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TWO DIFFERENT SPECIES OF *EUGLENA*, *E. GENICULATA* AND *E. MYXOCYLINDRACEA* (EUGLENOPHYCEAE), ARE VIRTUALLY GENETICALLY AND MORPHOLOGICALLY IDENTICAL[1]

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We investigated the similarity of a single *Euglena myxocylindracea* strain, isolated originally by Bold and MacEntee, to several *Euglena geniculata* strains on both morphological and DNA levels. We found the three DNA stretches, consisting of fragments coding for the parts of cytoplasmic and chloroplast small subunit rRNA, and the internal transcribed spacer (ITS2) of cytoplasmic rDNA, with the combined length of 4332 nucleotides, are identical in *E. myxocylindracea* and *E. geniculata*, strain SAG 1224-4b. Morphological differences between *E. myxocylindracea* and any *E. geniculata* strain examined were well within the range of *E. geniculata* variability as well. The only difference behind the distinction of *E. myxocylindracea* from *E. geniculata* is the presence of the second chloroplast in the latter. However, we were able to induce the appearance of the second chloroplast in the cells of *E. myxocylindracea* and its disappearance in the cells of *E. geniculata* by changing the composition of the culture media. We therefore conclude that *E. myxocylindracea* Bold and MacEntee should be regarded as an environmental form of *E. geniculata* Dujardin. For the first time the morphology of *E. geniculata* chloroplasts was shown as revealed by confocal laser microscopy.

Key index words: **16S rDNA; 18S rDNA; chloroplasts; *Euglena geniculata*; *Euglena myxocylindracea*; ITS2; molecular phylogeny; taxonomy**

Abbreviations: **ITS, internal transcribed spacer; SSU, small subunit**

After more than 150 years of floristic and systematic studies on euglenoid species, several systems of classification and over 1000 described taxa (species, varieties, and forms) have emerged. Recently, molecular data were introduced into the taxonomical debate, proving obsolete some classification systems based on morphological characters. The problem seems to be particularly acute in the case of euglenoid species, which are single celled and morphologically relatively simple organisms. In particular, many species of *Phacus, Lepocinclis*, and *Euglena* are more closely related to species from other genera rather than to those of the same genus (Leander and Farmer 2000, 2001, Linton et al. 2000, Milanowski et al. 2001, Müllner et al. 2001).

In this work we report on apparent genetic identity of two euglenoid species differentiated by a morphological criterion, namely the number of chloroplasts. *Euglena myxocylindracea* Bold and MacEntee (1973), with its one chloroplast, is distinguished from *E. geniculata* Dujardin (1841), which has two, based on a prevailing practice in *Euglena* systematics that attributes great weight to chloroplast number and morphology (Gojdics 1953, Huber-Pestalozzi 1955, Pringsheim 1956, Popova 1966, Popova and Safonova 1976, Zakryś 1986, Zakryś and Walne 1994, Kim et al. 1998, Shi et al. 1999).

Chloroplast number has been considered a primary criterion of classification, particularly in the subgenus *Euglena* (sensu Zakryś 1986), where axially located star-shaped chloroplasts are only few, numbering one to three, whereas other features, such as the size and shape of the cell and the presence, size, and shape of the mucocyst (bodies located close to periplast), were considered secondary. Based on these criteria, three groups were distinguished in the subgenus *Euglena*: 1) those with a single chloroplast located in front of the nucleus (*E. archaeoviridis* Zakryś and Walne, *E. cantabrica* Pringsheim, *E. cuneata* Pringsheim, *E. myxocylindracea* Bold and MacEntee, *E. stellata* Mainx, and *E. viridis* Ehrenberg), the so-called viridis group; 2) those

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with two chloroplasts on two opposite sides of the nucleus (*E. chadefaudii* Bourrelly, *E. dicentra* Skuja, *E. geniculata* Dujardin, and *E. schmitzii* (Schmitz) Gojdics), termed the geniculata group; and 3) those with three chloroplasts (*E. tristella* Chu).

This system of classification allowed easy distinction between the three groups mentioned above, whereas identification of particular species was difficult within the first two groups, because those species were discriminated on the basis of the secondary features (size and shape of the cell, as well as the mucocyst morphology and arrangement). By the above primary criterion, *E. myxocylindracea* (having only one chloroplast) is closely affiliated with the other species possessing one chloroplast. On the other hand, the only trait distinguishing *E. myxocylindracea* from *E. geniculata* is the number of chloroplasts. However, three different DNA stretches in *E. myxocylindracea* analyzed in this study proved to be identical to their homologues from one of the *E. geniculata* strains (having two chloroplasts). Following this finding, we asked whether the number of chloroplasts—a strict taxonomic criterion in *Euglena*—could be environmentally dependent and whether the second chloroplast could be induced in an *E. myxocylindracea* strain and eliminated in an *E. geniculata* strain by changing their growth conditions.

MATERIALS AND METHODS

Euglenoid strains and culture conditions. The strains used in this study are shown in Table 1. All strains were cultivated in 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 16:8-h light:dark, with approximately 27 μmol photons$\cdot m^{-2} \cdot s^{-1}$ provided by cool-white fluorescent tubes. Additionally, in experiments aimed at inducing changes in a cell chloroplast number, *E. geniculata* strain 4b, 4f, and *E. myxocylindracea* were cultivated in media, composed of mineral medium I and organic medium II, mixed in different proportions. Four variants, A, B, C, and D, containing, respectively, 95, 90, 75, and 50% of medium I were used. Medium I (pH 4.6) consisted of 600 mg$\cdot L^{-1}$ KH_2PO_4, 160 mg$\cdot L^{-1}$ $Ca(NO_3)_2 \times 4H_2O$, 800 mg$\cdot L^{-1}$ $MgSO_2 \times 7H_2O$, 2 g$\cdot L^{-1}$ $(NH_4)_2SO_4$, 12 mg$\cdot L^{-1}$ $FeCl_3$, 4 mg$\cdot L^{-1}$ $ZnSO_4 \times 7H_2O$, 1.4 mg$\cdot L^{-1}$ $MnSO_4 \times 5H_2O$, 0.4 mg$\cdot L^{-1}$ $CuSO_4 \times H_2O$, 0.6 mg$\cdot L^{-1}$ H_3BO_3, 0.6 mg$\cdot L^{-1}$ vitamin B_1, 0.004 mg$\cdot L^{-1}$ vitamin B_{12}, 50 mL$\cdot L^{-1}$ soil extract, and 20 mL$\cdot L^{-1}$ sphagnum extract. Medium II consisted of 1 g$\cdot L^{-}$ $NaC_2H_3O_2$, 2 g$\cdot L^{-1}$ trypton, 2 g$\cdot L^{-1}$ yeast extract, and 0.1 g$\cdot L^{-1}$ $CaCl_2$. Cultures of *E. myxocylindracea* and *E. geniculata* from medium 3c were transferred to four variants of liquid media and kept in test tubes in a light chamber for 3 days. Then, every 2 days for the next 14 days the cultures were sampled for microscopic analysis.

Biometric studies. Biometric measurements were performed using the LUCIA Measurement program (Laboratory Imaging s.r.o., Praha, Czech Republic). One hundred randomly chosen actively swimming cells from each of the eight strains of *E. geniculata* and one strain of *E. myxocylindracea* were analyzed. For each strain, four cellular parameters were measured: length, width,

TABLE 1. Strains and accession numbers for cytoplasmic and chloroplast SSU rDNA sequences. Those obtained in this study are in boldface.

		GenBank accession no.		
Taxon	Strain	16S rDNA	18S rDNA	ITS
Colacium vesiculosum	UW Łazienki	AF289238		
Colacium vesiculosum	UTEX LB1315		AF081592	
Euglena agilis	UTEX 1605		AF115279	
Euglena agilis	UW Pruszkow-1	AF289239		
Euglena gracilis	unknown		M12677	
Euglena gracilis	unknown	X12890		
Euglena geniculata	SAG 1224-4b	AF289241	**AY070249**	**AY070243**
Euglena geniculata	SAG 1224-4c			**AY070244**
Euglena geniculata	SAG 1224-4f	**AY070252**	**AY070248**	**AY070237**
Euglena geniculata	SAG 1224-4g			**AY070238**
Euglena geniculata	ACOI 66			**AY070241**
Euglena geniculata	ACOI 197			**AY070239**
Euglena geniculata	ACOI 530			**AY070240**
Euglena geniculata	ACOI 994			**AY070242**
Euglena myxocylindracea	UTEX 1989	**AY070251**	**AY070250**	**AY070245**
Euglena spirogyra	SAG 1224-13b	AF289243		
Euglena spirogyra	UTEX 1307		AF150935	
Euglena stellata	SAG 1224-14	AF289244		
Euglena stellata	UTEX 372		AF150936	
Euglena tripteris	UW OB	AF289245		
Euglena tripteris	unknown		AF286210	
Euglena tristella	SAG 1224-35	AF289246	**AY070247**	
Euglena viridis	SAG 1224-17d	AF289248	**AY070246**	
Lepocinclis fusiformis	ACOI 1025	AF289249		
Lepocinclis ovum	SAG 1244-8		AF110419	
Phacus pleuronectes	SAG 1261-3b	AF289251		
Phacus pleuronectes	unknown		AF081591	
Eutreptia viridis	SAG 1226-1c	AF289247	AF157312	

ACOI, Culture Collection of Algae at the Department of Botany, University of Coimbra, Portugal; SAG, Sammlung von Algenkulturen Pflanzenphysiologisches Institut der Universität Göttingen, Germany; UTEX, Culture Collection of Algae at the University of Texas at Austin, Texas, USA; UW, Department of Plant Systematics and Geography of Warsaw University, Poland.

circularity, and the area of the projection of the cell on a flat surface. Because these features were not independent, one-dimensional variance analyses were performed for each of them. After rejecting the null hypothesis that there is no difference between subpopulations, the differences between subpopulations were estimated by the Tukey honest significant difference test.

Confocal microscopy. All observations were done on material preserved with a 10% solution of glutaraldehyde by adding one drop of a fixative and one drop of water to the fresh material placed on the slide. The cells were then viewed on a Zeiss LSM 510 (Zeiss, Jena, Germany) confocal laser scanning microscope at an excitation wavelength of 543 nm.

DNA Isolation, amplification, and sequencing. Isolation of DNA (using Dneasy Tissue Kit-QIAgen, QIAgen GmbH, Hilden, Germany), amplification, purification of PCR products, and sequencing of chloroplast small subunit (SSU) rDNA was performed as previously described (Milanowski et al. 2001). Primers for PCR amplification and sequencing are shown in Table 2. For 18S rDNA amplification, a 50-mL reaction mixture was used, containing 1 U of Taq polymerase (MBI Fermentas, Vilnius, Lithuania), 0.2 mM dNTPs, 2.5 mM $MgCl_2$, 10 pmol of each primer, reaction buffer (MBI Fermentas), and 10–50 ng DNA. The PCR protocol consisted of 5 min of denaturation at 95° C, followed by five initial cycles comprising 0.5 min at 95° C, 0.5 min at 56° C, and 1.5 min at 72° C, then by 30 cycles comprising 0.5 min at 95° C, 20 s at 58° C, and 1.5 min at 72° C. The final extension step was performed for 7 min at 72° C. PCR products were sized on agarose gels and purified using QIAEXII gel extraction kit (QIAgen). Purified PCR products were sequenced with external and internal primers (slightly modified from Elwood et al. 1985) by cycle sequencing using BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA). The internal transcribed spacer (ITS2) DNA was amplified in a 50-mL mixture containing 1 U of Taq polymerase (MBI Fermentas), 0.2 mM dNTPs, 10 mM $MgCl_2$, 5 pmol of each primer, reaction buffer (MBI Fermentas), and 10–50 ng of DNA. The PCR protocol consisted of 5 min of denaturation at 95° C, followed by 7 initial cycles comprising 0.5 min at 95° C, 2 min at 50° C, and 1.5 min at 72° C, then by 35 cycles comprising 0.5 min at 95° C, 0.5 min at 57–60° C and 1.5 min at 72° C. The final extension step was performed for 10 min at 72° C. PCR products were purified either by precipitation with 98% ethanol and 0.3 M potassium acetate or by electrophoresis and excision of a predominant band, followed by purification with QIAEXII gel extraction kit (QIAgen). PCR products were sequenced from both strands with ITS2 forward and reverse primers by cycle sequencing using BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). All readings (16S rDNA, 18S rDNA, ITS2) from ABI Prism 310 or 377 DNA sequenator (Applied Biosystems, Foster City, CA, USA), after removal of primer sequences, were assembled into "contigs" by the SeqMan program of the LASERGENE package (DnaStar, Madison, WI, USA) and checked manually for consistency.

Table 2. Nuclear rDNA primers used for PCR amplification and sequencing.

Primer	Position of 3′ end	Sequence (5′-3′)
18S5′	89	CAGTGGGTCTGTGAATGGCTCC
18S382F	483	AGGGTTCGATTCCGGAG
18S557R	668	TTACCGCAGCTGCTGGC
18S570F	682	GTGCCAGCAGCTGCGGT
18S892R	1293	CTAAGAATTTCACCTCTG
18S1125R	1539	CAATTCCTTTAAGTTTC
18S1141F	1555	CAAACTTAAAGGAATTG
18S1263R	1677	GAGCGGCCATGCACCAC
18S3′	2127	CGACGGGCGGTGTGTACAAGT
ITS2F	48 (5.8S rDNA)	GCATCGATGAAGAACGCAGC
ITS2R	38 (28S rDNA)	TTCCTCCACTGAGTGATATGC

18S rDNA primers for PCR are from the sequence of *E. gracilis* (Sogin et al. 1996). ITS2 forward primer corresponds to the first component of large subunit rRNA–5.8S rRNA (E. Linton, unpublished data). ITS2 reverse primer corresponds to the second component of LSU rRNA—the first of the 13 small RNAs that form an equivalent of 25–28S rRNA in *E. gracilis* (Schnare and Gray 1990, Schnare et al. 1990).

Sequence accession numbers, alignment, and phylogenetic analysis. The GenBank accession numbers for SSU rDNA sequences reported here and the sequences used for phylogenetic analyses are shown in Table 1. Alignment of sequences was obtained using the Clustal W 1.8 program (Thompson et al. 1994) with default options and was manually checked and edited according to the secondary structure of *Euglena gracilis* as suggested by Van de Peer et al. (1999). Several regions that could not be unambiguously aligned were omitted from analyses. All nucleotides were treated as independent and unordered multistate characters of equal weight. The alignments used for analyses are available online at *http://www.blackwellpublishing.com/products/journals/suppmat/JPY/JPY02020/JPY02020sm.htm*. The Hasegawa-Kishino-Yano model (Hasegawa et al. 1985), with parameter values estimated by maximum likelihood, was used for distances calculations. Distance analyses, as well as maximum likelihood parameter estimation, were performed by PAUP, version 4.0b6 for Microsoft Windows (Swofford 1998). The heuristic search option, with MULPARS, tree-bisection-reconnection branch swapping, ACCTRAN optimization, and random addition with 100 replicates, was used to find the best tree. Bootstrap support of specific nodes (Felsenstein 1985) was estimated with 1000 replications and default options, as implemented in PAUP. The sequence of *Eutreptia viridis*, member of *Eutreptiaceae*, was used to root the trees. Trees were drawn by Tree View, Version 1.6.1 for Microsoft Windows (Page 1996).

RESULTS

Sequence analysis. We obtained almost complete sequences of chloroplast and cytoplasmic SSU rDNA from several species of *Euglena* subgenus *Euglena*, including *E. myxocylindracea* and two *E. geniculata* strains, 4b and 4f. The *E. myxocylindracea* rDNAs appeared to be identical to the ones from *E. geniculata*, strain 4b, raising the possibility that the two species are actually two somewhat different morphological forms of the same species.

Figures 1 and 2 show the minimum evolution trees with maximum likelihood distances obtained for SSU rDNA from chloroplast and cytoplasmic ribosomes, respectively, with the substitution model of Hasegawa-Kishino-Yano (1985). The tree shown in Figure 1 was obtained with values estimated for the following parameters: base frequencies (A = 0.2899, C = 0.1837, G = 0.2354, T = 0.2910), proportion of invariable sites (0.2341), gamma shape parameter (0.4442), and transition/transversion ratio = 2.6182. The values estimated for the tree from Figure 2 were as follws: base frequencies (A = 0.2325, C = 0.2614, G = 0.2482, T = 0.2578), proportion of invariable sites (0.2637), gamma shape parameter (0.5269), and transition/transversion ratio = 1.8169. The trees are similar with respect to the branching order relevant for this study. They both reveal several known facts about Euglenoid phylogeny observed earlier (Leander and Farmer 2000, 2001, Linton et al. 2000, Milanowski et al. 2001, Müllner et al. 2001). First, some relationships are poorly resolved. Second, several genera, including *Phacus*, *Lepocinclis*, and *Colacium*, are intermixed with *Euglena* species. Thus, the genus *Euglena*, represented here by more than one species, is not monophyletic. Third, a

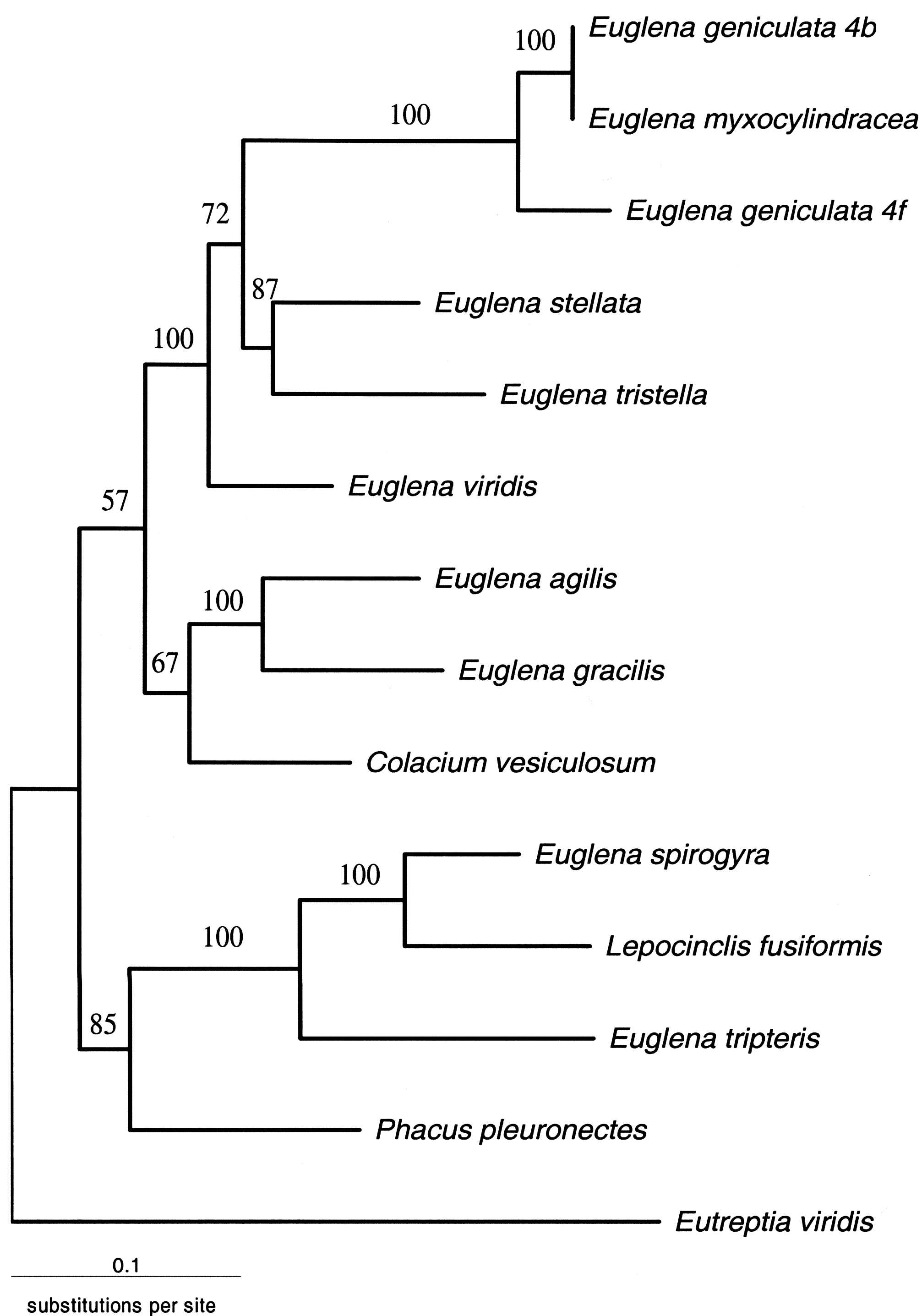

FIG. 1. The minimum evolution tree of selected euglenoid SSU rDNA sequences from chloroplast ribosomes based on maximum likelihood distances. Bootstrap values higher than 50% (1000 replications) are shown at the nodes. See text for parameter values of the Hasegawa-Kishino-Yano (1985) model of sequence evolution.

clade (100% bootstrap in Fig. 1 and 69% in Fig. 2) consisting of *Euglena* subgenus sensu Zakryś (1986) is present on the trees. Within this group there is a very well defined clade (bootstrap support of 100% on both trees) consisting of two *E. geniculata* strains and *E. myxocylindracea*. As expected, *E. myxocylindracea* and *E. geniculata* 4b, which have identical rDNA sequences for both compartments, form clades with a 100% bootstrap support on both trees.

To further assess the genetic similarity of the two species, we sequenced the fragment encompassing the part of 5.8 S rRNA and ITS2 from the original strain

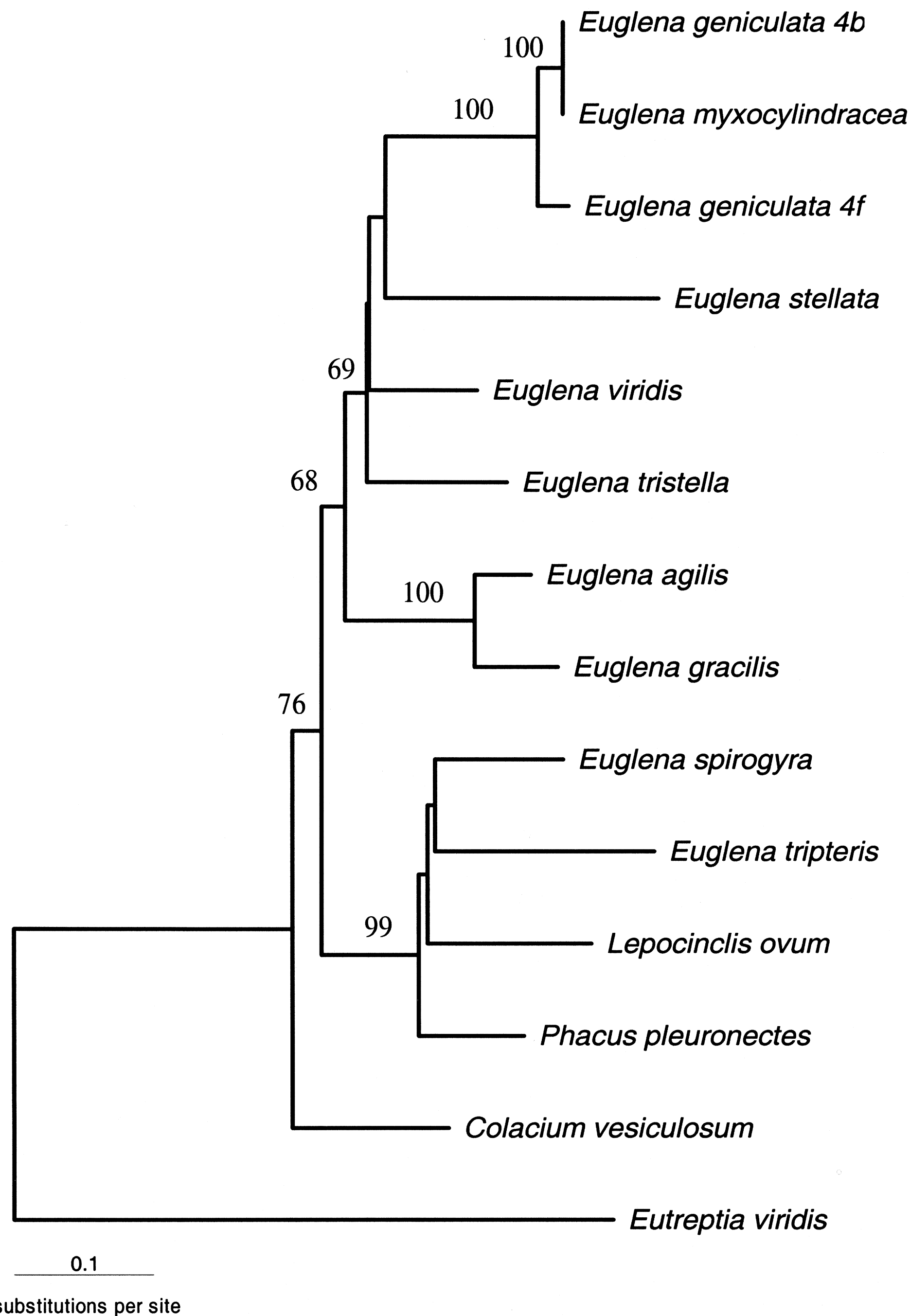

FIG. 2. The minimum evolution tree of selected euglenoid SSU rDNA sequences from cytoplasmic ribosomes based on maximum likelihood distances. Bootstrap values higher than 50% (1000 replications) are shown at the nodes. See text for parameter values of the Hasegawa-Kishino-Yano (1985) model of sequence evolution.

of *E. myxocylindracea* and several strains of *E. geniculata*. The obtained sequences form two groups consisting of 1) mutually very similar or identical sequences and 2) sequences substantially different from each other. The first group, represented in Figures 1 and 2 by the sequence *E. geniculata* 4f, also contains the sequences of *E. geniculata* strains: ACOI 66, 197, 530 and 994, and SAG 4c. The second group, represented in Figures 1 and 2 by the sequences of *E. geniculata* 4b and *E. myxocylindracea*, also contains the sequence of *E. geniculata* strain 4g. The lengths of the sequences are approximately 630 and 740 for the first and the second group, respectively. Unequivocal alignment of all these sequence fragments could only be done within the region corresponding to 5.8 S rRNA. The part corresponding to ITS2 could be aligned only

TABLE 3. Nucleotide differences per site between cytoplasmic fragments of circular DNA consisting of partial 5.8 sRNA and ITS2 regions of the *Euglena geniculata* and *Euglena myxocylindracea* strains in the alignment produced by Clustal W program under default parameters.

Strain	1	2	3	4	5	6	7	8
1 *E. geniculata* 66	—							
2 *E. geniculata* 530	0.000	—						
3 *E. geniculata* 994	0.000	0.000	—					
4 *E. geniculata* 4c	0.002	0.002	0.002	—				
5 *E. geniculata* 197	0.005	0.005	0.005	0.003	—			
6 *E. geniculata* 4f	0.005	0.005	0.005	0.003	0.000	—		
7 *E. geniculata* 4b	0.387	0.387	0.387	0.387	0.383	0.383	—	
8 *E. geniculata* 4g	0.387	0.387	0.387	0.387	0.383	0.383	0.000	—
9 *E. myxocylindracea*	0.387	0.387	0.387	0.387	0.383	0.383	0.000	0.000

within the two groups of strains. Table 3 shows the pair-wise differences between these sequences. Within the first group, the average difference per nucleotide is 0.003 ± 0.002, whereas the three sequences from the second group are identical. Thus, we were unable to find any genetic differences between *E. myxocylindracea* and *E. geniculata* strains 4b and 4g. The average distance between the two groups is 0.386 ± 0.002.

Light and confocal microscope observations. Observations under light and confocal microscope confirmed that after being kept for several months in medium 3c, each *E. myxocylindracea* cell had a single axial star-shaped chloroplast. The long branches of the chloroplast, radiating from the central section containing the pyrenoid, extended far into the distal and proximal ends of the cell (Fig. 3, A and B). As the terminal points of the branches reached the cell surface they became flattened, forming irregular lobes (Fig. 3, A and B) and even reticulate structures (Fig. 3A), which filled the space immediately under the periplast, along the entire length of the cell (Fig. 3, A and B). Cells containing a second chloroplast, initially small and localized behind the nucleus, first began to appear in a culture of *E. myxocylindracea* after 3 days of adaptation in medium C (Fig. 3C). The number of cells with the second plastid rose steadily in the course of the next several days. After 10 days about 90% of the cells contained two chloroplasts of similar size, but with branches much shorter than those of the initial single chloroplast. One of them was behind and the other in front of the nucleus forced into the central part of the cell by the growing distal chloroplast (Fig. 3, D and D′). Relatively short branches of those two chloroplasts were flattened and lobately expanded at the ends, filling the space under the periplast in each half of the cell. Using the medium with higher organic content (D) only slightly accelerated the rate of the emergence of the second plastid in the higher number of cells. Their number never exceeded 90%–95% and the above-mentioned changes in cell appearance were reversible upon transferring the *E. myxocylindracea* culture to the initial (3c) or less organic (A or B) medium.

Chloroplasts in both *E. geniculata* strains (4b and 4f) displayed the same spatial organization as those in *E. myxocylindracea*, which has two chloroplasts. One was in front of and the other behind the nucleus and their relatively short arms penetrated only that part of the cell, which contained the center of each chloroplast, outfitted with a large pyrenoid (Fig. 4A). There were no changes in the number of plastids in the cells of both strains when they were transferred from medium 3c to the medium with more organic content (C or D). However, the transfer to the more mineral medium (A or B) consequently led to the loss of the chloroplast located behind the nucleus (Fig. 4, A–C). A substantial reduction in size of the plastid located behind the nucleus could be observed in about 40% of the cells after only 6 days following the transfer from the initial medium 3c to the medium B (Fig. 4B′). Further reorganization of the photosynthetic apparatus took place over the next 10 days. As a consequence, two chloroplasts with short arms (Fig. 4A) were replaced by one chloroplast with long arms extending far into both ends of the cell (Fig. 4C).

In cultures that were not overloaded, most cells were dividing and no aberrations in cell divisions were observed. In the cells both with single and double chloroplasts, the division followed the path characteristic of species with axial chloroplasts, where the relative location of the nucleus and the chloroplasts is independent of the cell division, hence considered a diagnostic character (Popova 1966, Asaul 1975). The nucleus migrated only slightly toward the front of the cell, permitting the available space between the chloroplasts (in two-chloroplast cells, Fig. 3D′) or the space between the dividing chloroplast and greatly narrowed distal part of the cell (in single-chloroplast cells, Fig. 3B).

The results of the biometric measurements of four cell parameters (length, width, circularity, and area of projection of the cell on a flat surface) for all eight *E. geniculata* strains and *E. myxocylindracea* indicate that although these cell parameters are not diagnostically important, they differ significantly between some strains (Table 4).

DISCUSSION

The prevailing practice in *Euglena* systematics was to attribute great weight to chloroplast number and morphology. This was based on the belief that these features are genetically determined and thus unchangeable (Gojdics 1953, Huber-Pestalozzi 1955, Pring-

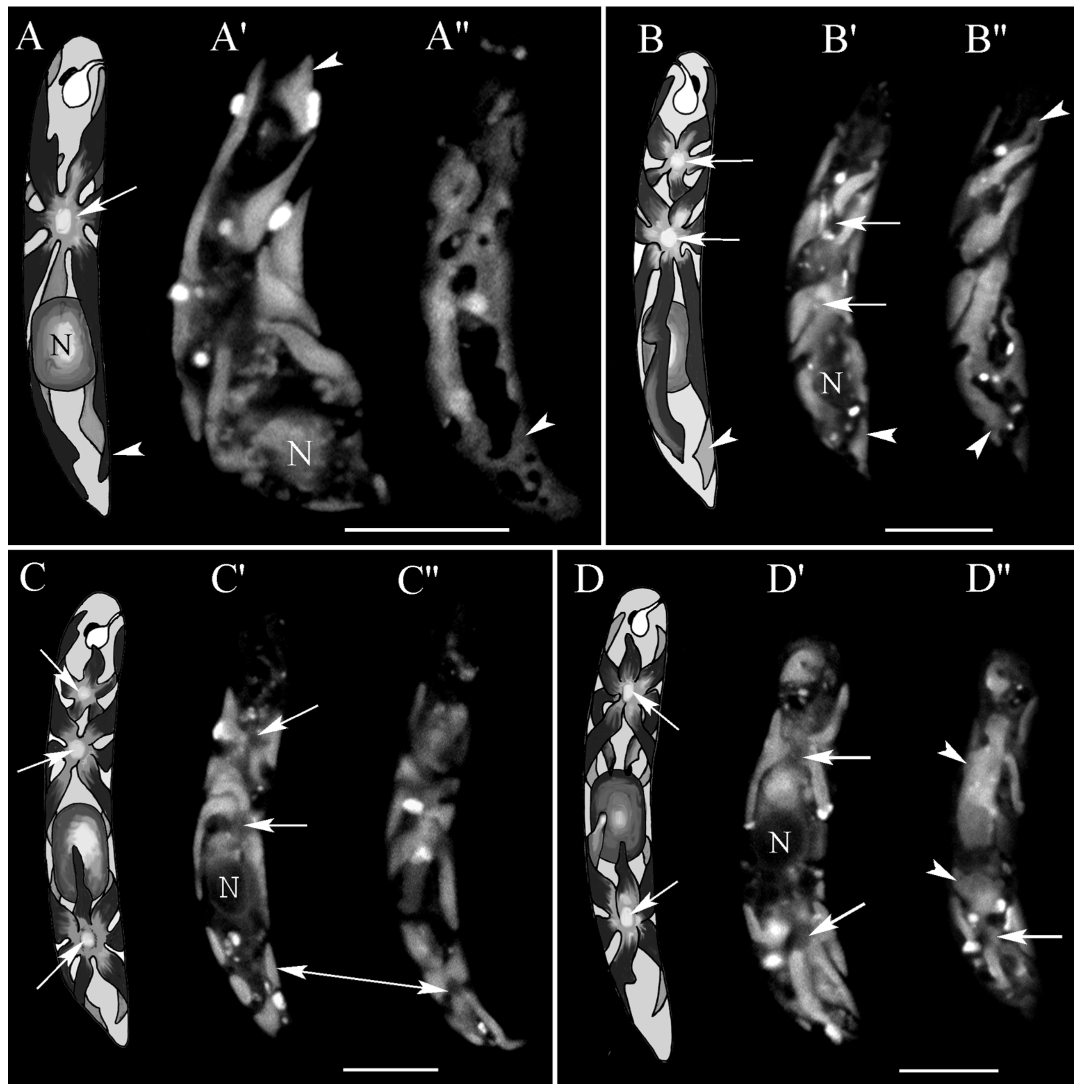

FIG. 3. Confocal microscope photographs and drawings showing the induction of a second chloroplast in *Euglena myxocylindracea.* (A) Transverse view: section through the cell with one large, stellate, axial chloroplast located in front of the nucleus (N). The chloroplast fragments twisting along the curvature of the periplast are taking the shape of reticulate structures. (B) Transverse view: section through the cell during division, with a single anteriorly located chloroplast. Two daughter chloroplasts are visible within the cell (arrows). Surface of the cell with long chloroplast branches going away toward the posterior and anterior ends (arrowheads). (C) Two daughter chloroplasts located in front of the nucleus (N), each with a visible pyrenoid (arrows), and one smaller chloroplast situated behind the nucleus (double-head arrow). Transverse view: section through the smaller chloroplast of the cell. (D and D′) Two large axial and stellate chloroplasts, each with a central pyrenoid (arrows), one situated in the front of the nucleus (N) and the other one behind it. The terminal lobately flattened fragments of the stellate chloroplasts extending to the periplast (arrowheads). Scale bars, 20 μm.

sheim 1956, Popova 1966, Popova and Safonova 1976, Zakryś 1986, Zakryś and Walne 1994, Kim et al. 1998, Shi et al. 1999). Recently however, molecular studies have shown that the species of colorless euglenoids, which apparently lost chloroplasts, such as *Astasia longa* and *Khawkinea quartana*, are very closely related to some members of the genus *Euglena* (Montegut-Felkner and Triemer 1997, Linton et al. 1999, 2000, Preisfeld et al. 2000, 2001, Müllner et al. 2001). On the other hand, some green euglenoids can lose their chloroplasts in response to stress conditions such as changes in the photoperiod, balance of minerals, UV irradiation, high temperature, or antibiotic treatment (Pringsheim and Pringsheim 1951, Lyman et al. 1961, Ben-Shaul et al. 1964, Schiff and Epstein 1965, Leff and Krinsky 1967, Uzzo and Lyman 1969, Marčenko 1970, 1973, 1974).

The occurrence of reversible changes in chloroplast morphology of euglenoids, such as disintegration, decay, and fragmentation, has long been known and described in the literature (Conrad 1943, Conforti 1991,

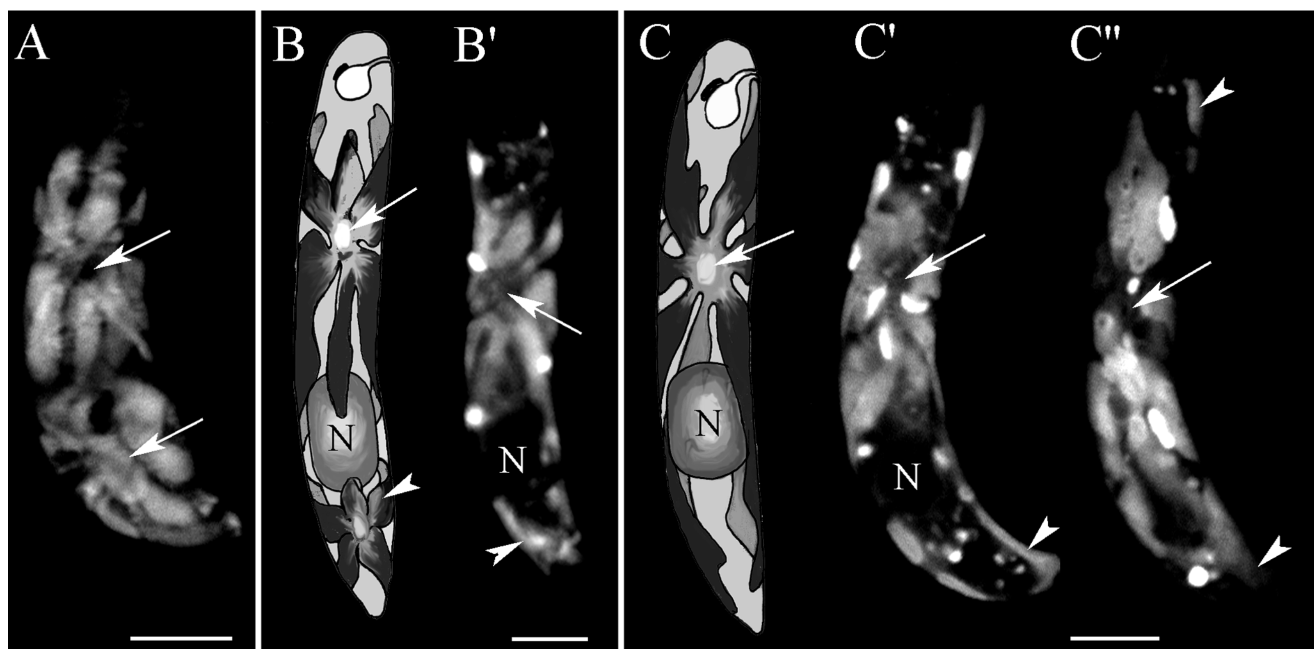

FIG. 4. Confocal microscope photographs and drawings showing the reduction of a second chloroplast in *Euglena geniculata.* (A) Two stellate axial chloroplasts with large centrally located pyrenoids (arrows). (B and B′) Two axial stellate chloroplasts of different sizes are visible in the cell. The large plastid anterior to the nucleus (N) contains a large central pyrenoid (arrow). The small chloroplast (arrowhead) is posterior to the nucleus. (C) The single, large, stellate chloroplast with a pyrenoid (arrows); ribbon-like branches radiate out toward the cell surface and extend far into both ends of the cell (arrowheads). The surface of the cell. Scale bars, 20 μm.

1998). Other changes include abnormality of karyo- and cytokinesis, decreased rate of cell division, changes in the shape and the size of the cell, and other developmental aberrations of strains maintained for a long time in synthetic media or in unfavorable environmental conditions (Conrad 1943, Zakryś 1980, 1983, 1988, 1997, Fasulo et al. 1982, Kiss et al. 1986, Conforti 1991). All the above descriptions refer to species with numerous parietal (or partially parietal) chloroplasts, such as *E. gracilis, E. acus,* or *E. spirogyra.* No information in the literature is available to our knowledge on the long- or even short-term loss of chloroplast number without the change of their morphology with respect to species with few (one to three) large axial chloroplasts of *Euglena* subgenus, where the number of chloroplasts is a primary and thus a diagnostic character. The circumstances of the description in 1973 by Bold and MacEntee of a new euglenoid species, *E. myxocylindracea,* indicate in light of our molecular studies that such a phenomenon of chloroplast number change also may be encountered in nature in *Euglena* subgenus.

Euglena myxocylindracea was isolated from soil samples from a vegetable garden and a white pine (*Pinus strobus*) forest (Maple Farm, Paupack, Pike Country, PA, USA) in March 1972. Before classifying the new organism as a new taxon, the authors submitted it for close examination over several months "in a variety of media, including BBM, BBM modified with urea as a nitrogen source, and biophasic soil-water tubes, with or without a pea cotyledon." These observations indicated that the new organism was "*Euglena* viridis-like in cellular organization, *i.e.*, with elongate, ribbon-like chloroplasts radiating from a single paramylon center; the nucleus post-median to posterior" (Bold and MacEntee 1973). Today, by ultrastructural standards, we define such an organization of a photosynthetic apparatus as an axial, single, star-like chloroplast with pyrenoid situated in its center, surrounded by numerous paramylon grains—a paramylon center (Dragos et al. 1979, Péterfi et al. 1979, Zakryś and Walne 1998, Zakryś et al. 2001).

According to Bold and MacEntee (1973) the strain of *E. myxocylindracea* was different from *E. viridis* "because of its characteristically prolonged, dominant, gelatinous palmelloid phase in a variety of liquid and solid culture media in which the cells secrete dichotomously branching slimy cylinders; more especially, because of its lack of an emergent flagellum, or when one is present, its abbreviated length, as compared with the body-length flagellum of *E. viridis.*" Moreover, the two species had different morphology of motile cells, which are spindle-shaped in *E. viridis* but cylindrical in *E. myxocylindracea.*

The proposal of a new taxon based on non-chloroplast features, such as the length of a flagellum, the palmella type, or the shape of motile cells, was further corroborated by the then-authority of euglenoid systematics, Dr. Gordon Leedale, who personally examined the material of Bold and MacEntee (1973). How-

TABLE 4. Average values of four cell parameters for *Euglena geniculata* and *Euglena myxocylindracea* strains.

Strains	Cell parameters			
	Length (μm)	Width (μm)	Area (μm^2)	Circularity
E. geniculata				
4b	75.09 ± 12.82	8.02 ± 1.56	595.4 ± 129.9	0.28 ± 0.06
4c	77.74 ± 8.08	7.09 ± 1.08	564.3 ± 121.7	0.24 ± 0.04
4f	71.64 ± 5.93	8.98 ± 1.18	643.9 ± 104.7	0.31 ± 0.03
4g	61.21 ± 5.59	7.13 ± 1.23	434.8 ± 75.6	0.29 ± 0.05
66	75.87 ± 9.85	7.32 ± 0.94	548.4 ± 98.2	0.25 ± 0.04
197	74.52 ± 8.93	8.08 ± 1.30	598.4 ± 95.7	0.28 ± 0.05
530	73.28 ± 11.34	7.31 ± 1.35	534.2 ± 118.9	0.26 ± 0.05
994	75.68 ± 10.06	7.62 ± 1.16	571.8 ± 88.3	0.26 ± 0.05
E. myxocylindracea	62.85 ± 6.82	6.72 ± 1.07	421.6 ± 75.7	0.28 ± 0.04

Values are means ± SD.

ever, despite a year-long observation, none of the researchers observed the presence of two chloroplasts in cells of the *E. myxocylindracea* strain maintained in different media. Molecular studies performed 20 years later in our laboratory prompted us to revisit the problem of *E. myxocylindracea.*

Growing strains on different media (more or less organic) reveals the characteristic entirely unknown in Euglenales, namely the ability of their cells to quickly and reversibly reorganize their photosynthetic apparatus depending on growing conditions. This reorganization is apparently not related to degenerative changes, such as partial fragmentation or disintegration of the entire chloroplast, but involves the replacement of one big structure, such as a single chloroplast, with two smaller ones. Moreover, this reconstruction is not coupled with any changes in the cell morphology or irregularity of karyo- or cytokineses, expressed by the emergence of gigantic multinuclear cells or existence of so-called multiple divisions (Asaul 1967, Zakryś 1980, 1983).

Our molecular studies indicate that *E. myxocylindracea* is contained within the *E. geniculata* clade. Moreover, there are two well-distinguished groups within the *E. geniculata* + *E. myxocylindracea* clade. One is represented here by *E. geniculata* strains (SAG: 1224-4c, isolated in Switzerland, and 4f, isolated in Austria; and ACOI: 66, 197, 530, and 994, isolated in Portugal). The other group consists of *E. myxocylindracea* (UTEX 1989, isolated in the United States) and *E. geniculata* strains (SAG: 1224-4b, isolated in Czechoslovakia, and 4g, isolated in England). The species within these two groups are mutually very similar to each other and probably represent two separate clones. Moreover, our data suggest that within five distinct groups of strains—four described as *E. geniculata* (1224-4c; 4f and 197; 66, 530, and 994; 1224-4b and 4g) and one as *E. myxocylindracea*—three of them (4f and 197; 66, 530, and 994; 1224-4b and 4g) may even be genetically mutually identical organisms. Therefore, the separation into the two larger and five smaller groups could not be attributed to geographical differentiation. Similarly high genetic polymorphism accompanied by the lack of morphological polymorphism was detected in natural populations of two other *Euglena* species, *E. agilis* and *E. gracilis* (Zakryś and Kucharski 1996, Zakryś et al. 1996, 1997, Zakryś 1997).

Precise biometric measurements did not reveal any substantial statistical differences in the size and the shape of the cell between all *E. geniculata* and *E. myxocylindracea* strains. The amount of detected polymorphism is contained well within the range of species diversity of *E. geniculata.* Therefore, given all above-described findings we propose that *E. myxocylindracea* Bold and MacEntee (1973) should merely be considered an environmental form of *E. geniculata* and included as a synonym of *E. geniculata* Dujardin (1841). Except for being discovered by Bold and MacEntee (1973), *E. myxocylindracea* was not found anywhere else, which suggests it is an extremely rare environmental form.

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