore of New England and much of northern Europe was covered with ice, shorelines were beyond the shelf edges, and tide pool habitats were either nonexistent or minimal (Dyke et al. 2002). Subsequent isolation due to sea level changes and occasional gene flow in ocean currents would re-establish pools through dispersal. Such events, occurring repeatedly over millions of years in both tide pools and freshwater habitats, may have led to periods of isolation that generated distinctive genetic clades.

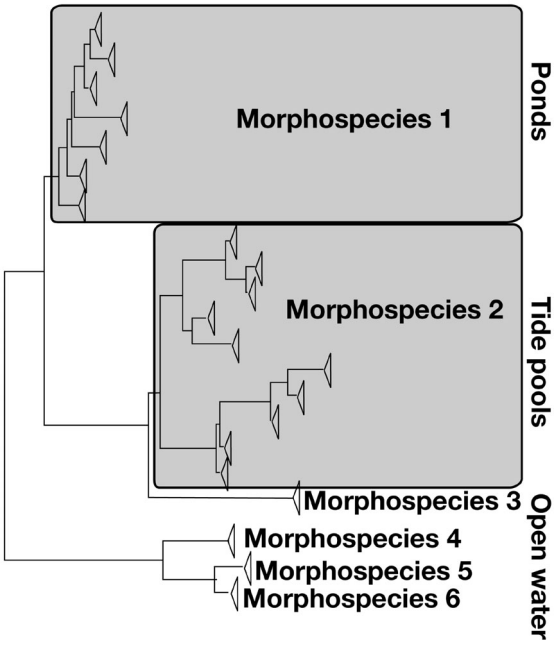

Fig. 5. Hypothesis of interaction of genetic diversity, morphospecies and habitat type. The genetic diversity of ciliate morphospecies appears to vary by habitat type. Ciliate morphospecies in evolutionary unstable habitats, tide pools and freshwater, contain considerable diversity. In contrast, morphospecies in the open ocean, where there have likely been fewer historic barriers to gene flow, contain more limited genetic variation

In this scenario, it is possible that the northeast Atlantic coast, where we see the greatest diversity of *Strombidium oculatum* haplotypes, served as a refugium in which diversity was preserved during periods of isolation due to sea level changes. Alternatively, the high level of genetic diversity within the Irish Sea could reflect recent gene flow/migration, perhaps even mediated by anthropogenic movement of ships and water. Regardless, the subsequent persistence of genetically isolated *Strombidium* spp. within tide pools suggests that some form of niche separation has occurred among the genetically distinct ciliates as ecologically identical clades are unlikely to persist over long time periods (Fig. 5).

Support for the idea that the ephemeral nature of habitats (tide pools, ponds) is related to the observed cryptic diversity within *Halteria* spp. and *Strombidium* spp. morphospecies comes from comparisons with open water representatives of the same orders of ciliates. Contrary to our observations on *Halteria* spp. and *Strombidium* spp., genetic divergence among clones of this same locus is low for previously published spatially and temporally separated populations of the planktonic ciliates *Eutintinnus pectinis*, *Tintinnopsis* sp., and *Favella ehrenbergii* (Snoeyenbos-West et al. 2002). Similarly, the small subunit rDNA sequences are virtually identical in *Laboea strobila* isolated from the Isle of Man (British Isles) (Agatha et al. 2004), Trieste (Italy) (Agatha et al. 2004) and Connecticut (USA) (Snoeyenbos-West et al. 2002). The low genetic diversity within these planktonic species suggests either that open water ciliates have not been subject to periods of past isolation, or that dominant haplotypes have replaced other genetic variants in this more continuous environment.

Our data indicate that future studies of ciliates from evolutionarily ephemeral habitats should include large sample sizes to test for the presence of cryptic diversity. For example, in our relatively limited sampling of tide pool *Strombidium* spp., we found 4 haplotypes each represented only a single time, suggesting that there are more rare haplotypes yet to be discovered (Fig. 3). Moreover, measures of abiotic factors and ecological roles may reveal environmental correlates for genetically distinct haplotypes. Finally, as with all single marker studies, additional data from elsewhere in the ciliate genome are required to confirm the

observed pattern of genetic diversity and to fully assess potential impact of sex/hybridization.

Evidence for greater diversity of eukaryotic microbes in general is beginning to emerge from molecular analyses of diverse lineages. For example, recent analyses of the flagellate genera *Ancyromonas*, *Cafeteria*, and *Caecitullus* from surface waters and deep-sea sediments revealed that individual morphospecies can be underlain by diverse genetic entities (Scheckenbach et al. 2005). Furthermore, cryptic species in some planktonic Foraminifera have been observed through DNA analysis, and have subsequently been supported by more detailed studies of morphology and ecology (de Vargas et al. 1999).

In sum, we have shown both high gene flow and high diversity in 2 clades of ciliates from ephemeral environments (Fig. 6). In one of those clades (*Strombidium oculatum/S. stylifer*), we have also found very high diversity in co-occurring populations. The presence of high genetic diversity concomitant with high gene flow among some populations of *S. oculatum/S. stylifer* and *Halteria* spp. suggests that understanding the phylogeography of ciliates, and perhaps protists in general, requires disentangling gene flow and genetic diversity as separate parameters (Fig. 6). Our study is consistent with the observation of cryptic species in the model ciliates *Tetrahymena* and *Paramecium* (Nanney 2004), both of which are restricted to freshwater habitats. Hence, it is likely that there are many more species of protists yet to be discovered (Foissner 1999, in press) and that current rates of gene flow may be high for many of these species.

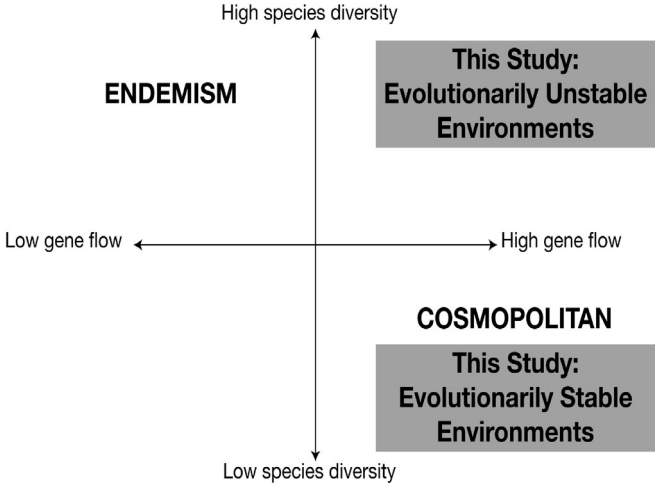

Fig. 6. Revised model of ciliate biogeography. Results from the *Halteria grandinella* and *Strombidium oculatum* data occupy the upper right quadrant (high gene flow, high species diversity), results from previously published data on marine ciliates the lower right quadrant

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