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Research Note

Morphology and reproduction of *Volvox capensis* (Volvocales, Chlorophyceae) from Montana, USA

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Abstract: *Volvox capensis* was recorded previously only from South Africa. Here we established culture strains of this species from a sample collected in Montana, USA. Morphological details of asexual and sexual spheroids and molecular phylogeny of these strains were studied. The present alga was identified as *V. capensis* on the basis of morphological characteristics of asexual spheroids and zygotes. However, differences between the Montana and South African materials were recognized in number of sperm packets in a sexual, monoecious spheroid as well as in mode of gametic union between sperm and eggs. Possible polyspermy was observed in eggs of *V. capensis* by 4′-6-diamidino-2-phenylidole staining. Genetic difference between these two entities was small based on sequences of internal transcribed spacer 2 region of nuclear ribosomal DNA.

Key Words: Monoecious species, Morphology, Polyspermy, Reproduction, *Volvox capensis*, *Volvox* sect. *Volvox*

Volvox is a genus of multicellular green flagellates, originally described by Linnaeus (1758). Smith (1944) classified the genus into four sections based on differences in asexual spheroid morphology. *Volvox* sect. *Volvox* (= *Euvolvox* sensu Smith 1944) has interesting morphological features that are not found in other members of the colonial Volvocales: the spheroids have thick cytoplasmic bridges between stellate or amoeboid somatic cells (Smith 1944). Recent molecular phylogenetic analyses demonstrated that *Volvox* sect. *Volvox* is separated from other members of *Volvox*, *Eudorina* and *Pleodorina* (Nozaki *et al.* 2000, 2014; Herron *et al.* 2009).

Approximately 10 species of *Volvox* sect. *Volvox* have been described (Smith 1944; Iyengar & Desikachary 1981; Isaka *et al.* 2012). Three species of this section (*V. capensis* Rich & Pocock, *V. amboensis* Rich & Pocock and *V. perglobator* Powers) were known only from the country of the original description (Smith 1944; Starr *et al.* 1980; Iyengar & Desikachary 1981; Nozaki 2003; Isaka *et al.* 2012). Recently, Isaka *et al.* (2012) found two new species of *Volvox* sect. *Volvox* in Japan (*V. ferrisii* N. Isaka *et al.* and *V. kirkiorum* Nozaki *et al.*) that have not been found elsewhere. Thus, the distribution of species of *Volvox* sect. *Volvox* appears restricted to small areas, although this may be a consequence of species rarity.

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Volvox capensis is one such endemic species; it was originally described based on material originating from South Africa (Rich & Pocock 1953). They characterized this species by morphological attributes in asexual and sexual spheroids. Starr *et al.* (1980) established a strain of *V. capensis* (K37) from South African material. They reported that sexual reproduction was induced by L-glutamic acid and that *V. capensis* grew equally well in light and in darkness in a medium supplemented with sodium acetate. Although morphological details of asexual and sexual spheroids in clonal culture, combined with molecular phylogenetic information, are important for species taxonomy in *Volvox* sect. *Volvox* (Isaka *et al.* 2012), Starr *et al.* (1980) only briefly described morphology of asexual and sexual spheroids of the South African *V. capensis*. However, this *V. capensis* strain is not available, and no other culture strains of *V. capensis* have been reported.

Recently, we established cultures of *V. capensis* from a soil sample collected in Montana, USA. The surprising discovery in North America of a species only previously described from South Africa may have important implications for biogeography, dispersal and rates of molecular and morphological evolution within *Volvox* sect. *Volvox*. Morphological details of asexual and sexual spheroids and molecular phylogeny of these strains are described in this report.

Soil samples were collected in a marsh connected to Ninepipe Reservoir, Montana, USA (47°26′20.9″N, 114°05′52.9″W) on 28 July 2014. The soils were imported to Japan by permission of the Minister of Agriculture,

Forestry and Fisheries, Japan, based on the Plant Protection Law. Clonal cultures (strains M1-2–7) were established with the pipette-washing method (Pringsheim 1946) from media obtained by rewetting a small amount of dried soil (*c*. 0.5 g) in Petri dishes (90 × 20 mm). The cultures were grown in screw-cap tubes (18 × 150 mm) containing 11 ml of VTAC medium [containing 200 mg l^{-1} sodium acetate $4H_2O$ in growth medium (Nozaki 1983)] at 20°C, 23°C or 25°C on a 14:10 light:dark (LD) schedule under cool-white fluorescent lamps at an intensity of 110–150 μmol m^{-2} s^{-1}. Asexual spheroids were observed by examining a small aliquot of spheroids grown continuously by inoculating 0.5–1.0 ml of actively growing culture into fresh medium every 2–5 d. Sexual spheroids developed spontaneously in a culture ≥ 2 d old with VTAC medium at 25°C. For maturation of sexual spheroids and their fertilized eggs or zygotes, 0.5–1.0 ml actively growing culture with sexual spheroids was inoculated into 11 ml USVT medium [VTAC medium supplemented with 40 mg l^{-1} urea and 40 ml l^{-1} soil extract medium (*c*. 0.5 mg paddy soil in 20 ml distilled water autoclaved for 10 min)].

For maintenance of cultures, the spheroids were grown in 11 ml AF-6/3 medium [AF-6 medium (Kato 1982; Kasai *et al*. 2009) diluted with two volumes of distilled water] at 20°C on LD or in 11 ml 600ACVT medium (VTAC with 600 mg l^{-1} sodium acetate) at 15°C or 20°C in darkness. Inoculations of 0.2–0.5 ml culture with fresh medium were performed every 1–3 wk.

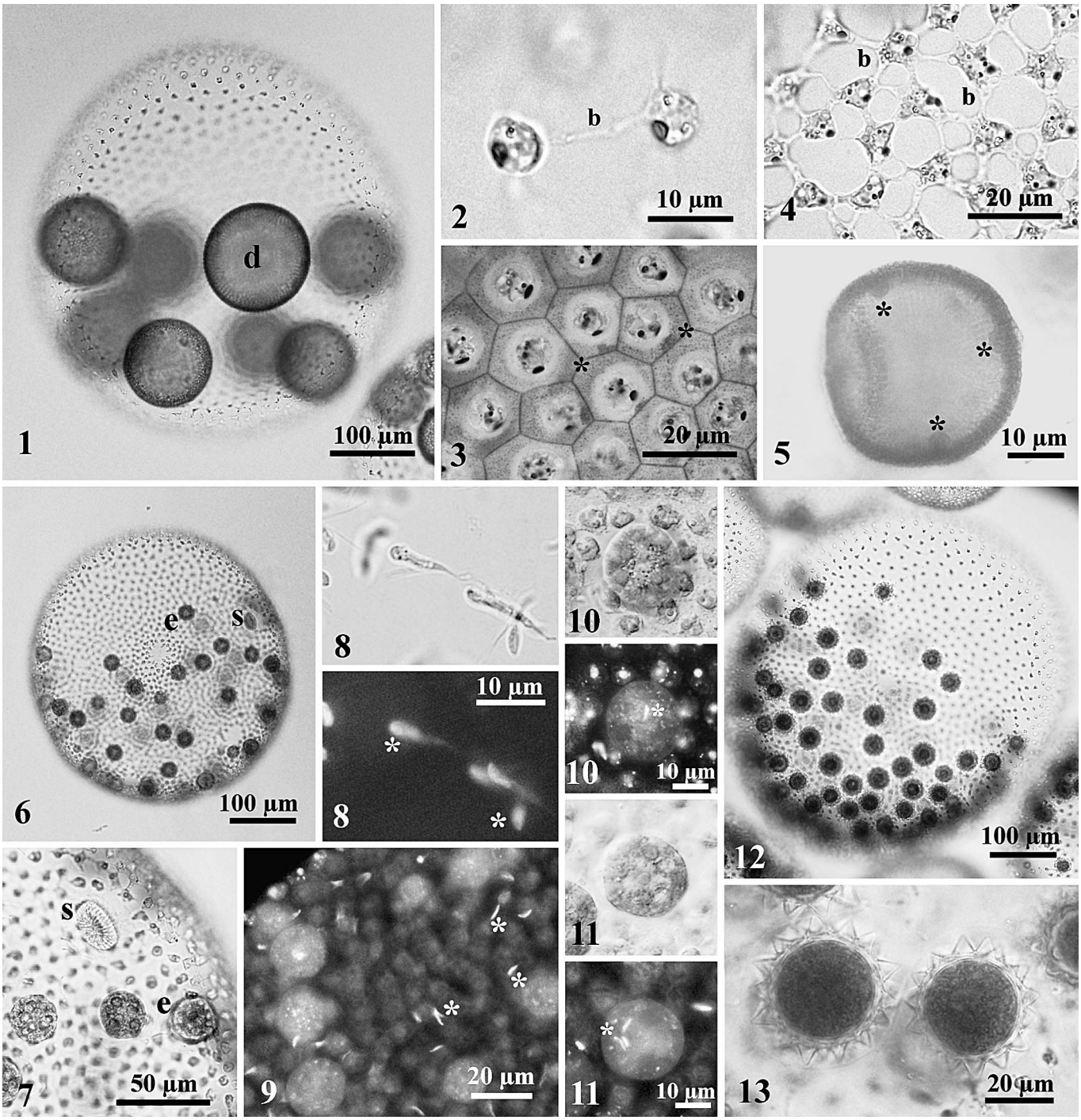
Light microscopy was carried out on a BX60 microscope (Olympus, Tokyo, Japan) equipped with Nomarski optics. Numbers of cells in spheroids were measured as described by Smith (1944) and Nozaki (1988). Individual cellular sheaths of the gelatinous matrix of the spheroids were examined after mixing approximately 10 μl of the cultured material with 2–5 μl 0.002% (w/v in distilled water) methylene blue (1B-429 WALDECK GmbH & Co. Division Chroma, Münster, Germany). For detecting nuclei of sperm, the cells were stained with 4′-6-diamidino-2-phenylidole (DAPI) as described previously (Nozaki *et al*. 1989) and observed with an epifluorescence microscope (Olympus BX-60).

For deducing the phylogenetic position of the alga, we used the large subunit of Rubisco (*rbc*L) plus photosystem II CP43 apoprotein (*psb*C) genes and the internal transcribed spacer (ITS) regions of nuclear ribosomal DNA (rDNA) (ITS-1, 5.8S *r*DNA and ITS-2) from operational taxonomic units (OTUs) listed in Table S1. Aligning of the two data sets (*rbc*L-*psb*C genes and ITS rDNA) was described previously (Isaka *et al*. 2012). Designation of the out-group was carried out based on the phylogenetic results by Isaka *et al*. (2012) and Nozaki *et al*. (2014). Maximum-likelihood (ML) analyses based on the selected models (GTR+5G and K2 models for *rbc*L-*psb*C genes and ITS rDNA, respectively) with 1000 replicates of bootstrap analyses (Felsenstein 1985) were performed by MEGA v5.2.2 (Tamura *et al*. 2011). In addition, 1000 replicates of bootstrap analyses were performed using maximum parsimony (MP) based on a branch-and-bound search by PAUP v4.0 (Swofford 2002).

As reported by Starr *et al*. (1980) for the South African strain of *V*. *capensis*, sexual spheroids developed spontaneously as the culture aged in AF-6/3 medium under LD; whereas, cultures in 600ACVT growing in darkness did not produce sexual spheroids. All of the spheroids on LD became sexual during 2 or 4 wk. Such sexual spheroids (even though they had eggs or zygotes) did not form new spheroids even when they were inoculated into new medium. Although cultures in darkness produced only asexual spheroids, the spheroids in 7–21-d-old cultures became immobile, accumulated in the bottom of culture tube and did not grow even when inoculated into new medium. Thus, inoculations must be carried out every 1–3 wk in AF-6/3 medium (LD) or in 600ACVT medium (darkness) in order to maintain the culture strains.

Asexual spheroids of the cultured material were spherical to ovoid in shape, were up to 600 μm long and contained 2000–6200 somatic cells and 7–20 (usually 8–10) gonidia distributed in the posterior two-thirds portion (Fig. 1). Somatic cells in the anterior region of the spheroid were pear-shaped, spherical or ovoid in side view, with approximately identical length and width or longer in length than width (Fig. 2). Somatic cells were up to 8 μm with two flagella, a single stigma and a cup-shaped chloroplast with a single pyrenoid and were enclosed by individual sheaths (Figs 2, 3). The cells were connected by one another via thick cytoplasmic bridges (Fig. 4). Gonidia were evident in juvenile spheroids or even during inversion stage of daughter spheroid formation (Fig. 5). Sexual spheroids were up to 500 μm long with 3200–6200 cells and were monoecious with 39–145 (usually 70–100) eggs and two to six sperm packets (Fig. 6). Sperm packets were compressed globoids composed of biflagellate male gametes (sperm; Fig. 7). Sperm packets did not escape from the parental sexual spheroid; at maturity they dissociated into individual sperm (Fig. 8). Fertilization between egg and sperm took place within the same sexual spheroid (Fig. 9). Penetration of the sperm nuclei into eggs was confirmed when stained with DAPI (Fig. 10). Sometimes, two or more elongate sperm nuclei were observed within the egg protoplast (Fig. 11). Mature zygotes were 32–35 μm in diameter (when measured excluding the spines) with a thick cell wall with spines (Figs 12, 13). Spines of zygote wall were 4–7 μm and almost straight with a blunt tip (Fig. 13).

This organism was similar to the original *V*. *capensis* samples and the culture strain [K37 (Starr *et al*. 1980) = zyg-6 (Mai & Coleman 1997); Coleman, personal communication] originating from South Africa (Rich & Pocock 1933; Starr *et al*. 1980) in shape of anterior somatic cells and number of gonidia in asexual spheroids, number of zygotes (eggs) in the monoecious sexual spheroid and shapes of zygote spines (Table S2). However, the present Montana strains were different from the South African materials in number of sperm packets in the sexual spheroid, modes of fertilization between sperm and egg and size of zygotes (Table S2). In the Cape Flats (South Africa) material of *V*. *capensis* (Rich & Pocock 1933) and the South African strain (Starr *et al*. 1980), the numbers of sperm packets in a monoecious colony were 5–19 and 10–15, respectively. The number of sperm packets in the monoecious spheroid of *V*. *capensis* var. *rhodesiensis* Rich & Pocock (from N'gamo and Old N'gamo, South Africa) ranged from 7 to 35 (Rich & Pocock 1933). In the South African materials, some of the



Figs 1–13. *Volvox capensis* Rich & Pocock from Montana, USA. Strain M1-2.

Fig. 1. Asexual spheroid with daughter colonies (d).

Figs 2–4. Somatic cells in asexual spheroids.

Fig. 2. Side view of anterior cells showing cytoplasmic bridge (b).

Fig. 3. Optical section of top view of cells surrounded by individual sheaths (asterisks). Stained with methylene blue.

Fig. 4. Top view of somatic cells interconnected by cytoplasmic bridges (b).

Fig. 5. Optical section of developing embryo during inversion. Note that gonidia (asterisks) of the next generation are evident.

Figs 6, 7. Monoecious sexual spheroids with eggs (e) and sperm packets (s).

Fig. 6. Mature spheroid.

Fig. 7. Portion of mature spheroid.

Figs 8–11. Fixed, DAPI-stained cells in sexual reproduction.

Fig. 8. Differential (upper panel) and epifluorescence (lower panel) images of dissociated, individual sperm showing elongate nuclei (asterisks).

Fig. 9. Epifluorescence image of sperm packets dissociating into individual sperm (asterisks) within monoecious sexual spheroid.

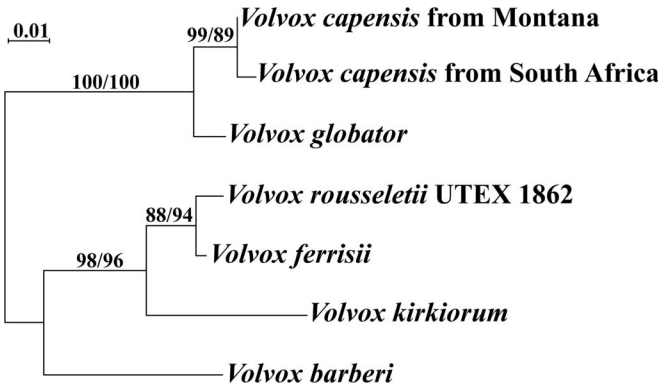


Fig. 14. ML tree of *Volvox* sect. *Volvox* based on ITS region of nuclear ribosomal DNA (ITS-1, 5.8S rDNA and TIS-2; Table S1). Bootstrap values from ML (left) and maximum parsimony (right) analyses are shown on the branches.

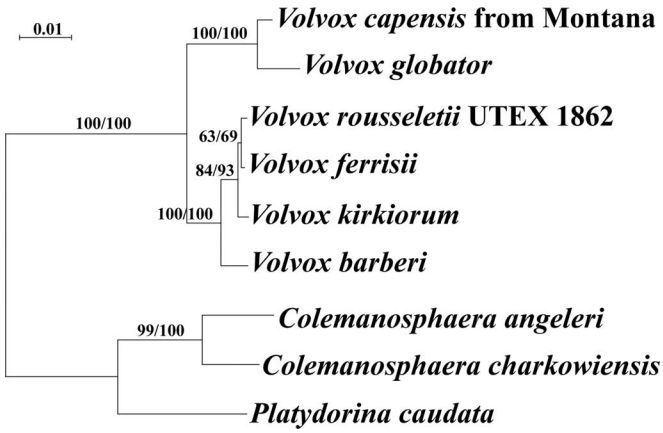


Fig. 15. ML tree of *Volvox* sect. *Volvox* and other colonial Volvocales based on *rbc*L and *psb*C genes (Table S1). Bootstrap values from ML (left) and maximum parsimony (right) analyses are shown on the branches.

sperm packets escaped from the parental monoecious spheroid (Rich & Pocock 1933; Starr *et al*. 1980), and the packets swam to the same or another sexual spheroid for fertilization (Starr *et al*. 1980). In contrast, the Montana strains of *V*. *capensis* had only two to six sperm packets in the monoecious spheroid, and the packets did not escape from the parental spheroid but dissociated into individual sperm within the parental monoecious spheroid for self-fertilization (Figs 6–9). Thus, sexual spheroids in our material contained abundant dissociated sperm inside the cell layers (Fig. 9) except for the early stage of development and the late stage of zygote formation (Fig. 12). In addition, zygotes in our strains were 32–35 μm in diameter (excluding spines); whereas the South African materials (including *V*. *capensis* var. *rhodesiensis*) were 37–53 μm in diameter (Table S2). Thus, the present Montana strains of *V*. *capensis* represent an entity that is distinct from the South African materials in sexual reproduction characteristics.

Possible polyspermy was observed in the Montana strains by DAPI staining (Fig. 11). However, dioecious strains of *V*. (sect. *Merrillosphaera*) *carteri* Stein did not exhibit such polyspermy even when stained with DAPI (Kuroiwa *et al*. 1993). Whether the penetration of multiple sperm nuclei into the egg (possible 'physiological polyspermy'; e.g. Iwao 2012) may be related to the monoecism in *V*. *capensis* needs further study of various species of *Volvox* sect. *Volvox*. Details of the physiological polyspermy will be described in a separate article.

Phylogenetic relationships within *Volvox* sect. *Volvox* resolved by the ITS rDNA tree (Fig. 14) were essentially the same as those of Isaka *et al*. (2012) except for the phylogenetic position of the additional Montana OTU of *V*. *capensis*, which is sister to *V*. *capensis* strain zyg-6 (= K37) from South Africa. These two OTUs exhibited only a single nucleotide difference in ITS-2 rDNA. Thus, a typical degree of within-species variation in ITS-2 rDNA (Coleman 2009) separates the Montana and South African strains. Both ITS rDNA and chloroplast *rbc*L-*psb*C gene trees demonstrated that *V*. *capensis* is sister to *V*. *globator* with 100% bootstrap values in ML and MP methods (Figs 14, 15).

The present Montana strain is different from the South African samples and *V*. *capensis* strain K37 in size of zygotes, number of sperm packets in sexual spheroids and mode of fertilization (Table S2). However, genetic difference between them (a single substitution in ITS-2 rDNA) appears minor, representing variation within a single biological species (see Coleman 2009). Thus, morphological evolution may be rapid in *V*. *capensis*.

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SUPPLEMENTARY DATA

Supplementary data associated with this article can be found online at http://dx.doi.org/10.2216/15-014.1.s1.

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Figs 10, 11. Differential (upper panels) and epifluorescence (lower panels) images of possible fertilized eggs.
Fig. 10. Sperm nucleus (asterisk) penetrating in egg.
Fig. 11. Three sperm nuclei (asterisk) detected in egg.
Fig. 12. Sexual colony with mature zygotes.
Fig. 13. Mature zygotes with spines developing on the walls.

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