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Life cycle and molecular phylogeny of *Vaucheria piloboloides* (Vaucheriales, Xanthophyceae) from Sado Island, Japan

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SUMMARY

The siphonous, yellow–green alga *Vaucheria piloboloides* (Vaucheriales, Xanthophyceae) is the type species of the section *Piloboloideae* and is widely reported worldwide. Despite its taxonomic importance and broad range distribution, its detailed life cycle and molecular phylogenetic position are unknown. In the present study, we established culture strains of *V. piloboloides* collected from Sado Island, Niigata Prefecture, Japan, and examined its morphology, life cycle and phylogenetic position based on *rbc*L sequences. The morphology of the gametangia and aplanosporangia (asexual sporangia) of our samples, along with their monoecious nature, resembled that of *V. piloboloides* from the Atlantic coast, including the type locality. The *rbc*L phylogenetic analysis demonstrated that our sample formed a clade with other *Piloboloideae* species, supporting the current sectional scheme of *Vaucheria*. Under culture conditions, our samples exhibited a monophasic life cycle. Development of gametangia was observed in any culture conditions, whereas development of aplanosporangia could be induced by low salinity (salinity = 24).

Key words: culture experiment, *Piloboloideae*, *rbc*L, sectional classification.

INTRODUCTION

Vaucheria de Candolle is a yellow–green alga with a siphonous thallus and oogamous reproduction. It belongs to the family Vaucheriaceae and includes 100 species ranging from freshwater to marine environments (Guiry & Guiry 2023). Most taxonomic works on *Vaucheria* have adopted a system of sections (Hoppaugh 1930; Ott & Hommersand 1974; Muralidhar *et al*. 2014; Huisman *et al*. 2024). The sectional classification is based on the morphology of sexual reproductive structures, and several sectional classification schemes have been proposed (Walz 1866; Rieth 1980; Entwisle 1988). Although the latest classification scheme by Entwisle (1988) has been shown to agree with molecular phylogeny (Andersen & Bailey 2002), over half of the *Vaucheria* species, including the type species of the sections, still lack molecular data.

The section *Piloboloideae* (Walz) Heering is characterized by multiporic and fusiform-cylindrical antheridia subtended by a wall-bound cavity (Entwisle 1988). *Vaucheria piloboloides* Thuret, the type species of the *Piloboloideae* (Walz 1866), was originally described from Normandy, France (Thuret 1854) and has been widely reported worldwide: Europe, Bermuda, Pakistan, India, Australia and Japan (Woronin 1869; Ernst 1904; Taylor & Bernatowicz 1952; Christensen 1987; Entwisle 1988; Yamamoto *et al*. 2013). Despite its taxonomic importance and broad distribution, its phylogenetic position and detailed life cycle are unknown. In 2023, we found a *Vaucheria* species resembling *V. piloboloides* in Sado Island, Niigata Prefecture, Japan, and successfully established culture strains. Leveraging this achievement, we carried out culture experiments and phylogenetic analyses to examine its reproduction, life cycle and phylogenetic position.

MATERIALS AND METHODS

Sample collection, culture experiments and morphological observation

The sampling was conducted in Lake Kamo, a brackish lake on Sado Island, Niigata Prefecture, Japan (see details in Results) on 12 November 2022, as well as 27 February, 1 April and 9 December 2023. Water temperature was monitored at the sampling sites from 21 July 2022 through 27 April 2023, using a MX2201 Pendant temperature logger (Onset Computer Corporation, Bourne, MA, USA); salinity was monitored from 28 April 2023 to 31 May 2023, using CTW-WF-L (JFE Advantech Co., Ltd, Nishinomiya, Japan).

To establish unialgal culture strains, small fragments were isolated from a single tiny tuft, which was collected at Akitsu (see details in Results) on 1 April 2023. These fragments

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were cultivated in a plastic Petri dish (90 × 20 mm) with full strength PESI medium (Tatewaki 1966). The medium was prepared with artificial sea water (MARINE ART SF-1; Tomita Pharmaceutical Co., Ltd, Naruto, Japan) with a salinity of 31. The established culture strains (strain codes: vau4, 5 and 6), were maintained in a 10°C short-day condition (SD; light : dark = 8:16 h) with dim light (5 μmol m^{-2} s^{-1} photon flux density), where the strains grew only vegetatively.

To examine the effects of photoperiod and temperature on the maturation of cultured thalli, the strains were subsequently transferred to five different photoperiod and temperature conditions with normal light (10–30 μmol m^{-2} s^{-1} photon flux density): 10°C SD, 10°C long-day (LD; light : dark = 16:8 h), 15°C LD, 15°C SD and 20°C LD conditions. In addition, because it has been reported that low salinity strongly induces the development of aplanosporangia (asexual sporangia) in *V. piloboloides* (Ernst 1904), the strains were cultured with low salinity PESI (salinity = 24) under the 10°C SD normal light condition. Culture medium was refreshed every 2 weeks.

Morphological observations were conducted using stereo (Stemi 305; Zeiss, Oberkochen, Germany), inverted (CKX53; Olympus, Hachioji, Japan) and compound light (BX51; Olympus) microscopy. Light micrographs were captured with an embedded camera under the stereo microscope, whereas a FLOYD-200 (Wraymer Inc., Osaka, Japan) and a BC-1200 camera (Sato Shouji Inc., Kawasaki, Japan) were used with the inverted and compound microscopes, respectively. Images of the field thalli and culture thalli, which grew in the 15°C LD condition, were analyzed using ImageJ, version 1.54d (NIH, Bethesda, MD, USA) (Schneider *et al.* 2012) for measurement.

Field thalli, as well as cultured thalli, were preserved as pressed specimens and deposited in the Herbarium of Faculty of Science, Hokkaido University (Sapporo, Japan) (SAP) and the National Museum of Nature and Science (Tsukuba, Japan) (TNS): SAP 115651 and TNS-AL 223720. In addition, part of the field thalli was preserved in a freezer or in formalin sea water for morphological observation. The established culture strains, vau4–6, were deposited in The Kobe University Macro-Algal Culture Collection (KU-MACC): KU-3509, KU-3510 and KU-3511.

Molecular phylogeny

To infer the phylogenetic position of the *Vaucheria* sp., a partial sequence of *rbc*L was newly generated. Total genomic DNA was extracted from the culture strain, vau4, using GenCheck® DNA Extraction Reagent (FASMAC, Atsugi, Japan) as described by Hoshino *et al.* (2020) and served as template for a PCR. We performed a nested PCR: the first PCR was carried out with the primers PRBF1 and RSPR, and 1 μl of the PCR product was used as template for the second PCR with PRBF1 and PRBR3A, as well as PRBF3 and RSPR (Kogame *et al.* 1999). The PCR was performed using KOD One® PCR Master-Mix (Toyobo, Osaka, Japan) and TaKaRa PCR Thermal Cycler Dice (Takara Bio, Kusatsu, Japan), and the PCR program consisted of a denaturation step at 98°C for 5 s, followed by 15 cycles (30 cycles for the second PCR) of 98°C for 10 s, 50°C for 5 s and 68°C for 5 s. Purification of the PCR products and cycle sequencing were carried out as described by Kogame *et al.* (2015) and Sanger sequencing was performed by a DNA sequencing service (FASMAC, Atsugi, Japan).

The obtained sequences were aligned with sequences retrieved from GenBank (see Supporting information, Table S1) using MAFFT, version 7.520 (Katoh & Standley 2013), and sites, except *rbc*L, were manually excluded. The alignment (70 operational taxonomic unit (OTUs), 1473 bp) was analyzed using IQ-TREE, version 2.2.2.7 (Chernomor *et al.* 2016; Kalyaanamoorthy *et al.* 2017; Minh *et al.* 2020) for maximum likelihood (ML) analysis and MrBayes, version 3.2.7 (Huelsenbeck & Ronquist 2001; Ronquist *et al.* 2012) for Bayesian inference (BI) analysis. The ML analysis by IQ-tree was performed with 1000 standard non-parametric bootstrap replicates and with the best nucleotide models inferred by the flag '-m MFP': TIM + F + I + R2 for the first, TNe + FQ + I for the second and TVM + F + I + G4 for the third codon. The BI analysis was performed with the partition and nucleotide models inferred by Kakusan4 (Tanabe 2011) based on the Bayesian information criterion (Schwarz 1978): GTR + G for the first and third and JC69 + G for the second codon. Monte Carlo Markov chains were run with the following parameters: 10000000 generations, two runs, four chains, sampling frequency of 100 and burn-in fraction of 0.25. Stationarity of the Monte Carlo Markov chain run was monitored using Tracer, version 1.7.1 (Rambaut *et al.* 2018).

RESULTS

Habit of field thalli and field conditions

The *Vaucheria* species was found at two sites in Lake Kamo: Akitsu (38°04′31.2″N, 138°25′23.9″E) and Ryotsu (38°04′21.8″N, 138°26′22.9″E). In February and April, the *Vaucheria* species covered the muddy lake bottom at a depth of approximately 2–3 m, forming green tufts (Fig. 1a), but was not found in November and December. The thallus exhibited oogonia and antheridia on the same filament, indicating their monoecy (Fig. 1b). Aplanosporangium was not observed. Water temperature was in the range 7–15°C (mean 12°C) in February to April and 9–18°C (mean 14.5°C) in November to December (see Supporting information, Fig. S1a). Salinity in May was 30.7 ± 0.68 (mean ± SD) at Akitsu and 31.2 ± 0.53 at Ryotsu (see Supporting information, Fig. S1b).

Effect of photoperiod and temperature under normal salinity conditions

Under the normal salinity (salinity = 31) and the normal light conditions, cultured thalli irregularly dichotomously branched and developed antheridia and oogonia in 1–3 weeks regardless of photoperiod and temperature (10°C LD, 10°C SD, 15°C LD, 15°C SD and 20°C LD) (Fig. 1c, d). The gametangia were produced terminally or laterally and the oogonia were typically adjacent to the antheridia (Fig. 1c–e). The vegetative filaments were 39.8–60.1 μm (mean 52.3 μm) in diameter and included numerous spindle-shaped chloroplasts with a pyrenoid (Fig. 1f). Preceding the development of antheridia,

the tips of the vegetative filaments turned grayish and tapered, and bore one to multiple protuberances (Fig. 1g–i). Eventually, they were separated from the vegetative filaments by a wall-bound cavity (Fig. 1j). The mature antheridia were cylindrical to fusiform, sometimes almost bifurcate, 182.1–333.1 μm (mean 248.2 μm) long, with one terminal pore and 0–3 lateral pores borne on cylindrical to conical protuberances (Fig. 1k and Table 1). The spermatozoids emerged through the terminal and lateral pores (Fig. 1l, m).

The oogonia developed as the tips of the vegetative filaments became globose and their cytoplasm accumulated towards the distal ends (Fig. 1n–p). Eventually, oogonia exhibited a lenticular oospore at their distal end, there was almost no protoplasmic mass in the proximal oogonial cavity (Fig. 1q) and it was unclear at which stage (Fig. 1m–q) fertilization occurred. The mature oogonia were claviform with an almost globose distal end, 357.3–564.3 μm (mean 443.0 μm) long (axial direction) and 144.1–223.0 μm (mean 189.8 μm) in diameter (radial direction) and the oospores were 88.1–127.5 μm (mean 105.7 μm) long and 126.3–164.6 μm (mean 147.1 μm) in diameter. The ratio of oospore diameter to oospore length was 1.4 on average, and the ratio of oogonia diameter to oospore diameter was 1.29 on average (Table 1).

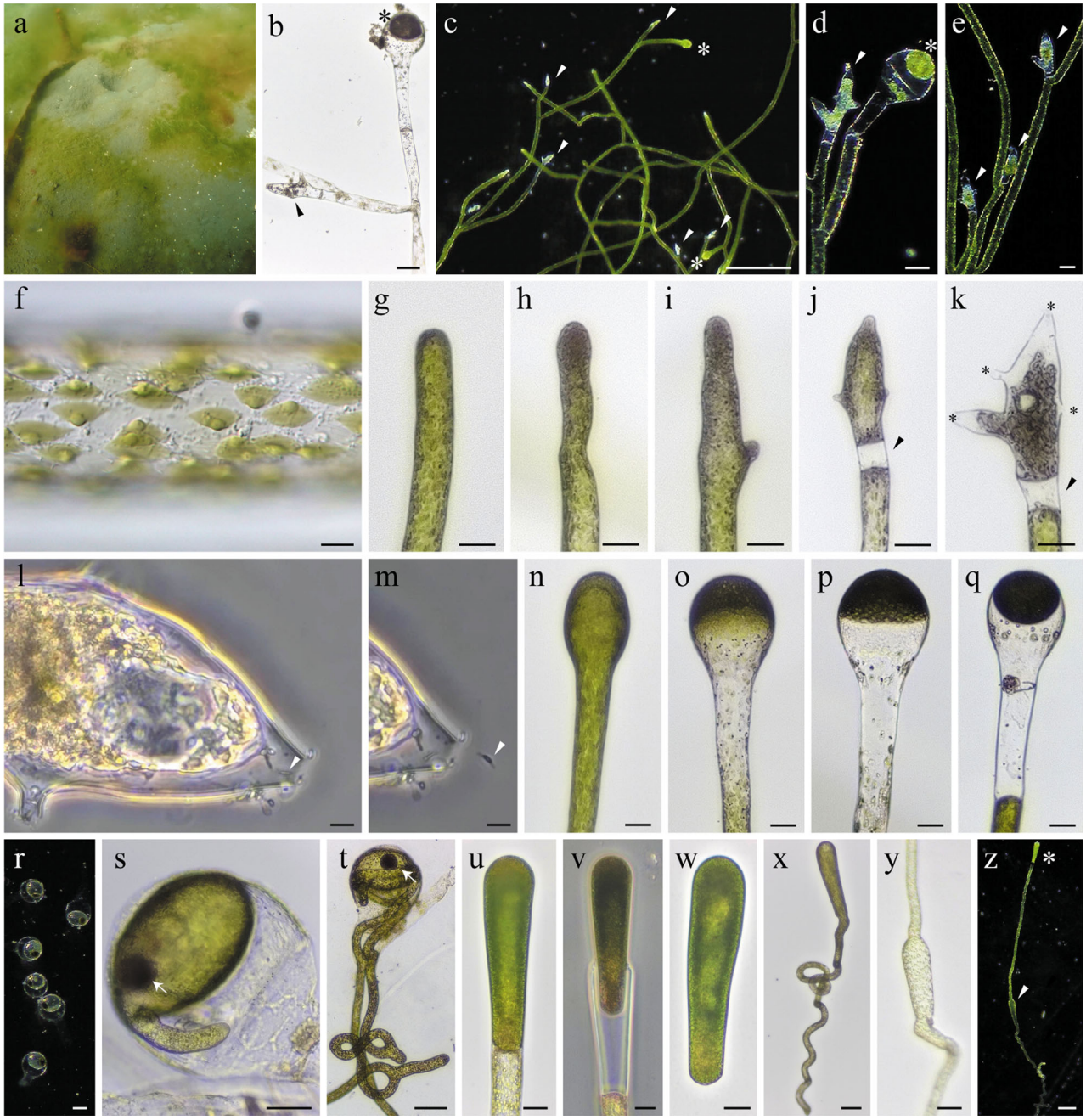

Fig. 1. Legend on next page.

The oogonia and oospores of the field thalli were also measured because they were in good condition (Table 1). These structures were found to be larger than those of the culture thalli, but similar in the ratios of oospore diameter to oospore length and oogonia diameter to oospore diameter (Table 1).

No instance of oospore discharge was observed, whereas the detachment of oogonia, along with their oospores, from the filaments was frequently observed (Fig. 1r). The base of the oogonia appeared to be very fragile, and the detachment of oogonia was easily induced by gently shaking the thallus in the culture medium using tweezers. The oospores within the detached oogonia were less pigmented and appeared more translucent compared to young oospores, and displayed brownish spherical structures of an unknown nature inside (Fig. 1r–t).

Germination of the oospores was observed only in 15°C LD. The oospores produced several germ tubes within the oogonia (Fig. 1s,t). The germling formed antheridia and oogonia, indicating that the species has a monophasic life cycle as is known in other *Vaucheria* species (Yamagishi 1993).

Aplanosporangia were observed only once in 10°C SD in vau4 when it did not have gametangia. The aplanosporangia were produced terminally, claviform, 401–436 μm long, with a maximum diameter of approximately 98 μm (Table 1), and deeply pigmented compared to vegetative filaments (Fig. 1u). Each sporangium released a single unflagellated spore (Fig. 1v) and the released spore (Fig. 1w) produced a sinuous germ tube from its base within 24 h (Fig. 1x). In several cases, aplanospores were observed extending germ tubes not only from the base, but also from the apex (Fig. 1y). The germlings transferred to 20°C LD developed antheridia and oogonia within 1 week.

Effect of low salinity

In the 10°C SD normal light condition, low salinity effectively induced the development of aplanosporangia. After 3 days to 4 weeks of cultivation at low salinity (salinity = 24), all strains developed numerous aplanosporangia. Furthermore, released aplanospores often developed new aplanosporangia immediately after germination (Fig. 1z). The morphology of the aplanosporangia was the same as that observed in vau4 under the normal salinity condition. The production of aplanosporangia continued for around 2 weeks, but gradually became space and transitioned to the production of gametangia. Aplanosporangia and gametangia could co-exist only during the transition.

*Rbc*L phylogeny

Our *rbc*L-based phylogenetic analysis supported the monophyly of the seven sections available in GenBank. Our *Vaucheria* sample (LC791471) was placed in the section *Piloboloideae* (Fig. 2) and closest to *V. litorea* C. Agardh (AF207527 and MW266050; *p*-distance = 4.1%).

DISCUSSION

The morphology of the antheridia (i.e. multiporic and fusiform-cylindrical antheridia subtended by a wall-bound cavity) indicates that our *Vaucheria* sample belongs to the section *Piloboloideae* (Entwisle 1988) and this sectional classification was supported by the *rbc*L phylogeny. Our sample, characterized by being monoecious with straight antheridia, claviform oogonia and oospores that do not completely fill the distal end of the oogonium, could be assigned to one of the following three marine species (Huisman *et al.* 2024): *V. piloboloides*, *Vaucheria bermudensis* Taylor & Bernatowicz, and *Vaucheria caloundrensis* Cribb. *Vaucheria piloboloides* is the oldest species among the three (Thuret 1854), and *V. bermudensis* and *V. caloundrensis* were described as species resembling *V. piloboloides* (Taylor & Bernatowicz 1952; Cribb 1960). *Vaucheria bermudensis* was originally described from Bermuda, North Atlantic (Taylor & Bernatowicz 1952). Compared

Fig. 1. Morphology of *Vaucheria piloboloides* from Sado Island, Japan. (a) *In situ* habit at the sampling site. *V. piloboloides* is covering the muddy lake bottom. (b) Formalin-fixed field thallus bearing an antheridium (arrowhead) and oogonium (asterisk). Observed using an inverted light microscope. (c) Cultured material showing irregularly dichotomous branching, antheridia (arrowheads), and oogonia (asterisks). Observed using a stereo light microscope. (d, e) Terminally or laterally bearing antheridia (arrowhead) and oogonium (asterisk). Observed using a stereo light microscope. (f) Surface view of a vegetative filament showing spindle-shaped chloroplasts with a pyrenoid. Observed using a compound light microscope. (g) Tip of vegetative branch. Observed using an inverted light microscope. (h–k) Development of antheridium. The tips of filaments gradually turn ashen gray, become dense in content, and develop projections (h, i). Upon maturation, the antheridia are separated from the vegetative filament by a wall-bound cavity (j, k; arrowheads) and release sperm through multiple openings at the tips of the projections (k; asterisks). Observed using an inverted light microscope. (l, m) Release of a sperm (arrowhead) from an antheridium. Observed using an inverted light microscope with phase contrast. (n–q) Development of an oogonium. The tips of filaments swell into a spherical shape, with cytoplasm accumulating at their apex (n–p). Eventually, a single lenticular oospore forms at the apex of the empty club-shaped the oogonium (q). Observed using an inverted light microscope. (r) Oogonia detached from filaments. Observed using a stereo light microscope. (s, t) Germination of an oospore. A germinated oospore (s) and its appearance five days later (t). Brownish spherical structures in oospores are indicated by arrows. Observed using an inverted light microscope. (u–y) Aplanosporangia, aplanospore, and germination of an aplanospore. Each aplanosporangium (u) releases a single aplanospore (v, w), which produces a germ tube from its base (x) and sometimes also from its apex (y). Observed using an inverted light microscope, and with phase contrast for (v). (z) A germling of an aplanospore (arrowhead) developing an aplanosporangium (asterisk). Observed using a stereo light microscope. Scale bars = 100 μm (b, d, e, r, t, x, y), 1 mm (c), 10 μm (f, l, m), 50 μm (g–k, n–q, s, u–w) and 250 μm (z).

Table 1. Morphological comparison between our material and the three species it most closely resembles

		Vaucheria from Sado Is.[†]	*V. piloboloides*	*V. bermudensis*[¶¶]	*V. caloundrensis*[††]
Vegetative filaments					
	Diameter (μm)	39.8–60.1 (52.3)[c]	40–60[‡]; 55–80[§]; 26–66[¶]; 60–112[§§]	18–51	26–60
Antheridia					
	Length (μm)	182.1–333.1 (248.2)[c]	160–200[¶]; 212–300[§§]	219–464 (331)	140–200
	Ratio of length to diameter of the supporting cell (wall-bound cavity)	0.2–2.4 (0.89)[c]	Usually 2.0[¶]; 0.7–1.3 (−1.9)[§§]	0.81	0.9–1.5, mostly 1.3
	The numbers of pores	1–4 (3.1)[c]	1 terminal and 1–4 (usually 2) lateral pores[§,§§]	1–3	Typically 2–3
Oogonia					
	Oogonia shape				
	Size of oogonia: length × diameter (μm)	357.3–564.3 (433.0) × 144.1–223 (189.8)[c]; 157.5–230.8 (195.3) diameter[f]	320–500 (380) × 140–210 (161)[¶]; 330–520 × 200–284[§§]	285–510 (388) × 116–153 (131)	280–440 × 210–266
	Oospore shape	Lenticular	Lenticular	Depressed-spherical to transversely elliptical	Compressed-globular
	Size of oospores: length × diameter (μm)	88.1–127.5 (105.7) × 126.3–164.6 (146.3)[c]; 92.6–138.0 (117.9) × 129.6–185.6 (159.2)[f]	90–100 × 150[‡]; 75–110 (85.7) × 105–145 (119.3)[¶]; 140–188 × 128–168[§§]; 130 diameter[¶¶]	90–136 (105) × 105–141 (120)	90–105 × 100–148
	Ratio of oospore diameter to oospore length	1.17–1.64 (1.4)[c]; 1.23–1.51 (1.37)[f]	1.5–1.67[‡]; 1.39[¶]	1.15	No data
	Ratio of oogonia diameter to oospore diameter	1.07–1.47 (1.29)[c]; 1.06–1.37 (1.22)[f]	1.35[¶]	1.08	1.6–2.2 (1.9)
Aplanosporangia					
	Shape	Claviform	Claviform[‡,§]	Claviform to obovoid	Claviform or obovate
	Length × diameter (μm)	401–436 × 98[c]	270 × 80[‡]; 200–400 (280) × 120[§]; 250 × 80[‡‡]	212–240 × 80–110	210 × 100
	Ratio of length to diameter	4.09–4.45[c]	3.38[‡]; 1.67–3.33[§]; 3.13[‡‡]	No data	2.1

[†]Present study. [c]Value from cultured thalli. [f]Value from field thalli.
[‡]Woronin (1869).
[§]Ernst (1904).
[¶]Taylor and Bernatowicz (1952).
[††]Cribb (1960).
[‡‡]Taylor (1960).
[§§]Entwisle (1988).
[¶¶]Yamamoto *et al.* (2013).
Mean values are shown in parenthesis.

to *V. piloboloides*, this species is reported to have oogonia with a smaller diameter and oospores that are oblate-spheroidal or somewhat compressed on the lower side (Taylor & Bernatowicz 1952) (Table 1). On the other hand, *V. caloundrensis* was originally described from Caloundra, Queensland, Australia, and was reported to be distinguishable from *V. piloboloides* by the larger diameter of its oogonia (Cribb 1960) (Table 1). Because of the differences in oogonium/oospore size, diagnostic characters among the three species have included the ratio of oospore diameter to oospore length and the ratio of oogonia diameter to oospore diameter (Taylor & Bernatowicz 1952; Cribb 1960; Rieth 1980). However, Entwisle (1988) argued that the above species distinctions are not clear-cut and referred his South Australian samples, which had large oogonia, to the oldest name *V. piloboloides* rather than to *V. caloundrensis* (Table 1).

Considering the characteristics of oogonia and oospores, our sample was most similar to *V. piloboloides* among the three species. In particular, the ratio of oospore diameter to length and the ratio of oospore diameter to oogonium diameter in our sample closely matched those of *V. piloboloides* (Table 1). In Japan, *V. piloboloides* was first reported from the Seto Inland Sea and the Japanese name 'Hiramame-fushinashimidoro' has been proposed for it (Yamamoto

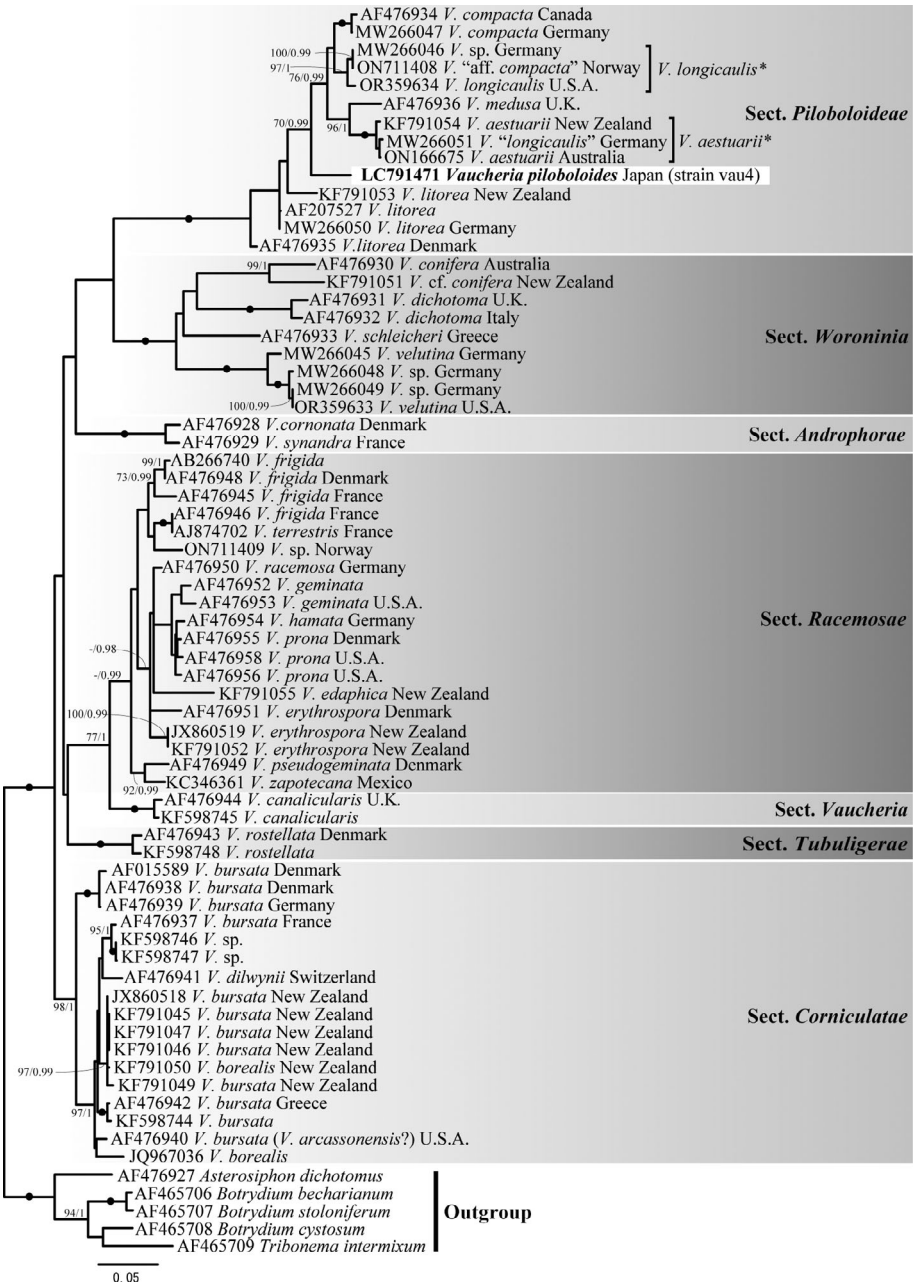

Fig. 2. Maximum likelihood tree based on *rbc*L sequences (248–1467 bp and 70 OTUs). Numbers on branches indicate bootstrap values from ML analysis (left) and posterior probabilities from BI analysis (right). The black circles indicate branches with full support (100/1.0). Only bootstrap values >70 and posterior probabilities >0.95 are shown. For *Vaucheria* sequences, geographic origin is given after the taxon name if known. Newly sequenced samples are indicated in bold. Regarding KF791051, KF791054 and KF791055, incorrect species names have been assigned in Muralidhar *et al.* (2014) and NCBI, but they have been corrected to the proper species names here (A. Muralidhar, 2023, personal communication). Species identification of *V. longicaulis* and *V. aestuarii* (asterisks) is according to Huisman *et al.* (2024). Scale bar = number of nucleotide substitutions per nucleotide site.

et al. 2013; Yamamoto 2017). The present study represents the second finding of the species in Japan. *Vaucheria piloboloides* from the Atlantic coast is characterized by three reproductive organs: oogonia, antheridia and aplanosporangia (Le Jolis 1863; Woronin 1869; Ernst 1904; Taylor and Bernatowicz 1952), but aplanosporangia have not been reported in Japanese specimens (Yamamoto *et al.* 2013). In the present study, aplanosporangia were not detected in the field material, but were successfully observed in culture. The morphology of the aplanosporangia closely resembled those of Atlantic *V. piloboloides* (Woronin 1869; Ernst 1904), except that they were longer (Table 1).

Besides *V. piloboloides*, some *Vaucheria* species have been reported to possess both sexual (gametangia) and asexual organs (aplanosporangia/zoosporangia) (Christensen 1987; Entwisle 1988). However, the effect of photoperiod and temperature on the reproductive mode has not been well examined, except by League and Greulach (1955). They examined *Vaucheria bursata* (as *Vaucheria sessilis* (Vaucher) De Candolle) and observed that gametangia and zoosporangia co-occurred under any culture conditions. Our samples resemble *V. bursata* in that gametangia formation is probably not photoperiodic, but they differ in the frequency of formation of asexual organs.

Development of the aplanosporangia in *V. piloboloides* had been intensively studied by Ernst (1904). He reported that, in the field populations of Naples, only one type of reproductive organ, either asexual or sexual, can be observed. Ernst examined freshly collected samples in spring and reported that aplanosporangia formation occurred for 4–6 weeks, and this was then replaced by a single, abundant production of

gametangia. These findings may be consistent with the observations of the present study and Yamamoto *et al*. (2013), where aplanosporangia were not found on field thalli having gametangia. In addition, Ernst (1904) brought completely sterile filed thalli to the laboratory and cultured them under various condition (light intensity, dryness, nutrient conditions and salinity) for several days, discovering that low salinity effectively induces the development of aplanosporangia without gametangia formation. His culture experiments were conducted at room temperature with natural light from windows, such that the specific photoperiod and temperature conditions are unclear. Nevertheless, his results were reproducible in the present study. Furthermore, our culture experiment, which continued for a longer period compared to that of Ernst (1904), demonstrated a similar developmental process of reproductive organs as observed in field populations by Ernst (1904), initially producing aplanosporangia, followed by formation of gametangia.

Vaucheria piloboloides probably reproduce either sexually or asexually depending on environmental conditions and developmental stage. Under culture conditions, low salinity could induce the aplanosporangia formation (Ernst 1904; present study). However, it is totally unclear whether the formation of aplanosporangia in the field also induced by low salinity. To understand the reproductive strategy of this species, it is necessary to investigate the correlation between reproductive modes and environmental factors in the field.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Water temperature and salinity of the sampling sites.

Table S1. List of *rbc*L sequences used for phylogenetic analyses.