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New species and species diversity of *Desmodesmus* (Chlorophyceae, Chlorophyta) in Saga City, Japan

Mikihide Demura

Desmodesmus **spp. are one of the most dominant components of phytoplankton, which are present in most water bodies. However, identification of the species based only on morphological data is challenging. The aim of the present study was to provide a comprehensive understanding of the actual distribution of the *Desmodesmus* species in Saga City, Saga Prefecture, Japan. In the present study, 38 water bodies were surveyed between June 2017 and March 2023. A total of 86 culture strains were established from the samples collected from the 21 sites, and identified by molecular phylogenetic analysis, comparison of ITS2 rRNA secondary structures, and observation of surface microstructure. In total, four new species, including *D. notatus* Demura sp. nov., *D. lamellatus* Demura sp. nov., *D. fragilis* Demura sp. nov., and *D. reticulatus* Demura sp. nov. were proposed and 17 *Desmodesmus* species were identified as described species. The present study revealed > 20 *Desmodesmus* species, exhibiting high genetic diversity in a small area.**

Keywords *Desmodesmus*, Genetic diversity, Microalgae, New species, Phylogeny

Desmodesmus (R. Chodat) S. S. An, T. Friedl & E. Hegewald (Chlorophyceae, Chlorophyta) is a green algal genus observed in most freshwater areas[1–3]. It is phytoplankton of approximately 10–50 µm. The morphology of *Desmodesmus* is mostly a colony (coenobium) of four cells in a row with a characteristic structure called "spine" at the edge of the outer cells[4,5]. However, the spine may be absent in certain species[6] or present in all cells of the colony[7]. In recent years, because of the rapid growth and easy cultivation of *Desmudesmus*, research on algal mass cultivation that combines the removal of nitrogen and phosphorus from wastewater with algal biomass production has increased[8,9].

Desmodesmus was an established genus, independent of *Scenedesmus* by An et al.[10].

Scenedesmus is a genus described nearly 200 years ago[11], and many species have been described only by morphological information. An et al.[10] performed a detailed analysis based on molecular phylogenetic and secondary structure analysis using internal transcribed spacer 2 (ITS2) sequences and showed that *Desmodesmus* is an independent genus from *Scenedesmus*.

Desmodesmus exhibits high morphological variation depending on environmental conditions[12–14]; therefore, descriptions based solely on optical microscopic morphological information led to numerous synonyms being attributed to it. However, since the study performed by An et al.[10], Hegewald et al. have facilitated the accurate and efficient identification by utilizing a method that combined molecular phylogenetic analysis using ITS2 sequences, ITS2 RNA secondary structure analysis, and detailed morphological observations using scanning electron microscopy (SEM)[e.g., Refs.[7,10,15,16]]. In the secondary structure analysis of ITS2 RNA, the compensatory base change (CBC) has been proven to be an important character. The CBC is a base mutation that occurs in both nucleotides in a paired structural position, whereas the hemi-CBC is a mutation of a single nucleotide in a paired structural position, in which the nucleotide bond is retained[17]. The CBC and hemi-CBC, which are predicted from the sequence of ITS2 RNA, have enabled classification that considers the biological species concept[17]. The CBC and hemi-CBC have been used for taxonomic systematics and identification in microalgae other than *Desmodesmus*, such as Chlorophyceae[17,18] and diatoms[19]. Nguyen et al.[20] have utilized specific sequences ITS2 RNA, considering CBC and hemi-CBC, for the identification of *Desmodesmus*.

Currently, distributional studies of *Desmodesmus* species, based on phylogenetic analysis and detailed morphological observation using SEM, are underway in various locations, including Romania[21], New Zealand[22], and Poland[23].

In Japan, *Desmodesmus* (recorded as *Scenedesmus* at the time) has been documented as a major phytoplankton genus in many lakes and marshes since the 1930s[24,25]. However, these studies only reported morphological

Faculty of Agriculture, College of Natural Sciences, Institute of Education and Research, Saga University, 1 Honjo, Saga 840-8502, Japan. email: st8148@cc.saga-u.ac.jp

observations using optical microscopy, and the exact distribution of the species remained unclear. Demura et al.[26] were the first to clarify the distribution of *Desmodesmus* species in Japan by utilizing a method that combined molecular phylogenetic analysis using ITS2 sequences, ITS2 RNA secondary structure analysis, and detailed morphological observations using SEM. Demura et al.[26] identified seven previously described species and one new species of *Desmodesmus* in five water bodies in Saga City, Japan.

Saga University and Saga City office are promoting a microalgae biomass production project and have focused on rapidly-growing *Desmodesmus* as a target microalgae. The search for *Desmodesmus* culture strains that could propagate faster was necessary, and a diversity survey of *Desmodesmus* in lakes and ponds in the city has been conducted. However, Demura et al.[26] surveyed only five water bodies, which is insufficient for comprehensively understanding the diversity and distribution of *Desmodesmus* in Saga City, Japan. Therefore, in this study, I aimed to provide a more detailed understanding of the actual distribution of the *Desmodesmus* species in Saga City, Japan, and determine the true diversity of *Desmodesmus* by expanding the study area within Saga City, and increasing the number of analyzed strains.

Results

Distribution surveys were conducted across 38 water bodies in Saga City, from June 2017 to March 2023. *Desmodesmus* species were observed at 21 sites (55.3%) (Fig. 1). In total, 86 *Desmodesmus* strains were established (Table 1, Fig. 1), and 21 species of *Desmodesmus* were identified including four new species (Table 1, Fig. 2). *Desmodesmus armatus* was the most widely distributed, being detected at 10 sites. In contrast, certain species were only found at a single site; for example, *D. communis* was only found at Hirao-yon-chome-ike; *D. insignis* was only detected at Kannonji-tsutsumi. *D. insignis* and *D. communis* were detected at Kannonji-tsutsumi and Hirao-yon-chome-ike, respectively, throughout the sampling period.

Molecular phylogenetic analyses of the maximum likelihood method and the Bayesian phylogenetic inference using ITS2 sequences derived almost identical phylogenetic trees (Fig. 3). In Clade 2, eight strains, including dSDes-Koumin3, dSgDes-Shizu1, and dSgDes-Ko2; dSgDes-ecoJ1, dSgDes-ecoOc3, and dSgDes-ecoApr(belonging to phylogenetic lineage D); and dSgDes-Nemu1and dSgDes-YO2-1 (belonging to phylogenetic lineage E), formed a clade that was sister to dSgDes-Hasu1, which showed 100% homology with the sequence represented by GenBank accession number MK447733, identified by *D. opoliensis*. Therefore, strain

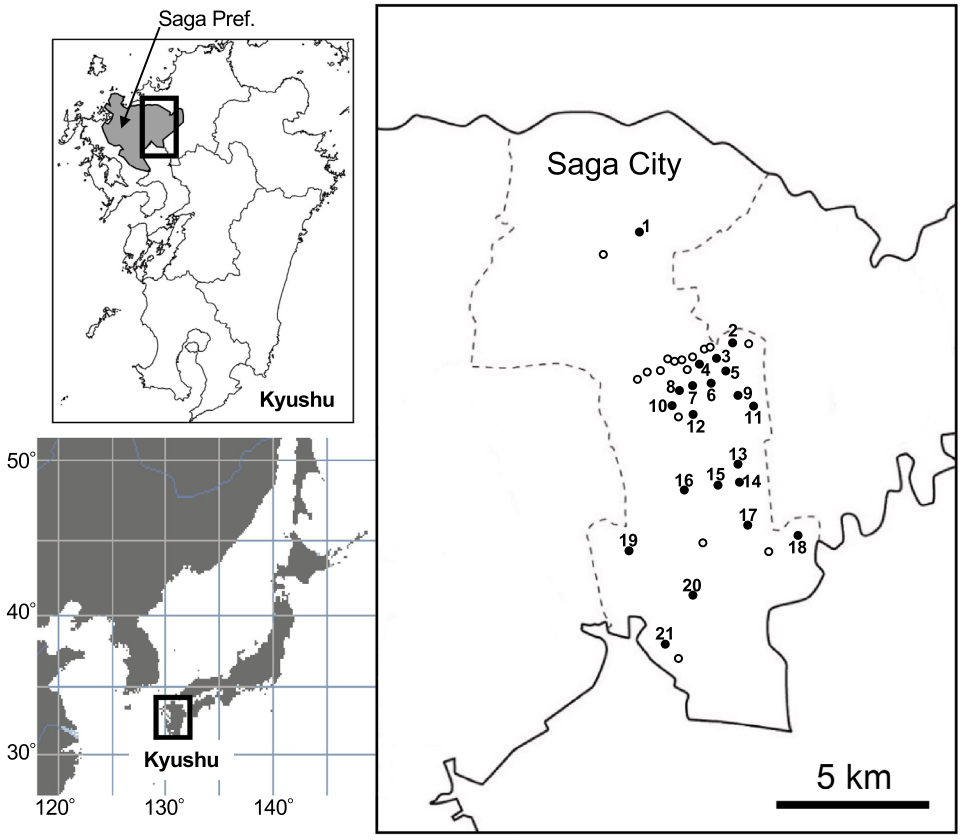

Figure 1. Survey and sampling sites in Saga City, Saga Prefecture, Japan. Sites (black dots) are numbered from north to south and represent areas from which *Desmodesmus* culture strains could be established (Table 1). White circles represent sites that were surveyed but where *Desmodesmus* was not detected. Sites 3, 5, 12, 17, 20 were the study sites referenced in Demura et al.[26].

Sampling site no. in Fig. 1	Locality	N, E (degree)	Sampling day	Strain name	Accession no	Alphabet in Fig. 3	Species name of *Desmodesmus*
1	The Hokuzan Dam	33.433855, 130.237797	20 May, 2022	dSgHokuzan1	LC777096	B	*D. armatus* (Chodat) E. Hegewald
				dSgHokuzan2	LC777097	G	*D. intermedius* (Chodat) E. Hegewald
2	Pond near Kinryu park	33.329538, 130.315569	13 March, 2023	dSgDes-High1	LC777098	P	*D. protuberans* (F. E. Fritsch & M. F. Rich) E. Hegewald
				dSgDes-High2	LC777099	–	*D. insignis* (West & G.S.West) E. Hegewald
				dSgDes-High3	LC777100	B	*D. armatus*
				dSgDes-High4	LC777101	B	*D. armatus*
3	Kinryu park (wetland)	33.329307, 130.306942	9 July, 2019	dSgDes-m	LC642119	C	*D. armatus*
4	Kannonji-tsutsumi (pond)	33.327028, 130.298031	12 December, 2017	dSgKDes1	LC777102	A	*D. serratus* (Corda) S. S. An, Friedl et E. H. Hegewald
				dSgKDes2	LC777103	A	*D. serratus*
				dSgKDes3	LC777104	F	*D. insignis*
				dSgKDes4	LC777105	A	*D. serratus*
			31 August, 2021	dSgKDesA1	LC777106	–	*D. arthrodesmiformis* (Schroder) S.S.An, Friedl & E.Hegewald
				dSgKDesA3 (Type)	LC777107	–	*D. lamellatus* Demura
			12 October, 2021	dSgKDesOc1	LC777108	–	*D. denticulatus* (Lagerheim) S. S. An, T. Friedl & E. Hegewald
			7 December, 2021	dSgKDes12/1	LC777109	F	*D. insignis*
				dSgKDes12/2	LC777110	I	*D. reticulatus* Demura
			16 February, 2022	dSgKDes2/1	LC777111	–	*D. insignis*
				dSgKDes2/2	LC777112	F	*D. insignis*
				dSgKDes2/3	LC777113	F	*D. insignis*
			22 April, 2022	dSgKDes4/1	LC777114	F	*D. insignis*
				dSgKDes4/2 (Type)	LC777115	I	*D. reticulatus*
5	Oya-tsutsumi (pond)	33.326024, 130.312058	28 November, 2018	dSgDes-b	LC642122	–	*D. dohacommunis* Demura
			13 March, 2023	dSgDes-Oya1	LC777116	–	*D. tropicus* (W.B.Crow) E. Hegewald
				dSgDes-Oya2	LC777117	J	*D. tropicus*
				dSgDes-Oya3	LC777118	O	*D. protuberans* var. *communioides* Hegewald
6	Ko-tsutsumi (pond)	33.325925, 130.311168	13 March, 2023	dSgDes-Ko1	LC777119	L	*D. maximus* (West & G.S.West) Hegewald
				dSgDes-Ko2	LC777120	–	*D. notatus* Demura
				dSgDes-Ko3	LC777121	L	*D. maximus*
7	Imamura-tame-ike (pond)	33.321029, 130.291052	22 April, 2022	dSgDes-Ima1	LC777122	J	*D. tropicus*
				dSgDes-Ima2	LC777123	O	*D. protuberans* var. *communioides*
				dSgDes-Ima3	LC777124	O	*D. protuberans* var. *communioides*
8	Ueno-tsutsumi (pond)	33.317081, 130.251355	20 May, 2022	dSgDes-Komin1	LC777125	C	*D. armatus*
				dSgDes-Komin2	LC777126	O	*D. protuberans* var. *communioides*
				dSgDes-Komin3	LC777127	-	*D. notatus*
9	Shizuka-ike (pond)	33.312350, 130.323276	17 Jun, 2022	dSgDes-Shizu1	LC777128	-	*D. notatus*
				dSgDes-Shizu2	LC777129	P	*D. protuberans*
10	Shoubu-en (artificial pond)	33.309727, 130.254533	20 May, 2022	dSgDes-Shoubu	LC777130	-	*D. pseudocommunis* Hegewald

Continued

Sampling site no. in Fig. 1	Locality	N, E (degree)	Sampling day	Strain name	Accession no	Alphabet in Fig. 3	Species name of *Desmodesmus*
11	Shiroishibaru (wetland)	33.308115, 130.336966	16 February, 2022	dSgDes-Shiro1	LC777131	B	*D. armatus*
				dSgDes-Shiro2	LC777132	P	*D. protuberans*
				dSgDes-Shiro3	LC777133	B	*D. armatus*
				dSgDes-Shiro4	LC777134	B	*D. armatus*
				dSgDes-Shiro5	LC777135	B	*D. armatus*
12	Hirao-yon-chome-ike (pond)	33.299276, 130.293793	15 Jun, 2017	dSgDes-0	LC642123	–	
			23 January, 2020	dSgDes-eco1	LC642120	M	*D. communis* (E. Hegewald) E. Hegewald
				dSgDes-eco2	LC777136	M	*D. communis*
				dSgDes-eco3	LC777137	M	*D. communis*
				dSgDes-eco4	LC777138	M	*D. communis*
				dSgDes-eco5	LC777139	M	*D. communis*
			13 February, 2020	dSgDes-eco6	LC642126	J	*D. tropicus*
				dSgDes-eco7	LC777140	M	*D. communis*
				dSgDes-eco8	LC777141	M	*D. communis*
				dSgDes-eco9	LC777142	M	*D. communis*
				dSgDes-eco10	LC777143	–	*D. protuberans*
			17 March, 2020	dSgDes-eco11	LC642121	M	*D. communis*
				dSgDes-eco12	LC642124	–	*D. lefevrei* (Deflandre) S.S.An, T.Friedl & E.H.Hegewald
				dSgDes-eco13	LC777144	M	*D. communis*
				dSgDes-eco14	LC777145	M	*D. communis*
				dSgDes-eco15	LC777146	M	*D. communis*
			17 Jun, 2021	dSgDes-ecoJ1	LC777147	D	*D. notatus*
				dSgDes-ecoJ2	LC777148	M	*D. communis*
				dSgDes-ecoJ3	LC777149	M	*D. communis*
			31 August, 2021	dSgDes-ecoA1	LC777150	–	*D. insignis*
				dSgDes-ecoA2	LC777151	D	*D. notatus*
				dSgDes-ecoA3	LC777152	J	*D. tropicus*
			12 October, 2021	dSgDes-ecoOc1	LC777153	M	*D. communis*
				dSgDes-ecoOc3 (Type)	LC777154	D	*D. notatus*
			7 December, 2021	dSgDes-eco12/1	LC777155	J	*D. tropicus*
			16 February, 2022	dSgDes-ecoFeb1	LC777156	–	*D. subspicatus* (Chodat) E. Hegewald & A.W.F. Schmidt
				dSgDes-ecoFeb2	LC777157	–	*D. subspicatus*
				dSgDes-ecoFeb3	LC777158	–	*D. subspicatus*
				dSgDes-ecoFeb4	LC777159	B	*D. armatus*
			22 April, 2022	dSgDes-ecoApr	LC777160	D	*D. notatus*
13	Hyotan-jima park (artificial pond)	33.278719, 130.319256	22 April, 2022	dSgDes-Hyo	LC777161	G	*D. intermedius*
14	Tonbo-ike (pond)	33.271471, 130.312316	13 March, 2023	dSgDes-Tonbo	LC777162	C	*D. armatus*
15	Nemunoki park (pond)	33.267596, 130.313495	13 March, 2023	dSgDes-Nemu1	LC777163	E	*D. notatus*
				dSgDes-Nemu2	LC777164	K	*D. tropicus*
				dSgDes-Nemu3	LC777165	H	*D. fragilis* Demura
16	Kouno park (wetland)	33.265989, 130.280567	20 May, 2022	dSgDes-Kami	LC777166	G	*D. intermedius*

Continued

Sampling site no. in Fig. 1	Locality	N, E (degree)	Sampling day	Strain name	Accession no	Alphabet in Fig. 3	Species name of *Desmodesmus*
17	Kose park (creek)	33.255848, 130.325748	21 April, 2020	dSgBigDeka1	LC642127	K	*D. tropicus*
				dSgBigDeka2	LC777167	J	*D. tropicus*
				dSgBigDeka4	LC642125	-	*D. protuberans*
				dSgBigChibi1	LC777168	N	*D. protuberans* var. *communioides*
			7 December, 2021	dSgBigDes12/1	LC777169	C	*D. armatus*
				dSgBigDes12/2	LC777170	–	*D. denticulatus*
				dSgBigDes12/3	LC777171	B	*D. armatus*
			22 April, 2022	dSgBigDes4/1 (Type)	LC777172	H	*D. fragilis*
				dSgBigDes4/2	LC777173	O	*D. protuberans* var. *communioides*
18	Hasu-ike park (pond)	33.243961, 130.358293	17 Jun, 2022	dSgDes-Hasu1	LC777174	–	*D. opoliensis* (P.G.Richter) E.Hegewald
				dSgDes-Hasu2	LC777175	–	*D. protuberans* var. *communioides*
				dSgDes-Hasu3	LC777176	–	*D. pseudoprotuberans* Hegewald
19	Shinrin park (pond)	33.238661, 130.249086	3 July, 2017	dSg-moriDes	LC777177	N	*D. protuberans* var. *communioides*
20	Creek in Kawazoe-cho	33.229067, 130.274588	21 August, 2018	dSgDes-i	LC642118	B	*D. armatus*
21	Creek in Yokamati	33.201545, 130.266397	22 April, 2022	dSgDes-YO1-1	LC777178	B	*D. armatus*
				dSgDes-YO1-2	LC777179	B	*D. armatus*
				dSgDes-YO2-1	LC777180	E	*D. notatus*
				dSgDes-YO2-2	LC777181	B	*D. armatus*

Table 1. Location of sampling sites, and strains analyzed in this study.

dSgDes-Hasu1 was identified as *D. opoliensis* based on the ITS2 sequence from GeneBank and on the morphology of naviculoid or fusiform inner cells (Fig. 4D), which is consistent with that of the species described previously[27] however, the inner cells of the eight strains were not naviculoid or fusiform, but were bale-shaped (Fig. 4A). The density of cell surface microprojections (warts) was 20–30/μm^2 for cells of the eight strains (Fig. 4C), and 1–2/μm^2 for dSgDes-Hasu1 cells (Fig. 4E). The ridges on cells of the eight strains had small fold-like structures (height: 0.2 μm; Fig. 4A, B) that were not present on those of dSgDes-Hasu1 cells (Fig. 4D). These morphologies, i.e., cell morphology, number of dots, ridges, etc., were stable at different growth stages and under different culture conditions. CBCs were detected between dSgDes-Hasu1 and the eight strains for 2–3 locations, but not among the eight strains (Table 2, Fig. 5A). The sequence difference (%) between *D. opoliensis* (strain dSgDes-Hasu1) and the eight strains ranged from 2.11 to 3.38% (Table 2). The sequence difference (%) among strains with undetectable CBC ranged from 0.42 to 3.38% (Table 2).

Clade 3-1 mainly comprised species with asymmetric spines at both ends of their cells (Fig. 6A, arrows). However, the cells of strains dSgDes-0 and dSgKDesA3 that formed a clade with a high bootstrap value (99) and posterior probability (1.00) did not have asymmetric spines at either end, although there was a short spine at the ends of all cells (Fig. 6B, C, arrows). In addition, cells of strains dSgDes-0 and dSgKDesA3 had tube-like structures on their cell ridges (Fig. 6B, arrowheads). The cells of strain dSgKDesA3 had a large fold-like structure on each of their ridges as stable morphology, which was not observed for cells of other *Desmodesmus* species (Fig. 5B). The strains dSgDes-0 and dSgKDesA3 formed a clade with *D. lefevrei* and *D. pirkollei* (Fig. 3). No CBCs were detected between the strain dSgDes-0 and *D. lefevrei* or *D. pirkollei* (Table 3). However, one CBC each was detected between the strain dSgKDesA3 and strains of *D. lefevrei* or *D. pirkollei* (Table 3, Fig. 5B). The number of hemi-CBCs detected between strain dSgDes-0 and the other strains being compared was the lowest for dSgKDesA3 (Table 3). The sequence difference (%) between dSgDes-0 and dSgKDesA3 was 1.29%, a low value among closely related strains (Table 3).

Although strain dSgDes-eco12 was identified as *D. pirkollei* by Demura et al.[26], it was classified as *D. lefevrei* in this study owing to the absence of branched warts characteristic of *D. pirkollei*[16], and to the ITS2 sequence being monophyletic (sister position) with that of *D. lefevrei* with a high bootstrap value (94) and posterior probalility (0.85) (Fig. 3).

In the Clade 3-2, the lineage H (dSgDes-Nemu3, dSgBigDes4/1) and G (dSgHokuzan2, dSgDes-Hyo, dSgDes-Kami), which was identified as *D. intermedius*, formed a robust clade supported by a high bootstrap value (100) and posterior probalility (1.00) (Fig. 3). This clade was monophyletic with the lineage I (dSgKDes12/2, dSgKDes4/2) with a high bootstrap value (99) and posterior probalility (0.99) (Fig. 3). The cells of strains in the lineage H were similar to those of *D. intermedius* in the presence of spines at both ends, but differed from those of *D. intermedius* in the absence of a reticulate pattern on the cell surface (Fig. 7A–D). Compared to those of other *Desmodesmus* strains, cells in the lineage H were often crushed during pretreatment for SEM. The cells of strains in the lineage I had a reticular pattern (size 0.1–0.3 μm, Fig. 7E, F) of surface structure similar to those

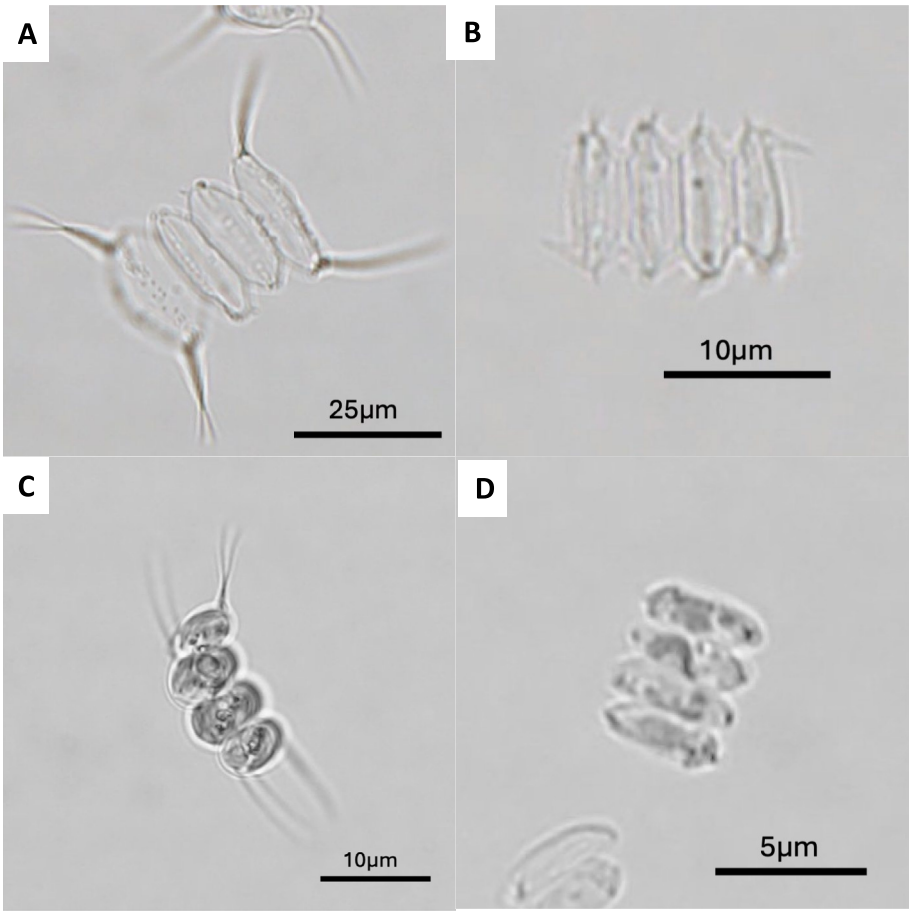

Figure 2. Light microscope photographs of the type species. (**A**) Strain dSgDes-ecoOc3 (*Desmodesmus notatus*). (**B**) Strain dSgKDesA3 (*D. lamellatus*). (**C**) Strain dSgBigDes4/1(*D. fragilis*). (**D**) Strain dSgKDes4/2 (*D. reticulatus*).

of *D. intermedius*; however, spines were absent (Fig. 7E), although fold-like structure were present at the outer edge of terminal cells (Fig. 7E, arrow). One or three CBCs were detected among the lineages G (*D. intermedius*), H, and I (Table 4, Fig. 5C, D). The sequence difference (%) among the three species ranged from 2.36 to 6.67%.

Descriptions of new taxa

Desmodesmus notatus Demura, sp. nov.

Holotype: TNS-AL-63142 in TNS (Department of Botany, National Museum of Nature and Science), Japan. This represents the dried algal material prepared from the strain dSgDes-ecoOc3 for SEM.

Ex-holotype strain: Strain dSgDes-ecoOc3, maintained in Demura Laboratory, Saga Algal Industry R&D Center, Saga City, Saga Prefecture, Japan. The strain was isolated from Hirano-yon-chome-ike, Saga City, Saga Prefecture, Japan (N33.299276, E130.293793).

DNA sequence: ITS2 (LC777154).

Description: coenobia size was 13.2 ± 1.2 μm × 19.9 ± 2.6 μm (Fig. 2A). Coenobium of four cells was observed with the outer cells bearing spines. The inner cells were bale-shaped. The size of spines was 11.9 ± 1.2 μm. Microprojections (warts) were present on the cell surface, at a density of 20–30/μm^2. Folds of height approximately 0.2 μm were present on the ridges of the cells. This species was monophyletic (sister taxon) with *D. opoliensis* in the phylogenetic tree constructed using ITS2 sequences; however, it can be distinguished from *D. opoliensis* by its bale-shaped inner cells, density of warts, ridges on the cells, and by the presence of CBC between both species.

Etymology: the specific epithet "notatus" is derived from the Latin word meaning "pattern of small dots".

Morphological keys that identify *D. notatus* and *D. opolienesis*.

1. Inner cell morphology: Bale-shaped.....*D. notatus*, Naviculoid or fusiform.....*D. opolienesis*.
2. Density of wall surface warts: High.....*D. notatus*, Low.....*D. opolienesis*.
3. Edges on the cells: Present.....*D. notatus*, Absent.....*D. opolienesis*.

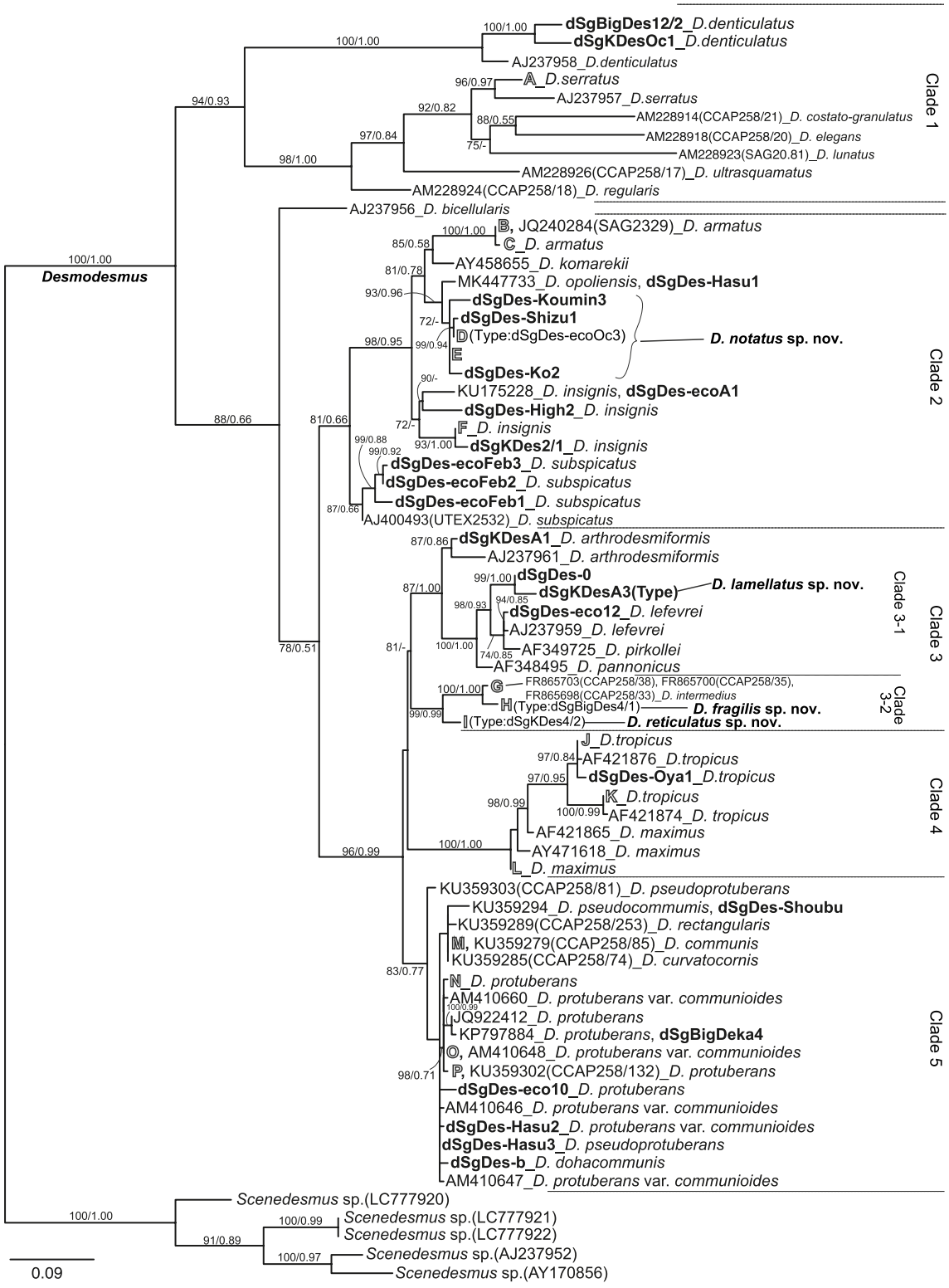

Figure 3. Phylogenetic tree constructed using maximum likelihood method using internal transcribed spacer 2 (ITS2) sequences. Bootstrap values greater than 70 are shown with posterior probability. Letters from A to P represent multiple culture strains that resulted in the same sequence (Table 1). Abbreviations for the public culture collections; CCAP, Culture Collection of Algae & Protozoa at the Scottish Association for Marine Science; SAG, The Culture Collection of Algae at the University of Göttingen; UTEX, Culture Collection of Algae at the University of Texas.

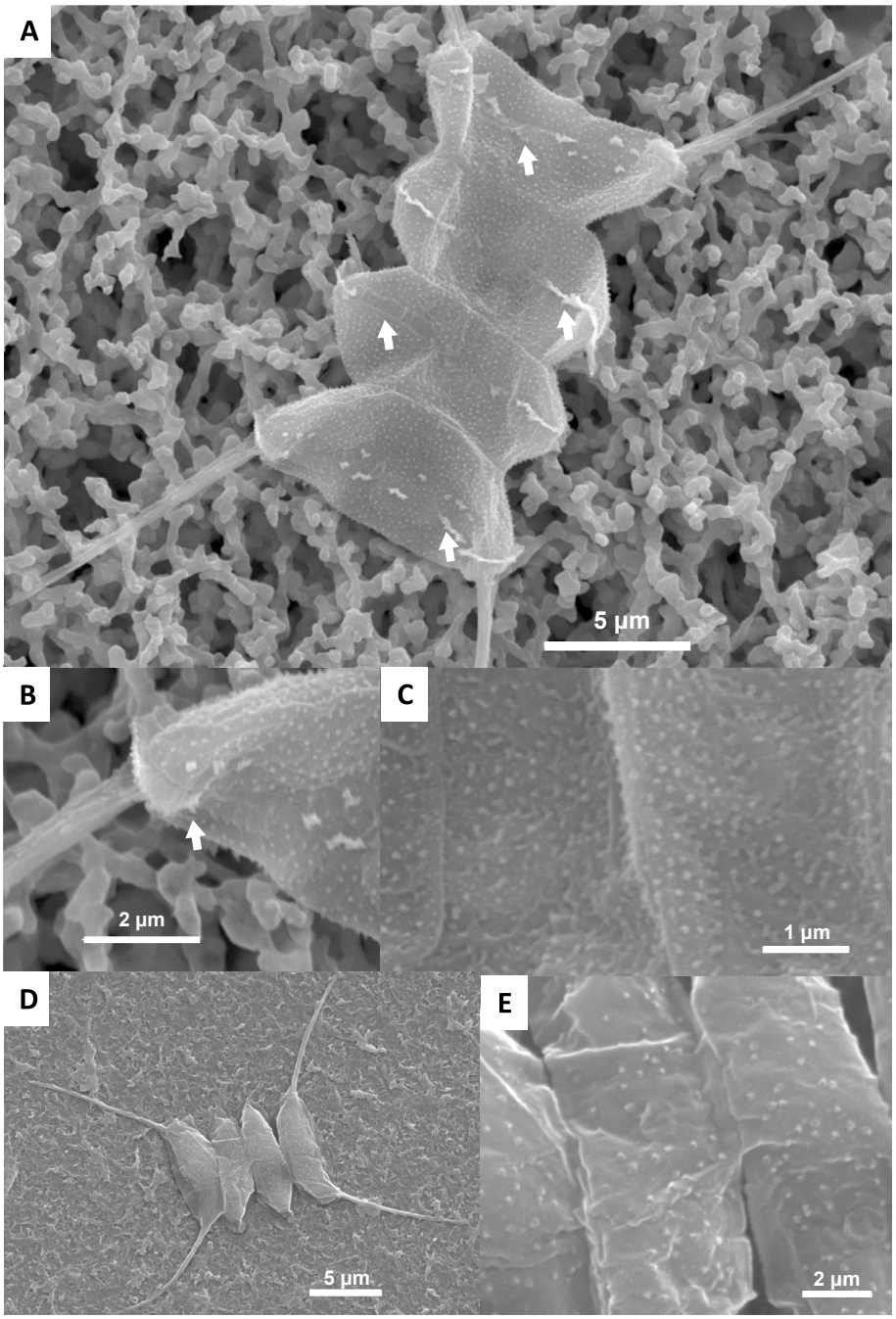

Figure 4. Scanning electron micrographs of the strain dSgDes-Oc3 (**A**,**B**,**C**), representing a type strain of *Desmodesmus notatus*; and the strain dSgDes-Hasu1 (**D**,**E**), identified as *D. opolinensis*. (**A**) Coenobium of the strain dSgDes-Oc3, with small folds on the ridges of each cell (indicated by arrows). (**B**) A part of the outer cell and small fold (indicated by arrows). (**C**) Magnified view of cell surface of the strain dSgDes-Oc3 showing a high density of microprojections (warts). (**D**) Coenobium of the strain dSgDes-Hasu1. (**E**) Magnified view of cell surface of the strain dSgDes-Hasu1 showing a low density of warts.

Desmodesmus lamellatus Demura, sp. nov.

Holotype: TNS-AL-63143 in TNS (Department of Botany, National Museum of Nature and Science), Japan. This represents the dried algal material prepared from the strain dSgKDesA3 for SEM.

Ex-holotype strain: Strain dSgKDesA3 maintained in Demura Laboratory, Saga Algal Industry R&D Center, Saga City, Saga Prefecture, Japan. The strain was isolated from Kannonji-tsutsumi, Saga City, Saga Prefecture, Japan (N33.327028, E130.298031).

DNA sequence: ITS2 (LC777107).

Description: coenobia size was $6.8 \pm 1.2\ \mu m \times 10.8 \pm 2.4\ \mu m$ (Fig. 2B). Coenobium of four cells was observed, which did not contain any long spines. The ridge of each cell was surrounded by a fold of approximately 1 µm

		1	2	3	4	5	6
1	dSgDes-Hasu1_*D. opoliensis*		2 (3.80)	2 (2.95)	2 (2.53)	2 (2.11)	3 (3.38)
D. notatus sp. nov							
2	dSgDes-Koumin3	2		0 (2.53)	0 (2.11)	0 (2.11)	0 (3.38)
3	dSgDes-Shizu1	2	4		0 (0.42)	0 (0.84)	0 (2.11)
4	Strains of phylogenetic lineage: D (contain type strain dSgDes-ecoOc3)	1	3	1		0 (0.84)	0 (1.69)
5	Strains of phylogenetic lineage: E	0	2	2	1		0 (1.27)
6	dSgDes-Ko2	1	4	3	2	2	

Table 2. Number of compensating base changes (CBCs) and sequence difference %(right upper) and hemi-CBCs (left lower) in the secondary structure of internal transcribed spacer 2 (ITS2) RNA among *D. opoliensis*-related strains.

length. This species exhibited a large fold-like structure on each of their ridges as a stable morphology, a feature not observed in cells of other *Desmodesmus* species. Tube-like structures were present within these folds. Short spines of 1–1.5 µm length were present at the ends of all cells. One CBC each was detected between this species and *D. lefevrei* and *D. pirkollei*.

Etymology: the specific epithet "lamellatus" is derived from the Latin word meaning "folded".

Morphological key that identifies *D. lamellatus* from *D. leferei* and *D. pirkollei*.

Large fold-like structure: Present……*D. lamellatus*, Absent……*D. leferei* and *D. pirkollei*.

Desmodesmus fragilis Demura, sp. nov.

Holotype: TNS-AL-63144 in TNS (Department of Botany, National Museum of Nature and Science), Japan. This represents the dried algal material prepared from the strain dSgBigDes4/1 for SEM.

Ex-holotype strain: Strain dSgBigDes4/1 maintained in Demura Laboratory, Saga Algal Industry R&D Center, Saga City, Saga Prefecture, Japan. The strain was isolated from Kose Park Creek, Saga City, Saga Prefecture, Japan (N33.255848, E130.325748).

DNA sequence: ITS2 (LC777172).

Description: coenobia size was 5.2 ± 0.8 µm × 13.0 ± 3.4 µm (Fig. 2C). Coenobium of four cells was observed, the outer cells of which contained spines. The size of the spines was 6.7 ± 0.7 µm. Except for small dots on the cell surface, no other structures were observed. This species was monophyletic (sister taxon) with *D. intermedius* in the phylogenetic tree constructed using ITS2 sequences. However, this species is clearly distinguished from *D. intermedius*, which has a reticulate structure on the cell surface. Two CBCs were detected between this species and *D. intermedius*.

Etymology: the specific epithet "fragilis" is derived from the Latin word meaning "fragile".

Desmodesmus reticulatus Demura, sp. nov.

Holotype: TNS-AL-63145 in TNS (Department of Botany, National Museum of Nature and Science), Japan. This represents the dried algal material prepared from the strain dSgKDes4/2 for SEM.

Ex-holotype strain: strain dSgKDes4/2 maintained in Demura Laboratory, Saga Algal Industry R&D Center, Saga City, Saga Prefecture, Japan. The strain was isolated from Kannonji-tsutsumi, Saga City, Saga Prefecture, Japan (N33.327028, E130.298031).

DNA sequence: ITS2 (LC777110).

Description: coenobia size was 3.5 ± 0.5 µm × 6.1 ± 0.9 µm (Fig. 2D). Coenobium of four cells was observed, which did not contain any spines. A reticulum of size 0.1–0.3 µm was present on the cell surface. Small fold-like structures of height 0.3–0.5 µm were present on the outer cells. This species was monophyletic (sister taxon) with *D. intermedius* and *D. fragilis* in the phylogenetic tree constructed using ITS2 sequences. However, this species is clearly distinguished from *D. intermedius* and *D. fragilis*, which have spines at the ends of the cell. Three CBCs were detected between this species and *D. intermedius* or *D. fragilis*.

Etymology: the specific epithet "reticulatus" is derived from the Latin word meaning "reticulated".

Morphological keys that identify *D. intermedius*, *D. fragilis* and *D. reticulatus*.

1. A reticulum structure on the cell surface: Present……*D. intermedius*, *D. reticulatus*, Absent……*D. fragilis*.
1a. Spines at the ends of the cell: Present…..*D. intermedius*, Absent…..*D. reticulatus*.
2. Small fold-like structures on the outer cells: Present……*D. reticulatus*, Absent……*D. intermedius* and *D. fragilis*.

Discussion

This study revealed the distribution of *Desmodesmus* species within the limits of Saga City (431.4 km^2). In the present study, *Desmodesmus* was present at more than half (55.3%) of the surveyed sites. The actual percentage is expected to be much higher because this survey was limited to 1 L of surface water, and many of the sites were surveyed only once. In addition, based on the habitat diversity of freshwater environments that includes ponds, wetlands, dams, artificial ponds, and creeks, and considering that *D. insignis* and *D. communis* can be found

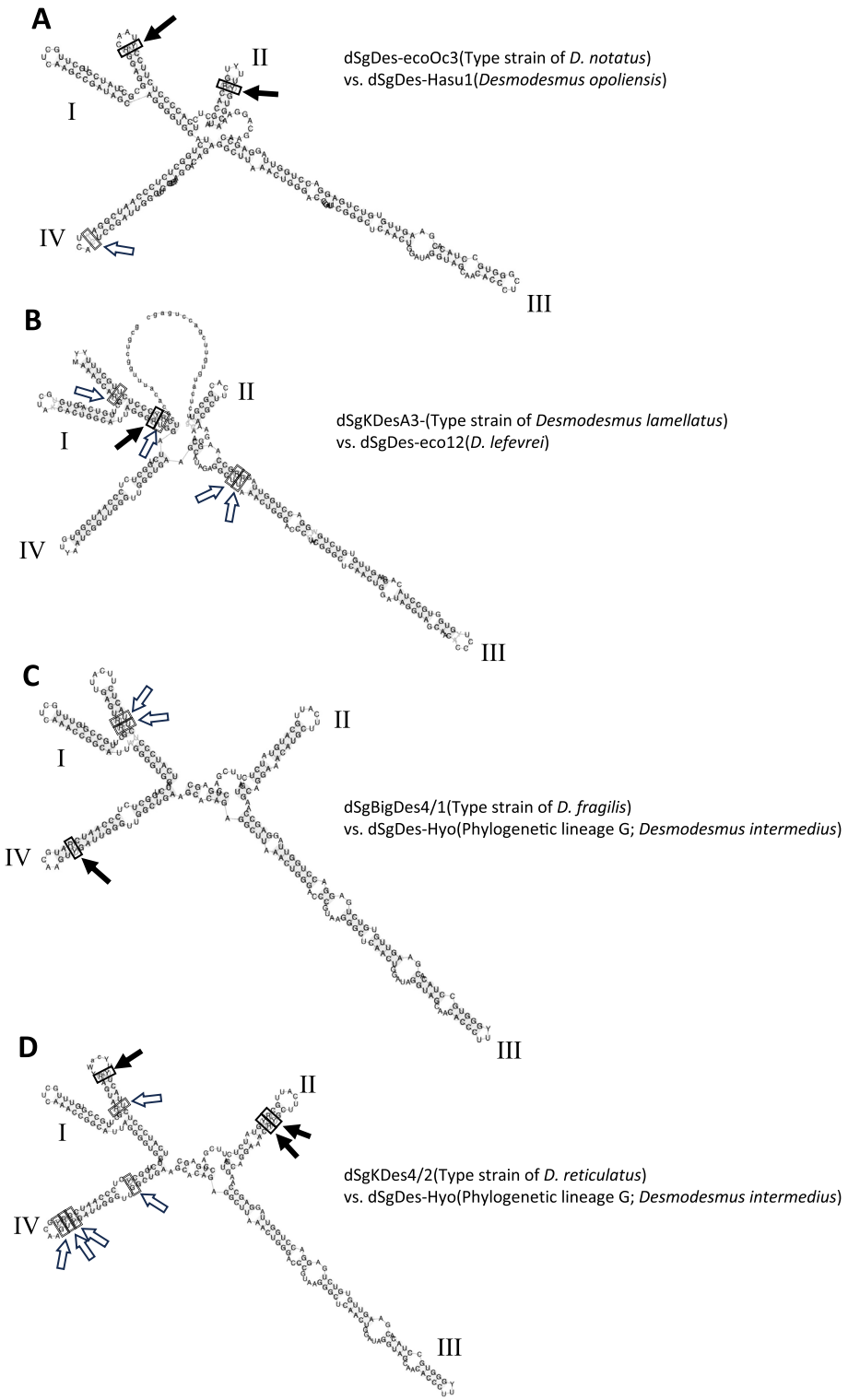

Figure 5. Secondary structures of the internal transcribed spacer 2 (ITS2) sequence. (**A**) secondary structure of the type strain dSgDes-ecoOc3 (*Desmodesmus notatus*) with high similarity to the ITS2 sequence of the *D. opoliensis* strain dSgDes-Hasu1. (**B**) secondary structure of the type strain dSgKDesA3 (*D. lamellatus*) with high similarity to the ITS2 sequence of the *D. lefevrei* strain dSgDes-eco12. (**C**) Secondary structure of the type strain dSgBigDes4/1 (*D. fragilis*) with high similarity to the ITS2 sequence of the *D. intermedius* strain dSgDes-Hyo. (**D**) Secondary structure of the type strain dSgKDes4/2 (*D. reticulatus*) with high similarity to the ITS2 sequence of the *D. intermedius* strain dSgDes-Hyo. I to IV indicated helix information. The black arrows in the secondary structure indicate the position of the compensating base changes (CBCs). The white arrows in the secondary structure indicate the position of the hemi-compensating base changes (hemi-CBCs).

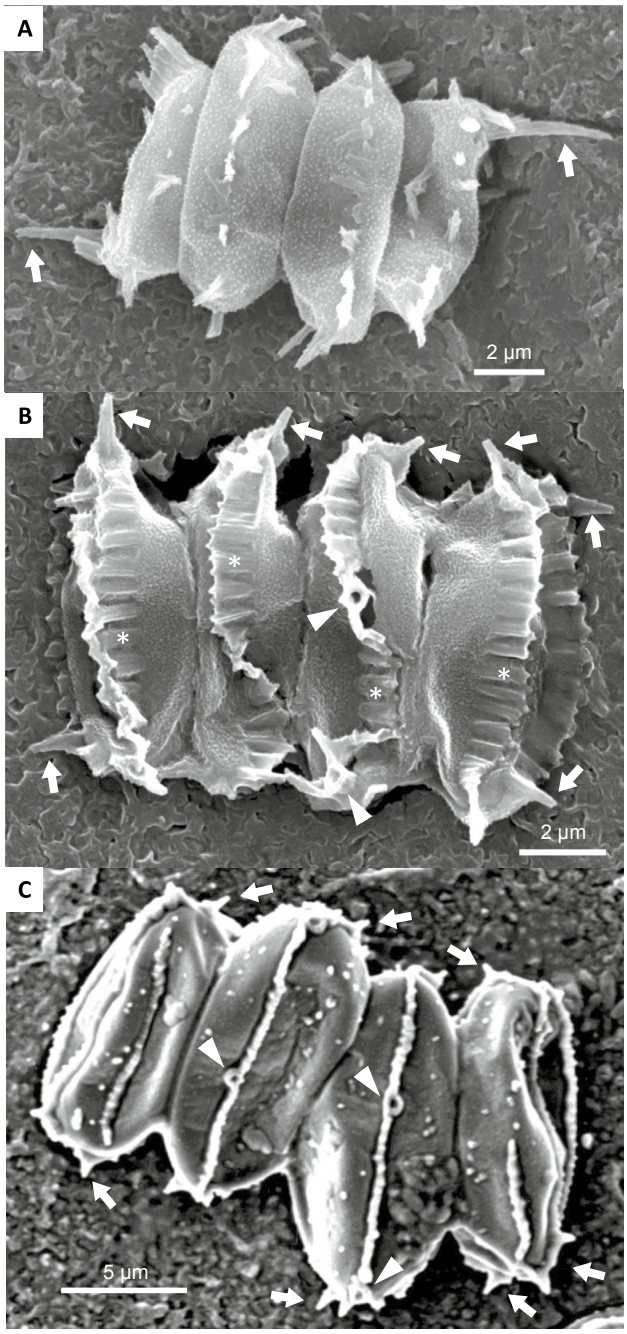

Figure 6. Scanning electron micrographs of the strain dSgDes-eco12 (**A**), identified as *Desmodesmus lefevrei*; the strain dSgKDesA3 (**B**), representing a type strain of *D. lamellatus*; and the strain dSgDes-0 (**C**). (**A**) Coenobium of the strain dSgDes-eco12 with asymmetric spines (arrows). (**B**) Coenobium of the strain dSgKDesA3 with a large fold-like structure on the ridges of the cells (asterisks), tube-like structures on the cell ridges (arrowheads), and short spines (arrows). (**C**) Coenobium of the strain dSgDes-0 with tube-like structures on the cell ridges (arrowheads), and short spines (arrows).

throughout the entire sampling period, *Desmodesmus* is considered a genus capable of adapting to a wide range of environmental conditions.

In the present study, *D. armatus* was identified at many sites, with no regular occurrence pattern being observed regarding sites, environments, or seasons. Vanormelingen et al.[28] reported genetic and morphological variations in *D. armatus* strains found in adjacent ponds in response to predation pressures and environmental differences. The fact that *D. armatus* was found in a highly variable environment in this study indicates its adaptive nature.

Of the species recorded in the present study, *D. communis* has the most documented occurrence worldwide; Hegewald & Braband[7] identified strains of this species from Europe, India, North America, South America, and

		1	2	3	4	5
1	dSgKDesA3_*D. lamellatus* sp. nov		0 (1.29)	1 (6.17)	1 (5.22)	1 (5.58)
2	dSgDes-0	1		0 (3.94)	0 (3.92)	0 (3.78)
3	dSgDes-eco12_*D. lefevrei*	4	5		0 (1.18)	0 (1.69)
4	AJ237959_*D. lefevrei*	3	4	1		0 (2.95)
5	AF349725_*D. pirkollei*	2	3	4	4	

Table 3. Number of compensating base changes (CBCs) and sequence difference % (right upper) and hemi-CBCs (left lower) in the secondary structure of internal transcribed spacer 2 (ITS2) RNA among *D. lamellatus*-related strains.

New Zealand. However, in the present study, *D. communis* was only found in one pond (Hirao-yon-chome-ike), where it was observed throughout the entire sampling period. Different species may exhibit varying distribution and diffusion patterns, and further studies are required to confirm this aspect.

In the present study, *D. notatus* was found to be genetically closely related to *D. opolinensis*; however, differences in cell morphology and surface structure, as well as in the presence of CBC between the two species, classified these as different species. The maximum sequence difference (3.38%) among *D. notatus* strains was identical to the sequence difference (3.38%) between the strain of *D. opolinensis* and strain dSgDes-Ko2 of *D. notatus*. Zou et al.[29] reported the presence of 1.1 to 21.7% interspecific sequence differences in ITS (probably a combined ITS1 and ITS2 sequence) among species of *Scenedesmus*, which is very closely related to *Desmodesmus*, indicating the difficulty of identifying species by sequence difference alone in the case of *Desmodesmus*.

Desmodesmus notatus was found in six ponds and creeks in Saga City, and showed high genetic diversity compared to other species; therefore, it is likely that, similar to *D. armatus*, it will be found in a wider area in the future.

The strains dSgDes-0 and dSgDes-eco12 were previously identified as *D. pirkollei* by Demura et al.[26]. However, in the present study, detailed morphological observations revealed that the strain dSgDes-eco12 was *D. lefevrei*. The strain dSgDes-0 could also be identified as *D. lamellatus* for the following two reasons: first, it formed a robust clade with the strain dSgKDesA3 (*D. lamellatus*), and second, no CBCs were identified between the strains dSgDes-0 and dSgKDesA3, and the number of hemi-CBCs was the lowest. However, large folds on the cell ridges, a morphological feature of *D. lamellatus*, were rarely observed for the dSgDes-0 strain, and additionally, no CBCs were identified between the dSgDes-0, *D. lefevrei*, and *D. pirkollei* strains. Undoubtedly, ITS2 is very effective in classifying *Desmodemus*; however, the limitations of analysis using ITS2 alone may be experienced. More detailed molecular phylogenetic analyses, with the addition of sequence data of ITS full length sequence (ITS1, the 5.8S rRNA gene, ITS2), chloroplast and mitochondrial genes are warranted for accurate identification of the dSgDes-0 strain in the future.

Desmodesmus fragilis cells exhibited an extremely limited surface structure, despite the presence of reticulate structures on the cell surfaces of the closely related *D. intermedius* and *D. reticulatus*. The role of "cell wall structures" of *Desmodesmus* is unknown, although some studies have been conducted on the "spines" that may influence buoyancy[30]. *Desmodesmus fragilis* cells are very fragile and are easily crushed compared with those of other *Desmodesmus* species, indicating that cell wall structures may play a role in protecting the cell structure.

The water bodies investigated in this study comprise a variety of environments that include ponds, wetlands, dams, artificial ponds, and creeks. Demura et al.[3] reported water quality surveys over a period of more than one year for the ponds Kannonji-tsutsumi and Hirao-yon-chome-ike, and found that the total nitrogen, total phosphorus, and organic matter concentrations for Hirao-yon-chome-ike exceeded those for Kannonji-tsutsumi, indicating that Hirao-yon-chome-ike is a highly eutrophic pond. In the present study, *D. serratus*, *D. arthrodesmiformis*, *D. lamellatus*, and *D. reticulatus* were detected from Kannonji-tsutsumi but not from Hirao-yon-chome-ike, whereas *D. communis*, *D. tropicus*, *D. notatus*, *D. subspcatus*, *D. protuberans*, and *D. lefervrei* were detected from Hirao-yon-chome-ike but not from Kannonji–tsutsumi, which suggests that the differences in species composition likely reflect differences in water quality.

Although the survey area in the present study was geographically limited, it is expected that extending this study will lead to a better understanding of the distribution of *Desmodesmus* throughout Japan and worldwide. To understand the true diversity of *Desmodesmus* and the evolutionary process of its distribution, it is necessary to investigate not only the water quality, but also the interrelationships with coexisting organisms, birds, wind, etc., all of which serve as vectors for its distribution.

Research on the utilization of microalgal biomass, not only for fuels but also for pharmaceuticals and food products, is developing extensively[31,32]. Microalgal biomass has also attracted attention from Sustainable Development Goals (SDGs) and carbon-neutrality perspectives[33]. *Desmodesmus* is a candidate microalga for algal biomass production owing to its high productivity and tolerance to various water qualities[8,9]. Although studies have reported the mass cultivation of *Desmodesmus*[34], actual commercialization of the genus has yet to begin. To use *Desmodesmus* for biomass production in the future, it is necessary to search for culture strains with properties such as rapid growth and ability to produce commercially useful substances. The present study revealed > 20 *Desmodesmus* species, exhibiting high genetic diversity, in a small area. The results of this study indicate that *Desmodesmus* possesses a variety of characteristics, and that useful culture strains can be established even in local area.

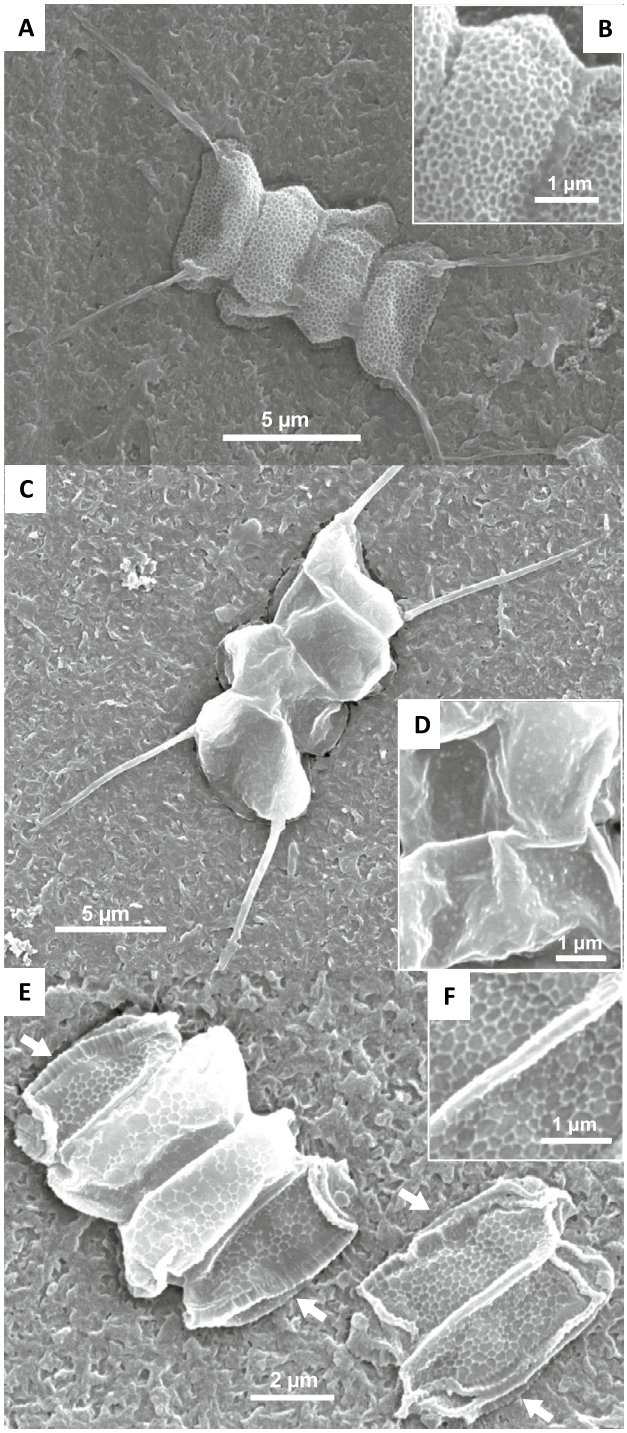

Figure 7. Scanning electron micrographs of the strain dSgDes-Hyo (**A**,**B**), identified as *Desmodesmus intermedius*; the strain dSgBigDes4/1 (**C**,**D**), representing a type strain of *D. fragilis*; and the strain dSgKDes4/2 (**E**,**F**), representing a type strain of *D. reticulatus*. (**A**) Coenobium of the strain dSgDes-Hyo. (**B**) A reticulate pattern on the cell surface of the strain dSgDes-Hyo. (**C**) Coenobium of the strain dSgBigDes4/1. (**D**) Cell surface of the strain dSgBigDes4/1. (**E**) Coenobium of the strain dSgKDes4/2 with small fold-like structures on the outer cells (arrows). (**F**) A reticulate pattern on the cell surface of the strain dSgKDes4/2.

Materials and methods

Distribution survey and strain establishment

Distribution surveys were conducted across 38 water bodies in Saga City, Saga Prefecture, Japan, from June 2017

Strains of phylogenetic lineage		1	2	3
1	**G**_*D. intermedius*		1 (2.36)	3 (6.67)
2	**H**_*D. fragilis* sp. nov	2		3 (6.27)
3	**I**_*D. reticulatus* sp. nov	5	5	

Table 4. Number of compensating base changes (CBCs) and sequence difference % (right upper) and hemi-CBCs (left lower) in the secondary structure of internal transcribed spacer 2 (ITS2) RNA among Clade 3–2 (Fig. 2) strains.

to March 2023 (Fig. 1, Table 1). Surface water samples (1 L) were collected in plastic bottles. These were brought back to the laboratory and placed at 10 °C for approximately 15 h. Thereafter, the sedimentary microalgae that settled at the bottom were observed under an inverted light microscope (CKX53; Olympus, Tokyo, Japan) to determine the presence or absence of *Desmodesmus*. When *Desmodesmus* was detected, the culture strain was established by the method described by Demura et al.[26]. In brief, the coenobium of *Desmodesmus* was isolated from the water sample using a micropipette[35] under an inverted light microscope (CKX53; Olympus). All established strains were maintained in 15 mL test tubes containing AF6 medium[36] at 25 °C under a 12 h light/12 h dark cycle using white fluorescent illumination (approximately 100 µmol photons m^{-2} s^{-1}). In total, 86 new strains were established in unialgal culture and in a clonal state (Table 1).

Observation by using scanning *electron* microscopy (SEM)

For SEM, 10 mL of the culture was centrifuged at 2000×*g* for 5 min (KUBOTA 3740, KUBOTA CO., Tokyo, Japan) at 25 °C. After removing the supernatant, the sedimented coenobia were resuspended in 500 µL of deionized water. Then, 5 µL of the resuspended mixture was pipetted onto a cellulose ester membrane filter (ADVANTEC, Tokyo, Japan) or RO membrane (ADVANTEC), following which 5 µL of 2% ionic liquid HILEM IL1000 (Hitachi High-Tech Corp., Tokyo, Japan) was added to it by pipetting. Thereafter, the samples were air dried for approximately 15 h at 25 °C, sputtered with platinum (JFC-1600; JEOL, Tokyo, Japan), and subjected to SEM using a JSM-6510 microscope (JEOL) with the following settings: voltage of 15 kV and working distance of "10".

Phylogenetic analysis and species identification

DNA was isolated from each sample and the ITS region, containing a part of the 18S ribosomal RNA gene, the ITS1, the 5.8S rRNA gene, the ITS2 and a part of the 28S rRNA gene, was amplified following the methods described by Demura et al.[26]. Since the study by An et al.[10], molecular phylogenetic and secondary structure analyses of *Desmodesmus* have been performed using only ITS2; therefore, the data that exist in GenBank are often ITS2-only. In addition, because taxonomic studies of *Desmodesmus* have been sufficiently successful with ITS2 sequences [e.g., Refs.[7,10,15,16]], ITS2-only sequences were used in the present study. A total of 127 sequences, including 86 new sequences and 46 database sequences from GenBank[37], were used for phylogenetic analysis of the ITS2 region (approximately 236 bp). Identical sequences are collectively presented as "lineages A to P" on the molecular phylogenetic tree.

Sequence alignment was performed using the analysis software MAFFT[38] with default settings, except for changing "L-INS-i" in "iterative refinement methods" in the "advanced settings" option. Aligned sequences were checked manually using AliView[39], and sites with gaps in more than half of the sequences were removed. Molecular phylogenetic trees were constructed using the maximum likelihood method and IQ-Tree[40]. The settings selected in "substitution model options" in IQ-Tree were "Substitution model Auto" and "FreeRate heterogeneity Yes [+R]." In addition, the bootstrap analysis standard and the number (100) of bootstrap alignments were chosen. The molecular phylogenetic tree was edited using FigureTree[41].

Bayesian phylogenetic inference was performed using PhyloBayes version 4.1[42] with the same dataset used for the maximum likelihood phylogeny estimation. Two independent Markov chain Monte Carlo (MCMC) chains were run under the general time-reversible (GTR) substitution model with discrete gamma-distributed among-site rate heterogeneity. Chain convergence and stationarity were assessed using the bpcomp program in PhyloBayes. The first 10,000 generations were discarded as burn-in, and trees were sampled every 10th generation from the subsequent 15,000 generations. The two chains were confirmed to have converged sufficiently (maxdiff < 0.1).

Identification of the species was performed by confirming that the ITS2 sequence of the strain established in this study was monophyletic with the database sequences of the described species, and that the morphological characteristics were consistent with those of the species reported in previous studies[5–7,10,15,16,23,26,27,43–56].

Comparison of RNA secondary structure

Some strains could not be identified by molecular phylogenetic analysis of ITS2 sequence or morphological observation using SEM; for these, the RNA secondary structures were compared using the CBC and hemi-CBC of related strains. A one-to-one structural comparison was performed using the software LocARNA[57] with default settings, and the CBC and hemi-CBC were visually counted. The ITS2 sequence diversity comparison (sequence difference, %) was calculated manually by comparing alignment sequences performed using LocARNA.

Data availability

The dataset used in this study in available upon reasonable request through the following email contact: st8148@cc.saga-u.ac.jp.

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Author contributions

This study was conducted by M. Demura.

Competing interests

The author declares no competing interests.

Additional information

Correspondence and requests for materials should be addressed to M.D.

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