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Research Article
原著論文

Monactinus biwaeensis, a new combination for *Pediastrum biwae* Negoro (Chlorophyceae) – characterization of its morphology, molecular phylogeny, and asexual reproduction

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緑藻類 *Pediastrum biwae* に対する新組み合わせ *Monactinus biwaensis* の形態，分子系統，無性生殖過程の特徴

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抄録：琵琶湖から新種記載され，これまで琵琶湖の固有種とされた緑藻類*Pediastrum biwae*について，その形態，分子系統，生活環の一部，を新たに確立された培養株を用いて再検討した．分子系統解析を行った結果，この藻類は*Monactinus*属に含まれたが，*M. simplex*や*M. sturmii*とは異なるクレードを形成することが示された．*Monactinus*属のこれらクレード間で，細胞表面の超微細構造，光学顕微鏡画像に基づく形態学的分析から，本種の辺縁細胞は明らかに非対称な突起を持つことが明らかになった．類似種である*M. simplex*や*M. sturmii*はほぼ対称的な突起を持っていた．確立した培養株の無性生殖過程の観察では，この藻類は中央の細胞の側面がコエノビウムの成長過程で凹状から直線状，凸状に形態が変化した．この３つの形態はこれまで記載されてきた*P. biwae* var. *triangulatum*，*P. biwae* var. *ovatum*，*P. biwae* var. *biwae*（自動名）に対応するが，これらの変化が連続的であることから分類学的に変種として妥当ではないと結論した．これらの結果を踏まえ，*Monactinus biwaensis* (Negoro) K.Matsuoka et M.Kayama comb. nov. を従来の*Pediastrum biwae* Negoroに対する新しい組み合わせとして指定した．

Abstract: *Pediastrum biwae* Negoro (Sphaeropleales, Hydrodictyaceae) formerly considered an endemic species of Lake Biwa, Japan was re-examined regarding its molecular phylogeny, morphology, and a part of its asexual life cycle, and geographical distribution based on the new strains established. The molecular phylogenetic analysis showed that this species is a member of *Monactinus*, and forms a different clade from *M. simplex* and *M. sturmii*. These different clades in the genus *Monactinus* are also differentiated on the basis of ultrastructure of the cell surface. Morphometrical analysis based on light microscopy revealed that the marginal cells of this species have distinctly asymmetrical projections. In contrast, the similar species *M. simplex* and *M. sturmii* have almost symmetrical ones. According to our observations of asexual reproduction, this alga changes the morphology from concave to convex via straight side of the central cells. These three types respectively correspond to the three varieties of the species previously described, i.e., *P. biwae* var. *triangulatum*, *P. biwae* var. *ovatum*, and *P. biwae* var. *biwae* (autonomy), and therefore, these varieties are not valid taxonomically. *Monactinus biwaensis* (Negoro) K. Matsuoka et M. Kayama comb. nov. was designated from a previous *Pediastrum biwae* Negoro as a new combination.

Key words: ITS2 rDNA; Lake BIWA; LSU rDNA; scanning electron microscopy; SSU rDNA

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Introduction

The genus *Monactinus* Corda, 1939, one of the common planktonic algae and is distributed globally in freshwater ponds and lakes, is belonging to the Family Hydrodictyaceae Dumoritier, 1829 of the Class Chlorophyceae Wille, 1884 (Guiry and Guiry, 2016). However, the precise taxonomy of these algae is needed in order to re-study their morphological characteristics together with molecular phylogenetic analysis. In the Family Hydrodictyaceae, *Lacunastrum*, *Parapediastrum*, and *Pseudopediastrum* have been newly proposed and *Monactinus* and *Pediastrum* have been re-established after molecular phylogenetic analysis and detailed morphological examinations.

It was not until the 1990s that molecular phylogenetic techniques were used to classify microalgae. Buchheim et al. (2005) and McManus and Lewis (2005) were the first to begin a molecular phylogenetic study of the diverse green alga *Pediastrum* sensu lato and to elucidate the relationship between morphological characteristics and molecular phylogeny. Buchheim et al. (2005) and McManus and Lewis (2005) found that *Pediastrum*, including the closely related *Hydrodictyon reticulatum* (Linnaeus) Bory de Saint-Vincent 1824 is polyphyletic. Then, Buchheim et al. (2005) proposed the division previously called the genus *Pediastrum* into *Pediastrum* Meyen 1928, *Stauridiuim* Corda 1839, *Monactinus* Corda 1839, *Pseudopediastrum* E. Hegewald, 2005, and *Parapediastrum* E. Hegewald, 2005. Subsequently, research on those taxa was actively continued, and then McManus et al. (2011) proposed another new genus, *Lacunastrum* H. McManus, 2011 separate from the former genus *Pediastrum*.

Among the above genera, the genus *Monactinus* was first proposed by Corda (1839) and was separated from other genera after molecular phylogenetic analysis by Buchheim et al. (2005). The genus *Monactinus* is characterized with a single projection on the marginal cell as shown in Fig. 1. At present, only three species, *M. simplex* (Meyen) Corda 1839, *M. asymmetricus* (Yamagishi and E.Hegewald) E.Hegewald, 2005 and *M. sturmii* (Teinsch) M.Jena and C.Bock, 2014 belong to the genus *Monactinus* (Jena et al., 2014; Guiry and Guiry, 2016; Lenarczyk and Saluga, 2018) after re-examination of molecular phylogenetic studies. *M. asymmetricus* is also a member of the genus *Monactinus* (Guiy, 2017; Hegewald and Yamagishi, 2005), but unfortunately a molecular phylogenetic study of this species has not yet been carried out.

Pediastrum biwae was first described as a new species from Lake Biwa of Japan by Negoro (1954). This alga is characterized by a single projection of the marginal cells, as is *Monactinus* simple. The single projection has one to three slender strings from the tip called "bristles" (Komárek and Jankovská, 2001) in plankton stage. Negoro (1954) separated *P. biwae* from *P. simplex* by the unequally-arranged boundary projections of marginal cells. Simultaneously the following variants, var. *triangulatum* Negoro, 1954 (slender cells and concave outer margin of the cell wall), var. *ovatum* Negoro, 1954 (thickened cells and convex outer margin of the cell wall), and var. *biwae* (autonomy; intermediate between the above mentioned two varieties and straight outer margin of the cell wall), were established according to the thickening state of all cells, including the marginal cells, by Negoro (1954) as illustrated in Fig. 2. After Negoro (1954), *Pediastrum biwae* has been considered as endemic species of the Lake Biwa - Yodogawa River system for long time.

P, biwae is also expected to belong to the genus *Monactinus* because of its single projection of the marginal cells. Recently Akbulut et al. (2019) subscribed *Monactinus simplex* var. *biwaense* (Fukushima) E.Taşkin et M.T.Alp. However, this new variety is not correctly proposed, because Fukushima (1953) did not properly subscribed *Pediastrum simplex*. var. *biwaense*. So far, neither molecular

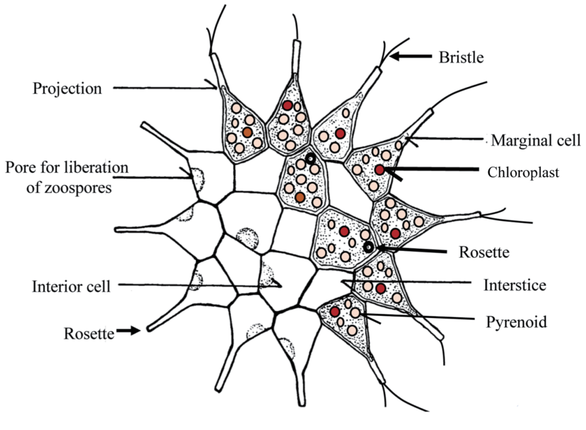
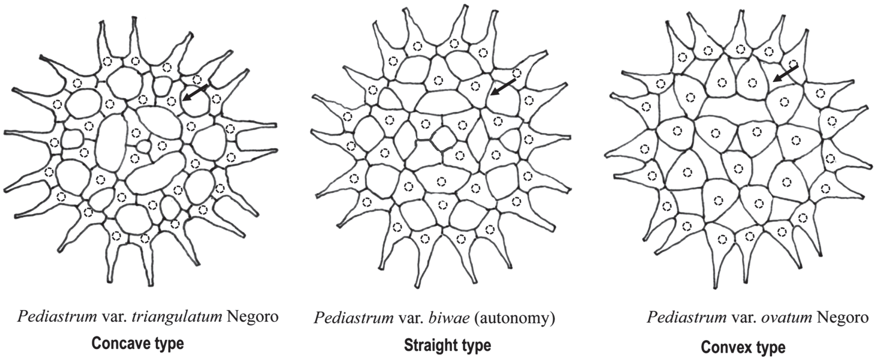


Fig. 1. Descriptive terms for the coenobium of the genus *Monactinus* (*Monactinus simplex* group).


Pediastrum var. *triangulatum* Negoro
Concave type

Pediastrum var. *biwae* (autonomy)
Straight type

Pediastrum var. *ovatum* Negoro
Convex type

Fig. 2. Three varieties recognized by Negoro (1954). A: *Pediastrum biwae* var. *triangulatum* Negoro, 1954. Redrawn from Iconotype of Negoro, 1954 Fig. 5. “Concave” was added by the present author as an aid for focusing on the characteristic morphology of interior cells (arrow). B: *Pediastrum biwae* var. *biwae* (autonomy). Redrawn from Iconotype of Negoro, 1954 Fig. 1. “Straight” was added by the present author as an aid for focusing on the characteristic morphology of interior cells (arrow). C: *Pediastrum biwae* var. *ovatum* Negoro, 1954. Redrawn from Iconotype of Negoro, 1954, Fig. 10. “Convex” was added by the present author as an aid for focusing on the characteristic morphology of interior cells (arrow).

phylogenetic nor detailed morphological studies have been conducted. Also, the taxonomic validity of *P. biwae* var. *triangulatum* and *P. biwae* var. *ovatum* has not been fully examined in morphology and molecular phylogeny.

In this paper, we will clarify the taxonomic position of *P. biwae* based on consideration of its morphological characteristics, focusing on the morphology of the marginal and central cells, the fine structure of the cell surface under the scanning electron microscope (SEM) and molecular phylogenetic analysis using the SSU, ITS2, and LSU rDNA regions. We will take account of the process of asexual reproduction and morphological changes during asexual reproduction observed during culturing of *P. biwae* and then discuss the validity of three varieties of *P. biwae*.

Material and Method

1. Sampling location and date

Lake Biwa, an ancient lake, is the largest lake in Japan. Its south end is located at 34°59.71’ N and 135°54.35′ E and north end at 35°29.95′ N and 136°10.4′ E, its area is 669 km^2, and average water depth is 41.2 m (Environmental Policy Division of Shiga Prefecture, 2019). The Seta-gawa River, the only outflow river, changes its name to the Uji-gawa River while flowing through mountains composed of Paleozoic to Mesozoic sedimentary and metamorphic rocks, changes its name to the Yodo-gawa River after merging with the Kizu-gawa River and Katsura-gawa River, and finally reaches Osaka Bay, the eastern part of the Seto Inland Sea.

The plankton samples were obtained with a plankton net of 20 µm opening mesh size (Rigosha, Japan) from several locations in Lake Biwa. The surface sediment samples used for the present study were collected with a gravity TFO corer type B (Rigosha, Japan) in Lake Biwa and the sampling dates were recorded (Fig. 3, Table 1).

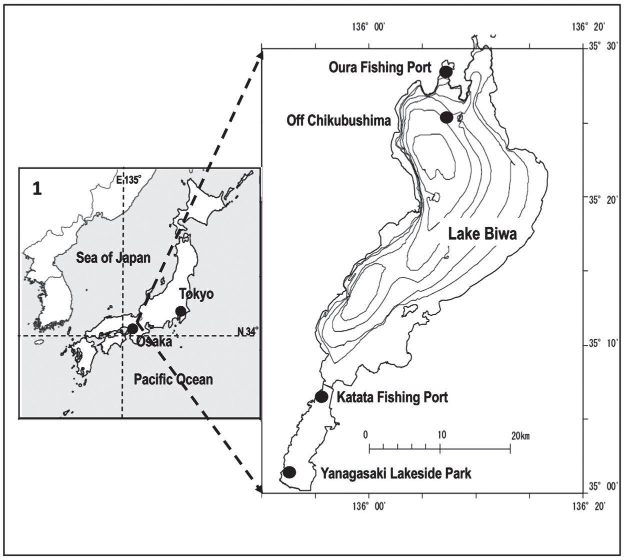


Fig. 3. Sampling sites in Lake Biwa, central Japan.

Table 1. Sampling location and date.

Location	Sampling date	Latitude (N)	Longitude (E)	Water depth	Sample type	Sampling device	Strain name
Yanagasaki Lakeside Park in Lake Biwa	28 June 2019	35°01.56’	135°52.08’		Plankton	Plankton net*	
Katata Fishing Port in Lake Biwa	30 May 2022	35°06.9’	135°55.45’		Plankton	Plankton net*	
Oura Fishing Port in Lake Biwa	10 June, 11 July, 24 August 2022	35°29.0’	136°07.43’		Plankton	Plankton net*	SeedBank20228-1, -2, 3
West off of Chikubu-shima Island in Lake Biwa	11 October 2021	35°25.08’	136°08.4’	-70m	sediment	TFO corer type II	

Plankton net*: 20 μm in opening size

2. Culture experiments

A total of 15 cases of unialgal culture experiments were conducted. To examine the morphological changes of *P. biwae* during culturing, several coenobia were picked up from the plankton net samples collected from Lake Biwa using glass capillary pipets under an inverted microscope (Leica DMi1 + MC120 HD, Leica Microsystems, Germany) and were used for unialgal culture experiments. Type C culture medium (Ichimura 1971) was mainly used. For obtaining suitable specimens for SEM observation, 1/2 concentration of Type C culture medium was used for further incubation experiments, because aberrant forms of coenobia sometimes appeared during the use of type C culture medium. The culture condition for both culture experiments were 12:12 h (light:dark) and 100 μmol/m^2/s-1 at 20° C. The culture strains provided for molecular phylogenetic analysis were named as SeedBank 20228-1, -2, -3.

3. Light microscopy

From the plankton net samples, a coenobium of *Pediastrum biwae* was picked up under an inverted microscope and then their morphologies were observed with an upright NIKON ECLIPSE E600 light microscope (Nikon, Japan) and photographs were taken using a NIKON Digital Camera DS-5Mc (Nikon, Japan).

4. Scanning electron microscopy (SEM)

Both a part of the specimens of the culture experiments and specimens picked up from surface sediments were observed under a SEM in order to detect fine morphology of the cell surface of *Pediastrum biwae*. The coenobia of *P. biwae* were collected and dehydrated in an ethanol series (50%, 70%, 80%, 90%, 95%) of final concentration 100 %. From 50% to 90% ethanol, the coenobia were soaked for 10 mins, in 95% ethanol for 15 mins, and in 100% ethanol for 15 mins twice. At the final stage, a drop containing these coenobia was placed on a cover slip and coated with platinum using MSP-1S (Vacuum Device Co. Ltd., Japan). SEM observation was carried out with a VE7800 Scanning Electron Microscope (Keyence Co. Ltd., Japan) with maximum magnification x1000〜5000 at Osaka Museum of Natural History, Osaka, Japan.

5. DNA extraction and molecular phylogenetic analysis

Some specimens named SeedBank 20228-1, -2, -3 from the culture experiments collected from Oura Fishing Port in Lake Biwa on 24 August 2022 were used for molecular phylogenetic analysis.

Total DNA of three strains was extracted using a Wizard® Genomic DNA Purification Kit (Promega Co. Ltd., USA) following the manufacturer’s instructions.

PCR amplifications were performed using KOD-plus-Ver2 (TOYOBO Co. Ltd., Osaka, Japan) and two primer sets: SSU (18S) rDNA gene (5′ -AACCTGGTTGATCCTGCCAGT-3′ , 5′ -TGATCCTTCTGCAGGTTCACCTAC-3′ ; Medlin et al., 1988) and LSU (26S) rDNA gene (5′ -GCATATCAATAAGCGGAGGA-3′ , 5′ -TCCCCTTGTCCGTACCAGT-3′ ; Buchheim et al., 2001), internal transcribed spacer 2 (ITS2) region gene (5′ -CGATACGTAGTGTGAATTGC-3′ , 5′ -GAGGGTACTTCTATAGACTACAATTCTCC-3′). This ITS2 primer is original for analyzing the sequence of *P. biwae* in this study. PCR amplification was conducted for 30 cycles of a denaturation step at 96° C for 30 s, an annealing step at 46° C for 20 s, and an elongation step at 68° C for 5 min.

For three strains of *P. biwae*, the SSU rDNA, ITS2 region and LSU rDNA of three were examined. These gene sequences were aligned with available sequence data of the Hydrodictyaceae in the GenBank nucleotide database using MAFFT (Katoh and Standley, 2013). Ambiguously aligned sites were removed manually using BioEdit (Hall, 1999). The SSU rDNA dataset comprising 40 taxa and 1590 sites, ITS2 region dataset comprising 31 taxa and 301 sites, and LSU rDNA dataset comprising 51 taxa and 1756 sites, were used for constructing phylogenetic trees. Three datasets were subjected to phylogenetic analysis using IQ-TREE 1.6.12 (Nguyen et al., 2015), under the TIM2+F+I+G4 and TN+F+I+G4 model with 100 bootstrap replicates.

6. Morphological analysis of *Pediastrum biwae*

Since the morphology of the marginal cell is one of the important characteristics for differentiating *P. biwae* and *M. simplex*, we measured the length of sides of marginal cells in order to determine the morphometric characteristics of these species as illustrated in Fig. 4. Since *M. simplex* and *P. biwae* have a single projection, the outer edges of the projection and the marginal cell were combined to form one side of a triangle, and the length of the triangle was measured to obtain the ratio of the two (Fig. 4). The lengths of the two sides were measured to obtain the ratio of the two sides (thereafter called “side ratio”). However, because the sides facing the projection of *P. biwae* were bent in the middle, they were divided into two parts. To determine the ratio per a coenobium, at least three peripheral cells were measured and the average value was calculated. Data from areas other than Lake Biwa were obtained from photographs of *Pediastrum simplex* and *Pediastrum simplex* var. *biwaensis* published in papers previously reported as shown Table 2. Those of *P. biwae* were collected at Oura Fishing Port, Katata Fishing Port and Yanagasaki Lakeside park of Lake Biwa in our own study.

In order to know if the two side ratios of *P. simplex* and *P. biwae* could be significantly different, we performed a discriminant analysis using SPSS (IBM, version 29) with the inverse sine square root-transformed side ratios of both groups (n = 18 for *P. simplex* and n = 12 for *P. biwae*).

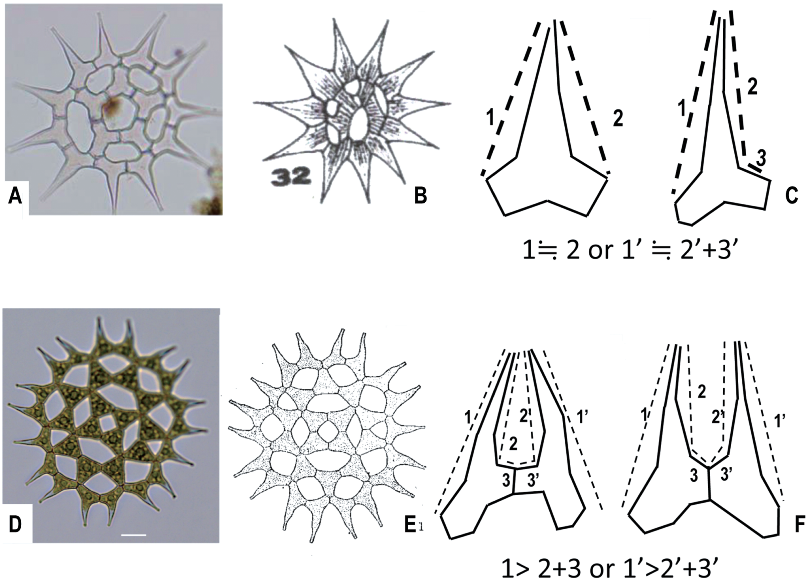


Fig. 4. Photographs, iconotypes and morphology of marginal cells of *Monactinus simplex* and *Pediastrum biwae*. A: *Monactinus simplex* collected from surface sediments of Shinjiko Lake in Shimane Prefecture, Japan. B: One of iconotypes of *Pediastrum simplex* (Re-drawn of Parra (1979, pl. 5 Fig. 32). This illustration was redrawn from Meyen (1829, Fig. 3). C: Measurements of side ratio of marginal cells of *Monactinus simplex* including several varieties. D: Specimen collected from plankton of Lake Biwa identical to *Pediastrum* (*Monactinus*) *biwae* (straight type). E: Illustration of *Pediastrum biwae* Negoro (re-drawn of Negoro, 1954, Fig. 1). F: Measurements of side ratio of marginal cells of *Pediastrum* (*Monactinus*) *biwae*.

Table 2. Ratio of the two sides of the marginal cells of *Monactinus* (*Pediastrum*) *simplex* and *Monactinus biwaensis* (= *Pediastrum biwae*) recorded from various locations in the world including specimens preserved in surface sediments off of western Chikubu-shima in Lake Biwa. Data of taxa marked with * were obtained from the original photographs or drawings.

Species	Variety	Ratio (1/ 2 or 2+3) or (1/ 2 + 3)	Reference
Pediastrum simplex	var. *simplex* (*echinulatum*?)*	0.95	Parra 1979
Pediastrum simplex	var. *pseudoglabrum**	0.96	Parra 1979
Pediastrum simplex	var. *echinulatum**	0.96	Lee *et al.* 2018
Pediastrum simplex	var. *echinulatum**	0.96	Echenique *et al.* 2013
Pediastrum simplex	var. *simplex* (*pseudoglabrum*?)*	0.93	Garduño-Solórzano *et al.* 2016
Monactinus simplex	var. *simplex* (*echinulatum*?)*	0.97	Jena *et al.* 2014
*Monactinus sturmii**		0.97	Jena *et al.* 2014
Pediastrum simplex	var. *simplex*?*	1	Kim & Kim 2012
Pediastrum simplex	probably var. *simplex**	0.93	Kim & Kim 2012
Pediastrum simplex	var. *simplex* (*echinulatum*?)*	0.99	Kim & Kim 2012
Pediastrum simplex	var. *sturmii**	1	Kim & Kim 2012
Pediastrum simplex	var. *simplex*?	1	Meyen 1829
Pediastrum simplex	probably var. *echinulatum**	0.9	Meyen 1828
Pediastrum simplex*		0.87	Khaw *et al.* 2020
Pediastrum simplex	var. *doudenarium**	0.96	Rai & Misra 2012
Pediastrum simplex	var. *echinulatum**	0.98	Martines-Goss *et al.* 2016
Pediastrum simplex	var. *clathratum**	0.97	Pasztaleniec & Poniewozik 2004
Pediastrum simplex	var. *echinulatum**	0.96	Pasztaleniec & Poniewozik 2004
Pediastrum biwae	var. *biwae**	0.73	Negoro 1954 (holotype; drawing)
Pediastrum biwae	var. *triangulatum**	0.71	Negoro 1954 (holotype; drawing)
Pediastrum biwae	var. *ovatum**	0.75	Negoro 1954 (holotype; drawing)
Pediastrum biwae	var. *triangulatum*	0.78	This study
Pediastrum biwae	var. *biwae*	0.76	This study
Pediastrum biwae	var. *biwae**	0.77	Naidu *et al.* 2018
Pediastrum biwae	var. *triangulatum**	0.88	Martinez-Goss *et al.* 2016
Pediastrum biwae	var. *triangulatum**	0.82	Loaiza-Restano 2013
Pediastrum biwae	var. *triangulatum**	0.84	Kim & Kim 2012
Pediastrum simplex	var. *clathratum**	0.81	Komárek & Jankovská 2001
Pediastrum simplex	var. *clathratum**	0.84	Pasztaleniec & Poniewozik 2004
Pediastrum simplex	var. *duodenarium**	0.83	Baykal *et al.* 2004

Results

1. Morphology under light microscope based on culture experiments

Pediastrum biwae was classified into three morphotypes; slender and concave cell type (Fig. 2A), straight and intermediate cell type (Fig. 2B), and fat and convex cell type (Fig. 2C). The following case using a single coenobium identical to *P. biwae* var. *triangulatum* collected at Oura Fishing Port on 11 of July, 2022 was a typical example for asexual reproduction as described below. As shown in *P. biwae* provided for a culture experiment (Fig. 5A), the coenobium of the slender cell type of which interior cells were concave became slightly fatter and rounded (Fig. 5B) on day 2 from starting the culture. The interior cells became larger. On day 3, each cell of the coenobium became enlarged, changing from a triangular form with concave side to an intermediate one with straight sides. (Fig. 5C). The interior cells continuously enlarged from 3.2 to 4 times in size and convex. On day 4, cells constituting the coenobium continuously enlarged (Fig. 5D). On day 5, daughter cells released from each cell of the original coenobium were observed forming several new coenobia, whose morphology was mainly identical to that of the intermediate cell type. Also, each cell produced many coenobia containing different cell numbers including many aberrant forms (arrows in day 5: Fig. 5E).

In another case, a single coenobium of *P. biwae* var. *triangulatum* was picked up from the sample collected at Oura Fishing Port on 11 of July, 2022 (Fig. 6A). On Day 3, cells constituting the coenobium became slightly larger (Fig. 6B)

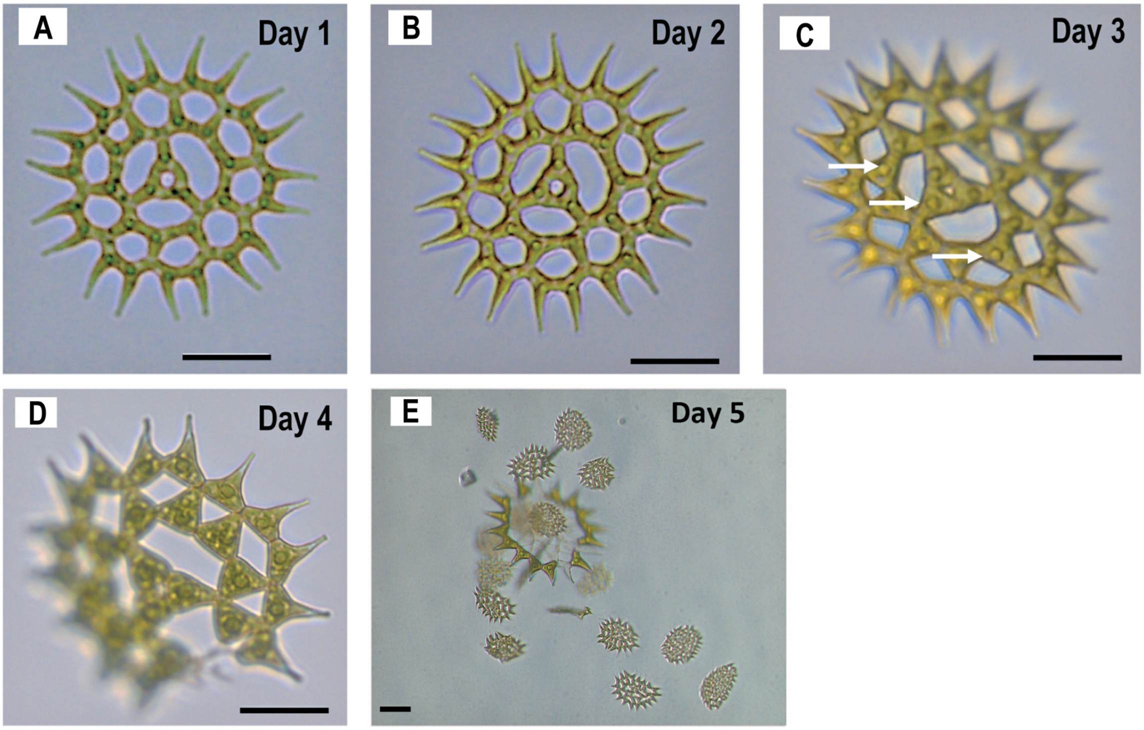


Fig. 5. Morphological changes of a single coenobium of *Pediastrum biwae* during asexual reproduction in culture experiments. A: *P. biwae* concave type (= *Pediastrum biwae* var. *triangulatum*) collected at Oura Fishing Port, Lake Biwa on 11 July 2022. B: Morphology of coenobium after 2 days from the incubation starting. C: Coenobium became larger than that of day 1 and white arrows indicate pyrenoids after 3 days. Judging from the straight side of the inner cells, the coenobium may be identical to *P. biwae* straight type (= *Pediastrum biwae* var. *biwae*). D: One of coenobia reproduced from Fig. 12 is identical to *P. biwae* straight type (= *Pediastrum biwae* var. *biwae*). E: Many aberrant daughter coenobia appeared.

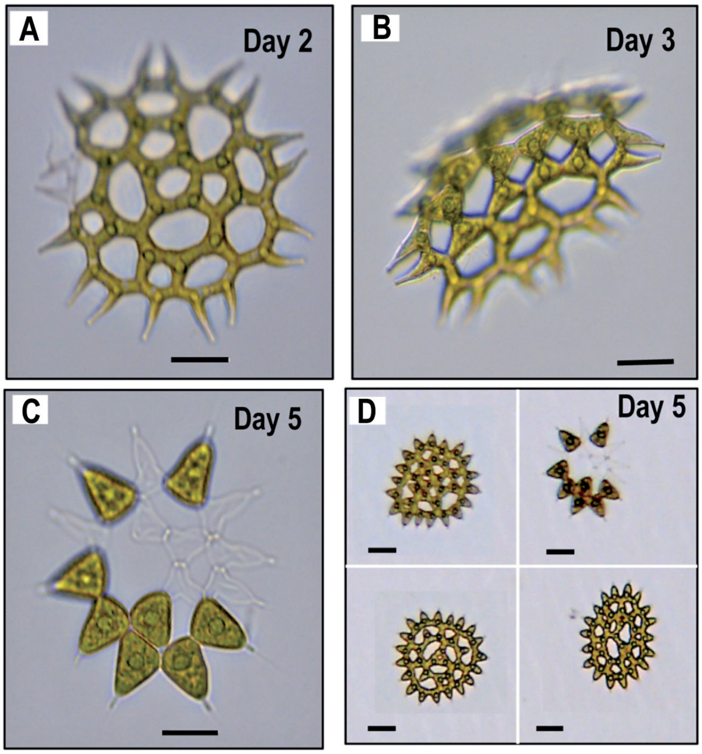


Fig. 6. Morphological changes of a single coenobium of *Pediastrum biwae* during asexual reproduction in culture experiments. A: *Pediastrum biwae* concave type (= *Pediastrum biwae* var. *triangulatum*) collected at Oura Fishing Port in Lake Biwa on 11 July 2022. B: A coenobium after 3 days from the culture starting. Note that the coenobium was slightly convex. C: Coenobium after 5 days became fatter than that of 2 days. The marginal cells of the coenobium of 5 days may be identical to *Pediastrum biwae* convex type (= *Pediastrum biwae* var. *ovatum*). D: Several daughter coenobia after 5 days. The marginal cells of the coenobia might be identical to *Pediastrum biwae* convex type (= *Pediastrum biwae* var. *ovatum*).

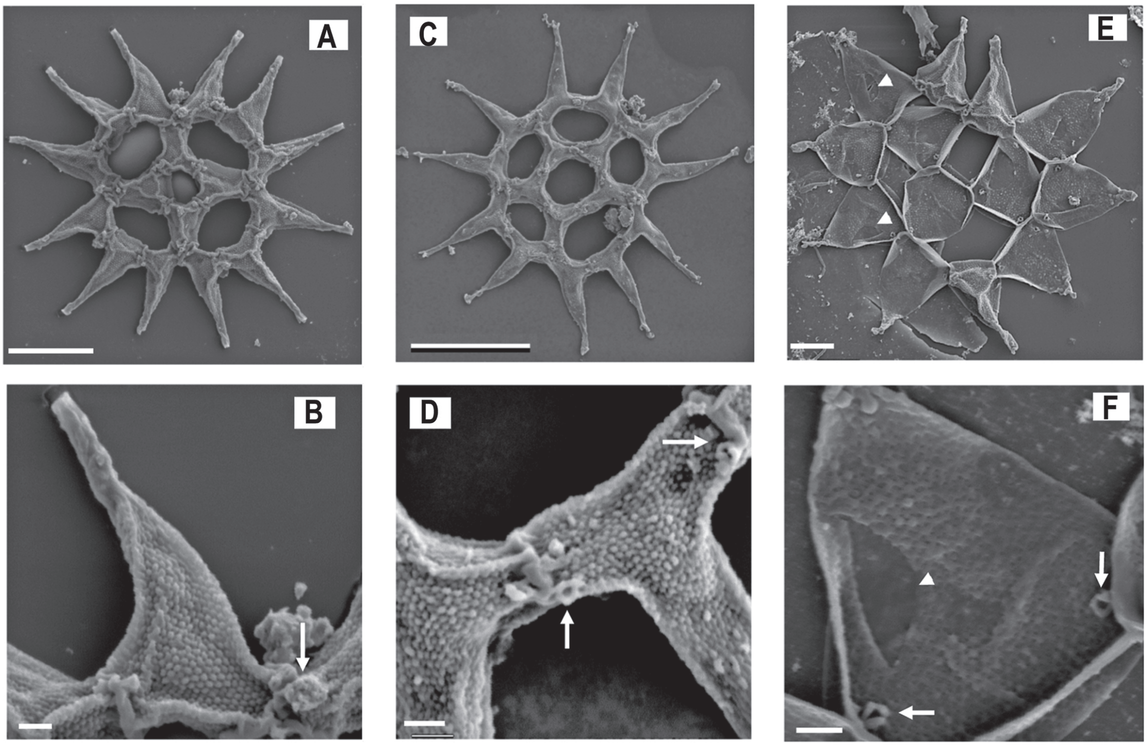


Fig. 7. SEM photographs of three different morpho types in *Monactinus biwaensis*, A: *Monactinus biwaensis* (=*Pediastrum biwae* straight type). Scale bar; 7 µm. B: Enlargement of A showing granular surface of the marginal cell. Arrow indicates a rosette. Scale bar; 2 µm. C: *Monactinus biwaensis* (= *Pediastrum biwae* concave type). Scale bar; 9 µm. D: Enlargement of C showing granular surface of the marginal cell. Scale bar; 2 µm. E: *Monactinus biwaensis* (= *Pediastrum biwae* convex type). Arrowheads indicate pores for liberation of zoospores. Scale bar; 8 µm F: Enlargement of showing granular surface of the marginal cell. Arrows indicate rosettes. Scale bar; 2 µm.

and on Day 5, all cells had a convex outline (Fig. 6C). This coenobium was identical to *P. biwae* var. *ovatum* and released many coenobia consisting of basically 32 cells, but sometimes 16 cells or less as shown in Figs. 5E, 6D.

As a summary of the findings by the culture experiments, asexual reproduction of *Pediastrum biwae* can be described as follows. On day 2 after starting the incubation, most coenobia had changed their morphologies from slender *P. biwae* var. *triangulatum* to normal (var. *biwae*). On day 3, each cell constituting a coenobium was enlarged and released a daughter coenobium asexually. On day 4, all cells of incubated coenobia produced daughter coenobia. In some case, daughter coenobia continued asexual reproduction and produced further grandchild cells.

In the plankton net samples collected at the different sites and months from Lake Biwa, a slender (concave) type identical to *P. biwae* var. *triangulatum* and an intermediate type identical as *P. biwae* var. *biwae* were usually observed, but *P. biwae* var. *ovatum* (convex type) was never encountered.

2. The side ratio of marginal cells

Measurement data of the side ratio of marginal cells of *Pediastrum simplex* and *P. biwae* is shown in Table 2. The ratio of *Pediastrum simplex* including var. *simplex*, var. *echinulatum*, var. *pseudoglabrum*, and var. *sturmii, P. simplex* var. *echinulatum* shown by Parra (1979), Kim and Kim (2012) and Jena et al. (2014) was 1 to 0.93. Even in SEM photographs, the side ratio of *M. simplex* and *M. sturmii* (Jena et al., 2014) was 0.97. Consequently, the average value in all these varieties of *P. simplex* (or *M. simplex*) was 0.93 to 1.

Pediastrum biwae also consisted of three morphotypes identical to var. *biwae*, var. *triangulatum*, and var. *ovatum*. The average side ratio of the marginal cells of the holotype drawing of *P. biwae* reported by Negoro (1954) were as follows: var. *biwae* 0.73, var. *triangulatum* 0.77, and var. *ovatum* 0.75. In the plankton collected from Lake Biwa in the present study, the average side ratio of var. *biwae* was 0.76 to 0.78. Several occurrences of *P. biwae* or of algae named "*P. simplex* var. *biwaense*" from various locations around the world other than the location of Lake Biwa were as follows: the side ratio was 0.77 (Naidu et al., 2018) to 0.84 (Kim and Kim, 2012) as shown in Table 2. Since the distribution of the ratios of the marginal cells of both *P. simplex* (*M. simplex*) and *P. biwae* is concentrated in a range close to 1, the ratios are presented as minimum, median, and maximum values. The range for *P. simplex* is 0.87, 0.96, and 1 ($n = 18$), and for *P. biwae* it is 0.71, 0.80, and 0.88 ($n = 12$), respectively.

To investigate whether there is a difference between the two groups in this ratio, the ratios of both groups were first transformed using the inverse sine square root transformation before being applied to discriminant analysis to improve data normality and homogeneity of variance. The F-value between the means of the two groups is 75.129, with a significance level of $p < 0.001$, indicating a significant difference in the population means. An eigenvalue of 0.854 suggests that the discriminant function model is highly effective. Wilks' Lambda of 0.271 and a Chi-square value of 35.857, both with a significance level of $p < 0.001$, indicate that the ratios of the two groups differ significantly (Table 2).

3. Morphology under scanning electron microscope (SEM)

In the light microscope, the cell surface of *Pediastrum biwae* appeared to be rather smooth. However, scanning electron microscopy revealed that its cell surface was covered with granules (Fig. 7B, D, F). These granules were also observed in even the coenobia extracted from sediments. The diameter of these granules of *P. biwae* concave type was 0.2 to 0.3 µm, the density of granules was 8 - 10 per / µm^{2}, and no reticulate structure was present (Fig. 7B, D). In *P. biwae* convex type provided from the culture strain (SeedBank 20228-1) (Fig. 7A), a faintly reticulate structure was observed, the number of granules was reduced to 0-3 / µm^{2}, and the size of granules was increased (0.5 to 0.6 µm) (Fig. 7B). In *P. biwae* convex type collected from the surface sediments collected from off-Chikubushima in Lake Biwa, granular ornaments were fewer or mostly none in number, a faintly reticulate structure was more clearly developed, and the cell wall became thinner (Fig. 7F). These morphological changes occurred in asexual reproduction. Near the boundary of marginal cells, short rods called "rosettes" were observed in all *P. biwae* (arrows in Fig. 7B, D, F). SEM observations of other *Pediastrum* (*Monactinus*) taxa in the literature for clarifying the fine morphology of the cells are summarized in Table 3.

Table 3. List of species for SEM observations were conducted.

New species name	Old species name	Fine morphology	Reference
Monactinus simplex* var. *simplex	*Pediastrum simplex* var. *simplex*	Reticulate with spines on trigonal or tetragonal mesh	Wu 1987
	Pediastrum simplex var. *simplex*		Parra 1979
	Pediastrum simplex var. *simplex*		Lenarczyk 2015
	Pediastrum simplex var. *simplex*		Jena *et al.* 2014
	Pediastrum simplex var. *echinulatum*	Reticulate with spines on trigonal or tetragonal mesh	Parra 1979
	Pediastrum simplex var. *echinulatum*		MacManus & Lewis 2005
	Pediastrum simplex var. *echinulatum*		Lenarczyk 2014
	Pediastrum simplex var. *echinulatum*		Garduño-Solórzano *et al.* 2016
	Pediastrum simplex var. *echinulatum*		Lenarczyk & Wolowski 2016
	Pediastrum simplex var. *clathratum*	Granulate	Hegwald & Yamagishi 1994
	Pediastrum simplex var. *clathratum*		Parra 1979
	Pediastrum simplex var. *clathratum*		Garduño-Solórzano *et al.* 2016
Monactinus sturmii	*Pediastrum simplex* var. *sturmii*	Reticulate with spines on trigonal or tetragonal mesh	Jena *et al.* 2014
	Pediastrum simplex var. *sturmii*		Garduño-Solórzano *et al.* 2016
Monactinus asymmetricum	*Pediastrum asymmetricum*	Reticulate with spines on trigonal or tetragonal mesh	Hegwald & Yamagishi 1994
Monactinus biwaensis		Granulate	This study
	Pediastrum biwae (fossil)	Granulate?	Harada 1984

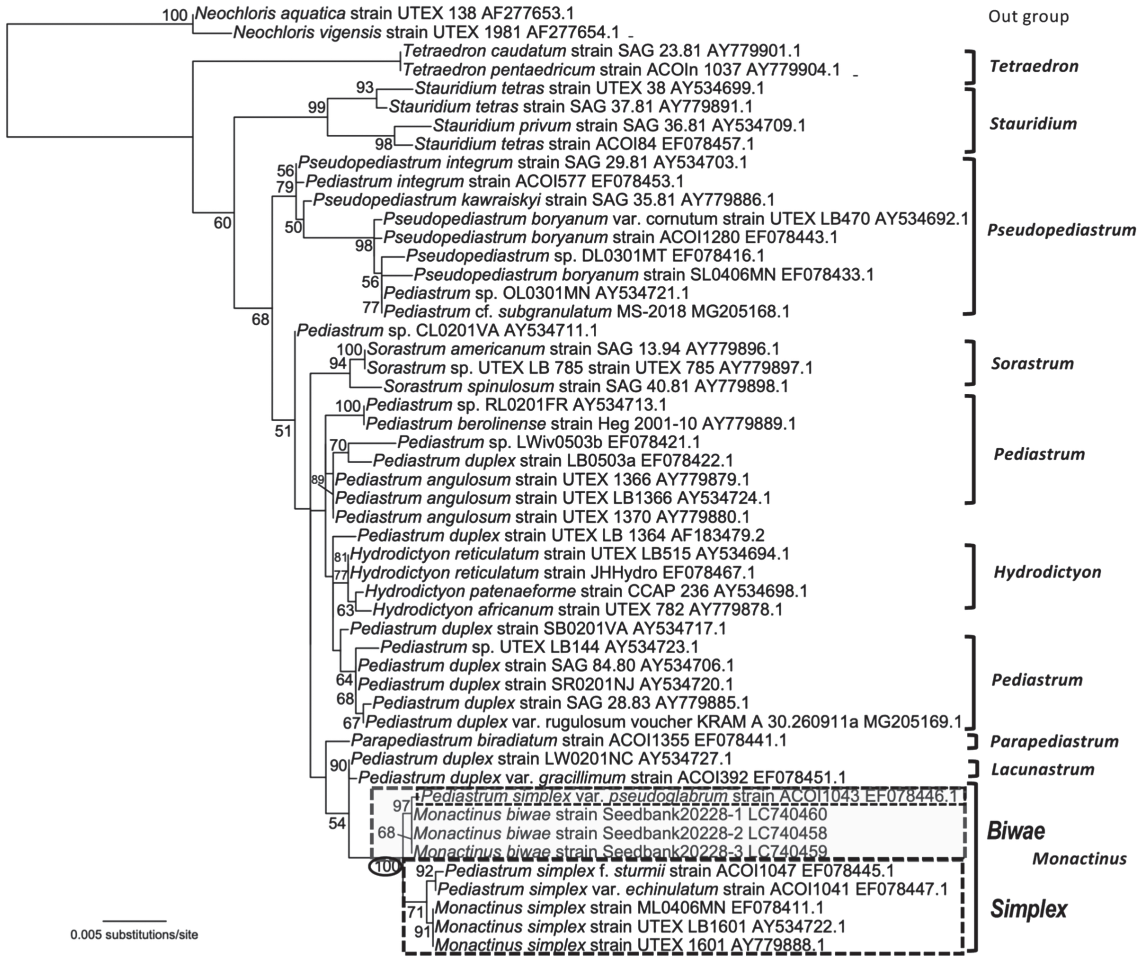


Fig. 8. Phylogenetic tree of the family Hydrodictyaceae based on the LSU rDNA. *Monactinus biwaensis* belongs to the Monactinus group, which is divided into two different clades: the Simplex clade and the Biwae clade.

4. Molecular phylogenetic analysis

Three strains of *Pediastrum biwae* (SeedBank 20228-1, -2, -3) collected from Oura Fishing Port in Lake Biwa for LSU rDNA were provided to make phylogenetic trees. As a result, *P. biwae* was included in the cluster of *Monactinus* as shown in Fig. 8. The *Monactinus* clade was strongly supported as monophyletic from other genera formerly classified into the genus *Pediastrum*. *Pediastrum biwae* formed a sister group with *M. simplex* (AY779888, EF078411, AY534722), *M. simplex* var. echinulatum (EF078447), *M. simplex* f. *sturmii* (EF078445) and *M. simplex* var. *pseudoglabrum* (EF078446), together forming a sister group in the LSU rDNA tree (Fig. 8).

In the tree based on SSU rDNA, the three strains of *P. biwae* were also grouped together with *M. simplex* (UTEX 1601; AY780664) and *M. sturmii* (HM021322, LC192141, HM021325, HM021324, HM021132) (Fig. 9). This group also clearly differed from other genera with 98 % bootstrap probability. In the *Monactinus* group, three clades were formed: a clade consisting of *P. biwae* (Accession numbers LC740455-LC740457) and a strain of *M. simplex* (UPMC-A0058), a clade consisting of a single strain designated as *P. simplex* (UTEX 1601), and a clade of *M. sturmii*.

In the tree of ITS2, the three strains of *P. biwae* were again grouped together with *M. simplex* (AY577726) and *M. sturmii* (HM021325, 021324, 021323, 021322, 021321) as the *Monactinus* group with high bootstrap probability. In addition, three clades were formed: the Sturmii, Simplex, and Biwae clades (Fig. 10).

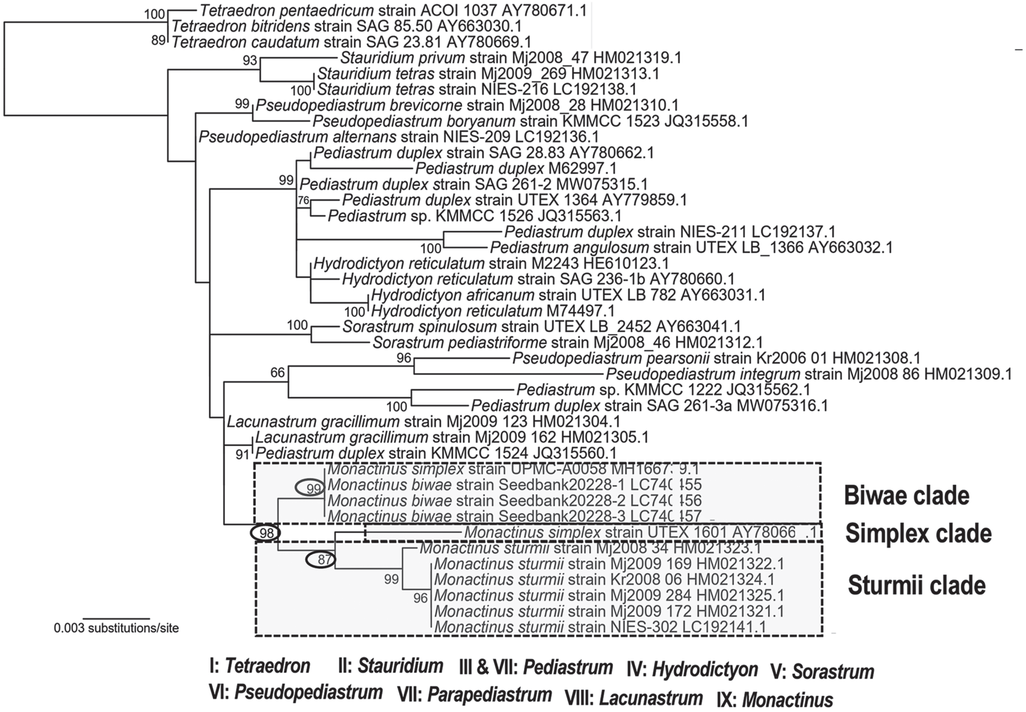


Fig. 9. Phylogenetic tree of the family Hydrodictyaceae based on the SSU rDNA. *Monactinus biwaensis* belongs to the Monactinus group. The Monactinus group consists of three clades: the Biwae clade, the Simplex clade, and the Sturmii clade. The strain of accession no. MH166739 was identified as *P. simplex*; however, this strain seems to be identical to *M. biwaensis* from the viewpoint of this molecular data.

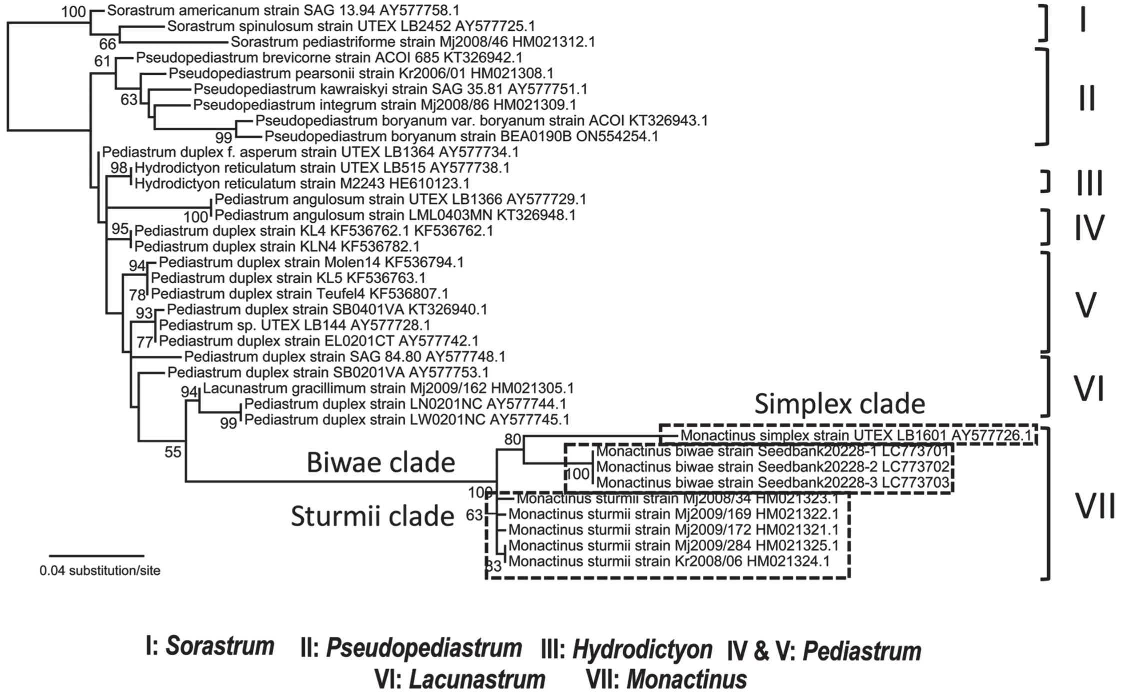


Fig. 10. Phylogenetic tree of the family Hydrodictyaceae based on the ITS2. *Monactinus biwaensis* definitely belongs to the Monactinus group. The Monactinus group consists of three clades: the Biwae clade, the Simplex clade, and the Sturmii clade.

5. Taxonomy of *Monactinus biwae*

Since the culture experiments, the coenobia identical to *Pediastrum biwae* var. *triangulatum* showed morphological changes from slender (var. *triangulatum*) to convex (var. *ovatum*) via straight (var. *biwae*) forms. These changes clearly indicate that varieties described by Negoro (1954) are not taxonomically justified.

Class Chlorophyceae Wille, 1884
Order Sphaeropleales Luerssen, 1877
Family Hydrodictiaceae Dumortier, 1829
Genus *Monactinus* Corda, 1839
Monactinus biwaensis* (Negoro, 1954) K. Matsuoka et M. Kayama *comb. nov.
（Figs. 5, 6, 7）

Basionym:

Pediastrum biwae Negoro, 1954, Acta Phytotaxonomica et Geobotanica 15: 135, Figs. 1-4. Kyoto, Japan

Pediastrum biwae var. *triangulatum* Negoro, 1954, Acta Phytotaxonomica et Geobotanica 15: 135, Figs. 5-8. Kyoto, Japan

Pediastrum biwae var. *ovatum* Negoro, 1954, Acta Phytotaxonomica et Geobotanica 15: Figs. 9-10. Kyoto, Japan

Description:

Monactinus biwaensis consists of mainly eight, sixteen, thirty-two, and rarely sixty-four slender cells which form a coenobium (Fig. 7A, C, D). The marginal cells possess a single projection with one or two bristles. These single projections are facing each other or are isolated (Figs. 5A, 6A). As the coenobium grows, the sides of the interior cells of the central part change from concave to straight and then convex (Figs. 5A, 5D, 6A, 6C), these cells have one to two rosettes (Fig. 7B, D, F). The cell yields many granular chloroplasts and a single pyrenoid (Figs. 5A, 6A). All of the cell surface seems to be rather smooth under light microscope; however, it is granular under scanning electron microscope (Fig. 7B, D, F). The empty cells contain a semicircular pore after liberation of zoospores (Fig. 7E, F).

Homotypic synonyms:

Pediastrum biwae var. *triangulatum* Negoro, 1954, Acta Phytotaxonomica et Geobotanica 15: 135, Fig. 5-8. Kyoto, Japan

Pediastrum biwae var. *ovatum* Negoro, 1954, Acta Phytotaxa et Geobotany 15: Fig. 9-10. Kyoto, Japan

Pediastrum biwae Negoro sensu Kokaji. Yurugi, Higashida, and Ishigami 1981, Fig. 1.

Pediastrum biwae Negoro sensu Loaiza-Restano 2013 MS Dissertação (Mestrado) -- Instituto de Botânica da Secretaria de Estado do Meio Ambiente: 50, Pl. 7, Fig. 51-55, Pl. 20, Fig. 148.

Pediastrum biwae var. *triangulatum* Negoro sensu Martinez-Goss M.R., Arguelles E.D..LR, Nacorda J.O.O. 2016, Philippine Scientists 53: 40, Pl. 1, Fig. 2.

Pediastrum biwae var. *triangulatum* Negoro sensu Ishigami, Ichise and Ohtsuka 2024, The Plankton in Lake Biwa, p. 147.

Heterotypic synonyms:

Pediastrum simplex var. *biwaense* T. Mizuno, 1952, Japanese Journal of Limnology 16: 34 (nomen nudum).

Pediastrum simplex var. *biwaensis* H. Fukushima, 1956, The Journal of Yokohama Municipal University Series C: 13 (46) p. 8 (nomen nudum; erroneous combination).

Pediastrum simplex Meyen sensu T. Yamagishi and M. Hirano, 1973, Contribution from the Biological Laboratory, Kyoto University: 24(2) 6I, p. 66, Pl 6, 1-6.

Pediastrum simplex var. *biwaense* Fukushima sensu C. González, 2009, Catálogo de las algas y cianoprocariotas dulciacuícolas de Cuba. Ed. Universo Sur, Universidad Cienfuegos: p. 58.

Pediastrum simplex var. *biwaense* Fukushima sensu M. Jena and S.P. Adhikary, 2011, Nelumbo: 53 p. 24. Pl. 2, Figs. 11 and 12.

Pediastrum simplex var. *biwaense* Fukushima in Y.J. Kim and H.S. Kim, 2012, Algal Flora of Kores, Freshwater Green Algae, Col. 6, No. 2 Chlorophyta: Chlorophyceae: Chlorococcales I: Micractiniaceae, Botryococcaceae, Characiaceae, Hydrodictyaceae: p. 66, Figs. 52, 53 A-F.

Pediastrum simplex var. *biwaense* Fukushima in O.M. Lee, G. Joh, M.R., Kim and S.W. Nam, 2018, Nakdong and National Biological Resource Center: p. 212, Fig. 93-2, A-F.

Pediastrum simplex var. *biwaense* Fukushima in R.B.V. Naidu, P.C. Raju and G.S. Ranganayakulu, 2018, Bulletin of Pure and Applied Sciences, 86–87, Fig. 1D.

Lectotype: Figure 1 of Negoro, 1954 as *Pediastrum biwae* Negoro

Type locality: Lake Biwa, Central, Japan

Remarks: *Monactinus biwaensis* is separated from *M. simplex* and *M. asymmetricus* in having adjacent projections that face each other and granulate surface of the cells. The varieties of *Pediastrum biwae*, var. *triangulatum* and var. *ovatum*, are not taxonomically independent, because these varieties appear during asexual reproduction in the unialgal culture experiments starting from a coenobium identical to concave type.

Following publications showed only photographs and illustrations identical to *Monactinus biwaensis*

Monactinus simplex var. *biwaense* (Fukusima) Taşkin E. et Alp M.T. sensu Akbulut et al., 2019 (erroneous combination).

Pediastrum simplex Meyen sensu Y.S. Kha w, N.M.H. Khong, N.A.Shaharuddin, and F.M. Yusoff, 2020 Journal of Microbiological Methods: 172, Fig. 4 F6.

Pediastrum simplex var. *biwaense* Fukushima sensu L. Xiang, X. Huang, C. Huang, X. Chen, H. Wang, J. Chen. M. Sun, and Y. Xiao 2021 Review of Palaeobotany and Palynology: 289, 104396, Fig. 2.

Pediastrum duplex var. *gracillimum* West & GS West sensu R.W. Bobiles, C. Cabiles, N.K. Torreta 2014 Philippine Scientest 51, Figs. 2, 3.

Genbank Accessions: SSU rDNA: LC740455, LC 404456, LC404457; LSU rDNA: LC740458, LC404459, LC740460; ITS2: LC773701, LC773702, LC773703.

Discussion

1. Morphological characteristics of *Monactinus biwaensis*

The morphological feature differentiating *M. biwaensis* from *M. simplex* is the characteristics of the direction of projection of the marginal cell. As the diagnostic feature for *Pediastrum biwae* described by Negoro (1954), the two projections adjacent to marginal cells are facing or separating. These features are well shown in the ratio of the marginal cells of *M. simplex* being nearly symmetrical vs those of *M. biwaensis* being irregularly triangular in general as shown in Table 2.

Regarding SEM observations of the cell surface, observations of several taxa of *P. simplex* or *M. simplex* including several varieties, have been performed so far and these are also summarized in Table 3. *Monactinus asymmetricus*, another species characterized by a single projection in the marginal cell, has a reticulate structure with spines on trigonal meshes and rosettes on the margin of the interior cells (Hegewald and Yamagishi 1994, Figs. a to d). One of the varieties of *M. simplex*, namely *P. simplex* var. *echinulatum*, shows a reticulate structure with spines on trigonal or tetragonal meshes (MacManus and Lewis 2005, Fig. 7; Parra 1979, pl. 50 Figs. a-d; Lenarczyk 2014, Figs. 4 I, J, and Fig. 6 E).

Another well-known variety, *M. simplex* var. *sturmii*, which is now treated as an independent species *M. sturmii*, also possesses a reticulate structure with spines (Jena et al., 2014, Fig. 3d). This structure with spines and some rosettes is also observed in *P. simplex* var. *simplex* (Parra 1979, pl. 47a; Lenarczyk 2014, Fig. 5E) and *M. simplex* (Jena et al., 2014, Fig. 3b).

Interestingly, the cell surface in these two groups, *M. simplex* and *M. biwaensis*, as defined by molecular analysis in the *Monactinus* group is morphologically different. Based on the illustration published by Lenaczyk (2014), the cell surface of *M. biwaensis* is called "reticulate with granules" vs that of *M. simplex* var. *simplex, M. sturmii*, and *P. simplex* var. *echinulatum*, which is called "reticulate spines on trigonal or tetragonal mesh" under SEM (Fig. 11). On the other hand, *M. simplex* var. *clathratum*, which is included in the clade of *M. biwaensis* in the LSU rDNA phylogenetic tree, shows a granulate surface of the cells (Garduño-Solórzano et al., 2016). Parra (1979, p. 115 and pl. 52 a-g) introduced *P. simplex* var. *pseudoglabrum* as another new variety. The photographs of *P. simplex* var. *pseudoglabrum* of Parra (1979, pl. 52 g) give a strange impression of the fine structure of its rather smooth surface. However, careful observations of the photographs of this variety showed a granular fine structure in Parra (1979, pl. 52 f). Komárek and Jankovská (2001) also suggested that *P. simplex* var. *pseudoglabrum* has a finely granular structure. On the other hand, *P. simplex* var. *clathratum* Shröder 1883 of Garduño-Solórzano et al. (2016, Figs 33–36) shows a granular surface with several rosettes. These two varieties, var. *pseudoglabrum* and var. *clathratum* seem to have the same fine structure of the cell surface. Therefore, *P. simplex* var. *pseudoglabrum* may be a heterotypic synonym of *P. simplex* var. *clathratum*.

As described above, the change of morphology of *M. biwaensis* as the density of granules is reducing and the reticulation is developing during asexual reproduction suggests that the surface ornament consists of two different layers: outer granular and inner reticulate layers. The granules can stretch and become flatter and finally only the reticulate layer remained as the fossil condition. However, the fine structure of the cell surface of *M. biwaensis* is basically granular with a few rosettes, as shown in Fig. 7B, D, F. Accordingly, both the molecular data and the morphological characteristics clearly show that these two taxa, *M. biwaensis* and *P. simplex* var. *clathratum*, should be separate and independent of each other.

Morphologically and molecular phylogenetically, *M. biwaensis* is clearly differentiated from *M. simplex* and *M. sturmii*. In conclusion, *M. biwaensis* is not a variety of *M. simplex* and rather is an independent species in the genus *Monactinus*.

2. Molecular phylogenetic position of *Monactinus biwae*

The genus *Monactinus* is morphologically characterized by a single projection on the marginal cell (Buchheim et al., 2005). This is concordant with the morphological features of *Monactinus biwaensis*. For clarifying the phylogenetic position of *M. biwaensis*, a molecular phylogenetic tree using all sequence data of species previously assigned to *Pediastrum* was constructed.

At the moment, *M. simplex*, *M. sturmii*, and *M. asymmetericum* are assibned to the genus *Monactinus* (Jena et al., 2014; Lenarczyk and Saluga, 2018; Guiry and Guiry, 2016; AlgaeBase checked on 24 February 2024). In the phylogenetic tree based on LSU rDNA, three strains of *M. biwaensis* form a cluster with *M. simplex*, but are clearly separated from *M. simplex* var. *pseudoglabrum* (Fig. 10). Three strains of *M. biwaensis* used for SSU rDNA analysis are also separated from the cluster consisting of *M. simplex* and *M. sturmii*. These data suggest that *M. biwaensis* is the independent species, as described as described by Negoro (1954) based on morphology (Fig. 11). Fukushima (1956) recorded this species as *P. simplex* var. *biwaense* (sic), as *P. biwae* is not an independent species but a variant of *P. simplex*, and later it was transferred to the genus *Monactinus* and named *M. simplex* var. *biwaensis* (Fukushima) Taşkin et Alp in Akbulut et al. (2019). However, this reassessment is not proper judging from the present results of the SSU, LSU, and ITS2 analysis. The *Monactinus* group is divided into three major clades: the Simplex clade, the Sturmii clade, and the Biwae clade, although in the LSU rDNA tree, the *sturmii* clade is not clearly separated due to the short data of *M. sturmii* (Figs. 8, 9, 10).

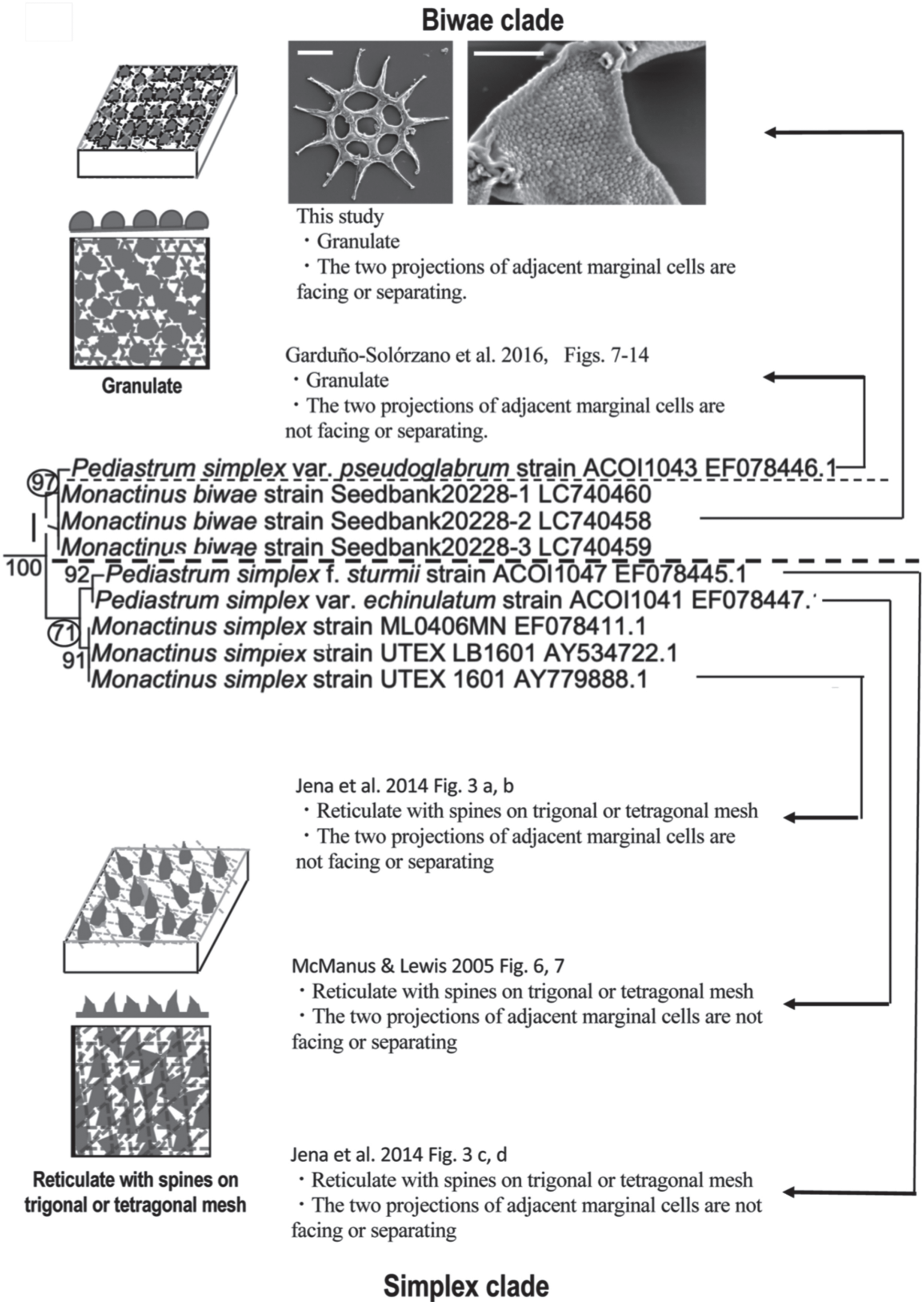


Fig. 11. The relationship between molecular phylogeny of LSU rDNA tree and fine structure of the cell surface in the *Monactinus* group. The Simplex clade including *M. simplex*, *M. simplex* var. *echinulatum*, and *M. simplex* f. *sturmii* has a cell surface that is reticulate with spines on a trigonal or tetragonal mesh and the Biwae clade including *M. biwaensis* and *P. simplex* var. *pseudoglabrum* possesses a granular cell surface. The photographs of these taxa are from the following publications: *M. simplex* AY534726 (Jena *et al.* 2014), *M. simplex* var. *echinulatum* EF078447 (Jena *et al*. 2014), *P. simplex* f. *sturmii* EF078445 (McManus and Lewis 2005), *P. simplex* var. pseudoglabrum EF078446 (Parra 1979), and *M. biwaensis* (this study).

In the SSU rDNA analysis, *M. biwaensis* forms a different clade consisting of *M. simplex* and *M. sturmii* except for *M. simplex* (accession no. MH166739) collected from Malaysia (Khaw et al., 2020). This specimen registered as accession no. MH166739 may be important for discussing the relationship between *M. simplex* and *M. biwaensis*. According to the result of the BLAST analysis, the most probable identification of this Malaysian strain was firstly *Lacunastrum gracillimum* and secondarily *M. simplex*. However, Khaw et al. (2020) concluded this specimen to be identical to *M. simplex* based on its single projection of the marginal cell. This might be due to the lack of molecular data of *M. biwaensis* at that time. We measured the side ratio of *M. simplex* shown by Khaw et al. (2020, Fig. 4 F6) and obtained a value of about 0.88. This is similar to that of the *M. simplex* group, as shown in Table 2. However, this ratio also overlaps that of the *M. biwaensis* group. Therefore, the molecular phylogenetic data is probably correct and this specimen may be identical to *M. biwaensis* rather than *M. simplex*.

In the phylogenetic trees based on the ITS2 analysis, the genus *Monactinus* was clearly separated from other genera with a high bootstrap probability (Fig. 10). In the SSU rDNA tree three clades consisting of *M. simplex*, *M. biwaensis*, and *M. sturmii* could be recognized within the *Monactinus* (Fig. 9). However, there is too little molecular data on *M. simplex*, which consists of many varieties and forma, to establish these clades. In addition, *M. simplex* and *M. sturmii* have very similar cell surface fine structure under SEM observation. The genus *Monactinus* is currently considered to be composed of the Simplex and the Biwae clades based on molecular phylogenetic data and fine structure of the cell surface. Consequently, *M. biwaensis* is clearly separated from *M. simplex* and *M. sturmii* molecular-phylogenetically.

3. Invalidation of two varieties of *Pediastrum biwae*

In general, morphological changes that appear in the life history of the same strain that are not genetic mutations are not treated as variants. Negoro (1954) proposed two varieties in *Pediastrum biwae*: *P. biwae* var. *biwae* (autonomy), *P. biwae* var. *triangulatum* and *P. biwae* var. *ovatum*, when he described *P. biwae* as a new species. Subsequent researchers have also accepted these varieties taxonomically, for example, Wakabayashi and Ichise (1982) and Ishigami et al. (2024). Honda (1973) also conducted incubation experiments of *P. biwae* and observed *P. biwae* var. *triangulatum* asexually reproducing *P. biwae* var. *ovatum* via *P. biwae* var. *biwae*. These morphological changes of *P. biwae* were not hereditary, and were caused by the age of the organisms and the nutritional condition as mentioned by Honda (1973). Kokaji et al. (1981) also conducted the culture experiments of *P. biwae* under various conditions and illustrated the morphological changes of *P. biwae* from the slender (var. *triangulatum*, Kokaji et al., 1981 Fig. 1a) to normal (var. *biwae*, Kokaji et al., 1981 Fig. 1b) forms during culture. These morphological changes suggest that the three varieties of *P. biwae* proposed by Negoro (1954) are not controlled by environmental conditions but rather probably represent a time series of asexual reproduction.

The morphological changes during the culture experiments are concordant with previous observations of the extremely rare occurrence of *P. biwae* var. *ovatum* in natural plankton in Lake Biwa (Wakabayashi and Ichise, 1982), because production of *P. biwae* var. *ovatum* is rare in asexual reproduction in culture. Also, molecular data were not different among these three types, which are morphologically equivalent to these three varieties.

In conclusion, three varieties of *P. biwae*, var. *triangulatum*, var. *biwae*, and var. *ovatum*, cannot be separated each other taxonomically. It is better to understand these as morphologically different coenobia produced in asexual reproduction and to call them the concave type for var. *triangulatum*, the straight type for var. *biwae*, and the convex type for var. *ovatum*.

4. Occurrence of *Monactinus biwae* in Japan and other countries

Monactinus biwaenseis (*=Pediastrums biwae*) has been reported from waters far from the Lake Biwa -Yodogawa River system in Japan (Negoro, 1959; Nasu, 1971). According to Negoro (1959), the range of the water system connected to Lake Biwa where *P. biwae* is found is as follows: Lake Yogo, the Yogo-gawa River, Lake Biwa, the Seta-gawa River, the Uji-gawa River, the Yodo-gawa River and finally into Osaka Bay. However, *M. biwaensis* has also been listed from the following locations: southwestern part (Banshu) of Hyogo Prefecture (Imazu, 1973), Regulation Pond of Isahaya

Reclamation of Nagasaki Prefecture (Ishizaki et al., 2001, 2002), and the Hitokura Dam Lake of Hyogo Prefecture (Kinki Regional Development Bureau, Ministry of Land, Infrastructure, Transport and Tourism, 2022) at the moment. Although these occurrence reports were not accompanied by any descriptions, photographs, or drawings, but rather were only included in lists of species, these lists suggested that *Monactinus biwaensis* is distributed outside of the Lake Biwa-Yodogawa River system in Japan. More than hundred years ago, fry of sweet fish inhabiting in Lake Biwa was commercially transported to other regions in Japan (Imura, 2013; Ishigami et al., 2024). Therefore, the occurrence of *M. biwaensis* outside the Lake Biwa-Yodo-gawa River System may be resulted by human commercial activities. It is expected that molecular phylogenetic analysis for these algae outside of the Lake Biwa.

There are several reports of the occurrence of *M. biwaensis* outside of Japan. However, in some of these reports, *P. biwae*, including *P. simplex* var. *biwaense*, from around the world seems not to be reliable without examination of the morphological characteristics of marginal cells and molecular phylogenetic analysis. After careful observation of the appearance of marginal cells in photographs and/or drawings, the following specimens seemed to be belonging to *M. biwaensis*; Korea (Kim and Kim, 2012), Cambodia (Yamagishi and Hirano, 1973), Thailand (Prasertsin et al., 2018), north-eastern India (Jena and Adhikary, 2011), the south-eastern coast of India (Naidu et al., 2018), and San Paulo district of Brazil (Loaiza Restano, 2013). In the Philipines, Martinez-Goss et al. (2016) recorded *M. biwaensis* from Laguna de Bay. Bobiles et al. (2014) also showed this alga under the different species name, *Pediastrum duplex* var. *gracillimum* from mountain lakelets of Mt. Asog and Mt. Maliano. Unfortunately, identification of the species was wrong, because a single projection was developing on the marginal cell, and these adjacent projections were faced each other or were separated. These morphological features are common with *M. biwaensis*. As seen in Malaysia (Khaw et al., 2020), the species identification of *M. biwaensis* requires molecular phylogenetic analysis. According to these data, *Monactinus biwae* have been mainly reported from warm temperate to tropical waters of East to Southeast Asia. Xiang et al. (2021) and González (2009) reported *Pediastrum simplex* var. *biwaense* from China and Cuba, respectively, without microphotographs, but it cannot be determined whether these algae are indeed *M. biwaensis*. However, species morphologically identical to *M. biwaensis* might have been reported from various locations in the world under the names *P. simplex* var. *clathratum* (Komárek and Jankovská, 2001, Fig. 12B; Pasztaleniec and Poniewozik, 2004, Figs. 12, 13) and *M. simplex* var. *duodenarium* (Baykal et al., 2004, Fig. 4 a). *Pediastrum simplex* var. *duodenarium* was originally described as *Monactinus duodinarius* by Baily (1854) and it was then transferred to a variety of *Pediastrum simplex* by Rabenhorst (1868). Further molecular phylogenetic analyses and SEM observations of the cell surface are desirable for understanding the taxonomic validity of this organism.

Conclusion

- Based on morphological measurements of a single projection of the marginal cells, it is confirmed that adjacent projections of the marginal cells of *Pediastrum biwae* Negoro are facing or separating each other.
- Under the SEM observations, the cell surface of *P. biwae* is ornamented with small granules.
- In molecular phylogenetic analysis using the LSU, ITS2, and SSU rDNA, *Pediastrum biwae* nested in a different position from *Monactinus simplex* and *M. sturmii*.
- Based on both morphological characteristics and molecular phylogenetic analysis, *Monactinus biwaensis* (Negoro) K.Matsuoka et M.Kayama is proposed for *P. biwae* as a new combination.
- The two varieties of *P. biwae* ; var. *triangulatum* and var. *ovatum*, proposed by Negoro (1954) are invalid because their equivalent forms occur during coenobium growth in asexual reproduction.

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Authorship contribution statement

MK: Writing, culture experiments, DNA analysis, AI: culture experiments, MLWT: sampling, Statistical analysis. KI: sampling, KM: Conceptualization, writing, DNA analysis, sampling, SEM observation, review of the manuscript.

Repository of materials used in this study

• Culture of *Monactinus biwaensis* collected at Oura Fishing Port on 20 September 2022: SeedBank 20228-1, SeedBank 20228-2, SeedBank 20228-3.

• Stubs for SEM observations: SEM of *Monactinus biwaensis* of the culture strains and of palynomorphs of the surface sediments made on 11 October 2022.

All culture strains and stubs for SEM are kept at SeedBank Co Ltd.

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