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TAXONOMY OF THE *PHACUS OSCILLANS* (EUGLENACEAE) AND ITS CLOSE RELATIVES—BALANCING MORPHOLOGICAL AND MOLECULAR FEATURES[1]

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The establishment of epitypes (together with emended diagnoses) for seven species of *Phacus* Dujard. [*Phacus oscillans* G. A. Klebs, *Phacus parvulus* G. A. Klebs, *Phacus pusillus* Lemmerm., *Phacus skujae* Skvortzov, *Phacus inflexus* (Kisselew) Pochm., *Phacus polytrophos* Pochm., and *Phacus smulkowskianus* (Zakryś) Kusber] was achieved by literature studies, verification of morphological diagnostic features (cell size, cell shape), as well as molecular characters (SSU rDNA). The investigated *Phacus* species are mostly well distinguished morphologically, with an SSU rDNA interspecific sequence similarity of 95.1%–99.0% and an intraspecific sequence similarity of 99.0%–99.9%. Some of the phylogenetic relationships among the seven species have not been resolved, but the topology obtained indicates their assignment into two sister clades. The first clade is composed of two sister groups (*P. parvulus* and *P. pusillus*), while the second constitutes an assemblage of the remaining five species. The relationships between the clades remain unresolved.

Key index words: **asexual organisms; Euglenaceae; Euglenida; Euglenophyta; molecular phylogeny; morphology; *Phacus;* rDNA; sequence variability; taxonomic revision**

Abbreviations: **BA, Bayesian analysis; bs, nonparametric bootstrap; di, decay index; ML, maximum likelihood; MP, maximum parsimony; NJ, neighbor joining; nt, nucleotide; pp, posterior probability**

In the last several years, great efforts have been made in the search for the best tool for species description, particularly regarding microscopic organisms, usually displaying a paucity of morphological features that could enable their proper identification (Stoeckle 2003, Tautz et al. 2003, Blaxter 2004). Recently, and with increasing frequency, molecular data have been used as diagnostic features (Sarkar et al. 2002, DeSalle et al. 2005).

In Euglenales, there were a few attempts to supplement diagnoses with molecular features for taxa of different ranks: families, orders, genera (Marin et al. 2003, Ciugulea et al. 2008), and species (Kosmala et al. 2007a,b, 2009). There have also been diagnostic descriptions of new species of Euglenales in which molecular features played a pivotal role (Kosmala et al. 2007b, 2009).

Following this approach, we deal with a group of *Phacus* species, which we tentatively call the ''*Phacus oscillans* clade,'' after the first species described. Species in the group are characterized by a small body size (<40 μm long). On recently published phylogenetic trees, they form a well-supported clade consisting of *P. oscillans*, *P. parvulus*, *P. pusillus*, *P. skujae*, *P. smulkowskianus,* and others (Milanowski et al. 2006, Triemer et al. 2006, Kosmala et al. 2007b). These species can be quite well distinguished at the morphological level, but not at the molecular level. Preliminary analyses indicated that intraspecies differences of the SSU rDNA sequence were ≤1.0%. It is not a typical situation in Euglenales, where strains displaying the same morphology usually show substantial molecular diversification (Shin and Triemer 2004, Kosmala et al. 2005, 2007b, 2009). An extreme example of such a situation is *Monomorphina pyrum*, in which intraspecific differences in the SSU rDNA sequence reach 6.6% (Kosmala et al. 2007a).

The aim of this study was (i) to learn the extent of variability within the ''*Phacus oscillans* clade,'' at both the morphological and molecular levels; (ii) to verify morphological diagnostic features of these species and supplement their diagnostic descriptions with molecular features (signature sequences); and (iii) to establish epitypes.

MATERIALS AND METHODS

Euglenoid strains and culture conditions. The strains used in this study are described in Table S1 (in the supplementary material). All strains were cultivated in a liquid soil-water medium, enriched by a small piece of garden pea (medium 3c, Schlösser 1994), under identical conditions, in a growth chamber maintained at 17°C and 16:8 light:dark (L:D), ~27 μmol photons $\cdot m^{-2} \cdot s^{-1}$ provided by cool-white fluorescent tubes (Philips, Amsterdam, the Netherlands).

LM observations. Observation of morphological features (cell size and shape, shape and number of large paramylon grains, periplast ornamentation) was performed using a light microscope (NIKON Eclipse E-600 with Nomarski differential interference contrast, Nikon, Tokyo, Japan) equipped with the

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software for image recording and processing. Photographic documentation was made possible with the use of the digital NIKON DX-1200 camera connected to a microscope. Cultures were sampled every 2 weeks, for periods of 3–4 months. Such sampling enabled us to observe all cells during their developmental stages, from the young cells (immediately after division) to the old.

Biometric studies. Performed using the LUCIA Measurement program (Laboratory Imaging s. r. o., Prague, Czech Republic). One hundred randomly chosen, actively swimming cells from each young dividing population were analyzed. All observations were performed on material that was preserved with a 5% glutaraldehyde solution, by adding one drop of a fixative to the fresh material placed on the slide. Two parameters were measured for each strain: cell length and cell width. The data were analyzed using Statistica software (StatSoft Inc., Tulsa, OK, USA).

DNA isolation, amplification, and sequencing. Isolation of total DNA, amplification of the SSU region, and purification and sequencing of PCR products were performed by standard methods as previously described (Milanowski et al. 2001, Zakryś et al. 2002, Kosmala et al. 2007b).

Sequence accession numbers, alignment, and phylogenetic analyses. The GenBank accession numbers for the SSU rDNA sequences reported here and used for phylogenetic analyses are shown in Table S1. The alignment of sequences, obtained by using ClustalX 1.83 (Thompson et al. 1997) with default options, was manually checked and edited according to the secondary structure of Euglenophyceae, as suggested by Marin et al. (2003), using the Genetic Data Environment (GDE 2.2) software program (Smith et al. 1994). The SSU rDNA data set of 2,098 characters was generated for phylogenetic analyses. After the removal of sites with uncertain homology, which could not be unambiguously aligned, 1,629 positions [1,169 of which were constant and 335 maximum-parsimony (MP) informative] were left in the SSU rDNA alignment of 33 sequences. The alignment used for analyses is shown in the supplementary material (Appendix S1). Distance [neighbor-joining (NJ)], maximum-likelihood (ML), and MP analyses were performed by PAUP*, Version 4.0b6 for Microsoft Windows (Swofford 1998). To find the best tree, in MP analysis the heuristic search option was used with MULTREES, tree-bisection-reconnection (TBR) branch swapping, ACCTRAN optimization, and random addition, with 1,000 replicates. Bootstrap support for specific nodes (Felsenstein 1985) was estimated using 1,000 replications; 10 heuristic searches (random stepwise addition) were used for each bootstrap replicate. In ML analysis, the heuristic search was used with MULTREES, TBR branch swapping and random stepwise addition, with 10 replications. Bootstrap support was estimated using 100 replications (fast stepwise addition option). The Bayesian analyses (BA) were performed, and values of their model parameters were estimated by MrBayes 3.1.2 software (Huelsenbeck and Ronquist 2001, Ronquist and Huelsenbeck 2003). Four Markov chains were run, with 1,000,000 generations per chain with the first 20% of trees discarded. Models of sequence evolution for ML, NJ, and BA methods and their parameter values for ML and NJ methods were estimated by Modeltest 3.7 (Posada and Crandall 1998). Auto decay indices (Bremer 1994) were calculated by AutoDecay 4.0.2 (Eriksson 1998) for MP analyses. The likelihood ratio test (hLRTs) of the Modeltest software program (Posada and Crandall 1998) suggested an SYM+I+G model (Zharkikh 1994), whereas the Akaike test chose a general-time-reversible GTR+I+G model (Lanave et al. 1984, Tavare 1986, Rodriguez et al. 1990). These models were applied to calculate NJ and ML trees, with parameter values drawn from Modeltest [for SYM + I + G model: BaseFreq = equal, RateMatrix = (0.7684 2.0210 0.9908 0.2698 3.8100), $G = 0.7559$, Invariant = 0.4691; for GTR + I + G model: BaseFreq = (0.2318 0.2517 0.2854) RateMatrix = (0.8278 2.0228 1.1643 0.2400 4.1292), $G = 0.7499$, Invariant = 0.4667]. For BA analyses, only model GTR + I + G was applied. The sequences from *P. acuminatus*, *P. longicauda* var. *torta*, *P. orbicularis*, *P. platyaulax*, and *P. pleuronectes* were used to root the trees (Milanowski et al. 2006, Triemer et al. 2006, Kosmala et al. 2007b), which were drawn by Tree View, Version 1.6.1 for Microsoft Windows (Page 1996).

RESULTS

LM observations. There was certain morphological variability in all strains investigated, both within as well as among strains. This differentiation concerned such diagnostic features as cell size and shape (especially the degree of flattening and twisting and the presence and depth of the furrow), the number and shape of large paramylon grains, and periplast ornamentation.

Cell size: According to this feature, the 18 strains were divided into three distinct groups: (i) strains with the average cell length of ~17 μm (ASW 08018, CCAC 0096, SAG 1261-5, UTEX 1282; finally identified as *P. polytrophos* and *P. pusillus*); (ii) strains with average cell length between 20 and 26 μm (ACOI 1312, ACOI 1339, ACOI 1336, ACOI 1093, ACOI 2954, ASW 08016, ASW 08058, ASW 08060, ASW 08139, AICB 323, CCAC 0089, UTEX 1285; finally identified as *P. skujae, P. oscillans, P. inflexus*, and *P. parvulus*); (iii) strains with average cell length >28 μm (ACOI 1226, ASW 08053; finally identified as *P. smulkowskianus*) (Table 1).

Substantial intrastrain diversification in the cell size, dependent mostly on the developmental stage, was observed, in addition to the cell-size differentiation between strains. In young and not exceedingly overcrowded cultures, small vigorously dividing cells dominated, as expected. As the populations aged and growth conditions deteriorated, the rate of cell divisions subsided, and most of the cells consequently were able to grow to a larger size, sometimes twice that of the young cells (Table 1).

Cell shape: With respect to this feature, seven groups were distinguished: (1) Strains ACOI 1093, ACOI 2954, ASW 08058, ASW 08060, ASW 08139 (finally identified as *P. parvulus*) had cells flattened dorsiventrally, longitudinally oval and without a furrow (Fig. 1, o and p). (2) Oval, slightly flattened cells with a shallow furrow (visible only during swimming) were observed in three strains (CCAC 0096, SAG 1261-5, UTEX 1282; finally identified as *P. pusillus*) (Fig. 1, q and r). (3) Five strains (ACOI 1312, ASW 08016, AICB 323, CCAC 0089, UTEX 1285; finally identified as *P. skujae*) had cells firmly flattened with a deep furrow, which in cross-section resembled the letter ''V'' (Fig. 1, j and k). (4) Two strains (ACOI 1226, ASW 08053; finally identified as *P. smulkowskianus*) had cells firmly flattened and distinctly, spirally twisted (two to three complete turns around a long axis) (Fig. 1, d–f). (5) Trapezoid cells

TABLE 1. Comparison of the cell morphology of *Phacus* strains.

Taxon	Strain	Furrow	Body anterior	Cell length (μm)		Cell width (μm)	
				Mean ± SD	Min.–max.	Mean ± SD	Min.–max.
Phacus inflexus	ACOI 1336 epitype	Absent	With short tailpiece	23.3 ± 1.9	17.8–27.0	12.0 ± 2.4	7.7–18.8
Phacus oscillans	ACOI 1339 epitype	Deep	Tapered and blunt	26.1 ± 0.7	24.5–27.6	9.2 ± 0.8	6.7–11.2
Phacus parvulus	ACOI 1093 epitype	Absent	Tapered and blunt	21.2 ± 1.1	18.0–23.2	10.5 ± 1.1	7.4–13.1
	ACOI 2954	Absent	Tapered and blunt	21.3 ± 1.0	19.1–23.3	10.3 ± 1.0	8.3–12.6
	ASW 08058	Absent	Tapered and blunt	20.1 ± 1.2	16.9–22.8	12.6 ± 1.4	10.0–15.8
	ASW 08060	Absent	Tapered and blunt	21.2 ± 0.5	20.0–22.2	10.9 ± 0.7	9.1–12.8
	ASW 08139	Absent	Tapered and blunt	21.2 ± 1.3	17.7–23.9	11.7 ± 1.6	8.8–15.6
Phacus polytrophos	ASW 08018 epitype	Shallow	Tapered and blunt	16.9 ± 1.4	13.0–19.6	9.3 ± 1.7	6.0–13.1
Phacus pusillus	UTEX 1282 epitype	Very shallow	Tapered and blunt	16.4 ± 0.9	14.3–18.0	8.0 ± 0.8	6.0–9.9
	CCAC 0096	Very shallow	Tapered and blunt	16.9 ± 1.0	13.8–18.6	10.1 ± 1.4	8.0–13.6
	SAG 1261-5	Very shallow	Tapered and blunt	16.5 ± 0.8	14.5–18.1	7.7 ± 0.7	6.5–9.2
Phacus skujae	ACOI 1312 epitype	Deep	Tapered and blunt	20.2 ± 0.8	18.3–22.1	8.2 ± 0.6	7.2–9.8
	AICB 323	Deep	Tapered and blunt	22.2 ± 0.9	19.2–24.1	10.2 ± 0.9	8.0–12.3
	ASW 08016	Deep	Tapered and blunt	25.0 ± 2.0	19.3–28.2	11.5 ± 2.6	8.3–17.8
	CCAC 0089	Deep	Tapered and blunt	25.0 ± 2.2	19.5–28.4	11.6 ± 3.0	8.0–18.9
	UTEX 1285	Deep	Tapered and blunt	22.4 ± 0.9	20.4–24.4	9.6 ± 0.7	8.2–11.3
Phacus smulkowskianus	ACOI 1226 epitype	Absent	With short tailpiece	28.1 ± 2.2	20.1–33.3	14.5 ± 2.7	10.4–23.3
	ASW 08053	Absent	With short tailpiece	28.6 ± 2.4	23.3–31.1	14.5 ± 3.0	10.2–22.9

with a shallow furrow were observed in the strain ASW 08018 (finally identified as *P. polytrophos*) (Fig. 1, m and n). (6) Strain ACOI 1339 (finally identified as *P. oscillans*) was distinguished by a deep and wide furrow (in cross-section having the shape of the letter ''C'') created due to the edges of a flat cell curling up together (Fig. 1, g–i). (7) The strain ACOI 1336 (finally identified as *P. inflexus*) had flat, s-shaped cells, bent in half along a long axis, slightly spirally twisted (one complete turn around a long axis) (Fig. 1, a–c). Body shape in aging populations was distinctively altered due to accumulation of a large number of paramylon grains.

Number and shape of large paramylon grains: This characteristic changed in relation to deteriorating living conditions. In all young (several days old) cultures growing in fresh media, the cells were dividing vigorously and possessed one or two paramylon grains that were larger than the rest and usually link-like or ring-like (Fig. 1, a, d, e, g–k, o, p, and r), although sometimes oval (Fig. 1q) or plate-like (Fig. 1, m and n). As the population aged, becoming more overcrowded, the grains were increasing in size, eventually nearly taking over the entire area of the cell (Fig. 1l), considerably changing the cell appearance and making strain identification impossible (with the exception of *P. smulkowskianus* and *P. inflexus* strains: ACOI 1226, ASW 08053, and ACOI 1339; the helicoidally twisted cells of which were distinct despite deformation). The cells changed their shape from flat to cylindrical, while the furrow was steadily becoming more shallow, ultimately disappearing completely.

Periplast ornamentation: This feature was not used to differentiate the strains under consideration, since the periplast in all of them was only slightly obliquely striated. This skewed striation was dependent on the cell shape (the extent of twisting) and became more distinct during swimming (when cells capable of metaboly are twisting due to the swirling motion).

After consideration of morphological characters and genetic similarity, we were able to assign species names to clades on a phylogenetic tree obtained from rDNA data. Diagnostic features that appropriately characterized clades (species) were the cell size and shape, specifically the degree of flattening and twisting and the presence and depth of a furrow.

Phylogenetic analyses. Nine new *Phacus* SSU rDNA sequences were obtained. The mean parameters estimated during BA analysis were r(A–C) = 0.087595, r(A–G) = 0.214359, r(A–T) = 0.118786, r(C–G) = 0.027673, r(C–T) = 0.456312, r(G–T) = 0.095275, pi(A) = 0.231848, pi(C) = 0.252565, pi(G) = 0.283130, pi(T) = 0.232457, a = 0.687452; the proportion of invariable sites was 0.412752. The trees obtained by all methods (NJ, MP, ML, and BA) have the same ingroup topology and similar branch support (bs, bootstrap; di, decay index; or pp, posterior probabilities). Figure 2 shows the tree obtained by MrBayes using the GTR+I+G model for the *Phacus* genus using the SSU rDNA sequences.

The tree was drawn with *P. acuminatus*, *P. longicauda* var. *torta*, *P. orbicularis*, *P. platyaulax,* and *P. pleuronectes* as an outgroup (Fig. 2), since they branched off first in published *Phacus* phylogenies (Milanowski et al. 2006, Triemer et al. 2006, Kosmala et al. 2007b). The rest of the species (the ''*Phacus oscillans* clade'') may be divided between two groups. The first very well-supported clade (BA = 1.00, MP = 99, NJ = 99, ML = 90) consists of two sister groups identified as *P. parvulus* without a furrow, comprising five strains (ACOI 1093, ACOI 2954, ASW 08058, ASW 08060, ASW 08139), and *P. pusillus* with a slightly distinguished furrow, consisting of three strains (CCAC 0096, SAG 1261-5, UTEX 1282).

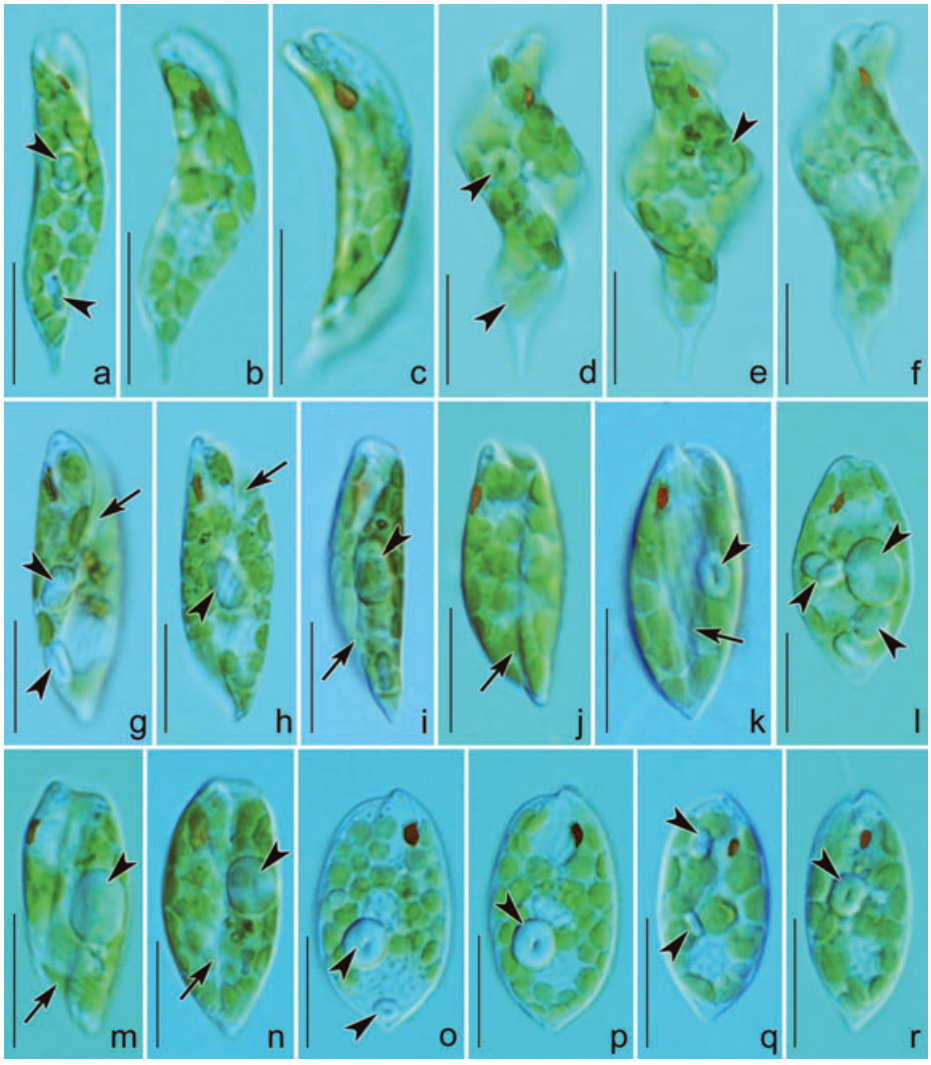

FIG. 1. Light microscope photographs showing an overview of the living cells of *Phacus inflexus, P. oscillans, P. parvulus, P. polytrophos, P. pusillus, P. smulkowskianus,* and *P. skujae.* (a–c) Cells of *P. inflexus* (strain ACOI 1336); flat, ''bent in half'' along the long axis, spirally twisted, s-like or c-like, bent from the side, ending with a short tail; two considerably sized, short, link-like/ring-like paramylon grains (arrowheads) are visible. (d–f) Cells of *P. smulkowskianus* (strain ACOI 1226); flat, helicoidally twisted, ending with a short tail; one can see one or two large, link-like/ring-like paramylon grains (arrowheads). (g–i) Cells of *P. oscillans* (strain ACOI 1339); a longitudinal, deep furrow (arrows); two considerably sized, short link-like/ring-like paramylon grains (arrowheads) are visible. (j–l) Cells of *P. skujae.* (j) Longitudinally ovoid cell of the strain AICB 323 with a visible, longitudinal furrow (arrow). (k, l) Cells of the strain ACOI 1312. (k) Longitudinal furrow (arrow) and one large, link-like paramylon grain (arrowhead) are visible. (l) A deformed (inflated) cell with many oval and plate-like paramylon grains (arrowheads). (m, n) Trapezoidal-rhomboidal cells of *P. polytrophos* (strain ASW 08018); shallow furrow (arrows) and one considerably sized paramylon grain are visible (arrowheads). (o, p) Dorsiventrally flattened, elliptical cells of *P. parvulus* (strain ACOI 1093); one or two considerably sized, ring-like/link-like paramylon grains are visible (arrowheads). (q, r) Longitudinally ovoid cells of the strain *P. pusillus* (strain UTEX 1282) with one or two considerably sized, oval/ring-like/link-like paramylon grains (arrowheads). Scale bars, 10 μm.

The second clade with strong support (BA = 1.00, MP = 83, NJ = 97, ML = 83) consists of five branches representing different species with distinct morphology, the mutual relationships of which are not resolved. Two branches consist of very well-supported clades. The first clade (BA = 1.00, MP = 98, NJ = 99, ML = 93) consists of five *P. smulkowskianus* strains (ACOI 1226, ASW 08053, CCAC 0042, CCAC 0097, SAG 58.81). Their distinctively helicoidally twisted cells distinguish these strains very well from the rest of the species. The second clade (BA = 1.00, MP = 99, NJ = 100, ML = 98) combines six strains of *P. skujae* (ACOI 1312, AICB 323, ASW 08016, CCAP 1261/10, CCAC 0089, UTEX 1285). Their cells have a deep furrow, which in a cross-section has the shape of the letter ''V.'' Each of the remaining three branches consists of a single strain. *P. inflexus* (ACOI 1339) has s-shaped, slightly helicoidally twisted cells bending in half along the longer body axis. *P. oscillans* (ACOI 1336) has flat cells with

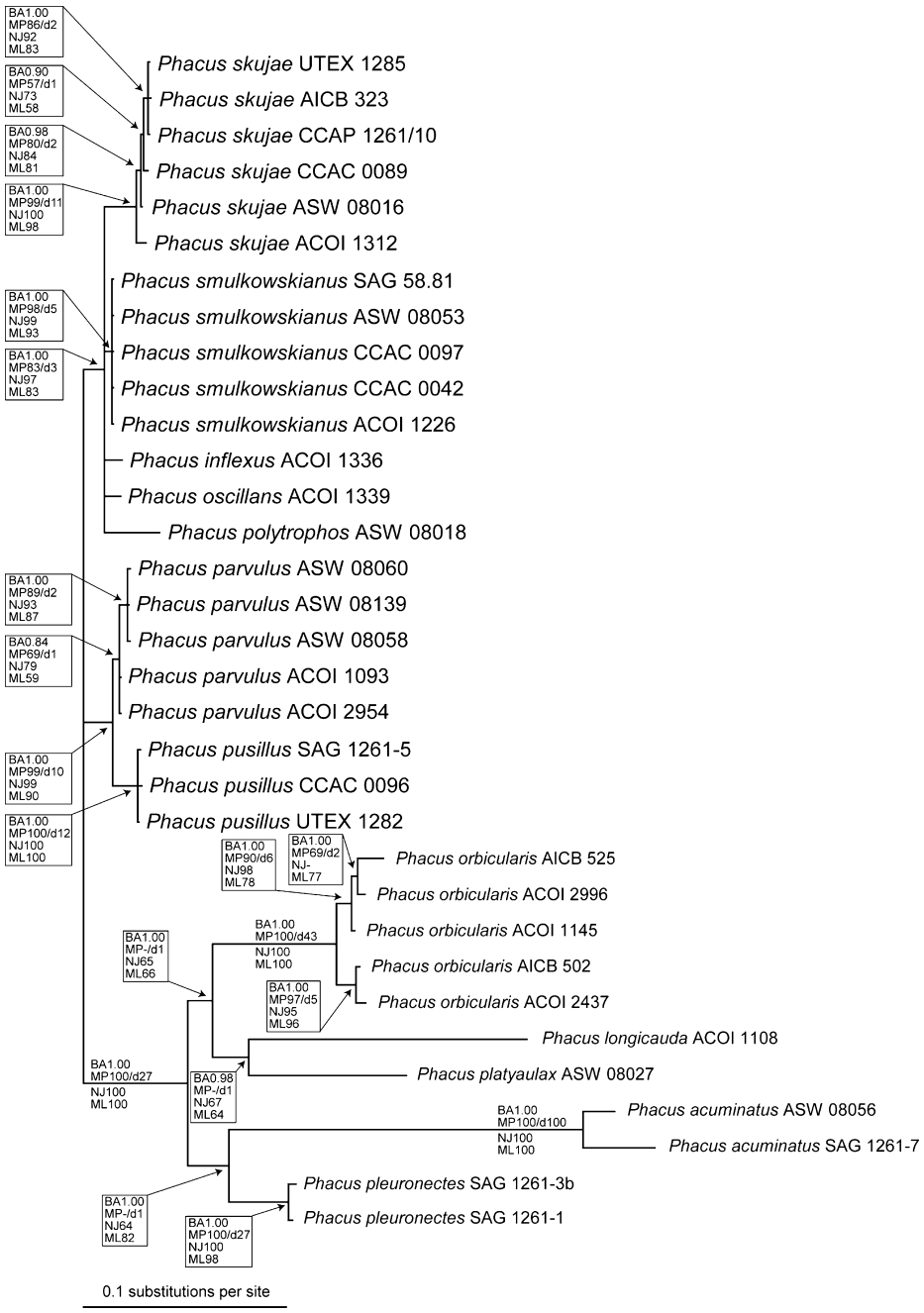

FIG. 2. The phylogenetic tree of SSU rDNA sequences obtained by Bayesian inference (model: GTR+G+I). Numbers at the nodes show posterior probabilities of the tree bipartitions, as well as the bootstrap values/decay indices obtained by MP analysis, and bootstrap values obtained by NJ and ML analyses (model GTR+I+G). Clades not present in the particular analysis are indicated by a ''–.'' GTR, general time reversible.

edges curling in, thus creating a deep furrow resembling the letter ''C'' in cross-section. *P. polytrophos* (ASW 08018) has trapezoid cells with a shallow furrow.

Genetic variability. Interestingly, all strains of the ''*Phacus oscillans* clade'' display a high degree (95.1%–99.9%) of SSU rDNA similarity (Table 2). Among the four species represented by more than one strain, the greatest variability within a single species is found in *P. skujae*, where the sequence of the ACOI 1312 strain differs from those of UTEX 1285 and AICB 323 by 1.0%. At the same time, differences between the two very well morphologically distinguished species, *P. oscillans* ACOI 1336 and *P. smulkowskianus* ACOI 1226, were only 0.9%. Thus, similarity between strains of the same and different species is in some cases of the same magnitude.

The situation is entirely different with respect to outgroup species of *Phacus* (Table 3). One of the well-known cases is *P. orbicularis*, which is represented here by the five most diverged strains. SSU rDNA sequence similarity of these strains is in the range of 97.6%–99.6%. The species most similar to *P. orbicularis*, both morphologically and genetically, is *P. pleuronectes*. Its similarity is within the 91.8%–92.1% range.

TABLE 2. SSU rDNA sequence similarity between strains from the ''*Phacus oscillans*'' clade (strains with 100% similarity are excluded).

Strain	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
(1) *P. inflexus* ACOI 1336	–														
(2) *P. oscillans* ACOI 1339	98.6	–													
(3) *P. parvulus* ACOI 1093	97.4	97.4	–												
(4) *P. parvulus* ASW 08058	97.2	97.1	99.6	–											
(5) *P. parvulus* ASW 08060	97.2	97.1	99.6	99.9	–										
(6) *P. parvulus* ASW 08139	97.3	97.2	99.7	99.9	99.9	–									
(7) *P. polytrophos* ASW 08018	97.2	97.2	95.8	95.6	95.6	95.8	–								
(8) *P. pusillus* SAG 1261-5	96.9	96.9	98.8	98.6	98.5	98.6	95.2	–							
(9) *P. pusillus* CCAC 0096	96.8	96.8	98.7	98.6	98.5	98.5	95.1	99.8	–						
(10) *P. pusillus* UTEX 1282	96.9	96.9	98.8	98.7	98.6	98.6	95.3	99.9	99.9	–					
(11) *P. skujae* ACOI 1312	97.3	97.2	96.3	96.3	96.2	96.3	96.7	95.7	95.6	95.7	–				
(12) *P. skujae* AICB 323	97.6	97.4	96.6	96.4	96.4	96.6	96.7	96.1	96.0	96.1	99.0	–			
(13) *P. skujae* ASW 08016	97.9	97.7	96.8	96.7	96.7	96.8	96.9	96.1	96.0	96.1	99.2	99.8	–		
(14) *P. skujae* CCAC 0089	97.7	97.5	96.6	96.6	96.5	96.6	96.7	96.0	95.9	96.1	99.1	99.7	99.8	–	
(15) *P. skujae* UTEX 1285	97.7	97.5	96.6	96.5	96.5	96.6	96.7	96.1	96.1	96.2	99.0	99.9	99.8	99.8	–
(16) *P. smulkowskianus* ACOI 1226	98.8	99.1	97.5	97.3	97.3	97.4	97.4	96.9	96.9	97.0	97.7	98.0	98.3	98.1	98.1

TABLE 3. SSU rDNA sequence similarity between the *Phacus* strains outgroup.

Strain	1	2	3	4	5	6	7	8	9	10	11
(1) *P. acuminatus* ASW 08056	–										
(2) *P. acuminatus* SAG 1261-7	96.0	–									
(3) *P. longicauda* var. *torta* ACOI 1108	84.3	83.7	–								
(4) *P. orbicularis* ACOI 1145	85.8	85.2	88.2	–							
(5) *P. orbicularis* ACOI 2437	86.1	85.3	88.2	98.4	–						
(6) *P. orbicularis* ACOI 2996	85.6	85.0	88.0	99.5	98.1	–					
(7) *P. orbicularis* AICB 502	85.8	85.1	88.1	98.6	99.6	98.5	–				
(8) *P. orbicularis* AICB 525	85.2	84.7	87.7	98.8	97.6	98.8	97.8	–			
(9) *P. platyaulax* ASW 08027	85.5	84.7	88.3	90.8	90.6	90.4	90.6	90.7	–		
(10) *P. pleuronectes* SAG 1261-1	87.4	86.8	89.3	92.1	91.8	91.9	92.1	91.9	90.4	–	
(11) *P. pleuronectes* SAG 1261-3b	87.6	87.0	89.3	92.0	91.7	91.8	91.9	91.7	90.4	99.5	–

Signature sequences. The characteristic sequence designated for the investigated *Phacus* species corresponds to the helix E 23_2 (Marin et al. 2003). Both the nucleotide sequence and the length of the helix (from 74 to 86 nucleotides in analyzed species) constitute a diagnostic feature. This region is the most diversified part of analyzed sequences and, despite overall similarity, is characteristic for particular species.

TAXONOMIC REVISIONS

1. ***Phacus oscillans*** G. A. Klebs, Unters. Bot. Inst. Tübing. p. 313, pl. 3, fig. 6, 1883. Emend. Zakryś et Karnkowska.

Emended diagnosis: Cells (17–33 μm x 7–11 μm; 26 × 9 μm on average) with a deep furrow created by edges of a flat cell curling inward; in cross-section the furrow has the shape of the letter ''C''; the whole body conspicuously spirally twisted along the longer axis resulting in asymmetry, particularly of its frontal part. In general, the outline of swimming cells longitudinally oval or cylindrical, with a posterior end slightly tapering to a small, bluntly rounded short extension. With the 84 nucleotide-long SSU rDNA signature sequence:

helix E 23_2: CTGCCAGGTGGCTGGTGGCTG-TGGTGTTGTTCACTGGTTCGCTGGTGGGCATAG-CCATGGCTGCTGGAACTCAACCCCTGGCAG

Lectotype: Here designated in fig. 6, table 3 in Klebs, d. c.

Epitype: Permanently preserved material (cells in resin, for EM) from the strain ACOI 1339, deposited at the Herbarium of the Biology Faculty at the University of Warsaw, Al. Ujazdowskie 4. PL-00478 Warszawa, Poland (http://www.zielnik.biol.uw.edu.pl/zsigr.html). Figure 1, g–i, shows illustrations of the epitype.

Strain type: ACOI 1339 available at the Culture Collection of Algae at the Department of Botany, University of Coimbra, Portugal, and at the Culture Collection of Algae at the University of Cologne (CCAC), Germany.

Synonym: Phacus oscillans G. A. Klebs var. *curta* Skvortzov, Berichte Deutsh. Bot. Ges. 46: 121, pl. 2, fig. 64, 1928.

2. ***Phacus parvulus*** G. A. Klebs, Unters. Bot. Inst. Tübing. p. 313, pl. 3, fig. 5, 1883. Emend. Zakryś et Karnkowska.

Emended diagnosis: Cells (17–24 μm x 7.4–15.8 μm; 21 x 10 μm on average), broadly ovoid or elliptic, without a furrow, posterior end tapered and blunt.

With the 74 nucleotide-long SSU rDNA signature sequence:

helix E 23_2: CTGCCAGGTGGCTGGTGGCTG-CGGTTGTGGCTCTCGGGTCATGGCTGTWGTCGC-TGGAACTCAACCCCTGGCAG

Lectotype: Here designated in fig. 5, table 3 in Klebs, d. c.

Epitype: Permanently preserved material (cells in resin, for EM) from the strain ACOI 1093 deposited at the Herbarium of the Biology Faculty at University of Warsaw, Al. Ujazdowskie 4. PL-00478 Warszawa, Poland (http://www.zielnik.biol.uw.edu.pl/zsigr.html). Figure 1, o and p, shows illustrations of the epitype.

Strain type: ACOI 1093 available at the Culture Collection of Algae at the Department of Botany, University of Coimbra, Portugal, and at the Culture Collection of Algae at the University of Cologne (CCAC), Germany.

3. ***Phacus pusillus*** Lemmerm., Krypt. M. Brand. p. 514, 1910. Emend. Zakryś et Karnkowska.

Emended diagnosis: Cells (14–20 μm x 6–13 μm; 16 × 8 μm on average) flattened (but not dorsiventrally), oval with a posterior end tapered and blunt, having a very shallow furrow running along the full length of the cell; sides of the cell on both sides of the furrow conspicuously convex (''pillow-like''); the whole body slightly twisted along the long axis. With the 80 nucleotide-long SSU rDNA signature sequence:

helix E 23_2: CTGCCAGGTGGCTGGTGGCTGG-TGGTTGTGTCTGGTTCGCCAGGCATGGCTGTCG-GCTGCTGGA-ACTCAACCCCTGGCAG

Basionym: Phacus alatus G. A. Klebs, Stammform in Hübner, Programm d. Realgymnasiums Stralsund, p. 6, fig. 7, a–b, 1886.

Lectotype: Here designated in fig. 223 in Lemmermann in Pascher's Süssw., Fl., 2, 1913.

Epitype: Permanently preserved material (cells in resin, for EM) from the strain UTEX 1282 deposited at the Herbarium of the Biology Faculty at the University of Warsaw, Al. Ujazdowskie 4. PL-00478 Warszawa, Poland (http://www.zielnik.biol.uw.edu.pl/zsigr.html). Figure 1, q and r, show illustrations of the epitype.

Strain type: UTEX 1282, deposited at the Culture Collection of Algae at the University of Texas at Austin, Texas, USA.

Synonym: Phacus parvulus var. *pusillus* (Lemmerm.) Popova, Opred. Presnov. Vodor. CCCP 7: 213, pl. 86, fig. 15, 1955.

4. ***Phacus skujae*** Skvortzov, Ber. Deutsch. Bot. Gesellsch 46: 116, pl. 2, fig. 42, 1928. Emend. Zakryś et Karnkowska.

Emended diagnosis: Cells distinctively flattened (18–29 μm × 7–19 μm; 19 × 11μm on average), in general, outline longitudinally oval; posterior end tapered and blunt. The whole body slightly asymmetrical and slightly spirally twisted. A deep furrow running along the whole body length, in cross-section having the shape of the letter ''V.'' With the 86 nucleotide-long SSU rDNA signature sequence:

helix E 23_2: CTGCCAGGTGGCTGGTGGCTG-TGGTGCTGTYYRCTGGTTCTTACYGGTGGGYBGT-GYCATGGCTGCTGGAACTCAACCCCTGGCAG

Lectotype: Here designated fig. 42, pl. 2 in Skvortzov, d. c.

Epitype: Permanently preserved material (cells in resin, for EM) from the strain ACOI 1312 deposited at the Herbarium of the Biology Faculty at the University of Warsaw, Al. Ujazdowskie 4. PL-00478 Warszawa, Poland (http://www.zielnik.biol.uw.edu.pl/zsigr.html). Figure 1, j–l, shows illustrations of the epitype.

Strain type: Strain ACOI 1312, deposited at the Culture Collection of Algae at the Department of Botany, University of Coimbra, Portugal, and at the Culture Collection of Algae at the University of Cologne (CCAC), Germany.

5. ***Phacus inflexus*** (Kisselew) Pochmann, Arch. Protistenk. 95: 133, fig. 20, a–h, 1942. Emend. Zakryś et Karnkowska.

Emended diagnosis: Cells (18–31 μm x 7–19 μm; 23 x 12 μm on average) in general, outline widely oval, flat but bent in half along the longer axis, slightly spirally twisted (usually in one turn); s- or sickle-shaped (in the shape of the letters S or C), slightly asymmetrical. Posterior end gradually narrowing to a short, blunt tailpiece. With the 80 nucleotide-long SSU rDNA signature sequence:

helix E 23_2: CTGCCAGGTGGCTGGTGGCTGT-GGTTGTTCACTGGTTCGCCAGGGGGCTGCCATG-GCTGCTGGAACTCAACCCCTGGCAG

Basionym: Euglena inflexa I. Kisselew, Trudy Sr.-Az. Gos. Univ. Ser. 12a, 9: 76, pl. 2, fig. 14, 1931.

Lectotype: Here designated fig. 20a in Pochmann, d. c.

Epitype: Permanently preserved material (cells in resin, for EM) from the strain ACOI 1336 deposited at the Herbarium of the Biology Faculty at the University of Warsaw, Al. Ujazdowskie 4. PL-00478 Warszawa, Poland (http://www.zielnik.biol.uw.edu.pl/zsigr.html). Figure 1, a–c, shows illustrations of the epitype.

Strain type: ACOI 1336 available at the Culture Collection of Algae at the Department of Botany, University of Coimbra, Portugal, and at the Culture Collection of Algae at the University of Cologne (CCAC), Germany.

Synonym: P. inflexus var. *minor* Bourr. et Manguin, Soc. Edit. Enseign. sup. Paris, p.179, table 21, figs. 211–213, 1952.

6. ***Phacus polytrophos*** Pochm., Arch. Protistenk. 95: 128, fig. 15, a–d, 1942. Emend. Zakryś et Karnkowska.

Emended diagnosis: Cells (13–30 μm × 6–13 μm; 17 × 9 μm on average) cylindrical, in a general

outline trapezoidal-rhomboidal (wide and truncated slantwise in the anterior, tapered and blunt in the posterior end) with a shallow furrow running along the whole body length. Cells slightly flattened and slightly spirally twisted during movement. With the 85 nucleotide-long SSU rDNA signature sequence:

helix E 23_2: CTGCCAGGTGGCTGGTGGCTGTGGTGTTGTTCACTGGTTTACTGGTGGGCATAGCCATGGCTGCTGGAACTGAGGGCGCTGGCAG

Lectotype: Here designated in fig. 15c in Pochmann, d. c.

Epitype: Permanently preserved material (cells in resin, for EM) from the strain ASW 08018 deposited at the Herbarium of the Biology Faculty at the University of Warsaw, Al. Ujazdowskie 4. PL-00478 Warszawa, Poland (http://www.zielnik.biol.uw.edu.pl/zsigr.html). Figure 1, m and n, shows illustrations of the epitype.

Strain type: Strain ASW 08018, deposited at the Culture Collection of Algae at the University of Cologne (CCAC), Germany.

Synonym: Phacus neosulcatus Z. X. Shi, Acta Hydrobiologica Sinica 11: 11, fig. 1, l–m, 1987.

7. ***Phacus smulkowskianus*** (Zakryś) Kusber, Willdenowia 28: 246, 1998. Emend. Zakryś et Karnkowska.

Emended diagnosis: Cells (20–41 μm x 7–23 μm; 28 x 14 μm on average) extremely flattened and helicoidally twisted (two to three rounds), generally broadly spindle-shaped; truncated straight or slightly slantwise in the frontal part, tapered in the posterior and ending with a short tail. Periplast only longitudinally striated without struts (small, transversal striae located between the longitudinal striation). With the 84 nucleotide-long SSU rDNA signature sequence:

helix E 23_2: CTGCCAGGTGGCTGGTGGCTGTGGTGTTGTTCACTGGTTCGCCGGTGGGCATAGCCATGGCTGCTGGAACTCAACCCCTGGCAG

Basionym: Euglena smulkowskiana Zakryś, Nova Hedwigia 42: 524, pl. 4, fig. 6, 1986 (Zakryś 1986).

Lectotype: Here designated fig. 6 in Zakryś, d. c.

Epitype: Permanently preserved material (cells in resin, for EM) from the strain ACOI 1226 deposited at the Herbarium of the Biology Faculty at the University of Warsaw, Al. Ujazdowskie 4. PL-00478 Warszawa, Poland (http://www.zielnik.biol.uw.edu.pl/zsigr.html). Figure 1, d–f, shows illustrations of the epitype.

Strain type: Strain ACOI 1226, deposited at the Collection of Algae at the Department of Botany, University of Coimbra, Portugal, and at the Culture Collection of Algae at the University of Cologne (CCAC), Germany.

Synonyms: Phacus similis Christen, Revue Algologique 6: 164, pl. 1, figs. 3 and 4, 1961, nom. inval. (Kusber 1998); *P. similis* f. *minor* Bourr. & Couté, Revue Algologique 4: 296, pl. 1, fig. 3, 1978, nom. inval. (Kusber 1998).

DISCUSSION

Despite considerable molecular similarities, most of the investigated species are morphologically well distinguished. However, mistakes in identification are made frequently (see Table S1), which might be the result of changes in cell morphology caused by inclement habitat conditions. Even such a seasoned scholar as Drežepolski (1925) identified the distinctively characteristic species *P. inflexus* as *P. pusillus* (Drežepolski 1925, p. 233, fig. 128). Kisselew, relying only on Drežepolski's drawing, described it in 1931 (Kisselew 1931) as a new species (*P. inflexus* Kisselew).

The helicoidally twisted cells of a rare species, *P. smulkowskianus*, known in the literature as *P. similis* Christen, are very similar to those of *P. helicoides* Pochmann and *P. longicauda* var. *tortus* Lemmermann [=*P. tortus* (Lemmerm.) Skvortzov], and therefore all perceived by many authors as coming from closely related species (Christen 1961, Tell and Conforti 1986, Kusber 1998) or belonging to the same species (Popova and Safonova 1976, Starmach 1983). *P. smulkowskianus* and *P. longicauda* var. *tortus* belong to two distant clades on contemporary phylogenetic trees, which indicates that they are not closely related (Marin et al. 2003, this study). *P. smulkowskianus* belongs to the same clade together with taxa of a different shape but similar, although slightly smaller, body size (Fig. 2). Relationships within the ''*Phacus oscillans* clade'' are not resolved, but the close affiliation between the two sister strains having flat, straight cells (*P. parvulus* and *P. pusillus*) is well supported. Cells of *P. pusillus* have a shallow furrow and are 16 × 8 μm on average, while those of *P. parvulus* are devoid of a furrow and are 21 x 10 μm on average (Table 1). For this reason, Popova (1955) considered *P. pusillus* to be a variety of *P. parvulus* (*P. parvulus* var. *pusillus*). Her conjecture concerning the close relationship of the two taxa is confirmed by contemporary phylogenetic analyses. We argue that, given molecular and morphological differences among all species from this clade, *P. parvulus* and *P. pusillus* should be given the status of separate species. We believe that verification of diagnostic features, including a signature sequence of the SSU rRNA helix E 23_2, and the establishment of epitypes, will help avoid mistakes in identification. The problem is illustrated by the large number of misidentified strains deposited in the culture collections of algae (Table S1).

A similar situation involves the three remaining species of this clade (*P. oscillans*, *P. skujae*, *P. polytrophos*). Their common features are the presence of a furrow, the depth and shape of which are subject to deformation, caused by the accumulation of

paramylon grains (see Results). For this reason, the three species are easily identified only in young cultures with vigorously dividing cells. Of the three, *P. oscillans* (Klebs 1883) was described first, then *P. skujae* (Skvortzov 1928), and finally *P. polytrophos* (Pochmann 1942). As diagnostic features of the latter, Pochmann lists: trapezoidal-rhomboidal shape, cylindrical (not flattened) cells, the presence of a short furrow and a large paramylon grain, filling almost the whole cell. According to Pochmann, the same forms were identified by Drežepolski (1925) as *P. parvulus* (p. 232, fig. 127) and by Swirenko (1915) as *P. oscillans* (p. 55, pl. 3, fig. 5). Shi (1987) described *P. neosulcatus*, which, because of the cell size (23–24 x 12–13 μm), cylindrical (not flattened) body, and a shallow furrow, was deemed to be a synonym of *P. polytrophos*. In light of the studies presented herein, the short furrow and the presence of a very large paramylon grain are the consequences of physiological changes (population aging and inclement habitat conditions). In young and not overcrowded populations, one or two large paramylon grains take up only a small part of the cell, and a furrow runs along the entire length of the body. Features intermediate between *P. polytrophos* and *P. oscillans* are displayed by *P. skujae*. Numerous strains of the latter are deposited in culture collections, enabling its molecular and morphological analyses. In combination with literature studies, this has made possible the verification of diagnostic features and epitype establishment for the two remaining species, even though each is represented in all world collections by only a single strain.

Species identification and the delimitation of species' boundaries are the subject of a vigorous discussion among taxonomists. Two opposing methods, using exclusively either molecular or morphological criteria, have been advocated (Lipscomb et al. 2003, Tautz et al. 2003, Will and Rubinoff 2004). There is a growing consensus, however, that the two approaches should be used concomitantly. We follow this approach. The advantages of taxa identification based on unique DNA sequences are simplicity and speed, while its main disadvantages are the difficulty in drawing universal boundaries between taxa of a different rank, and in delimiting a universal level of DNA diversity characteristic of a taxon rank (Moritz and Cycero 2004, Wheeler et al. 2004, DeSalle et al. 2005, Witt et al. 2006). Irrespective of the approach, the most difficult cases involve sister species, particularly closely related ones. Comparison of different levels of SSU rDNA sequence diversification within the ''*Phacus oscillans* clade'' to those within other clades representing different *Phacus* species is a good illustration of the problem. Similarity of different *Phacus* species varies from 83.7%, for *P. longicauda* and *P. acuminatus*, to 99.1%, for *P. oscillans* and *P. smulkowskianus*, while the smallest intraspecies similarity is 96% (Table 3). Thus, intraspecific SSU rDNA sequence diversity could exceed that between species. Therefore, it is impossible to delineate a universal level of diversity for this sequence, in the case of the *Phacus* species and other species of green euglenoids.

DNA sequences are increasingly being used as a diagnostic feature (Sarkar et al. 2002, Seberg et al. 2003, DeSalle et al. 2005). This approach has a particular appeal when examining Euglenales, since the paucity of diagnostic features calls for additional criteria for species delineation. The species investigated herein display a high level of SSU rDNA sequence similarity, hampering the identification of the appropriate section to serve as the diagnostic feature. The helix E 23_2, chosen based on the structure proposed by Marin et al. (2003), differed in length among most of the species. Only for the pairs *P. inflexus/P. pusillus* and *P. oscillans/P. smulkowskanus* was its length the same. In both cases, morphologies of species from the same pair differed substantially, enabling easy identification. In fact, *P. inflexus* and *P. smulkowskianus* have features so highly characteristic that they can hardly be confused with other *Phacus* species. Moreover, their helix E 23_2 sequences also differed. We therefore consider the length of the helix E 23_2 as a diagnostic feature. In the case of *P. skujae* (and to a lesser extent, *P. parvulus*), there are some intraspecific differences within the E 23_2 helix. However, these differences are less than those between sequences from different species. There is a possibility that such diversity exists within a species herein represented by a single strain. We have searched world collections for more strains of these species but to no avail.

In certain situations, one category of data—either morphological or sequential—functions to differentiate the taxa, while the other does not. Only combining the two approaches makes it possible to uncover such a situation. This is exactly the type of situation encountered within the ''*Phacus oscillans* clade.'' The combination of inferences based on molecular and morphological data affords the proper discrimination of species and also carries the potential to avoid mistakes persistently being made in species identification.

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Supplementary Material

The following supplementary material is available for this article:

Table S1. The euglenoid strains and the corresponding SSU rDNA GenBank accession numbers for the taxa used in this study. Accession numbers of new sequences are in boldface. Strains used in biometric studies are underlined.

Appendix S1. The alignment of SSU rDNA used for analyses (with information about excluded positions).

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