Morphological and Molecular Identification of Colonial Volvocalean Green Algae

Introduction

Algae are an important group of organism which includes photosynthetic prokaryotes such as cyanobacteria and photosynthetic eukaryotes such as green algae which are commonly found in lakes and ponds. Algae, which replenish themselves every single day, providing an entire new plankton for water animals, supply nearly fifty-percent of the total oxygen on earth. Volvocalean green algae are an order of algae which includes many families such as Volvocaceae and Chlamydomonadaceae. This order includes members that are closely related organisms with difference in cellular organization and complexity as well as mode of sexual reproduction; therefore, they form an evolutionary time clock and have become a model system for the study of the origin of male-female sexuality and the evolution of multicellularity. However taxonomy of this group of organism is still incomplete and additional members are still waiting to be discovered to help delineate the phylogeny, filling the missing links and helping us to paint a clearer picture of the evolutionary process. In this project, I tried to identify new colonial volvocalean green algae base on molecular phylogeny analysis and morphological comparison. In summary, a strain belonging to *Volvox africanus* was found to be new to Japan. A strain sharing morphological similarity to *Volvox africanus* representing a previously undescribed species belonging to *Volvox sect. Merrillosphaera* was identified. And a strain representing a new genus within the family Volvocaceae was identified.

Material and Methods

Cultures

Strain 20130615zp211 was isolated by pipette-washing method (Pringsheim 1946) from water samples collected at Lake Isanuma (N35°55.361' E139°30.914'; water temperature 27 °C; pH 6.3) on 15 June 2013. Strains 20130703vo2 and 20130703vo4 were also established using water samples collected in Lake Biwa (N 35°04.439' E 135°55.923'; water temperature 24.5°C; pH 8.4) on 3 July 2013. The cultures were grown in screw-cap tubes (18×150mm) containing about 10ml MG medium (Ichimura, 1973). The cultures were cultivated at 25 ℃, on a 10:14 h light–dark cycle, under cool-white fluorescent lamps at 150–200 $\mu molm^{-2}s^{-1}$.

Light microscopy

To observe vegetative morphology, about 1ml of each actively growing culture was inoculated into fresh medium every 14 days. Light microscopy was carried out using an OLYMPUS BX60 microscope (KS OLYMPUS, Tokyo, Japan) equipped with Nomarski interference optics.

Molecular phylogenetic analyses

The protocol developed by Fawley and Fawley (2004) modified by Nakada and Nozaki (2007) was used to prepare the total DNA of all strains. The methods for sequencing the chloroplast gene (*rbcL*) for the large subunit of RuBisCO of all strains were essentially the same with those of previous studies (Nozaki et al., 1995, 1997, 2000, 2002). For phylogenetic analyses, identical

sequences were treated as a single operational taxonomic unit (OTU). The coding regions of the sequences, together with the sequences of several reference strains were aligned by Clustal W using software BioEdit (Hall, 1999). From this alignment, a distance matrix was calculated by applying the General Time Reversibility method by MEGA version 5 (Tamura *et al.*, 2011). A phylogenetic tree was constructed using the neighborjoining (NJ) algorithm (Saitou & Nei, 1987) and maximum likelihood (ML) algorithm by MEGA version 5 (Tamura *et al*., 2011), and the robustness of the resulting lineages was tested using bootstrap analysis (Felsenstein, 1985) with 1,000 replications.

Results and Discussion

***Identification of* V. africanus *and a new species belonging to* Volvox sect. Merrillosphaera**

After field collection in Lake Biwa and strain isolation, several strains captured our attention. They share some common morphological characteristics, which include: mature colony size is smaller compared with other *Volvox* species; both parental and daughter colonies are oval shaped; and no cytoplasmic bridges were present between somatic cells (Figure 1). The presence of paired daughter colony in strain 20130703vo2 (Figure 1 A) identifies this strain as *V. africanus* (Smith, 1994; Nozaki and Coleman 2011). Molecular phylogenic analysis further confirmed this as the phylogenetic position of this strain was found to be sister to *V. africanus* (Figure 4).

However another strain (20130703vo4), although sharing similarity with *V. africanus,* possesses unpaired daughter colonies (Figure 1C), and its morphological characteristics are unique (with 3 to 7 posterior gonidia) compared with other species within *Volvox sect. Merrillosphaera* (Nozaki and Coleman, 2011). Furthermore, phylogenetic position of this strain is distant from that of *V.africanus* (Figure 4); therefore, we postulate that it is probably a new species. However, as characteristics of sexual reproduction and zygote germination are important in *Volvox* taxonomy (Smith 1944; Nozaki and Coleman 2011), detailed characterization of these two processes in this strain should be performed to describe a new species taxonomically.

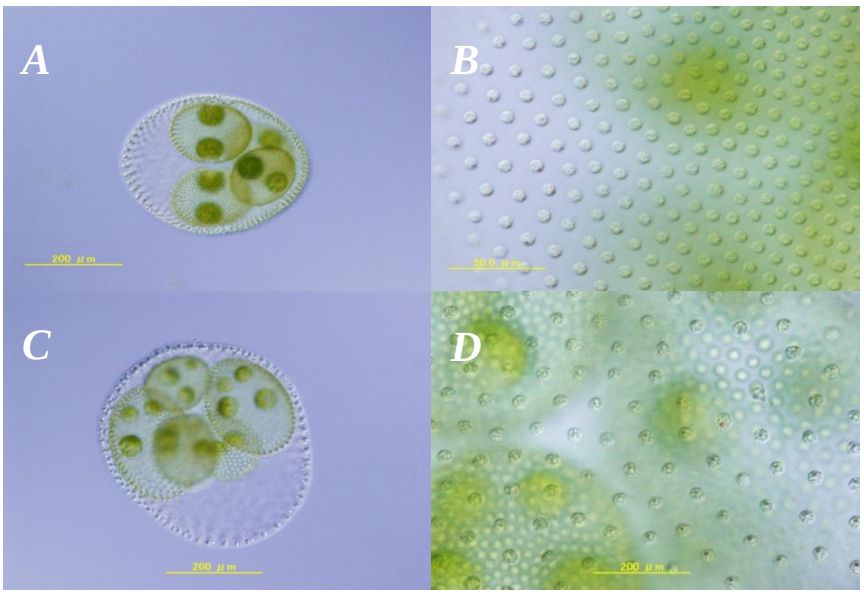

Figure 1. Morphology of vegetative colony of *Volvox africanus* (Strain designation:20130703vo2; A and B) and *Volvox.* sp. (Strain designation: 20130703vo4; C and D), both of which were isolated from Lake Biwa.

Discovery of a strain belonging to a new genus

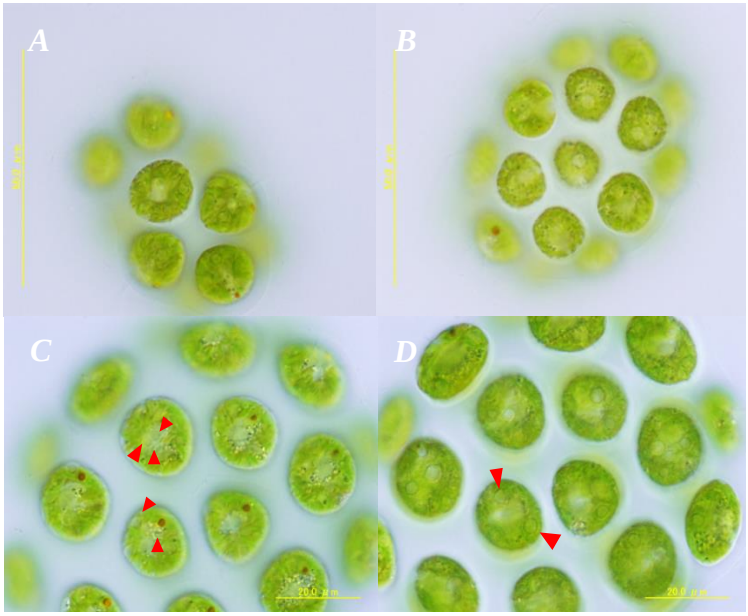

Figure 2. Morphological characteristics of strain of interest (20130615zp211) in comparison to related genus. A to D: Strain 20130615zp211. 16-celled colonies are cylindrical (A) while 32-celled colonies are oval shaped (B).Strain isolated from Lake Isanuma, Japan. Multiple anterior contractile vacuoles (C) and two major pyrenoids (D) were observed.

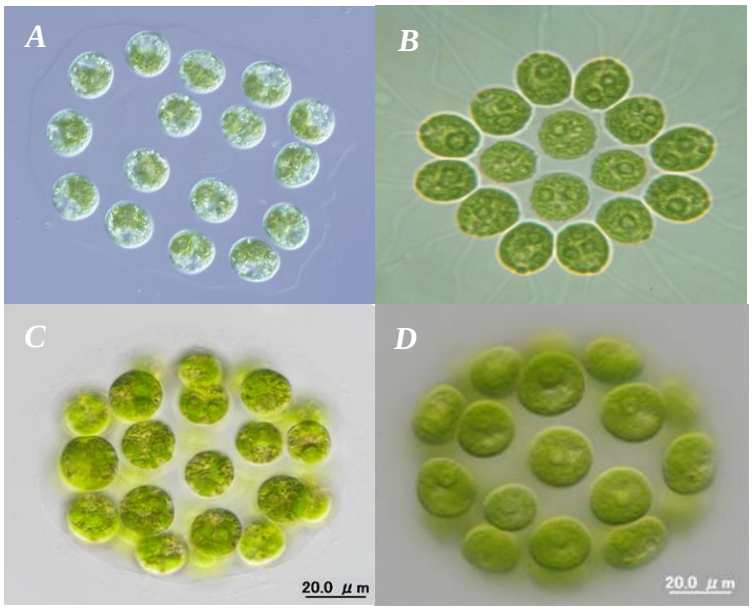

Figure 3. Morphological characteristics of *A: Platydorina kofoid; B: Gonium multicoccum; C: Eudorina* sp.; D: *Yamagishiella* sp.; (Photos were obtained from Nozaki Hisayoshi)

During the field collection, another strain (20130615zp211) with cylindrical shape and compact 16- or 32-celled colonies without differentiated small somatic cells was isolated. This vegetative morphology is very similar to the volvocacean genera *Yamagishiella* and *Eudorina* (Yamada et al. 2008), Molecular phylogenetic analysis showed that this strain is closest to the genus *Platidorina* but with low bootstrap values (51-52%). However, morphologically, this strain is cylindrical or oval instead of flattened as observed in *Gonium* and *Platydorina; (*Figure 2A, 2B, 3A and 3B). Therefore, this strain does not belong to these two genera. Despite the similarity in colony shape between this strain and the genus *Eudorina* and *Yamagishiella* (Figure 2A, 2B, 3C and 3D), detailed microscopic examination revealed that somatic cells of parental colony possess multiple contractile vacuoles distributed mostly at the anterior face (Figure 2C) and two major pyrenoids in the chloroplast (Figure 2 D), which are different from those of *Eudorina* which has well-distributed contractile vacuoles in each cell and those of *Yamagishiella* which has two anterior contractile vacuoles in each cell and a single basal pyrenoid in the cup-shaped chloroplast (Yamada *et al,* 2008). Therefore, based on these major morphological dissimilarities on genus level and the large evolutionary distance deduced from molecular phylogenetic analysis (Figure 4), we conclude that the strain belongs to a new genus.

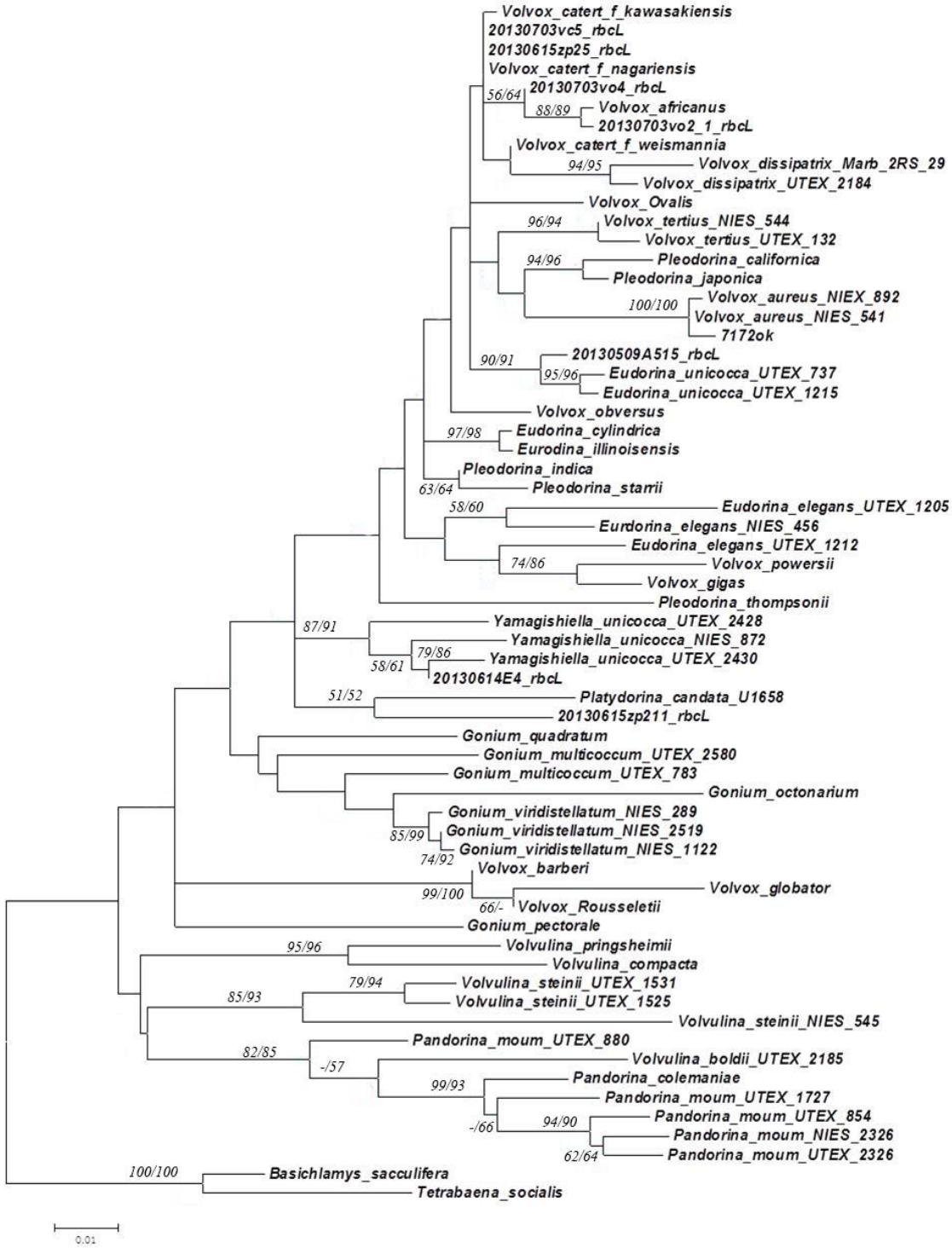

Figure 4. Maximum Likelihood (ML) tree based on *rbcL* genes of newly isolated strains and reference strains. Branch lengths are proportional to evolutionary distance estimated by the General Time Reversible model and are indicated by the scale bar besides the tree. Numbers above or below the branches represent 50% or more bootstrap values based on 1,000 replications of the Maximum Likelihood and Neighbor Joining analyses respectively.

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