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PHYLOGENY AND SYSTEMATICS OF *EUGLENA* (EUGLENACEAE) SPECIES WITH AXIAL, STELLATE CHLOROPLASTS BASED ON MORPHOLOGICAL AND MOLECULAR DATA—NEW TAXA, EMENDED DIAGNOSES, AND EPITYPIFICATIONS[1]

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Morphological and molecular studies, as well as original literature reexamination, necessitate establishment of five *Euglena* species with a single axial, stellate chloroplast [*Euglena viridis* (O. F. Müller) Ehrenberg 1830, *Euglena pseudoviridis* Chadefaud 1937, *Euglena stellata* Mainx 1926, *Euglena pseudostellata* sp. nov., and *Euglena cantabrica* Pringsheim 1956], three species with two chloroplasts (*Euglena geniculata* Dujardin ex Schmitz 1884, *Euglena chadefaudii* Bourrelly 1951, and *Euglena pseudochadefaudii* sp. nov.), and one species with three chloroplasts (*Euglena tristella* Chu 1946). The primary morphological features, allowing distinction of the considered species are the presence and the shape of mucocysts, as well as the number of chloroplasts. Spherical mucocysts occur in *E. cantabrica* and *E. geniculata*, while spindle-shaped mucocysts are present in *E. stellata*, *E. pseudostellata*, *E. chadefaudii*, *E. pseudochadefaudii*, and *E. tristella*. No mucocysts are observed in *E. viridis* and *E. pseudoviridis*. Two new species (*E. pseudochadefaudii* sp. nov. and *E. pseudostellata* sp. nov.) differ from the respective species, *E. chadefaudii* and *E. stellata*, only at the molecular level. Molecular signatures and characteristic sequences are designated for nine distinguished species. Emended diagnoses for all and delimitation of epitypes for seven species (except *E. viridis* and *E. tristella*) are proposed.

Key index words: **asexual organisms; *Euglena pseudochadefaudii* sp. nov.; *Euglena pseudostellata* sp. nov.; *Euglena viridis*; Euglenaceae; Euglenozoa; mucocysts; phylogeny; rDNA; taxonomical 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**

The problem of ''critical groups of species'' (i.e., species morphologically similar) is one of the issues being vigorously investigated in euglenoid systematics. Morphological and molecular comparative studies of cultured strains, enabling verification of diagnostic features, have resolved many controversial issues present in the literature, sometimes for many decades. Recent successful studies on the subject encompass species such as *Euglena agilis* (Zakryś and Kucharski 1996, Zakryś et al. 1996, 2004, Zakryś 1997); *Euglena myxocylindracea* (Zakryś et al. 2002); *Lepocinclis spirogyroides* (Marin et al. 2003, Kosmala et al. 2005); *Lepocinclis fusca* (Kosmala et al. 2005); *Monomorphina pyrum* (Kosmala et al. 2007b); and *Phacus pleuronectes, P. orbicularis*, and *P. hamelii* (Kosmala et al. 2007a). However, the attempt at taxonomical verification of a group of *Euglena* species with axial, stellate chloroplasts was only partially successful (Shin and Triemer 2004) since the strains with a single chloroplast (morphologically identified as *E. stellata* and *E. viridis*) belonged to several distinct clades (Shin and Triemer 2004). We believe that the cause of these difficulties lies in the small number of strains considered, as well as in some inconsistencies in description of cell morphology of the epitype species *E. viridis* (strain NJ 001) and the strain *E. viridis* SAG 1224-17c rooted in the confusing historical description of different types of mucous-producing bodies in euglenoids. In both strains, the authors reported the presence of spherical mucocysts (Shin and Triemer 2004), whereas according to our observations, these strains do not have true mucocysts (i.e., uniform in size, small, subpellicular bodies, with openings located between periplast folds, which excrete mucus outside the cell). The true mucocysts have been described by Mignot (1966) in cells of *E. stellata* (under the name ''corps muciféres''), by Arnott and Walne 1967 (as ''pellicle-pores'') in cells of *E. granulata*, and by other authors in cells of species with star-shaped chloroplasts (Dangeard 1928, Mainx 1926, Gojdics 1953, Buetow 1968). They have also been reported by Shin and Triemer for many species with star-shaped chloroplasts (fig. 1, B–D; 2; 3, A–E in Shin and Triemer 2004). Therefore, reexamination of these controversial taxonomical issues seemed necessary. The issue is especially important because it concerns a group of common and cosmopolitan species, as well as a recently established epitype of *E. viridis* Ehrenberg.

One of the main criteria, so far, for distinguishing species with axial, stellate chloroplasts has been their

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number (from one to three). Based on this criterion, three groups of species had been described: (1) species with one chloroplast, located anterior the nucleus (*E. viridis* Ehrenberg 1830, *E. stellata* Mainx 1926, *E. pseudoviridis* Chadefaud 1939, *E. cantabrica* Pringsheim 1956, *E. cuneata* Pringsheim 1956, and *E. archaeoviridis* Zakryś et Walne 1994); (2) species with two chloroplasts, one located anterior and the other located posterior to the nucleus [*E. geniculata* (Dujardin) Schmitz 1884, *E. chadefaudii* Bourrelly 1951 and *E. dicentra* Skuja 1948]; and (3) species with three chloroplasts (*E. tristella* Chu 1946).

Another important diagnostic feature is the presence and the shape of mucous bodies (mucocysts) present under the periplast. Consequently, the lack of mucocysts defined *E. pseudoviridis;* spherical mucocysts were characteristic of *E. viridis*, *E. cantabrica, E. cuneata*, and *E. geniculata*; and spindle-shaped (i.e., fusiform) mucocysts were observed in *E. stellata*, *E. chadefaudii,* and *E. tristella.*

Other features, such as color, size and shape of cells, habitat requirements, production of mucilage at different division stages, presence of pyrenoids, or chloroplast morphology, were historically important for description of different varieties and forms of *E. viridis* (var. *olivacea* Klebs 1883, var. *lacustris* Francé 1897, var. *stagnalis* Francé 1897, var. *mucosa* Lemmermann 1910, var. *lefevrei* Chadefaud 1937, var. *maritima* Pringsheim 1953, var. *halophila* Pringsheim 1953, fo. *salina* Popova 1947, var. *maxima* Philipose 1982), *E. stellata* (f. *terricola* Pringsheim 1956), and *E. geniculata* (var. *terricola* Dangeard 1901, var. *guttula* Playfair 1923, var. *juvenilis* Playfair 1923, var. *dangeardii* Pringsheim 1953, var. *anglesia* Pringsheim 1953).

Cultured strains of species with two chloroplasts and spherical mucocysts (*E. geniculata, E. myxocylindracea* Bold et Mac Entee 1973) were the subject of taxonomic studies, which produced preliminary proposals. Distinction of *E. myxocylindracea* as a species has been deemed unwarranted, and its rank was rendered as a synonym of *E. geniculata* (Zakryś et al. 2002). The studies of other species, particularly those having a single chloroplast, conducted by Chadefaud (1937), Pringsheim (1956), and recently by Shin and Triemer (2004), helped to provide valuable data but did not produce unequivocal taxonomical resolutions. Therefore, our aims in this study were (1) assessing morphological and genetic variability range of the taxa having one or two axial and stellate chloroplasts, as well as establishing their relationships; (2) verification of morphological diagnostic features, with particular emphasis on species with one chloroplast in a cell; (3) epitype establishment and amendment of diagnostic descriptions of species forming well-established clades on a molecular phylogenetic tree; and (4) amendment of diagnostic description of *E. viridis* Ehrenberg on the basis of the epitype established by Shin and Triemer (2004).

MATERIALS AND METHODS

Euglenoids 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 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 light:dark (L:D), ~27 μmol photons·m^{-2}·s^{-1} provided by cool-white fluorescent tubes.

LM observations. Observation of morphological features was performed using a light microscope (Nikon Eclipse E-600 with Nomarski contrast; Nikon, Tokyo, Japan), equipped with the software for image recording and processing. Photographic documentation was made by the 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 all the cells during their developmental stages, from the young (immediately after division) to the steadily aging to the old.

Biometric studies. They were performed using the LUCIA Measurement program (Laboratory Imaging s. r. o., Prague, Czech Republic). One hundred randomly chosen actively swimming cells from each of the strains 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. Two parameters were measured for each strain—length and width. The data were analyzed using the Statistica program (StatSoft Inc., Tulsa, OK, USA).

DNA isolation, amplification, and sequencing. Isolation of total DNA, amplification of 16S rDNA and 18S rDNA regions, and purification and sequencing of PCR products were performed as previously described (Milanowski et al. 2001, Zakryś et al. 2002).

Sequence accession numbers, alignment, and phylogenetic analysis. The accession numbers of sequences used for phylogenetic analyses are shown in Table S1. Sequence alignments were obtained by using the ClustalX program (Thompson et al. 1997) set to default, manually checked and edited according to the secondary structure of *Euglena gracilis* (Wuyts et al. 2002). Several regions of uncertain homology, which could not be unambiguously aligned, were omitted from the analyses and calculations of sequence similarity. The alignments used for analyses are available in EMBL [16S: ALIGN_001219; 18S: ALIGN_001218; 16S/18S: ALIGN_001220]. All nucleotides were treated as independent and unordered, multistate characters of equal weight. The alignments were edited, and uncorrected sequence similarities were calculated using the program GenDoc 2.6 (Nicholas and Nicholas 1997).

Phylogenetic analyses for all 37 strains were performed separately for 16S rDNA, 18S rDNA, and for combined data. Distance (NJ), maximum likelihood (ML), and maximum parsimony (MP) analyses, as well as homogeneity test (chi-squared) of nucleotide distribution and partition homogeneity test were performed by PAUP*, Version 4.0b10 for Microsoft Windows (Swofford 1998). To find the best tree, the heuristic search option was used with MULPARS, tree-bisection-reconnection (TBR) branch swapping, ACCTRAN optimization, and random addition, with the number of replicates depending on the method used (1,000 for NJ and MP, and 10 for ML). Bootstrap support for specific nodes (Felsenstein 1985) was estimated with the default options using 1,000, 1,000, and 100 replications for MP, NJ, and ML analyses, respectively, as implemented in PAUP*. Models of sequence evolution and their parameters for ML and NJ methods were chosen, separately for each of the three alignments, by Modeltest 3.7 program (Posada and Crandall 1998). Auto decay indices (Bremer 1994) were calculated by AutoDecay 4.02 program

(Eriksson 1998) for MP analyses. Bayesian analyses (BA) were performed by MrBayes 3.1 (Huelsenbeck and Ronquist 2003). Models of sequence evolution for Bayesian analyses were chosen separately for 16S rDNA and 18S rDNA (not shown) as well as for combined data by MrModeltest 2.2 (Nylander 2004). For each chosen model, two types of analysis were performed where the covariant model of evolution was used or not. In the case of combined sequences, two partitions were specified for 18S and 16S rDNA, respectively; however, analyses without partitioning were also performed. The sequences of *E. anabaena* were used to root the trees (see Milanowski et al. 2006), which were drawn by Tree View, Version 1.6.1 for Microsoft Windows (Page 1996).

RESULTS

Light microscope observations. Biometric measurements and observation under light microscope of 27 strains indicated some stable morphological differences between strains, concerning features such as the presence and the shape of mucocysts and the shape of cells. Some inter- and intrastrain variations, dependent on the ontogeny stage and physiological condition of cells, regarding the size of cells, morphology, and the number of chloroplasts and mucocysts, were also observed (Table 1).

Mucocysts number and morphology: We begin the verification of this feature in the strain *E. viridis* NJ 001, from which comes the epitype established by Shin and Triemer (2004). We were unable to detect any mucocysts in cells of the strain NJ 001, or in two related strains (SAG 1224-17d and SAG 1224-17c). In all remaining strains, the mucocysts were present and fell into two categories: (i) spherical, present in all strains of *E. geniculata* (with two chloroplasts) and in some strains with a single chloroplast (ACOI 192, ACOI 1095, SAG 26.93, SAG 1224-25, and SAG 1224-40); and (ii) spindle shaped, present in *E. tristella* strain SAG 1224-35 (with three chloroplasts) and in some strains with a single chloroplast (SAG 1224-14, ACOI 1158, ACOI 2951, and ACOI 2956), as well as in strains with two chloroplasts (CCAP

TABLE 1. Comparison of morphological characters among *Euglena* species.

Taxa	Strain	Cell length (μm)		Cell width (μm)		Length/ width	Cell shape	Chloroplasts number in the culture	Mucocyst shape
		Mean ± SD	Min.–Max.	Mean ± SD	Min.–Max.				
E. cantabrica	ACOI 192	48.0 ± 5.6	36.5–61.2	17.8 ± 1.8	13.7–22.5	2.7	Widely fusiform	1	Spherical
	ACOI 1095	46.4 ± 4.0	37.2–58.6	19.5 ± 2.1	13.7–24.9	2.4	Widely fusiform	1	Spherical
	SAG 26.93	50.4 ± 5.5	38.6–66.3	18.8 ± 1.7	14.3–23.1	2.6	Widely fusiform	1	Spherical
	SAG 1224-25	59.3 ± 6.1	41.3–76.1	23.6 ± 2.3	17.9–29.1	2.5	Widely fusiform	1	Spherical
	SAG 1224-40	65.2 ± 6.5	48.3–84.1	22.7 ± 2.4	16.4–29.6	2.8	Widely fusiform	1	Spherical
E. chadefaudii	CCAP 1224-17p	63.9 ± 3.7	54.5–73.1	16.4 ± 1.5	13.9–20.8	3.9	Fusiform	2 or 1	Spindle
	CCAP 1224-17g	61.0 ± 4.7	50.0–71.2	15.0 ± 1.7	11.5–19.3	4.0	Fusiform	2 or 1	Spindle
	IAM E-11	52.3 ± 3.8	43.3–60.0	15.0 ± 1.7	11.4–19.0	3.5	Fusiform	1, rarely 2	Spindle
E. geniculata	SAG 1224-4b	71.6 ± 4.7[a]	59.5–78.3	9.7 ± 0.9[a]	7.8–11.9	7.4	Cylindrical	2	Spherical
	SAG 1224-4f	71.6 ± 5.9[a]	55.5–84.0	9.0 ± 1.2[a]	5.4–12.0	7.9	Cylindrical	2	Spherical
	UTEX 1989	62.8 ± 6.8[a]	49.0–73.3	6.7 ± 1.1[a]	4.5–8.9	9.4	Cylindrical	1, rarely 2	Spherical
	SAG 1224-4c	77.7 ± 8.0[a]	60.0–91.3	7.0 ± 1.0[a]	5.4–9.2	11.1	Cylindrical	2	Spherical
	SAG 1224-4g	61.2 ± 5.6[a]	49.6–71.3	7.1 ± 1.2[a]	4.9–9.8	8.6	Cylindrical	2	Spherical
	ACOI 66	75.9 ± 9.8[a]	57.3–97.5	7.3 ± 0.9[a]	5.5–9.6	10.4	Cylindrical	2	Spherical
	ACOI 197	74.5 ± 8.9[a]	59.6–90.0	8.1 ± 1.3[a]	5.0–11.0	9.2	Cylindrical	2	Spherical
	ACOI 530	73.3 ± 11.3[a]	56.2–91.7	7.3 ± 1.3[a]	5.6–11.5	10.0	Cylindrical	2	Spherical
	ACOI 994	75.7 ± 10.0[a]	54.0–94.4	7.6 ± 1.2[a]	4.8–10.3	9.9	Cylindrical	2	Spherical
	MI-61[b]		47.0–60.0[b]		10.0–12.0[b]		Cylindrical[b]	1[b]	Spherical[b]
E. pseudochadefaudii	MI-00	66.0 ± 4.5	55.7–77.4	13.2 ± 1.1	10.4–16.0	5.0	Fusiform	1, rarely 2	Spindle
E. pseudostellata	ACOI 2951	56.0 ± 4.0	46.0–63.0	15.2 ± 1.3	12.7–17.0	3.7	Fusiform	1	Spindle
	ACOI 2956	54.2 ± 4.6	45.7–65.3	12.4 ± 1.3	9.8–16.1	4.4	Fusiform	1	Spindle
E. pseudoviridis	SAG 1224-17c	52.9 ± 5.5	42.5–66.8	13.0 ± 1.1	10.9–15.6	4.2	Fusiform or cylindrically fusiform	1	Absent spherical[b]
E. stellata	ACOI 1158	35.9 ± 2.6	28.5–40.0	8.9 ± 0.7	7.2–10.9	4.0	Fusiform	1	Spindle
	SAG 1224-34b	40.0 ± 2.8	33.5–44.8	9.0 ± 0.9	6.5–11.2	4.4	Fusiform or cylindrically fusiform	1	Spindle
	SAG 1224-14	34.1 ± 2.5	27.8–39.7	11.0 ± 0.9	8.7–12.9	3.1	Fusiform	1	Spindle
	NJ "sandy"[b]		57.0–75.0[b]		8.0–12.0[b]	6.7	Cylindrical[b]	1	Spindle[b]
E. tristella	SAG 1224-35	57.3 ± 6.2	44.0–71.9	15.0 ± 1.4	12.2–17.6	3.8	Fusiform	3 or 2	Spindle
E. viridis	NJ 001	41.6 ± 4.2	33.7–51.0	11.9 ± 1.4	9.0–15.3	3.5	Fusiform or cylindrically fusiform	1	Absent spherical[b]
	SAG 1224-17d	45.7 ± 4.0	37.8–54.0	12.2 ± 1.1	9.3–14.7	3.7	Fusiform or cylindrically fusiform	1	Absent

[a]Data from Zakryś et al. (2002).
[b]Data from Shin and Triemer (2004).

1224/17g, CCAP 1224/17p, IAM E-11, and MI-00) (Table 1).

Spherical mucocysts were significantly smaller (~0.5–0.8 μm in diameter: Fig. 1, r and s) than spindle-shaped ones (~2–4 μm long and 0.5–0.8 μm wide: Fig. 1, w and x) and more numerous (uncountable), whereas the spindle-shaped mucocysts numbers observed in a single cell were less numerous (countable) (Fig. 1, r, s, w, and x). The number of mucocysts, which were always present in living cells, was dependent on the organism's physiological condition and ontogeny stage. Mucocysts were the most numerous in organisms undergoing cell division with a reduced rate, in cultures that were overcrowded, yet enjoying comfortable environmental conditions. They were significantly less numerous in vigorously dividing cells present in young cultures and only sparsely distributed in dying, several-month-old cultures, showing pronounced signs of disintegration.

Both spherical and spindle-shaped mucocysts were organized in rows spirally arranged parallel to the periplast striation; they were massed at the front and at the end of the cell (Fig. 1w). Characteristic organization of the rows was conspicuous especially when the number of mucocysts was large (Fig. 1, r and w). Their distribution appeared more randomly scattered as the number of mucocyst rows diminished (Fig. 1, s and x).

Cell metaboly: All investigated strains had cells capable of metaboly, but the extent of cell plasticity was dependent to a considerable degree on the presence, shape, and number of mucocysts. Accordingly, strains possessing spindle-shaped mucocysts were the most rigid. Those relatively large and elongated structures arranged in rows effectively made the periplast firm, especially when they were very numerous and the cells were small. Therefore, cells with spindle-shaped mucocysts easily maintained their shape during slow movement and even for a short time after they stopped moving. In the case of broadly fusiform cells of *E. cantabrica*, such a stiffness was not imposed by minute, spherical mucocysts. This situation resulted in rounding of the body end in slowly swimming cells, which were quickly changing shape to ellipsoidal and then spherical after they stopped their movement (Fig. 1, i–l). Such a pronounced metaboly was not displayed by the longitudinally cylindrical cells of *E. geniculata* (Fig. 1u).

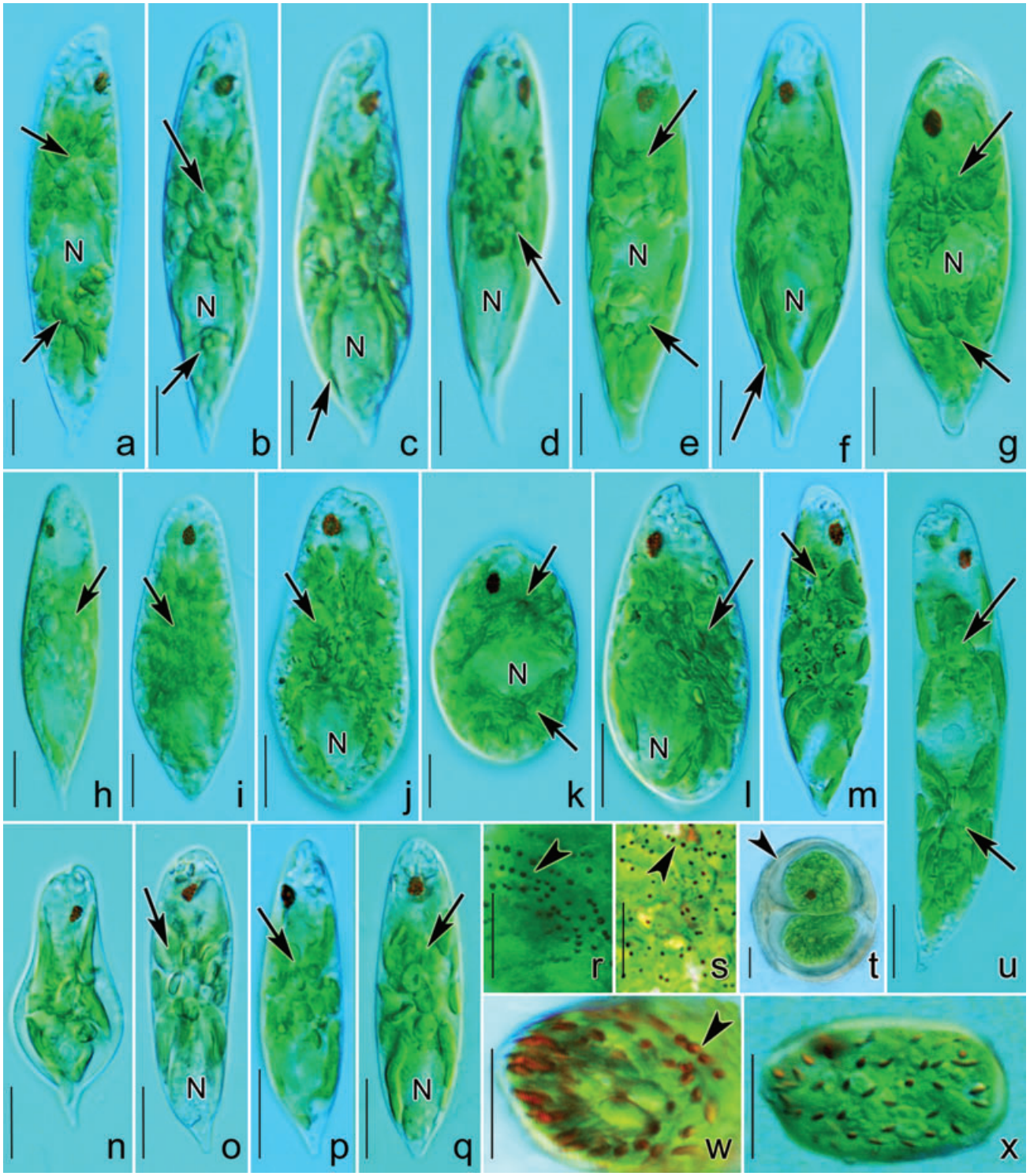
The strains with a single chloroplast (NJ 001, SAG 1224-17d, and SAG 1224-17c), with no mucocyst present, displayed the most pronounced metaboly among all investigated species. It was manifested by characteristic inflation of the central part of the cell while its ends remained practically unchanged (Fig. 1n).

Palmella stage: The ability to divide, both of the free-swimming, flagellated cells and of those being in a nonmotile, palmelloid state, was observed in all strains investigated. Cell divisions were much more prevalent at the palmella stage only in *E. cantabrica*, in cells usually surrounded by a thick envelope of mucilage (Fig. 1t), when they were entering palmella stage and changing their shape to elliptical or spherical (Fig. 1, k and t).

Chloroplasts: Substantial morphological differentiation, concerning the size—how much of the cell volume was taken by it—and the number of chloroplast bands was observed in all strains having a single chloroplast. The size of the chloroplast was strongly correlated with ontogeny stage and habitat conditions. Before division, the chloroplast was considerably elongated, reaching up to 2/3 of the cell's length (Fig. 1, m and q), while immediately after division it formed a smaller, shorter structure (Fig. 1o). In young cultures, the chloroplast possessed numerous, long bands extending to the very ends of the cell. In overcrowded, aging cultures, the chloroplast began to shrink and deform to eventually become disintegrated into singular bands. In broadly fusiform cells of *E. cantabrica,* the chloroplast formed the largest structure, developing lengthwise, independent of changes resulting from ontogeny (Fig. 1, i, j, and l).

In strains with two chloroplasts, most of the cells in young populations had both chloroplasts of similar size (one located anterior and the other posterior to the nucleus) (Fig. 1, a, e, g, and u). Only one feature was clearly dependent on ontogeny stage. Bands of both chloroplasts were substantially shortened before cell division. As the culture grew older and habitat conditions deteriorated, a disintegration of photosynthetic apparatus ensued. It was manifested by steady reduction (Fig. 1b) and finally disappearance of the posterior chloroplast, located behind the nucleus, with concomitant elongation of chloroplast's bands of the anterior one (Fig. 1, c, d and f). Not all strains were equally susceptible to changes in habitat conditions. Cells with two chloroplasts predominated in the *E. chadefaudii* strain CCAP 1224/17g, independent of the age of the culture and environmental conditions (Fig. 1g). It was the case only in young cultures of the *E. chadefaudii* strains CCAP 1224/17p and IAM E-11, whereas in old cultures, the cells with two chloroplasts were in the minority (Fig. 1, a–c, e, and f). Cells with a single chloroplast was generally encountered in the strain MI-00 (Fig. 1d), while the formation of two chloroplasts was only sporadically observed in young populations. The strain of *E. tristella* (with three chloroplasts) was also susceptible to deteriorating habitat conditions. This resulted in loss of the smallest, third chloroplast, located in the vicinity of stigma in the anterior portion of the cell. However, loss of a chloroplast located behind the nucleus was never observed in this species.

Biometry: Precise measurements of strains cultured in identical conditions showed that those with a single chloroplast had cells of diverse sizes. The



smallest cells were observed in *E. stellata* (34–40 × 9–11 µm, on average), with a single exception of the strain ''sandy'' (57–75 × 8–12 µm; Shin and Triemer 2004), in *E. viridis* and *E. pseudoviridis* (42–53 × 12–13 µm, on average). Slightly bigger cells were observed in *E. pseudostellata* (54–56 × 12–15 µm, on average) and in some *E. cantabrica* strains, such as ACOI 192, ACOI 1095, and SAG 26.93 (46–50 × 18–19.5 µm, on average), while the two remaining *E. cantabrica* strains, SAG 1224-25 and SAG 1224-40, had the biggest cells (59–65 × 23–24 µm, on average) (Table 1).

Cell size and shape of species with two chloroplasts (*E. geniculata, E. chadefaudii,* and *E. pseudochadefaudii*)

FIG. 1. Light microscope photographs showing an overview of living cells, chloroplasts, and true mucocysts of *Euglena* species. (a–c) Cells of the *E. chadefaudii* strain IAM E-11. (a) Cylindrically fusiform cell with two star-shaped chloroplasts, one located in front of the nucleus (N) and the other behind it. Short chloroplast bands indicate that the cell is preparing itself for division. A cluster of small paramylon grains (arrows) is shielded by each chloroplast center. (b) A fusiform cell with two chloroplasts, one of a typical size is located in front of the nucleus, the other behind the nucleus is in a remnant form. A cluster of paramylon grains (arrows) is visible in the center of each chloroplast. (c) A fusiform cell with a single chloroplast located in front of the nucleus. Long chloroplast bands are extending to the very ends of the cell (arrow). (d) An *E. pseudochadefaudii* MI-00 cell with a single chloroplast (arrow). (e–g) *E. chadefaudii.* (e) A cylindrically fusiform cell of the strain CCAP 1224/17p with two chloroplasts, located in front of and behind the nucleus. Chloroplast centers are veiled by paramylon grains (arrows). (f) A fusiform cell of the strain CCAP 1224/17p with a single chloroplast located in front of the nucleus. Long chloroplast bands are extending to the very ends of the cell (arrow). (g) An *E. chadefaudii* CCAP 1224/17g cell with two chloroplasts, whose centers are marked by clusters of paramylon grains (arrows). (h) An *E. pseudostellata* ACOI 2956 cell with a single chloroplast and a cluster of paramylon grains in its center (arrow). (i–l) *E. cantabrica.* (i, j) Widely fusiform cells of the ACOI 192 strain with a single, large chloroplast developing along the long axis (arrows) and pushing the nucleus (N) toward the end of the cell. (k) A cell of the strain ACOI 1095 preparing itself to divide. Two offspring chloroplasts are visible on both sides of the nucleus (arrows). (l) A deformed (with rounded end), widely fusiform cell of the strain SAG 1224-25 in the arrested stage. A large chloroplast (arrow) is taking virtually all space of the cell and pushing away the nucleus (N). (m) A fusiform cell of the *E. pseudoviridis* strain SAG 1224-17c with a visible large chloroplast, whose wide and long bands are reaching the periplast and are bending alongside its curvature (arrow). (n, o) The *E. viridis* strain NJ 001. (n) A capable of metaboly cell in the arrested stage with a characteristically inflated central part. (o) The cell assuming cylindrically fusiform shape while swimming. A single, star-shaped chloroplast (arrow) is visible in front of the nucleus (N). (p, q) *E. stellata.* (p) A star-like, single chloroplast (arrow) is visible in a cell of the SAG 1224-14 strain. (q) A cluster of paramylon grains marking the center of the single chloroplast (arrow) in a cell of the strain ACOI 1158. (r, s) Spherical true mucocysts of the *E. cantabrica* strain ACOI 192. (r) Numerous true mucocysts forming entirely filled rows (arrowhead). (s) In cells with the decreased number of mucocysts their rows are less filled (arrowhead) and appear irregularly distributed. (t) A palmella stage of the *E. cantabrica* SAG 1224-25 cell with a thick mucilage envelope (arrowhead). (u) A cylindrical cell of the *E. geniculata* strain SAG 1224-4b, with two chloroplasts, of which one is located in front of and the other behind the nucleus (arrows). (w) *E. pseudostellata* ACOI 2956 showing rows of spindle-shaped true mucocysts (arrowhead). (x) In cells of the *E. stellata* strain SAG 1224-14, spindle-shaped true mucocysts are few, and therefore rows are not conspicuous.

differed noticeably. Cylindrical cells of *E. geniculata* were longer and narrower (61–78 × 6.7–9.7 μm, on average; length/width = 8–11) than fusiform cells of *E. chadefaudii* and *E. pseudochadefaudii* (52–66 × 13–16 μm, on average; length/width = 3.5–5).

The only species with three chloroplasts, *E. tristella* SAG 1224-35, had cells of average dimensions, equal to 57 × 15 μm (Table 1).

Cell shape: A strictly cylindrical cell shape was characteristic of only *E. geniculata* strains (length/width ratio = 8–11). Cells of all other strains were fusiform, but more or less cylindrical in the middle portion, and the amount of this length extension, as well as the proportion of cells displaying it, showed some intrastrain diversity, depending on the ontogeny stage. The effect of changing cell shape from strictly fusiform to cylindrically fusiform was caused by growth of one or more chloroplasts in cells preparing to divide (Fig. 1, m and q).

Cells of *E. cantabrica*, with its broadly fusiform (length/width = 2.4–2.8) shape, were noticeably wider and conically narrowed at the posterior, yet with a tendency for rounding (Fig. 1, i–l). They differed considerably from cells of other species in this large group of strains. In all other strains, a body ended with more or less pointed hyaline tail (Fig. 1, a–h, m–q).

Phylogenetic analysis. After the removal of sites of an uncertain homology, which could not be unambiguously aligned, 2,816 positions were left in the combined 18S and 16S rDNA alignment (1,440 sites from the 18SrDNA and 1,307 sites from the 16S rDNA) of 37 sequences (1,636 of which were constant and 888 MP informative). The likelihood ratio test of the Modeltest 3.7 program (Posada and Crandall 1998) has suggested a TrNef + I + G model (Tamura and Nei 1993) with a fraction of unchangeable nucleotides (I) and a gamma (G) distribution of nucleotide substitution rates, while the Akaike test has chosen 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 NJ, ML, and BA trees, with parameters drawn from Modeltest results (NJ, ML) or estimated by the phylogeny-inferring program (BA) (Table 2). Figure 2 shows the tree obtained by MrBayes program for the *Euglena* genus using the combined 16S rDNA and

TABLE 2. Parameters estimated by 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	0.2603	0.2164	0.2719	0.2513	0.3502	0.5452	1.2101	3.5218	1.2120	0.5929	5.6849	1.0000
	SYM + I + G	0.2500	0.2500	0.2500	0.2500	0.3535	0.5524	1.0136	3.5554	1.1741	0.5101	4.7085	1.0000
Modeltest	GTR + I + G	0.2603	0.2164	0.2719	0.2513	0.3502	0.5452	1.2101	3.5218	1.2120	0.5929	5.6849	1.0000
	TrNef + I + G	0.2500	0.2500	0.2500	0.2500	0.3497	0.5413	1.0000	3.8578	1.0000	1.0000	5.0699	1.0000

A,C,G,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, G–T: rates of reversible nucleotide substitutions.

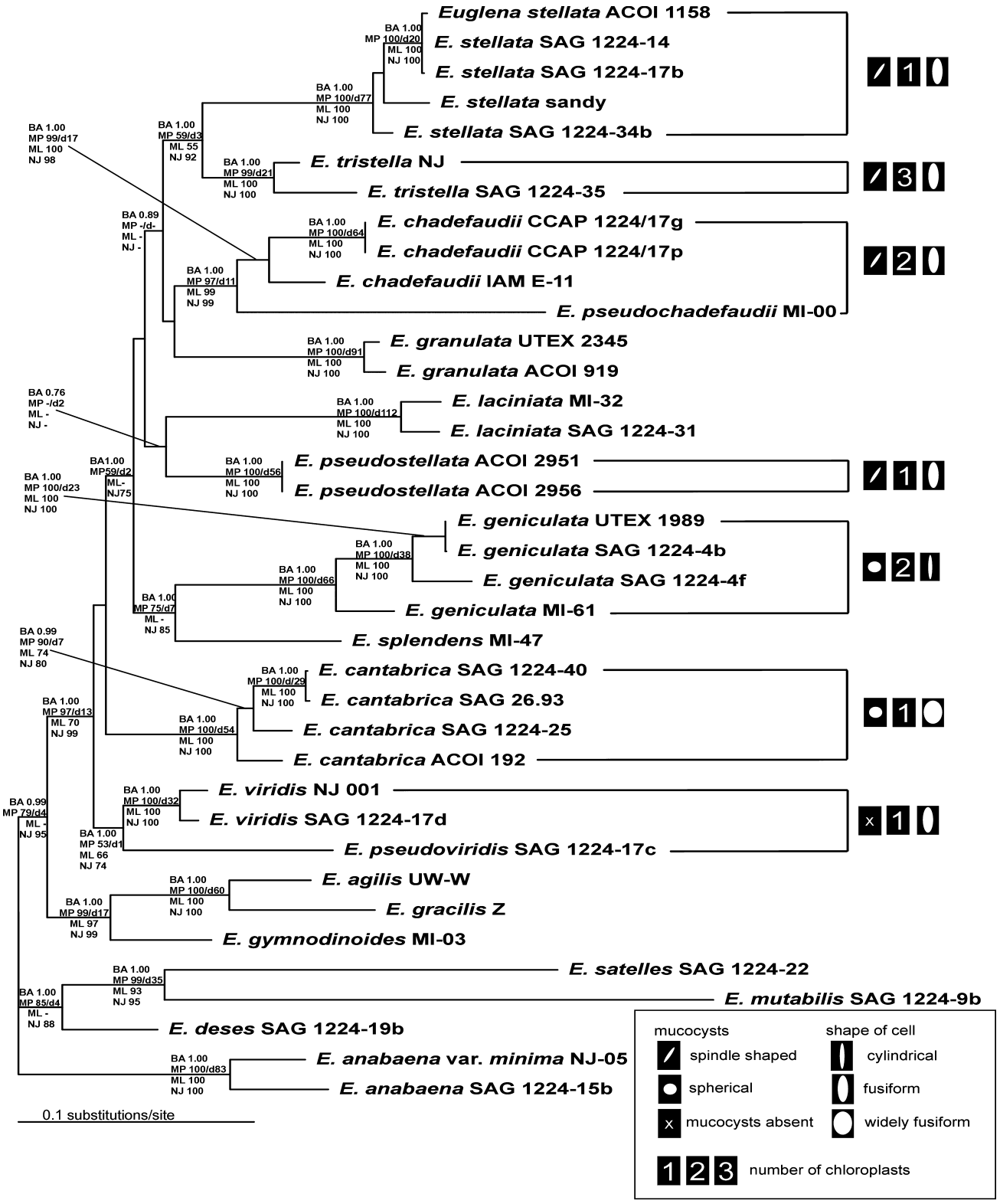


FIG. 2. The phylogenetic tree of the combined 16S and 18S rDNA sequences obtained by Bayesian inference (partition = yes, model for part 18S and 16S: GTR + G + I). Numbers at the essential nodes show posterior probabilities of the tree bipartitions, as well as the bootstrap values/decay indices obtained for the main clades by MP analysis and bootstrap values obtained by NJ and ML analysis analyses (model GTR + I + G). Probabilities <75% are not shown. ML, maximum likelihood; MP, maximum parsimony; NJ, neighbor joining; GTR, general time reversible.

18S rDNA sequences. The trees obtained separately for 16S rDNA and 18S rDNA sequences were almost identical (see below), concerning clades with a strong support (pp > 0.95).

The tree is drawn with *E. anabaena,* the most distantly related species of the *Euglena* clade (Milanowski et al. 2006), as an outgroup, while the *E. satelles, E. deses,* and *E. mutabilis* clade branches off next, followed by *Euglena agilis, E. gracilis,* and *E. gymnodinoides* clade. The rest of the species, the subject of this study, form a well-supported clade and could in turn be divided among three clades, relationships among which are not resolved. The first two very well-supported clades are *E. cantabrica* clade, comprising three strains (SAG 1224-25, SAG 26.93, and ACOI 192), and *E. viridis/E. pseudoviridis* clade consisting of two *E. viridis* strains (NJ 001 and SAG 1224-17d) and a single *E. pseudoviridis* strain (SAG 1224-17c). Similar branching order is observed on the 18S tree (see also Shin and Triemer 2004), while on the 16S tree, these two clades are clustered together with *E. splendens, E. granulata,* and *E. chadefaudii/pseudochadefaudii* (pp = 0.95, data not shown). The third clade has strong support in Bayesian analysis (BA = 1.00, NJ = 75%) and consists of nine very strongly supported clades (BA = 1.00, bs ≥ 99%), corresponding to nine separate species, relationships among which are not fully resolved. The first clade consists of five *E. stellata* strains (ACOI 1158, SAG 1224-14, SAG 1224-17b, SAG 1224-34b, and NJ sandy) and forms a sister relationship (BA = 1.00, bs = 55%–92%) with the second clade, comprising two *E. tristella* strains (NJ and SAG 1224-35). The third clade containing a single *E. pseudochadefaudii* strain MI-00 forms a sister relationship with the fourth group of three *E. chadefaudii* strains (CCAP 1224/17p, CCAP 1224/17g, and IAM E-11). Each of the fifth, sixth, and seventh groups has two strains: *E. granulata* (UTEX 2345 and ACOI 919), *E. laciniata* (MI-32 and SAG 1224-31), and *E. pseudostellata* (ACOI 2951 and ACOI 2956). Their phylogenetic association is uncertain. Finally, the seventh branch, consisting of a single *E. splendens* strain (MI-47), has a sister relationship (BA = 1.00, NJ = 85%) with the eighth group of three *E. geniculata* strains (UTEX 1989, SAG 1224-4b, and SAG 1224-4f). All the above mentioned nine clades and their sister relationships are also present in the 18S tree (not shown). The only difference in the 16S tree is that *E. laciniata* joins the *E. stellata/tristella* clade (pp = 1.0), forming together an unresolved trichotomy (not shown). Additionally, *E. splendens* forms a sister relationship with *E. geniculata* on the 18S tree (pp = 1.0). Taxonomy of *E. granulata, E. laciniata,* and *E. splendens* is beyond the scope of this work.

Taxa-to-clades assignment. After consideration of morphological characters and genetic similarity, we were able to assign species names to clades in a phylogenetic tree obtained from rDNA data. Three species (*E. pseudoviridis, E. pseudochadefaudii* sp. nov., and *E. pseudostellata* sp. nov.) were distinguished only on the basis of molecular data (Fig. 2 and Table 3). Thus, 18S rDNA sequences within species showed 95% similarity (Table 3), while between species it was at most 94.1% (the value for *E. viridis* and *E. pseudostellata,* Table 3). Strains with a single, star-shaped chloroplast form four well-supported clades in a combined 16S and 18S rDNA sequence tree (Fig. 2), while one (*E. pseudoviridis*) is represented by a single strain. Four of these groups were ascribed to the taxa already known from the literature (*E. viridis, E. pseudoviridis, E. stellata, E. cantabrica*), whereas one was assigned to a new species (*E. pseudostellata* sp. nov.). Two of these species (*E. pseudoviridis* and *E. pseudostellata* sp. nov.) were distinguished on the basis of their differences at the molecular level, both with respect to 16S (not shown) and 18S rDNA sequences (Table 3). Similarity between 18S rDNA sequences of *E. viridis* and *E. pseudoviridis* was ~91%, while for *E. stellata* and *E. pseudostellata,* it was ~89%. At the same time, similarity within *E. viridis* was 97.7% and within *E. stellata* was >95.3% (Table 3). Strains with two chloroplasts are represented by another two well-supported clades, which were identified as two already described species (*E. geniculata* and *E. chadefaudii*) and a single strain, which was given the name of a new species (*E. pseudochadefaudii* sp. nov.). *Euglena pseudochadefaudii* sp. nov., was distinguished from *E. chadefaudii* only molecularly. Similarity of 18S rDNA sequences between these two species was 85%, whereas within *E. chadefaudii* it was >95.2% (Table 3). Two strains, representing a species with three chloroplasts (*E. tristella*), have an unstable position in a tree. Similarity of 18S rDNA sequences within *E. tristella* was 99.4% (Table 3).

Signature sequences. Unique SSU rDNA signature sequences were chosen to identify investigated species (Table 4). These signature sequences are in the conserved region of rDNA and can be easily compared with homologous sequences from other taxa.

TAXONOMIC REVISIONS

Euglena viridis (O. F. Müller) Ehrenberg, Physic. Abh. Akad. Wiss. Berlin, p. 82, pl. VI, fig. III, 1830 (1832). Emend. Zakryś et Kosmala.

Emended diagnosis: Cells without true mucocysts (subpellicular bodies uniform in size with openings located between periplast folds), fusiform or cylindrically fusiform, with a very diversified size (30–60 × 9–15 μm, on average), capable of a pronounced metaboly, with ability to inflate its central part. Chloroplast single, axial, situated in front of the nucleus, in the shape of a star, whose arms extend far toward the ends of the cell and the periplast, bending with the curvature of the latter. Central chloroplast part containing a pyrenoid is obscured by a cluster of numerous, small paramylon

TABLE 3. 18S rDNA sequence similarity among *Euglena* strains.

Strain	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
1 *E. cantabrica* ACOI 192	–																						
2 *E. cantabrica* SAG 1224-25	96.0	–																					
3 *E. cantabrica* SAG 1224-40	96.0	97.3	–																				
4 *E. cantabrica* SAG 26.93	95.9	97.2	99.8	–																			
5 *E. chadefaudii* CCAP 1224/17g	89.5	89.8	89.5	89.5	–																		
6 *E. chadefaudii* CCAP 1224/17p	89.5	89.8	89.5	89.5	100.0	–																	
7 *E. chadefaudii* IAM E-11	89.9	89.9	90.1	89.9	95.2	95.2	–																
8 *E. geniculata* MI-61	89.3	89.7	89.2	89.1	89.0	89.0	89.7	–															
9 *E. geniculata* UTEX 1989	89.4	90.2	89.5	89.3	89.1	89.1	89.7	96.8	–														
10 *E. geniculata* SAG 1224-4b	89.4	90.2	89.5	89.3	89.1	89.1	89.7	96.8	100.0	–													
11 *E. geniculata* SAG 1224-4f	88.6	89.3	88.7	88.6	88.8	88.8	89.1	96.3	98.0	98.0	–												
12 *E. pseudochadefaudii* MI-00	83.6	83.2	83.1	83.2	85.0	85.0	85.9	82.4	83.7	83.7	83.7	–											
13 *E. pseudostellata* ACOI 2951	90.4	90.9	91.0	90.9	92.3	92.3	93.1	90.7	91.2	91.2	90.6	86.2	–										
14 *E. pseudostellata* ACOI 2956	90.4	90.9	91.0	90.9	92.3	92.3	93.1	90.7	91.2	91.2	90.6	86.2	100.0	–									
15 *E. pseudoviridis* SAG 1224-17c	89.4	90.3	89.7	89.6	88.5	88.5	88.3	87.9	88.1	88.1	87.9	83.1	89.7	89.7	–								
16 *E. stellata* ACOI 1158	86.7	86.8	86.3	86.2	86.3	86.3	87.0	88.3	87.7	87.7	87.5	82.4	88.5	88.5	86.1	–							
17 *E. stellata* NJ sandy	86.8	87.6	86.5	86.4	85.8	85.8	86.0	87.2	87.2	87.2	87.1	82.4	88.4	88.4	85.8	95.3	–						
18 *E. stellata* SAG 1224-17b	86.9	87.0	86.5	86.4	86.5	86.5	87.2	88.4	87.8	87.8	87.6	82.5	88.7	88.7	86.2	99.6	95.3	–					
19 *E. stellata* SAG 1224-14	86.9	87.1	86.6	86.5	86.5	86.5	87.3	88.4	87.9	87.9	87.7	82.5	88.7	88.7	86.2	99.6	95.4	99.9	–				
20 *E. stellata* SAG 1224-34b	87.2	87.4	87.2	87.0	87.0	87.0	87.7	88.4	88.2	88.2	88.0	82.5	89.2	89.2	86.3	96.6	95.8	96.7	96.8	–			
21 *E. tristella* NJ	91.5	91.3	90.9	90.7	91.5	91.5	91.8	90.7	90.9	90.9	90.8	85.2	93.3	93.3	90.3	89.1	89.1	89.2	89.3	90.2	–		
22 *E. tristella* SAG 1224-35	91.2	90.8	90.5	90.4	91.3	91.3	91.6	90.5	90.5	90.5	90.5	85.1	93.1	93.1	90.2	89.0	89.0	89.1	89.2	89.9	99.4	–	
23 *E. viridis* NJ 001	91.1	91.0	90.8	90.7	90.8	90.8	91.7	90.9	91.0	91.0	90.8	84.5	93.8	93.8	91.2	88.0	87.1	88.2	88.3	88.9	93.3	93.0	–
24 *E. viridis* SAG 1224-17d	91.0	91.1	90.8	90.7	90.9	90.9	92.1	90.8	91.1	91.1	90.5	84.7	94.1	94.1	90.8	88.3	87.6	88.5	88.6	88.5	92.8	92.5	97.7

TABLE 4. SSU rDNA signature sequences for *Euglena* species.

Taxon	Location	Sequence
E. viridis	Helices 16, 16′, 4′, 17′, 18 and 18′ and regions between helices 5′ and 16, 16 and 16′, 16′ and 4′, between 4′ and 17, 17 and 17′, 17′ and 18	**EV1:** 5′-CGC GCA AAT TGC CCA ATG CAA RAA CAT CCT GCG AGG CAG CGA CGA ACT GCA GCA ATC CTG CTGG TAT CTT CAC CAG CAG GAC TTG GAA TGG ATG CAA TCC AAA CAC AGT G-3′
	Helices 24′, 23′, 27	**EV2:** 5′-TGC AAG TGC ATG TTC GTC GAT CAA GGA TGA GAG TCC GGG GAG CMA AGA TG-3′
E. pseudoviridis	Helices 16, 16′, 4′, 17′ and 18 and regions between helices 5′ and 16, 16 and 16′, 16′ and 4′, between 4′ and 17, 17 and 17′, 17′ and 18	**EPV1**: 5′-GCG CAA ATT GCC CAA TGC AAA AAC ATC TGC GAG GCA GCG ACG AAC CGC AGC AAT CCC GCT GGT GCT CTG CGC TGG CGG GAC TTG GAA TGG ATG CAA TCC AAA CAC AGT G-3′
	Helices 24′, 23′ and 27	**EPV2**: 5′-CGC AGG AGC CTG TCC GTC GAC CAA GGA TGA GAG TTT GGG GAG CAA AGA TGA TC-3′
	Helix 39′, 42 and 43 and regions between helix 39′ and 42, 43 and 43′	**EPV3**: 5′-AGT GAG ATA TCT GCC TCC CAA TAG TCT CGT GGC TCG CAT CTG GTA GGG AC-3′
E. stellata	Helices 17′, 18 and regions between helix 17′ and 18, and helix 18 and 18′	**ES1**: 5′-GCC AGT GGB CTT TGG AAT GGA TGC AAC CCA AAC ACA GC-3′
	Helices 24′, 23′ and 27	**ES2**: 5′-TGC RGG CCT GKG TTC RTC GAT CAA GGA TGA GAG TCT GGG GAA CGA AGA TG-3′
	Helices 39′, 42 and 43 and regions between helix 39′ and 42, 43 and 43′	**ES3**: 5′-AGT GAG ATC TCT GCC TCC CAG TAG CCC CTG GCT CGC ATY TGG TAG GGC-3′
E. pseudostellata	Helices 16, 16′, 4′, 17′ and 18 and regions between helices 5′ and 16, 16 and 16′, 16′ and 4′, between 4′ and 17, 17 and 17′, 17′ and 18	**EPS1**: 5′-GCG CAA ATT GCC CAA TGC AAA AAC ATT TCT GTG AGG CAG CGA CGA TCC GCA GCA ATC CTG CCA ACT CAT TTG TTG GTG GGA CTT GGA ATG GAT GCA ATC CAA ACA CAG T-3′
E. cantabrica	Helices 16, 16′, 4′, 17′ and 18 and regions between helices 5′ and 16, 16 and 16′, 16′ and 4′, between 4′ and 17, 17 and 17′, 17′ and 18	**EC1**: 5′-CAC GCA AAT TGC CCA ATG GAG AAA CAC MGT CCG AGG CAG CGA CGA ACC GTA GCA AT CCC GCC GGG RTT CTTC CCC ARY GGG ACT TGG RAT GGA TGC AAT CCA AAC ACA GTG A-3′
	Helices 26′, 24′, 23′ and 27 and regions between helix 26 and 26′, helix 26′ and 24′, helix 23′ and 27	**EC2**: 5′-GAA GGC ARG TGC GTG TTC GTT CGA TCA AGG ATG AGA GTT CGG GGA GCM AAG ATG-3′
	Helices 39′, 42 and 43 and regions between helix 39′ and 42, helix 42 and 43 and helix 43 and 43′	**EC3**: 5′-AAT GAG ATA TCT GCC TYC CAG TAG CCC CGG GCT CGT ATC CGG TAG GGC-3′
E. geniculata	Helices 17′, 18 and regions between helix 17 and 17′, helix 17′ and 18 and helix 18 and 18′	**EG1**: 5′-TCA YCC AGT GGG ACT TGG AAT GGA TGC AAT CCA AAC ACA G-3′
	Helices 24′, 23′, 27 and regions between helix 23′ and 27	**EG2**: 5′-TGC AAG GAY GTG TTC GTC GAT CAA GGA TGA GAG TCT GGG GAG CGA AGA TG-3′
E. chadefaudii	Helices 16, 16′ and 4′ and regions between helix 5′ and 16, helix 16 and 16′, helix 16′ and 4′ and helix 4′ and 17	**ECH1**: 5′-CGC GCA AAT TGC CCA RTG CAG ACA CCC CTG TGA GGC AGC GAC GAW CCG CAG-3′
	Helices 17′, 18 and regions between helix 17′ and 18, helix 18 and 18′	**ECH2**: 5′-CTC TGY YGG TGG GAC TTG GAA TGG ATG CAA TCC AAA CAC AGY GAT-3′
E. pseudochadefaudii	Helices 16, 16′, 4′, 17′ and 18 and regions between helices 5′ and 16, 16 and 16′, 16′ and 4′, between 4′ and 17, 17 and 17′, 17′ and 18	**EPC1**: 5′-CGC GTA ACT TGC CCA ATG CCA ACT CAA CTT TGG TGA GGC AGC GAC GAA TTG CAA CAATCCTGCCGACA CTC TTG TCT GTG GGA CTT GTG ATG GAT GCA ATC CAA AAA CCG T-3′
	Helices 24′, 23′, 27, E 23-1′and region between helix 23′ and 27	**EPC2**: 5′-TGC AAG ACT GTG TCC GTC AAT CAA GGA TGA GAG TTC GGG GAG CAA AGA GG-3′
	Helices 42 and 43 and regions between helix 39′ and 42, helix 42 and 43 and helix 43 and 43′	**EPC3**: 5′-CAT CTG CCT CCC AGT AGC CTG AGT CTC GCA AAT TGT AGG GGA GT-3′
E. tristella	Helices 16′, 4′, 17, 17′, 18 and regions between helix 16′ and 4′, helix 4′ and 17, helix 17 and 17′, helix 17′ and 18, and helix 18 and 18′	**ET1**: 5′-TGT GAG GCA GCG ACG AAC CGC AGC AAT CCT GC TGG CCC TCT TAG CCA GTG GGA CTT GGA ATG GAT GCA ATC CAA ACA CAG TG-3′
	Helices 42 and 43 and regions between helices 39′ and 42, helices 42 and 43 and helices 43 and 43′	**ET2**: 5′-ATC TCT GCY TCC CAG TAG CCT GRG GCT CGC ATC TGG TAG GGT C-3′

grains. Cytoplasm-encoded SSU rDNA molecule with A as second nucleotide of helix E23_8, T as second nucleotide of helix E23_8′, and A as fourth nucleotide of helix 31 (counting from 3′ end of helix).

Basionym: Cercaria viridis O. F. Müller, Animalcuta infusoria fluviatilia et marina, p. 126, pl. XIX, figs. 6–13, 1786.

Lectotype: (see Shin and Triemer, J. Phycol. 40:770, 2004): O. F. Müller, op. cit., fig. 10.

Epitype: (see Shin and Triemer, loc. cit. 2004): Lyophilized sample deposited at the Michigan State University Herbarium (Shin NJ 001). The figure 3H in Shin and Triemer (2004) and Figure 1, n and o, in this study are illustrations of the epitype.

Strain-type: Deposited in the American Type Culture Collection with the number SHIN NJ 001 ATC PRA-110; the epitype is described from the strain.

Synonyms: *E. viridis* var. *mucosa* Lemmermann, Kryptogamenflora der Mark Brandenburg, p. 492, 1910; *E. viridis* var. *purpurea* Playfair, Prock. Linn. Soc. New South Wales 46:117, 1921; *E. viridis* var. *lefevrei* Chadefaud, *Le Botaniste* 28:170, 1937; *E. viridis* var. *maritima* Pringsheim, Arch. Microbiol. 18:156, 157, fig. 7, 1953; *E. viridis* var. *halophila* Pringsheim, op. cit. 157, 158, fig. 8, 1953; *E. viridis* f. *salina* Popova, Izv. Zapadno-Sibirsk. Fil. Akad. Nauk. SSSR. Ser. Biol. 2:53, pl. 1, fig. 7, 1947; *E. archaeoviridis* Zakryś et Walne, Algol. Stud. 72:75, 76, fig. 5 (a–f), 1994.

Euglena pseudoviridis Chadefaud, Le Botaniste 28:108–113, fig. 9 (I–IV), 1937. Emend. Zakryś et Kosmala.

Emended diagnosis: Differs from *E. viridis* only at the molecular level. Cytoplasm-encoded SSU rDNA molecule with C as third nucleotide of helix 23′, A as first nucleotide of the spacer between helices 13 and 13′, and T as first nucleotide of the spacer between helices 46′ and 45′.

Lectotype: Here designated fig. 9 (I) in Chadefaud, d. c.

Epitype: Permanently preserved material (cells in resin, for EM) from the strain SAG 1224-17c, deposited at the herbarium of the Biology Faculty at Warsaw University, Al. Ujazdowskie 4. PL-00478 Warszawa, Poland (http://www.zielnik.biol.uw.edu.pl/zsigr.html). Figure 1m is the illustration of the epitype.

Strain-type: SAG 1224-17c is available at the Sammlung von Algenkulturen Pflanzenphysiologisches Institut der Universität Göttingen, Germany.

Euglena stellata Mainx, Arch. Protistenkd. pp. 159–160, fig. Da, 1926. Emend. Zakryś et Kosmala.

Emended diagnosis: Cells with spindle-shaped mucocysts, occuring in regular rows parallel to the periplast striation. If mucocysts are few, they appear irregularly distributed. Cells of diverse size (28–75 × 9–12 μm, on average), fusiform or cylindrically fusiform. One star-shaped, axial chloroplast located in front of the nucleus. Pyrenoid in the chloroplast center obscured by a cluster of paramylon grains. Cytoplasm-encoded SSU rDNA molecule with C as seventh nucleotide of helix 25′(counting from 3′ end of helix), C as third nucleotide of helix 38′ (counting from 3′ end of helix), and C as fourth nucleotide of helix 37′.

Lectotype: Here designated fig. Da in Mainx, d. c.

Epitype: Permanently preserved material (cells in resin, for EM) from the original strain isolated by Mainx (SAG 1224-14), deposited at the herbarium of the Biology Faculty at Warsaw University, Al. Ujazdowskie 4. PL-00478 Warszawa, Poland (http://www.zielnik.biol.uw.edu.pl/zsigr.html). Figure 1p is the illustration of the epitype.

Strain-type: SAG 1224-14 (the original strain isolated by Mainx) available at the Sammlung von Algenkulturen Pflanzenphysiologisches Institut der Universität Göttingen, Germany, and at the Culture Collection of Algae and Protozoa at Center for Ecology and Hydrology, Cumbria, UK (with the number CCAP 1224/14).

Synonyms: *E. stellata* f. *terricola* Pringsheim, Nova Acta Leopoldina 18:106, fig. 28D, 1956.

Euglena pseudostellata sp. nov. Zakryś et Kosmala

Diagnosis: Cellulae fusiformes, 45–65 × 12–15 μm, processu brevi hyalino terminatae. Mucocystae fusiformes, seriatim dispositae. Chloroplastus pyrenoidum includens, unicus, stellaris, ante nucleum dispositus. Nucleotida sequentiae SSU rDNA ita locuta sunt: nucleotidum T sextum in spatio inter helicis 47′ et 33′ et nucleotidum C quintum in helice 27′ (ab extremitate 3′ hujus helices).

Description: Differs from *E. stellata* only at the molecular level. Cells 46–65 × 12–15 μm on average; mucocysts spindle-shaped, in rows. One star-shaped chloroplast, located in front of the nucleus, with a pyrenoid obscured by a cluster of small paramylon grains. Differs from *E. stellata* only at the molecular level: cytoplasm-encoded SSU rDNA molecule with T as sixth nucleotide of the spacer between helices 47′ and 33′ and C as fifth nucleotide of helix 27′(counting from 3′ end of helix).

Type: Permanently preserved material (cells in resin, for EM) from the strain ACOI 2956 deposited at the herbarium of the Biology Faculty at Warsaw University, Al. Ujazdowskie 4. PL-00478 Warszawa, Poland (http://www.zielnik.biol.uw.edu.pl/zsigr.html). Figure 1h is the illustration of the type.

Type locality: Freshwater, small pond near Coimbra, Portugal.

Strain-type: ACOI 2956 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.

Euglena cantabrica Pringsheim, Nova Acta Leopoldina 18, pp. 110–111, fig. 32 (A–H), 1956. Emend. Zakryś et Kosmala.

Emended diagnosis: Cells with a pronounced metaboly, widely fusiform to widely cylindrically fusiform (36–84 × 14–24 μm, length/width ratio = 2.5–3), narrowed conically at the end, with a tendency for rounding during slow swim or arrest. Small, spherical mucocysts (~0.5–0.8 μm in diameter, visible only after staining, e.g., with neutral red), occurring in regular rows parallel to the periplast striation. If they are few, they appear irregularly distributed (scattered). Division in palmelloid state, surrounded by thick mucilage envelope, is dominant. A single, large, star-shaped chloroplast developing along the long axis, situated in front of the nucleus, with centrally located pyrenoid, which is obscured by a cluster of small paramylon grains.

Chloroplast arms reaching far toward the frontal and distal parts of the cell and bending their ends along the periplast curvature. Cytoplasm-encoded SSU rDNA molecule with inserted T after second nucleotide in helix 24′ (counting from 3′ end of helix), deletion of fifth nucleotide in helix 27′ and A as second nucleotide of helix 39′.

Lectotype: Here designated fig. 32A in Pringsheim, d. c.

Epitype: Permanently preserved material (cells in resin, for EM) from the original strain SAG 1224-25, deposited at the herbarium of the Biology Faculty at Warsaw University, Al. Ujazdowskie 4. PL-00478 Warszawa, Poland (http://www.zielnik.biol.uw.edu.pl/zsigr.html). Figure 1l is the illustration of the epitype.

Strain-type: SAG 1224-25 (the original strain isolated by Pringsheim) available at the Sammlung von Algenkulturen Pflanzenphysiologisches Institut der Universität Göttingen, Germany, and at the Culture Collection of Algae and Protozoa at Center for Ecology and Hydrology, Cumbria, UK (with the number CCAP 1224/33A).

Synonyms: *E. viridis* var. *olivacea* Klebs, Unters. Bot. Inst. Tübing., 1:297, 1883. *E. viridis* f. *olivacea* (Klebs) Popova, Flora sporovych rastenij SSSR, 7:244, 1966. *E. dicentra* Skuja, Symbolae Botanicae Upsalienses 9(3):184, 185, pl. 21, fig. 15, 1948. *E. cuneata* Pringsheim, Nova Acta Leopoldina 18:109, 110, fig. 31 (A–I), 1956. *E. viridis* var. *maxima* Philipose, Proc. Indian Acad. Sci (Plant Sci.) 91:593, fig. 23, 1982.

Euglena geniculata Schmitz, Jahrb. wiss. Bot., 15:11, pl. 1, fig. 11, 1884. non *E. geniculata* Dujardin, Hist. Natur. Zoophyten-Infusoires, Paris: 362, 1841. Emend. Zakrýs et Kosmala.

Emended diagnosis: Cells cylindrical, narrowed at the rear and ending with a tail of varying size (47–95 × 10–28 μm; length/width ratio = 8–11). Spherical mucocysts (~0.5–0.8 μm in diameter, visible only after staining, e.g., with a neutral red), occurring in rows located between the periplast striation. If mucocysts are few, they appear irregularly distributed (scattered). Two star-shaped, axial chloroplasts, one located in front of and the other behind the nucleus, each with a pyrenoid in the center, obscured by a cluster of small paramylon grains. The chloroplast located behind the nucleus tends to disappear in unfavorable habitat conditions. Cytoplasm-encoded SSU rDNA molecule with A as third nucleotide of helix 4′.

Lectotype: Here designated fig. 11, pl. 1 in Schmitz, d. c.

Epitype: Permanently preserved material (cells in resin, for EM) from the strain SAG 1224-4b deposited at the herbarium of the Biology Faculty at Warsaw University, Al. Ujazdowskie 4. PL-00478 Warszawa, Poland (http://www.zielnik.biol.uw.edu.pl/zsigr.html). Figure 1u is the illustration of the epitype.

Strain-type: SAG 1224-4b available at the Sammlung von Algenkulturen Pflanzenphysiologisches Institut der Universität Göttingen, Germany, and at the Culture Collection of Algae and Protozoa at Center for Ecology and Hydrology, Cumbria, UK (with the number CCAP 1224/4B).

Synonyms: *E. geniculata* Dujardin, Hist. Natur. Zoophyten-Infusoires, Paris: 362, pl. 5, fig. 15, 16, 1841. nomen dubium.; *E. geniculata* Dujardin ex Schmitz var. *terricola* Dangeard, Botaniste 8:153, fig. 5, 1901. *E. terricola* (Dang.) Lemmermann, Krypt. M. Brand., 3:493, fig. 6, 1910, *E. schmitzii* Conrad et Van Meel, Mém. Inst. R. Sc. nat. Belg., 124:153, 1952. *E. schmitzii* Gojdics, Madison, The Univ. of Wisc. Press. p. 75, pl. 5, fig. 2, a–c, 1953, *E. myxocylindracea* Bold and MacEntee, J. Phycol. 9:155, fig. 1, 1973.

Euglena chadefaudii Bourrelly 1951, Bull. Soc. Bot. France, 98:145, fig. A (A–I), 1951. Emend. Zakryś et Kosmala.

Emended diagnosis: Cells 40–112 μm long, 12–24 μm wide (length/width ratio = 3.5–4), fusiform or cylindrically fusiform, ending with a short, hyaline tail. Mucocysts spindle shaped, in rows parallel to the periplast striation. If mucocysts are few, they appear irregularly scattered. Two star-shaped, axial chloroplasts, one located in front of, the other behind the nucleus, each with a pyrenoid in the center, which is obscured by a cluster of small paramylon grains. The chloroplast located behind the nucleus has a tendency to disappear in inclement habitat conditions. Differs from *E. geniculata* in body and mucocyst shape. Cytoplasm-encoded SSU rDNA molecule with T as second nucleotide of helix 12 and A as second nucleotide of helix 12′(counting from 3′ end of helix).

Lectotype: Here designated fig. A (A) in Bourrelly, d. c.

Epitype: Permanently preserved material (cells in resin, for EM) from the strain CCAP 1224/17p deposited at the herbarium of the Biology Faculty at Warsaw University, Al. Ujazdowskie 4. PL-00478 Warszawa, Poland (http://www.zielnik.biol.uw.edu.pl/zsigr.html). Figure 1, e and f, is the illustration of the epitype.

Strain-type: CCAP 1224/17p deposited at the Culture Collection of Algae and Protozoa for Center of Ecology and Hydrology, Cumbria, UK.

Euglena pseudochadefaudii sp. nov. Zakryś et Kosmala

Diagnosis: Cellulae fusiformes vel cylindrice-fusiformes, 55–77 × 10–16 μm, processu brevi hyalino terminatae. Mucocystae fusiformes, seriatim dispositae. Chloroplasti stellares bini, uterque unicum pyrenoidum includens, primus ante, secundus post nucleum dispositus.

Nucleotida sequentiae SSU rDNA ita locuta sunt: nucleotidum A secundum in helice 13, nucleotidum T secundum in helice 13′ (ab extremitate 3′ hujus helices), nucleotidum C secundum in spatio inter helicis 5′ et 16 (ab extremitate 3′ hujus spatii),

nucleotidum A undecimum in helice 25′, nucleotidum T octavum in helice 25′ (ab extremitate 3′ hujus helices) et nucleotidum A tertium in spatio inter helicis 38′ et 36′.

Description: Differs from *E. chadefaudii* only at the molecular level. Cells 55–77 μm long × 10–16 μm wide (length/width ratio = 5.0), fusiform or cylindrically fusiform. Mucocysts spindle shaped, organized in rows. Two star-shaped, axial chloroplasts, one located in front of and the other behind the nucleus, each with a pyrenoid in its center obscured by a cluster of small paramylon grains. The chloroplast located behind the nucleus has a strong tendency to disappear in stressful habitat conditions. Differs from *E. chadefaudii* only at the molecular level: cytoplasm-encoded SSU rDNA molecule with A as second nucleotide of helix 13, T as second nucleotide of helix 13′(counting from 3′ end of helix), C as second nucleotide of the spacer between helices 5′ and 16 (counting from 3′ end of the spacer), A as 11 nucleotide of helix 25′, T as eighth nucleotide of helix 25′ (counting from 3′ end of helix), and A as third nucleotide of spacer between helices 38′ and 36′.

Type: Permanently preserved material (cells in resin, for EM) from the strain MI-00 deposited at the herbarium of the Biology Faculty at Warsaw University, Al. Ujazdowskie 4. PL-00478 Warszawa, Poland (http://www.zielnik.biol.uw.edu.pl/zsigr.html). Figure 1d is the illustration of the type.

Type locality: Small pond, East Lansing, Michigan, USA.

Strain-type: MI-00 deposited at Department of Plant Biology, Michigan State University, East Lansing, USA, and at the Culture Collection of Algae and Protozoa at Center for Ecology and Hydrology, Cumbria, UK.

E. tristella Chu, Sinensia 17, pp. 101–102, fig. 10, 1946. Emend. Zakryś et Kosmala.

Description: Cells 40–80 μm long × 11–23 μm wide (length/width ratio = 3.8), fusiform or cylindrically fusiform. Mucocysts spindle shaped, organized in rows. Three star-shaped chloroplasts; two of them axial (one located in front of and the other behind the nucleus), the third chloroplast located in the vicinity of stigma in frontal part of the cell. Each chloroplast with a pyrenoid in its center obscured by a cluster of small paramylon grains. Cytoplasm-encoded SSU rDNA molecule with T as fifth nucleotide of helix 25′(counting from 3′ end of helix) and G as first nucleotide of the spacer between helices E23_7′ and E23_8.

Lectotype: Here designated fig. 10 in Chu, d. c.

Commentary for taxonomic revision. We have not considered the two varieties of *E. geniculata* (var. *guttula* Playfair 1923, and var. *juvenilis* Playfair 1923) as synonyms of this species, because they have a different chloroplast organization than that found in *E. geniculata* (''chloroplast a thin, parietal lamina'').

We have not considered the species *E. circularis* Gojdics 1953 and *E. centralis* Gojdics 1953, which were described because of having circular paramylon grains, since we were unable to observe cells with such a shape of paramylon grains.

DISCUSSION

Mucocysts. There are several types of cellular structures secreting mucous in euglenoids, most likely of distinct evolutionary origin and having different functions, such as swimming or crawling, building stalks (in *Colacium*), or protecting palmella. They are collectively called mucocysts and often described in the literature very vaguely (Mignot 1966, Arnott and Walne 1967, Mignot and Hovasse 1973, Hausmann and Mignot 1977, Ward and Willey 1981, Hilenski and Walne 1983, Willey 1984). In species with axial, star-shaped chloroplasts, they are always uniform in size, small, subpellicular bodies with openings located between periplast folds (Dangeard 1901, Mainx 1926, Gojdics 1953, Mignot 1966, Arnott and Walne 1967, Buetow 1968). We consider this type to be true mucocysts. Such true mucocysts have been described in cells of *E. stellata* (under the name ''corps muciféres'') by Mignot (1966) and by Arnott and Walne (1967) under the name ''pellicle-pores'' in cells of *E. granulata.*

Therefore, true mucocysts are always aligned with more or less helically arranged periplast folds (Dangeard 1901, Mainx 1926, Gojdics 1953, Mignot 1966, Buetow 1968). If mucocysts in a cell are few, their rows become thinned, and they may appear as scattered, which was sometimes considered a diagnostic feature (Gojdics 1953, Shin and Triemer 2004). Our observations show that the number of true mucocysts depends on the overall physiological shape of the cell and its ontogeny stage. The true mucocysts are the most numerous in cells dividing slowly, yet in a good physical condition, and slightly less numerous in vigorously dividing cultures, as a consequence of sharing the organelles between progenitor cells. In old and overcrowded populations, when the habitat conditions are very unfavorable and signs of cell dying, such as hematochrome and paramylon accumulation in the cytoplasm as well as chloroplast disintegration become apparent, the number of true mucocysts also decreases. However, in species where true mucocysts are present, their size and shape are always the same, and they never disappear completely. Therefore, we have determined that the shape and the presence or absence of true mucocysts are good diagnostic features as previously indicated (Mainx 1926, Shin and Triemer 2004).

Chloroplast number and morphology. Historically, the chloroplast number and morphology were considered the most important diagnostic features in species with large, axial chloroplasts (Gojdics 1953, Huber-Pestalozzi 1955, Pringsheim 1956, Popova

1966, Popova and Safonova 1976, and many other floristic studies). Recent observations indicate, however, that this view is not entirely warranted, because in species with two or three chloroplasts within the cell, the chloroplast number depends heavily on the physiological condition of the cell, which in turn relies on habitat conditions (this study, Zakryś et al. 2002, Shin and Triemer 2004).

Therefore, several features, such as the number of chloroplasts, the presence and the shape of mucocysts, and, additionally, the size and the shape of cells, should be taken into account simultaneously for species identification. Only all these features taken together allow correct identification in most cases. Molecular studies, particularly concerning strains growing in cultures, are the method of choice when discrimination is still impossible, in spite of considering multiple characters. Thus, distinguishing large forms of *E. stellata* and/or *E. pseudostellata* (with a single chloroplast) from *E. chadefaudii* and/or *E. pseudochadefaudii*, which have lost one of their two chloroplasts, is easy at the molecular level. Distinguishing *E. tristella* with two chloroplasts, after having lost the third one, from *E. chadefaudii* and/or *E. pseudochadefaudii*, which retained both chloroplasts, is another example. Molecular studies are also necessary to distinguish *E. stellata* from *E. pseudostellata*, *E. viridis* from *E. pseudoviridis*, and *E. chadefaudii* from *E. pseudochadefaudii* because these pairs of species apparently do not differ morphologically. We decided to give these pairs of species separate names since they differ considerably at the molecular level, with respect to both 16S (not shown) and 18S rDNA sequences (Table 3).

Morphological chloroplast features, such as the size, shape, and length of chloroplast bands, depend on ontogeny stage and habitat conditions (see Results), and therefore none of these features could be considered separately as diagnostic.

Taxa with a single chloroplast: E. stellata was described by Mainx in 1926 as possessing a single, axial chloroplast, similar to *E. viridis*. As the main diagnostic features distinguishing it from *E. viridis*, Mainx recognized smaller cell size, smoother periplast, less conspicuous, stellate chloroplast, and preference for a more acidic nourishment. This description was amended by Chadefaud (1939), who pointed out the presence of fusiform true mucocysts in the cells of the original *E. stellata* strain, sent to him by Mainx. At the same time, he noticed spherical mucocysts present in *E. viridis* and proposed to distinguish another species, *E. pseudoviridis*, which was devoid of mucocysts altogether (Chadefaud 1937). Many authors, including those who published comprehensive monographs (Gojdics 1953, Huber-Pestalozzi 1955, Pringsheim 1956), agreed later with Chadefaud. Only Popova (1966, p. 242) and Chu (1946, p. 98) questioned the validity of distinguishing *E. stellata*, since they considered it an *E. viridis* variety. Shin and Triemer (2004) observed neutral-red-staining bodies in the strain NJ 001 of *E. viridis*, for which they established the epitype. We would argue that the structures visible in cells of the strain NJ 001 (3-H inset, Shin and Triemer 2004) are not true mucocysts for two reasons. First, the picture represents an optical cross-section through the middle of the cell and therefore is not showing the surface of it, nor is it showing the periplast stripes, between which true mucocysts are located. Second, the structures are too large (>1 μm in diameter) and not uniform in size to be considered spherical true mucocysts (Arnott and Walne 1967). Even though it is difficult to estimate their size precisely (the scale is missing), their considerable size could indirectly be inferred from a small magnification of the picture showing the entire cell. We have similar reservations regarding fig. 3-F inset in Shin and Triemer (2004), showing neutral-red-staining bodies in cells of the strain SAG 1224-17c. Our observations do not confirm the presence of true mucocysts there. Our experience with neutral red tells us that mucocysts are not the only structures being stained in the cytoplasm of a living cell. True mucocysts differ from other stained structures mainly in their localization and distribution. They lay between periplast stripes, forming helical rows parallel to periplast folding. Therefore, the stained structures recognized by Shin and Triemer (2004) in figures 3-H and 3-F are apparently not true mucocysts, as they do not fit their distribution and localization pattern. Buetow (1968, p. 142) warned against similar situations, pointing out that small, stained vacuoles could be mistaken for mucocysts.

Strains NJ 001, SAG 1224-17c, and SAG 1224-17d have spindle-shaped cells ending with a conspicuous, colorless tail. If mucocysts are not taken into account, they, with respect to their shape, most closely resemble the cells that Ehrenberg (1830, 1838) immortalized as *E. viridis* and Müller (1786) as *Cercaria viridis* (Ehrenberg does not mention mucocysts at all—see Ehrenberg 1830/1831, p. 82, and Ehrenberg 1831/1832, p. 71). The striking similarity comes from the fact that cells devoid of mucocysts display extensive plasticity, characterized by symmetrical inflation of a central, but not rear or front part of the cell. This plasticity is very similar to metaboly of *Discoplastis* (*Euglena*) *spathirhyncha* Skuja and may be very helpful by allowing an experienced observer to distinguish *E. viridis* from other species with a single chloroplast, without resorting to mucocyst staining. We therefore believe that the epitype of *E. viridis* was appropriately chosen, even though the strain has no spherical mucocysts, whose presence in the cells of this species was commonly accepted (e.g., Gojdics 1953, Huber-Pestalozzi 1955, Pringsheim 1956, Popova 1966, and many floristic studies) since Chadefaud (1937) amended its diagnostic description by including the feature. To avoid further confusion, we do not question the epitype of

E. viridis established by Shin and Triemer (2004), but we amend its diagnostic description. At the same time, we recognize *E. pseudoviridis* Chadefaud 1937, whose cells are devoid of mucocysts, as a separate species, differing from *E. viridis* only at the molecular level (difference of 18S rDNA sequences between *E. pseudoviridis* and *E. viridis* equals ~9%, Table 3).

Moreover, we do not think that it is justified to distinguish *E. archaeoviridis* Zakryś et Walne (Zakryś and Walne 1994) as a separate species. The shape of mucocysts was not considered, and its diagnostic feature, distinguishing it from *E. viridis*, is the presence of a pyrenoid. However, later studies have shown that pyrenoids are present in all species having axial, stellate chloroplasts, irrespective of their number, even though they are not always visible under the light microscope (Dragos et al. 1979, Péterfi et al. 1979, Zakryś and Walne 1998, Zakryś et al. 2001). Consequently, we consider *E. archaeoviridis* Zakryś et Walne a synonym of *E. viridis*.

We regard all other strains having cells with a single chloroplast and spherical mucocysts, which form a clade together with *E. cantabrica* (SAG 1224-25 original strain) and *E. cuneata* (SAG 26.93 original strain), such as ACOI 192 and SAG 1224-40, as representatives of *E. cantabrica*. Their 18S rDNA sequences differ only by up to 4% (Table 3). We thus give priority to the name ''cantabrica'' over ''cuneata,'' even though both species were described at the same time (Pringsheim 1956). Our decision comes from the fact that the diagnostic description of *E. cantabrica* is more precise, since Pringsheim is giving its cell dimensions as ''on the average 60 × 20 μm,'' while the cell size of the two species differs in our judgment only slightly. In our measurements, cell dimensions of the SAG 1224-25 strain of *E. cantabrica* are 59 × 24 μm, on average, while those of the *E. cuneata* strain SAG 26.93 are 50 × 19 μm. This has no diagnostic bearing and is further corroborated by the clade topology, in which strains with smaller and larger cells are intermixed. Cells of *E. cantabrica* are ''usually spindle-shaped'' and ''often cylindrical in the middle portion'' and those of *E. cuneata* ''often resembling a cylinder rather than a spindle,'' according to Pringsheim (1956, pp. 109–11). We believe that all features that supposedly differentiate the two species are rather subjective and not very precise and therefore have no diagnostic value. Strains constituting the ''cantabrica'' clade differ substantially with respect to cell size (36.5–84 × 13.7–29.6 μm), and two of them (SAG 1224-25 and SAG 1224-40) have the largest cells among species possessing a single chloroplast (Table 1). Their unifying features are broadly fusiform body shape and strong inclination for divisions in a palmelloid state, having a thick mucilaginous envelope. The presence of such a palmella in *E. viridis* var. *olivacea* Klebs [=*E. viridis* f. *olivacea* (Klebs) Popova 1966] was pointed out by Klebs (1883), Dangeard (1901), and Popova (1966), and therefore we have included these taxa into synonyms of *E. cantabrica*. Additionally, *E. viridis* f. *olivacea* strains from Russia had relatively large cells (65–70 × 14–16 μm). Because of its cell size (69–94 × 28–40 μm), *E. viridis* var. *maxima* Philipose, described in 1982 from India, was also regarded by us as a form of *E. cantabrica*. The size of Indian strains having a single, stellate chloroplast was the largest given so far. We conclude that, independent of the size and shape of broadly fusiform cells, the presence of spherical mucocysts and the preference for division in the lamella stage accurately distinguish *E. cantabrica* from other species with a single chloroplast.

Besides the *E. viridis* clade, characterized by lack of mucocysts, and the *E. cantabrica* clade, characterized by spherical mucocysts, there are two other clades of strains with a single chloroplast in the tree, both characterized by the presence of spindle-shaped true mucocysts (Fig. 2). The first group consists of the original strain *E. stellata* (SAG 1224-14) and other strains having small cells (34–40 × 9–11 μm, on average). The only exception here is the strain NJ ''sandy'' (57–75 × 8–12 μm, according to Shin and Triemer 2004). Another group consists of two strains (ACOI 2951 and ACOI 2956) with cells 54–56 μm long and 12–15 μm wide, on average. They differ from other strains only in SSUrDNA sequence. The position of this clade is very unstable. In most cases, it forms a sister relationship with the *E. stellata* or *E. chadefaudii* clades. We have given the taxon a new name *E. pseudostellata*, because cells of these two strains have only one chloroplast and two have never been observed. Differences between 18S rDNA sequences of *E. stellata* and *E. pseudostellata* are quite prominent and range from 10.7% to 11.6%, depending on the strain (Table 3).

With respect to the species with a single chloroplast, topology of our tree is very similar to that of Shin and Triemer (2004), in which there are the same three clades: *E. stellata, E. viridis,* and *E. cantabrica*. Our tree differs from theirs by having the forth clade, consisting of *E. pseudostellata*, and by different localization of the strain without mucocysts, *E. viridis* SAG 1224-17c, which in Shin and Triemer's tree (figs. 4, 5 in Shin and Triemer 2004) belongs to the *E. cantabrica* clade, characterized by the presence of spherical mucocysts.

Our studies did not confirm the validity of other features commonly used to diagnose species, such as chloroplast morphology, mucilage production in division stages, or habitat requirements, even though there was a large diversification of considered strains, regarding these features. Therefore, we wholly agree with Pringsheim (1956, p. 104), who questioned the validity of distinguishing many forms and varieties of *E. viridis* (var. *olivacea*, var. *lacustris*, var. *stagnalis*, var. *mucosa*, var. *purpurea*, var. *lefevrei*, var. *maritima*, var. *halophila*, f. *salina*) on the basis of

such characters, by arguing that ''the number of only slightly different forms is great in this group, and giving names to all of them would only increase confusion.''

Taxa with two chloroplasts: Four species have been described so far: *E. geniculata* (Dujardin) Schmitz 1884, *E. dicentra* Skuja 1948, *E. chadefaudii* Bourrelly 1951, and *E. myxocylindracea* Bold and MacEntee1973. The last one has been recently recognized as a synonym of *E. geniculata* (Zakryś et al. 2002).

E. geniculata has cylindrical cells with spherical mucocysts, while both cells and true mucocysts of *E. chadefaudii* are spindle shaped. Therefore, these two species may appear as easily distinguishable. Nevertheless, Pringsheim (1956) was skeptical about the validity of distinction of *E. chadefaudii* on the basis of a mucocyst shape, thus questioning the importance of spheroid mucocysts as a diagnostic feature in *E. geniculata* (Pringsheim 1956, p. 112). He maintained that he had observed many times in nature *E. geniculata* cells devoid of mucocysts and that sufficient criteria for distinguishing species with two chloroplasts are the body shape, presence of pyrenoids, and organization of stellate chloroplasts (Pringsheim 1956, p. 114). For this reason, he agrees with the validity of distinction of *E. geniculata* var. *terricola*, described as having ellipsoid cells and whose chloroplasts do not form star-shaped structures. No data on mucocysts were considered then. However, he is not consistent in his opinions, since he illustrates his own finding of *E. geniculata* var. *terricola* with a drawing of the cell with multiple, spindle-shaped mucocysts stained with neutral red (fig. 34C). He is not questioning this feature in species with a single chloroplast, either. Our studies have not confirmed Pringsheim's observations and therefore do not validate distinguishing *E. geniculata* var. *terricola* or *E. dicentra*. We have found that all investigated strains of *E. geniculata* had spherical mucocysts, regardless of their physiological status and habitat conditions. In the case of *E. geniculata* var. *terricola,* we noted chloroplast disintegration caused by unfavorable habitat conditions, while in the case of *E. cantabrica*, there exists a specific division form observable in all strains of this species. Widely fusiform cells of *E. cantabrica* with a profound metaboly, preparing for division, change their shape into ellipsoid. Therefore, when there is more available space, the two chloroplasts in a dividing cell often place themselves anterior and posterior to the nucleus. This configuration may be perceived as the presence of two chloroplasts instead of a transient state of a single chloroplast, resulting from ontogeny (Fig. 1k). In other species with a single chloroplast, such as *E. stellata* or *E. viridis*, the offspring chloroplasts do not change their location in the frontal part of a cylindrical or fusiform cell because they are not able to ''squeeze'' themselves along a large nucleus. Such an ellipsoid cell of *E. cuneata* with two chloroplasts located on both sides of a nucleus was succinctly described by Pringsheim (1956, fig. 32D, p. 110) as a ''contracted specimen with chromatophore bands radiating from two points.'' This description may suggest that he did not realize it was a dividing cell.

Our studies support the view of the existence of two distinct species with two chloroplasts. They are easily distinguished by different mucocyst shape. *E. geniculata* has spherical mucocysts, while in *E. chadefaudii* they are spindle-shaped. The two species form well-supported and separated clades in a phylogenetic tree (Fig. 2). Similar topology is found on Shin and Triemer's trees (figs. 4, 5 in Shin and Triemer 2004). The only difference is that they call the ''chadefaudii'' clade (strains ASW 08134, UTEX 85, and MI-00) ''stellata II.'' The reason for the name *E. stellata* was the presence of only a single chloroplast in the cells of these strains. Our studies have shown that these strains react to unfavorable culture conditions by losing a chloroplast located in a rear part of the cell. Only using less-sensitive strains of *E. chadefaudii*, such as CCAP 1224/17g or CCAP 1224/17p, which retain both chloroplasts in laboratory cultures, allows proper identification of species from the clade. Strain MI-00 differs considerably at rDNA sequence level from other strains of the clade to which it belongs (~15%, see Table 3, Fig. 2). We were unable to find a common signature sequence (Ekelund et al. 2004) for all the strains from this clade and decided to give the strain MI-00 a separate species name—*E. pseudochadefaudii*. We made a similar decision regarding two species of *Monomorphina*—*M. pyrum* and *M. pseudopyrum* (Kosmala et al. 2007b). All available descriptions show large diversification of *E. chadefaudii* populations, regarding its cell size: [54–67 × 15–18 μm (Gojdics 1953), 64–85 × 16.8–19.6 μm (Zakryś and Walne 1994), 65–90 × 18–24 μm (Kato 1982), 87–112 × 19–21 μm (Bourrelly 1951), 40–112 × 12–21 μm (Tell and Conforti 1986)]. We therefore took into account data from the literature in our diagnostic description, even though our own strains were smaller than those observed by Bourrelly and others.

Similarly, substantial diversification of cell size was observed between natural populations of *E. geniculata*: [35–89 × 11–22 μm (Chu 1946), 51–95 × 11–17 μm (Skuja 1948), 50–80 × 12–23 (Pringsheim 1956), 50–87 × 10–28 μm (Popova 1966), 50–95 × 12–22 μm (Németh 1997)]. This finding was further confirmed by studies of cultured strains (Zakryś et al. 2002, Shin and Triemer 2004), although the strains were identical on the molecular level (sequences of ITS2 were identical for all studied strains, Zakryś et al. 2002). The smallest cells in culture (47–60 × 10–12 μm) had the strain MI-61 (Shin and Triemer 2004, Table 1). Again, the literature data were taken into account in amended diagnosis.

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Arnott, H. J. & Walne, P. L. 1967. Observations on the fine structure of the pellicle pores of *Euglena granulata*. *Protoplasma* 64:330–44.

Bourrelly, P. 1951. *Euglena chadefaudii*, une nouvelle espèce d'Euglène viridoide. *Bull. Soc. Bot. France* 98:143–5.

Bremer, K. 1994. Branch support and tree stability. *Cladistics* 10:295–304.

Buetow, D. M. 1968. Morphology and ultrastructure of *Euglena*. *In* Buetow, E. D. [Ed.] *The Biology of Euglena*. Academic Press, New York, pp. 110–81.

Chadefaud, M. 1937. Recherches sur l' anatomie comparée des Eugléniens. *Le Botaniste* 28:86–185.

Chadefaud, M. 1939. Sur l'organisation d'*Euglena stellata* Mainx et sur la discrimination des Euglénes viridoides. *Arch. Zool. Exp. Gén. Notes Rev.* 80:49–54.

Chu, S. P. 1946. Contributions to our knowledge of the genus *Euglena*. *Sinensia* 17:75–134.

Dangeard, P. A. 1901. Reserches sur les Eugleniens. *Le Botaniste* 8:97–360.

Dangeard, P. A. 1928. L'appareil mucifere et le vacuome chez les Euglenes. *Ann. Protist.* 1:69–74.

Dragos, N., Péterfi, L. S. & Craciun, C. 1979. Fine structure of *Euglena*. II. *Euglena stellata* Mainx and *Euglena viridis* Ehrenberg. *Nova Hedwigia* 31:223–46.

Dujardin, F. 1841. *Historie naturalle des Zoophytes-Infusories*. Roret, Paris, 684 pp.

Ehrenberg, C. G. 1830. Beiträge zur Kenntnis der Organisation der Infusorien und ihrer geographischen Verbreitung, brsonders in Sibirien. *Physik. Könıgl. Akad. Wiss. Berlin* 1830:1–88.

Ehrenberg, C. G. 1838. *Die Infusionsthierchen als vollkommene Organismen. Ein Blick in das tiefere organische Leben der Natur. Nebst einem Atlas von vierundsechszig coloriten Kupfertafeln, gezeichnet vom Verfasser*. Leopold Voss, Berlin, 548 pp.

Ekelund, F., Daugbjerg, N. & Fredslund, L. 2004. Phylogeny of *Heteromita*, *Cercomonas* and *Thaumatomonas* based on SSU rDNA sequences, including the description of *Neocercomonas jutlandica* sp. nov., gen. nov. *Eur. J. Protistol.* 40:119–35.

Eriksson, T. 1998. *AutoDecay 4.0.2*. A program distributed by the author. Department of Botany, Stockholm University, Stockholm, Sweden.

Felsenstein, J. 1985. Confidence limits on phylogenies: approach using the bootstrap. *Evolution* 39:783–91.

Francé, R. 1897. Protozoen. *In* Entz, G. [Ed.] *Resultate der wissenschaftlichen Erforschung des Balatonsees. 2. Band. Die Biologie des Balatonsees*. Commissionsverlag Von. Ed. Hölzel, Wien, Germany, pp. 29–32.

Gojdics, M. 1953. *The Genus* Euglena. The University of Wisconsin Press, Madison, Wisconsin, 253 pp.

Hausmann, K. & Mignot, J. P. 1977. Unterschungen an den mucocysten von *Euglena splendens* Dangeard 1901. *Protistologica* 13:213–7.

Hilenski, L. L. & Walne, P. L. 1983. Ultrastructure of ejectile mucocysts in *Peranema trichophorum* (Euglenophyceae). *J. Protozool.* 30:491–6.

Huber-Pestalozzi, G. 1955. *Das Phytoplankton des Süsswassers IV. Euglenophyceen. E.* Schweizerbart'sche Verlagsbuchhandlung, Stuttgart, Germany, 1135 pp.

Huelsenbeck, J. P. & Ronquist, F. 2001. MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics* 17:754–5.

Kato, S. 1982. Taxonomic studies on the genus *Euglena* in Japan (1). *J. Jpn. Bot.* 57:217–21.

Klebs, G. 1883. Über die Organisation einiger Flagellaten-Gruppen und ihre Beziehungen zu Algen und Infusorien. *Untersuchung Botan. Inst. Tübingen* 1:233–362.

Kosmala, S., Bereza, M., Milanowski, R., Kwiatowski, J. & Zakryś, B. 2007a. Morphological and molecular examination of relationships and epitype establishment of *Phacus pleuronectes, Phacus orbicularis* and *Phacus hamelii*. *J. Phycol.* 43:1071–82.

Kosmala, S., Karnkowska, A., Milanowski, R., Kwiatowski, J. & Zakryś, B. 2005. The phylogenetic and taxonomic position of *Lepocinclis fusca* comb. nova (=*Euglena fusca*) (Euglenaceae). Morphological and molecular justification. *J. Phycol.* 41:1258–67.

Kosmala, S., Milanowski, R., Brzóska, K., Pękala, M., Kwiatowski, J. & Zakryś, B. 2007b. Phylogeny and systematics of the genus *Monomorphina* (Euglenaceae) based on cytoplasmic SSU sequence analysis. *J. Phycol.* 43:171–85.

Lanave, C., Preparata, G., Saccone, C. & Serio, G. 1984. A new method of calculating evolutionary substitution rate. *J. Mol. Evol.* 20:86–93.

Lemmermann, E. 1910. Beiträge zur Kenntnis der Planktonalgen. *Arch. Hydrobiol.* 5:291–338.

Mainx, F. 1926. Einige neue Vertreter der Gattung *Euglena* Ehrenberg. *Arch. Protistenk.* 54:150–64.

Marin, B., Palm, A., Klingberg, M. & Melkonian, M. 2003. Phylogeny and taxonomic revision of plastid-containing Euglenophytes based on SSU rDNA sequence comparisons and synapomorphic signatures in the SSU rRNA secondary structure. *Protist* 154:99–145.

Mignot, J. P. 1966. Structure et ultrastructure de quelques Euglenomonadines. *Proistologica* 2:51–117.

Mignot, J.-P. & Hovasse, R. 1973. Nouvelle Contribution a la Connaissance des Trichocystes Les Organites Grillages *d'Entosiphon sulcatum* (Flagellata, Euglenida). *Protistologica* 9:373–91.

Milanowski, R., Kosmala, S., Zakryś, B. & Kwiatowski, J. 2006. Phylogenetic analysis of photosynthetic euglenophytes based on combined chloroplast and cytoplasmic SSU rDNA sequences. *J. Phycol.* 42:721–30.

Milanowski, R., Zakryś, B. & Kwiatowski, J. 2001. Phylogenetic analysis of chloroplast small-subunit rRNA genes of the genus *Euglena* Ehrenberg. *Int. J. Syst. Evol. Microbiol.* 51:773–81.

Nicholas, K. B. & Nicholas, H. B. 1997. *GeneDoc: Analysis and Visualization of Genetic Variation*. Program distributed by authors. Available at: http://www.nrbsc.org/downloads (accessed 01 February 2009).

Németh, J. 1997. *Az Ostoros Algák Kishatározója 1 (Euglenophyta). Vizügyi Hidrobiológia 8*. Környezetgazdálkodási Intézet, Budapest, 319 pp. (in Hungarian).

Nylander, J. A. A. 2004. *MrModeltest v2*. Program distributed by the author. Evolutionary Biology Centre, Uppsala University, Uppsala, Sweden.

Page, R. D. M. 1996. TREEVIEW: an application to display phylogenetic trees on personal computers. *Comp. Appl. Biosci.* 12:357–8.

Philipose, M. T. 1982. Contributions to our knowledge of Indian algae-III. Euglenaceae-Part-I. The genus *Euglena* Ehrenberg. *Proc. Indian Acad. Sci. (Plant Sci.)* 91:551–99.

Playfair, G. J. 1921. Australian freshwater flagellates. *Proc. Linn. Soc. N.S. Wales, Sydney* 46:99–146.

Playfair, G. J. 1923. Notes on freshwater algae. *Proc. Linn. Soc. N. S. Wales, Sydney* 48:206–28.

Popova, T. G. 1947. Sistematiceskije zametki po evglenovym. [Taxonomical note about euglenophytes]. *Izv. Zap. Sib. Fil. AN SSSR Ser. Biol. (Novosibirsk)* 2:47–71 (in Russian).

Popova, T. G. 1966. *Euglenophyta 1. Flora Plantarum USSR. 8*. Nauka, Moskva-Leningrad, 411 pp.

Popova, T. G. & Safonova, T. A. 1976. *Flora Sporovych Rastenij SSSR, 9*. [Flora plantarum cryptogamarum URSS]. Euglenophyta. 2. Izd. Nauka, Leningrad, 286 pp.

Posada, D. & Crandall, K. A. 1998. MODELTEST: testing the model of DNA substitution. *Bioinformatics* 14:817–8.

Pringsheim, E. G. 1953. Salzwasser-Eugleninen. *Arch. Microbiol.* 18:149–64.

Pringsheim, E. G. 1956. Contributions towards a monograph of the genus *Euglena*. *Nova Acta Leopoldina* 18:1–168.

Péterfi, L. S., Dragos, N. & Craciun, C. 1979. Fine structure of *Euglena*. I. *Euglena tristella* Chu. *Nova Hedwigia* 31:197–221.
Rodriguez, F., Oliver, J. L., Marin, A. & Medina, J. R. 1990. The general stochastic model of nucleotide substitution. *J. Theor. Biol.* 142:485–501.
Ronquist, F. & Huelsenbeck, J. P. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 19:1572–4.
Schlösser, U. G. 1994. SAG-Sammlung von Algenkulturen at University of Göttingen. Catalogue of strains 1994. *Bot. Acta* 107:111–86.
Schmitz, F. 1884. Beiträge zur Kenntnis der Chromatophoren. *Pringsh. Jahrb. Wiss. Bot.* 15:1–175.
Shin, W. & Triemer, R. E. 2004. Phylogenetic analysis of the genus *Euglena* (Euglenophyceae) with particular reference to the species *Euglena viridis*. *J. Phycol.* 40:759–71.
Skuja, H. 1948. Taxonomie des Phytoplanctons einiger Seen in Uppland. *Schweden. Symb. Bot. Uppsal.* 9:1–399.
Swofford, D. L. 1998. *PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods)*. Version 6. Sinauer Associates, Sunderland, Massachusetts.
Tamura, K. & Nei, M. 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Mol. Biol. Evol.* 10:512–26.
Tavare, S. 1986. Some probabilistic and statistical problems on the analysis of DNA sequences. *Lec. Math. Life Sci.* 17:57–86.
Tell, V. & Conforti, G. 1986. *Euglenophyta Pigmentadas de la Argentina*. J. Cramer in der Gebrüder Borntraeger Verlegsbuchhandlung, Berlin-Stuttgart, Germany, 31 pp.
Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* 24:4876–82.
Ward, K. A. & Willey, R. L. 1981. The development of a cell-substrate attachment system in a euglenoid flagellate. *J. Ultrastruct. Res.* 74:165–74.
Willey, R. L. 1984. Fine structure of the mucocysts of *Colacium calvum* (Euglenophyceae). *J. Phycol.* 20:426–30.
Wuyts, J., Van de Peer, Y., Winkelmans, T. & De Wachter, R. 2002. The European database on small subunit ribosomal RNA. *Nucleic Acids Res.* 30:183–5.
Zakryś, B. 1997. On the identity and variation of *Euglena agilis* Carter (=*E. pisciformis* Klebs). *Algol. Stud.* 86:81–90.
Zakryś, B., Empel, J., Milanowski, R., Gromadka, R., Borsuk, P., Kędzior, M. & Kwiatowski, J. 2004. Genetic variability of *Euglena agilis* (Euglenophyceae). *Acta Soc. Bot. Pol.* 73:305–9.
Zakryś, B., Jaume-Sanchez, J. & Walne, P. L. 2001. Chloroplast ultrastructure of *Euglena cantabrica* Pringsheim, *E. cuneata* Pringsheim, *E. deses* Ehrenberg, *E. mutabilis* Schmith (Euglenophyceae): taxonomic significance. *Acta Protozool.* 40:161–7.
Zakryś, B. & Kucharski, R. 1996. Microevolutionary processes in *Euglena pisciformis*. Genetic drift or adaptation? *Algol. Stud.* 81:23–37.
Zakryś, B., Kucharski, R. & Moraczewski, I. 1996. Genetic and morphological variability among clones of *Euglena pisciformis* based on RAPD and biometric analysis. *Algol. Stud.* 81:1–21.
Zakryś, B., Milanowski, R., Empel, J., Borsuk, P., Gromadka, R. & Kwiatowski, J. 2002. Two different species of *Euglena*, *E. geniculata* and *E. myxocylindracea* (Euglenophyceae), are virtually genetically and morphologically identical. *J. Phycol.* 38:1190–9.
Zakryś, B. & Walne, P. L. 1994. Floristic, taxonomic and phytogeographic studies of green Euglenophyta from southeastern United States, with emphasis on new and rare species. *Algol. Stud.* 72:71–114.
Zakryś, B. & Walne, P. L. 1998. Comparative ultrastructure of chloroplasts in the subgenus *Euglena* (Euglenophyta): taxonomic significance. *Cryptogam. Algol.* 19:3–18.

Supplementary Material

The following supplementary material is available for this article:

Table S1. The euglenoid strains and the corresponding 16S rDNA and 18S rDNA GenBank accession numbers for the taxa used in this study. Accession numbers of new sequences are in bold face. Strains used in morphological studies are underlined.

This material is available as part of the online article.