

## Research Article

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# Morphological and genetic diversity of *Euglena deses* group (Euglenophyceae) with emphasis on cryptic species

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The *Euglena deses* group are common freshwater species composed of *E. adhaerens*, *E. carterae*, *E. deses*, *E. mutabilis*, and *E. satelles*. These species are characterized by elongated cylindrical worm-like cell bodies and numerous discoid chloroplasts with a naked pyrenoid. To understand the cryptic diversity, species delimitation and phylogenetic relationships among members of the group, we analyzed morphological data (light and scanning electron microscopy) and molecular data (nuclear small subunit [SSU] and large subunit [LSU] rDNAs and plastid SSU and LSU rDNAs). Bayesian and maximum likelihood analyses based on the combined four-gene dataset resulted in a tree consisting of two major clades within the group. The first clade was composed of two subclades: the *E. mutabilis* subclade, and the *E. satelles*, *E. carterae*, and *E. adhaerens* subclade. The *E. mutabilis* subclade was characterized by a lateral canal opening at the anterior end and a single pellicular stria, whereas the *E. satelles*, *E. carterae*, and *E. adhaerens* subclade was characterized by an apical canal opening at the anterior end of the cell and double pellicular striae. The second clade consisted of 20 strains of *E. deses*, characterizing by a subapical canal opening at the anterior end and double pellicular striae, but they showed cell size variation and high genetic diversity. Species boundaries were tested using a Bayesian multi-locus species delimitation method, resulting in the recognition of five cryptic species within *E. deses* clade.

**Key Words:** *Euglena deses* group; molecular phylogeny; morphology; phylogeny; species delimitation

**Abbreviations:** LSU, large subunit; ML, maximum likelihood; MLBT, ML bootstrap support value; nr, nuclear; nt, nucleotide; PP, posterior probability; pt, plastid; SEM, scanning electron microscopy; SSU, small subunit

## INTRODUCTION

The species delimitation is essential for both biologists and the general public because species are fundamental units to understand ecosystem and biodiversity assessments in most fields with living organisms. In addition, taxonomic rank is a language used by scientists to help the public recognize the diversity, ecology, distribution, and evolutionary history of living organisms. However, the morphologically similar and simple features in uni-

cellular algal species can make it extremely difficult to perform species identification and delimitation. In microalgae, an insufficient number of morphological features that could be used to clearly distinguish one species from another enhances the problem of species delimitation. Hidden or cryptic, i.e., morphologically highly similar species, are frequently described in green algae (Lewis and Flechtner 2004, Fawley et al. 2011, Demchenko et al.



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2012), chrysophytes (Škaloud et al. 2012, 2014, Jo et al. 2013), and diatoms (Mann et al. 2004, Lundholm et al. 2012). This problem is particularly difficult in euglenoids where authors often struggle to differentiating hidden species morphologically, and thus define cryptic species according to the phylogenetic species concept (Kosmala et al. 2007b, 2009, Kim et al. 2013a, 2013b, Kim and Shin 2014).

Traditionally, the species of the *Euglena deses* group (i.e., *E. adhaerens*, *E. carterae*, *E. deses*, *E. mutabilis*, and *E. satelles*) were classified according to taxonomic systems based on morphological characteristics. Chu (1946) sorted these species into group II under the genus *Euglena* using the characteristics of chloroplast shape and number and the presence of a naked pyrenoid. Gojdics (1953) categorized these species into B or E groups according to the number, arrangement and shape of the chloroplasts. Pringsheim (1956) classified the *E. deses* group into the subgroup *Serpentes* based on cell shape, metabolic degree and chloroplast type. More recently, Zakryś (1986) arranged these species into both subgenera *Discoglana* and *Calliglana* based on characteristic features of the chloroplast, for example small, numerous discoid chloroplasts without pyrenoid and fewer discoid chloroplasts with a naked pyrenoid. Unfortunately, the taxonomy of these euglenoids has been depended mainly on morphological characteristics recognizable by light microscopy. However, for many of these species and varieties, it is difficult to distinguish details because of strong metabolic movements and similar morphological features. In addition, in old cells crowded with paramylon grains, the diagnostic features of the chloroplasts used to identify these species are masked, and a definitive identification of pyrenoids is inhibited. The use of fixatives and dyes as methods to identify these cells remains limited. However, transmission electron microscopy and scanning electron microscopy (SEM) have been increasingly applied to clarify details of cell organelles (Leedale 1967, 1982, Triemer 1980, Walne et al. 1986, Shin and Boo 2001, Zakryś et al. 2001, Kim and Shin 2007, Kusel-Fetzmann and Weidinger 2008, Monfils et al. 2011).

Molecular analyses have also been attempted to understand the phylogenetic relationships among euglenacean species, including species of the *E. deses* group (Marin et al. 2003, Shin and Triemer 2004, Triemer et al. 2006, Kim and Shin 2008, Kim et al. 2010, Linton et al. 2010, Karnkowska-Ishikawa et al. 2011). Recently, Karnkowska-Ishikawa et al. (2011) synonymized all morphological varieties of *E. deses* to only one species, based on a morphologically recognizable light microscopy character

(periplast mucus papillae) and nuclear-encoded small subunit (nr SSU) rDNA sequence data, but recognized high genetic diversity and extreme variation in cell dimensions. However, that study was limited to only 10 cultured strains of the *E. deses* group and analyzed only partial nr SSU rDNA sequence data.

*Euglena deses* is a dominant freshwater species with many morphological varieties characterizing by an elongated cylindrical cell shape, strong metaboly, numerous discoid chloroplasts with a naked pyrenoid and rod-shaped paramylon grain in the cytoplasm. Since Ehrenberg (1835, 1838) first established the species *E. deses* in drawings, other investigators have described many taxa with morphology similar to that of *E. deses* (Klebs 1883, Schmitz 1884, Lemmermann 1910, Playfair 1921, Wermel 1924, Mainx 1927, Fritsch et al. 1930, Braslavskaja-Spectrova 1937, Matvienko 1938, Pringsheim 1953, 1956, Popova 1955, 1966, Zakryś 1986, Shi 1989). However, there was little known about species limitation and phylogeny of *E. deses* based on morphological and molecular data. In this study, we focused on species delimitation of *E. deses*, one of the most common members of the genus *Euglena*. In particular, we aimed to 1) morphologically and genetically differentiate among the *Euglena deses* group, using 32 strains; 2) examine phylogenetic relationships of the species within group; 3) explore cryptic diversity of *E. deses* using species delimitation analyses. The present study will contribute to our understanding of the cryptic diversity of the *E. deses* group.

## MATERIALS AND METHODS

### Strains and cultures

The strain information and GenBank accession numbers are listed in Supplementary Table S1. Strains were either obtained from culture collections or were collected using a 20 µm mesh plankton net (Bokyeong Co., Busan, Korea) from small ponds in the USA, the Philippines, Japan and Korea. All of the strains were grown in modified AF-6 medium (Watanabe and Hiroki 1997) and were maintained at 20-22°C under conditions of a 14 : 10 light : dark cycle with 30 µmol photons m<sup>-2</sup> s<sup>-1</sup> from cool white fluorescent tubes.

### Strain identification and observation

Culture strains were observed and identified under an Axio Imager A2 microscope (Carl Zeiss Inc., Hallberg-

moos, Germany) equipped with differential interference contrast optics. Images were captured with an AxioCam HRc (Carl Zeiss Inc.) photomicrographic system attached to the microscope. For morphological measurements, we used 3-4-week-old cultures of each species. Cellular dimensions were determined by measuring 25-35 cells of each taxon from photographic images. The morphological features, the cell size and shape, paramylon type, canal position, caudal shape and pellicle structure, were analyzed (Table 1).

### Scanning electron microscopy

To obtain fully extended cells, a small volume of cells in liquid medium (1-2 mL) was transferred into the bottom of a small petri dish containing a piece of filter paper saturated with 2% OsO<sub>4</sub> and mounted on the inner surface of the lid. The lid was then placed over the chamber, and the cells were fixed by OsO<sub>4</sub> vapors for 10 min. Four to five drops of 2% OsO<sub>4</sub> were added directly into the liquid medium, and the cells were fixed for another 30 min. The cells were transferred onto 0.45- $\mu$ m nylon membrane filters (Whatman International Ltd., Maidstone, UK), dehydrated with a graded series (50, 60, 70, 80, 90, and 100%) of ethyl alcohol or acetone and dried in a critical point dryer (HSP-2; Hitachi, Tokyo, Japan) with CO<sub>2</sub>. The filters were mounted on stubs and sputter-coated with platinum. The cells were viewed under a LEO-1530 FE-SEM (Carl Zeiss Inc., Hallbergmoos, Germany).

### DNA extraction, amplification, sequencing, and sequence alignments

All 78 strains, including six outgroup taxa, were grown and harvested, and DNA was extracted from cultured cells as previously described (Kim and Shin 2008, Kim et al. 2010). The four genes were sequenced from plasmid-like chromosomes found in cytoplasmic SSU and large subunit (LSU) (Greenwood et al. 2001) and plastid-encoded SSU (pt SSU) and LSU rDNA genes (Kim and Shin 2008, Kim et al. 2013b). A total of 96 new sequences were generated, including 19 sequences of nr SSU, 21 sequences of nr LSU, 24 sequences of pt SSU, and 21 sequences of pt LSU rRNA genes. All sequences were aligned by eye using the Genetic Data Environment (GDE 2.4) program (Smith et al. 1994), using the secondary structure of the cytoplasmic SSU and LSU rRNA molecules of *Euglena gracilis* Klebs (Wuyts et al. 2001, Schnare and Gray 2011) as a guide. The conserved regions of the four genes were readily alignable across taxa and were used for the phy-

logenetic analyses. All ambiguous positions excluded for the phylogenetic analyses and pair-wise comparisons.

### Phylogenetic analyses

A combined dataset of 5,384 nucleotides (nr SSU, 1,653; nr LSU, 766; pt SSU, 1,336; pt LSU, 1,629) was generated for the phylogenetic analyses. The sequences of the six species of the family Phacaceae were used as outgroup taxa because these taxa were shown to represent a basal clade in previous molecular studies of photosynthetic euglenoids (Triemer et al. 2006, Kim and Shin 2008, Kim et al. 2010, Linton et al. 2010, Kim et al. 2015).

Bayesian analyses were performed using MrBayes 3.2 (Ronquist et al. 2012). A combined data analysis was performed using the four Metropolis-coupled Markov chain Monte Carlo (MC<sup>3</sup>) with 10 million cycles for each chain. The trees were saved to a file every 1,000 cycles, and the burn-in point was identified graphically by tracking the likelihood values (TRACER v. 1.6; <http://tree.bio.ed.ac.uk/software/tracer/>).

The maximum likelihood (ML) phylogenetic analyses were performed using RAxML 8.1.20 (Stamatakis 2014) with the general time reversible plus Gamma (GTR + GAMMA) model. We used 1,000 independent tree inferences using the *-#* option of the program to identify the best tree. The *-f a* option was used in RAxML for the simultaneous search for the best likelihood tree and rapid bootstrap analysis with *"-# 1,000"* (1,000 bootstrap replications) with default options. Gamma correction values of the combined dataset were obtained automatically with the program (Supplementary Table S2). ML bootstrap support values (MLBT) of each monophyletic node calculated using 1,000 bootstrap replications under the same ML setting.

### DNA-based species delimitation

We used a recently developed Bayesian method, Bayesian Phylogeography and Phylogenetics (BP&P v3), which aims to detect signals of species divergence in multiple gene trees, even in the absence of monophyly, based on models combining species phylogeny and the ancestral coalescent process, and assuming no admixture following the speciation event (Yang and Rannala 2014). The marginal posterior probability of 5-species scenario suggested by molecular data was estimated using the program. BP&P gives the posterior probability of each delimited species and the posterior probability for the number of delimited species. A gamma prior G (1, 10), with mean

**Table 1.** Comparison of cell morphology of *Euglena deses* complex

Taxon	Strain (No. of individual)	Cell length Mean ± SD (min-max)	Cell width Mean ± SD (min-max)	Ratio of length / width	Chloroplast (diameter)	Pellicle shape	Paramylon shape	Position of canal opening	Shape of posterior end	Reference
<i>E. adhaerens</i>	Songdang042007A (n = 28) ASW08138	92.8 ± 9.0 (76.3-110.3) 124.1 ± 8.2 (107.2-135.3)	13.9 ± 1.4 (11.3-16.9) 9.3 ± 1.1 (7.2-12.1)	6.7 13.3	4.9 ± 0.7 (4.0-6.6)	Single Single	Short-rod -	Apical Apical	Wedge -	This study Karnkowska-Ishikawa et al. (2011)
<i>E. carterae</i>	SAG1224-22	98.4 ± 7.9 <sup>b</sup> (81.5-134.9)	10.4 ± 1.3 <sup>b</sup> (7.6-14.3)	9.5	11.0 ± 1.0 <sup>a</sup> (9.5-13.2)	Narrow <sup>a</sup> Double	Small <sup>a</sup> Ellipse	Apical <sup>a</sup>	Round <sup>a</sup>	-
<i>E. deses</i> D1 clade	Saeraewool103109C (n = 30)	121.4 ± 6.3 (111.4-132.1)	10.3 ± 2.8 (7.6-17.4)	11.8	6.8 ± 1.2 (5.9-7.6)	Double	Ellipse	Subapical	Acute	This study
	Saeraewool102007R	102.6 ± 12.5 (78.7-131.4)	12.1 ± 1.5 (9.5-14.9)	8.5	-	Double	-	Subapical	-	This study
	Yongsu042007D (n = 31)	137.3 ± 15.2 (100.1-164.1)	10.4 ± 1.2 (8.1-12.5)	13.2	7.8 ± 1.3 (7.1-10.1)	Double	Small ellipses	Subapical	Acute	This study
<i>E. deses</i> var. <i>deses</i> D2 clade	SAG1224-19b (n = 26) Daeriji010910A (n = 28) ASW08074	83.2 ± 7.2 (69.9-96.2) 77.5 ± 5.3 (66.2-92.2) 163.8 ± 13.9 (133.5-199.2)	7.7 ± 0.8 (6.0-8.9) 10.0 ± 1.4 (7.1-12.3) 16.1 ± 2.0 (10.5-22.7)	10.8 7.8 10.2	6.0 ± 0.5 (5.0-6.8) 4.9 ± 0.4 (4.1-5.5) 8.7 (4.9-13.3)	Double Double Double Double	Short-rod Rod Rod	Subapical Subapical Subapical	Wedge Wedge? acute? Wedge? acute?	This study This study Karnkowska-Ishikawa et al. (2011)
<i>E. deses</i> D3 clade	Dongsan101709A (n = 26)	160.2 ± 13.2 (137.3-186.4)	12.1 ± 1.9 (7.5-15.7)	13.2	6.6 ± 0.8 (5.1-7.7)	Double	Ellipse	Subapical	Acute	This study
	Jeongsan110104-5 (n = 25) SAG1224-23	192.3 ± 18.3 (173.2-246.2) 163.9 ± 15.9 (119.5-206.3)	16.1 ± 2.6 (12.2-23.2) 18.5 ± 2.3 (14.3-24.0)	11.9 8.9	4.9 ± 0.6 (4.0-6.3) 6.9 (3.2-12.4)	Double Double	Ellipse -	Subapical Subapical	Wedge Wedge	This study Karnkowska-Ishikawa et al. (2011)
	Sinweoljunje120807A (n = 31)	144.9 ± 8.5 (130.7-158.4)	13.0 ± 1.4 (10.3-15.7)	11.1	7.06 ± 0.81 (5.4-8.6)	Double	Rod	Subapical	Wedge	This study
<i>E. deses</i> D4 clade	Bihaksan031007A (n = 35)	107.5 ± 5.8 (98.5-117.9)	10.7 ± 1.3 (8.7-12.8)	10.1	9.0 ± 0.8 (7.8-10.3)	Double	Short-rod	Subapical	Acute	This study
	Jeongsan092004A (n = 25) MI104, USA (n = 27) SAG1224-20	98.1 ± 8.3 (86.6-118.4) 90.6 ± 5.6 (82.0-103.2) 83.4 ± 12.8 (53.3-123.9)	10.4 ± 1.4 (8.6-14.4) 7.8 ± 0.8 (6.5-9.2) 10.3 ± 1.2 (8.0-12.8)	9.4 11.7 8.1	7.2 ± 0.8 (5.9-7.9) 6.2 ± 0.6 (5.4-7.3) 7.6 (5.0-11.7)	Double Double Double Double	Short-rod Short-rod Short-rod -	Subapical Subapical Subapical Subapical	Acute Acute Round Round	This study This study This study Karnkowska-Ishikawa et al. (2011)
<i>E. deses</i> var. <i>inter-media</i> D5 clade	ASW08044	171.9 ± 9.0 (98.0-229.1)	17.2 ± 3.5 (9.7-25.3)	10.0	8.8 (5.9-10.9)	Double	Ellipse	Subapical	Wedge	Karnkowska-Ishikawa et al. (2011)
<i>E. mutabilis</i>	NJ, Sandy, USA (n = 25) SAG1224-9b	52.3 ± 10.3 (39.1-83.3) 81.1 ± 9.2 (61.6-96.7)	10.4 ± 1.2 (8.3-13.1) 8.2 ± 3.7 (6.8-11.6)	5.0 9.9	- 19.0 ± 2.0 (16.3-22.3)	Single Single	- Short-rod	Subapical Subapical	- -	This study This study
	Mulyoungari091209A	81.5 ± 5.2 (71.6-88.1)	7.9 ± 0.4 (7.2-8.8)	10.3	13.3 ± 0.9 (12.2-14.9)	Single	Short-rod	Subapical	Acute	This study
<i>E. satelles</i>	ASW08093	128.3 ± 9.5 (105.2-154.1)	10.3 ± 1.3 (6.8-13.7)	12.5	-	Double	-	Apical	Round	Karnkowska-Ishikawa et al. (2011)

Strains labeled: ASW, Algenkulture-Sammlung an der Universität Wien, Vienna, Austria; SAG, Sammlung von Algenkulturen Pflanzenphysiologisches Institut der Universität Göttingen, Germany.

<sup>a</sup>This study.<sup>b</sup>Karnkowska-Ishikawa et al. (2011).

1/10 = 0.1 (one difference per 10 bp) was used on the population size parameters (s). The age of the root in the species tree ( $\tau_0$ ) was assigned the gamma prior G (2, 2000) which means 0.1% of sequence divergence, while the other divergence time parameters were assigned the Dirichlet prior. Each analysis was run twice with  $10^6$  generations to confirm consistency between runs. The burn-in point was identified graphically by tracking the likelihood values (TRACER v. 1.6; <http://tree.bio.ed.ac.uk/software/tracer/>).

## RESULTS

### Phylogenetic analyses

The phylogenetic trees with supported values constructed from the Bayesian and RAxML analyses had identical tree topologies (Fig. 1). The *Euglena deses* group (*E. adhaerens*, *E. carterae*, *E. deses*, *E. mutabilis*, and *E. satelles*) was monophyletic with strong support values within the genus *Euglena* (posterior probability [PP], 1.00; MLBT, 100) and divided into two major clades. One clade consisted of the *E. mutabilis* subclade and the *E. satelles* and *E. adhaerens* subclade (PP, 1.00; MLBT, 83). The *E. mutabilis* subclade was composed of 8 strains and formed a monophyletic lineage with strong support values (PP, 1.00; MLBT, 100). The *E. satelles* and *E. adhaerens* subclade consisted of two strains of *E. adhaerens*, one strain of *E. carterae* and *E. satelles* respectively, and formed a strongly supported monophyletic lineage (PP, 1.00; MLBT, 100). The other clade consisted of 20 strains of *E. deses* and formed a monophyletic lineage with strong support values (PP, 1.00; MLBT, 100), and subdivided further into five lineages with strong support values except for the branch between D1 and D2 subclades (PP, 0.64; MLBT, 58).

### Species delimitation

We used a species delimitation method (BP&P) for 20 strains of *E. deses* based on the combined four-gene sequence dataset. Species delimitation results indicated in the species phylogeny (Fig. 1) and supported four cryptic species of *E. deses* (D1-D4; with SPP = 1.0 for each clade) with one additional single taxa D5. The result obtained from the species delimitation method was completely congruent with cell length parameter (Table 1, Fig. 1).

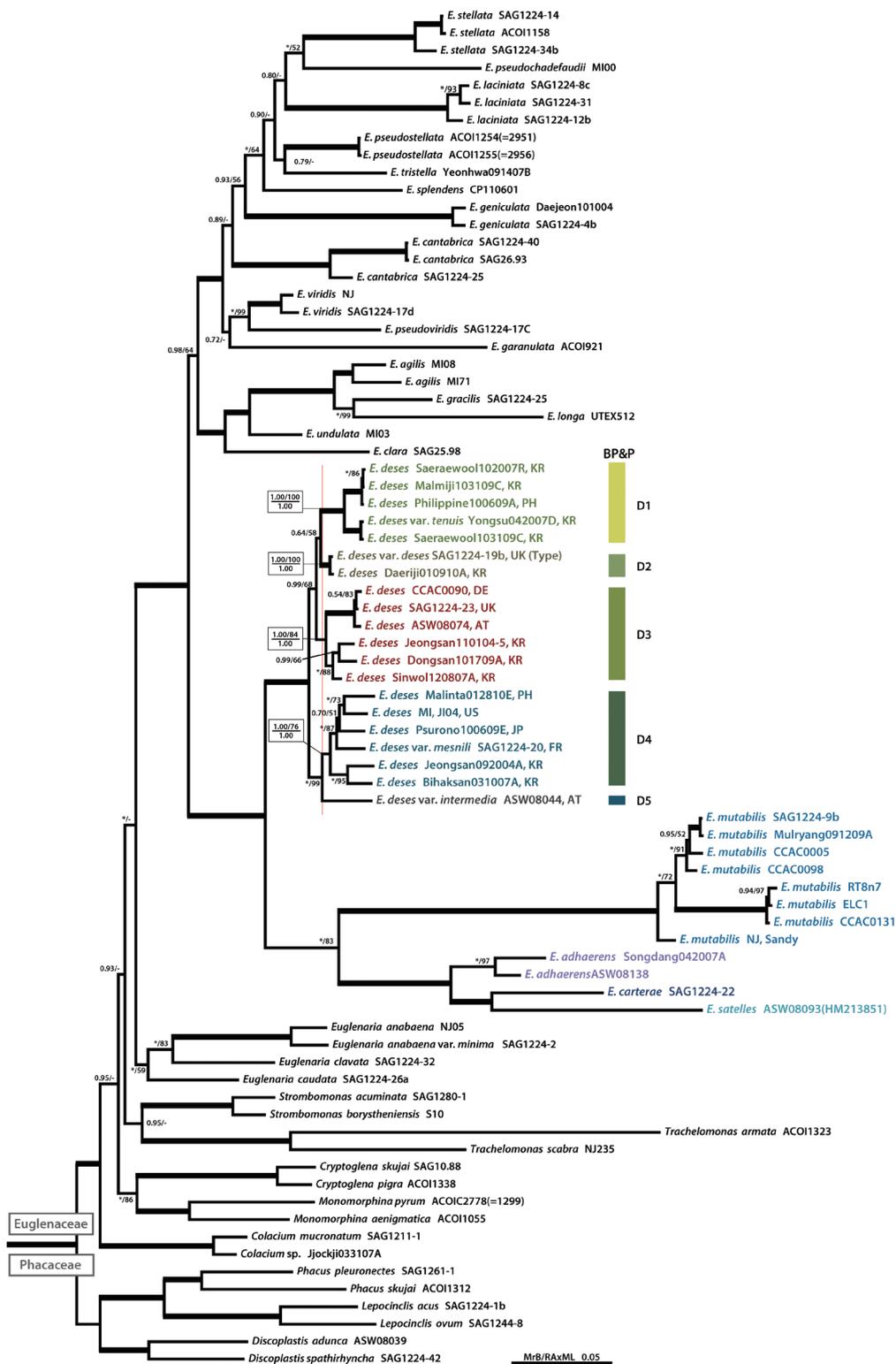
### Morphological observations

The 32 strains of the *E. deses* group were observed using light microscopy and SEM (Table 1, Figs 2 & 3). The following morphological features for species identification in this group were analyzed: size and shape of the cell, size and shape of the chloroplast, paramylon type, location of the canal opening, shape of posterior end, presence of pyrenoid, and ornamentation of the pellicle (Table 1). All strains had common morphological features such as a cylindrically elongated cell shape, numerous discoid chloroplasts with a naked pyrenoid, and pronounced metaboly.

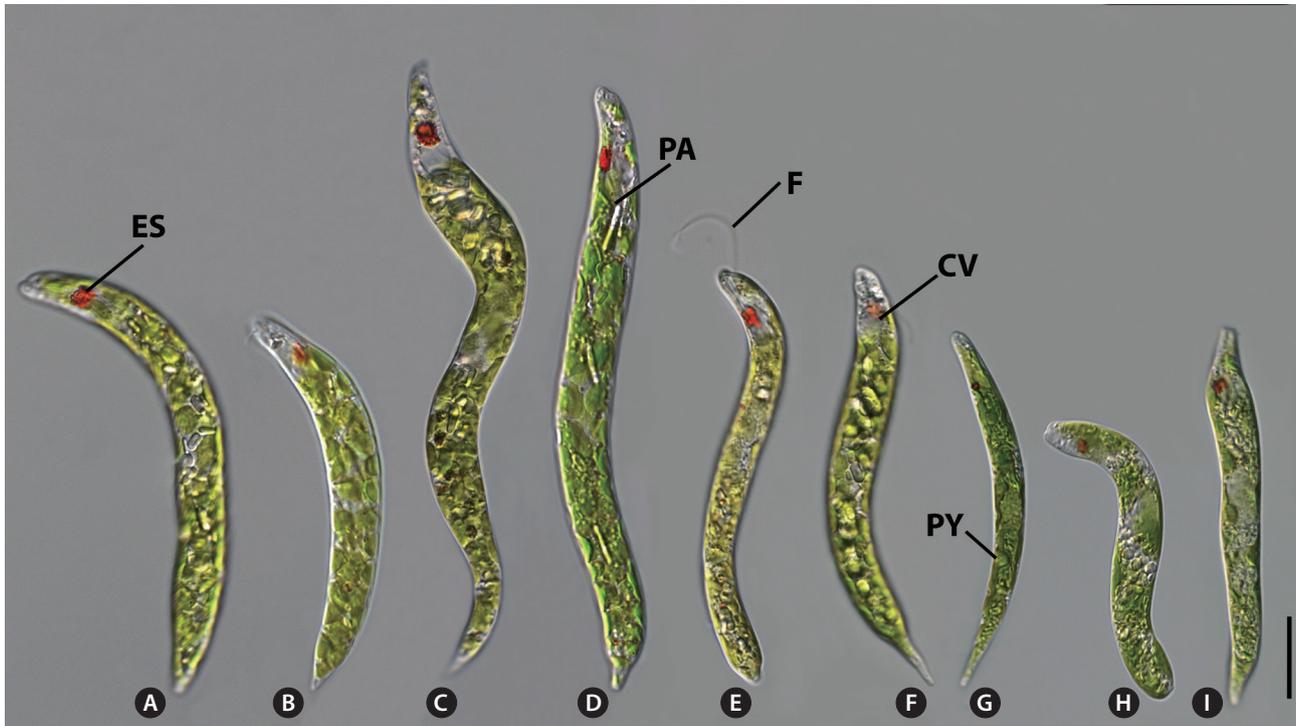
All of *E. deses* cells were worm-like and moved by metabolically bending or swimming. The anterior end was rounded with a laterally oriented canal opening (Figs 2A-F & 3A-L). Free-swimming cells demonstrated a flagellum that was either nearly the same size of the body length or very short. The posterior end was rounded and tapered as acute or wedge. The diameter of the chloroplasts ranged from  $4.9 \pm 0.4 \mu\text{m}$  of the Daeriji010910A strain in D2 clade to  $9.0 \pm 0.8 \mu\text{m}$  of the Bihaksan031007A strain in D4 clade, and pyrenoid presence / visibility depended on the developmental state of the cell. Paramylon grains were rod-like or thick and brick-shaped and filled in the cytoplasm of the *E. deses* cells.

The *E. deses* cells were well grouped by cell dimension in each subclade resulted from the molecular phylogeny and species delimitation analysis. The D1 subclade was composed of five strains with  $(102.6 \pm 12.5-137.3 \pm 15.2) \times (10.3 \pm 2.8-12.1 \pm 1.5) \mu\text{m}$  in dimension (Fig. 2A). The D2 subclade was consisted of two smallest strains with  $(77.5 \pm 5.3-83.2 \pm 7.2) \times (7.7 \pm 0.8-10.0 \pm 1.4) \mu\text{m}$  in dimension (Fig. 2B). The D3 subclade was consisted of six biggest strains with  $(144.9 \pm 8.5-192.3 \pm 18.3) \times (12.1 \pm 1.9-18.5 \pm 2.3) \mu\text{m}$  in dimension (Fig. 2C & D). The subclade D4 was consisted of six strains with  $(83.4 \pm 12.8-107.5 \pm 5.8) \times (7.8 \pm 0.8-10.7 \pm 1.3) \mu\text{m}$  in dimension (Fig. 2E & F). The subclade D5 was composed of only one strain with  $(171.9 \pm 9.0) \times (17.2 \pm 3.5) \mu\text{m}$  in dimension. The pellicular strips were very fine and spirally arranged (Fig. 3A-L). Adjoining strips were fused in the anterior end upon entering the canal opening (Fig. 3B, E & H, arrowheads). The pellicle structure showed that the wide frame was depressed between two sharp processes; the strips therefore looked distinctly like double-tracked strips by SEM (Fig. 3C, F, I & L, arrowheads).

The *E. mutabilis* cells were  $(52.3 \pm 10.3-81.5 \pm 5.2) \times (7.9 \pm 0.4-10.4 \pm 1.2) \mu\text{m}$  in dimension (Table 1, Fig. 2G). The canal opening was placed sub-apically, pellicular strips



**Fig. 1.** Consensus Bayesian tree of the *Euglena deses* group based on combined nuclear small subunit, partial-large subunit, plastid small subunit, and partial-large subunit rDNA sequences. The tree was rooted with six family Phacaceae species as outgroups. The Bayesian posterior probability (PP) and maximum-likelihood bootstrap support value (MLBT) are shown above or below the branches. The bold branches indicate strongly supported values (PP, 1.00 and MLBT, 100%). An asterisk refers to supported values (PP, 1.00 or MLBT, 100%), and a dash indicates support values <50%. The bold branches indicate strongly supported values (PP, 1.00 and MLBT, 100%). The speciation probabilities are provided for each node of BP&P in box below the divider (speciation posterior probability for number of species; SPP[5], 1.00). The vertical red line indicates the speciation point, as estimated by the BP&P.



**Fig. 2.** Morphology of the *Euglena deses* species group. Cells demonstrated an eyespot (ES), a single emergent flagellum (F), a contractile vacuole (CV), numerous rod-shaped paramylon grains (PA), and numerous discoid chloroplasts with naked pyrenoids (PY). (A-F) *E. deses*. (A) Saeraewool103109C in D1 clade. (B) Daeriji010910A in D2 clade. (C) Jeongsan110104-5 in D3 clade. (D) Sinwoljunje120807A in D3 clade. (E) MI J04 in D4 clade. (F) Jeongsan092004A in D4 clade. (G) *E. mutabilis* Mulyoungari091209A. (H) *E. carterae* SAG1224-22. (I) *E. adhaerens* Songdang042007A. Scale bars represent: 20  $\mu$ m.

reached into the canal, and the flagellum ended inside (Figs 2G & 3M-O). The few chloroplasts were discoid-shaped and  $13.3 \pm 0.9$ - $19.0 \pm 2.0$   $\mu$ m in diameter. By SEM, the anticlockwise spirals of pellicular strips showed strict single stria in the middle part of the cell (Fig. 3M-O).

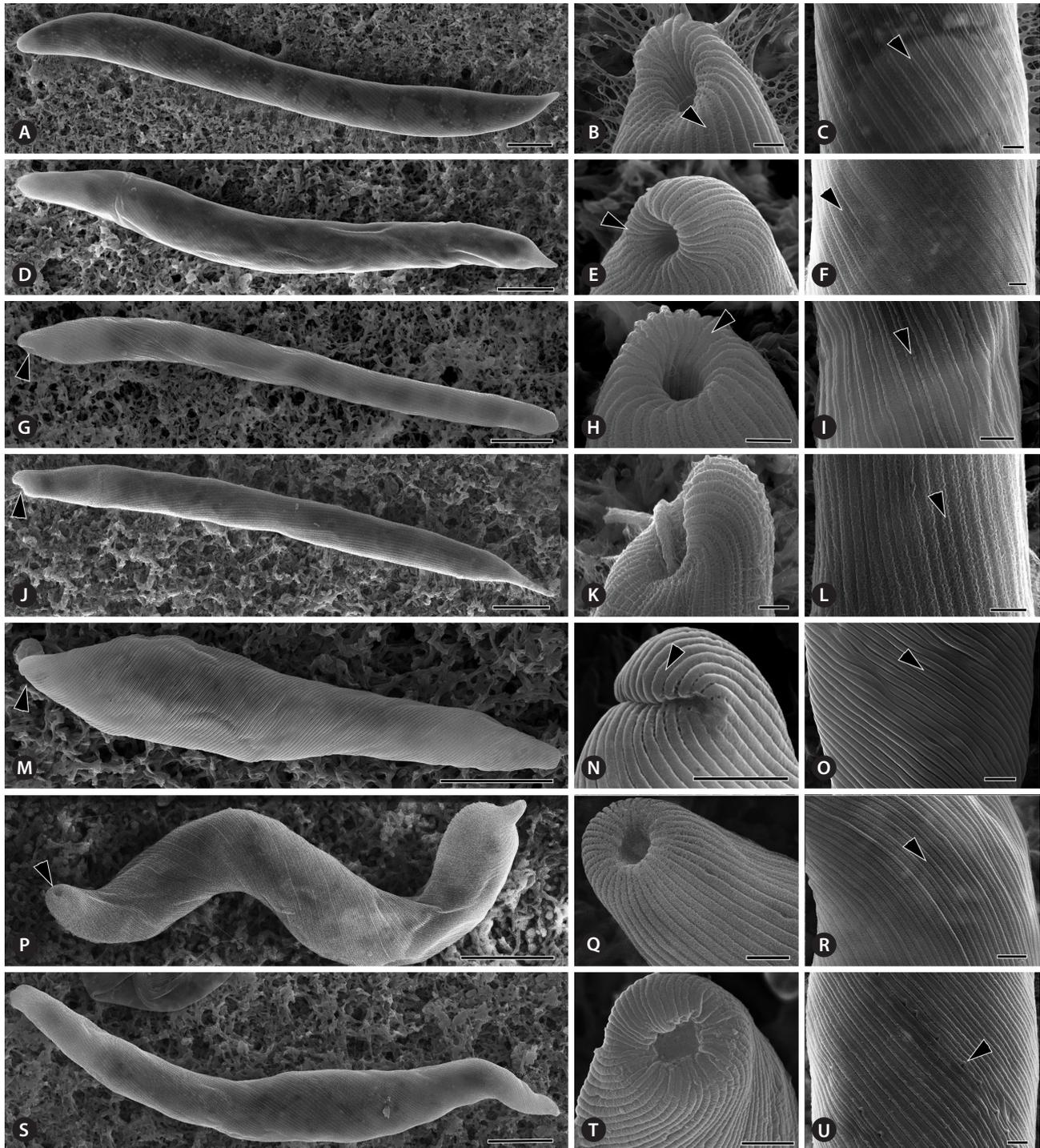
The *E. carterae* cells were  $98.4 \pm 7.9$   $\mu$ m in length and  $10.4 \pm 1.3$   $\mu$ m in width and were oblong and worm-like with a broad, obtuse and rounded posterior region (Fig. 2H). The *E. carterae* SAG1224-22 cells demonstrated an apical canal opening and pellicular strips with double striae. Their movement was metabolic, bending and slowly creeping. The flagellum never exited the canal opening. Small ellipses paramylon grains filled the cell. The canal opening was positioned apically. The pellicular strips were not visible by light microscopy but showed faintly double strips under SEM (Fig. 3P-R, arrowheads). SEM revealed an anticlockwise arrangement of strips that became narrower near the canal opening, and pairs of strips became confluent before entering the canal (Fig. 3P & Q).

The *E. adhaerens* cells were  $92.8 \pm 9.0$ - $124.1 \pm 8.2$   $\mu$ m in length and  $9.3 \pm 1.1$ - $13.9 \pm 1.4$   $\mu$ m in width. The few chlo-

roplasts were discoid-shaped and  $4.9 \pm 0.7$   $\mu$ m in diameter. The canal opening was positioned apically, and the canal opening was terminal and clearly visible (Figs 2I, 3S & T). Under SEM, the anticlockwise spirals of pellicular strips showed weak double striae in the middle part of the cell (Fig. 3S-U). The pellicle appeared more delicately striped near the anterior and posterior end. The strips became narrower but were not confluent before entering the canal.

## DISCUSSION

Cryptic diversity in euglenoids has commonly been unveiled by molecular markers and in combination with morphological data (Kosmala et al. 2007b, 2009, Kim et al. 2013a, 2013b, Kim and Shin 2014). In this study, molecular reassessment revealed the presence of five cryptic lineages among 20 strains of *E. deses* using the Bayesian multi-locus species (BP&P) delimitation method. Each lineage was adequately distinct from the others to regard them as separately evolving lineages. We validate the five



**Fig. 3.** Morphology of the *Euglena deses* group by scanning electron microscopy. All strains demonstrated cell shape, enlarged anterior end, and pellicular strips. (A-L) *E. deses* bearing an subapical anterior end (G & J, arrowheads) double striae (B, C, E, F, H, I & L, arrowheads) in pellicle strips. (A-C) *E. deses* Yongsu042007D in D1 clade. (D-F) *E. deses* Jeongsan110104-5 in D3 clade. (G-I) *E. deses* MI J04 in D4 clade. (J-L) *E. deses* Jeongsan092004A in D4 clade. (M-O) *E. mutabilis* Mulyoungari091209A bearing a subapical anterior end (M, arrowhead) and a single stria (N & O, arrowheads) in pellicle strips. (P-R) *E. carterae* SAG1224-22 bearing an apical anterior end (P, arrowhead) and double striae (R, arrowhead) in pellicle strips. (S-U) *E. adhaerens* Songdang042007A bearing an apical anterior end and a single (U, arrowhead) pellicle strip. Scale bars represent: A-U, 1  $\mu$ m.

species scenario for *E. deses*, and five clades (D1-D5) was also suggested by molecular data (as phylogeny and speciation probability results). Based on our data, this study largely supported a traditionally recognized and circumscribed *E. deses sensu stricto* species (D2 clade, included SAG1224-19b strain) as a distinct lineage. In addition, larger cell-sized strains were divided into four distinct lineages (D1, D3, D4, and D5 clades). The phylogenetic results were nearly congruent with that of species delimitation result suggested by species delimitation analysis. Therefore, our data supported that the size diversity of *E. deses* correspond to five clades by species delimitation method and may thus indeed represent closely related species.

Many taxonomic issues of *E. deses* has occurred as it is one of the most common species in the genus. Since being established by Ehrenberg (1835, 1838), Klebs (1883) erected a new variety, *E. deses* fo. *intermedia*, based on lack of pyrenoid. After that, many new forma and varieties were described based on cell dimension, chloroplast morphology with or without pyrenoid, paramylon morphology, and habitat (Lemmermann 1910, 1913, Playfair 1921, Wermel 1924, Fritsch et al. 1930, Deflandre and Dusi 1935, Pringsheim 1953, Popova 1955, Zakryś 1986, Shi 1989). Marin et al (2003) rejected the distinction of the authentic strain (SAG1224-20) of *E. deses* var. *mesnili* at the species level (*E. mesnili*) because the strain was positioned within the *E. deses* clade. More recently, Karnkowska-Ishikawa et al. (2011) synonymized many forma and varieties (*E. deses* var. *intermedia*, *E. intermedia*, *E. intermedia* var. *klebsii*, *E. deses* var. *tennuis*, *E. deses* var. *gracilis*, *E. deses* var. *minuta*, *E. klebsii*, *E. intermedia* var. *brevis*, *E. deses* fo. *mesnili*, *E. intermedia* fo. *major*, *E. deses* fo. *klebsii*, *E. deses* fo. *digrana*, *E. intermedia* var. *acidophila*) to only a species, *E. deses*. However, in this study *E. deses* strains were divided further into more than four lineages (Fig. 1, Supplementary Fig. S1) and matched well with size class (D1 clade, 103-137 µm; D2, 78-83 µm; D3, 145-192 µm; D4, 83-108 µm; D5, 172 µm). Pringsheim (1956) also tried to categorize his culture strains of *E. deses* based on cell size to four groups (group I-IV). However, he was not convinced about these size ranks because his clones seemed to show different dimensions of cell body and chloroplast. In euglenoids, morphological variations can occur in culture over long periods or in different environmental conditions (Popova 1966, Zakryś et al. 2002, 2004, Kosmala et al. 2005, 2007a, 2007b, 2009, Karnkowska-Ishikawa et al. 2011). Therefore, our data largely coincided with that of Pringsheim (1956) and support that cell size is one of most important characters within the *E. deses* clade. In

addition, many authors have recognized huge size differences in populations of *E. deses* (Ehrenberg 1835, 1838, Lemmermann 1910, Playfair 1921, Wermel 1924).

Other diagnostic characters, such as shape of posterior end and paramylon have been used as important characters to identify forma or varieties within *E. deses* (Pringsheim 1956, Zakryś 1986). In this study, the caudal and paramylon grain shape were not considered important characters because the strains with an acute, wedge, or rounded posterior ends were intermixed in D2-D4 clades, and the various paramylon grain shapes were not limited to a specific clade.

Recently, the papillae, forming rows of mucus along with pellicular strip, was used as a key character differentiating *E. deses* from other species within the group (Karnkowska-Ishikawa et al. 2011). When we observed the cells of *E. deses*, we could not see any papillae (dots or darken dots) even if the cells were stained with neutral red (Supplementary Fig. S1). However, when the cells were pressed by a coverslip as water becomes dry out, the mucus-like cytoplasmic fluids were produced (Supplementary Fig. S1B & S1G). Therefore, the papillae may be an artifact and not a reliable diagnostic character for *E. deses*.

Another notable morphological feature among species of the *E. deses* group is the position of the canal opening. This morphological characteristic was congruent with our molecular phylogeny. Both *E. deses* and *E. mutabilis* demonstrated a lateral canal opening with a slight depression or lip. However, *E. deses* has a double pellicle strips with a wide median depression between the keel and the overhang, whereas *E. mutabilis* had a single stria. *E. carterae*, *E. satelles*, and *E. adhaerens* presented an apical canal opening. In particular, the anterior part of *E. carterae* was narrowly extended, and the canal opening was centrally positioned at the anterior end. Additionally, the strips were broader in *E. satelles* than *E. carterae*, and *E. adhaerens* demonstrated a plateau-like, flat frame of the strip along the entire cell (Fig. 3U).

*E. deses* var. *carterae* was established first by Pringsheim (1953) as a variety characterized by cell division in brackish water, the lack of a flagellum and the presence of a small stigma. Later, Marin et al. (2003) raised the variety *E. deses* var. *carterae* to the species *E. carterae* based on a molecular phylogenetic analysis showing its location outside of the *E. deses* clade as an independent clade. The morphological features of this species also indicated that *E. carterae* could be differentiated from *E. deses* by the presence of a pellicular ridge with a narrow strip (A or M-type) (Kusel-Fetzmann and Weidinger 2008). More

recently, Karnkowska-Ishigawa et al. (2011) suggested that two species, *E. carterae* SAG1224-22 and *E. satelles* ASW08093, could be combined into *E. satelles*. However, our results did not support this idea, as these two stains were clearly separated according to morphological and molecular data. *E. carterae* SAG1224-22 and *E. satelles* ASW08093 showed a sister relationship in our tree (Fig. 1) and demonstrated unique pellicle strips; *E. carterae* had more narrow double striae in the pellicle strips than that of *E. satelles*. In addition, both species differ by 30 µm in cell length (*E. carterae* SAG1224-22,  $98.4 \pm 7.9$ ; *E. satelles* ASW 08093,  $128.3 \pm 9.5$ ).

We analyzed detailed morphological and molecular data among the *E. deses* group and observed a high morphological diversity among strains of *E. deses* in terms of cell size and tail shape. This study provides clear diagnostic data to identify the following five species by morphological and molecular analysis: *E. deses* (subapical anterior part, double striae), *E. adhaerens* (apical, weak double striae), *E. carterae* (apical, narrow double striae), *E. satelles* (apical, double striae), and *E. mutabilis* (apical, single stria). Molecular reassessment revealed the presence of five cryptic lineages in *E. deses* using the BP&P species delimitation method and congruent with molecular phylogenetic result. This study supported five species of *E. deses*-like by morphological diagnostic characters, and assessed cryptic diversity within *E. deses* species.

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## SUPPLEMENTARY MATERIAL

**Supplementary Table S1.** Strains of the *Euglena deses* complex used in this study and the GenBank accession numbers for their nuclear SSU, LSU and plastid SSU, LSU rDNA gene sequenced (www.e-algae.org).

**Supplementary Table S2.** Evolutionary models, log likelihood values (-lnL), and model parameters resulted from each phylogenetic analyses for the combined data sets (www.e-algae.org).

**Supplementary Fig. S1.** Light micrographs of *Euglena*

*deses* strains showing eyespot (ES), chloroplast (CP), paramylon (PA), pyrenoid (PY), pellicular strip (arrowheads), and papillae (arrows) (www.e-algae.org).

## REFERENCES

- Braslavska-Spectorova, E. P. 1937. Pro novii vid biezdgoutivkovich evglen [Regarding a new species of *Euglena* without flagella]. J. Inst. Bot. Acad. Sci. R. S. S. Ukraine 11:91-99.
- Chu, S. P. 1946. Contributions to our knowledge of the genus *Euglena*. Sinensia 17:75-134.
- Deflandre, G. & Dusi, H. 1935. Description d'une Euglène nouvelle. *Euglena mesnili* nov. spec. Arch. Zool. Exp. Gén. 77:12-14.
- Demchenko, E., Mikhailyuk, T., Coleman, A. W. & Pröschold, T. 2012. Generic and species concepts in *Microglena* (previously the *Chlamydomonas monadina* group) revised using an integrative approach. Eur. J. Phycol. 47:264-290.
- Ehrenberg, C. G. 1835 (1833). Dritter Beitrag zur Erkenntnis grosser Organisation in der Richtung des kleinsten Raumes. Phys. Abh. K. Akad. Wiss. Berlin 1833:145-336.
- Ehrenberg, C. G. 1838. *Die Infusionsthierchen als Vollkommene Organismen. Ein Blick in das Tiefere Organische Leben der Natur. Nebst Einem Atlas von Vierundsechzig Colorirten Kupfertafeln. Gezeichnet vom Verfasser.* Verlag von Leopold Voss, Leipzig, 547 pp.
- Fawley, M. W., Fawley, K. P. & Hegewald, E. 2011. Taxonomy of *Desmodesmus serratus* (Chlorophyceae, Chlorophyta) and related taxa on the basis of morphological and DNA sequence data. Phycologia 50:23-56.
- Fritsch, F. E., Rich, F. & Stephens, M. E. L. 1930. Contribution to our knowledge of the freshwater algae of Africa. 7. Freshwater algae (exclusive of diatoms) from Griqualand West. Trans. R. Soc. S. Afr. 18:1-92.
- Gojdics, M. 1953. *The Genus Euglena*. The University of Wisconsin Press, Madison, WI, 268 pp.
- Greenwood, S. J., Schnare, M. N., Cook, J. R. & Gray, M. W. 2001. Analysis of intergenic spacer transcripts suggests 'read-around' transcription of the extrachromosomal circular rDNA in *Euglena gracilis*. Nucleic Acids Res. 29:2191-2198.
- Jo, B. Y., Shin, W., Kim, H. S., Siver, P. A. & Andersen, R. A. 2013. Phylogeny of the genus *Mallomonas* (Synurophyceae) and descriptions of five new species on the basis of morphological evidence. Phycologia 52:266-278.
- Karnkowska-Ishikawa, A., Milanowski, R. & Zakryś, B. 2011. The species *Euglena deses* (Euglenaceae) revisited: new

- morphological and molecular data. *J. Phycol.* 47:653-661.
- Kim, J. I., Linton, E. W. & Shin, W. 2015. Taxon-rich multigene phylogeny of the photosynthetic euglenoids (Euglenophyceae). *Front. Ecol. Evol.* 3:98.
- Kim, J. I. & Shin, W. 2007. Ultrastructure of *Cryptoglena pigra* from Korea. *Algae* 22:325-331.
- Kim, J. I. & Shin, W. 2008. Phylogeny of the Euglenales inferred from plastid LSU rDNA sequences. *J. Phycol.* 44:994-1000.
- Kim, J. I. & Shin, W. 2014. Molecular phylogeny and cryptic diversity of the genus *Phacus* (Phacaceae, Euglenophyceae) and the descriptions of seven new species. *J. Phycol.* 50:948-959.
- Kim, J. I., Shin, W. & Triemer, R. E. 2010. Multigene analyses of photosynthetic euglenoids and new family, Phacaceae (Euglenales). *J. Phycol.* 46:1278-1287.
- Kim, J. I., Shin, W. & Triemer, R. E. 2013a. Cryptic speciation in the genus *Cryptoglena* (Euglenaceae) revealed by nuclear and plastid SSU and LSU rRNA gene. *J. Phycol.* 49:92-102.
- Kim, J. I., Shin, W. & Triemer, R. E. 2013b. Phylogenetic reappraisal of the genus *Monomorphina* (Euglenophyceae) based on molecular and morphological data. *J. Phycol.* 49:82-91.
- Klebs, G. 1883. Über die Organisation einiger Flagellaten-Gruppen und ihre Beziehungen zu Algen und Infusorien. *Untersuchungen Bot. 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.* 43:1071-1082.
- Kosmala, S., Karnkowska, A., Milanowski, R., Kwiatowski, J. & Zakryś, B. 2005. Phylogenetic and taxonomic position of *Lepocinclis fusca* comb. nov. (= *Euglena fusca*) (Euglenaceae): morphological and molecular justification. *J. Phycol.* 41:1258-1267.
- Kosmala, S., Karnkowska-Ishikawa, A., Milanowski, R., Kwiatowski, J. & Zakryś, B. 2009. Phylogeny and systematics of *Euglena* (Euglenaceae) species with axial, stellate chloroplasts based on morphological and molecular data: new taxa, emended diagnoses and epitypifications. *J. Phycol.* 45:464-481.
- Kosmala, S., Milanowski, R., Brzóska, K., Pękala, M., Kwiatowski, J. & Zakryś, B. 2007b. Phylogeny and systematics of the genus *Monomorphina* (Euglenaceae) based on morphological and molecular data. *J. Phycol.* 43:171-185.
- Kusel-Fetzmann, E. & Weidinger, M. 2008. Ultrastructure of five *Euglena* species positioned in the subdivision Serpentes. *Protoplasma* 233:209-222.
- Leedale, G. F. 1967. *Euglenoid flagellates*. Concepts of modern biology series. Prentice-Hall Inc., Englewood Cliffs, NJ, 242 pp.
- Leedale, G. F. 1982. Ultrastructure. In Buetow, D. E. (Ed.) *The Biology of Euglena, Vol. III. Physiology*. Academic Press, New York, pp. 1-27.
- Lemmermann, E. 1910. *Kryptogamenflora der Mark Brandenburg. III. Algen*. Verlag von Gebrüder Borntraeger, Leipzig, 712 pp.
- Lemmermann, E. 1913. Eugleninae. In Pascher, A. (Ed.) *Die Süßwasserflora Deutschlands, Österreichs und der Schweiz. H. 2: Flagellatae II*. Verlag von Gustav Fisher, Jena, pp. 115-174.
- Lewis, L. A. & Flechtner, V. R. 2004. Cryptic species of *Scenedesmus* (Chlorophyta) from desert soil communities of western North America. *J. Phycol.* 40:1127-1137.
- Linton, E. W., Karnkowska-Ishikawa, A., Kim, J. I., Shin, W., Bennett, M. S., Kwiatowski, J., Zakryś, B. & Triemer, R. E. 2010. Reconstructing euglenoid evolutionary relationships using three genes: nuclear SSU and LSU, and chloroplast SSU rDNA sequences and the description of *Euglenaria* gen. nov. (Euglenophyta). *Protist* 161:603-619.
- Lundholm, N., Bates, S. S., Baugh, K. A., Bill, B. D., Connell, L. B., Léger, C. & Trainer, V. L. 2012. Cryptic and pseudo-cryptic diversity in diatoms: with descriptions of *Pseudonitzschia hasleana* sp. nov. and *P. fryxelliana* sp. nov. *J. Phycol.* 48:436-454.
- Mainx, F. 1927. Beiträge zur Morphologie und Physiologie der Eugleninen. I. Morphologische Beobachtungen. Methoden und Erfolge der Reinkultur. *Arch. Protistenkd.* 60:305-414.
- Mann, D. G., McDonald, S. M., Bayer, M. M., Droop, S. J. M., Chepurinov, V. A., Loke, R. E., Ciobanu, A. & du Buf, J. M. H. 2004. The *Sellaphora pupula* species complex (Bacillariophyceae): morphometric analysis, ultrastructure and mating data provide evidence for five new species. *Phycologia* 43:459-482.
- 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.
- Matvienko, O. M. 1938. Materiali do vivotchennia vodorostii URSSR (Contributions to the study of the algae of the Ukraine USSR). *Tr. N. D. Inst. Bot. Kharkov* 3:29-70.
- Monfils, A. K., Triemer, R. E. & Bellairs, E. F. 2011. Characterization of paramylon morphological diversity in photosynthetic euglenoids (Euglenales, Euglenophyta). *Phy-*

- cologia 50:156-169.
- Playfair, G. I. 1921. Australian freshwater flagellates. Proc. Linn. Soc. N. S. W. 46:99-146.
- Popova, T. G. 1955. *Euglenovyye vodorosli. Opredelitel' prosvodnykh vodoroslej SSSR, 7* [*Euglenophyta. The handbook of freshwater algae of the USSR, 7*]. Sovetskaya Nauka, Moskva, 267 pp. (in Russian).
- Popova, T. G. 1966. *Euglenophyta 1. Flora Plantarum USSR. 8*. Nauka, Moskva-Leningrad, 411 pp. (in Russian).
- Pringsheim, E. G. 1953. Salzwasser-Eugleninen. Arch. Microbiol. 18:149-164.
- Pringsheim, E. G. 1956. Contributions towards a monograph of the genus *Euglena*. Nova Acta Leopold. 18:1-168.
- Ronquist, F., Teslenko, M., van der Mark, P., Ayres, D. L., Darling, A., Höhna, S., Larget, B., Liu, L., Suchard, M. A. & Huelsenbeck, J. P. 2012. MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. Syst. Biol. 61:539-542.
- Schmitz, F. 1884. Beiträge zur Kenntnis der Chromatophoren. Pringsh. Jahrb. Wiss. Bot. 15:1-175.
- Schnare, M. N. & Gray, M. W. 2011. Complete modification maps for the cytosolic small and large subunit rRNAs of *Euglena gracilis*: functional and evolutionary implications of contrasting patterns between the two rRNA components. J. Mol. Biol. 413:66-83.
- Shi, Z. 1989. A new variety of *Euglena intermedia* inhabiting acid water. Acta Hydrobiol. Sin. 13:287-288.
- Shin, W. & Boo, S. M. 2001. Ultrastructure of *Phacus trypanon* (Euglenophyceae) with an emphasis on striated fiber and microtubule arrangement. J. Phycol. 37:95-105.
- Shin, W. & Triemer, R. E. 2004. Phylogenetic analysis of the genus *Euglena* (Euglenophyceae) with particular reference to the type species *Euglena viridis*. J. Phycol. 40:759-771.
- Škaloud, P., Kynčlová, A., Benada, O., Kofroňová, O. & Škaloudová, M. 2012. Toward a revision of the genus *Synura*, section Petersenianae (Synurophyceae, Heterokontophyta): morphological characterization of six pseudo-cryptic species. Phycologia 51:303-329.
- Škaloud, P., Škaloudová, M., Procházková, A. & Němcová, Y. 2014. Morphological delineation and distribution patterns of four newly described species within the *Synura petersenii* species complex (Chrysophyceae, Stramenopiles). Eur. J. Phycol. 49:213-229.
- Smith, S. W., Overbeek, R., Woese, C. R., Gilbert, W. & Gillevet, P. M. 1994. The genetic data environment: an expandable GUI for multiple sequence analysis. Comput. Appl. Biosci. 10:671-675.
- Stamatakis, A. 2014. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics 30:1312-1313.
- Triemer, R. E. 1980. Role of Golgi apparatus in mucilage production and cyst formation in *Euglena gracilis* (Euglenophyceae). J. Phycol. 16:46-52.
- Triemer, R., Linton, E., Shin, W., Nudelman, A., Monfils, A., Bennett, M. & Brosnan, S. 2006. Phylogeny of the Euglenales based upon combined SSU and LSU rDNA sequence comparisons and description of *Discoplastis* gen. nov. (Euglenophyta). J. Phycol. 42:731-740.
- Walne, P. L., Moestrup, Ø., Norris, R. E., & Ettl, H. 1986. Light and electron microscopical studies of *Eutreptiella eupharyngea* sp. nov. (Euglenophyceae) from Danish and American waters. Phycologia 25:109-126.
- Watanabe, M. M. & Hiroki, M. 1997. *NIES-Collection: list of strains. 5th ed.* National Institute for Environmental Studies, Tsukuba, 127 pp.
- Wermel, E. 1924. Neue order wenig bekannte Protisten XII. Neue order wenig bekannte Flagellaten XI. Beschreibung neuer Flagellaten aus Russland. Arch. Protistenkd. 48:204-206.
- Wuyts, J., De Rijk, P., Van de Peer, Y., Winkelman, T. & De Wachter, R. 2001. The European large subunit ribosomal RNA database. Nucleic Acid Res. 29:175-177.
- Yang, Z. & Rannala, B. 2014. Unguided species delimitation using DNA sequence data from multiple loci. Mol. Biol. Evol. 31:3125-3135.
- Zakryś, B. 1986. Contribution to the monograph of Polish members of the genus *Euglena* Ehrenberg 1830. Nova Hedwig. 42:491-540.
- Zakryś, B., Cambra-Sanchez, J. & Walne, P. L. 2001. Chloroplast ultrastructure of *Euglena cuneata* Pringsheim, *E. deses* Ehrenberg and *E. mutabilis* (Euglenophyceae): taxonomic significance. Acta Protozool. 40:161-167.
- Zakryś, B., Empel, J., Milanowski, R., Gromadka, R., Borsuk, P., Kędzior, M. & Kwiatkowski, J. 2004. Genetic variability of *Euglena agilis* (Euglenophyceae). Acta Soc. Bot. Pol. 73:305-309.
- Zakryś, B., Milanowski, R., Empel, J., Borsuk, P., Gromadka, R. & Kwiatkowski, J. 2002. Two different species of *Euglena*, *E. geniculata* and *E. myxocylindracea* (Euglenophyceae), are virtually genetically and morphologically identical. J. Phycol. 38:1190-1199.