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A taxonomic study of *Eudorina unicocca* (Volvocaceae, Chlorophyceae) and related species, based on morphology and molecular phylogeny

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Colonial volvocacean algae engage in two types of sexual reproduction: isogamy and anisogamy/oogamy with sperm packets. This difference is an important generic diagnosis within the Volvocaceae. Although *Yamagishiella* differs from the anisogamous genus *Eudorina* in its isogamous sexual reproduction, the vegetative morphology and asexual reproduction characteristics of the two genera are indistinguishable, especially between *Eudorina unicocca* G. M. Smith and *Yamagishiella unicocca* (Rayburn et Starr) Nozaki. We re-examined morphological characteristics of *E. unicocca* and related species, using multiple strains of *E. unicocca* and *Y. unicocca* and molecular phylogenetic analyses. Strains from two Japanese lakes, which produced aplanospores and were solely asexual, could be assigned to either *E. unicocca* or *Y. unicocca*, based on traditional morphological diagnoses. However, a new morphological diagnosis (the difference in the distribution and number of contractile vacuoles on the cell surface) and molecular phylogenetic analyses demonstrated that all were *E. unicocca*. Furthermore, *E. unicocca* can be divided into two species on the basis of the presence or absence of individual cellular sheaths in the colonial gelatinous matrix, which are observable with methylene blue staining. These two species, *E. peripheralis* (Goldstein) T. K. Yamada stat. nov. (= *E. unicocca* var. *peripherialis* Goldstein) and *E. unicocca* (including the Japanese aplanosporic strains), formed two robust monophyletic groups, based on chloroplast gene sequences for the large RuBisCO subunit and internal transcribed spacer regions of nuclear ribosomal DNA.

Key words: aplanosporic strain, *Eudorina unicocca*, *Eudorina peripheralis* stat. nov., Chlorophyceae, molecular phylogeny, morphology, taxonomy, Volvocales, *Yamagishiella*

Introduction

Eudorina Ehrenberg is a cosmopolitan volvocacean genus, comprising about eight species (Goldstein, 1964; Nozaki & Krientz, 2001). The genus is distinguished from *Pandorina* Bory de St. Vincent, *Yamagishiella* Nozaki and *Pleodorina* Shaw by the presence of cellular envelopes in the gelatinous (extracellular) matrix, anisogamous sexual reproduction with sperm packets and the absence of obligately somatic cells (Nozaki *et al*., 1989; Nozaki & Kuroiwa, 1992; Nozaki & Ito, 1994). However, in recent molecular phylogenetic analyses of multiple chloroplast genes (Nozaki *et al*., 2000; Nozaki, 2003), *Eudorina* forms a non-monophyletic, basal lineage within the clade containing anisogamous/oogamous members of the colonial Volvocales (*Volvox*, excluding *Volvox* sect. *Volvox*; *Pleodorina*; and *Eudorina*).

Yamagishiella Nozaki in Nozaki & Kuroiwa (1992) is a monotypic genus containing *Y. unicocca* (Rayburn et Starr) Nozaki, which was originally described as *Pandorina unicocca* Rayburn et Starr (1974). This genus is distinguished from *Pandorina* by its cellular envelopes and 32-celled colonies (Nozaki & Kuroiwa, 1992). Although *Yamagishiella* differs from *Eudorina* in its isogamous sexual reproduction, the vegetative morphology and asexual reproduction characteristics of the two genera are indistinguishable (Nozaki & Kuroiwa, 1992; Nozaki & Ito, 1994). For example, like *Y. unicocca* (Rayburn & Starr, 1974), *Eudorina unicocca* G. M. Smith is recognized by the presence of a single, basal pyrenoid in the chloroplast of vegetative cells (Smith, 1930; Goldstein, 1964). Thus, *E. unicocca* cannot be distinguished from *Y. unicocca* without molecular markers when the

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mode of sexual reproduction is unknown (Nozaki *et al*., 1998).

Goldstein (1964) established a species-level taxonomic system for the genus *Eudorina*, based on comparative morphological observations of cultured material. However, he did not observe sexual reproduction in several strains of *E. unicocca* that produced parthenospores or aplanospores (Goldstein, 1964). These 'aplanosporic' strains are impossible to re-identify, as they were not maintained in culture collections (Starr & Zeikus, 1993; Kasai *et al*., 2004). Therefore, new strains of the aplanosporic algae and new generic diagnoses of *Eudorina* and *Yamagishiella* are needed to establish a clear morphological distinction between the two genera and a straightforward identification system. We re-examined the morphological characteristics of *E. unicocca* and related species by examining many strains that could be identified as *E. unicocca* or *Y. unicocca* by their vegetative morphology. Several strains originating from Japanese lakes produced aplanospores and were solely asexual, but a new morphological diagnosis and molecular phylogenetic analyses demonstrated their identity as *E. unicocca*, morphologically distinguishable from *E. peripheralis* (Goldstein) T. K. Yamada stat. nov. (= *E. unicocca* var. *peripheralis* Goldstein).

Materials and methods

Cultures

We isolated aplanosporic strains from water samples collected from Lake Tsukui (TKI-C-1, -2 and -3), Tsukui-machi, Sagamihara, Kanagawa Prefecture, Japan, in July 2005 (water temperature 24.7°C; pH > 8.4) and Lake Sagami (990601-IE-3, -4, -5 and -6), Fujino-machi, Tsukui-gun, Kanagawa Prefecture, Japan, in June 1999 (water temperature 20.0°C; pH 8.3). Three *Y. unicocca* strains (Hasu-1, -2 and -4) were isolated from soil samples collected from a lotus paddy field in Noumi-cho, Etajima, Hiroshima Prefecture, Japan, in August 2004. Using the pipette-washing method (Pringsheim, 1946), clonal cultures were established directly from the water sample or from Petri dishes (90 × 20 mm) in which a small amount (~0.5 g) of dried soil had been wetted with distilled water. Four strains of *E. unicocca* (NIES-724, -725, -726 and UTEX 1221; Goldstein 1964), *E. elegans* NIES-717, *E. cylindrica* Korshikov NIES-722, *E. illinoisensis* (Kofoid) Pascher NIES-723, and five strains of *Y. unicocca* (NIES-578, -666, -762, -870 and -872) were obtained from culture collections (National Institute for Environmental Studies, Japan, NIES, or the Culture Collection of Algae at the University of Texas at Austin, UTEX) (Kasai *et al*., 2004; Starr & Zeikus, 1993; Table 1). The cultures were grown in screw-cap tubes (18 × 150 mm) containing about 11 ml AF-6 medium (Kato, 1982), modified by the elimination of $CaCO_3$ and addition of 400 mg l^{-1} MES (Kasai *et al*., 2004). The cultures were cultivated at about 20–25°C, on a 10 : 14 h light–dark cycle, under cool-white fluorescent lamps at 150–200 μmol m^{-2} s^{-1} intensity.

Light microscopy

To observe vegetative morphology and asexual reproduction, about 0.5 ml of each actively growing culture was inoculated into fresh medium every 3 to 12 days. To induce sexual reproduction, 11 ml of actively growing culture were concentrated to 0.3–0.5 ml by centrifugation. The concentrated cultures of two complementary mating types (e.g., *Y. unicocca* Hasu-1 and -4, Table 1) were mixed and added to 1.5 ml of nitrogen-deficient AFM medium (Nakazawa *et al*., 2001) in watch glasses (60 mm diameter) supported on glass depressions in Petri dishes. To minimize evaporation, about 0.5 ml of distilled water was added to the bottom of the Petri dishes. The Petri dishes were cultured under the usual conditions (14 : 10-h light–dark cycle; 20–25°C). For aplanospore formation, a concentrated culture (0.3–0.5 ml) of a single strain was mixed with 1.5–2.0 ml AFM medium and cultured as described above. In order to examine individual cellular sheaths of the gelatinous matrix of the vegetative colonies, about 10 μl of the cultured material were mixed with 2–5 μl 0.002% (w/v in distilled water) methylene blue (1B-429 Methylene Blue med., Puriss., WALDECK GmbH & Co Division Chroma, Münster, Germany). Light microscopy was carried out using an OLYMPUS BX60 microscope (KS OLYMPUS, Tokyo, Japan), equipped with Nomarski interference optics.

Molecular phylogenetic analyses

The protocol of Fawley & Fawley (2004) was used to prepare the total DNA of seven strains (Table 1), with some modifications (see Nakada & Nozaki, 2007). The method for sequencing the chloroplast gene (*rbc*L) for the large subunit of RuBisCO of two strains of *Y. unicocca* (Hasu-1 and NIES-870), *E. unicocca* UTEX 1221 and two aplanosporic strains (TKI-C-2 and 990601-IE-5) was essentially identical to that described previously (Nozaki *et al*., 1995, 1997, 2000, 2002). The region sequenced corresponded to *rbc*L positions 31–1159 in *Chlorella vulgaris* Beijerinck (Yoshinaga *et al*., 1988; Wakasuagi *et al*., 1997). For phylogenetic analyses, identical sequences were treated as a single operational taxonomic unit (OTU). The coding regions (1,129 bp) of the sequences were aligned by Clustal X (Thompson *et al*., 1997), including 14 other *Eudorina* strains, six other *Yamagishiella* strains, 17 *Volvox* strains, five *Pleodorina* strains, one *Platydorina* strain and two *Pandorina* strains (Table 1). From this alignment, a distance matrix was calculated by applying the two-parameter method (Kimura, 1980) in Clustal X. A phylogenetic tree was constructed using the neighbor-joining (NJ) algorithm (Saitou & Nei, 1987) with Clustal X, and the robustness of the resulting lineages was tested using bootstrap analysis (Felsenstein, 1985) with 1,000 replications. Based on the alignment data, a maximum

Table 1. List of *rbc*L gene and ITS sequences used in this study.

Taxa	Strain designation	Origin and DDBJ/EMBL/GenBank accession number	
		*rbc*L	*r*DNA ITS
Yamagishiella unicocca	NIES[a]-666 (UTEX[b] 2428)	D86823	
	UTEX 2430	D86825	
	NIES-872	AB044168	
	UTEX 2031	D86822	
	UTEX 840	D86826	
	UTEX 2127	D86824	
	NIES-870	AB359064[e]	
	Hasu-1[c] (NIES-1859)	AB359065[e]	
Eudorina unicocca	NIES-724 (UTEX 737, Goldstein[d]:1m "*E. unicocca* var. *unicocca*")	D86829	AB359069[e]
	TKI-C-2 (NIES-1858, From Lale Tsukui)	AB359066[e]	AB359070[e]
	990601-IE-5 (NIES-1855, From Lake Sagami)	AB359067[e]	AB359071[e]
Eudorina peripheralis	NIES-725 (UTEX 1215, Goldstein:93f "*E. unicocca* var. *unicocca*")	D63434	AF486525
	NIES-726 (UTEX 1218, Goldstein:100f "*E. unicocca* var. *peripheralis*")	D86830	AB359072[e]
	UTEX 1221 (Goldstein:4sm "*E. unicocca* var. *peripheralis*")	AB359068[e]	AB359073[e]
Eudorina cylindrica	NIES-722 (UTEX 1197, Goldstein[d]:47f)	D86833	AF182439
Eudorina illinoisensis	NIES-460	D63433	
	NIES-723 (UTEX 808)	D88809	
Eudorina elegans	NIES-717 (UTEX 1193, Goldstein[d]:56f)	D88803	
	NIES-456	D63432d	
	NIES-718 (UTEX 1195, Goldstein:44f)	D88810	
	NIES-719 (UTEX 1199, Goldstein:40f)	D88804	
	NIES-720 (UTEX 1205, Goldstein:60f)	D88805	
	NIES-568	D88808	
	NIES-721 (UTEX 1212)	D88806	
Eudorina minodii	NIES-856	AB047074-6	
Pleodorina californica	UTEX 809	D63439	
Pleodorina japonica	NIES-577	D63440	
Pleodorina indica	UTEX 1990	D86834	
Pleodorina thompsonii	UTEX 2804	AB214408	AF486540
Pleodorina starii	NIES-1362	AB214427	
Volvox aureus	NIES-541	D63445	
	NIES-891	AB076096	
	NIES-892	AB076086	
Volvox tertius	UTEX 132	AB076098	
	NIES-544	AB086174	
Volvox gigas	UTEX 1895	AB076084	
Volvox obversus	UTEX 1865	AB076085	
Volvox africanus	UTEX 1891	AB076101	
Volvox carteri			
f. *kawasakiensis*	NIES-732	D63446	
f. *nagariensis*	UTEX 1885	AB076099	
f. *weismannia*	UTEX 1875	AB076100	
Volvox powersii	UTEX 1863	AB214415	
Volvox dissipatrix	UTEX 2184	D63447	
	Marb. 2RS 29	AB214420	
Volvox rousseletii	UTEX 1862	D63448	
Volvox barberi	UTEX 804	D86835	
Volvox globator	UTEX 955	D86836	
Platydorina caudata	UTEX 1658	D86828	
Pandorina colemaniae	NIES-572	D63441	
Pandorina morum	NIES-887	AB044166	

[a]Microbial Culture Collection at the National Institute for Environmental Studies (Kasai *et al*., 2004). [b]Culture Collection of Algae at the University of Texas at Austin (Starr & Zeikus 1993). [c]Exhibiting isogamous sexual reproduction when mixed with Hasu-4 (see Materials and Methods). [d]Strains used by Goldstein (1964). [e]Sequenced for the present study.

parsimony (MP) analysis (including bootstrap analysis based on 1,000 replications of the general heuristic search using the tree-bisection-reconnection branch-swapping algorithm) was performed using PAUP* 4.0b10 (Swofford, 2003). In addition, the internal transcribed spacer (ITS) regions of nuclear ribosomal DNA from three strains of *E. unicocca* (NIES-724, NIES-726 and UTEX 1221) and two aplanosporic strains (TKI-C-2 and 990601-IE-5) were sequenced, as described by Sakayama *et al.* (2004), except for the primers used for polymerase chain reaction (PCR) and sequencing. The primers for the ITS regions were those of Coleman *et al.* (1994), with newly designed PCR primers (ITS-F1: 5′ GTGCTGCCGTACAGCCCTCAGTCG 3′; ITS F2: 5′ AACTAACCAACCAACTCCAAACCA 3′; ITS R3: 5′ GCCTCGAGCGCAATTTGCGT TCAA3′; and ITS R4: 5′ AGTGAGTATTAACCCGACGCTGAG 3′). The coding regions were aligned with those of *E. cylindrica* (NIES-722) and *Pleodorina thompsonii* Ott *et al.* (UTEX 2804), using Clustal X, taking account of their secondary structure (Mai & Coleman, 1997; Coleman, 2002). The sequence alignment is available upon request from the corresponding author (H.N.). After removing gaps in the ingroup OTUs, the data matrix (548 bp) was subjected to the same phylogenetic analyses as for *rbc*L, described above. Based on recent phylogenetic results for the colonial Volvocales (Nozaki *et al.*, 2000, 2006; Nozaki, 2003), two strains of *Pandorina* were designated as the outgroup for the *rbc*L phylogeny, and *E. cylindrica* and *P. thompsonii* as the outgroups for the ITS phylogeny.

Results and discussion

Morphology of aplanosporic strains from Japan

The vegetative colonies of strains isolated from Japanese lakes (Table 1) were ovoid or ellipsoidal in shape, up to 160 μm long, containing 16 or 32 cells of almost identical size embedded in the periphery of the gelatinous matrix (Figs 1, 2). Cells were broadly ovoid or subspherical, measuring up to 25 μm in diameter (Figs 3, 4). Each cell had two equal flagella, a massive cup-shaped chloroplast, a stigma and a nucleus. In addition to the two apical contractile vacuoles, several (up to nine) contractile vacuoles were distributed over the entire surface of the protoplast (Figs 3, 4). The size of the stigmata gradually diminished from the anterior to posterior pole of the colony. The chloroplast had a single large pyrenoid at the bottom (Fig. 4). No additional pyrenoids developed around the large pyrenoid, even in mature cells (Fig. 4). When stained with methylene blue, the gelatinous matrix of the colony revealed individual cellular sheaths that were rectangular in surface view (Fig. 6).

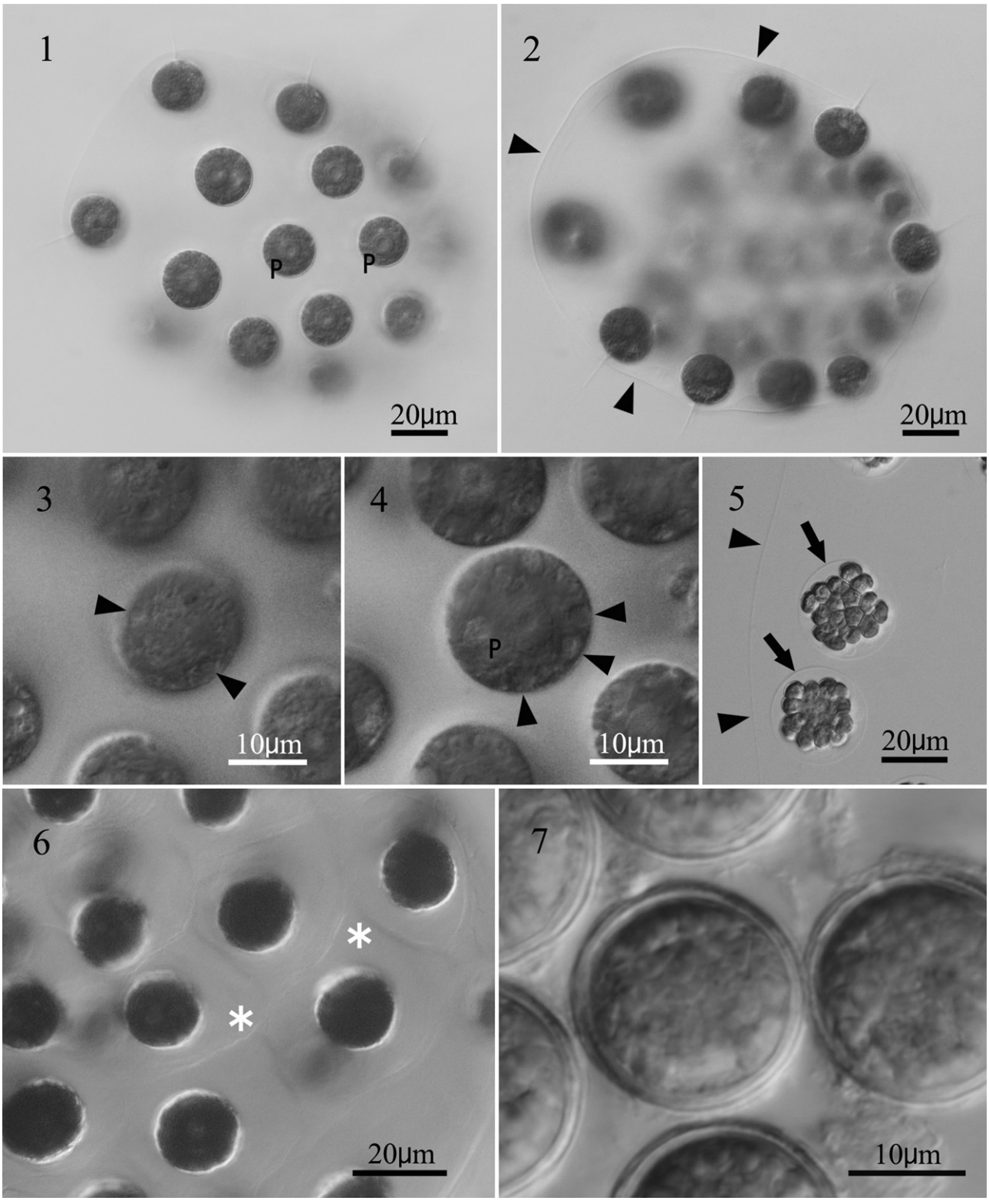
Asexual reproduction was accomplished by autocolony formation. Each cell divided four or five times successively to form a 16- or 32-celled plate, which inverted to form a compact spheroidal daughter colony within a transparent vesicle (i.e., cellular envelope; Nozaki & Kuroiwa 1992) in the parental gelatinous matrix (Fig. 5).

Sexual reproduction did not occur, even when all possible combinations of the seven strains were mixed in nitrogen-deficient AFM medium. In each strain, the cells lost their flagella and secreted a heavy cell wall within the colonial envelope 48–72 hours after transference to AFM medium. The aplanospores were spherical, subsequently turned reddish-brown and measured 17–25 μm in diameter (Fig. 7). Germination of the aplanospores was not observed.

The presence of a 32-cell, ovoid-to-ellipsoidal, colony without somatic cells, cellular envelopes and a single large pyrenoid in the chloroplast (Figs 4, 6, 8) identifies this alga as *E. unicocca* or *Y. unicocca* (Goldstein, 1964, Nozaki *et al.*, 1998). Because sexual reproduction did not occur, based on morphology alone, we could not determine whether the alga belonged to *Eudorina* or *Yamagishiella*. However, the number and distribution of contractile vacuoles in the vegetative cells of the aplanosporic alga appeared to differ from that in *Yamagishiella*. According to Rayburn & Starr (1974) and Nozaki *et al.* (1998), the latter only has two contractile vacuoles near the base of the flagella. In contrast, Goldstein (1964) described "two apical and generally several randomly-distributed contractile vacuoles" for *Eudorina*. However, distinguishing *Yamagishiella* vegetative cells by the presence of the two contractile vacuoles (Rayburn & Starr, 1974; Nozaki *et al.*, 1998) appears unreliable, as Smith (1950) reported two contractile vacuoles near the base of the flagella for *Eudorina*. The individual cellular sheaths in the colonial gelatinous matrix, revealed by methylene blue staining (Figs 6, 8), have not been reported for either *E. unicocca* or *Y. unicocca* (Smith, 1930; Goldstein, 1964; Rayburn & Starr, 1974; Nozaki *et al.*, 1998).

Molecular phylogenetic analyses of rbc*L and nuclear rDNA ITS sequences*

Based on phylogenetic analyses of *rbc*L sequences, two aplanosporic strains (TKI-C-2 and 990601-IE-5) and four *E. unicocca* strains (NIES-724, -725, -726 and UTEX 1221) formed a robust monophyletic group (with 100% bootstrap values for both NJ and MP analyses) that was firmly positioned within the anisogamous/oogamous members of the Volvocaceae (*Volvox*, excluding



Figs. 1–7. Light microscopy of an aplanosporic strain of *Eudorina unicocca* originating from Lake Tsukui, Kanagawa, Japan (TKI-C-2). Fig. 1. Surface view of 32-celled vegetative colony. Note a single, basal pyrenoid (P) in the chloroplast of each cell. Fig. 2. Optical section of 32-celled vegetative colony. Arrowheads indicate colonial envelope surrounding whole colony. Fig. 3. Surface view of vegetative cell, showing contractile vacuoles (arrowheads) distributed in the protoplast surface. Fig. 4. Optical section of vegetative cell, showing a single large pyrenoid (P) in the chloroplast, and contractile vacuoles (arrowheads) distributed in the protoplast surface. Fig. 5. Daughter colony formation in asexual reproduction. Note each cell is enclosed by a transparent vesicle or cellular envelope (arrows) within the parental gelatinous matrix (arrowheads). Fig. 6. Vegetative colony stained with methylene blue. Individual cellular sheaths of the colonial gelatinous matrix (asterisks) are apparent. Fig. 7. Mature aplanospores 27 days after transferring into nitrogen-deficient medium.

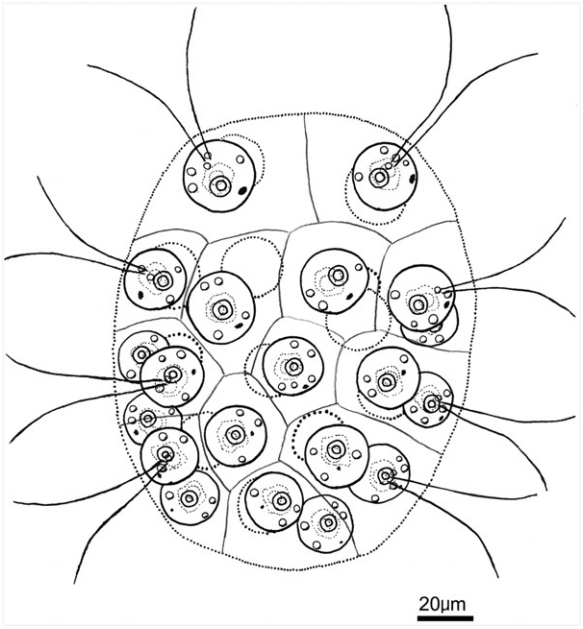


Fig. 8. Line drawing of 32-celled vegetative colony of an aplanosporic strain of *Eudorina unicocca* originating from Lake Tsukui, Kanagawa Prefecture, Japan.

Volvox sect. *Volvox*; *Pleodorina*; and *Eudorina*), but distantly separated from the clade comprising eight *Yamagishiella* strains (Fig. 9). Furthermore, the latter group was subdivided into two well-resolved clades, with 87–92% and 92–100% bootstrap values for the NJ and MP analyses, respectively. One clade comprised two aplanosporic strains and NIES-724, and the other contained NIES-725, NIES-726 and UTEX 1221.

Phylogenetic analyses of the ITS sequences showed two aplanosporic strains (TKI-C-2, 990601-IE-5) and four *E. unicocca* strains (NIES-724, -725, -726 and UTEX 1221) forming two robust monophyletic groups (Fig. 10), identical to the clades resolved in the *rbc*L gene phylogeny (Fig. 9).

Thus, our *rbc*L phylogeny places the aplanosporic strains in *Eudorina*, based on their phylogenetic position within the anisogamous/oogamous Volvocaceae members (Fig. 9). Furthermore, these strains can be classified as *E. unicocca*, on the basis of the single basal chloroplast pyrenoid in mature vegetative cells, the ovoid or ellipsoidal colony form (Figs 1, 2, 4; Goldstein 1964) and the sister phylogenetic relationship to NIES-724 that identified (Goldstein, 1964) as *E. unicocca* var. *unicocca* (Figs 9, 10; Table 1). As no additional small pyrenoids developed around the basal pyrenoid in this aplanosporic alga (Fig. 4), we identified it as *E. unicocca* var. *unicocca*, according to Goldstein's taxonomic system (1964).

Morphological differences in vegetative colonies of Eudorina *and* Yamagishiella

To elucidate the taxonomic significance of differences in the number and distribution of contractile vacuoles in vegetative cell protoplasts, we performed comparative morphological observations of four strains of *E. unicocca* (NIES-724, -725, -726 and UTEX 1221), *E. elegans* (NIES-717), *E. cylindrica* (NIES-722), *E. illinoisensis* (NIES-723) and five strains of *Y. unicocca* (NIES-578, -666, -762, -870 and -872). All *Eudorina* strains had several contractile vacuoles distributed over the entire surface of vegetative colony protoplasts (Figs 11–14), whereas *Yamagishiella* strains only had two contractile vacuoles near the base of the flagella (Figs 15–18).

Several or many contractile vacuoles throughout the surface of vegetative cell protoplasts have been observed in *E. illinoisensis* (NIES-459, NIES-460) (Nozaki, 1986), *E. minodii* (Chodat) Nozaki et Krienitz (NIES-856) (Nozaki & Krienitz, 2001) and *E. elegans* strains (NIES-456, -457 and -458) (Nozaki, 1984, 1985). Thus, based on the number and the distribution of contractile vacuoles, *Eudorina* and *Yamagishiella* can be clearly distinguished, indicating that the phylogenetic difference between these two genera (Nozaki *et al.*, 1998; Nozaki, 2003; Fig. 9) reflects not only modes of sexual reproduction (Nozaki & Kuroiwa, 1992) but also basic vegetative morphology.

Reclassification of Eudorina unicocca *based on morphological and molecular data*

To elucidate the taxonomic significance of the individual cellular sheaths in the colonial gelatinous matrix observed in the aplanosporic strains of *E. unicocca* (Figs 6, 8), we examined the gelatinous matrices using methylene blue on four other strains of *E. unicocca* (NIES-724, -725, -726 and UTEX 1221) that formed a monophyletic group with the aplanosporic strains in our *rbc*L phylogenetic analyses (Fig. 9). The stain revealed individual cellular sheaths in the colonial gelatinous matrix of NIES-724 (Fig. 19), as well as our aplanosporic strains (Figs 6, 8). However, NIES-725, NIES-726 and UTEX 1221 did not have this structure (Figs 20–22). Based on our phylogenetic analyses, the six strains of *E. unicocca* form two clades, one containing aplanosporic strains from two Japanese lakes and NIES-724, and the other comprising NIES-725, NIES-726 and UTEX 1221 (Figs 9, 10). Thus, the presence or absence of individual cellular sheaths in the colonial gelatinous matrix distinguishes these groups

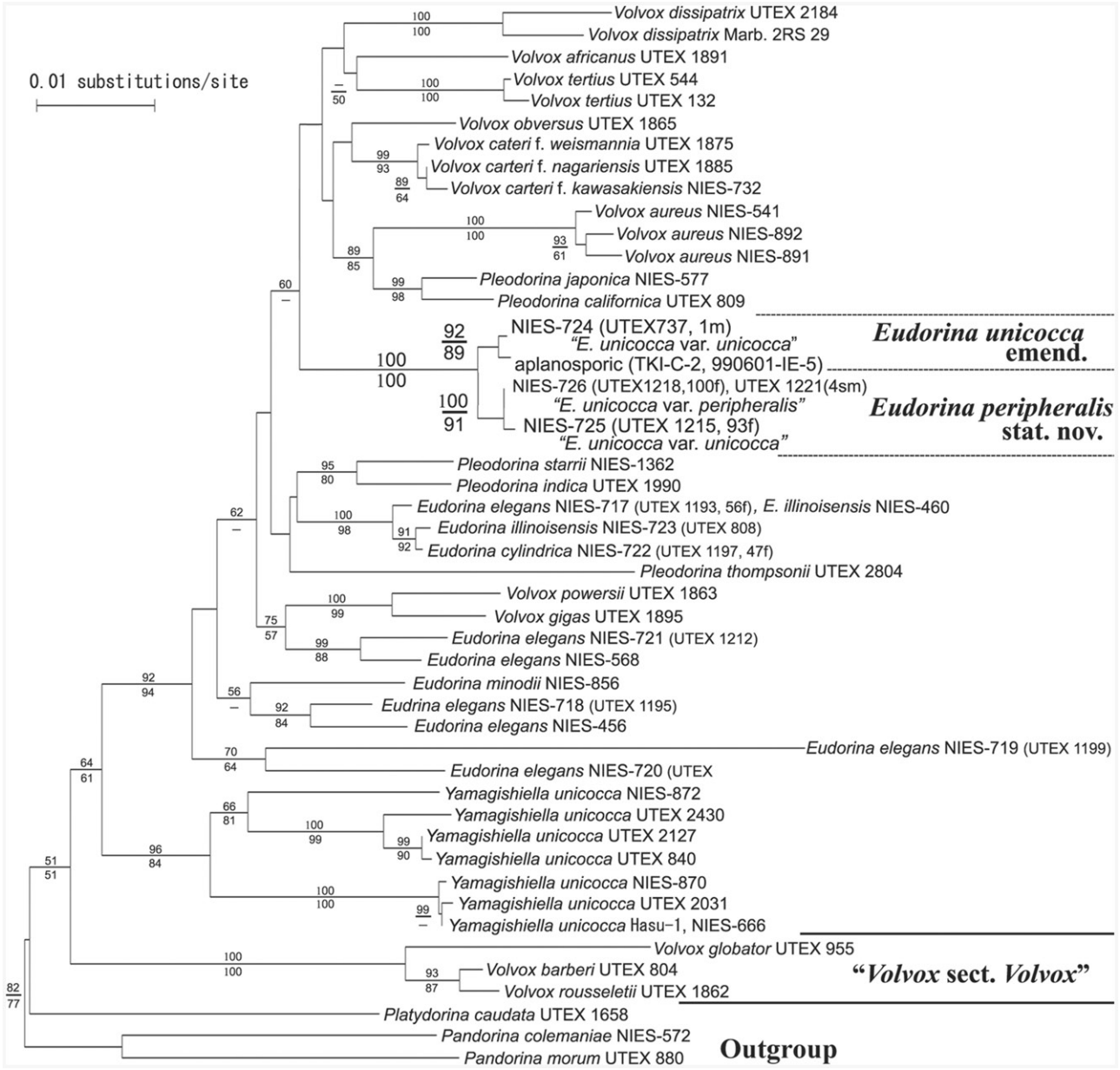


Fig. 9. Neighbor-joining (NJ) tree based on *rbc*L genes from 17 strains of *Eudorina* species, 17 strains of *Volvox*, five strains of *Pleodorina*, one strain of *Platydorina*, eight strains of *Yamagishiella*, and two strains of *Pandorina* (Table 1). Branch lengths are propotional to Kimura (1980) distances, which are indicated by the scale bar besides the tree. Numbers above or below the branches represent 50% or more bootstrap values based on 1,000 replications of the NJ or maximum parsimony analyses, respectively.

within *E. unicocca*. Goldstein (1964) reported almost complete sexual isolation between heterothallic strains belonging to these two groups. In *Pleodorina*, the presence or absence of similar individual cellular sheaths is a taxonomic criterion for distinguishing species (Nozaki *et al*. 1989, 2006). Thus, the two groups within *E. unicocca* could be classified as two species. However, both lineages contain strains (Japanese aplanosporic strains, NIES-724 and NIES-725) that contain only a single, basal pyrenoid in mature vegetative cell chloroplasts (i.e., *E. unicocca* var. *unicocca*, Goldstein, 1964) (Figs 1, 4), as in the original description (Smith, 1930). Therefore, either could be designated as *E. unicocca*. Here, we designate the monophyletic group comprising strains of *E. unicocca* var. *unicocca sensu* Goldstein (1964) as *E. unicocca*, and designate the other group, including *E. unicocca* var. *peripheralis* (NIES-726 and UTEX 1221; Table 1), as *E. peripheralis* (Figs 9, 10). Therefore, *E. unicocca*, as emended here, is characterized by having a single, basal pyrenoid

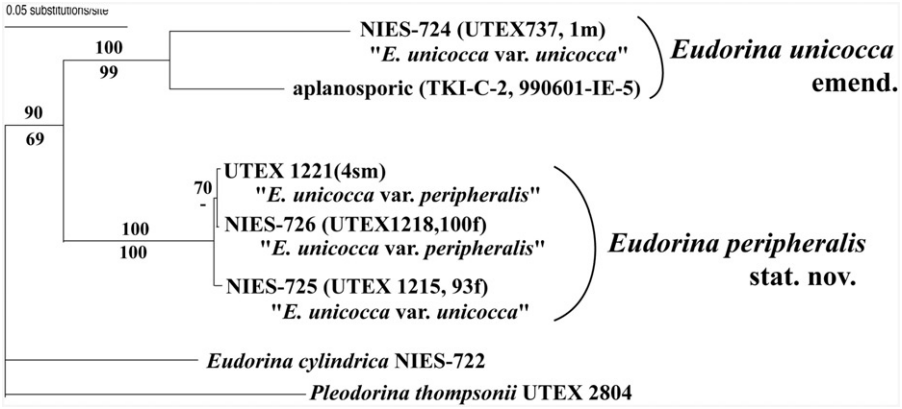
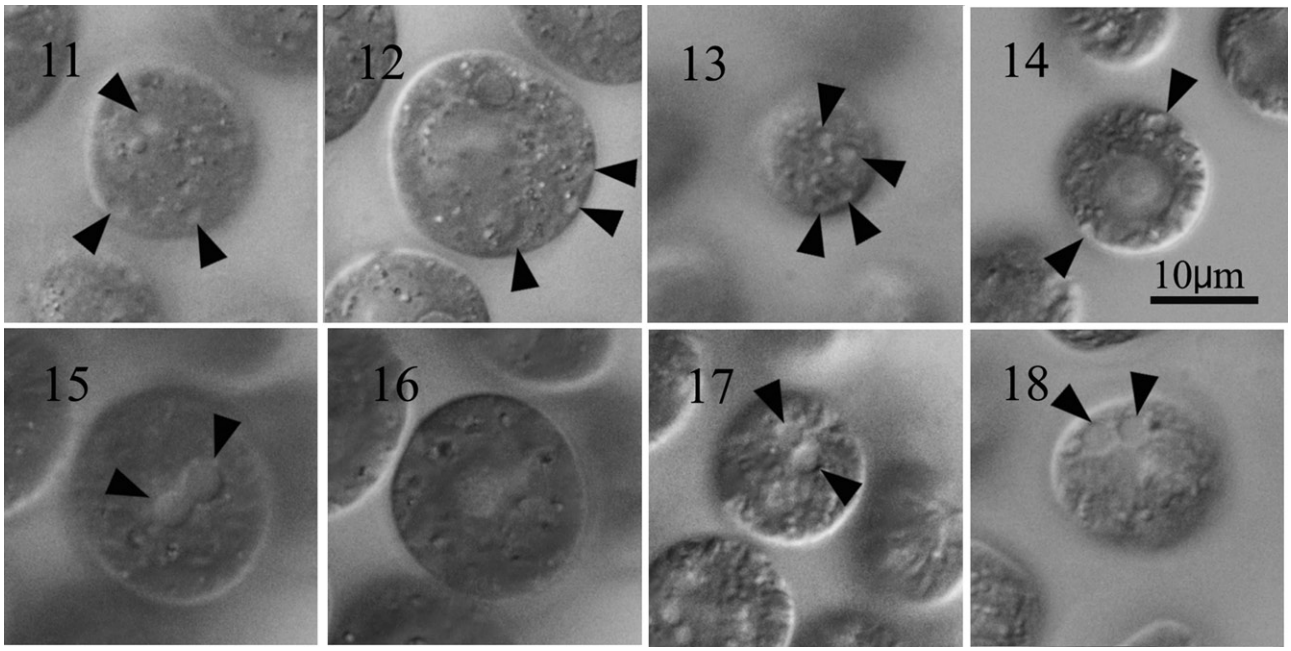
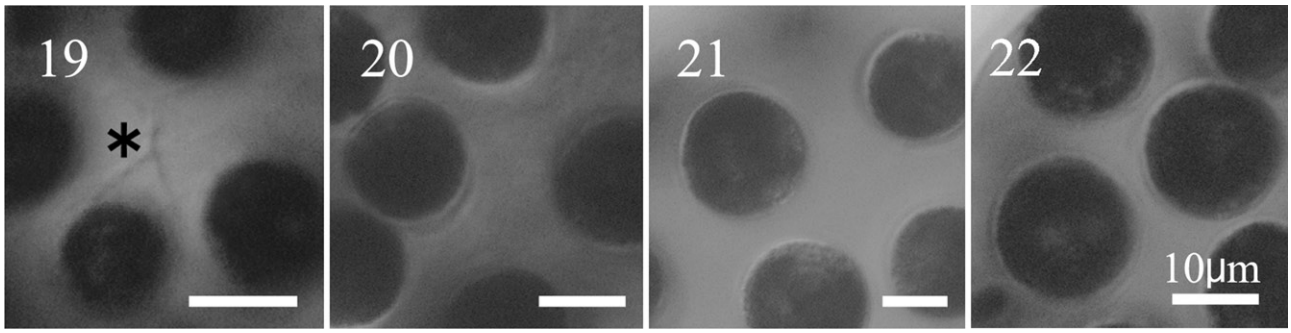


Fig. 10. Neighbor-joining (NJ) tree based on ITS sequences from 6 strains of *Eudorina* species and ones strain of *Pleodorina* (Table 1). Branch lengths are proportional to Kimura (1980) distances, which are indicated by the scale bar besides the tree. Numbers above or below the branches represent 50% or more bootstrap values based on 1,000 replications of the NJ or maximum parsimony analyses, respectively.



Figs 11–18. Vegetative cells of *Eudorina* and *Yamagoshiella* showing two types of distribution of contractile vacuoles in the protoplasts. Figs 11–14. *Eudorina*. Note each cell has several contractile vacuoles (arrowheads) distributed in the protoplast surface. Figs 11, 13 give surface views of protoplasts and Figs 12, 14 provide optical sections. Figs 11, 12. *E. unicocca* NIES-724 (UTEX 737, 1m). Fig. 13. *E. peripheralis* NIES-725 (UTEX 1215, 93f). Fig. 14. *E. illinoisensis* NIES-723. Figs 15–18. *Yamagishiella unicocca*. Note only two anterior contractile vacuoles (arrowheads) in the protoplast surface. Figs 15, 17, 18 are surface views of anterior regions of protoplasts and Fig. 16 an optical section of the protoplast. No contractile vacuoles are visible in the periphery. Figs 15, 16. Hasu-2. Fig. 17. NIES-762. Fig. 18. NIES-870.



Figs. 19–22. Vegetative colonies of *Eudorina unicocca* and *E. peripheralis* stained with methylene blue. Fig. 19. *E. unicocca* NIES-724 (UTEX 737,1m), showing individual cellular sheaths (asterisk). Fig. 20. *E. peripheralis* NIES-725 (UTEX 1215, 93f). Fig. 21. *E. peripheralis* NIES-726 (UTEX 1218, 100f). Fig. 22. *E. peripheralis* UTEX 1221 (4sm). Scale bars: 10 μm.

in mature vegetative cell chloroplasts, ovoid to ellipsoidal colonies and individual cellular sheaths in the gelatinous matrix of the colony. *Eudorina peripheralis* differs from *E. unicocca* in lacking individual sheaths. According to Goldstein (1964), *E. unicocca* var. *peripheralis* is characterized by having small pyrenoids around the large basal pyrenoid in mature vegetative cells. However, *E. peripheralis* includes NIES-725 (Figs 9, 10), which Goldstein (1964) identified as *E. unicocca* var. *unicocca*. Therefore, the presence or absence of small pyrenoids in mature cells represents intraspecific variability within *E. peripheralis*.

Taxonomic treatment

Eudorina peripheralis (Goldstein) T. K. Yamada stat. nov. (Figs 13, 20–22)

Basionym: *Eudorina unicocca* G. M. Smith var. *peripheralis* Goldstein, 1964, J. Protozool. 11: 324, Figs 11, 12.

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