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PHYLOGENETIC AND TAXONOMIC POSITION OF *LEPOCINCLIS FUSCA* COMB. NOV. (= *EUGLENA FUSCA*) (EUGLENACEAE): MORPHOLOGICAL AND MOLECULAR JUSTIFICATION[1]

Sylwia Kosmala, Anna Karnkowska, Rafał Milanowski, Jan Kwiatowski, and Bożena Zakrys[2]

Department of Plant Systematics and Geography, Warsaw University, Al. Ujazdowskie 4, PL-00-478 Warszawa, Poland

We studied the morphological diversity and analyzed the small subunit rDNA sequences of two taxa formerly known as *Euglena spirogyra* Ehr. and *Euglena fusca* (Klebs) Lemmermann. Our studies confirmed that the two should have the rank of a species, namely *Lepocinclis spirogyroides* (Ehr.) Marin et Melkonian and *Lepocinclis fusca* (Klebs) Kosmala et Zakryś comb. nov. (Euglenophyceae). We are defining new diagnostic features for these species, namely the size and the shape of the cells and the shape of the papillae, as well as designating epitypes for them.

Key index words: **Euglena fusca; Euglena spirogyra; Euglenida; Euglenophyta; *Lepocinclis fusca*; *Lepocinclis spirogyroides*; molecular phylogeny; morphology**

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

Enormous morphological plasticity of euglenoid cells has been well known for a long time, but recently there have been more thorough studies. It was shown experimentally that changes in physicochemical conditions are responsible for changes in cell morphology (Conforti 1991, Conforti et al. 1995, Zakryś and Kucharski 1996, Zakryś et al. 1996, 2002, 2004, Zakryś 1997). Similar processes are, without doubt, taking place in nature. In astatic pools, where euglenoids live, changing conditions are the norm. This causes enormous morphological diversity, which, together with ontogenetic variability, was the main cause for the creation of a plethora of descriptions of taxa of different rank but similar morphology (so-called critical groups of taxa), the distinction of which is often quite impossible. Verification of diagnostic features for taxa contained in such groups is possible because of morphological and molecular studies performed on strain cultures. Four euglenoid species have been studied in this way: *Euglena agilis* Carter (Zakryś 1997, Zakryś et al. 2004), *E. geniculata* Duj. (Zakryś et al. 2002), *E. viridis* Ehr. (Shin and Triemer 2004), and *Monomorphina pyrum* (unpublished data).

Continuing on this path, we present here the morphological and genetic (18S rDNA) variability studies of *Lepocinclis spirogyroides* Marin et Melkonian (= *Euglena spirogyra* Ehrenberg 1832). Morphological variability of this species has attracted investigators' attention for a long time (Klebs 1883, Lemmermann 1910, Playfair 1921, Lefèvre 1934, Szabados 1938, Johnson 1944, Chu 1946, Gojdics 1953, Leedale et al. 1965a,b, Pringsheim 1956, Popova 1966, Zakryś 1986). Nine varieties of this species have been described so far (var. *fusca* Klebs 1883, var. *laticlavius* Hübner 1886, var. *abrupte–acuminata* Lemm.1913, var. *elegans* Playfair 1921, var. *fusiformis* Deflandre 1924, var. *minor* Allorge and Lefèvre 1925, var. *suprema* Skuja 1932, var. *torta* Prijmačenko 1963 and var. *compressa* Shi 1994). One variety, var. *fusca* Klebs 1883, was elevated to the rank of species *E. fusca* (Klebs) Lemmermann 1910, for which two varieties were created: *E. fusca* var. *marchica* Lemmermann 1910 and var. *minima* Szabados 1949. Additionally, one species from China, *E. pseudospirogyra* (Shi 1994), was described in the last decade.

When describing new taxa, authors consider the following features as diagnostic: the shape, size, and degree of flatness of the cells; the color and ornamentation pattern of the periplast; the number of large paramylon grains; and the length of the flagella. Many authors, including those of critical monographic treaties, are questioning the logic of forming so many taxa (Chu 1946, Pringsheim 1956, Popova 1966). Some also present differing positions on whether there should be two species—*E. spirogyra* and *E. fusca* (Klebs 1883, Lemmermann 1913, Dreżepolski 1925, Lefévre 1934, Gojdics 1953)—or only one—*E. spirogyra* and its varieties (Chu 1946, Pringsheim 1956, Leedale et al. 1965a,b, Popova 1966). Marin et al. (2003) did not take a stand as to the position of *E. fusca* nor did they make a new classification and synonymization system, even though they did so in the case of numerous other species, for example, *L. platydesma* (= *E. platydesma*), *Lepocinclis spiroides* (= *E. spiroides*), *M. costata* (= *Ph. costatus*), *M. cochleata* (= *Ph. cochleatus*), *Monomorphina lepocincloides* (= *Ph. lepocincloides*), *M. trypanon* (= *Ph. trypanon*), *M. strongyla* (= *Ph. strongylus*), and many others.

Taking history into account, we have aimed to 1) delineate the range of morphogenetic variability of

[1]Received 23 February 2005. Accepted 29 August 2005.
[2]Author for correspondence: e-mail zakrys@biol.uw.edu.pl.

Lepocinclis spirogyroides; 2) revise the taxonomy of *L. spirogyroides* (= *E. spirogyra* Ehr.), including *E. fusca*; 3) verify the morphological diagnostic features of the taxa considered; and 4) designate the epitypes of taxa being reclassified.

MATERIALS AND METHODS

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

LM observations. Observation of morphological features were performed using a light microscope (Eclipse E-600 with Nomarski contrast, Nikon, Tokyo, Japan), equipped with software for image recording and processing. Photographic documentation was made with a digital camera (Nikon DX-1200) connected to a microscope. Cultures were sampled every 2 weeks for periods of 3 to 4 months. Such sampling enabled us to observe cells during all developmental stages, from the young (immediately after division) to the old.

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

DNA isolation, amplification, and sequencing. The total DNA was isolated from 20 to 30 mg of the centrifuged cells by using the Dneasy Tissue Kit (Qiagen GmbH, Hilden, Germany) in accordance with the manufacturer's protocol (with the addition of proteinase K). Primers for PCR amplification and sequencing are shown in Table 2. Fifty milliliters of a reaction mixture, containing 1 U of Taq Polymerase (Qiagen), 0.2 mM dNTPs, 2.5 mM $MgCl_2$, 10 pmol of each primer, reaction buffer (Qiagen), and 10–50 ng DNA, was used. The PCR protocol consisted of an initial 5 min at 95° C, followed by five initial cycles comprising 1 min at 95° C, 90 s at 40–54° C, and 45–105 s at 72° C, and then by 30 cycles comprising 30 s at 95° C, 30 s at 48–56° C, and 30–90 s at 72° C. The final extension step was performed for 7 min at 72° C. The PCR products were sized on agarose gels and purified using the

TABLE 1. Strains and accession numbers for cytoplasmic small subunit rDNA sequences.

Taxon name	Strain origin	18s rDNA GenBank accession number[a]
Euglena limnophila Lemmermann	ASW 08039	AJ 532453
Euglena spathirhyncha Skuja	SAG 1224-42	AJ 532454
Lepocinclis acus (Müller) Marin et Melkonian	SAG 1224-1a	AJ 532459
Lepocinclis acus (Müller) Marin et Melkonian	ASW 08037	AJ 532458
Lepocinclis acus (Müller) Marin et Melkonian	SAG 1224-1d	AJ 532457
Lepocinclis acus (Müller) Marin et Melkonian	Isolated in Korea	AF 090871
Lepocinclis bütschlii (Lemmermann)	Isolated in Korea	AF 096993
Lepocinclis fusca (Klebs) comb. nov.	ACOI 1032	**AY935690**
Lepocinclis fusca (Klebs) comb. nov.	ACOI 1414 (as *E. spirogyra*)	**AY935691**
Lepocinclis fusiformis (Carter) Lemmermann	ACOI 1025	**AY935697**
Lepocinclis oxyuris Schmarda	Isolated in Korea	AF 090869
Lepocinclis ovum (Ehr.) Minkevich	AICB 278	AJ 532455
Lepocinclis ovum (Ehr.) Minkevich	SAG 1244-8	AF110419
Lepocinclis spirogyroides (Ehr.) Marin et Melkonian	SAG 1224-10b (as *L. oxyuris*)	AJ 532464
Lepocinclis spirogyroides (Ehr.) Marin et Melkonian	ACOI 1027	**AY935692**
Lepocinclis spirogyroides (Ehr.) Marin et Melkonian	ACOI 1227	**AY935693**
Lepocinclis spirogyroides (Ehr.) Marin et Melkonian	ACOI 1413 (as *E. fusca*)	**AY935695**
Lepocinclis spirogyroides (Ehr.) Marin et Melkonian	SAG 1224-13b	**AY935694**
Lepocinclis spirogyroides (Ehr.) Marin et Melkonian	ASW 08002	AJ 532463
Lepocinclis spirogyroides (Ehr.) Marin et Melkonian	ASW 08026	AJ 532461
Lepocinclis spirogyroides (Ehr.) Marin et Melkonian	UTEX 1307	AF150935
Lepocinclis tripteris (Dujardin) Marin et Melkonian	SAG 1224-16	AJ 532456
Lepocinclis tripteris (Dujardin) Marin et Melkonian	UW-OB	**AY935696**
Phacus orbicularis Hübner	AICB 502	**AY935698**
Phacus orbicularis Hübner	AICB 525	**AY935699**
Phacus orbicularis Hübner	ASW 08054	AF 283315
Phacus oscillans Klebs	UTEX 1285	AF 181968
Phacus oscillans Klebs	CCAC-0089	AJ 532468
Phacus pleuronectes (Ehr.) Dujardin	SAG 1261-3b	AJ 532475
Phacus pleuronectes (Ehr.) Dujardin	Isolated in Korea	AF 081591
Phacus pusillus Lemmermann	SAG 1261-5	AJ 532471
Phacus pusillus Lemmermann	ACOI 1093	AJ 532472
Phacus similis Christen	SAG 58.81	AJ 532467

[a]Numbers obtained in this study are in boldface.

ACOI, Culture Collection of Algae at the Department of Botany, University of Coimbra, Portugal; AICB, Culture Collection of Algae at the Institute of Biological Research Cluj-Napoca, Romania; ASW, Algenkultur-Sammlung an der Universität Wien, Vienna, Austria; CCAC, Culture Collection of Algae at the University of Cologne, Cologne, Germany; SAG, Sammlung von Algenkulturen Pflanzenphysiologisches Institut der Universitvät Göttingen, Germany; UTEX, Culture Collection of Algae at the University of Texas at Austin, TX, USA; UW-OB, Department of Plant Systematics and Geography of Warsaw University, Poland.

TABLE 2. Primers for PCR amplification and sequencing of euglenoid 18S rDNA (Elwood et al. 1985, slightly modified).

Primer	Position of 3′ end	Sequence (5′–3′)
18S5′	89	CAGTGGGTCTGTGAATGGCTCC
18S382F	483	AGGGTTCGATTCCGGAG
18S557R	668	TTACCGCAGCTGCTGGC
18S570F	682	GTGCCAGCAGCTGCGGT
18S892R	1293	AGAATTTCACCTCTG
18S906F	1307	CAGAGGTGAAATTCT
18S1125R	1539	CAATTCCTTTAAGTTTC
18S1141F	1555	CAAACTTAAAGGAATTG
18S1263R	1677	GAGCGGCCATGCACCAC
18S1891F	1910	TGCATGCTAGAGCCAACAGC
18S3′	2127	CGACGGGCGGTGTGTACAAGT

QIAEXII Gel Extraction Kit (Qiagen). Purified PCR products were sequenced using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA). After the removal of primer sequences, all readings from the ABI Prism 310 DNA sequenator (Applied Biosystems) were assembled into contigs by the SeqMan program from the LASERGENE package (DnaStar, Madison, WI, USA) and checked manually for consistency.

Sequence accession numbers, alignment, and phylogenetic analysis. The GenBank accession numbers for the small subunit rDNA sequences reported here and for the sequences used for phylogenetic analyses are shown in Table 1. The alignment of sequences, obtained by using the Clustal X 1.8 program (Thompson et al. 1997) with default options, was manually checked and edited according to the secondary structure of *Euglena gracilis* as suggested by Wuyts (2002). Some regions that could not be unambiguously aligned were omitted from the analyses. Distance (neighbor joining [NJ]), maximum likelihood (ML), and maximum parsimony (MP) analyses and a homogeneity test (χ^2) of nucleotide distribution were performed by PAUP*, version 4.0b6, for Microsoft Windows (Swofford 1998). To find the best tree, the heuristic search option was used with MULPARS, tree-bisection-reconnection branch swapping, ACCTRAN optimization, and random addition, with the number of replicates depending on the method used (1000 for MP and 20 for ML). Bootstrap support for specific nodes (Felsenstein 1985) was estimated by the default options using 1000, 1000, and 100 replications for MP, NJ, and ML analyses, respectively, as implemented in PAUP*. Models of sequence evolution and their parameters for the ML and NJ methods were chosen by the Modeltest 3.6 program (Posada and Crandall 1998). Auto decay indices (Bremer 1994) were calculated by the AutoDecay 4.0.2 program (Eriksson 1998) for MP analyses. The Bayesian analyses (BAs) were performed and their model parameters were estimated by the MrBayes 3.04B program (Huelsenbeck and Ronquist 2001, Ronquist and Huelsenbeck 2003). Trees were drawn by TreeView, version 1.6.1, for Microsoft Windows (Page 1996).

RESULTS

Biometry. Precise measurements of cells grown in identical physicochemical conditions point to the existence of two groups of strains with significantly different sizes. The first group, consisting of strains ACOI-1227, ACOI-1027, SAG-1224/13b, and ACOI-1413, on average have cells 100–116 μm long and 7.6–8.6 μm wide. The cells of the second group, containing strains ACOI-1414 and ACOI-1032, are on average 178–189 μm long and 15–17 μm wide (Table 3 and Fig. 1L). The length of the tail constituted 7%–11% of the cell's length and was 8.4–18.2 μm long for both groups (Table 3).

Microscope observations. There were some consistent morphological differences between strains as well as some intrastrain diversity, dependent mostly on the culture age. The following most likely diagnostic features might be invoked here.

Cell shape: (i) Cells cylindrical, sometimes slightly flattened during movement (strains ACOI-1227, ACOI-1027, SAG-1224/13b, and ACOI-1413). (ii) Cells ribbon-like, significantly flattened (strains ACOI-1414 and ACOI-1032). In each strain there was some consistent diversity of the size of the cell's frontal part and relatively large diversity of the tail. The cell shape perturbations took place in aging colonies and were related to the accumulation of paramylon grains, subsequently leading to cell deformation (widening and shortening).

Periplast ornamentation: In the case of only two strains (ACOI-1027 and ACOI-1227) there were never papillae present, only pellicle strips. In the remaining strains (SAG-1224/13b, ACOI-1413, ACOI-1414, and ACOI-1032) the cells were covered with rows of papillae of different sizes, besides pellicle strips. The numbers of pellicle strips and rows made of small and large papillae as well as the number and their relative positioning were highly variable within

TABLE 3. Comparison of cell morphology of *Lepocinclis fusca* and *L. spirogyroides* (cell length includes tail).

Strain	Cell shape	Papillae shape	Cell length (μm)		Cell width (μm)		Tail (μm)	
			Mean ± SD	Min.–Max.	Mean ± SD	Min.–Max.	Mean ± SD	Min.–Max.
L. spirogyroides ACOI-1027	Cylindrical	Cuboid	112.31 ± 10.19	89–137	9.69 ± 1.58	6–10	8.42 ± 2.43	6.3–14.6
L. spirogyroides ACOI-1227	Cylindrical	Cuboid	116.72 ± 7.42	98–137	9.85 ± 1.22	6–10	11.52 ± 1.90	7.3–15.6
L. spirogyroides ACOI-1413	Cylindrical	Cuboid	100.05 ± 7.15	80–114	9.74 ± 1.32	6–11	10.91 ± 1.84	4.8–15.2
L. spirogyroides SAG-1224/13b	Cylindrical	Cuboid	115.88 ± 6.57	97–130	11.06 ± 0.99	7–11	12.04 ± 1.67	7.9–17.3
L. fusca ACOI-1032	Ribbon-like	Triangles or truncated triangles	189.39 ± 12.61	160–223	20.68 ± 3.07	11–19	18.20 ± 2.04	15.0–24.6
L. fusca ACOI-1414	Ribbon-like	Triangles or truncated triangles	177.95 ± 17.85	144–241	24.17 ± 3.38	12–24	15.59 ± 3.22	7.7–22.8

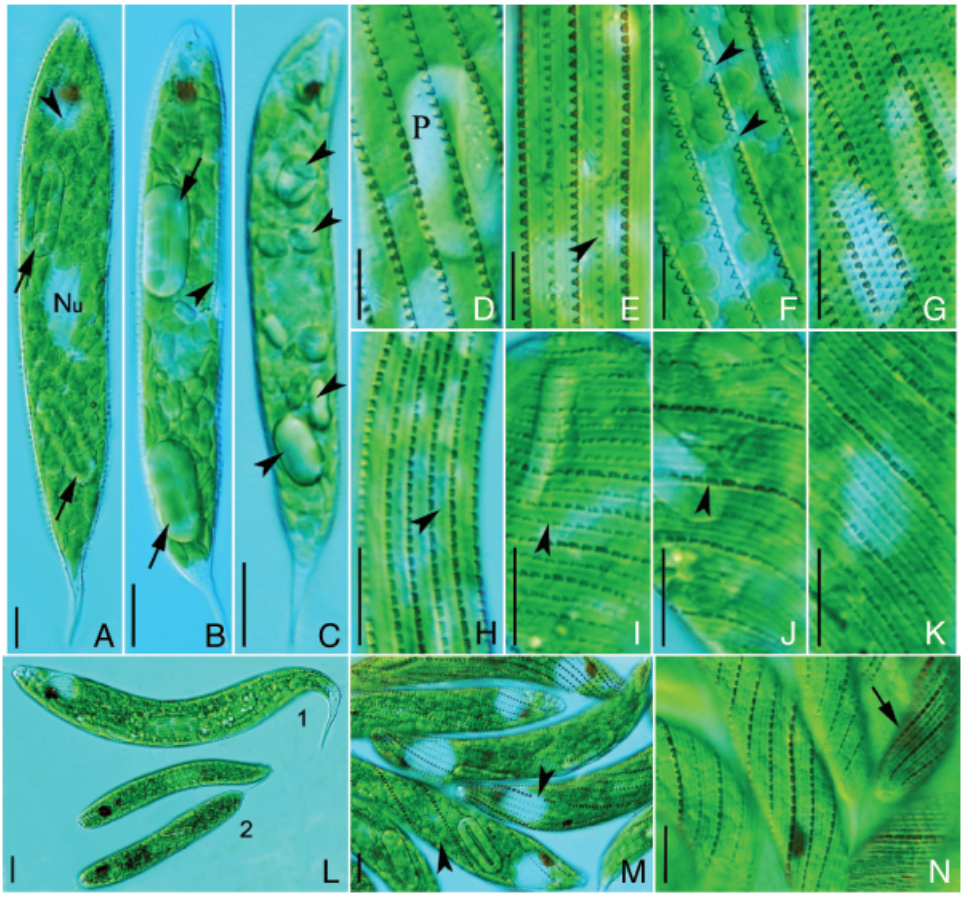

FIG. 1. Light microscope photographs showing an overview of living cells and periplast ornamentation of *Lepocinclis fusca* and *Lepocinclis spirogyroides*. (A) An overview of a ribbon-like cell of *L. fusca* ACOI-1414, ending with a sharp tail and covered with papillae rows (arrowhead); the nucleus is visible in the cell center with two large, ring-shaped, paramylon grains (arrows), one behind and the other in front of the nucleus. (B) An overview of a cylindrical cell of *L. spirogyroides* SAG-1224/13b; periplast ornamentation (arrowhead) and two large, ring-shaped, paramylon grains (arrows), one behind and the other in front of the nucleus, are visible. (C) Several paramylon grains of different shapes and distinctive sizes in the cell of *L. spirogyroides* ACOI-1413 (arrowheads). (D–G) Periplast ornamentation in *L. fusca* ACOI-1414. (D) Rows of papillae of equal size with the paramylon grain in the background. (E) Rows of large papillae alternating with rows of smaller papillae (arrowhead). (F) Widely spaced papillae rows with individual missing papillae visible in rows (arrowheads). (G) Periplast densely covered with rows of papillae of various size; from the side they appear as triangles or truncated triangles. (H–K) Periplast ornamentation in *L. spirogyroides* SAG-1224/13b. (H) Rows of cuboid papillae (appearing from the side as rectangles) alternating with single pellicle strips (arrowhead). (I) The periplast covered with numerous alternating rows of large and small papillae (arrowhead). (J) The periplast ornamented with a small number of rows of large cuboid papillae (arrowhead) alternating with numerous pellicle strips. (K) The periplast covered with papillae rows of different sizes. (L) The size of *L. fusca* ACOI-1032 cells (1) relative to that of *L. spirogyroides* SAG-1224/13b (2). (M) Differing periplast ornamentation in *L. fusca* ACOI-1032; some cells having densely packed papillae rows resulting in the darker color of the cells and some cells becoming light in color by steadily losing single papillae or whole rows of papillae (arrowheads). (N) Cells of *L. spirogyroides* SAG-1224/13b with differing periplast ornamentation; the darkest cell is densely covered with rows of cuboid papillae (arrow). Nu, nucleus; P, paramylon grain. Scale bars, 10 μm.

all populations (Fig. 1, D–K, M, and N). This variability was dependent on the stage of ontogeny and the age of the population. Thus, in young cultures the rows of papillae were complete, and cells covered with numerous alternating rows of small and large papillae significantly outnumbered those with papillae rows of similar size (Fig. 1, D, E, and H). As the population aged, there was a decrease of papillae size in some cells, whereas others were loosing papillae steadily, first one by one and then by rows (Fig. 1, F and M). Consequently, the number of cells with diversified and complex ornamentation, as well as those completely devoid of papillae, grew within populations. The loss of papillae also resulted in a change of color (see below and Fig. 1, M and N).

The shape of the papillae proved to be independent of the stage of ontogeny and environmental conditions. In strains SAG-1224/13b and ACOI-1413, the papillae had the shape of cuboids (they looked like rectangles when viewed from the side or from above) (Fig. 1, B, H–K, and N), whereas strains ACOI-1414 and ACOI-1032 had the shape of pyramids or truncated pyramids and looked like triangles or truncated triangles when viewed from the side (Fig. 1, D–G and M).

Periplast color: In two strains (ACOI-1227 and ACOI-1027) the cells were green, whereas the peri-

plast had no ornamentation (papillae) and was always colorless, independent of the population age. In the remaining strains (SAG-1224/13b, ACOI-1413, ACOI-1414, and ACOI-1032) both green and brown cells as well as cells intermediate in color were observed. The color intensity of the cells was dependent on the presence and the size of papillae. Cells covered densely with rows of large papillae were dark brown and became steadily lighter in color in the wake of the loss of papillae or decrease in size (Fig. 1, M and N). Cells completely devoid of papillae were intensely green.

Paramylon grain morphology: In the intensely dividing cells (particularly young cells growing on fresh media) there were relatively few and small paramylon grains scattered throughout the cytoplasm and two large grains in the shape of rings, one of which was located in front of the nucleus and the second behind (Fig. 1, A and B). As the populations aged, the number of small grains increased in all cultures; the large grains decreased their size and changed their shape to rod-like or ovoid (Fig. 1C). Some cells happen to have one or three large paramylon grains, and sometimes the rings become shorter and rounded up to the point that the central opening was no longer visible.

Flagellum length: The cells of strains ACOI-1227, ACOI-1027, SAG-1224/13b, and ACOI-1413 usually had a flagellum that was half the cell length, whereas in those of strains ACOI-1032 and ACOI-1414 the flagellum was significantly shorted, reaching at most one-tenth of the cell's length. The age of the cell culture had an impact on this feature. Numerous cells with very short flagella were observed in all the young and intensely dividing populations.

Phylogenetic analysis. After the removal of sites of an uncertain homology, which could not be unambiguously aligned, 1619 positions were left in the 18S rDNA alignment of 33 sequences (1075 of which were constant and 426 MP informative). The χ^2 tests showed homogenous nucleotide distributions ($P = 1.00$), permitting reliable phylogenetic analyses.

The likelihood ratio test of the Modeltest 3.6 program (Posada and Crandall 1998) suggested a symmetrical SYM + I + G model (Zharkikh 1994) with a fraction of unchangeable nucleotides (I) and a gamma (G) distribution of nucleotide substitution rates, whereas the Akaike test chose a general time reversible GTR + I + G model (Lanave et al. 1984, Tavare 1986, Rodriguez et al. 1990) for phylogenetic analyses. These models were applied to calculate the NJ, ML, and BA trees, with parameters drawn from Modeltest results (NJ, ML, BA) or estimated by the phylogeny inferring program (BA) (Table 4) and produced virtually identical trees with respect to the *Lepocinclis* branch. Figure 2 shows the tree obtained by the MrBayes program using the GTR + I + G model for the "Discoglena" branch of Euglenophyta, containing the genera of *Phacus*, *Lepocinclis*, and the former *Euglena* subgenus *Discoglena* sensu Zakryś (1986). Other methods (NJ, MP, and ML) produced congruent trees with respect to the nodes that had substantial statistical support (bs, bootstrap; di, decay index; or pp, posterior probabilities).

On the tree drawn with *E. spathirhyncha* and *E. limnophila* as an outgroup, the rest of the species were divided between two well-defined sister groups, *Phacus* (pp = 0.98, bs = 51–73, di = 1) and *Lepocinclis* (pp = 1.00, bs = 85–94, di = 6). The *Lepocinclis* clade was unresolved and consisted of six groups: 1) *Lepocinclis oxyuris* and *Lepocinclis fusca*, ACOI-1032 and ACOI-1414 (pp = 1.00, bs = 100, di = 57); 2) *L. spirogyroides* ACOI-1227, ACOI-1027, SAG-1224/13b, ACOI-1413 ASW-08026, ASW-08002, UTEX-1307, and SAG-1224/10b marked in the collection as *L. oxyuris* (pp = 1.00, bs = 99–100, di = 22); 3) former *Lepocinclis ovum* SAG 1244-8; 4) three remaining strains of the former *Lepocinclis* (pp = 100, bs = 99–100, di = 17); 5) *Lepocinclis acus* (pp = 1.00, bs = 100, di = 17); and 6) *L. tripteris* (pp = 100, bs = 100, di = 43). On the ML and BA trees, the clades *L. spirogyroides* and the former *Lepocinclis* showed up as sister groups (although without statistical significance), whereas there is no apparent affiliation between the clades *L. spirogyroides* and *L. fusca* (= *Euglena fusca*), strongly suggesting they should be considered a separate species. Additionally, the *L. oxyuris* strain SAG-1224/10b appeared deeply within the *L. spirogyroides* clade, suggesting that it has been incorrectly identified, whereas the Korean strain of *L. oxyuris* (Acc. AF090869) appears to be closely related to *L. fusca* (= *Euglena fusca*). Our observations of the SAG-1224/10b strain's cells under the light microscope did not reveal a longitudinal groove, characteristic of *E. oxyuris*, and it is therefore a strain of *L. spirogyroides*, whose cells do not form papillae, as in the case of strains ACOI-1027 and ACOI-1227. Because of its similarity in morphology and rDNA sequence, we renamed the strain SAG-1224/10b of *E. oxyuris* as *L. spirogyroides*.

We looked for molecular features distinguishing *L. spirogyroides* from *L. fusca* (= *Euglena fusca*) and found several synapomorphic changes among sequences subjected to analysis. When a wider range of available small subunit rDNA sequences of Euglenophyceae were considered, no nonhomoplasious synapomorphies (Marin et al. 2003) were found for *L. spirogyroides* clade and one nonhomoplasious synapomorphy (helix 24 forward: insertion "C" after 10 nt) were found for *L. fusca* + Korean strain of *L. oxyuris*.

Taxonomic revision. The topology obtained for phylogenetic trees without apparent affiliation of *E. fusca* to *L. spirogyroides* (both firmly enclosed within the *Lepocinclis* clade, additionally containing the well-defined clades of *L. acus*, *L. tripteris*, *L. ovum*) as well as the results of our microscope observations call for the transfer of *E. fusca* to the genus *Lepocinclis* and the delineation of two taxa of the rank of species: *Lepocinclis spirogyroides* and *Lepocinclis fusca* (= *E. fusca*). In the wake of the absence of unequivocal criteria in the original description of the two species considered, we

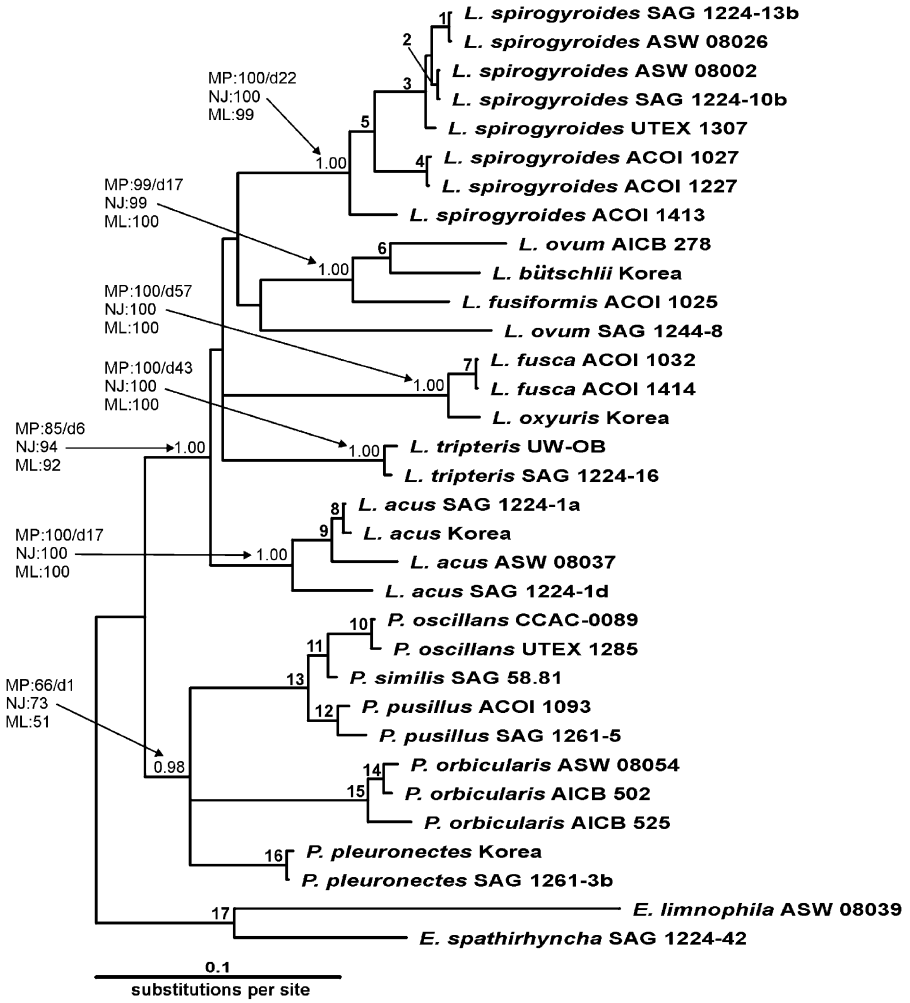

FIG. 2. The phylogenetic tree of the 18S rDNA sequence obtained by Bayesian inference (model GTR + G + I). Numbers at the essential nodes show posterior probabilities of the tree bipartitions as well as the bootstrap values/decay indices obtained for the main clades by MP analysis and bootstrap values obtained by NJ and ML analysis (model GTR + I + G). The support for the remaining nodes (numbered) is listed in Table 5. Branches leading to nodes with support of less than 50% are collapsed, and those without substantial support (pp < 0.95, bs < 0.75) are not listed.

define the epitypes for *L. spirogyroides* and *Lepocinclis fusca*.

Lepocinclis spirogyroides (Ehr.) Marin et Melkonian 2003, Protist 154, p. 104

Emended diagnosis: Cells 80–137 μm long, 6–11 μm wide, cylindrical, slightly flattened when swimming, ending with a tail. Periplast striped and often additionally covered with rows of papillae in the shape of cuboids, with varying numbers of papillae rows and papillae size.

Basionym: Euglena spirogyra Ehrenberg 1830, Physik. Abh. Akad. Wiss. Berlin 1830 (1832), p. 83, Pl. 6, Figure 4.

Lectotype: Figure 4 in Beiträge zur Kenntnis der Organisation der Infusorien und ihrer geographischen Verbreitung, brsonders in Sibirien; plate 6, 1830 (1832).

Epitype: Lepocinclis spirogyroides strain SAG-1224/13b, permanently preserved material (cells in resin, for electron microscopy) deposited at the Herbarium of the Department of Plant Systematic and Geography at Warsaw University, PL-00-478 Warsaw, Poland. Figure 1B is an illustration of the epitype.

Synonyms: Euglena spirogyra var. *laticlavius* Hübner 1886, Progr. Realg. Stralsund, p. 10, Fig. 12b; *Euglena spirogyra* var. *abrupte–acuminata* Lemm. 1913,

TABLE 4. Parameters estimated by the Modeltest (Posada and Crandall 1998) and MrBayes (Huelsenbeck and Ronquist 2001) programs for chosen models of sequence evolution.

Method	Model	A	C	G	T	I	G(α)	A-C	A-G	A-T	C-G	C-T	G-T
MrBayes	GTR + I + G	0.2206	0.2555	0.2954	0.2286	0.4453	0.6933	1.2273	3.0993	1.0790	0.2984	4.5896	1.0000
	SYM + I + G	0.2500	0.2500	0.2500	0.2500	0.4428	0.6720	1.0609	2.9575	0.8209	0.3500	4.1301	1.0000
Modeltest	GTR + I + G	0.2205	0.2558	0.2963	0.2274	0.4715	0.7158	1.2151	3.1172	1.0915	0.2985	4.5236	1.0000
	SYM + I + G	0.2500	0.2500	0.2500	0.2500	0.4675	0.6856	1.0455	2.9593	0.8190	0.3457	4.0296	1.0000

A, C, G, and T, frequency of nucleotides; I, fraction of unchangeable nucleotides; G(α), shape parameter (α) of gamma (G) distribution of nucleotide substitution rates; A-C, A-G, A-T, C-G, C-T, and G-T, rates of reversible nucleotide substitutions.

TABLE 5. Node support for the tree in Figure 2.

Node number	BA (pp)	MP (bs/di)	NJ (bs)	ML (bs)
1	1.00	99/d7	100	100
2	0.99	78/d1	76	79
3	1.00	100/d19	100	100
4	1.00	100/d16	100	100
5	0.97	88/d4	85	68
6	1.00	79/d4	90	87
7	1.00	99/d12	100	99
8	0.98	99/d5	99	87
9	1.00	98/d9	97	100
10	1.00	100/d16	100	100
11	1.00	81/d2	99	98
12	1.00	99/d9	92	98
13	1.00	100/d26	100	100
14	0.99	98/d6	89	86
15	1.00	100/d46	100	100
16	1.00	100/d27	100	100
17	1.00	100/d33	100	100

Arch. Hydrob. 8, p. 574; *Euglena spirogyra* var. *elegans* Playfair 1921, Proc. Linn. Soc. N. S. Wales, 40, p. 120, Pl. 4, Fig. 4; *Euglena spirogyra* var. *fusiformis* Deflandre 1924, Bull. Soc. Bot. France, 71, p. 118, Fig. 8; *Euglena spirogyra* var. *suprema* Skuja 1932, Act. H. Bot. Univ. Latv., 7, p. 34, Fig. 13; *Euglena spirogyra* var. *torta* Prijmačenko 1963, Ukr. Bot. Journal, 20, 2, p. 74, Fig. 3; *Euglena spirogyra* var. *compressa* Shi 1996, Acta Phytot. Sinica 34, p. 115, Figs. 1–5; *Euglena pseudospirogyra* Shi *in* Shi et al. 1994, Com. rep. surveys algal resources South-West. China, p. 148, Pl. 2, Figs. 4, 5.

Lepocinclis fusca (Klebs) Kosmala et Zakryś, comb. nov.

Emended diagnosis: Cells 144–241 μm long, 11–24 μm wide, notably flattened, ribbon-like, ending with a sharp tail. Periplast striped, in most cases additionally covered with rows of papillae shaped like a pyramid or a truncated pyramid (never as a regular cuboid), with varying numbers of papillae rows and papillae sizes.

Basionym. *Euglena spirogyra* var. *fusca* Klebs 1883, Unters. Bot. Inst. Tübing. 1, 2, p. 307, Pl. 3, Fig. 13, 1883.

Lectotype: Figure 13 in Über die Organisation einiger Flagellatengruppen und ihre Beziehungen zu Algen und Infusorien, Plate 3, 1883.

Epitype: Lepocinclis fusca strain ACOI 1414, permanently preserved material (cells in resin, for electron microscopy) deposited at the Herbarium of the Department of Plant Systematic and Geography at Warsaw University, PL-00-478 Warsaw, Poland. Figure 1A is an illustration of the epitype.

Synonyms: Euglena fusca (Klebs) Lemmermann 1910, Krypt. M. Brand., p. 498; *Euglena fusca* var. *marchica* Lemmermann 1913, in Pascher's Süssw., FI., 2, 1913, p. 131.

Commentary for taxonomic revision. We did not consider the varieties *E. spirogyra* var. *minor* Allorge and Lefèvre (1925) and *E. fusca* var. *minima* Szabados (1949), distinguished on the basis of the cell size (45–50 × 7–9 μm and 31–53 × 5–7 μm, respectively), because we did not observe such small cells in the populations studied. However, it may be assumed that *E. fusca* var. *minima* is *E. spirogyra* var. *minor* that was described earlier because of its periplast ornamentation pattern and the fact that the small size of the cells affiliates it with *E. spirogyra* Ehrenberg rather then with *E. fusca* (Klebs) Lemmermann.

DISCUSSION

Describing the new species *Euglena spirogyra*, Ehrenberg (1832) defined its cell shape as cylindrical, with the possibility of forming ribbon-like structures ("Es ist cylindrisch, kann sich aber bandförmig machen"). He had seen papillae as small granulations forming helical rows within the cell ("Im Innern sind spiralförmig gewundene Reihen kleiner Körnchen") and had shown them on his drawings as rectangular structures with rounded corners. The color of the cells is intensely green in his drawings (Ehrenberg 1832, Pl. 6, fig. 4; 1838, Pl. 7, fig. 10 and # 557 available on the website of Institut für Paläontologie, Museum für Naturkunde, Humboldt Universität zu Berlin, Ehrenberg Collection http://download.naturkundemuseum-berlin.de/Ehrenberg).

In 1883 Klebs distinguished the variety β, giving it the name *fusca*, which differed from the typical form α (*E. spirogyra*) by having a larger flagellum (about as long as the body), larger ribbon-like cells (form α: 91 × 8 μm; form β: 170 × 23 μm), a brown to almost black color, and substantially larger papillae ("Membran dunkelbraun bis fast schwarz; die Höcker sehr groβ. Körper breit bandförmig. Cilie von der Länge des Körpers. Durchschnittlich gröβer als α").

In 1910, Lemmermann elevated this variety to the rank of a species and between 1910 and 1913 specified and amended the criteria for distinguishing the two species: *E. spirogyra* had a size of 80–125 × 8–15 μm and a flagellum as long as 1/4 of the cell body, whereas *E. fusca* had a size of 225 × 25–27.5 μm and a flagellum as long as the body. He had applied these criteria and the size of papillae in 1913, when he published the guide for determining the taxa known at that time (*E. fusca*, *E. spirogyra*, *E. spirogyra* var. *laticlavius*, *E. spirogyra* var. *abrupte-acuminata*).

The description of *E. fusca*, created by Lemmermann, was later amended by other authors with respect to cell size and the larger diversification of the shape of the papillae of *E. fusca* relative to *E. spirogyra*. Most authors agreed on a distinctive difference in the cell lengths of the two, in favor of *E. fusca* (Dreżepolski 1925, Skuja 1932, Lefèvre 1934, Czosnowski 1948, Gojdics 1953, Popova 1966). Our findings concur. The cells of *E. spirogyra* (80–137 × 6–11 μm; Fig. 1L) are smaller than those of *E. fusca* (144–241 × 11–23 μm; Fig. 1L), and their size is consistent with the sizes reported earlier (80–130 × 8–25 μm; Lemmermann 1913, Dreżepolski 1925, Lefèvre 1934, Pringsheim 1956, Popova 1966). Likewise, the cell lengths we

observed for *E. fusca* are similar, although slightly larger than those reported previously (122–226 × 13–36 μm; Dreżepolski 1925, Skuja 1932, Lefèvre 1934, Czosnowski 1948, Gojdics 1953, Popova 1966). These small differences in size may be the consequence of the new methods used, allowing for measurements of the cells that are not entirely straight.

Regarding the shape of the cells, our observations are also consistent with those of Ehrenberg, Klebs, and other authors. *Euglena spirogyra* has cylindrical cells, which show a tendency to become slightly flattened while swimming, whereas those of *E. fusca* are always ribbon-like and very flat (Ehrenberg 1832, 1838, Klebs 1883, Dreżepolski 1925, Skuja 1932, Lefèvre 1934, Czosnowski 1948, Gojdics 1953, Popova 1966). However, the shape of the cell is somewhat variable, depending on population age. In aging cultures and/or deteriorating environmental conditions there is an accumulation of small paramylon grains, and the cells become deformed—shortening and widening take place. Such deformation was also noticed earlier by Pringsheim (1955), Perty (1852), and Stein (1878).

Differences in periplast ornamentation were the cause for describing six varieties (*E. spirogyra* var. *laticlavius*, *E. spirogyra* var. *abrupte–acuminata*, *E. spirogyra* var. *elegans*, *E. spirogyra* var. *suprema*, *E. spirogyra* var. *fusiformis*, *E. fusca* var. *marchica*). On the basis of our findings, we conclude that this feature is rather capricious, depending on growth conditions and the stage of ontogeny. In all strains in which we were able to observe papillae, there were cells with papillae forming variable patterns. We observed cells with equal papillae sizes in all rows as well as cells having alternating rows of small and large papillae. The latter pattern was found only in young and not overcrowded populations. We therefore suggest that this alternating pattern is associated with the process of periplast expansion, caused in turn by the widening of the cell after division. In aging populations, the number of cells with equivalent rows of papillae, as well as cells that partially or completely lacked papillae, grew. In 3- to 4-month-old cultures, the papillae were missing in practically all cells. Their absence was therefore an effect of the deteriorating environmental conditions. Klebs (1883) was first to describe the loss and rebuilding of papillae in the cells of the two forms (*spirogyra* and *fusca*). He observed the cells with and without papillae as well as those in which "there [were] numerous places without papillae." Similar observations of strains in growing cultures were made by Chu (1946), Pringsheim (1956), and Leedale et al. (1965a). Therefore, we believe that the papillae pattern cannot be considered a diagnostic feature, as it is a characteristic changing with the stage of ontogeny and growth conditions.

On the other hand, we observed that in all strains in which there were papillae present, their shape remained unchanged, independent of population age or growth conditions. In the strain identified as *E. spirogyra*, the papillae were always cuboid in shape (and appeared as rectangles from side and as squares from above, with their edges slightly rounded). They were similarly depicted in drawings made by Ehrenberg (1832, 1838). However, in *E. fusca*, the papillae had the shape of pyramids or truncated pyramids (from the side they appear as triangles or as truncated triangles). Klebs (1883) described them as "short, sharp bars," whereas Lefévre (1934) described them as "more complicated" than in *E. spirogyra*, that is, "they are headed by a little crown, formed by small fibers radiating fan-wise." Johnson (1944), who did not distinguish the variety *fusca*, ascribed as many as four shapes ("truncated pyramidal, pyramidal, truncated conical or conical processes") to the varieties of *E. spirogyra*. It therefore seems that differences in papillae shape are a good diagnostic feature.

We found that a helical (slanted) arrangement of the rows of papillae is the result of cell movement. Similar observations were made by Gojdics (1953), who reported that "In the broad extended organism, the striae are nearly parallel with the long axis of the body, but in twisting they assume varying degrees of slanting."

The length of the flagellum (in proportion to the body of the cell) was the first diagnostic feature in the Lemmermann (1913) guide, discriminating between *E. spirogyra* (short flagellum, one-fourth to one-half of the body length) and *E. fusca* (long flagellum equaling the body length). The observations of the representatives of *E. spirogyra* made herein are not entirely consistent with the literature. In strains ACOI-1227, ACOI-1027, ACOI-1413, and SAG-1224/13b, the flagellum was about one-half of the cell body, which fits within the limits given by Lemmermann (1913). It is longer than it should be according to Lefévre (1934) or Gojdics (1953) but slightly shorter than it should be according to Pringsheim (1955). The length of the flagellum (one-tenth of the body) in strains ACOI-1032 and ACOI-1414 (identified here as *E. fusca*) is even less concurrent with the literature. According to Klebs (1883), Lemmermann (1913), and Lefévre (1934), this species' flagellum should be almost as long as the body, whereas according to Gojdics (1953) some individuals may not posses a flagellum at all, because it is very fragile and may detach, to regrow later. As a consequence, there are always a certain number of cells within each population that have a very short flagellum. Therefore, we do not believe that flagellum length is a good diagnostic feature in this case and agree with Gojdics (1953) and Pringsheim (1955), who were of the opinion that Klebs (1883), Lemmermann (1913), and Lefévre (1934) wrongfully deemed flagellar length as discriminating *E. spirogyra* and *E. fusca*.

According to Klebs (1883), the dark brown color of cells was one of several features by which *E. spirogyra* differed from the *fusca* form. A similar position was taken by Lefévre (1934), Gojdics (1953), and Popova (1966). Our findings indicate that this is correct, but only with respect to the young populations. Indeed, in fresh cultures and favorable growing conditions, most *E. fusca* cells were dark brown. However, the color of

the cell was dependent on its periplast ornamentation, that is, the presence of papillae, their size and the number of rows, with the cells steadily darkening concomitantly with the increase in the number of papillae and the number of rows. Thus, the strains ACOI-1027 and ACOI-1227, whose cells were devoid of ornamentation, were always green. In all other strains, the number of brown and dark cells decreased as the population aged, because the cells were losing papillae and were becoming increasingly light in color.

This is consistent with previous observations made by Chu (1946), Pringsheim (1956), Leedale et al. (1965a,b), Zakryś (1986), and many others, pointing to the fact that the color of the cells is dependent on the presence of papillae. However, no one has tried to explain the nature of this dependence. Only Lefévre (1932) hypothesized that their dark color may be caused by the excretion of papillae, whereas Skuja (1932) connected it to the color of the papillae themselves. In our view, the papillae are colorless and the effect exists due to light diffraction, because the cells appear brown under smaller magnifications, but under larger magnifications the effect disappears and the cells appear green.

To summarize, in light of our findings and the literature cited, the only reliable diagnostic features for discriminating *Lepocinclis spirogyroides* (= *E. spirogyra*) and *Lepocinclis fusca* appear to be the shape and the size of the cells and the shape of the papillae. All other features, such as flagellum length, cell color, and periplast ornamentation, are changeable in populations and depend on growing conditions as well as on the stage of ontogeny and are therefore unreliable diagnostic features.

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