Evidence for Geographic Isolation and Signs of Endemism within a Protistan Morphospecies†

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The possible existence of endemism among microorganisms resulting from and preserved by geographic isolation is one of the most controversial topics in microbial ecology. We isolated 31 strains of “Spumella-like” flagellates from remote sampling sites from all continents, including Antarctica. These and another 23 isolates from a former study were characterized morphologically and by small-subunit rRNA gene sequence analysis and tested for the maximum temperature tolerance. Only a minority of the Spumella morpho- and phylotypes from the geographically isolated Antarctic continent follow the worldwide trend of a linear correlation between ambient (air) temperature during strain isolation and heat tolerance of the isolates. A high percentage of the Antarctic isolates, but none of the isolates from locations on other continents, were obligate psychrophilic, although some of the latter were isolated at low ambient temperatures. The drastic deviation of Antarctic representatives of Spumella from the global trend of temperature adaptation of this morphospecies provides strong evidence for geographic transport restriction of a microorganism; i.e., Antarctic protistan communities are less influenced by transport of protists to and from the Antarctic continent than by local adaptation, a subtle form of endemism.

MATERIALS AND METHODS

Media, isolation of strains, and cultivation conditions. The standard medium for the maintenance of strains and for the isolation process, except for the first step, was an artificial inorganic basal medium (NSY basal inorganic medium [18]). The ultramicrobacterial (<0.1-μm) strain MWH-NR1 (Betaproteobacteria; Polynucleobacter group) and the bacterial strain Listonella pelagia CBS (both available from M. Hahn [17]), representatives of typical free-living aquatic bacteria, were used as food for isolating the flagellates.

Samples originating from terrestrial and aquatic habitats in polar, temperate, subtropical, and tropical regions (Fig. 1A and B) were collected. Samples were transported to the laboratory in sealed tubes and processed immediately upon arrival. All treatments after sampling were carried out under aseptic conditions. Flagellates were isolated by serial dilution using either sterile filtrated water from the sampling site or artificial media following an acclimatization approach (4). The flagellates were counted using a Sedgewick-Rafter chamber, and a sub-
sample was diluted to a final flagellate abundance of 0.5 to 1 flagellate ml\(^{-1}\) and subsequently transferred to 24-well cell culture plates. Wells were supplemented with food bacteria at a concentration of 3 x 10\(^{5}\) to 5 x 10\(^{6}\) bacteria ml\(^{-1}\). Wells were checked every second day for a period of at least 2 weeks for positive growth under the microscope, using a total magnification of \( \times 1000 \). When flagellate growth was detected, the medium was transferred to a 50-ml Erlenmeyer flask containing fresh medium and bacteria. This procedure was repeated until pure cultures were established, but at least four times. Pure cultures were acclimatized to 16\(^\circ\)C and transferred to permanent culture with NSY medium at 16\(^\circ\)C and pH 7.8, for at least 3 months. All experiments were run in triplicate. Using this general strategy, the upper tolerance limits were determined.

**Morphological and phylogenetic analyses.** Cells were checked for the occurrence of chloroplasts. In addition, cells were checked for autofluorescence as follows. Cells were fixed with formaldehyde (final concentration, 2\% and stained with DAPI (4,6-diamidino-2-phenylindole) (final concentration, 10 \( \mu \)g ml\(^{-1}\)) for 30 min. The cells were then filtered onto a black Nuclepore 0.2-\( \mu \)m filter backed by a 0.45-\( \mu \)m cellulose nitrate filter and examined under an epifluorescence microscope, using UV and blue light excitation for DAPI and for chlorophyll autofluorescence, respectively. Representative strains were, in addition, checked by scanning electron microscopy (SEM) for tripartite hairs on the long flagellum and for occurrence of scales. Briefly, cells fixed with a mixture of Lugol’s solution and formaldehyde were allowed to sediment onto glass coverslips covered with poly-l-lysine solution (0.1\%, wt/vol). The coverslips were then recovered from the chambers and rinsed in distilled water or sodium cacodylate buffer. Subsequently, the slides were fixed for 30 min in an osmium tetroxide solution (2\% final concentration), rinsed again, and subsequently dehydrated using increasingly concentrated ethanol baths and a final wash in xanthydrolisulphazane.

Finally, the slides were glued to an SEM stub with a thin layer of Araldite glue, metal coated, and inspected. The phylogenetic affiliation of strains based on small-subunit (SSU) \( rRNA \) gene sequences was as described in a previous study (4). PCR products were either directly sequenced or used for subsequent cloning into the vector pGem-T Easy (Promega). Sequencing reactions were performed with an ABI Prism BigDye Terminator version 3.0 Ready Reaction cycle sequencing kit (Applied Biosciences) and an ABI PRISM model 3100 automated sequencer. Sequences were submitted to the BLAST search program of the National Center for Biotechnology Information (NCBI) and to generate phylogenetic trees by the neighbor-joining method (36). Parsimony trees were calculated using the program package PHYLIP (version 3.5; J. Felsenstein, Department of Genetics, University of Washington, Seattle). One hundred bootstrapped replicate resampling data points were generated with SEQBOOT (PHYLIP).

**Maximum temperature tolerance.** The isolated colorless chrysophytes were characterized regarding their temperature tolerance. During permanent culture, all strains were acclimatized to the same conditions, i.e., NSY inorganic basal medium at 16\(^\circ\)C and pH 7.8, for at least 3 months. All experiments were run in triplicate in 4 ml in 12-well tissue culture plates with the bacterial strain Listonella pelagia CBS as the food source. The strains were transferred to the experimental conditions, and growth was checked every 2 to 3 days by inverted light microscopy at a magnification of \( \times 200 \). The experiments ran usually for 3 days but up to 8 days until growth was observed. If no growth was observed after 8 days, the treatment was accepted not to support growth of the tested strain. If growth was observed, an aliquot was transferred to fresh medium and food at the next-higher temperature treatment in steps of 1 to 2\(^\circ\)C (the temperatures tested were 17.5, 18.0, 19.2, and 20.8\(^\circ\)C). Parsimony trees were calculated using the program package PHYLIP (version 3.5; J. Felsenstein, Department of Genetics, University of Washington, Seattle). One hundred bootstrapped replicate resampling data points were generated with SEQBOOT (PHYLIP).

**Nucleotide sequence accession numbers.** The almost-full-length 18S \( rRNA \) gene sequences determined in this study have been deposited in the NCBI database under accession numbers DQ388538 to DQ388568.
RESULTS

Morphological and 18S rRNA gene analyses. We isolated 31 colorless chrysophyte flagellates identified as Spumella spp. These isolates and, in addition, 23 isolates from our culture collection (4) were included in this study. The Spumella cells were colorless and solitary and had a spherical to ellipsoid cell form. From the front end emerged two flagella, both visible in

FIG. 2. Neighbor-joining tree showing the affiliation of SSU rRNA gene sequences from Spumella isolates with the Chrysophyceae sensu stricto. The numbers at the nodes of the tree indicate the percentage of bootstrap values for each node out of 100 bootstrap resamplings (values above 50 are shown). The scale bar indicates 2% estimated sequence divergence.
the light microscope. SEM investigations proved that the long one bears tripartite hairs. The cells either swim free in the medium or attach to the substrate with the posterior end. The cells feed on small particles, specifically bacteria, and the general sequence of feeding follows that described by Boenigk and Arndt (3). The cells were between 4 and 8 µm in diameter. Despite a detailed morphological investigation of our Spumella strains, a classification to one of the described taxa was not possible due to the poor quality of the existing taxon descriptions (2). We isolated Spumella spp. from all continents from contrasting freshwater and soil sites (Fig. 1). Based on morphology, the investigated species therefore seem to be ubiquitous, i.e., present in different habitat types and distributed worldwide. Both neighboring and parsimony 18S rRNA gene trees showed that most of our Spumella isolates were affiliated with the clusters C1, C2, and C3 as described previously (4) (Fig. 2; see Fig. S1 in the supplemental material). As the phylogenetic analysis indicates polyphyly of Spumella spp., we further use the term “Spumella-like” flagellates.

**Ecophysiological differentiation and indications for geographic restriction.** The “Spumella-like” flagellates differed significantly regarding their maximum tolerated growth temperature (by analysis of variance, \( P < 0.001 \)) (Fig. 2), ranging between 17.3°C for a strain originating from Heywood Lake, Signy Island, Maritime Antarctic, and 34.6°C for several strains from Africa and Asia (see Table S1 in the supplemental material). Except for one strain originating from Antarctica and tolerating 24.4°C, all strains affiliated with the Spumella subclusters C1 and C3 tolerated maximum temperatures of between 28 and 34.6°C and of between 32 and 34.6°C, respectively. Strains from subcluster C2 were split between the Antarctic strains, tolerating between 17.3 and 28°C, and strains from other regions, which tolerated between 30.7 and 32°C (Fig. 2; see Fig. S1 and Table S1 in the supplemental material).

The temperature tolerance of the isolates was correlated with the mean monthly and mean annual air temperatures at the isolation sites (Spearman rank order correlations, \( r = 0.857 \ [P < 0.001] \) and \( r = 0.867 \ [P < 0.001] \), respectively). Similar correlation with monthly and annual temperatures at the sampling sites indicates little seasonality.

The habitat type had no effect on this correlation (for soil versus freshwater, by analysis of covariance [ANCOVA], \( P = 0.624 \)).

The temperature tolerance of the isolates, excluding those from Antarctica, rose linearly with ambient air temperature (by linear regression, adjusted \( r^2 = 0.36 \ [P < 0.001] \)) (Fig. 3). Most of the strains from Antarctic samples deviated significantly from the global trend in that they were more strongly adapted to cold conditions (Fig. 3), including several psychrophilic strains (temperature maxima of \(<20°C\)). This was significant when assuming either a linear or a logarithmic model (for a linear model, by ANCOVA, \( P = 0.003 \); for log-transformed data, by ANCOVA, \( P < 0.001 \)). The test for the two different model assumptions was done because regression analysis yielded similar regression coefficients for both models (\( r^2 = 0.685 \); log \((X + 5)\) transformed, \( r^2 = 0.729 \)).

**DISCUSSION**

Strains affiliated with the same morhotype differ considerably with respect to ecophysiology. Morphological analysis of heterotrophic protistan communities from several marine and freshwater sites around Antarctica yielded taxa of which most had been previously reported from geographic locations elsewhere (6, 35), thus supporting the view that species may be worldwide distributed. Counter to this view of limited endemism are observations that the Antarctic region is more isolated than other parts of the world and that there has been environmental selection for specific adaptive strategies over a period of several million years (37). The morphological approach, indicating a cosmopolitan and ubiquitous distribution of most protists (12), may indeed be inadequate, as molecular studies provide evidence for considerable microdiversity and a certain geographic isolation of organisms in Antarctica. For protists (23), cyanobacteria (33), and heterotrophic bacteria (37), molecular data indicate a high level of geographic isolation. This study provides evidence for ecophysiological differentiation below the level of morphospecies but also below the level of SSU rRNA clusters. Similarly, a high functional diversity has been demonstrated for several flagellate taxa, specifically for *Oxyrris marina* (26), *Neobodo designis* (21, 38), and *Spumella* (5). These studies agree that ecophysiological parameters vary within a morphospecies. So far, the available studies do not support a clear correlation between ecophysiological and molecular differences, even though several studies indicate a habitat specificity on the level of SSU rRNA clusters (4, 21, 26, 38). All these studies provide indications that morpholog-
ical characters may be insufficient for a valid taxonomic classification.

Based on the current knowledge a solution of the species problem in protists seems to still be a distant prospect. In the meantime, we suggest that all available data be considered, including morphology, molecular data, and ecophysiological data, to define protistan species. Our data indicate that strains identical in the SSU rRNA gene sequence seem to have similar ecophysiological characteristics as well. Ecophysiological variation, however, seems generally to be high even between closely related flagellate strains (5; this study). Even slight differences in the SSU rRNA gene sequence may already be affiliated with very different ecophysiological adaptations. Further, we have to keep in mind that eventually any species concept, i.e., a classification of organisms in distinct units, may be inappropriate or at least problematic to describe the continuous transitions that we observe in the ecophysiology of protistan strains and taxa.

Antarctic strains are cold adapted. Our data clearly indicate an adaptation of Antarctic strains to the cold environment, even though the tolerated temperatures are far above the realized temperatures in the respective environments. We followed a conservative protocol; i.e., all strains were acclimatized to 16°C before the experiments. The deviation of the Antarctic strains from the global trend is therefore likely to be even stronger than observed in our experiment. Further, the relatively high tolerated temperatures of the cold-adapted flagellates, i.e., between 17.3 and 28°C, correspond to theoretical considerations: low-temperature adaptation of enzymes is regarded as an ongoing process, and optimal adaptation is therefore not to be expected (9). Cold adaptation of Antarctic chrysophyte flagellates has already been demonstrated for *Paraphysomonas* (7) and is further supported by observations of community growth of Antarctic flagellates at low temperature (25). In contrast, based on low realized growth rates of the Antarctic heterotrophic nanoflagellate community in Crooked Lake at 2°C, Laybourn-Parry et al. (24) concluded that these flagellates were not adapted. Those authors, however, did not exclude predation in their experiments, and the bacterial food concentration was below 5 × 10^5 bacteria ml^{-1} with a mean bacterial cell volume of around 0.1 μm^3. Under such conditions our isolates, originating from warmer habitats, would even die back (5; J. Boenigk et al. unpublished data), and we therefore do not see a conflict with the earlier study. For the investigated strains, temperature adaptation seems not to be linked to habitat characteristics, and SSU rRNA data may not provide sufficient resolution to separate ecophysologically different clusters. Even flagellate strains identical in SSU rRNA sequence (5) and flagellate strains originating from the same habitat (this study) may differ considerably in their ecophysiology. We therefore advise caution in the ecological interpretation of experiments based on a single strain.

Geographic barriers do matter for the distribution of protists. The west wind zone and the mainly wind-driven Antarctic circumpolar current, i.e., the strongest oceanic current (1), are the main reasons for the (bio-)geographic isolation of Antarctica and its stable cold climate. Thus, if “microbial endemism” is possible at all, then Antarctica would be a promising place to find such organisms (37). In fact, the drastic deviation of the temperature adaptation of Antarctic *Spumella* morphospecies from the global trend provides strong evidence for geographic transport restriction of evolutionary and, consequently, biogeographic significance. Assuming unlimited exchange of protists, one would expect that the global trend of a steady decrease in temperature tolerance with environmental temperature includes the Antarctic continent. This does not question the possibility that protistan cells are transported by air between geographic regions (37). Intercontinental transport of protists, as proposed by Finlay and Fenchel (10, 12), is very likely (cf. references 2 and 4) and is supported by the observation of significant air travel of spores and pollen to Antarctica (27). Further, the phylogenetic similarity of isolates from Antarctic and non-Antarctic sampling sites indicates recent (in evolutionary time scales) exchange. Our data show, however, that protistan transport to Antarctica is sufficiently restricted to allow the local protistan population to adapt (not only acclimatize) to local environmental conditions and thus to build up biogeographically restricted populations. The predominance of autochthonous strains from Antarctica that are identifiable from their deviation from the global trend of temperature adaptation (Fig. 2) demonstrates that successful colonization of Antarctic habitats by allochthonous strains is rare.

Based on our findings, we expect subtle cases of protistan endemism to exist also in other isolated habitats shielded by geographic barriers, as proposed by those critical of the ubiquity theorem (8, 16, 29). We assume, however, that the resolution of current taxonomic methods, including molecular analyses, may overlook endemic ecophysiological traits as well as variance at the whole-genome level. Applying finer taxonomic resolution, we may eventually find evidence for other geographic barriers to microbes. The existence of endemic morphospecies appears unlikely, since rates of global transport of microorganisms, although restricted, are too much higher than evolutionary speciation into morphologically distinct organisms. Whether microbes can be endemic or have a biogeography depends on whether we continue to restrict these terms to evolutionary distances separating species or higher taxa. It should be noted, however, that there are less permissive and durable barriers, such as geological formations or ice caps, that may give rise to geographic speciation of microbes as is known for plants and animals.

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REFERENCES


18. Foissner, W.

16. 15. Finlay, B. J., and K. J. Clarke. Finlay, B. J.


13. Finlay, B. J.

12. Coleman, A. W.

11. Finlay, B. J.


### 2.2 Molecular diversity

The molecular diversity of microbial eukaryotes has been extensively studied using various molecular techniques. These methods have allowed the identification of many new species and the discovery of novel lineages that were previously unknown. The use of molecular techniques has also revealed unexpected levels of diversity within certain groups of microorganisms. For example, the analysis of SSU rRNA gene composition in freshwater habitats has revealed a rich diversity of microeukaryotes that were previously unappreciated.

### 2.3 Ecological diversity

The ecological diversity of microbial eukaryotes is vast, ranging from unicellular organisms to complex multicellular structures. The diversity of these organisms extends across a wide range of environmental conditions, from the extreme cold of polar regions to the high temperatures of hot springs. This diversity has profound implications for our understanding of the role of these organisms in ecosystems. For instance, the diversity of microeukaryotes in Antarctic lakes has been shown to be high, with many species exhibiting tolerance to extreme environmental conditions.

### 2.4 Functional diversity

Microbial eukaryotes play a crucial role in the functioning of ecosystems. They are involved in processes such as nutrient cycling, energy turnover, and the decomposition of organic matter. For example, the presence of microeukaryotes in marine sediments has been shown to contribute to the degradation of organic carbon, which is a key process in the carbon cycle. The functional diversity of these organisms is critical for the maintenance of ecosystem health.

### 2.5 Evolutionary diversity

The evolutionary diversity of microbial eukaryotes is a topic of ongoing research. The analysis of mitochondrial DNA sequences has provided insights into the evolutionary relationships among different groups of microeukaryotes. For instance, the analysis of COI sequences has revealed the presence of several divergent lineages within the Ciliophora and the Stramenopiles.

### 2.6 Conclusion

Microbial eukaryotes are a diverse and important group of microorganisms. The study of their diversity across different environmental conditions has provided valuable insights into the ecology, evolution, and function of these organisms. As our understanding of microbial eukaryotes continues to grow, so too will our appreciation of their ecological and evolutionary significance.