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Unveiling hidden diversity in the *Synura petersenii* species complex (Synurophyceae, Heterokontophyta)

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With 7 figures and 2 tables

Abstract: *Synura petersenii* is a common freshwater colonial flagellate. Taxonomy of the species is traditionally based on morphology of the silica scales covering its cells. Studies of scales that vary in morphology imply that *S. petersenii* may include several separate but closely related species. In this study we used the ITS region for a molecular analysis of several clonal cultures of *S. petersenii*. Analyses of these data divide the strains among six distinct clades. Identification of compensatory base changes (CBCs) and hemi-CBCs characterizing each of the six clades unequivocally confirmed the results of our ITS analysis and implied the high probability of reproduction barriers among the clades. Furthermore, morphological analyses including both traditional methods and geometric morphometrics revealed unambiguous differences in scale structure among the six clades. All of these findings allow us to consider *S. petersenii* a complex of species. The results also indicate that the real hidden diversity of *S. petersenii* is very probably much higher.

Key words: *Synura petersenii*, phylogeny, morphology, ITS, CBC

Introduction

Synura petersenii Korshikov, the most frequently encountered taxon in the Synurophyceae (Wee 1982, Siver 1987, Pichrtová et al. 2007), is a colonial, freshwater, golden-brown flagellate with characteristic siliceous scales covering the cell surface. As a member of sectio *Petersenianae* (Petersen & Hansen 1956) the scales have a characteristic raised, hollow ridge along their middle, with the pointed, front end projecting obliquely forwards and upwards (Kristiansen & Preisig 2007). Species identification in this section is based upon scale features such as keel shape as well as the presence and number of struts and their interconnections. Morphological features typical for *S. petersenii* can be seen in Fig. 1.

The conspicuous morphological variability of *S. petersenii* scales is outstanding among *Synura* species and has led several authors to establish new varieties and formae (Asmund 1968, Cronberg & Kristiansen 1980, Kristiansen 1995, Petersen & Hansen 1958, Siver 1987, 1988, Vigna 1979). The high level of morphological variability observed in the scales always has evoked clues and doubts of the existing species concept suggesting that *S. petersenii* is a complex of species (Kristiansen, 1986, 2005, Řezáčová & Škaloud 2005). However, this variability still is regarded as plasticity within the single species (Kristiansen & Preisig 2007). Similar problems recently have

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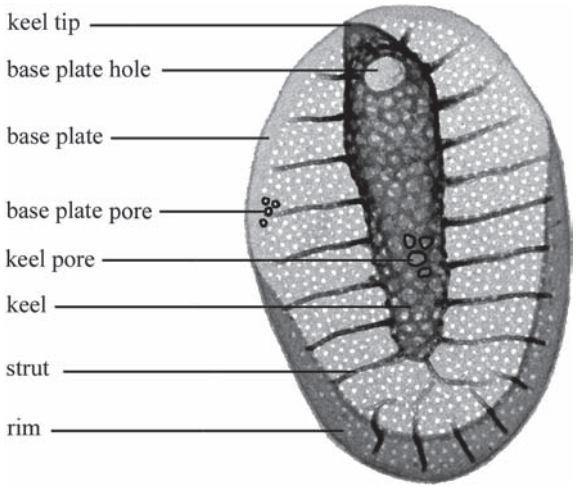


Fig. 1. Characteristic features of *S. petersenii* scale. The nomenclature was adopted from Wee (1997) and the keel pore is newly defined in this study.

been exposed in the diatoms, a heterokont group of autotrophic protists related to the chrysophytes. Notabley, diatom species are very similar morphologically but can be distinguished by minute morphological differences (e. g. Lundholm et al. 2006, Mann et al. 2004, Sarno et al. 2005). However, several molecular studies of diatoms imply that many traditionally-defined species are comprised of several to many entities and should be recognized as independent species (Amato et al. 2007, Behnke et al. 2004, Mann et al. 2004, Sato et al. 2009, Vanormelingen et al. 2008). Significantly, the same trend might exist for *S. petersenii*.

Currently, the diversity of silica-scaled chrysophyte species is based on morphological data obtained by transmission (TEM) or scanning electron microscopy (SEM) of scales and, to a lesser degree, other siliceous structures when they are present (e. g. bristles). However, *S. petersenii* only has scales. The first molecular analysis of *S. petersenii* was performed by Wee et al. (2001) and revealed significant intraspecific variability in ITS regions of *S. petersenii*. However, these data were not compared with scale morphology. Here, we explore further the molecular diversity in the *Synura petersenii* species complex by analyzing ITS rDNA sequences for 21 new strains isolated from localities in the Czech Republic. To determine if the molecular diversity corresponds with traditional morphological criteria we investigated the morphological variability of the scales using both traditional and modern landmark-based morphometric analyses. Finally, we explored the ITS2 secondary structure transcripts for the presence of compensatory base changes (CBCs; nucleotide changes at both sides of paired bases) and hemi-CBCs (change at only one side of nucleotide pair, but still preserving pairing) to find the hypothetical reproductive barriers between the organisms as proposed by Coleman (2000).

Materials and methods

Origin and cultivation of strains

The origins of all strains used in this study are provided in Table 1. We used both our own isolates (designated as S) as well as strains from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton in Maine, USA (designated as CCMP). To establish our isolates, samples were taken using a 25 μm mesh plankton net at different localities in the Czech Republic. Sin-

Table 1. Strains included in this study with their source localities and GenBank accession numbers of the ITS rDNA sequence data. Sequences of strains marked with an asterisk were obtained from GenBank. Note that live cultures of CCMP 862 and CCMP 866 were obtained for the morphometric analyses, while the source of the ITS sequences for these two strains was GenBank.

Clade	Strain identifier	Collection informaton	Accession No.
1	CCMP858 *	Lake Itaska State Park, Itaska County, MN	AF308837
1	CCMP861 *	Winter's Creek, Keeweenaw County, MI	AF308841
1	CCMP862 *	Winter's Creek, Keeweenaw County, MI	AF308842
1	CCMP863 *	ditch near Winter's Creek, Keeweenaw County, MI	AF308846
1	CCMP866 *	Newfoundland, Canada	AF308840
1	CCMP868 *	Fox River, McHenry County, IL	AF308839
1	*	White Springs Road Pond, Geneva, NY (Sandgren 2)	AF308838
1	*	Winter's Creek, Keeweenaw County, MI ("And" 2549)	AF308843
1	*	Big Betsy Pond, MI ("And" 5482)	AF308844
2	S 5.1	Aluvial pool, Modřany, Prague, Czech Republic	FM178494
2	S 5.2	Aluvial pool, Modřany, Prague, Czech Republic	FM178495
2	S 5.3	Aluvial pool, Modřany, Prague, Czech Republic	FM178496
2	S 14.2	Peatbog, Swamp NR, North Bohemia, Czech Republic	FM178497
3	CCMP864 *	road ditch near Gay, MI (CCMP 864)	AF308836
3	CCMP872 *	Winter's Creek, Keeweenaw County, MI	AF308835
3	CCMP873 *	Bluff Lake, Lake County, IL	AF308832
3	S 1.1	Zlatá stoka canal, Třeboň, Czech Republic	FM178498
3	S 1.2	Zlatá stoka canal, Třeboň, Czech Republic	FM178499
3	S 1.3	Zlatá stoka canal, Třeboň, Czech Republic	FM178500
3	S 6.4	Aluvial pool, Horní Lužnice NR, South Bohemia, Czech Republic	FM178501
3	S 6.5	Aluvial pool, Horní Lužnice NR, South Bohemia, Czech Republic	FM178502
3	S 4.19	Kladský pond, West Bohemia, Czech Republic	FM178503
3	S 7.7	Babín pool, Žďárské vrchy PLA, Czech Republic	FM178504
3	S 16.2	Xerr pond, South Bohemia, Czech Republic	FM178505
3	SAG 120.79 *	Lüneburger Heide, Germany	AF308834
3	*	Yarra River, Melbourne, Victoria, Australia	AF308833
4	S 7.10	Babín pool, Žďárské vrchy PLA, Czech Republic	FM178506
4	S 10.2	Huťský pond, Novohradské hory, South Bohemia, Czech Republic	FM178507
5	S 15.3	Peatbog, Úpské rašeliniště, Krkonoše NP, Czech Republic	FM178508
5	S 15.5	Peatbog, Úpské rašeliniště, Krkonoše NP, Czech Republic	FM178509
5	S 15.9	Peatbog, Úpské rašeliniště, Krkonoše NP, Czech Republic	FM178510
6	S 8.1	Kyjský pond, Prague, Czech Republic:Prague	FM178511
6	S 9.1	Confluence of the Morava and Dyje rivers, South Moravia, Czech Republic	FM178512
6	S 9.2	Confluence of the Morava and Dyje rivers, South Moravia, Czech Republic	FM178513
6	S 14.1	Peatbog, Swamp NR, North Bohemia, Czech Republic	FM178514
–	*	roadside ditch, Keeweenaw County, MI ("And" 2555)	AF308845

gle *Synura* colonies were removed from the natural sample by micropipetting and each single colony was placed into a separate culture vessel with DY IV medium (Andersen et al. 1997). The same method was used to move strains CCMP 862 and CCMP 866 into fresh medium for morphometric analyses. The cultures were kept at 15 °C (cooling box Helkama C5G). After one month of cultivation volumes of 200 µl of exponentially growing cultures were pipetted into a 1mL Eppendorf tubes, that were then kept frozen (–20 °C). The remaining culture was used for TEM or further cultivation. Only strains identified as *S. petersenii* on the basis of TEM observation were used in the study.

PCR amplification and ITS rDNA analysis

Thawed cultures were used directly as a template to amplify the entire ITS rDNA region (ITS1, 5.8S rDNA and ITS2), using terminal primers ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3'; White et al. 1990) and Kn4.1 (5'-TCA GCG GGT AAT CTT GAC TG-3'; Wee et al. 2001). All PCR were performed in 20 µl reaction volumes (15.1 µl sterile Milli-Q Water, 2 µl 10´ PCR buffer (Sigma), 0.4 µl dNTP (10 µM), 0.25 µl of primers (25 pmol/ml), 0.5 µl Red Taq DNA Polymerase (Sigma) (1U/ml), 0.5 µl of MgCl2, 1 µl of DNA (not quantified). PCR was performed in a XP thermal cycler (Bioer). PCR amplification began with 35 cycles of denaturing at 94 °C for 1 min, annealing at 54 °C for 1 min and elongation at 72 °C for 1 min 30 s, with a final extension at 72 °C for 10 min. The PCR products were quantified on a 1 % agarose gel stained with ethidium bromide and cleaned either with the JetQuick PCR Purification Kit (Genomed) or with QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's protocols. The purified amplification products were sequenced with the PCR primers at Macrogen, Inc. (Seoul, Korea, http://dna.macrogen.com). The acquired Czech sequences with all other available *S. petersenii* sequences deposited in the GenBank database were used for the ITS rDNA analysis (Table 1). Although cultures of CCMP 862 and CCMP 866 were obtained for the morphometric experiments, the ITS sequences for these two strains came from Genebank (see Table 1, Fig. 3).

ITS sequences (ITS1, 5.8 rDNA and ITS2 regions) were aligned visually on the basis of their rRNA secondary structure information (see below) using MEGA 3.1 (Kumar et al. 2004). To improve the alignment quality, positions with deletions in a majority of sequences were removed from the alignment, resulting in an alignment comprising 496 base positions. Phylogenetic trees were inferred by maximum likelihood (ML) and weighted parsimony (wMP) criteria using PAUP*, version 4.0b10 (Swofford 2002), and by Bayesian inference (BI) using MrBayes version 3.1 (Ronquist & Huelsenbeck 2003). A substitution model was estimated using the Akaike Information Criterion (AIC) with the program PAUP/MrModeltest 1.0b (Nylander 2004)and a GTR+Γ model was deemed best. Maximum likelihood analyses consisted of heuristic searches with 1,000 random sequence addition replicates and Tree Bisection Reconnection. Reliability of the resulting topology was tested using bootstrap analysis (100 replications) consisting of heuristic searches with 10 random sequence addition replicates, Tree bisection reconnection swapping, and a rearrangement limit of 5,000 for each replicate. The wMP bootstrapping was performed using heuristic searches with 100 random sequence addition replicates, tree bisection reconnection swapping, random addition of sequences (the number limited to 10,000 for each replicate), and gap characters treated as a fifth character state. In BI analysis, two parallel MCMC runs were carried out for 2 million generations, each with one cold and three heated chains employing the above-stated evolutionary model. Trees and parameters were sampled every 100 generations. Convergence of the two cold chains was checked and burn-in was determined using the "sump" command.

ITS1 and ITS2 rRNA secondary structure reconstruction

All of the sequences used for the ITS rDNA analysis (Table 1) also were used for the reconstruction of ITS1 and ITS2 rRNA secondary structures. The secondary structures were constructed using the mfold computer program (version 2.3; Walter et al. 1994; Zuker 2003) with folding temperature set to 25 °C. The boundaries between ITS1-5.8S rDNA and 5.8S-ITS2 rDNA regions were estimated according to the position of pairing between SSU-5.8 rDNA and 5.8-LSU rDNA regions. The common secondary structure was created using RnaViz (version 2; De Rijk et al. 2003) and used to identify compensatory base changes (CBCs) and hemi-CBCs.

Traditional morphological analysis

For the traditional morphological measurements, two strains were chosen randomly from each of the clades identified by the previous molecular study (clade 1: CCMP 862, CCMP 866, clade 2: S 5.3, S 14.1, clade 3: S 1.1, S 7.7, clade 4: S 10.2, S 7.10, clade 5: S 15.3, S 15.9, clade 6: S 9.2, S 14.1). Scales of those strains then were examined and photographed using a transmission electron microscope JEOL 1010. All the preparations for TEM were made after one month of cultivation in a fresh medium, so that the scale-silicification level was comparable. The samples were dried onto formvar-coated copper grids, rinsed with distilled water and examined with TEM. According to the position of the scales on the cell, 10 random body scales of each chosen strain were photographed with a digital camera at15,000x. Morphological characteristics were then measured in Adobe Photoshop Elements CS4 Extended version 11.0 with picture analysis tools. The characteristics measured were: scale length to width ratio, keel pore area, base plate pore area, and base plate hole area. The keel pores (Fig. 1) were defined as the larger pores on the keel visible on TEM pictures. They are covered with a layer of scale material, and usually cannot be seen in SEM micrographs. To obtain the area of keel pore and base plate pore, ten pores of each scale were measured and the data averaged. The significance of intergroup morphological differences was tested using one-way ANOVA and Tukey's pairwise comparisons. Then, PCA (principle component analysis) was made. Statistical analyses were carried out with the program PAST (Hammer et al. 2001). Next, canonical discriminant analysis (CDA) was performed using STATISTICA (StatSoft, Inc. 1998) and SigmaPlot, Systat Software, Inc. was used for construction of plots.

Geometric morphometrics

All *Synura* cells used in our morphometric analyses were cultivated for one month under identical conditions. Altogether, 353 body scales from 11 strains (S 1.1, S 5.3, S 7.7, S 7.10, S 9.2, S 10.2, S 14.1, S 14.2, S 15.9, CCMP 862, and CCMP 866) were photographed at the same magnification. Twenty-four landmarks were defined on each of the investigated scales using the TpsDig ver. 2.05 (Rohlf 2004a) program to delimit outlines of the base plate and the keel. Twenty of the semilandmarks were allowed to slide along the outline (Fig. 2). Landmark configurations were superimposed by generalized Procrustes analysis (Bookstein 1991) in tpsRelw ver.1.42 (Rohlf 2004b) and canonical variate analysis (CVA)/Manova was performed in IMP (Sheets 2002).

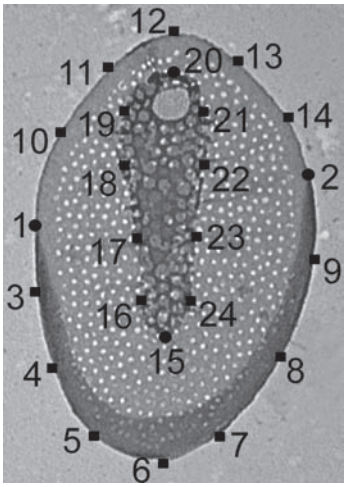


Fig. 2. Position of landmarks (circles) and semilandmarks (squares).

Results

ITS1–5.8S–ITS2 rDNA phylogeny

Different tree construction methods produced similar trees, resolving identical, highly supported clades, as well as the relationships among clades. The trees differed only in the organization of internal branches within clades. To assess the position of the root, a second alignment that included the sequence for *Synura uvella* (AF308847.1) was produced on the basis of common ITS1 and ITS2 secondary structures, and analyzed separately. With moderate support the analysis revealed the basal position of the clade consisting of the following strains: S 8.1, S. 9.1, S 9.2 and S 14.1 (tree and ITS1 secondary structure not shown, alignment can be downloaded at http://botany.natur.cuni.cz/algo/align/01_Synura_petersenii.fas). The maximum likelihood (ML) phylogram, rooted with the above-mentioned clade, is presented in Fig. 3. In this phylogeny, the *S. petersenii* strains clustered into six groups: clade 1 – GenBank sequences AF308837-AF308844, AF308846; clade 2 – S 5.1, S 5.2, S 5.3, S 14.2; clade 3 – S 1.1, S 1.2, S 1.3, S 4.19, S 6.4, S 6.5, S 7.7, S 16.2, and GenBank sequences AF308832-AF308836; clade 4 – S 7.10, S 10.2; clade 5 – S 15.3, S 15.5, S 15.9; and clade 6 – S 8.1, S 9.1, S 9.2, S 14.1. A single GenBank sequence (AF308845) occupied an isolated position, related to clades 1 and 2. All six groups of sequences received high statistical support, with a MrBayes PP ≥ 0.99, ML bootstrap ≥ 86, and MP bootstrap ≥ 96. Moreover, results also indicated a highly supported relationship between clades 1 and 2; as well as clades 1, 2, and 3.

ITS2 rDNA secondary structure and compensatory base changes

In all six of the *S. petersenii* clades compared, a common overall organization of the ITS2 rDNA secondary structure could be identified (Fig. 4A). The secondary structure consisted of three paired regions (helices I-III). Helix I was the most conservative region, having no observed nucleotide changes. In two clades (1 and 3), intraclade ITS2 variation (up to 4 nucleotide changes per clade) was detected. However, this variation was concentrated either on the loop region, or the nucleotide change broke a pairing between the nucleotides in the helix region. Thus, neither CBCs nor hemi-CBCs were present among sequences from the same clade (see Coleman 2000, 2003). In contrast

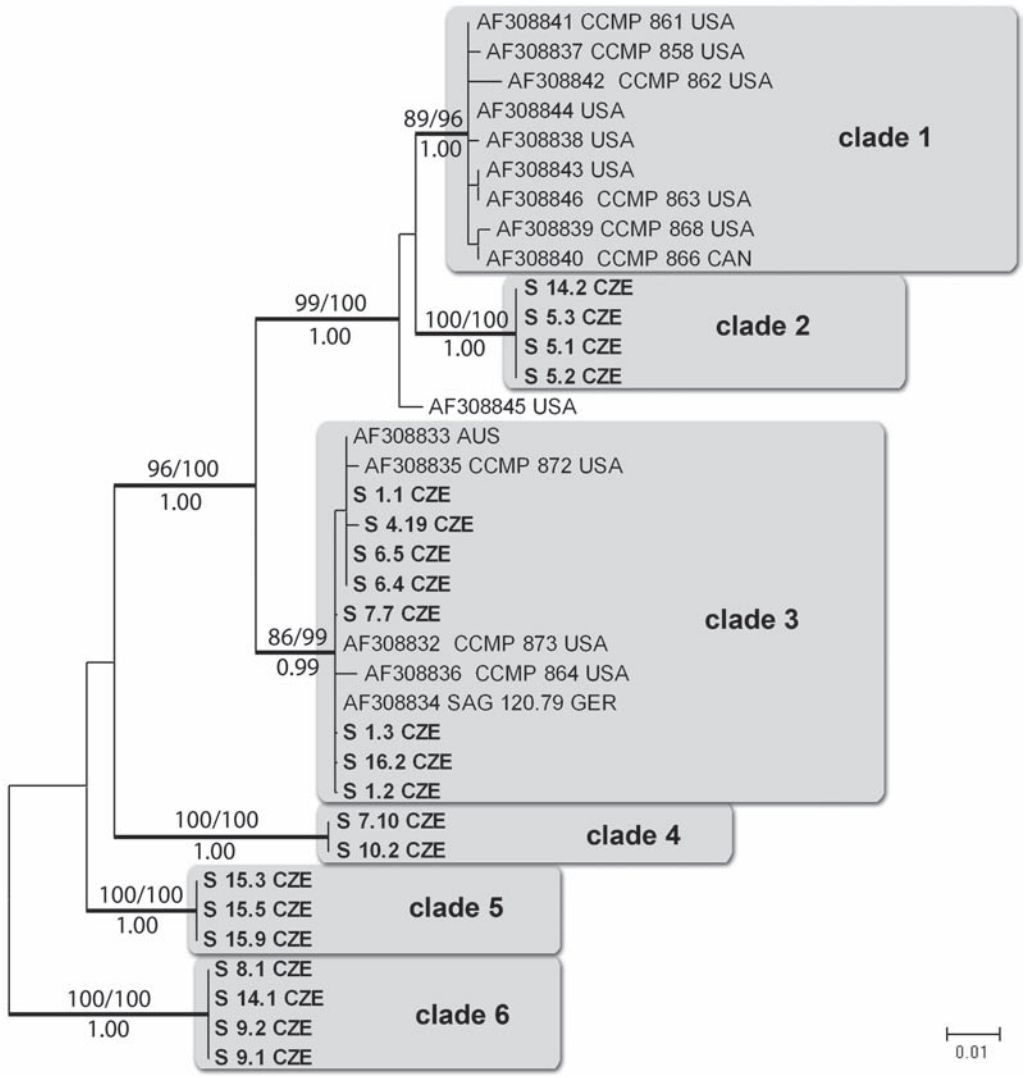
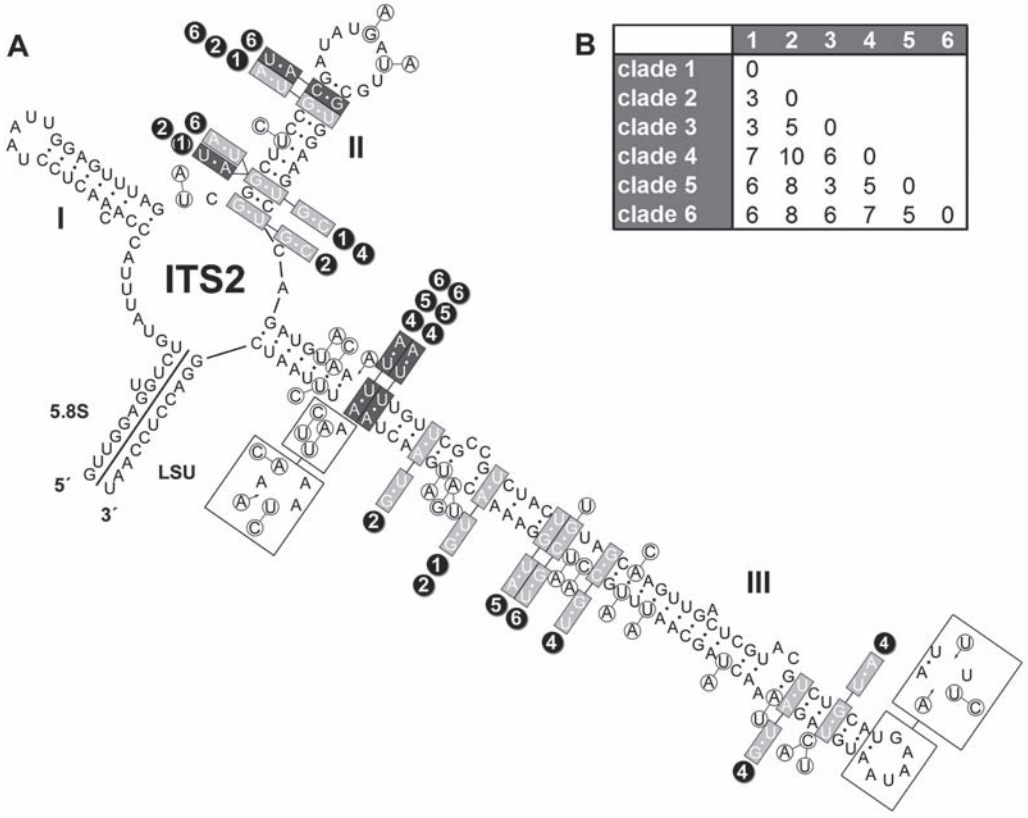


Fig. 3. Maximum likelihood tree based on ITS sequences. Values at the nodes indicate statistical support estimated by three methods – ML bootstrap (top left), MP bootstrap (top right) and MrBayes posterior node probability (lower). ITS sequences determined in this study are given in bold face. Scale bar – substitutions per site.

to this finding, the number of CBCs and hemi-CBCs varied from 3 to 10 among the different clades (Fig. 4B). The highest number of CBCs and hemi-CBCs was determined between clades 2 and 4, differing by three CBCs and seven hemi-CBCs. The regions with the most extensive interclade variation were helix II and the basal region of helix III.



	1	2	3	4	5	6
clade 1	0					
clade 2	3	0				
clade 3	3	5	0			
clade 4	7	10	6	0		
clade 5	6	8	3	5	0	
clade 6	6	8	6	7	5	0

Fig. 4. A. Predicted secondary structures of the ITS2 transcripts of *Synura petersenii* (strain S 1.3). Indicated base changes between the different *S. petersenii* s.l. genotypes are: the base pair marked in a dark-grey box indicates compensatory base changes (CBCs); base pairs marked in grey boxes indicate hemi-CBCs; single base changes are marked in circles; changes of the helix parts are indicated in large boxes. Affiliation of (hemi-)CBCs to particular clades is marked as white numbers in black spots. B. Numbers of (hemi-)CBCs differing each clade pair.

Traditional morphological analyses

Morphological analyses were performed on 12 strains (2 strains from each clade were examined). Measured morphological features (scale length and width, keel pores, base plate pores, and base plate hole) are depicted in Fig. 1. All six clades were found to be distinguishable on the basis of morphological characters (Fig. 5). The length to width ratio divided the clades into two distinct groups (Fig. 5A), one of them consisting of the clades with rounded scales (clades 1, 2, and 6), and the second one including the clades with long and narrow scales (clades 3, 4 and 5). Base plate pores of clades 3 and 5 were rather small, whereas in clade 2 they were relatively large, and in the case of the other clades (1, 4, and 6) they are medium-sized (Fig. 5B). Compared to the other clades, clade 5 had a notably large base plate hole (Fig. 5C). Clades 2 and 4 had a large average keel pore area, while keel pores of clades 1, 3, 5, and 6 were smaller (Fig. 5D). In clade 6, large base plate area to keel area ratio was noticed (Fig. 5E). The keel tip of most of the clades was acute, except for clade 5 where it was rounded, sometimes with a very thin tip or teeth on top.

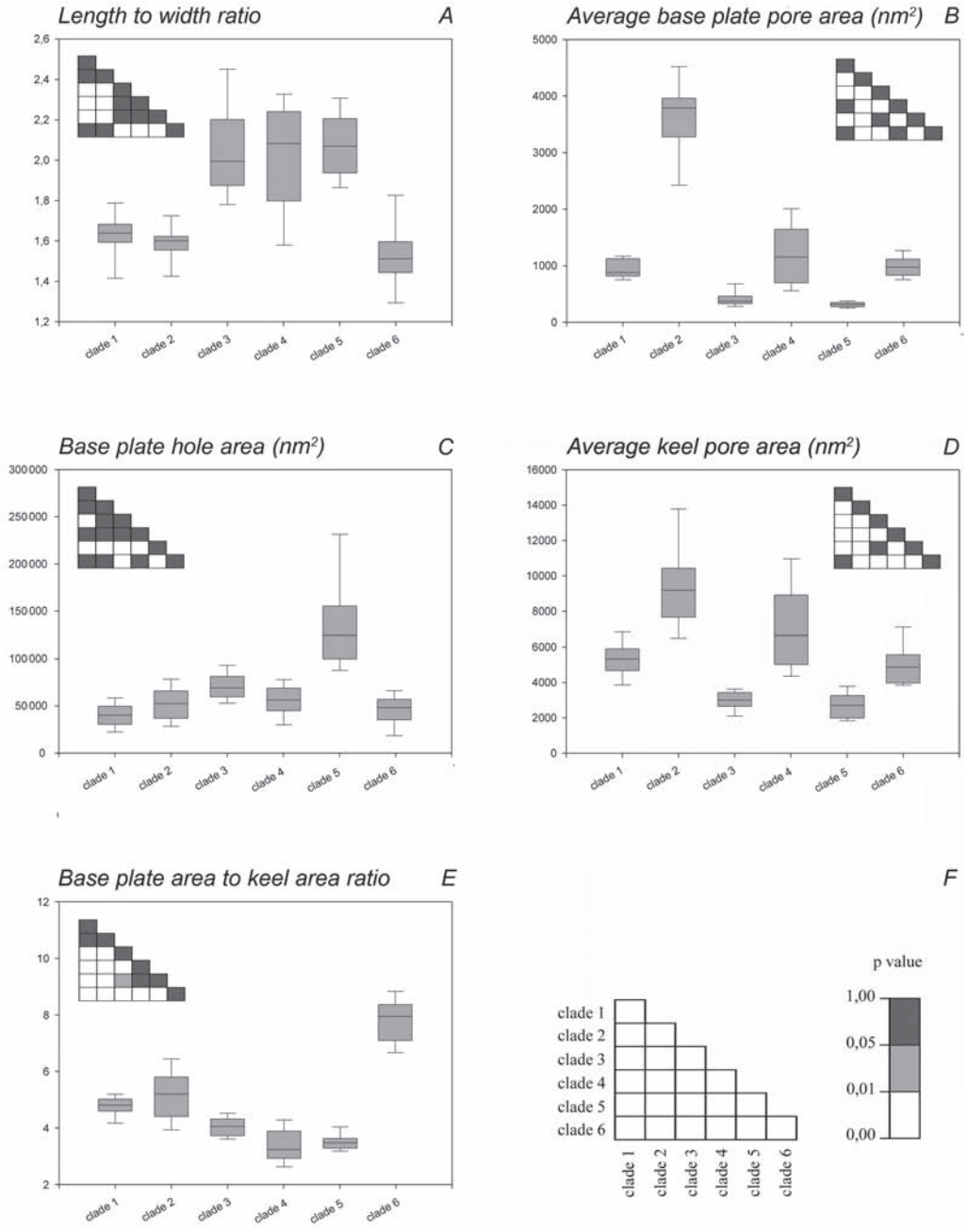


Fig. 5. Box plots of morphometric data for *Synura petersenii* scales. The morphometric data comprise length to width ratio of the scales (A), average base plate pore area (B), base plate hole area (C), average keel pore area (D), and base plate to keel area ratio (E). The grey areas of the boxes indicate 25 % and 75 % percentiles, the line within the fields is the median. The error bars indicate the 10 % and 90 % percentiles. The tables (F) represent the results of Tukey's pairwise comparisons for all pairs of clades. The p values are represented by different colors: white = 0.00 to 0.01; light grey = 0.01 to 0.05; dark grey = 0.05 to 1.00.

The principal component analysis (PCA) divided the measured scales into groups similar to those found in the molecular analysis (Fig. 6A). The first three axes described 47 %, 23 % and 13 % of variability. The first principal component axis was correlated with length to width ratio (compare Figs 5A and Fig 6A). Similarly to PCA, the canonical discriminant analysis (CDA) clearly separated all the six groups revealed by molecular analysis, with almost no overlap among them (Fig. 6B). According to the classification matrix (Tab. 2) the scales could be successfully placed into the matching phylogenetic groups in 95–100 % of the cases. In the case of clades 2, 4, and 5, all analyzed scales were classified correctly. The scales of clades 1, 3, and 6 were assigned correctly in 19 cases out of 20.

Table 2. Classification matrix showing the percentage of the scales correctly assign to the matching clade. Rows: observed classification; collumns: predicted classification.

	Percent correct	**clade 1** p = ,16667	**clade 2** p = ,16667	**clade 3** p = ,16667	**clade 4** p = ,16667	**clade 5** p = ,16667	**clade 6** p = ,16667
clade 1	95	19	0	0	1	0	0
clade 2	100	0	20	0	0	0	0
clade 3	95	1	0	19	0	0	0
clade 4	100	0	0	0	20	0	0
clade 5	100	0	0	0	0	20	0
clade 6	95	1	0	0	0	0	19
Total	97.5	21	20	19	21	20	19

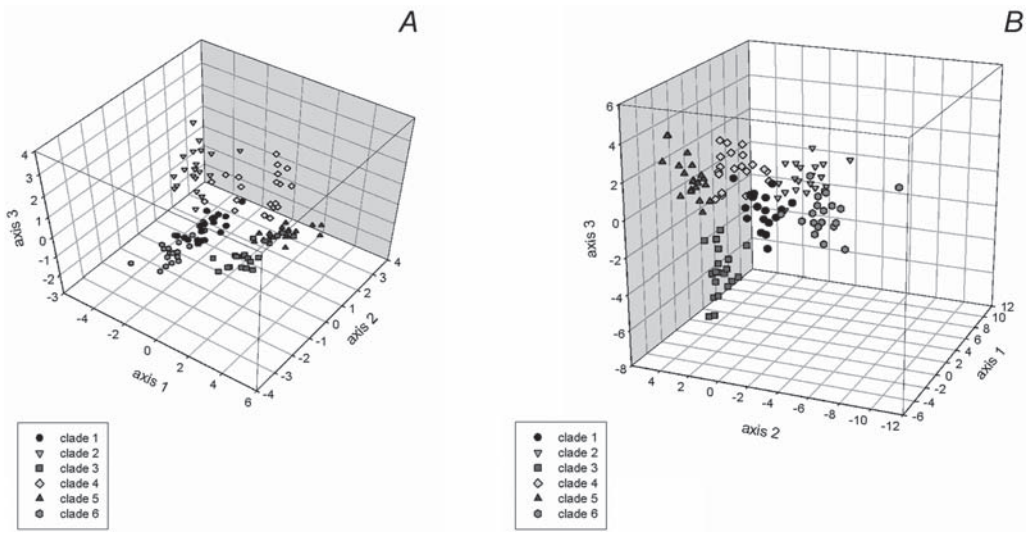


Fig. 6. Ordination diagrams showing the results of the principal component analysis (A) and the canonical discriminant analysis (B) of *S. petersenii* scale features.

Geometric morphometrics

Our analysis of 353 *S. petersenii* body scales from 11 strains revealed that all six genetically delimited clades were distinguishable on the basis of the geometric morphometric data. Statistically significant differences among all analyzed strains were revealed by Manova/CVA analysis (five significant canonical variates: $\lambda = 0.0122$; $p < 0.0001$; $\lambda = 0.1276$; $p < 0.0001$; $\lambda = 0.3143$; $p < 0.0001$; $\lambda = 0.5305$; $p < 0.0001$; $\lambda = 0.8095$; $p = 0.0029$). Along the first CV axis there are three groups separated from each other by the shape of their outline and keel (Fig. 7). Clade 6 has more rounded scales with a less developed keel. Base plates of clades 1 and 2 are similarly

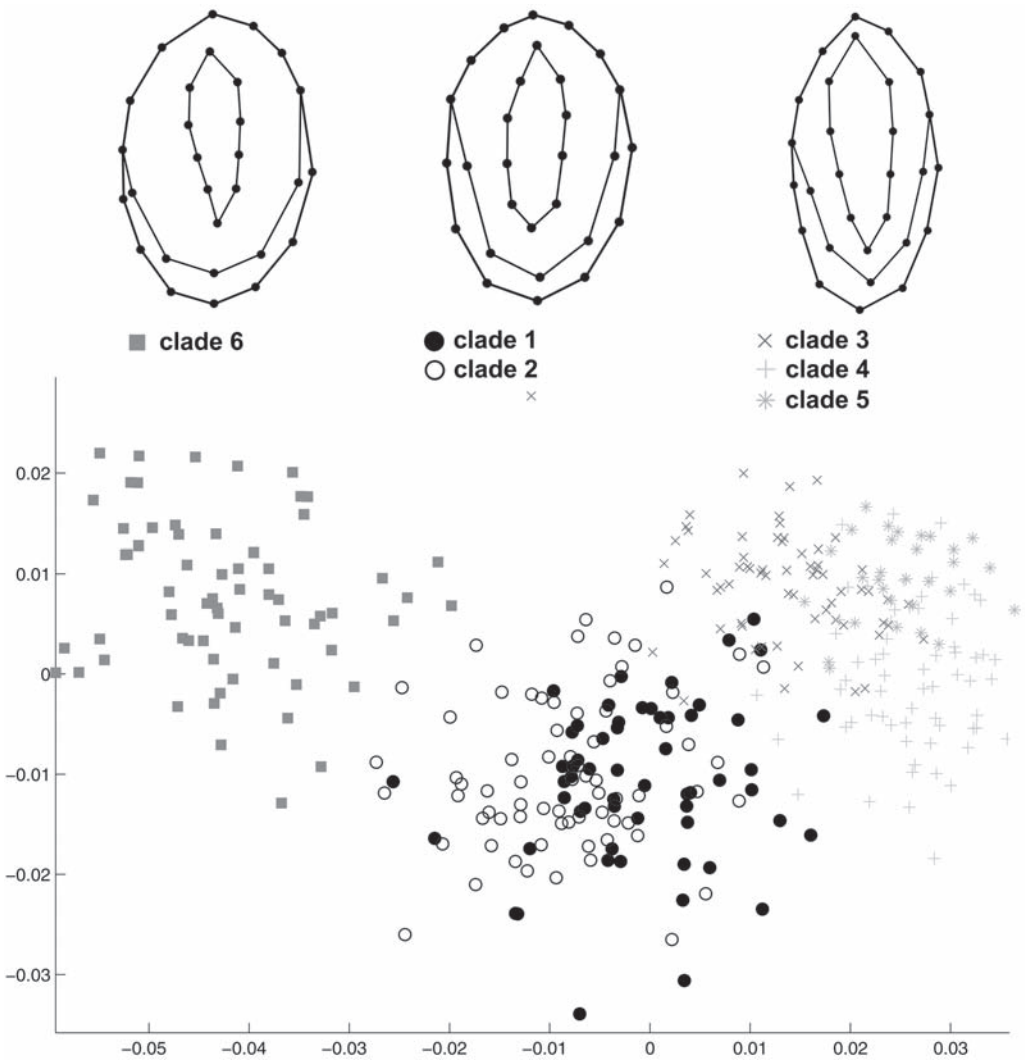


Fig. 7. The scatter plot of Manova/CVA analysis of 353 *S. petersenii* scales. Individual clades are grouped along the first CV axis and mean landmark configurations of three distinct groups are depicted.

rounded, but have an augmented keel and the rim of the scales is typically longer. The last group has more prolonged and rather lanceolate scales, with a wide and well-developed keel.

Discussion

Our results underscore the hidden diversity in the *S. petersenii* species complex. We have discovered congruence among ITS rDNA sequence diversity, the distribution of CBCs and hemi-CBCs in the ITS2 rDNA secondary structure, and the morphological variability of silica scales in several *Synura petersenii* clones.

We have found a large variation in the ITS sequences of our samples. The ITS rDNA region has now become the single most frequently utilized DNA region in taxonomic studies of protists. For example, ITS sequences were recently used in discovery of genetic variability and hidden diversity within several genera of green algae (Kroken & Taylor 2000, Lewis & Flechtner 2004, Vanormelingen et al. 2007) and diatoms (Behnke et al. 2004, Lundholm et al. 2006, Amato et al. 2007, Vanormelingen et al. 2008). As compared to the above-mentioned reports, the comparable differences in ITS rDNA sequences led authors either to describe separate clades as new species, or to consider them as separate species entities without formally describing them as new taxa.

The presence of at least one compensatory base change (CBC) or hemi-CBC in the secondary structure of spacer region ITS2 has been correlated with the occurrence of two different species under the biological species concept, i. e. the presence of reproductive barriers between the organisms (Coleman 2000). Recently, this hypothesis has been supported in various groups of protists, especially in diatoms (e. g. Coleman & Mai 1997, Behnke et al. 2004, Amato et al. 2007, Casteleyn et al. 2007, Müller et al. 2007). Comparing the secondary structures of ITS2 rRNA molecules, we always found at least 3 CBCs or hemi-CBCs between strains of different clades, and no CBCs or hemi-CBCs between any pair of strains belonging to the same clade. Accordingly all clades examined should be sexually incompatible. Because the CBC analysis has not been used previously to find hypothetical reproductive barriers between the chrysophycean organisms, there is no prior evidence that the presence of CBCs is really correlated with the presence of reproductive barriers between the organisms. However, this concept is widely accepted in several groups of protists, and there is no reason to think that *Synura* should be an exception. Actually, Müller et al. (2007) analyzed more than 1300 closely related eukaryotic species and revealed that the correlation between the presence of CBC in ITS2 rDNA and sexual incompatibility is generally applicable.

There is congruence between the morphology of the clones and their position in the ITS rDNA phylogeny. Members of different clades are distinguished by both conventional morphological characters and modern geometric morphometric analyses (Fig. 5; Fig. 7). To determine the morphological differences among the clades objectively we cultivated all clones under identical conditions. However, because we studied only those populations growing in culture, we have no information about morphological variability that occurs in nature. Indeed, in a natural sample the differences could be less distinctive due to ecomorphic variability. The dependence of scale variability on environmental conditions was studied by Martin-Wagenmann & Gutowski (1995) and Gavrilova et al. (2005). Although the scale dimensions significantly differed in various conditions, their length/width ratio remained the same. Similarly, the base plate hole diameter, as well as both base plate pore and keel pore areas were stable under all conditions studied. Thus, our morphological analyses were based on those characters not influenced by various environmental conditions (see Fig. 5). In *Synura* species, significant morphological variability of the scales is determined by the position of the scale on the cell. In general, scales found on the portion of the cell near where the cell attaches to the colony have a greater length to width ratio, while those scales located near the flagellum may have a smaller length-to-width ratio than the body scales (Siver 2002, Wee 1982, 1997). Even though we have tried to analyze the body scales only, some

portion of detected morphological variability was certainly influenced by scale position. However, grouping of scales in both PCA (Fig. 6A) and Manova/CVA (Fig. 6B) analyses clearly demonstrates that the effect of variability caused by the scale position had almost no effect compared to the genetically based differences in scale morphology.

The congruence of ITS rDNA phylogeny, CBC in the ITS2 rDNA, and scale morphology led us to the opinion that we have revealed the existence of several species within *S. petersenii*. In the past, many species concepts based on different criteria had been proposed to define species. Recently, the unifying general lineage species concept was proposed by de Queiroz (1998, 2007). This species concept is based on the hypothesis that every species is a distinct evolutionary lineage. Existence of the lineage should be supported with several species criteria, which can be based on morphology, monophyly, reproduction, ecology, or other characteristics. The different criteria that demark the species are formed during the long-term speciation process, and thus it is not necessary to find differences in all the criteria to be able to describe a new species. Instead, congruence of only several criteria is sufficient for species description. Our data reveal the congruence of three independent criteria (phenetic criterion, criterion of monophyly, and CBC criterion) therefore highly support the real existence of several pseudo-cryptic species (i. e. genetically distinct entities with minor morphological differences only detectable by very accurate morphological analyses; Mann & Evans 2007) in *S. petersenii*.

Because most of our data originated in a limited geographical area, we propose that many additional species will be found in the *S. petersenii* species complex on the basis of molecular and morphological investigation of clones isolated from diverse geographical areas. Morphological data retrieved from a number of floristic studies indicate the probable existence of an extensive hidden diversity of species that differ slightly in the morphology of their silica scales.

Conclusions and prospects

Using a combination of morphological and molecular data, the *Synura petersenii* clones used in this study grouped into six groups. Since these groups probably represent individual species, their delimitation and morphological differentiation could highly increase the value of *S. petersenii* as an ecological indicator in biomonitoring and paleolimnological studies. Further investigation of dispersal potencies could clarify obvious differences in biogeography of newly found clades (e. g. clade 1 restricted to the North America). Detailed investigation of niche preferences or seasonal fluctuation could explain the sympatric occurrence of particular clades (e. g., clade 3 and clade 4 were isolated from the same sample taken from the same pool). Finally, further studies of molecular diversity in Synurophyceae will be able to determine whether similar hidden diversity is also present in other *Synura* species, or if it is restricted to *S. petersenii*.

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