Rediscovery of the species of 'ancestral *Volvox*': morphology and phylogenetic position of *Pleodorina sphaerica* (Volvocales, Chlorophyceae) from Thailand

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Abstract: *Pleodorina sphaerica* Iyengar was considered to be a phylogenetic link between *Volvox* and the type species *Pleodorina californica* Shaw because it has small somatic cells distributed from the anterior to posterior poles in 64- or 128-celled vegetative colonies. However, cultural studies and molecular and ultrastructural data are lacking in *P. sphaerica*, and this species has not been recorded since 1951. Here, we performed light and electron microscopy and molecular phylogeny of *P. sphaerica* based on newly established culture strains originating from Thailand. Morphological features of the present Thai species agreed well with those of the previous studies of the Indian material of *P. sphaerica* and with those of the current concept of the advanced members of the Volvocaceae. The present *P. sphaerica* strains exhibited homothallic sexuality; male and facultative female colonies developed within a single clonal culture. Chloroplast multigene phylogeny demonstrated that *P. sphaerica* was sister to two other species of *Pleodorina* (*P. californica* and *Pleodorina japonica* Nozaki) without posterior somatic cells, and these three species of *Pleodorina* formed a robust clade, which was positioned distally in the large monophyletic group including nine taxa of *Volvox* sect. *Merrillosphaera* and *Volvox* (sect. *Janetosphaera*) *aureus* Ehrenberg. Based on the present phylogenetic results, evolutionary losses of posterior somatic cells might have occurred in the ancestor of *P. californica* and *P. japonica*. Thus, *P. sphaerica* might represent an ancestral morphology of *Pleodorina*, rather than of *Volvox*.

Key Words: Molecular phylogeny, Morphology, *Pleodorina*, *Pleodorina sphaerica*, Sexuality, Somatic cell, *Volvox*

INTRODUCTION

The volvocine algae constitute a model lineage for studying both the evolution of multicellularity and the evolution of sex (Kirk 1998; Hiraide *et al.* 2013). Traditionally, the most advanced member of the volvocine algae was considered to be *Volvox*, and *Pleodorina* was the direct ancestral form of *Volvox* (Shaw 1922; Kirk 1998). Although colony cell number differs between *Volvox* (>500) and *Pleodorina* (≤128), both algae exhibit differentiation of small somatic cells in the spheroidal colony or spheroid (Nozaki & Ito 1994; Nozaki *et al.* 2006, 2015a). However, distribution of somatic cells in these two genera is distinct: in all of the species of *Volvox*, somatic cells are distributed along anterior to posterior poles of the spheroid; whereas, in all species of *Pleodorina* except *Pleodorina sphaerica* Iyengar, somatic cells are distributed only in the anterior region or half of the colony (Smith 1944; Nozaki *et al.* 2006, 2015a). Vegetative colonies of *Pleodorina sphaerica* are 64- or 128-celled, as in

Pleodorina californica Shaw, but it has small somatic cells distributed from anterior to posterior poles of the colony (Iyengar 1933; Iyengar & Ramanathan 1951). Thus, Iyengar (1933) suggested that *P. sphaerica* might be classified under *Volvox* if one ignored its small cell number. However, since Iyengar (1933) and Iyengar & Ramanathan (1951) studied field-collected samples of *P. sphaerica* from India, this species has not been recorded or cultured. Thus, phylogenetic position of this interesting taxon remained unresolved.

During a recent field collection of freshwater green algae in Thailand, we fortunately encountered *Pleodorina sphaerica*. Morphology, reproduction and phylogenetic position of cultured material of *P. sphaerica* originating from Thailand are described in this report.

MATERIAL AND METHODS

Water samples (pH 6.6; 29.0°C) were collected in Phon Thong Lake, Phon Thong District, Roi Et Province, Thailand (16°16′43.3″N, 104°00′0.02″E), on 28 November 2015. Clonal cultures of *Pleodorina sphaerica* (strains 2015-1128-2P-4 and 2015-1128-2P-12) were established with the pipette-washing method (Pringsheim 1946) from the water

sample. The cultures were grown in screw-cap tubes (18 × 150 mm) containing 11 ml of AF-6 medium (Kato 1982; Kasai *et al.* 2009) or AF-6/3 medium (Nozaki *et al.* 2015b) at 20°C or 25°C on a 14-h light:10-h dark schedule under cool-white fluorescent lamps at an intensity of 110–150 μmol m^{-2} s^{-1}. Vegetative and asexual colonies were observed by examining a small aliquot of colonies grown continuously by inoculating 0.5–1.0 ml of actively growing culture into fresh medium every 7–10 d. Sexual male and female colonies developed spontaneously in old cultures; for enhancing the production of sexual colonies, the culture was grown in Volvox thiamin acetate(VTAC)/3 medium (VTAC medium diluted with two volumes of distilled water) (Nozaki *et al.* 2016) at 20°C or 25°C.

Light microscopic examinations were carried out using a BX60 microscope (Olympus, Tokyo, Japan) equipped with Nomarski interference optics. For transmission electron microscopy (TEM), vegetative colonies were subjected to the double fixation (with 1.0% glutaraldehyde and 2% OsO$_4$ in pre- and post-fixations, respectively), enblocked and examined as previously described (Nozaki & Kuroiwa 1992) except using a JEM-1010 electron microscope (JEOL, Tokyo, Japan).

Extracting total DNA of *Pleodorina sphaerica* strain 2015-1128-2P-12 and *Volvox tertius* Meyer strain 1-3k-4 (Supplemental Material, Figs S1–S6) was performed as described by Nakada & Nozaki (2007). Sequencing the five chloroplast genes [large subunit of RuBisCO (*rbc*L), adenosine triphosphate (ATP) synthase beta subunit (*atp*B), Photosystem I P700 chlorophyll a apoprotein A1 (*psa*A), Photosystem I P700 chlorophyll a apoprotein A2 (*psa*B) and Photosystem II CP43 reaction centre protein (*psb*C) genes] was performed as described previously (Nozaki *et al.* 1995, 1999, 2006) except that five primers specific to three chloroplast genes of *P. sphaerica* strain 2015-1128-2P-12 and 12 specific primers of the *V. tertius* strain 1-3k-4 *psa*A gene (Supplemental Material, Table S1) were also used to complete the sequencing. Coding regions of these five genes of *P. sphaerica* (DDBJ/GenBank accession numbers LC215633∼LC215637) and *V. tertius* strain 1-3k-4 (DDBJ/GenBank accession numbers LC215627∼LC215631) were manually aligned to the 6021 base pairs of 38 operational taxonomic units (OTUs) of the advanced members of the colonial Volvocales (Eudorina group plus outgroup OTUs) previously studied (Nozaki *et al.* 2015a; available from TreeBASE: http://www.treebase.org/treebase-web/home.html; study ID: S18363). The alignment was subjected to phylogenetic analyses with 1000 replicates of bootstrap analyses (Felsenstein 1985), by maximum likelihood (ML) method (with the general time reversible + invariable sites + gamma distribution [GTR + I + G] model selected) using MEGA6.06 (Tamura *et al.* 2013) and by the maximum parsimony (MP) method using a heuristic search with stepwise addition of 10 random replicates (with the tree bisection-reconnection branch-swapping algorithm) using PAUP 4.0b10 (Swofford 2002). *Yamagishiella*, *Platydorina*, *Colemanosphaera* and *Volvox* sect. *Volvox* (*V. barberi* Shaw, *V. globator* Linnaeus and *V. rousseletii* G.S.West) were treated as the outgroup because their phylogenetic positions are very close to the Eudorina group (Nozaki *et al.* 2014).

Four new strains [*Pleodorina sphaerica* strains 2015-1128-2P-4 and 2015-1128-2P-12 and *Volvox tertius* strains 1-3k-4 and 1-3k-5 (Supplemental Material, Figs S1–S6)] were deposited to Microbial Culture Collection at the Institute for National Environmental Studies (Kasai *et al.* 2009) (http://mcc.nies.go.jp/localeAction.do?lang=en) as NIES-4066–4069.

RESULTS

Light microscopy

Vegetative colonies of *Pleodorina sphaerica* strains 2015-1128-2P-4 and 2015-1128-2P-12 were broadly ovoid or spherical in shape and contained 64 or 128 biflagellate cells arranged at the periphery of the gelatinous matrix, measuring up to 270 μm long (Fig. 1). The 64-celled or 128-celled colony contained 8–15 or 15–20 reproductive cells, respectively. The reproductive cells were spherical or subspherical in shape and were randomly distributed among the small somatic cells in the posterior half of the colony (Figs 1, 2). Somatic cells were spherical or ovoid in shape, measuring up to 13 μm in lengthwise diameter. Each somatic cell had a cup-shaped chloroplast with single basal pyrenoid and a stigma (Fig. 3). One to three contractile vacuoles were observed in the lateral side of the somatic cells. There was gradual reduction in stigma size in the somatic cells from anterior to posterior poles of the colony. Sometimes, one or two additional small stigmata could be seen in the chloroplast of the somatic cell. Each reproductive cell had a massive cup-shaped chloroplast, several or more contractile vacuoles randomly distributed in the protoplast surface and a nucleus, measuring up to 30 μm in diameter. Three to five large vacuoles were observed in the centre of the protoplast of mature reproductive cells (Fig. 4). Stigma is lacking in the chloroplast of reproductive cells (Fig. 2). Four to ten pyrenoids were randomly distributed in the chloroplast of mature reproductive cells (Fig. 4). When stained with methylene blue, no apparent structures separating cells within the gelatinous matrix (individual sheaths; Nozaki *et al.* 1989, 2006) could be recognized (Fig. 5).

Asexual reproduction was accomplished by daughter colony formation; successive divisions of each reproductive cell within a transparent vesicle inside the parental gelatinous matrix formed a cup-shaped plakea that inverted to develop into a compact spheroidal colony (Figs 6–8). During the inversion, each daughter protoplast grew two flagella, one of which became markedly longer than the other in the newly formed daughter colony (Fig. 9); the shorter one was embedded within the new gelatinous matrix of the daughter colony; whereas, the longer one appeared to move the whole daughter colony. The newly formed daughter colony did not show differentiation between somatic cells and reproductive cells (Figs 7–9). As the colony grew, the two flagella became equal in length, and the difference in cell size between somatic and reproductive cells gradually became apparent, as did the stigma in somatic cells.

Sexuality of the strains was homothallic: both male and female colonies developed within a single clonal culture, and zygotes were formed within the female colony. Number and arrangement in reproductive cells were indistinguishable among asexual, male and female colonies. Reproductive cells in the male colony performed successive divisions and partial
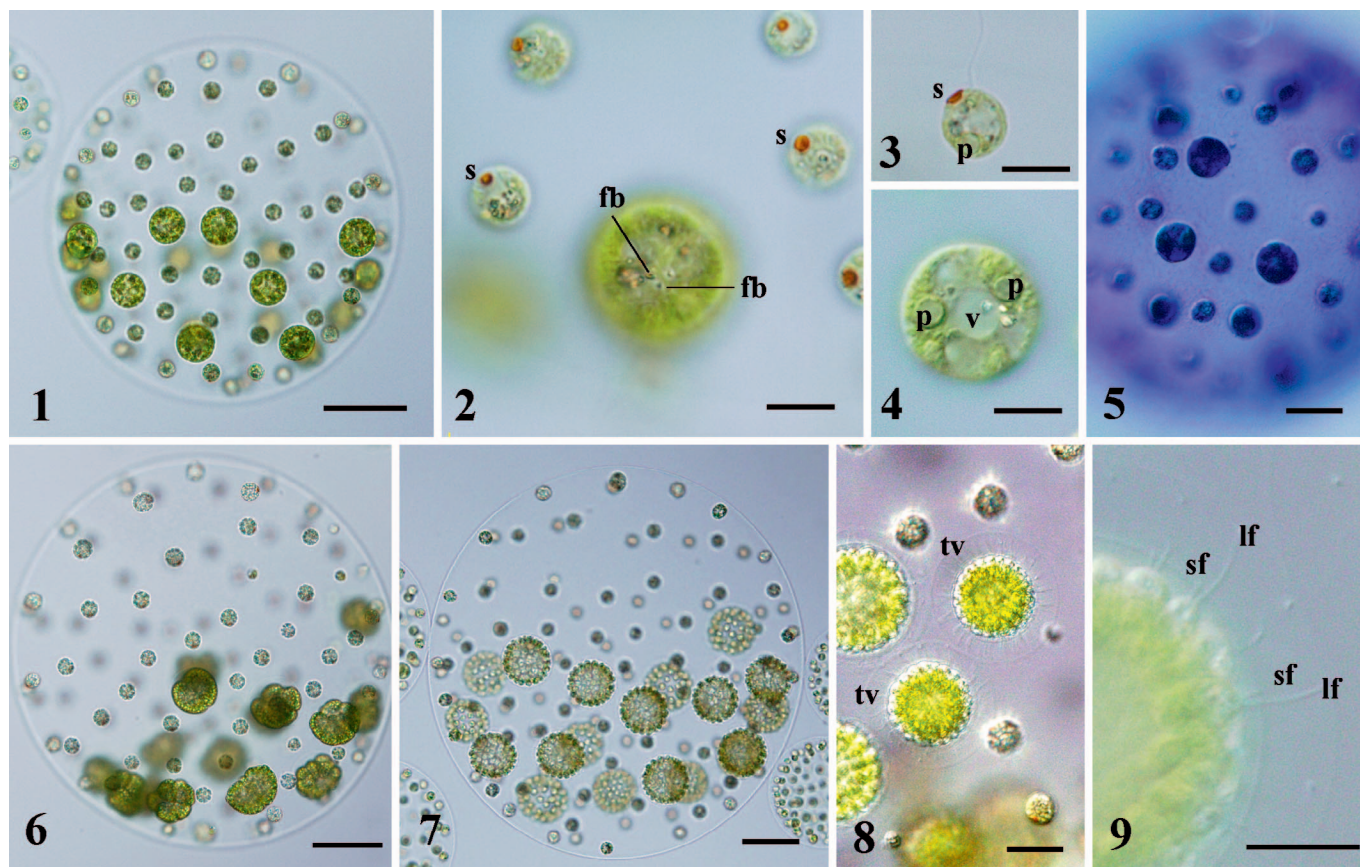
Figs 1–9. Light microscopy of *Pleodorina sphaerica* Iyengar strains 2015-1128-2P-4 (Figs 1–7) and 2015-1128-2P-12 (Figs 8, 9) originating from Thailand.

    Figs 1–5. Vegetative phase. Abbreviations: fb, flagellar base; p, pyrenoid; v, vacuole; s, stigma.

    Fig. 1. 128-celled colony. Scale bar = 50 μm.

    Fig. 2. Surface view of colony showing large reproductive cells and small somatic cells. Note that reproductive cell has two flagella and lacks stigma. Scale bar = 10 μm.

    Fig. 3. Optical section of somatic cell. Scale bar = 10 μm.

    Fig. 4. Semi-optical section of top view of reproductive cell. Scale bar = 10 μm.

    Fig. 5. Colony stained with methylene blue. Note that distinct individual sheaths are lacking in the gelatinous matrix. Scale bar = 20 μm.

    Figs 6–8. Asexually reproducing colonies.

    Fig. 6. Two- or four-celled stage. Scale bar = 50 μm.

    Fig. 7. Daughter colonies developing within the parental colony. Scale bar = 50 μm.

    Fig. 8. Portion of colony showing a transparent vesicle (tv) encompassing each daughter colony. Scale bar = 20 μm.

    Fig. 9. Portion of newly formed daughter colony showing short flagellum (sf) and long flagellum (lf) in each protoplast. Scale bar = 10 μm.

inversion to form packets of 128 (possibly) spindle-shaped cells or male gametes (Figs 10–12). Sperm packets escaped from the parental male colony and swam to female colonies then dissociated into individual male gametes that penetrated into the female colony for conjugation. The male gamete was spindle-shaped and had two equal flagella (Fig. 13). No prominent cytoplasmic protuberances were observed near the base of the flagella even using phase contrast microscopy (Fig. 13). Morphologically, female colonies could not be distinguished from asexual colonies except that large reproductive cells (gamete) in the female colonies became thick-walled possible zygotes after the penetration of male gametes into the female colony (Fig. 14). The zygotes were spherical in shape and had a double-layered smooth wall when immature (green in colour) (Fig. 15). Such a double-layered structure was not

evident in fully matured reddish zygotes, which measured 28–30 μm in diameter (Figs 16, 17).

Transmission electron microscopy

The ultrastructure of the *Pleodorina sphaerica* extracellular matrix agreed well with that of the advanced genera of the Volvocaceae, such as *Yamagishiella*, *Eudorina*, *Pleodorina* and *Volvox* (Nozaki & Kuroiwa 1992). The entire colony was surrounded by the tripartite layer (colonial boundary; Nozaki & Kuroiwa 1992) of the extracellular matrix (Figs 18–20). Each protoplast was enclosed tightly by the dense, fibrillar extracellular matrix (cellular envelope; Nozaki & Kuroiwa 1992) (Figs 19–21).

    The cell had a massive cup-shaped chloroplast, centrally located nucleus and mitochondrial profiles that were mainly
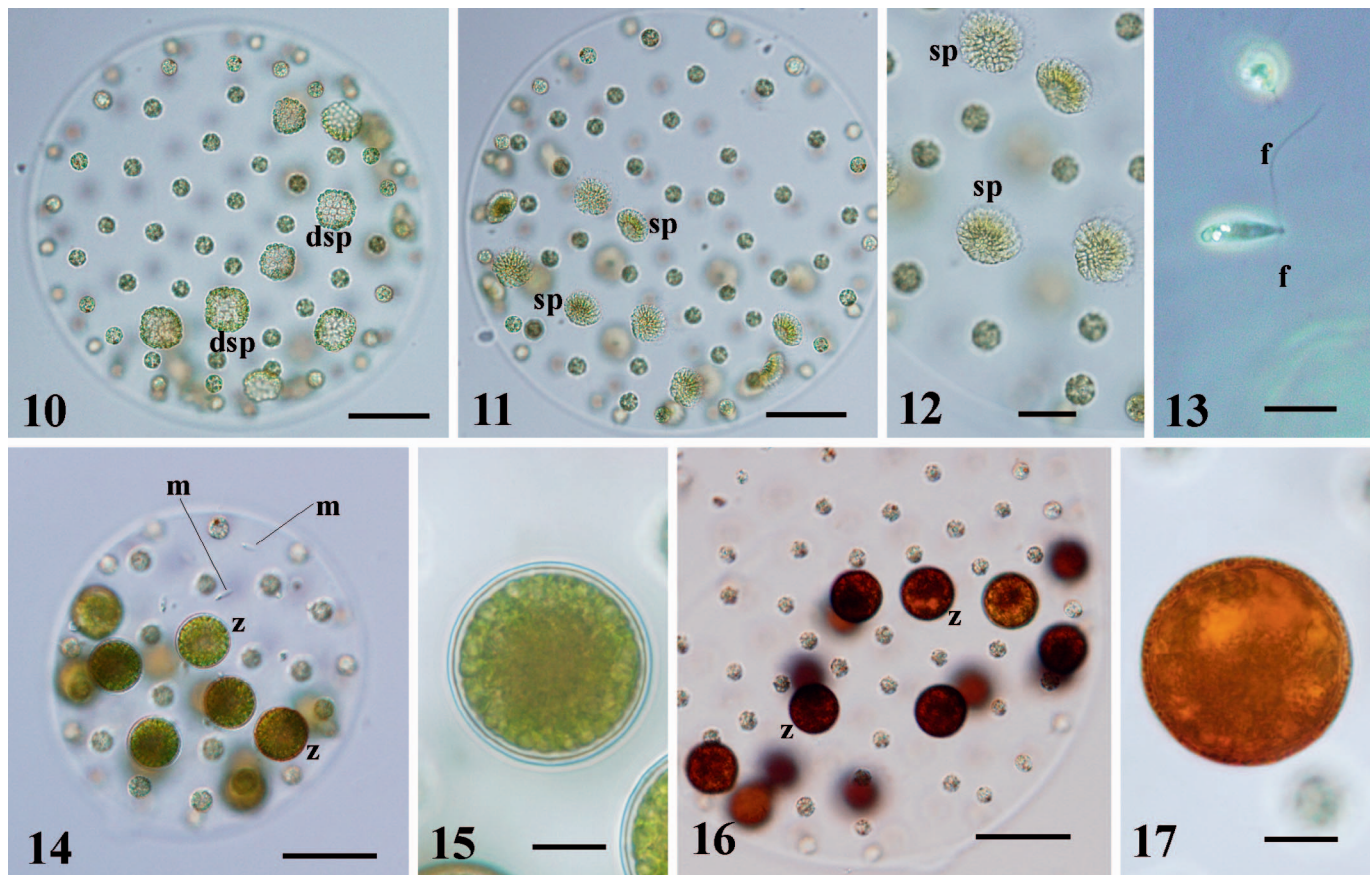
Figs 10–17. Light microscopy of sexual reproduction in *Pleodorina sphaerica* Iyengar strains 2015-1128-2P-4 (Figs 11, 12) and 2015-1128-2P-12 (Figs 10, 13–17) originating from Thailand.

  Fig. 10. Male colony with developing sperm packets (dsp). Scale bar = 50 µm.
  Fig. 11. Male colony with sperm packets (sp). Scale bar = 50 µm.
  Fig. 12. Sperm packets (sp) developing within the male colony. Scale bar = 20 µm.
  Fig. 13. Male gamete. Note no cytoplasmic protuberance at the same of the flagella (f). Scale bar = 10 µm.
  Fig. 14. Female colony with immature zygotes (z) and penetrating male gametes (m). Scale bar = 50 µm.
  Fig. 15. Immature zygote with double-layered cell wall. Scale bar = 10 µm.
  Fig. 16. Female colony with mature zygotes (z). Scale bar = 50 µm.
  Fig. 17. Mature zygote. Scale bar = 10 µm.

distributed in the cytoplasm between cell membrane and the chloroplast (Figs 18–21). Pyrenoids were observed within the chloroplast, surrounded by starch granules. Tubular thylakoid lamellae penetrated the pyrenoid matrix from various directions between starch grains (Fig. 21). Large vacuoles were evident within the cytoplasm especially in reproductive cells (Figs 18, 19).

Molecular phylogeny

Chloroplast multigene phylogenetic analyses demonstrated a large monophyletic group [Volvox–Pleodorina clade or clade I of Nozaki *et al.* (2015a)] composed of *Volvox* sect. *Merrillosphaera* sensu Nozaki *et al.*, *Volvox* sect. *Janetosphaera* (composed of only *Volvox aureus* Ehrenberg) and three species of *Pleodorina* (*P. californica*, *P. japonica* Nozaki and *P. sphaerica*); the grouping was supported by 94–100% bootstrap values in MP and ML analyses (Fig. 22). This monophyletic group was subdivided into two sister clades, one

composed of nine taxa of *V.* sect. *Merrillosphaera*, the other containing *V. aureus* and the three species of *Pleodorina*. These three species of *Pleodorina* constituted a robust clade (with 100% bootstrap values in MP and ML analyses) in which *P. sphaerica* diverged basally from the other two species (Fig. 22). Other phylogenetic relationships were essentially the same as those reported by Nozaki *et al.* (2015a).

DISCUSSION

The present alga can be clearly identified as *Pleodorina sphaerica* in having 64- or 128-celled vegetative colonies in which small somatic cells are distributed from anterior to posterior poles of the colony (Iyengar 1933; Iyengar & Ramanathan 1951; Nozaki *et al.* 2006). While this type of somatic cell distribution is similar to that of all species of *Volvox*, *Volvox* has more than 500 cells in a vegetative or asexual colony/spheroid (Smith 1944; Isaka *et al.* 2012;
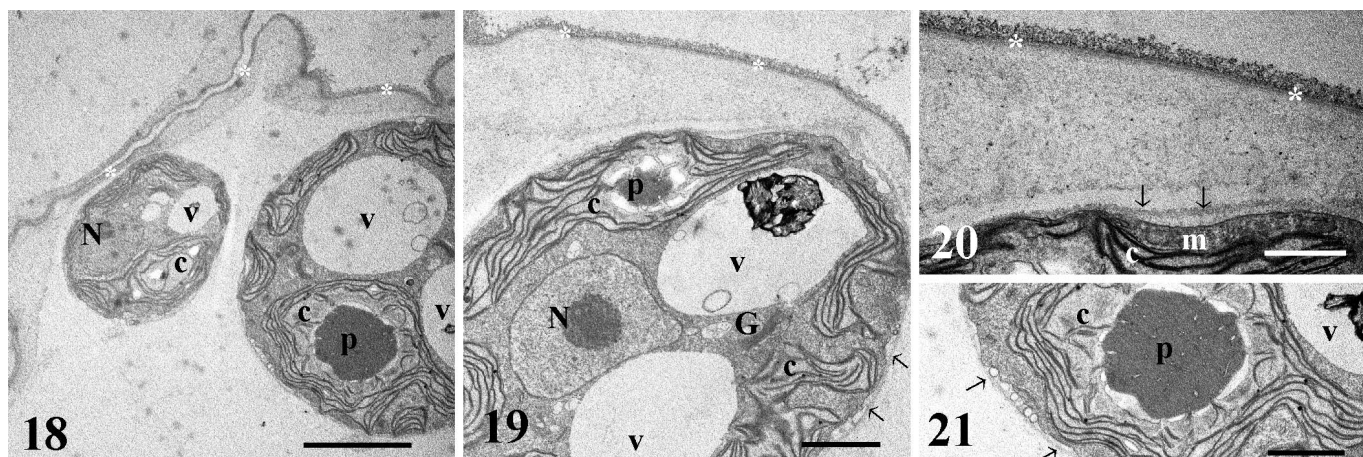
Figs 18–21. Transmission electron microscopy of vegetative colonies of *Pleodorina sphaerica* Iyengar strain 2015-1128-2P-12 originating from Thailand. Each protoplast is enclosed by a dense fibrillar layer (cellular envelope; arrows) of the extracellular matrix inside a tripartite layer (colonial boundary; asterisks) encompassing whole colony. Abbreviations: c, chloroplast; G, Golgi body; m, mitochondrion; N, nucleus; p, pyrenoid; v, vacuole.
  Fig. 18. Part of colony showing somatic cell (left side) and reproductive cell (right side). Scale bar = 2 μm.
  Fig. 19. Median section of reproductive cell showing large vacuoles. Scale bar = 2 μm.
  Fig. 20. Peripheral portion of colony. Scale bar = 1 μm.
  Fig. 21. Part of cell showing pyrenoid in the chloroplast. Scale bar = 2 μm.

Nozaki *et al.* 2015a). By contrast, other species of *Pleodorina*, such as the type species *Pleodorina californica*, have no somatic cells that are distributed in the posterior pole of the colony (Nozaki *et al.* 2006). Thus, Iyengar (1933) considered *P. sphaerica* to represent an intermediate taxon between *P. californica* and *Volvox*.

The present phylogeny demonstrated that three species of *Pleodorina* (*P. californica*, *P. japonica* and *P. sphaerica*), nine taxa of *Volvox* sect. *Merrillosphaera* and *Volvox* (sect. *Janetosphaera*) *aureus* formed a large monophyletic group (Volvox–Pleodorina clade). These three species of *Pleodorina* constituted a small clade that was sister to *V. aureus* within the Volvox–Pleodorina clade, and *P. sphaerica* was basal to two other species of *Pleodorina* without posterior somatic cells (*P. californica* and *P. japonica*; Nozaki *et al.* 1989). Because only *P. californica* and *P. japonica* lack posterior somatic cells within the Volvox–Pleodorina clade (Nozaki *et al.* 1989, 2015a), evolutionary loss of posterior somatic cells might have occurred in the ancestor of *P. californica* and *P. japonica* (Fig. 20). Thus, evolution of *Pleodorina californica*/ *japonica* from a *P. sphaerica*–like ancestor might have occurred via loss of posterior somatic cells. *Pleodorina sphaerica* may therefore represent an ancestral morphology of *Pleodorina*, rather than of *Volvox*.

Both male and female colonies developed to form zygotes within a single clonal culture of *Pleodorina sphaerica*. Such homothallic and dioecious sexuality is observed in *Volvox aureus*, *Pleodorina californica* and *Pleodorina japonica* (Goldstein 1964; Darden 1966; Nozaki 1984; Nozaki *et al.* 1989), which constitute a robust monophyletic group with *P. sphaerica* (Fig. 22). Furthermore, female colonies of these species are facultative; i.e. female colonies are morphologically indistinguishable from asexual or vegetative colonies. Within the Volvox–Pleodorina clade, *Volvox tertius* also exhibits homothallic and dioecious sexuality with facultative females

(Starr 1968; Kasai *et al.* 2009; Supplemental Material, Figs S1– S6). Although sexuality is unknown in *Volvox ovalis* (Nozaki & Coleman 2011), other *Volvox* taxa within *Volvox* sect. *Merrillosphaera* sensu Nozaki *et al.* (2015a) analyzed in the present phylogeny (Fig. 22) produce 'special females' [sexual female spheroids are morphologically different from asexual spheroids (Starr 1968; Nozaki 1988; Nozaki & Coleman 2011; Nozaki *et al.* 2015a)], and their sexual spheroids may be monoecious, monoecious plus males, homothallic dioecious or heterothallic dioecious (Starr 1968, 1971; Nozaki *et al.* 2015a). Thus, the sexual type 'homothallic, dioecious with facultative females' appears to have evolved in parallel within Volvox– Pleodorina clade (Fig. 22). Although not included in the present chloroplast multigene phylogeny (due to the lack of available culture strains), *Volvox pocockiae* Starr and *Volvox spermatosphaera* Powers are also characterized by facultative females (Starr 1970). *Volvox spermatosphaera* is closely related to *Volvox ovalis* and *Volvox tertius* based on the sequences of internal transcribed spacer region 2 (ITS-2) of nuclear ribosomal DNA (Nozaki & Coleman 2011). However, phylogenetic position of *V. pocockiae* is not well resolved based on ITS-1 and ITS-2 sequences (Coleman 1999).

The present phylogenetic tree includes three other species of *Pleodorina* (*P. indica* Iyengar, *P. starrii* H.Nozaki, F.D.Ott & A.W. Coleman and *P. thompsonii* F.D.Ott, H.Nozaki, A.W.Coleman) lacking posterior somatic cells (Nozaki *et al.* 1989, 2006). They are separated from Volvox– Pleodorina clade (Fig. 22). Both *Pleodorina indica* and *Pleodorina starrii* exhibit heterothallic sexuality in culture (Starr & Zeikus 1993; Nozaki *et al.* 2006); whereas, whether the sexuality is homothallic or heterothallic is unknown in *Pleodorina thompsonii* (Nozaki *et al.* 2006). Thus, *P. indica* and *P. starrii* are fundamentally different from *P. californica*, *P. japonica* and *P. sphaerica* in phylogeny and sexuality.
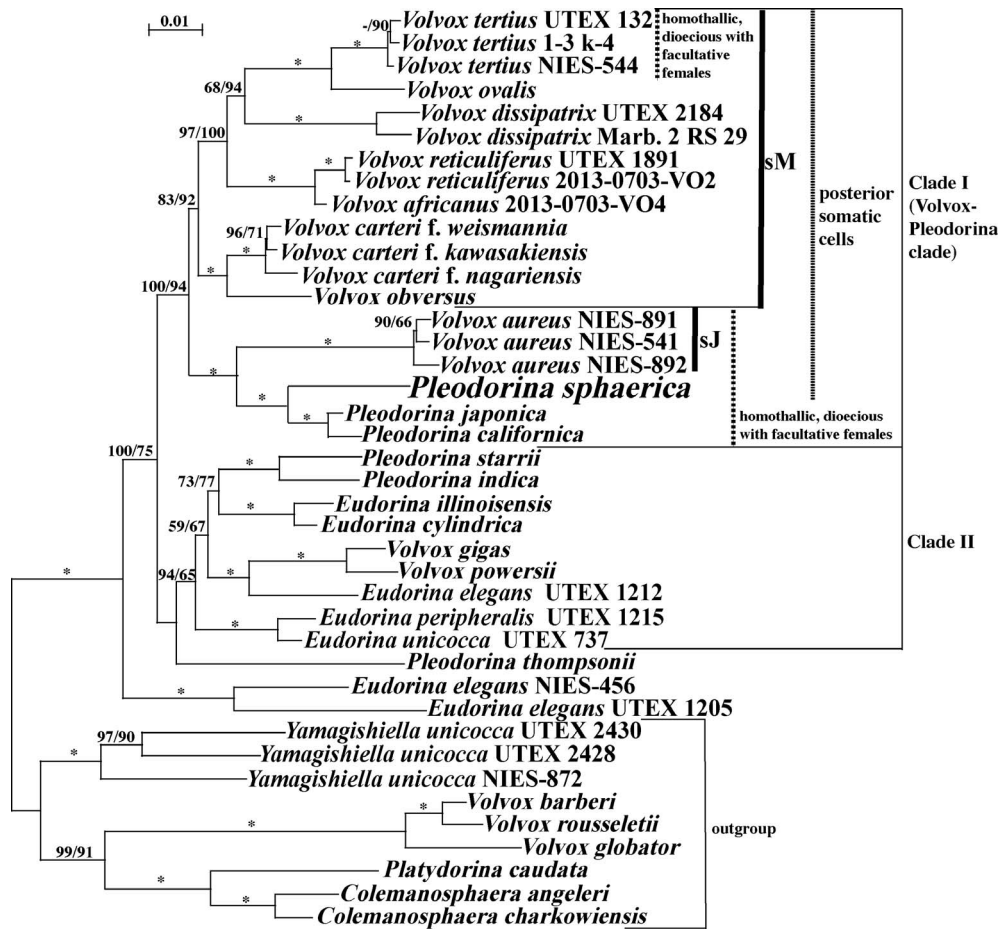
Fig. 22. Phylogenetic position of *Pleodorina sphaerica* Iyengar within the advanced members of the Volvocaceae [Eudorina group (Nozaki *et al.* 2000)], as inferred from 6021 base pairs of five chloroplast genes. The tree was constructed by maximum likelihood (ML) method. Branch lengths are proportional to the genetic distances, which are indicated by the scale bar above the tree. Numbers on the left or right side at the branches represent bootstrap values (50% or more, based on 1000 replicates) obtained with the ML and maximum parsimony analyses, respectively. Asterisks at the branches indicate 100% bootstrap values by the two methods. For details of the methods, see the text. Clade I and clade II are essentially the same as those in Nozaki *et al.* (2015a) except for the addition of *P. sphaerica* and *Volvox tertius* Meyer strain 1-3k-4 in the present study. 'sM' and 'sJ' in clade I represent *Volvox* sect. *Merrillosphaera* and *Volvox* sect. *Janetosphaera*, respectively, based on Nozaki *et al.* (2015a).

ACKNOWLEDGEMENT

SUPPLEMENTARY DATA

Supplementary data associated with this article can be found online at http://dx.doi.org/10.2216/17-3.1.s1.

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