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Virescentia asiatica sp. nov. (Batrachospermales, Rhodophyta), a new freshwater red alga from East Asia

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ABSTRACT
This study introduced a new species of freshwater red alga discovered in Taiwan, *Virescentia asiatica* sp. nov. Using genetic and morphological analyses, we determined its taxonomic status and phylogenetic relationship among other species of *Virescentia* (Sirodot) Necchi, D.C.Agostinho & M.L.Vis. While *Virescentia* taxonomy and species diversity have been extensively explored in Europe and the Americas, Asia remained less studied, with only one new species described in China. This study was the first record of *Virescentia* in Taiwan and confirmed it as a new species, *Virescentia asiatica* sp. nov. The investigation was based on comparing morphological characteristics and genetic markers *rbc*L and COI-5P in collections from around the world. Molecular evidence showed what we describe as *V. asiatica* sp. nov. forming a well-supported clade with four previously unidentified *Virescentia* sequences from Japan, and conspecific with at least three of these. We considered it likely that *V. asiatica* sp. nov. inhabited a wider geographic range in East Asia, including other regions besides Taiwan and Japan. Morphologically, *Virescentia asiatica* sp. nov. was distinct only in habit, differing from other *Virescentia* species in having an apparently diminutive thallus and curved branching. This study enhanced our understanding of the classification and phylogenetic relationships within *Virescentia*, contributing to the knowledge of freshwater red algae biodiversity in East Asia.

KEY WORDS
Taiwan,
freshwater red algae,
morphology,
*rbc*L,
COI-5P,
new species.

RÉSUMÉ

Virescentia asiatica *sp. nov. (Batrachospermales, Rhodophyta), une nouvelle algue rouge d'eau douce d'Asie de l'Est.*

Cette étude présente une nouvelle espèce d'algue rouge d'eau douce découverte à Taïwan, *Virescentia asiatica* sp. nov. À l'aide d'analyses génétiques et morphologiques, nous avons déterminé son statut taxonomique et sa relation phylogénétique avec d'autres espèces de *Virescentia* (Sirodot) Necchi, D.C.Agostinho & M.L.Vis. Alors que la taxonomie et la diversité des espèces de *Virescentia* ont été largement explorées en Europe et en Amérique, l'Asie est restée moins étudiée, avec seulement une nouvelle espèce décrite en Chine. Cette étude constitue le premier signalement de *Virescentia* à Taïwan et confirme qu'il s'agit d'une nouvelle espèce, *Virescentia asiatica* sp. nov. L'enquête s'est basée sur la comparaison des caractéristiques morphologiques et des marqueurs génétiques *rbc*L et COI-5P dans des collections du monde entier. Les preuves moléculaires ont montré que ce que nous décrivons comme *V. asiatica* sp. nov. forme un clade bien soutenu avec quatre séquences de *Virescentia* précédemment non identifiées du Japon, et est conspécifique avec au moins trois d'entre elles. Nous considérons qu'il est probable que *V. asiatica* sp. nov. occupe une aire de répartition géographique plus large en Asie de l'Est, comprenant d'autres régions que Taiwan et le Japon. Morphologiquement, *Virescentia asiatica* sp. nov. n'était distincte que par son port, différant des autres espèces de *Virescentia* par un thalle apparent de petite taille et une ramification courbée. Cette étude a permis d'améliorer notre compréhension de la classification et des relations phylogénétiques au sein de *Virescentia*, contribuant ainsi à la connaissance de la biodiversité des algues rouges d'eau douce en Asie de l'Est.

MOTS CLÉS
Taiwan,
algue rouge d'eau douce,
morphologie,
*rbc*L,
COI-5P,
espèce nouvelle.

INTRODUCTION

Red macroalgae, comprising over 7000 described species worldwide, are essential primary producers in aquatic ecosystems (Guiry & Guiry 2023). Although the majority of them thrive in marine environments, around 5% inhabit freshwater habitats, and three orders, Balbianiales, Batrachospermales, and Thoreales, are found exclusively in freshwater environments (Kumano 2002; Sheath & Vis 2015; Vis & Necchi 2021). The order Batrachospermales is the most species-rich group accounting for two-thirds of all freshwater members (Vis & Necchi 2021) and the paraphyletic genus *Batrachospermum sensu lato* historically underwent many taxonomic revisions. Based on various molecular and morphological studies, most of the sections were combined and/or raised to distinct genera of Batrachospermales, as follows: *Acarposporophycos* Necchi (section *Acarposporophytum*; Necchi *et al.* 2019a), *Atrophycus* Necchi & Rossignolo (section *Setacea*; Rossignolo & Necchi 2016), *Kumanoa* Entwisle, M.L.Vis, W.B.Chiasson, Necchi & A.R.Sherwood (sections *Contorta* and *Hybrida*; Entwisle *et al.* 2009), *Montagnia* Necchi, M.L.Vis & A.S.Garcia (section *Macrospora*; Necchi *et al.* 2019b), *Paludicola* Necchi & M.L.Vis (section *Turfosa*; Vis *et al.* 2020), *Sheathia* Salomaki & M.L.Vis (section *Helminthoidea*; Salomaki *et al.* 2014), *Virescentia* (Sirodot) Necchi, D.C.Agostinho & M.L.Vis (section *Virescentia*; Sheath *et al.* 1994; Necchi *et al.* 2018), and *Visia* Necchi (section *Aristata*; Necchi *et al.* 2019a).

The genus *Virescentia* is distinguished from other genera of Batrachospermales by several key features, including well-developed whorls, carpogonial branches composed of short cells that are well-differentiated from the fascicle cells, generally straight carpogonial branches that occasionally exhibit slight curvature, carpogonia with long and stalked trichogynes, and large, densely arranged, axial carposporophytes inserted centrally within the whorl (Necchi *et al.* 2018; Vis & Necchi 2021). To differentiate species in *Virescentia*, the following features are used (Necchi *et al.* 2018; Vis & Necchi 2021; Fang *et al.* 2021): the shape of fascicles composing the whorl (curved, audouinelloid or straight), the shape of carpogonial branches (slightly curved or straight), the length of carpogonia (short or long), the abundance of secondary fascicles in the internode, the presence of special expansion cell in the fascicles, and biogeographic range. To date, seven species of *Virescentia* are recognized (Agostinho & Necchi 2014; Necchi *et al.* 2018; Vis & Necchi 2021; Fang *et al.* 2021): *V. crispata* (Kumano & Ratnasabapathy) Necchi, Agostinho & Vis, *V. guangxiensis* K.P.Fang, F.R.Nan & S.L.Xie, *V. gulbenkiana* (Reis) Necchi, Agostinho & Vis, *V. helminthosa* (Bory) Necchi, Agostinho & Vis (type species), *V. viride-americana* Necchi, Agostinho & Vis, *V. viride-brasiliensis* (Necchi & Agostinho) Necchi, Vis & Agostinho, and *V. vogesiaca* (Schultz ex Skuja) Necchi, Agostinho & Vis.

A lineage reported from Japan remains taxonomically unresolved. Analysis of *rbc*L sequence data by Hanyuda *et al.* (2004) revealed five *rbc*L haplotypes within specimens collected from Japan, reported as *Batrachospermum helminthosum* Bory (synonym: *V. helminthosa*). However, it was suggested that four of the five Japanese haplotypes represent a new *Virescentia* species, and the fifth one as an additional, different species, thus necessitating further taxonomic investigation (Necchi *et al.* 2018; Vis & Necchi 2021). Additionally, Fang *et al.* (2021) indicated that one of the five Japanese haplotypes shows a high similarity with *V. guangxiensis*; however, the lack of voucher specimens of the Japanese material reported by Hanyuda *et al.* (2004) for morphological observation impeded formal description of the new species.

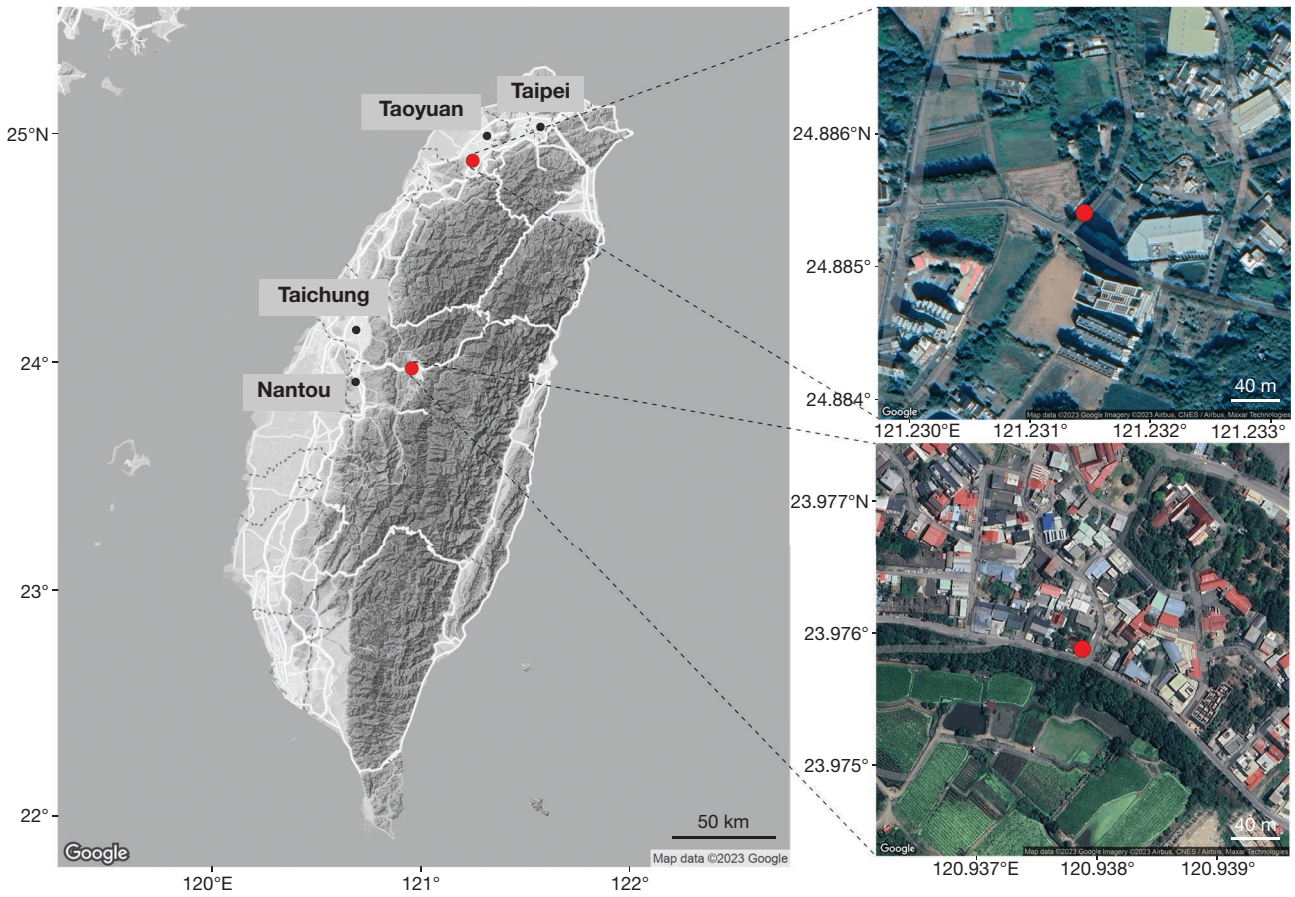

FIG. 1. — Sampling locations for the two *Virescentia asiatica* sp. nov. specimens collected in Taiwan and reported in this study. The two sampling sites are denoted by **red dots**.

Taiwan is a geographically interesting connection between the Philippines, China, and Japan, but had been little explored for freshwater red algal diversity. A comprehensive analysis of plastid *rbc*L barcodes of freshwater red macroalgae in Taiwan unveiled a significant number of previously undiscovered species (Zhan *et al.* 2021), leading to the identification of two new *Kumanoa* species (Aristya *et al.* 2022). This research has also revealed an undescribed *Virescentia* species closely related to the four "*B. helminthosum*" *rbc*L haplotypes from Japan, which are awaiting taxonomic revision as advocated by Necchi *et al.* (2018) and Vis & Necchi (2021).

To officially designate the newly discovered *Virescentia* species found by Zhan *et al.* (2021) as a distinct entity, we used two main approaches. Firstly, we rigorously analysed the phylogenetic relationship among the recently found *Virescentia* specimen (Zhan *et al.* 2021) and other *Virescentia* species, employing both the plastid *rbc*L and mitochondrial COI-5P markers. COI-5P is widely used as a barcode marker in red algae, hence our sequencing will contribute to the freshwater red macroalgae barcode database. Secondly, we conducted a comprehensive morphological examination of the specimens, making detailed comparisons with other recognized *Virescentia* species. This research enhances our knowledge of the diversity and geographical distribution of freshwater red macroalgae in Taiwan and East Asia.

MATERIAL AND METHODS

SAMPLE COLLECTION

Gametophytic specimens were collected from an irrigation ditch in Taoyuan, northern Taiwan on March 17, 2015 (voucher: HAST-146271; Fig. 1; Appendix 1). This material was found growing with another freshwater alga, *Sheathia dispersa* Necchi, J.A.West, E.K.Ganesan & S.K.Rai. A second population was found in Puli Township, Nantou, central Taiwan on February 4, 2018 (voucher: THU.569b; Fig. 1; Appendix 1). This second material collection was of '*Chantransia*' (i.e., sporophytic stage) mixed with *S. dispersa* and was confirmed as the same unidentified *Virescentia* species found in Taoyuan, northern Taiwan, through Sanger sequencing of *rbc*L. During our collection at the irrigation ditch in Taoyuan, we also detected the presence of other freshwater red algae (e.g. *S. dispersa* and *Montagnia macrospora* (Montagne) Necchi, M.L.Vis & A.S.Garcia that were common in aquarium shops, the latter being an invasive species; Zhan *et al.* 2021; Fontana *et al.* 2022). Upon collection, a small portion of the thalli was preserved in 95% ethanol or silica gel for subsequent molecular analyses, while the rest was preserved in 10%-15% formalin for further morphological observations. The remaining materials were preserved at the Herbarium of Academia Sinica, Taiwan (HAST), Taiwan. The pH, nutrients, and light

TABLE 1. — The pairwise uncorrected genetic distance (%) based on *rbc*L between *Virescentia asiatica* sp. nov. from Taiwan and its phylogenetically related samples from Japan.

	Virescentia asiatica **Taiwan (MH835533)**	***Virescentia asiatica*** **Japan (AB114644)**	***Virescentia asiatica*** **Japan (AB114645)**	***Virescentia asiatica*** **Japan (AB114642)**	***Virescentia* sp.** **Japan (AB114643)**
Virescentia asiatica Taiwan (MH835533)	0%	–	–	–	–
Virescentia asiatica Japan (AB114644)	0%	0%	–	–	–
Virescentia asiatica Japan (AB114645)	0.14%	0.14%	0%	–	–
Virescentia asiatica Japan (AB114642)	1.37%	1.37%	1.51%	0%	–
Virescentia sp. Japan (AB114643)	1.78%	1.78%	1.93%	2.05%	0%

conditions are reported in Fontana *et al.* (2022). The map showing sampling locations (Fig. 1) was generated using the ggmap package (Kahle & Wickham 2013) in R version 4.2.3 (R Core Team 2021).

Morphological observation and analysis

Morphological analyses of *Virescentia* were conducted with a focus on characteristics considered to be diagnostic in previous taxonomic research (Necchi *et al.* 2018; Vis & Necchi 2021) such as gross morphology, sexuality (monoicous, dioicous, or polyoicous), origin of carpogonial branches, size of carpogonia, shape and number of cells in primary fascicles, density of secondary fascicles, and shape and size of carposporangia. For morphological observations, materials were stained with an aceto-haematoxylin-chloral hydrate solution (Wittmann 1965). The images were captured using a Canon EOS600D digital camera mounted on a Leica DM 750 microscope (Leica Microsystems, Wetzlar, Germany).

Sequence amplification and analysis

For DNA extraction, tissue dried in silica gel was homogenized by grinding in liquid nitrogen with a mortar and pestle. Total DNA was extracted using the commercial Quick-DNAT-MPlant/Seed Miniprep Kit (Zymo Research, California, United States), according to the manufacturer's protocols. For gene-specific amplifications, combinations of primers were used as follows: for plastid *rbc*L, primers F160 + R753, F492 + R1150, and F993 + Rsst (Freshwater & Rueness 1994; Vis *et al.* 1998; Geraldino *et al.* 2006); and for mitochondrial COI-5P, primers cox143F + C880R (Yang *et al.* 2008) and GazF1 + GazR1 (Saunders 2005). The PCR amplification was performed according to the cycling conditions described in Lin *et al.* (2001) for *rbc*L and in Saunders & Moore (2013) for COI-5P. The Sanger sequencing of the PCR products was carried out using Applied Biosystems 3730xl DNA Analyzer at Mission Biotech and Genomics Company (Taipei, Taiwan). The newly generated *rbc*L and COI-5P sequences were deposited in GenBank (Appendix 1). For subsequent phylogenetic analyses, we retrieved 50 *rbc*L and 59 COI-5P sequences from GenBank that include all known sequences from *Virescentia* species as ingroup and three taxa as outgroups (Appendix 1).

Phylogenetic analyses

A concatenated alignment (*rbc*L + COI-5P) was produced using MUSCLE v3.8.31 (Edgar 2004) in MEGA version X (Kumar *et al.* 2018), resulting in an aligned concatenated sequence matrix with a total of 71 sequences and 1315 bp (730 bp for *rbc*L and 585 bp for COI-5P). The sequence matrix was then used as input for subsequent phylogenetic analyses. The phylogenetic inference was conducted with two methods: maximum likelihood (ML) and Bayesian inference (BI). Prior to ML analysis, we partitioned our data by gene and codon position to select the best nucleotide substitution model. The ML phylogenetic tree was then inferred using IQ-Tree v1.6.10 (Nguyen *et al.* 2015; Hoang *et al.* 2017), with '-s TEST-B 1000' parameters. Statistical support for each node of the ML tree was determined based on 1000 replications of bootstrap analysis. The BI phylogenetic tree was reconstructed using MrBayes v3.2.6 (Ronquist *et al.* 2012), with 'lset nst = 6 rates = invgamma' parameters. Statistical support for each node of the BI tree was based on two Markov Chain Monte Carlo (MCMC) runs (nchains = 4) for 20 000 000 generations with sampling every 20 000 generations. The posterior probability for each node was summarized from the 50% post- burn-in trees after discarding those below the convergence (i.e., the average standard deviation of split frequencies across two runs less than 0.01).

Species delimitation analysis and p-distance estimation

Estimation of p-distances and single-locus algorithmic species delimitation analyses were performed for each locus (i.e., *rbc*L and COI-5P) separately. Two alignment matrices were produced: *rbc*L, comprised of 51 sequences and 730 bp; and COI-5P, comprised of 60 sequences and 585 bp. In the two alignment matrices mentioned above, gaps were trimmed to prevent systematic errors in phylogenetic inference caused by missing data, leading to a shorter sequence length in the final dataset. The p-distances were computed using MEGA version X (Kumar *et al.* 2018). For the single-locus species delimitation, we determined the species barcode gap using the Automated Barcode Gap Discovery method (ABGD; Puillandre *et al.* 2012). Analytical procedures were done according to the descriptions in Liu *et al.* (2015). ABGD was performed

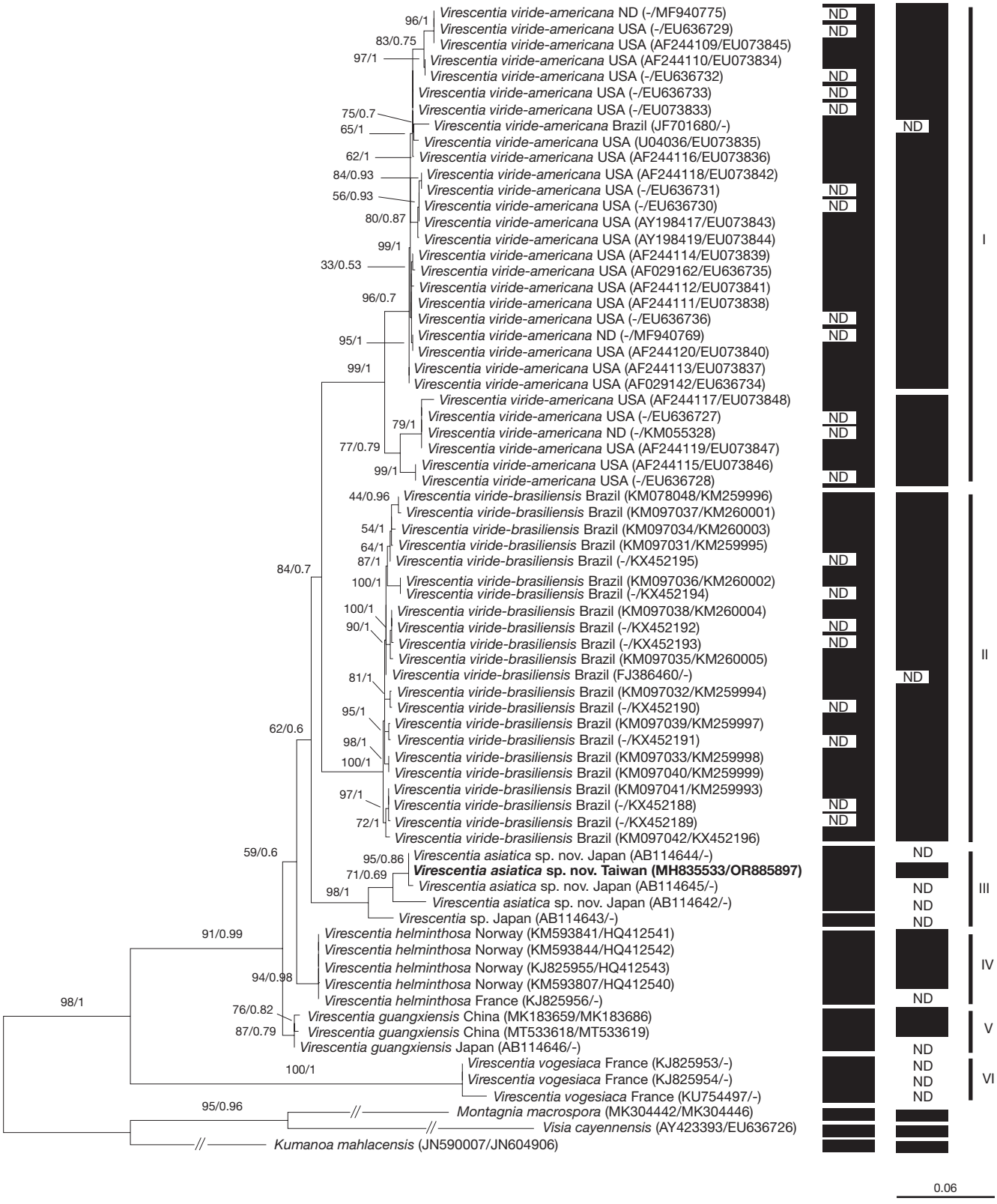

FIG. 2. — Maximum likelihood (ML) tree of *Virescentia* (Sirodot) Necchi, D.C.Agostinho & M.L.Vis inferred from the concatenation of *rbc*L+COI-5P (**left panel**), and results of ABGD species delimitation analysis based on *rbc*L (**left side of right panel**) and COI-5P (**right side of right panel**). Sequences produced from this study are highlighted in **bold**. Nodal support presented as percentage bootstrap from ML and as posterior probabilities from Bayesian Inference (BI), respectively. Abbreviations: **ABGD**, Automatic Barcode Gap Discovery; **ND**, not determined.

using the online analysis web tool (https://bioinfo.mnhn.fr/abi/public/abgd/abgdweb.html). This method determines the putative species number according to the gap threshold between intraspecific and interspecific genetic distance. The pairwise genetic distance was calculated using Kimura-2 parameters and the relative gap width (X) was set to 1. Other settings were left as defaults. Only the results from the initial partition were considered, as suggested by Kekkonen & Hebert (2014).

RESULT

Phylogenetic analyses and species delimitation

The concatenated gene tree topologies, derived from both ML and BI methods, exhibited a high degree of congruence and, consequently, only the ML tree is presented (Fig. 2). Six well-supported clades (I to VI) were identified. Clade I featured the North American species, *V. viride-americana*, with one sample from Brazil. Clade II included only *V. viride-brasiliensis* from Brazil, and was a sister lineage to Clade I. Notably, clade III constituted a distinct monophyletic group including sequences of our Taiwanese *Virescentia* samples (two identical sequences) and four of the haplotypes from Japan (AB114642-AB114645) (Fig. 2). Clade IV comprised *V. helminthosa* from Norway and France, while clade V included *V. guangxiensis* from China and haplotype AB114646 from Japan. Finally, clade VI included only *V. vogesiaca* from France (Fig. 2).

The species validation of all *Virescentia* samples using single-locus data via the ABGD for *rbc*L sequences resulted in the most conservative outcome. Seven putative *Virescentia* species were identified: five aligning with clades I, II, IV, V, VI, and two species within clade III. One species within clade III corresponded to the Taiwanese specimen analyzed in this study (subsequently characterized as *V. asiatica* sp. nov.) and AB114642, AB114644, and AB114645 sequences from Japan. The second species within clade III corresponded to the AB114643 sequence from Japan. The analysis based on COI-5P, instead, was less conservative, estimating three putative cryptic species within *V. viride-americana* (Fig. 2).

We assessed the genetic divergences (uncorrected p-distance) within clade III, based solely on *rbc*L (due to the unavailability of COI-5P data for the Japanese samples). The Taiwanese sample matched identically with the Japanese haplotype AB114644, while displaying 0.14% and 1.37% variation with haplotypes AB114645 and AB114642, respectively. The Japanese haplotype AB114643 showed higher divergence compared to the others within clade III, ranging from 1.78% to 2.05% (Table 2; Appendices 2; 3).

Morphological observations

Considering that the molecular analyses revealed that the undescribed *Virescentia* species from Taiwan was distinct from all other known *Virescentia* species, the only distinguishing characters we could find were in gross habit (size and curvature of thallus). The diminutive size may be due to the age of plants but the curved branches seem consistent in the material examined. These differences are documented in the description below, where we propose *V. asiatica* sp. nov. as a new species.

Family Batrachospermaceae Fr.
Genus *Virescentia* (Sirodot)
Necchi, D.C.Agostinho & M.L.Vis

Virescentia asiatica sp. nov.
(Fig. 3A-M; Table 2)

Type material. — **Taiwan** • Taoyuan, Longtan District, Longping Road, irrigation ditch in front of "HuangNiTangFuDe" Temple; 24°53'07.483"N, 121°13'53.234"E; 17.III.2015; Shao-Lun Liu; holotype: HAST-146271; GenBank: MH835533, MH835534 (*rbc*L) and OR885897 (COI-5P).

Etymology. — The epithet "asiatica" refers to the currently known distribution (Japan and Taiwan).

Description

The gametophyte is monoicous. The thallus reaches approximately 2 cm in height, displaying a black or dark brown coloration with a subtle mucilage (Fig. 3A, B). The branches of the thallus are strongly curved, showing an irregular and abundantly curved branching pattern (arrowheads in Fig. 3A, B). The terminal and older branches exhibit sparse fascicles, indicative of their deciduous nature during growth (Fig. 3A, B). The well-developed whorls are obconic or pear-shaped, measuring between 220 and 1060 µm in diameter (Fig. 3B-D), usually appearing distinct and contiguous or occasionally separated (Fig. 3B-D). Primary fascicles are composed of 7-14 cells, straight and display dichotomous branching (Fig. 3C). Secondary fascicles consist of 6-8 cells and cover the entire internode (Fig. 3D). In both primary and secondary fascicles, proximal cells are ellipsoidal or cylindrical, while distal cells are ellipsoidal or obovoidal (Fig. 3E). Spermatangia are 5-8 µm in diameter, are spherical or obovoidal and found terminally or laterally on primary and secondary fascicles (Fig. 3E). Carpogonial branches are relatively short and straight (Fig. 3F, G, I), consist of 3-4 disc-shaped or barrel-shaped cells (Fig. 3F), originating from periaxial cells of primary fascicles (Fig. 3J) with several short-branched involucral filaments comprising 1-4 cells (Fig. 3H). The carpogonium is 65-80 µm long (Fig. 3F-J), with trichogynes stalked and varying in shape such as cylindrical, clavate, or ellipsoidal (Fig. 3F-J). Carposporophytes are hemispherical and dense, axial, and either lower than or equal to the whorl radius with an average of 1-2 (occasionally up to 4) per whorl (Fig. 3D, K), and dimensions ranging from 140 to 800 µm in diameter and 100 to 750 µm in height (Fig. 3D, K). Gonimoblast filaments consist of 3-6 cylindrical cells (Fig. 3L). Carposporangia are obovoidal and their dimensions when mature are 34-40 µm in length and 18-20 µm in diameter (Fig. 3L, M).

Habitat and seasonality

The sample was collected on March 17, 2015, during the Spring season. We discovered the plant growing on stones

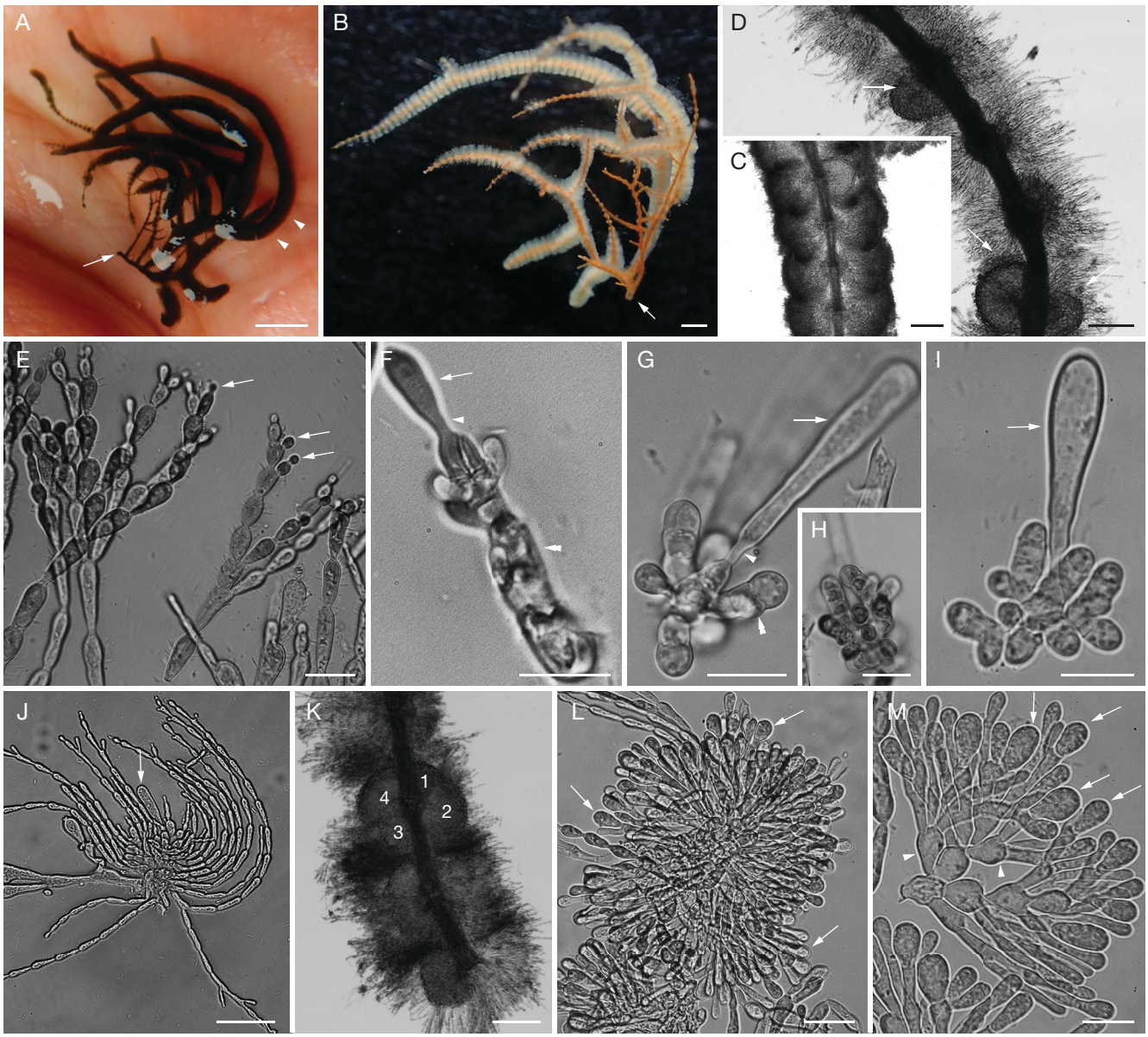

Fig. 3. — Morphological features of *Virescentia asiatica* sp. nov.: **A**, *in situ* gross morphology with diminutive holdfast (**arrow**). **Arrowheads** indicate the curvature of the branch; **B**, habit of the thalli with diminutive holdfast (**arrow**). **Arrowheads** indicate the curvature of the branch; **C**, contiguous barrel-shaped whorls formed by primary fascicles at nodes on the branch; **D**, branch showing whorls with hemispherical carposporophyte (**arrow**) at nodes and abundant secondary fascicles covering the entire internode; **E**, spherical spermatangia (**arrow**) at the tips of the fascicle cells; **F**, young straight carpogonial branch (**double arrowhead**) with carpogonium and unstalked (**arrowhead**) trichogyne (**arrow**); **G**, straight mature carpogonial branch with carpogonium (**double arrowhead**), cylindrical and stalked (**arrowhead**) trichogyne (**arrow**); **H**, compact and dense involucral branch; **I**, compact involucral branch with carpogonium and clavate trichogyne (**arrow**); **J**, carpogonial branch with trichogyne (**arrow**) arising from the basal periaxial cells of primary fascicle; **K**, four hemispherical carposporophyte at nodes; **L**, obovoidal carposporangia (**arrows**) at the tips of the gonimoblast filaments; **M**, close-up view of obovoidal carposporangia (**arrows**) at the tips of the gonimoblast filaments (**arrowheads**). Scale bars: A, 5 mm; B, 1 mm; C, 200 µm; D, K, 300 µm; E, F, M, 40 µm; G-I, 20 µm; J, L, 80 µm.

within the sun-exposed section of the stream, coexisting with the gametophyte of *S. dispersa*, which was notably abundant and widespread in that area at the time of collection. Prior to the first collection of *V. asiatica* sp. nov., we conducted monthly visits to the type locality over a year and a half (from February 2012 to October 2013), as a part of an ecological survey monitoring the population dynamics of an introduced freshwater red alga *M. macrospora* at the same site (Fontana *et al.* 2022). Throughout this period, there had been no visible growth of *V. asiatica* sp. nov. It was detected for the first time during a later visit on March 17, 2015, where it appeared in extremely limited numbers, with only a single thallus located. The ecological conditions at the type locality in early spring 2013 (i.e., during the same season of the later *V. asiatica* sp. nov. collection) were 23.1°C of water temperature with a pH of 7.82.

Geographic Distribution

Currently known from a few localities in Taiwan and Japan.

TABLE 2. — A list showing the morphological comparison between *Virescentia asiatica* sp. nov. and other known species of *Virescentia* (Sirodot) Necchi, D.C.Agostinho & M.L.Vis.

Character		*Virescentia asiatica*	*Virescentia crispata*	*Virescentia guangxiensis*	*Virescentia gulbenkiana*	*Virescentia helminthosa*	*Virescentia viride-americana*	*Virescentia viride-brasiliensis*	*Virescentia vogesiaca*
Thallus branch type		Curve	Straight	Straight	Straight	Straight	Straight	Straight	Straight
Whorl	Shape	Contiguous, obconic or pear-shaped	Contiguous, obconic, or pear-shaped	Contiguous or separated, barrel-shaped, or spherical	Contiguous or separated, barrel-shaped, or spherical	Contiguous or separated, barrel-shaped, spherical or pear-shaped	Contiguous or separated, barrel-shaped, spherical or pear-shaped	Contiguous or separated, barrel-shaped, spherical, pear-shaped or obconic	Contiguous or separated, barrel-shaped, obconic, pear-shaped or spherical
	Diameter (µm)	220-1060	150-350	250-350	430-810	300-800	280-940	280-1100	340-700
	Primary fascicle cell no.	7-14	6-13	5-7	8-13	8-15	8-19	7-18	7-14
Fascicle shape		Straight	Curved	Straight	Straight	Straight	Straight	Straight	Straight
Carpogonial branches shape		Straight	Slightly curved	Straight	Straight	Slightly curved	Straight	Straight	Straight
Carpogonium	Length (carpogonium basal cell + trichogyne)	65-80	54-75	Not observed	20-39	40-79	(30-)40-77	40-110	20-45
	Composed cell number (composed cell no. in twisted)	3-4	3-4	Not observed	3-12	3-11	1-7	1-7	(4-)8-20
	Shape	Disc or barrel-shaped	Disc- or barrel-shaped	Not observed	Disc-shaped	Disc-shaped	Disc-shaped	Disc-shaped	Disc-shaped
Carposporophyte	No. per whorl	1-2(-4)	1-2	Not observed	1-3	1-2	1-2	1-2	1-2
	Diameter	140-800	140-300	Not observed	120-300	150-420	(130-)140-390	200-550	140-330
	Height	100-750	100-250	Not observed	60-150	100-320	70-200(-240)	100-300	95-175
	Shape	Hemispherical	Spherical	Not observed	Cylindrical	Hemi-spherical	Spherical	Spherical	Spherical
Gonimoblast filament	Cell layer no.	3-6	3-5	Not observed	2-5	3-7	3-5	3-6	4-7
	Shape	Cylindrical	Cylindrical	Not observed	Cylindrical	Cylindrical	Cylindrical	Cylindrical or barrel-shaped	Cylindrical or ellipsoidal cells
Carposporangium	Length (µm)	34-40	14-30	Not observed	10-18	14-20.5	15-24	19-35	13-19
	Diameter (µm)	18-20	8-10	Not observed	6.5-10	8.5-12.5	8.5-12	10-24	8.5-13
	Shape	Obovoid	Clavate or obovoid	Not observed	Obovoid or pear-shaped	Obovoid or pear-shaped	Obovoid or pear-shaped	Obovoid, pear-shaped or ellipsoidal	Obovoid
Trichogyne	Shape	Stalked, cylindrical, clavate or ellipsoidal	Stalked, cylindrical	Not observed	Stalked, clavate, or cylindrical	Stalked, cylindrical, sometimes bifurcated or with knobs	Stalked, clavate or cylindrical	Stalked, clavate or sup-cylindrical	Stalked, clavate or ellipsoidal
Spermatangia	Diameter (µm)	5-8	6-8	Not observed	4-6	5-7	4-8	4-9	5-8
	Shape	Spherical or obovoid	Spherical or obovoid	Not observed	Spherical	Spherical	Spherical	Spherical or obovoid	Spherical
	Growth placement	Primary or Secondary fascicle	Primary or Secondary fascicle	Not observed	Primary fascicle	Primary or secondary fascicle	Primary or Secondary fascicle	Primary or secondary fascicle	Primary fascicle or secondary fascicle
Special expansion cells		Absence	Absence	Presence	Absence	Absence	Absence	Absence	Absence
References		This study	Necchi *et al.* (2018)	Fang *et al.* (2021)	Necchi *et al.* (2018)	Necchi *et al.* (2018)	Necchi *et al.* (2018); Krueger-Hadfield *et al.* (2024)	Necchi *et al.* (2018)	Necchi *et al.* (2018)

REMARKS

Compared to other known *Virescentia* species, *V. asiatica* sp. nov. has small thallus size (although may be attributable to the age of the material collected) with apparently curved branches (Table 2). Apart from its diminutive size and the curvature of the branches, few other morphological traits were found to distinguish this species from others in the genus *Virescentia*. Notably, unlike *V. asiatica* sp. nov., *V. crispata* and *V. helminthosa* have slightly curved carpogonial branches and *V. guangxiensis* has distinct expansion in the penultimate cells of primary or secondary fascicles (Table 2).

DISCUSSION

Our study, guided by molecular insights, expands the known species diversity within the genus *Virescentia* to eight. We conducted a comprehensive survey complemented by in-depth phylogenetic and morphological assessments of *V. asiatica* sp. nov. in comparison to the other recognized species in the genus. Nevertheless, exploring the hidden biodiversity in freshwater red algae remains a challenge. As demonstrated by Sherwood *et al.* (2014), the identification of new freshwater red algal species has primarily relied on traits in the gametophytic stage, which is notably scarce in some freshwater red

algae. Our findings align with this, as we discovered only one gametophyte individual of *V. asiatica* sp. nov. during extensive island-wide surveys, highlighting the challenge of specimen-based species inventories for freshwater red algae. Consequently, we anticipate that emerging technologies such as environmental DNA metabarcoding (e.g. Schulte *et al.* 2024) hold great promise for offering more extensive and efficient assessments of hidden freshwater red algal diversity, especially in scenarios similar to our own.

The combination of molecular and morphological tools is pivotal in uncovering cryptic species, particularly in groups like freshwater red algae where morphological distinctions are challenging. In this study, we present both molecular and morphological evidence supporting the proposal of the new species *V. asiatica* sp. nov., validating the "new *Virescentia* species hypothesis" introduced by Necchi *et al.* (2018). Necchi *et al.* (2018) suggested that the haplotypes reported as *V. helminthosa* from Japan (Hanyuda *et al.* 2004) represent at least one new species. One of the haplotypes was shown to be closely related to *V. guangxiensis* (Fang *et al.* 2021), while we suggest that three other haplotypes correspond to *V. asiatica* sp. nov. Our species delimitation analysis suggests the potential existence of a cryptic species close to *V. asiatica* sp. nov., represented by an additional haplotype from Japan. Furthermore, our estimate revealed three potential species within *V. viride-americana* and two within the *V. asiatica* lineage based on COI-5P and *rbc*L analyses, respectively, indicating the need for more comprehensive investigations. Identifying these entities at the species level poses challenges, potentially indicating incipient speciation with closely similar morphology. To tackle this, we advocate for an integrative taxonomy approach, aligned with the one proposed by Schlick-Steiner *et al.* (2010). This method involves utilizing a spectrum of evidence including multilocus (or genomic) data, physiological traits, biogeographical distribution, and ecological niche distinctions. By employing this integrative framework, we anticipate finding solutions to unravel these intricate taxonomic issues within *Virescentia*.

Two potential mechanisms have been suggested for the dispersal of freshwater macroalgae: aquarium-mediated and waterfowl-mediated dispersal. The former mechanism enables freshwater red algae to disperse over long distances across different continents by the aquarium trade (Zhan *et al.* 2021). Conversely, the latter mechanism primarily facilitates dispersal over shorter distances, such as between islands within a geographically confined region (Vis 2016). Unlike *S. dispersa* and *M. macrospora* that are common in aquaria, *V. asiatica* sp. nov. was not found in aquaria during our prior large-scale survey (Zhan *et al.* 2021). This finding suggests that the dispersal of *V. asiatica* between Japan and Taiwan is less likely due to the aquarium trade. Instead, considering the geographical proximity between Japan and Taiwan, it is more probable that waterfowl have mediated its presence. A similar idea was proposed by Hanyuda *et al.* (2004), suggesting that waterfowl facilitated the distribution of *Virescentia* species across various islands in Japan. However, it remains unclear whether *V. asiatica* sp. nov. can withstand short-term desiccation for transportation by the feet of waterfowl or survive the exposure to stomach acid via waterfowl feces. Further manipulative experiments are required to test these hypotheses. Nevertheless, with a more extensive sampling effort, the geographical distribution of *V. asiatica* sp. nov. is expected to considerably expand.

Overall, our study contributes significantly to understanding species diversity within the *Virescentia* genus, highlights challenges in identifying and inventorying freshwater red algae, and sets a direction for potential future research avenues, especially in the context of dispersal mechanisms and technological advancements for studying their hidden diversity.

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APPENDICES

Appendix 1. — A list showing the information of specimens and sequences used in this study. https://doi.org/10.5852/cryptogamie-algologie2024v45a7_s1

Appendix 2. — Pairwise p-distance of *rbc*L. https://doi.org/10.5852/cryptogamie-algologie2024v45a7_s2

Appendix 3. — Pairwise p-distance of COI-5P. https://doi.org/10.5852/cryptogamie-algologie2024v45a7_s3