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SSU rRNA gene variation resolves population heterogeneity and ecophysiological differentiation within a morphospecies (*Stramenopiles, Chrysophyceae*)

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Abstract

The objective of this study was to analyze the extent of ecophysiological and molecular homogeneity, or heterogeneity, of distinct protist populations and to test for correlations between molecular distance and ecophysiological adaptation in a widespread and ubiquitous protistan taxon. We selected heterotrophic nanoflagellates of the *Spumella* morphotype as a model organism, because these flagellates are widespread and among the dominant bacterivores in many microbial communities. We investigated the molecular microdiversity and the ecophysiological tolerances for a total of 13 strains originating from two freshwater samples. We further investigated the affiliation of different ecotypes with distinct small subunit ribosomal ribonucleic acid (SSU rRNA) genotypes, or molecular operational taxonomic units. These regional population studies were further compared to the molecular and ecophysiological diversity of 28 strains originating from remote sampling sites. None of the investigated populations are homogenous, but are rather heterogeneously composed of different ecotypes and genotypes, possibly corresponding to cryptic species. This population heterogeneity may partly explain the deviations between studies on single strains and populations in both laboratory and field studies. The molecular distance between the strains was correlated with the salinity and temperature adaptation of the respective strains, contradicting the assumption that SSU rRNA variation reflects accumulated neutral mutations. Independent of whether this correlation reflects adaptation above the (biological) species level or variation between asexually reproducing lineages, we demonstrate the unsuitability of the current classification system (species concept) for the investigated organisms, at least with respect to ecological and ecophysiological investigations.

The ecology of protistan species has usually been assigned to morphospecies and until recently, many ecologists have basically assumed a sufficient phylogenetic and ecophysiological resolution of the morphological characters (Finlay and Fenchel 2004). The multitude of studies inferring the ecology and physiology of protistan species from experiments based on single strains, or populations, reflects this line of thinking (Caron 1990; Ekelund 1996; Boenigk and Arndt 2000).

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The relatively recent introduction of comparative sequence analysis of phylogenetic marker genes (specifically ribosomal ribonucleic acid [rRNA] genes) has disclosed a high molecular variation within many nominal species (e.g., *Oxyrrhis marina* [Alveolata, Dinoflagellata; Lowe et al. 2005], *Neobodo designis* [Euglenozoa, Kinetoplastida; Koch and Ekelund 2005; von der Heyden and Cavalier-Smith 2005], *Micromonas pusilla* [Chloroplastida, Prasinophyceae; Šlapeta et al. 2005], and *Spumella* sp. [Chromista, Stramenopiles; Boenigk et al. 2004]). The findings of high molecular variation have stimulated research into a "cryptic" functional diversity reflecting particular adaptations among morphologically indistinguishable members of nominal species. Several studies indicate a habitat specificity for cryptic species at the level of small subunit ribosomal ribonucleic acid (SSU rRNA) gene clusters (Koch and Ekelund 2005; Lowe et al. 2005; van der Heyden and Cavalier-Smith 2005). Ecophysiological variation below the morphospecies level has become apparent in recent studies (Koch and Ekelund 2005; Lowe et al. 2005; Boenigk et al. 2006*a*). The degree of similarity between (clonal) strains, or

populations, affiliated with the same morphospecies and their ecophysiological adaptation to different habitats has, however, not been sufficiently addressed.

Consequently, several key questions in protist ecology cannot currently be satisfactorily answered. For instance, are protists with similar morphotypes from a particular environment homogeneous or are they composed of different distinct genotypes and ecotypes? Furthermore, are rRNA gene sequences suitable markers for identifying ecophysiologically distinct strains (i.e., does the SSU rRNA sequence variation correlate with ecophysiological diversity within morphologically indistinguishable protists)? A promising way to tackle these questions is through comprehensive studies on intraspecific ecophysiological variation based on a large set of strains and its correlation to genetic distances.

In order to resolve these questions, we investigated 13 clonal strains isolated from two distinct populations of the widely distributed and abundant flagellate morphospecies *Spumella*. *Spumella* spp. are among the most thoroughly investigated bacterivorous flagellate model taxa (Boenigk et al. 2006*c*). The *Spumella* morphotype most probably evolved independently several times within the chrysomonads and the distinct monophyletic lineages show a considerable molecular and ecophysiological variation both within and between sequence clades (Boenigk et al. 2006*a*; Boenigk 2008). It is, however, still unclear to what extent distinct populations of a protist morphospecies are composed of different molecular lineages and to what extent these lineages may be differently adapted. We combined a molecular analysis with an ecophysiological characterization of the strains. We hypothesized that protist populations (i.e., organisms affiliated with the same morphotype from the same sample) are not homogenous, but are rather composed of different genotypes reflecting different ecophysiological adaptations, and that the SSU rRNA gene variation is indicative of ecophysiological differentiation.

Methods

Sampling sites—Strains were isolated from two different sample sites (i.e., Lake Fuschlsee and the Fuschler Ache stream) in July 2004. Lake Fuschlsee is located in the Salzkammergut, at 47°48′10″N and 13°16′20″E. Its surface area is ~2.7 km^2 and its maximum depth is 66 m. The Fuschler Ache is a small stream (the mean discharge is 4.4 m^3 s^{-1}) with a total length of 22 km connecting the Fuschlsee and Mondsee lakes. The conductivity was 309 μS cm^{-1} and 396 μS cm^{-1}, the potential Hydrogen (pH) was 8.0 and 8.6 and the temperature 22.2°C and 20.5°C in Lake Fuschlsee and the stream sampling site, respectively. For comparative reasons, the strains originating from the remote global sampling sites were also included in the analysis. The latter strains have been described in former studies (Boenigk et al. 2005, 2006*b*). Data on the habitat characteristics are not available for these latter samples.

Isolation of the flagellates—Water originating from each respective sampling site was immediately filtered through 1.2-μm gauze. The obtained medium contained a natural bacterial assemblage, which was later used as a food resource. Prior to isolation, the medium was checked for the occurrence of flagellates which may have passed the filter. The medium was then directly checked by microscopy of the water and of the water collected on the nucleopore filters after staining with 4′,6-diamidino-2-phenylindole (DAPI). As a further control, a subsample of the filtered lake water was supplemented with bacteria and monitored for the growth of flagellates. We did not detect flagellates in the filtered lake water (i.e., either there were no flagellates present or the abundance was <0.05 cells mL^{-1}). Such slight potential contaminations, if present, did not affect the outcome of the isolation procedure. Two differently sized bacterial strains were added as an additional food source at a concentration of ~10^6 bacteria mL^{-1}. These were the bacterial strain *Listonella pelagia* CB5 (0.38 ± 0.2 μm^3) and the ultramicrobacterial strain MWH-MoNR1 (0.04 ± 0.017 μm^3); the closest known relative *Clavibacter michiganensis* ([Microbacteriaceae] Hahn and Höfle 1998; Hahn 2003).

The flagellate strains were isolated by serial dilution; we applied at least two to four subsequent isolation steps. In fresh samples, the flagellates were counted using a Sedgewick–Rafter chamber, and a subsample was diluted stepwise with filtered lake water to a final abundance of 0.5 flagellates mL^{-1}. Aliquots of 1 mL were subsequently transferred to 24-well cell culture plates (Becton Dickenson) and stored at room temperature in the dark. The wells were checked for the growth of flagellates every second day for a period of at least 2 weeks using a magnification of 20×. When growth was detected, the flagellates were transferred to a 50-mL Erlenmeyer flask containing 25 mL filtered lake water and 25 mL inorganic basal medium (IBM; Hahn et al. 2003) that was enriched with fresh food bacteria and the strains MWH-MoNR1 and *Listonella pelagia* CB5 at a concentration of ~10^6 bacteria mL^{-1}. After 2–6 d, the samples were further diluted to a final concentration of 0.05 flagellates mL^{-1} and 0.1 flagellates mL^{-1} using solely the IBM medium enriched with the strains MWH-MoNR1 and *Listonella pelagia* CB5 at a concentration of ~10^6 bacteria mL^{-1}. Each of these dilutions were transferred to wells of sterile 24-well cell culture plates (1 mL per well) and incubated at room temperature in the dark. A screening of the wells for the growth of flagellates was again performed by direct microscopical investigation every second day. This second isolation step was repeated one to three times, depending on the isolate. Finally, when growth was detected, the *Spumella*-like flagellates were transferred to a permanent culture using IBM medium supplemented with a wheat grain.

The isolation efficiency was roughly similar for the *Spumella*-like flagellates as compared to other protists. Roughly 15% of the isolated strains were *Spumella*-like flagellates (all between 4 μm and 6 μm in diameter). These were inspected by light microscopy and prepared for sequence analysis. However, some of the *Spumella*-like flagellate strains died in culture after the first few weeks, so that only 13 (out of 20) were kept in permanent culture

afterwards. These strains were ecophysiologically characterized and the SSU rRNA gene was sequenced.

Molecular characterization of the flagellates—For the molecular characterization of the strains, 20 μL of the flagellate culture (corresponding to 50–200 cells) was transferred into 0.2-mL polymerase chain reaction (PCR) tubes, heated for 5 min at 95°C and stored at −70°C for at least 24 h for subsequent direct PCR-amplification. We have previously demonstrated successful amplification, even of single cells (Auinger et al. 2008), and the mentioned quantity resulted in a strong product. The flagellate 18S rRNA genes were amplified using the forward primers SSU1Fa (5′-CCTGGTTGATYCTGCCAGTAG-3′) and ATKcfw (5′-CTGCCAGYAGTCATATGCTT-3′) and the respective reverse primers 1840Rb (5′-TCCTTCYGCAGGTTCACCTACG-3′) and ATKdre (5′-ACCTTGTTACGACTTTTGCTT-3′). Each PCR reaction contained 1.25 U Taq Polymerase (Quiagen), 1× PCR buffer, 200 nmol L^{-1} of each primer, 200 μmol L^{-1} deoxynucleotid triphospate, 20 μL aliquoted flagellate culture, and the respective amount of deionized, diethylpyrocarbonate (DEPC) treated water, resulting in a total reaction volume of 50 μl. The amplification was performed in a Primus Thermal Cycler (MWG-Biotech) after denaturation at 95°C for 3 min, followed by 35 cycles of 94°C for 1 min, 52°C for 1 min, 72°C for 2 min, and a final extension at 72°C for 10 min. The PCR products were purified using the QIAquick PCR purification kit (Qiagen) and were subsequently sequenced (~1700 base pairs [bp]). The sequencing reactions were performed with an ABI Prism® Big Dye™ Terminator version 3.0 ready reaction cycle sequencing kit (Applied Biosciences) and an ABI PRISM 3100 automated sequencer. The sequences were initially aligned using the "Clustal W" option (Thompson et al. 1997) in the BioEdit 5.0.9 sequence analysis software (Hall 1999). For further phylogenetic analyses we added the 13 almost complete sequences obtained in this study to an alignment available in the ARB program package. The new sequences were aligned by using the aligning tool of this package (http://www.arb-home.de; Ludwig et al. 2004). The alignment was based on primary and secondary structures, which improved the recognition of homologous positions. Where necessary, we included reference sequences not present in the ARB database from GenBank. The sequences were added to the ARB tree using the Quick Add Parsimony tool and by applying a Stramenopile-specific filter, as implemented in the same package. The alignment was verified by an automatic alignment to the next relative sequences and was corrected manually. The final position within the ARB tree and the bootstrap values were calculated using the Parsimony Interactive Tool. For a final tree calculation, a maximum likelihood analysis was performed using PhyML (Guindon and Gascuel 2003) and the implemented GTR substitution model. The reliability of the internal branches was assessed with the implemented bootstrap method. The sequences have been deposited in the GenBank database under the accession No. EU787413-EU787424.

Ecophysiological characterization of strains—Thirteen strains of the *Spumella* morphospecies collected on a local scale, as well as 28 additional strains from remote sampling sites (Boenigk et al. 2005, 2006*a*), were characterized with regard to their tolerance to key ecophysiological parameters (i.e., pH, temperature, and salinity). Prior to the experiments, all of the strains were acclimatized to the same conditions (i.e., IBM medium at 16°C and pH 7.8) for ≥3 months. All of the experiments were run in 4 mL of medium placed in 12-well tissue culture plates using the bacterial strain *Listonella pelagia* CB5 as a food source (Boenigk et al. 2004). The initial food concentration was adjusted to near-satiating food conditions (i.e., 2×10^7 bacteria mL^{-1}; Pfandl and Boenigk 2006). This strain remained the dominant bacterial strain, at least during the initial 2 d of the experiment. Potential shifts in the food spectrum, which may have developed later on, are not expected to affect the tolerances of the investigated flagellates, because the food conditions remained satiating (Pfandl and Boenigk, unpubl.) and the food spectrum of the *Spumella*-like flagellates is broad (Boenigk et al. 2004; Boenigk and Arndt 2000).

The experiments followed an acclimatization approach (i.e., the strains were stepwise acclimatized to the respective experimental conditions). During the experiments, growth was checked every 2–3 d by inverted light microscopy at 200× magnification. The experiments usually ran for 3 d but on occasions, were run up to 14 d until growth was observed. If no growth was observed after 14 d, the respective treatment was defined as not supporting the growth of the tested strain. If growth was observed, the strains were subcultured to either the next higher or lower treatment. All of the experiments were run in triplicate.

Despite the generally similar setup of the experiments, the medium and temperature conditions differed due to the demands of the respective test series. Temperature tolerance experiments were carried out in IBM medium. The maximum temperature tolerance limits were tested starting at 16°C and increased in steps of 1°C to 1.5°C. The pH tolerance experiments were carried out at 16°C using modified $IBM_{buffered}$ medium. The treatments were tested in a pH range from 2.5 to 12. An increase in steps of 0.3 pH units was applied. The pH remained stable in all of the experiments for all of the pH values below 8, whereas in the high pH treatments (a pH between 10.6 and 11.5), a permanent decrease by roughly 0.6 pH units per day was observed until the pH stabilized between 8 and 9. As the strains, at least initially, survived the high pH conditions, the starting values were used for the analysis. However, we also provide the pH conditions at the termination of the experiments (Table 1). The salinity tolerance experiments were carried out at 16°C in low light conditions. These experiments were run using a basal medium supplemented with different amounts of NaCl, starting with 1 g NaCl L^{-1} (corresponding to 1‰ and increasing in steps of 1 g NaCl L^{-1}). All of the experiments were replicated and the respective tolerance was calculated as the mean of all of the replicates.

Correlation of molecular and ecophysiological data—We tested for correlations between molecular divergence and

Table 1. Strains, GenBank sequence accession numbers, key variables of the strains ecophysiological adaptation, and origin of the strains.

Strain	Accession number	Cluster	Maximum temperature (°C)	Maximum pH tolerated (initially)	Maximum pH tolerated (stabilized)	Minimum pH tolerated (stabilized)	Maximum salinity (g L^{-1})	Origin
1-8-A1	EU787413	C3	28.7	10.9	8.5	3.15	2	Austria, Lake Fuschlsee
1-13-F1	EU787414	C3	30.7	11.2	8.68	3.15	5	Austria, Lake Fuschlsee
1-13-F4	EU787415	C3	32	10.9	8.5	3.15	3	Austria, Lake Fuschlsee
1-18-F1	EU787416	C3	28.7	10.9	8.5	3.15	3	Austria, Lake Fuschlsee
9-1-D1	EU787420	C3	31.7	10.9	8.5	3.15	5	Austria, Fuschler Ache
9-2-A2	EU787421	C1	28.7	10.9	8.5	3.15	6	Austria, Fuschler Ache
9-7-F1	EU787422	C1	30.7	10.9	8.5	3.15	6	Austria, Fuschler Ache
9-8-D3	EU787417	C3	32	11.2	8.68	3.15	5	Austria, Fuschler Ache
9-10-C1	EU787425	C2	30.7	10.9	8.5	3.15	5	Austria, Fuschler Ache
9-12-B3	EU787418	other	28	10.9	8.5	3.15	3	Austria, Fuschler Ache
9-14-C1	EU787419	C3	30.7	10.9	8.5	3.15	5	Austria, Fuschler Ache
9-14-D1	EU787423	C1	28.7	10.9	8.5	3.15	4	Austria, Fuschler Ache
9-20-C4	EU787424	C1	28.7	10.9	8.5	3.15	6	Austria, Fuschler Ache
JBC 07	AY651097	C3	33.6	11.2	8.68	3.15	6	China, aquatic habitat
JBM 08	AY651098	other	30.7	10.9	8.5	3.15	1	Austria, aquatic habitat
JBM 09	AY651087	C2	31.7	10.9	8.5	3.15	5	Austria, aquatic habitat
JBM 10	AY651074	C3	34.6	11.2	8.68	3.15	5	Austria, aquatic habitat
JBM/S 11	AY651083	C1	30.7	11.2	8.68	3.15	6	Austria, soil
JBM/S 12	AY651085	C1	28	11.2	8.68	3.15	6	Austria, soil
JBC 13	AY651080	C1	30.7	11.2	8.68	3.15	6	China, aquatic habitat
JBL 14	AY651086	C2	32	11.2	8.68	3.15	6	Austria, aquatic habitat
JBM 18	AY651092	other	28	11.2	8.68	3.15	3	Austria, aquatic habitat
JBM 19	AY651084	C1	28.7	10.9	8.5	3.15	4	Austria, aquatic habitat
JBC 20	DQ388539	other	31.7	10.9	8.5	3.15	5	China, aquatic habitat
JBC 21	DQ388540	other	30.7	10.9	8.5	3.15	5	China, aquatic habitat
JBC 22	AY651078	other	32	11.2	8.68	3.15	5	China, aquatic habitat
JBC/S 23	AY651081	C1	32	11.2	8.68	3.15	5	China, soil
JBC/S 24	AY651082	C1	32	11.2	8.68	3.15	6	China, soil
JBC27	AY651093	other	33.6	10.9	8.5	3.15	3	China, aquatic habitat
JBM 28	AY651089	other	31.7	10.9	8.5	3.15	5	Austria, aquatic habitat
JBC 30	AY651073	C3	33.6	11.2	8.68	3.15	6	China, aquatic habitat
JBC 31	AY651072	C3	33.6	11.2	8.68	3.15	6	China, aquatic habitat
JBAF32	AY651076	C3	34.6	11.2	8.68	3.15	6	Africa, aquatic habitat
JBAF33	AY651077	C3	34.6	10.9	8.5	3.15	4	Africa, aquatic habitat
JBAF35	AY651071	other	31.7	10.9	8.5	3.15	4	Africa, aquatic habitat
JBAS36	AY651079	C1	34.6	11.2	8.68	3.15	4	Australia, aquatic habitat
JBAS38	DQ388538	C3	32	10.9	8.5	3.15	4	Australia, aquatic habitat
JBNZ39	AY651088	C2	30.7	10.9	8.5	3.15	4	New Zealand, aquatic habitat
JBNZ41	AY651075	C3	34.6	10.9	8.5	3.15	4	New Zealand, aquatic habitat
JBNA45	DQ388541	C2	32	10.9	8.5	3.15	4	Hawaii, aquatic habitat
JBNA46	DQ388542	other	36.4	10.9	8.5	2.6	6	Hawaii, aquatic habitat

differences in the ecophysiological tolerances by dividing the isolates into two groups: One group, comprising the 13 strains of the *Spumella* morphotype, was used for analyses on a local scale. The second group, consisting of 28 strains of the *Spumella* morphotype from remote sampling sites, was used for analyses on a global scale. The uncorrected divergence (%; *p* distances) of the 18S rRNA gene sequences between the *Spumella*-like flagellates was calculated using BioEdit version 7.0.1 (Hall 1999), resulting in an identity matrix with a proportion of identical residues between the pairwise aligned sequences. The pairwise distances between the ecophysiological tolerances were calculated separately for each parameter. Correlations were tested using the Mantel option in the software package GenAlEx6 (Peakall and Smouse 2006).

Testing for such correlations is, in general, not trivial. Closely related strains should generally show similar ecophysiological adaptations. In contrast, distantly related flagellates may strongly differ in their ecophysiological adaptation, but they may also be adapted to similar conditions. Consequently, the pairwise adaptive difference between the strains does not necessarily need to be large for distantly related flagellates. In the present study, strong deviations in the ecophysiological adaptation occurred between some of the distantly related flagellates, whereas this was not observed between the more closely related

strains. Normally, measures of variance (i.e., standard deviation [SD]) should be high for groups of distantly related flagellates and low for groups of closely related flagellates. Therefore, we also tested the correlations between molecular distance and variation in ecophysiological similarity based on groups of pairs of flagellates with increasing molecular distances: the variance (in terms of SD) was calculated for the groups with a molecular distance of 0%, 0.1–1%, 1.1–2%, etc. The Mantel test was applied to the data in order to test the correlation between the molecular distance and variances.

Results

Molecular diversity—All of the *Spumella* morphotype isolates were affiliated to clade C of the Chrysophyceae sensu stricto (Andersen et al. 1999). Four strains were affiliated with subcluster C1, 1 strain with subcluster C2, and 7 strains with subcluster C3 (Fig. 1). One strain was not affiliated with any of the three subclusters. In some cases, the uncorrected genetic distances within the *Spumella* morphotype were quite high (up to 8.7%), but the sequence divergence within the subclusters was lower (e.g., $<1\%$ [C1] and $<2\%$ [C3]).

With respect to the two investigated populations, all of the four isolated clonal strains from Lake Fuschlsee were only affiliated with the C3 subcluster. The strains isolated from the stream sample (Fuschler Ache) were distributed within all of the subclusters (Fig. 1): 4 strains affiliated with the C1 subcluster, 1 strain affiliated with the C2 subcluster, 3 strains affiliated with the C3 subcluster and 1 strain did not group with any of the subclusters (9 strains in total).

Ecophysiological characterization of the strains—Thirteen regional and 28 global strains (Boenigk et al. 2005, 2006*b*) were selected for the ecophysiological experiments. All of the isolated organisms were allowed to acclimatize to identical starting conditions. We investigated the maximum salinity tolerance, the minimum and maximum pH tolerance and the maximum temperature tolerance. The data on the maximum temperature tolerance for the strains originating from the remote global sampling sites were taken from Boenigk et al. (2006*b*; *see* Methods).

pH tolerance: For all of the strains investigated, both on a regional and global scale, the pH tolerance was rather similar, irrespective of the genetic distance. The minimum pH tolerance was 3.15 for all of the strains except one (pH 2.6), and the maximum pH tolerance was between 10.9 (8.35) and 11.2 (8.68) for all of the strains (Table 1).

Temperature tolerance: On a regional scale, the maximum temperature tolerances ranged between 28.0°C and 31.7°C. Despite the strain-specific differences, we found no significant difference between the SSU rRNA sequence subclusters (ANOVA on ranks, $p > 0.05$), indicating that the strains affiliated with distinct subclusters may be adapted to similar conditions. On a global scale, the maximum temperature tolerances of all of the flagellates ranged between 28.0°C and 36.4°C (Table 2). Regarding the subclusters, the temperature tolerances of the strains grouped within C3 were significantly higher than for the strains affiliated with C1 (ANOVA on ranks, $p = 0.010$), indicating various distribution centers with respect to the different climatic zones.

Salinity tolerance: In general, the salinity tolerance increased slightly after acclimatization. However, acclimatization did not affect the general pattern of 'low' and 'high' salinity tolerance (Spearman rank order correlation, $p < 0.001$). We therefore concentrated on the direct transfer experiments for data analysis. On a regional scale, the salinity tolerances ranged between 2 g L^{-1} and 6 g L^{-1}. Within the subclusters, the salinity tolerances of the C1 strains were significantly higher than the tolerances of the strains affiliated with the C3 subcluster (ANOVA, *t*-test, $p = 0.048$). On a global scale, the salinity tolerances ranged between 1 g L^{-1} and 6 g L^{-1}. However, the differences between the subclusters were not significant (ANOVA on ranks, $p \gg 0.05$).

Correlations between ecophysiological variation and molecular distance—regional scale—Molecular distance was not significantly with either the pairwise difference of the flagellates' temperature tolerance limits or the pairwise difference of the flagellates' salinity tolerance limits (Table 3; Fig. 2). When the analysis was restricted to subcluster C3, molecular distance was correlated to both the temperature and salinity tolerances (Table 3). The analysis of the ecophysiological parameter variances of the flagellates grouped by distinct molecular distances (i.e., 0%, 0.1–1%, 1.1–2%, etc.) showed that the variances in temperature tolerance limits were correlated to molecular distance while the variances in salinity tolerance limits were not (Table 3; Fig. 3). Again, when the analysis was restricted to the C3 subcluster, the variances in the temperature and salinity tolerance limits were correlated to molecular distance (Table 3).

Global scale—Molecular distance was correlated to temperature and salinity tolerances (Table 3; Fig. 2). Molecular distance within the C3 subcluster was also correlated to temperature tolerance, but not to salinity tolerance. No correlations were found within the C1 subcluster (Table 3). Similarly, the variances in the temperature tolerance limits and salinity tolerance were correlated to molecular distance (Table 3; Fig. 3). Within the subclusters, only the variances in temperature tolerance were correlated to molecular distance for the C1 and C3 subclusters (Table 3).

Discussion

SSU rRNA gene variation within flagellate morphospecies indicate habitat specificity—Many protistan morphospecies, in particular those lacking a sufficient number of morphological features, contain a high number of genetically distinct lineages and in fact, comprise numerous cryptic species (Nanney et al. 1998; Zettler et al. 1998; Šlapeta et al. 2005). Accordingly, the isolated flagellates of the *Spumella* morphospecies showed a high 18S rRNA gene diversity and were, in most cases, affiliated with the previously described subclusters C1, C2, and C3 (Boenigk et al. 2005). However, it remains unclear whether this

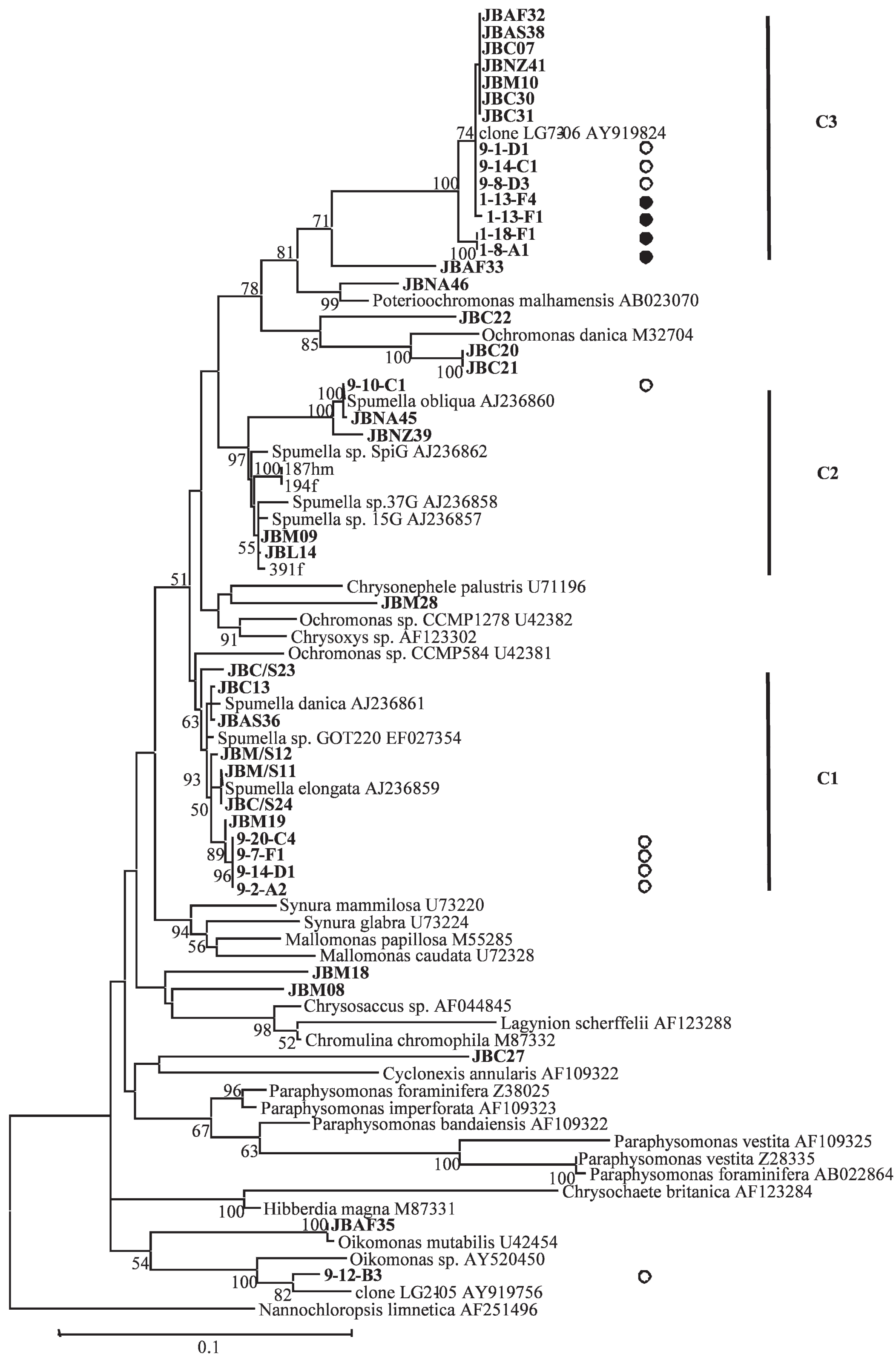

Fig. 1. Maximum likelihood tree showing the phylogenetic affiliation of the *Spumella*-like isolates to the Chrysophyceae sensu stricto. The numbers at the nodes of the tree indicate the percentage of bootstrap values for each node out of 100 bootstrap resamplings (values above 50 are shown). Habitat origins of the isolates on a regional scale are marked by either black (Lake Fuschlsee) or white circles (Fuschler Ache stream). Isolates which were included in the ecophysiological analysis are printed in bold.

Table 2. Temperature and salinity tolerance ranges and means for all of the flagellates originating from remote global and regional sampling sites, together with specific subclusters. *Spumella*-like flagellates not affiliated with any cluster are summarized as "other". n.d. = not determined.

		Regional scale		Global scale	
		Tolerance range	mean	Tolerance range	mean
Temperature (°C)	all flagellates	28.0–31.7	30.7±1.3	28.0–36.4	32.2±2.06
	C1	28.7–30.7	29.2±1.0	28.0–34.6	31.0±2.27
	C2	n.d.	30.7	30.7–32.0	31.6±0.62
	C3	28.7–31.7	30.6±1.4	32.0–34.6	33.9±0.91
	other flagellates	n.d.	28.0	28.0–36.4	31.8±2.27
Salinity (g L^{-1})	all flagellates	2–6	4.0±1.3	1–6	4.8±1.23
	C1	4.0–6.0	5.5±1.0	4–6	5.3±0.95
	C2	n.d.	5.0	4–6	4.7±0.96
	C3	2.0–5.0	3.8±1.2	4–6	5.1±0.99
	other flagellates	n.d.	3.0	1–6	4.1±1.54

molecular diversity reflects an ecophysiological differentiation or is, in fact, rather neutral.

Habitat specificity and ecophysiological differentiation between phylotypes have repeatedly been observed (Kim et al. 2004; Boenigk et al. 2005, 2006*a*, *b*) and a high functional diversity has already been demonstrated for several flagellate taxa (e.g., *Oxyrris marina* [Lowe et al. 2005], *Neobodo designis* [Koch and Ekelund 2005] and *Spumella* [Boenigk et al. 2004]). Our study confirms this observation and provides evidence for the habitat specificity within a morphospecies on a local scale. All of the *Spumella* flagellates in the C3 subcluster were exclusively found in aquatic environments (i.e., in either the lake or the stream). In most cases, the flagellates in the C1 subcluster were previously obtained from soil sites (Belcher and Swale 1976; Boenigk et al. 2005, 2006*b*). In this study, we isolated the strains affiliated with the C1 subcluster from the Fuschler Ache stream, indicating that these strains may have been introduced into the stream from the surrounding soils.

Flagellate populations are heterogeneously composed of different genotypes and different ecotypes—The *Spumella*-like flagellates investigated in this study all belong to one morphospecies; yet, they represent a polyphyletic group (Boenigk et al. 2005, 2006*b*). A high molecular variation, and possibly polyphyly, is known for many flagellate taxa (van der Heyden and Cavalier-Smith 2005). However, it is still unknown whether distinct flagellate morphospecies (i.e., flagellates living simultaneously at the same place) are composed of different genotypes and ecotypes or are instead homogeneous. We therefore investigated the molecular and ecophysiological variation within a flagellate morphospecies for clonal strains originating from the same sample. Sequence variation within the SSU of clonal strains is unknown for chrysophytes, but may occur in other protists (Thompson et al. 1999). Therefore, we cloned some of the strains (data not shown); however, we did not find any sequence variation. The sequence variation found in this study therefore reflects variation between individuals

Table 3. Correlations between the pairwise molecular and ecophysiological distances (upper line) and between the variances in ecophysiological tolerances and molecular distance (lower line). Correlation coefficients and *p* values are given for all of the flagellates on a regional and global scale and, in addition, for the distinct subclusters. Correlations were undertaken using a one-tailed Mantel test (*see* the Methods section for an explanation). Bold numbers indicate significant correlations between the molecular and ecophysiological distances and between the variances in ecophysiological tolerances and molecular distances. n.d. = not determined.

		Regional scale		Global scale	
		p	*r*	*p*	*r*
Temperature (°C)	all flagellates	0.091	0.150	**0.044**	**0.182**
	all flagellates	**0.009**	**0.376**	**0**	**0.429**
	C1	n.d.	n.d.	0.684	−0.147
	C1	n.d.	n.d.	**0**	**0.711**
	C3	**0.048**	**0.788**	**0.004**	**0.494**
	C3	**0**	**0.693**	**0**	**0.936**
Salinity (g L^{-1})	all flagellates	0.150	0.129	**0.006**	**0.272**
	all flagellates	0.451	−0.02	**0**	**0.640**
	C1	n.d.	n.d.	0.983	−0.281
	C1	n.d.	n.d.	0.999	−0.710
	C3	**0.046**	**0.493**	0.474	−0.001
	C3	**0**	**0.692**	1	−0.845

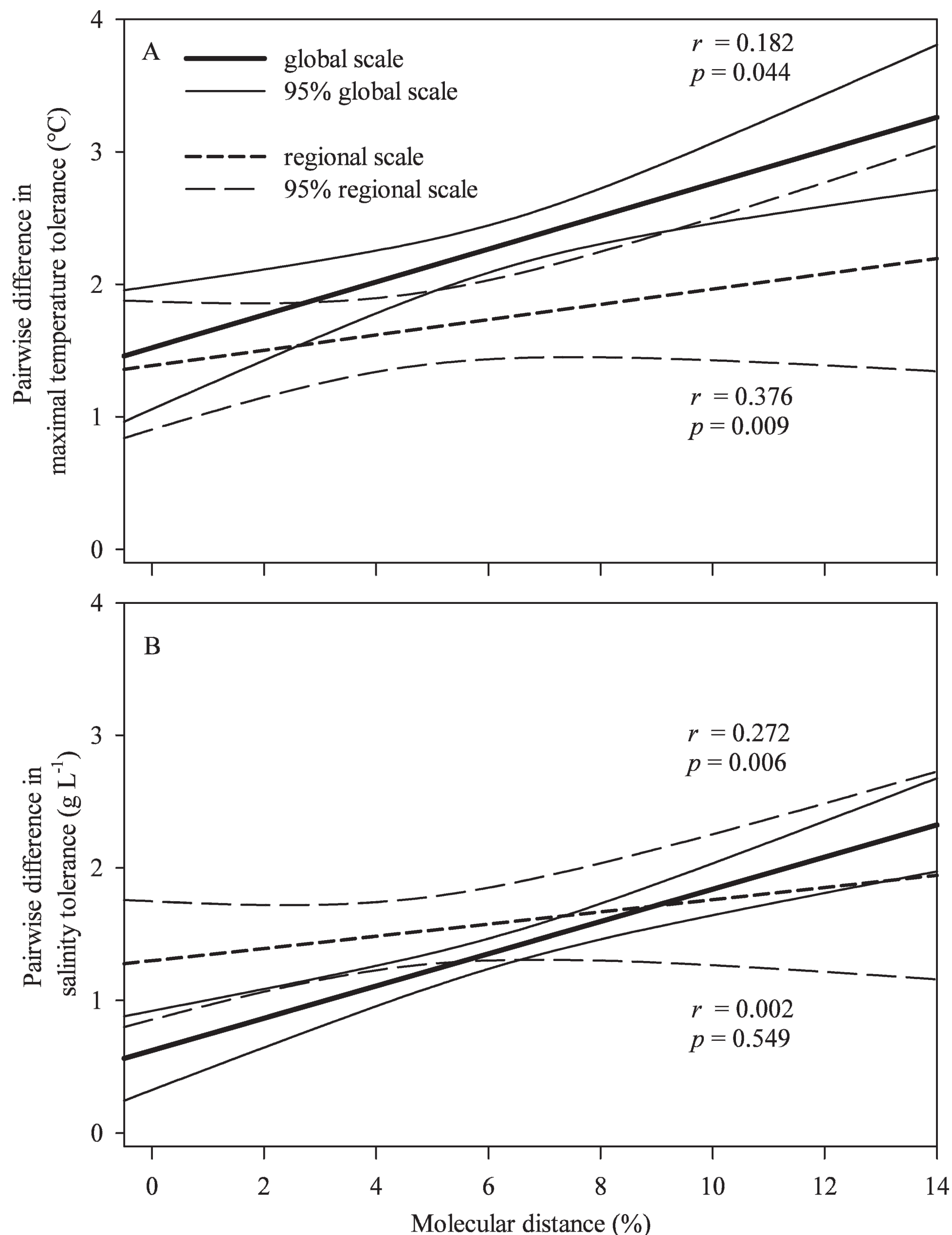

Fig. 2. Ecophysiological differentiation as a function of molecular distance. Regression lines and 95% confidence intervals are shown for flagellates originating from global remote sampling sites and the local sampling sites. (A) Maximum temperature tolerance, (B) maximum salinity tolerance. The correlation coefficients (r) and significance (p) based on the Mantel tests are given in the figure. Despite the low correlation coefficients (as expected – *see* Methods), all of the correlations were highly significant.

rather than sequence variation among ribosomal operons within a single strain.

The stream population was composed of different genotypes with different ecophysiological adaptations. This could be expected because the stream community was influenced by communities from the surrounding soil environments, as well as by the flagellate community in Lake Fuschlsee. However, even the Lake Fuschlsee population was composed of different genotypes. Furthermore, the strains originating from the same population also differed with respect to their ecophysiological tolerance limits. This general finding of morphospecies heterogeneity highlights the importance of niche and ecophysiological differentiation in maintaining the prevalence of the seemingly ubiquitous flagellate morphotypes.

The diversity and prevalence of individual variants within protist populations implies that generalizations about field populations based on single strains are problematic, or even inappropriate. Specifically, the assumption of a certain reaction norm and a certain

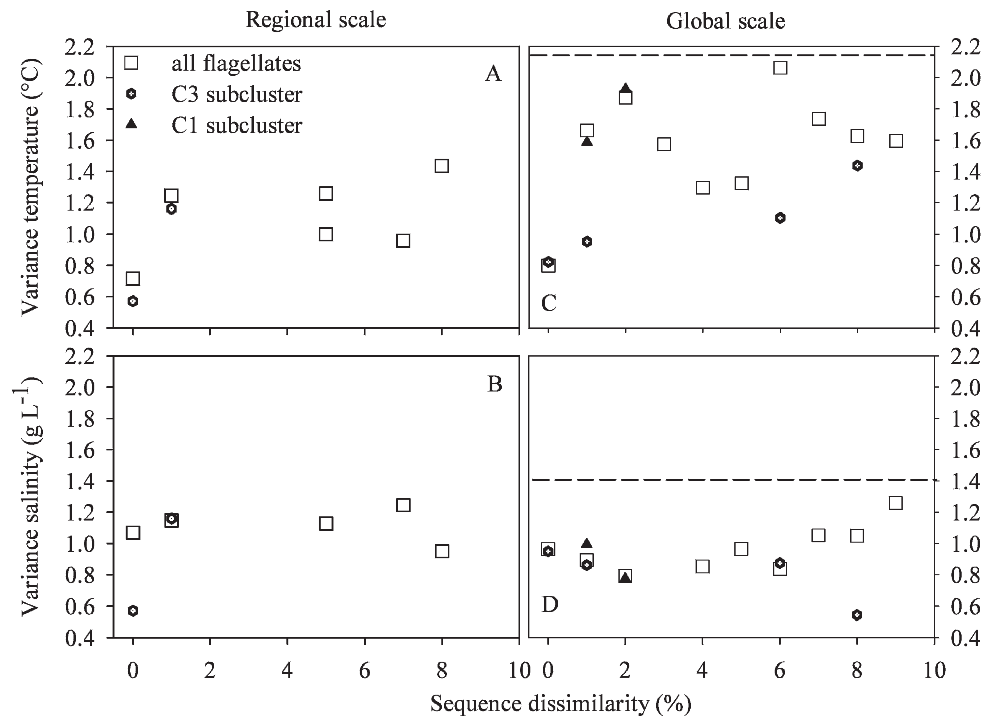

Fig. 3. Variances in the ecophysiological tolerance limits (temperature and salinity) as a function of molecular distance for flagellates (A, B) on a regional scale and (C, D) on a global scale based on groups of increasing molecular distances in steps of 1%. The reference line (dashed) refers to the mean ecophysiological difference between distantly related strains (i.e., strains with a molecular distance of ≥10%).

adaptation, which is intrinsic in the extrapolation of laboratory findings to field situations, seems problematic (Boenigk et al. 2004; Koch and Ekelund 2005; Lowe et al. 2005).

Protist ecophysiological differentiation is related to molecular distance—The ecophysiological relevance of high levels of molecular variation within protist morphospecies has been controversially discussed during recent years. While some authors consider molecular diversity to be an indication of functional differentiation and possibly speciation (von der Heyden and Cavalier-Smith 2005; Boenigk et al. 2006*a*), other authors consider molecular variation within protist morphospecies as being neutral (Fenchel 2005). Even though several studies have already indicated different ecophysiological adaptations (Boenigk et al. 2006*b*) and habitat preferences of different lineages (Boenigk et al. 2005; von der Heyden and Cavalier-Smith 2005; Boenigk et al. 2006*b*), an approximate linear correlation between molecular and ecophysiological variation has not yet been demonstrated. Such correlations, if present, would contradict Fenchel's assumption (Fenchel 2005) that variation in rRNA gene sequences only reflects accumulated neutral mutations and, at least for the investigated taxon, proves the unsuitability of the current morphospecies concept in protist ecology. The current evidence, based on light microscopy and scanning electron microscopy (Boenigk et al. 2005), does not allow for a sufficient taxon resolution. Even though further studies based on scanning and transmission electron microscopy may yield a higher morphological resolution (Amato et al. 2007), morphology alone may not suffice to differentiate species (Boenigk 2008).

The SSU rRNA gene seems to be differently suited for the analyses of the factors tested (salinity, pH, and temperature). The pH tolerance hardly differed at all between any of the strains tested. In contrast, the variation in salinity tolerance was generally high and hardly allowed for a correlation with molecular distance. This possibly indicates that the salinity adaptation may be a much faster process than the evolution of the SSU rRNA gene sequence variation. Consequently, SSU rRNA gene sequence data may be too conservative to correlate with salinity adaptation. However, temperature tolerance was correlated to molecular distance in terms of the SSU rRNA gene sequence variation. This correlation is partly due to an increasing variance of the pairwise temperature tolerances with increasing molecular distances (Fig. 3). The actual temperature adaptation of two distantly related strains is therefore not necessarily different, but the realized range of temperature adaptations increases with increasing molecular distance. Even though we are not aware of such findings in other protist taxa, such correlations are likely to be present in other taxa as well.

Implications for taxon resolution, species richness estimates, and the ecophysiological characterization of protist taxa—In sexually reproducing populations, alleles

at different gene loci either spread or go extinct more or less independently of each other (Normark et al. 2003). In contrast, different gene loci should have a shared, congruent phylogenetic history, both in asexual lineages and above the species level (Normark et al. 2003). In turn, within sexually reproducing populations, the variation in 'neutral' phylogenetic markers, such as the SSU rRNA gene, should be independent of ecophysiological variation (which is based on variation in a number of different gene loci). The correlation between ecophysiological and molecular variation in the investigated strains therefore provides evidence that the investigated strains affiliated with a distinct sequence clade are either not sexually reproducing or that the resolution of the SSU rRNA gene is above the species level. Because chrysomonad flagellates are generally assumed to be capable of sexual reproduction, the observed correlation indicates that even the monophyletic clades most probably represent numerous cryptic species. Whichever explanation is correct, the correlation between the SSU rRNA gene sequence data and ecophysiology demonstrates that the current species definition (morphospecies) is unsuitable for the investigated organisms with respect to their ecology. Evidence is accumulating that this criticism may hold true for many flagellate lineages (von der Heyden and Cavalier-Smith 2005). Consequently, the resolution of the (morpho-) species concept needs to be increased and will most certainly yield considerably higher species richness estimates.

We provide evidence contradicting the often postulated assumption that morphology sufficiently resolves protist ecophysiology (Finlay 1998; Finlay and Fenchel 2004). The morphotype reflects neither the genetic diversity nor the ecophysiological diversification among the morphospecies we investigated. Such taxonomically and ecophysiologically undersplit taxa are probably one of the most severe limitations to the future progress of flagellate ecology (de Vargas et al. 1999; Boenigk 2008).

Our results further demonstrate that protist populations in the field may be composed of different lineages with possibly different ecophysiological adaptations. The heterogeneous population structure of protist populations implies fundamental problems in ecological studies and may partly explain the deviations between field observations and laboratory studies, because the latter are usually dominated, or exclusively composed, of a single genotype.

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