Phytotaxa 369 (3): 200–210
http://www.mapress.com/j/pt/

Article

ISSN 1179-3155 (print edition)
PHYTOTAXA
ISSN 1179-3163 (online edition)

https://doi.org/10.11646/phytotaxa.369.3.3

Diversity and phylogeny of *Sargassum* (Fucales, Phaeophyceae) in Singapore

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Abstract

Sargassum species play key ecological roles on coral reefs, yet their diversity remains poorly known. Precise identification of *Sargassum* species, however, is improving with molecular genetic tools, though these have yet to be applied rigorously in Singapore. Historical records list 41 species, but no more than ten were verified based on herbarium vouchers, and even fewer (five species) were confirmed in the field based on a single nuclear gene marker in a previous study. Here, we revised the diversity of *Sargassum* in Singapore by examining all the morphologically distinct forms collected from the local coral reef environment. A total of six morphotypes, *Sargassum aquifolium* (Turner) C.Argardh (1820), *S.* cf. *granuliferum* C.Argardh (1820), *S. ilicifolium* (Turner) C.Argardh (1820), *S. swartzii* C.Argardh (1820), *S. polycystum* C.Argardh (1824), and an undescribed taxon '*Sargassum* sp.' (Mattio and Payri 2009), were delineated based on morphological characteristics. The morphotypes were placed in five molecular clades based on phylogenetic analyses of the nuclear *ITS-2*, chloroplastic partial RuBisCO operon *rbc*LS, and mitochondrial *cox*3. *Sargassum* cf. *granuliferum*, though morphologically distinct from all other species, is not phylogenetically distinct from *S. polycystum*. Our results provide a species list for Singapore that will be valuable for future studies on macroalgal biogeography and species-specific ecological relationships with other reef organisms, particularly corals.

Keywords: Brown macroalgae, Genetics, Morphology, Southeast Asia, *ITS-2, cox*3, *rbc*LS

Introduction

Globally, the macroalgal genus *Sargassum* C.Agardh (1820) comprises over 350 valid species (Guiry & Guiry 2018), and is especially diverse in tropical and subtropical marine environments (Phillips 1995). *Sargassum* has a characteristic non-filamentous thallus with a holdfast that branches to form many main axes. They have distinct leaves, receptacles, and the vesicles which are found on the axes near the leaves keep the algal structure upright when submerged.

Diversity assessments of *Sargassum* species are challenging due to difficulties in species identification using morphological characteristics (Mattio *et al.* 2013). One particular issue is that many species exhibit morphological plasticity in response to environmental change, age and reproductive state (Kilar *et al.* 1992). This variation can be large enough for a single species to be misidentified as two or more species (Mattio & Payri 2011). Resolving such taxonomic obstacles is important for precise quantification of biodiversity, planning for conservation and management, as well as for phytochemical research (Mattio & Payri 2011). For example, on coral reefs ecological interactions involving macroalgae can have considerable effects on coral community structure (Hughes 1994), coral diversity (Jones *et al.* 2004), and ecosystem services and function (Bellwood *et al.* 2004). Thus, to characterise and understand species-specific interactions, it is necessary to first be able to reliably identify *Sargassum* species and estimate their diversity.

Sargassum taxonomy has traditionally been based on morphology (Mattio & Payri 2011); however, with the recent application of DNA sequencing tools, a rich set of molecular character data are now available to supplement

 Accepted by Jeffery Hughey:23 Aug. 2018; published: 18 Sept. 2018

morphological datasets (Mattio *et al.* 2013). The use of genetic markers such as the internal transcribed spacer II (*ITS-2*), cytochrome c oxidase subunit III (*cox*3), and ribulose-1,5-bisphosphate carboxylase/oxygenase spacer (*rbc*LS) to reconstruct phylogenetic history has helped resolve species relationships and identification for many regions, including the western and central Pacific and western Indian Ocean (Mattio & Payri 2010; Mattio *et al.* 2013). Phylogenetic data from highly biodiverse areas within Southeast Asia, however, remain scarce with only a few studies conducted in Thailand (Kantachumpoo *et al.* 2015) and Vietnam (Nguyen 2014), thus hampering a more complete understanding of *Sargassum* biodiversity and biogeography in the Indo-Pacific region (Mattio *et al.* 2015).

Singapore, situated just over 1º north of the equator, is a Southeast Asian city state of 714 km^2 with 46 small offshore islands (Tun 2012, Fig. 1). *Sargassum* periodically dominates reef flats fringing Singapore's southern shores and islands (26.5–54.2% cover) (Low & Chou 2013). Seasonal growth of *Sargassum* begins in August, with peak extension (and biomass) in December to January, and it starts to decline at the end of February until only the perennial portions of the algae are left by March (Low & Chou 2013).

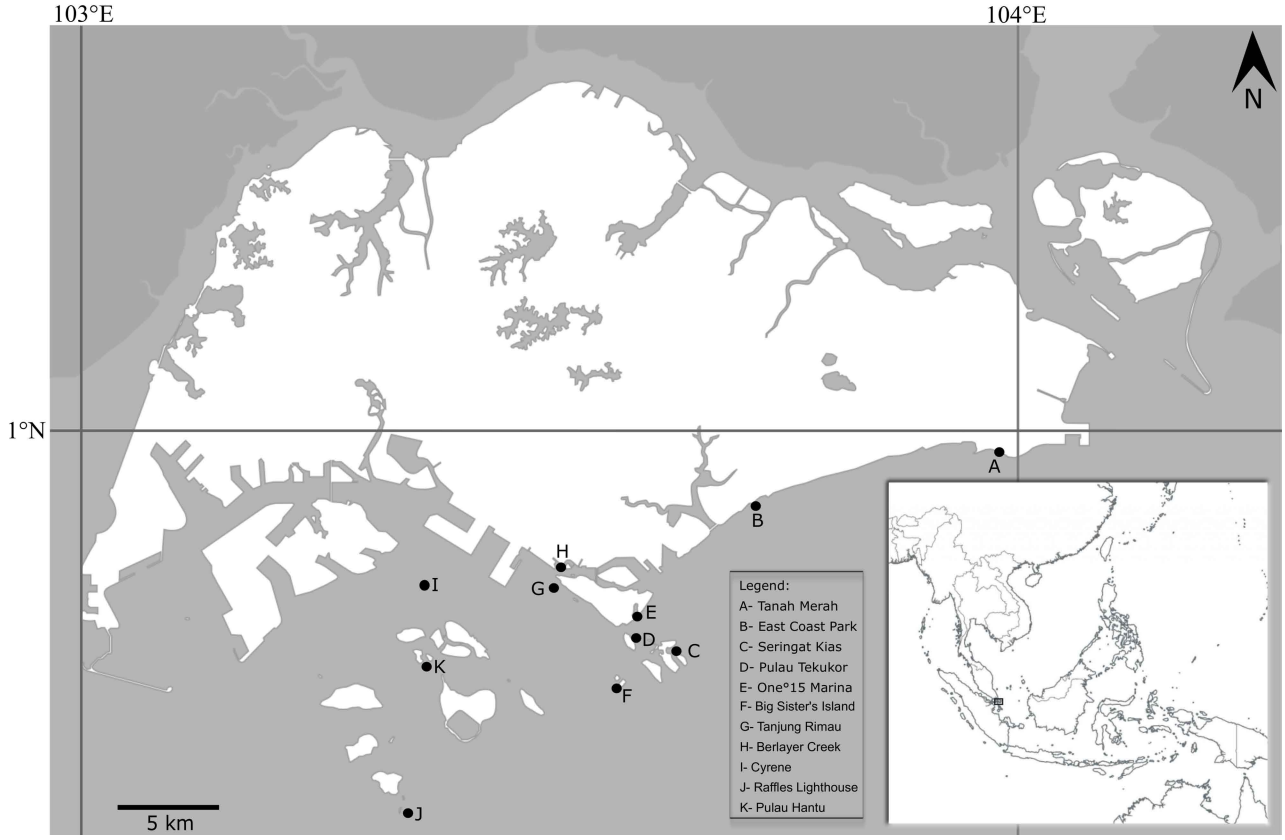

FIGURE 1. Map of Singapore with inset showing its location within Southeast Asia. Collection sites are denoted by circles.

In a literature review, Low (2015) recorded an initial total of 41 *Sargassum* species in Singapore, a number that was then reduced to 10 valid species following verification based on herbarium records at the Singapore Botanic Gardens Herbarium (SING) and the Lee Kong Chian Natural History Museum herbarium (SINU) (Table S1). Low (2015) eventually recovered only five species from field collections based on morphology and phylogenetic analysis of a nuclear molecular marker. Presently, *Sargassum* in Singapore is found primarily in coral reef and seagrass habitats, which are exposed to chronic turbidity and sedimentation as a result of coastal reclamation and shipping activities (Bauman *et al.* 2015, 2017). Since *Sargassum* species are known to be sensitive to anthropogenic environmental changes, these activities may have led to the decline and even extirpation of certain species (Phillips & Blackshaw 2011). Alternatively, it is possible that many of the species listed in the literature for Singapore are synonyms or misidentifications due to the failure to account for the full range of morphological variation within each species (see Mattio *et al.* 2013).

To address this shortfall in the understanding of *Sargassum* diversity in Singapore, we analysed a new molecular phylogenetic dataset from nuclear, mitochondrial and chloroplast genes. Furthermore, the incorporation of previously published sequences from a comprehensive and widespread collection of *Sargassum* species by Mattio *et al.* (Table S2) in our analyses puts the local diversity in a global context for uncovering phylogenetic patterns associated with this

ubiquitous macroalgal genus. Specifically, our aims were to: (1) update the species inventory of *Sargassum* in Singapore based on morphological and molecular evidence, as well as past collections and observations, and (2) reconstruct the phylogeny of local *Sargassum* species by incorporating sequences from the wider Indo-Pacific region.

Materials and methods

Sampling sites and collection

Sargassum specimens were collected during intertidal walks and by SCUBA from the southern shores of mainland Singapore and among the Southern Islands between December 2016 and July 2017 (Fig. 1; Table S3). Initial identification was performed according to previously studied morphological characteristics (Low 2015; Mattio & Payri 2009; Table 1), such as the shape of axis (flattened or cylindrical), shape of leaves (lanceolate, oblong or elliptical), margin of leaves (undulate or serrated), shape of vesicles (globular to elliptical), and vesicle stipule feature (simple or winged). No reproductive features (receptacles) were observed on the *Sargassum* samples as the collection period did not overlap with the reproductive season. Nevertheless, morphological identification was possible because multiple diagnostic characters were still associated with the non-fertile material collected (Table 1).

A total of 44 samples from 11 sites representing five morphotypes were collected and processed while fresh. Specimens were imaged using an Olympus TG3 camera. For each sample, part of the apical tip of a frond was cleaned of epiphytes, cut into smaller fragments of approximately 0.5 cm × 0.5 cm, preserved in 100% molecular-grade ethanol (Bressan *et al.* 2014), and stored in a -80˚C freezer. In total, 25 out of the 44 samples collected were pressed as herbarium vouchers and deposited at the SINU Herbarium.

DNA extraction, PCR amplification and sequencing

For each sample, three fragments of the preserved sample were dried and subsequently placed in a solution of 900µL of cetyltrimethylammonium bromide (CTAB) and 20µL of proteinase K (Doyle & Doyle 1987), then incubated at 55˚C overnight (minimum of six hours). DNA extraction of the digested tissues samples was conducted using phenol-chloroform-isoamyl alcohol according to Doyle & Doyle (1987).

Three genetic loci, nuclear *ITS-2*, chloroplastic *rbc*LS and mitochondrial *cox*3 were amplified using published primers (Table S4). Polymerase chain reaction (PCR) was carried out using a reaction mix containing GoTaq DNA polymerase (Promega), nuclease-free water, forward and reverse primers and template DNA. The reaction profile comprised an initial denaturation step at 95 ˚C for 5 min, followed by 40 cycles of denaturation at 94 ˚C for 40 s, primer annealing (Table S4) for 30 s, extension at 72 ˚C for 45 s, and a final extension step at 72 ˚C for 7 min (Mattio *et al.* 2008).

PCR products were purified using SureClean solution (Bioline) and cycle sequenced in both directions separately using the BigDye™ terminator method. The cycle sequenced products were precipitated, and sequenced using the ABI 3130 XL Genetic Analyser. A total of 44 sequences were obtained each for *rbc*LS and *ITS-2*, and 41 sequences for *cox3*. In addition, four *ITS-2* sequences from the *S. swartzii* morphotype—collected at two sites by Low (2015) (Table S2)—were included in our analyses.

Alignment of sequences and phylogenetic analyses

Sequences were assembled in Geneious v9.1.6 (Kearse *et al.* 2012) and compiled by gene in Mesquite 3.2 (Maddison & Maddison 2017). In addition to data obtained in the current study, sequences published by Mattio *et al.* (2008, 2009) and Mattio and Payri (2009) downloaded from NCBI GenBank (Table S2), as well as *S. swartzii ITS-2* sequences from Low (2015) were included in the analyses. Confamilial *Turbinaria ornata* was included as an outgroup as suggested by Stiger *et al.* (2003). Gene matrices were aligned separately using 'auto' default settings in MAFFT 7.304 (Katoh & Standley 2013), resulting in 748 base pairs (bp) each in the alignments of *ITS-2* and *rbc*LS, and 434 bp for *cox3*.

Data were analysed separately and concatenated for combined analyses, each carried out under maximum parsimony (MP), maximum likelihood (ML) and Bayesian methods to infer the phylogeny of *Sargassum*. Tree searches under MP were performed using TNT 1.5 (Goloboff & Catalano 2016) with 10000 random addition sequence replicates, each running under 100 cycles of tree fusing, drifting and ratcheting. Support for nodes were assessed by bootstrapping with 1000 pseudoreplicates (Felsenstein 1985). ML analyses were conducted using RAxML 8.2.4 (Stamatakis 2014) with the GTRGAMMA model and 10 random starting trees, and clade stability was tested using 1000 bootstrapped pseudoreplicates. Bayesian analysis was conducted by first estimating the best-fit model for each alignment using

TABLE 1. Morphological features of *Sargassum* morphotypes in Singapore (Low 2015; Mattio & Payri 2009)

Trait	*S. aquifolium*	*S. swartzii*	*S. polycystum*	*S. ilicifolium*	*Sargassum* sp.	*S.* cf. *granuliferum*
Axis	Compressed to flattened, smooth	Compressed to flattened, smooth	Cylindrical with stolon-like axis, heavily muricate	Cylindrical, smooth	Compressed, smooth	Cylindrical, sparsely muricate
Leaf shape	Thick and coriaceous, lanceolate	Linear, lanceolate to spatulate	Linear to lanceolate or oblong	Ovate to spatulate	Oblong-linear to ovate	Oblong
Leaf margin	Coarsely dentate to serrate	Undulate or slightly dentate	Irregularly and finely serrate or coarsely dentate	Denticulate or biserrate	Serrated to deeply dentate	Finely serrate
Leaf midrib	Well conspicuous, running halfway to the apex	Inconspicuous or percurrent	Percurrent	Thin midrib running halfway to apex	Midrib evanescent near apex	Percurrent
Vesicle shape	Ovoid, mucronate with leaf-like crown	Globular or ovate, smooth	Globular or ovate, smooth or mucronate	Globular or ovate, smooth. Occurs singly or paired; sometimes winged	Spherical or ovate, sometime with mucronate	Globular and in bunches
Vesicle size (mm)	Length: 5–10; Width: 4.5 –6	Length: 6–10; Width: 5–6	Length: 3–5; Width: 2–3	Length: 5–12; Width: 5–12	Length: 5–6; Width: 4–5	Length: 2–3; Width: 2–3
Vesicle pedicel	Flattened or leaf-like, longer or shorter than the vesicle	Compressed or terete, smooth, shorter or the same size as the vesicle	Cylindrical, thin and longer than the vesicle	Cylindrical to compressed, shorter than the vesicle	Flattened, shorter than the vesicle, smooth or with a few spines	Terete, shorter than the vesicle

jModelTest2 (Darriba *et al.* 2012; Guindon & Gascuel 2003) and the Akaike information criterion (*ITS-2*: GTR + Γ; *cox3*: HKY + Γ; *rbc*LS: GTR + I + Γ). Bayesian inference was performed on the partitioned dataset using MrBayes v3.2 (Huelsenbeck *et al.* 2001; Ronquist & Huelsenback 2003; Ronquist *et al.* 2012; Altekar *et al.* 2004), with two runs implemented over four Markov chains of 12 million generations, logging one tree per 100 generations. The first 20001 trees were discarded as burn-in after assessments of stationarity in Tracer v1.6 (Rambaut & Drummond 2013). Phylogenetic trees were visualised in FigTree v1.4.3 (Drummond & Rambaut 2007).

Results

Species identification based on morphology

A total of six morphotypes of *Sargassum* were collected in Singapore. These were identified as *S. aquifolium, S. swartzii, S. polycystum, S.* cf. *granuliferum, S. ilicifolium*, and '*Sargassum* sp.' based on published morphological characteristics and descriptions (Table 1; Mattio & Payri 2009; Low 2015). *Sargassum aquifolium* and *S. swartzii* were separated from other taxa by their characteristically flattened stems, versus cylindrical to compressed stems in the remaining taxa. *Sagassum polycystum* and *S.* cf. *granuliferum* were differentiated from *S. ilicifolium* based on their muricated stems as the latter had smooth stems. Abundant and widespread vesicles distinguished *S. polycystum* from *S.* cf. *granuliferum*, as the latter had vesicles that bunched together (Fig. 2). A morphotype referred to as *Sargassum* sp. (*sensu* Mattio & Payri 2009) was only slightly differentiated from *S. ilicifolium* based on stem structure and midrib patterns on the leaves (Table 1; Fig. 2).

Phylogenetic analyses

Molecular phylogenetic analyses of the concatenated dataset recovered trees that were generally concordant between the Bayesian inference (BI), maximum likelihood (ML) and maximum parsimony (MP) optimality criteria used (Fig. 3). Individual gene trees for *rbc*LS, *cox*3 and *ITS-2* (Supplementary material Figs S1–S3) were congruent among the genes, recovering strongly supported clades at the same positions on the phylogeny. Due to the topological consistency, the combined tree was used for inference to capture the full range of phylogenetic resolution. The designated outgroup, genus *Turbinaria*, was recovered unequivocally as sister to the rest of the species analysed. A monophyletic clade belonging to the genus *Sargassopsis,* with high MP/ML/BI support values (100/100/1), diverged as the sister group to genus *Sargassum* (MP/ML/BI: 99/100/1). Two distinct clades, sister groups within genus *Sargassum* (MP/ML/BI: 99/100/1), were recovered and formed subgenera *Sargassum* (*Sargassum*) (ML/MP/BI: 93/90/1) and *Sargassum* (*Bactrophycus*) (MP/ML/BI: 80/78/1).

A total of eight divergent clades were recovered within the subgenus *Sargassum* (*Sargassum*): (i) *S. aquifolium* IRD1546 distinct from (ii) the large *S. aquifolium* cluster*,* (iii) *S. swartzii*, (iv) *S. carpophyllum* J.Agardh (1848), (v) a large cluster grouping at least seven morphospecies, (vi) *S. polycystum* including *S.* cf. *granuliferum*, (vii) *S. ilicifolium*, and (viii) *Sargassum* sp. These are also represented as five sections each forming distinct clades. The present collection from Singapore was recovered in sections *Binderianae, Polycystae* and *Ilicifoliae* (Fig. 3). Six *Sargassum* species were recovered across these clades—*Sargassum aquifolium* and *S. swartzii* from section *Binderianae*, *Sargassum ilicifolium* and *S*. sp. from section *Ilicifoliae,* as well as *Sargassum polycystum* and *S*. cf. *granuliferum* from section *Polycystae*. Sections *Polycystae* and *Ilicifoliae* were well-supported monophyletic groups with node values of 99/100/1 and 99/100/1 (MP/ML/BI) respectively, and together formed a strong clade (100/100/1). Similarly, section *Binderianae*—sister group to the rest of the subgenus *Sargassum* (*Sargassum*)—was recovered as a distinct and strongly supported group with high MP/ML/BI support values of 99/100/1. Within *Binderianae*, the clade comprising *S. swartzii* was strongly supported (92/100/1) while the clade containing *S. swartzii* along with its sister clade of mainly *S. aquifolium* was moderately supported (51/53/0.9).

Section *Polycystae* (MP/ML/BI: 99/100/1) comprised *S. polycystum, S.* cf. *granuliferum* and *S. plagiophyllum* sequences (Fig. 3). *Sargassum plagiophyllum* C.Agardh (1824) was nested within *S. polycystum* sequences from this study but the clade was not well supported (-/88/-). Sequences ZT047, ZT050, ZT053, ZT069, ZT070, ZT074 and ZT076, identified morphologically as *S. cf. granuliferum*, did not form a monophyly and were nested within *S. polycystum* sequences.

Section *Sargassum* was only moderately supported (MP/ML/BI: 63/87/1). Several valid species recovered in the clade, with no apparent resolution among taxa, included *S. pacificum* Bory (1828), *S. polyporum* C.Montagne (1842), *S. scabridum* J.D.Hooker & W.Harvey (1845), *S. spinuligerum* G.Sonder (1845), *S. obtusifolium* J.Agardh (1848),

S. polyphyllum J.Agardh (1848) and *S. howeanum* A.H.S.Lucas (1935). Its sister group, section *Zygocarpicae* was a well-supported monophyletic group (99/100/1). *Sargassum carpophyllum* recovered in section *Zygocarpicae* formed a well-supported clade with MP/ML/BI values of 95/100/1, and was genetically distinct from *Sargassum turbinarioides* Grunow (1915).

There were generally low branch supports for groups descendant to the section-level clades (MP/ML<50/50), with unresolved shallow nodes at the species level. Two morphotypes within section *Ilicifoliae* formed a well-supported sister group (99/100/1). One of these morphotypes was unequivocally *S. ilicifolium* as samples from Singapore were nested within many *S. ilicifolium* sequences from New Caledonia, Fiji and the Solomon Islands (97/99/1). Its sister clade comprised sequences from specimens referred to as *Sargassum sp.* by Mattio and Payri (2009) (MP/ML/BI: 99/100/1) from Vanuatu, New Caledonia, the Solomon Islands, and Singapore.

Discussion

Recent taxonomic revisions of *Sargassum* have led to many nominal species being synonymised because of the high levels of intraspecific phenotypic variation (e.g. Mattio *et al.* 2008; Mattio & Payri 2011). These revisions integrated the results of gene sequencing and phylogenetic inferences to delimit *Sargassum* species and subgenera. Here, we use a concatenated DNA dataset comprising *ITS-2*, *cox*3 and *rbc*LS alignments to infer the phylogeny and species diversity of *Sargassum* in Singapore. These markers have been utilised to resolve the identities and molecular phylogeny of *Sargassum* elsewhere (Mattio *et al.* 2008). In particular, they have been used to support the division of the genus into two subgenera, *Sargassum* (*Sargassum*) and *Sargassum* (*Bactrophycus*), and eight sections—*Binderianae, Ilicifoliae, Polycystae, Sargassum [=Malacocarpicae], Zygocarpicae, Johnstonii, Lapazaenum and Sinicola*—included in subgenus *Sargassum* (*Sargassum*) (Dixon *et al.* 2014).

Our results reveal a total of six morphotaxa belonging to the subgenus *Sargassum* (*Sargassum*). *Sargassum polycystum, S.* cf. *granuliferum, S. aquifolium, S. swartzii, S. ilicifolium* and *Sargassum* sp. have been identified based on their morphological features. *Sargassum* sp. is morphologically distinct from the other morphotypes based on descriptions provided by Mattio and Payri (2009), and its identity as an unknown species is further confirmed by molecular analyses. *Sargassum* cf. *granuliferum*, though morphologically distinct from all other species, is not phylogenetically distinct as it does not form a reciprocally monophyletic group. The remaining taxa are supported by both morphological examinations and the molecular phylogeny.

The recently described section *Polycystae* (Mattio *et al.* 2009) comprises *S. polycystum* and *S. plagiophyllum* which have been recovered as a strongly supported monophyletic clade here (Fig. 3). Sequences from Singapore identified as *S. polycystum* and *S.* cf. *granuliferum* (Fig. 2), are nested within this section. However, the latter species has not been sequenced elsewhere, and thus our assignment requires further verification. Despite having morphological traits distinct from *S. polycystum* (Low 2015), the *S.* cf. *granuliferum* specimens contain sequences that are nested within *S. polycystum* phylogenetically. As the latter has been sequenced and positively identified from elsewhere, *S.* cf. *granuliferum* could be considered as a morphotype under *S. polycystum*. We note that *S. granuliferum* would take priority if *S.* cf. *granuliferum* here is positively assigned to the species. More genetic markers and sampling of both morphotypes from their type localities would be necessary to ascertain their identities and verify if they indeed represent distinct species.

Our results reveal that section *Binderianae* includes a well-supported monophyletic group of *S. swartzii* (MP/ML/BI: 92/100/1) that is a sister group to *S. aquifolium*. *Sargassum swartzii* is morphologically distinct from *S. aquifolium* based on the slender leaves with shallow dentate margins and small vesicles with terete stalks of *S. swartzii* (Noiraksar & Ajisaka 2008; Table 1; Fig. 2). Furthermore, *ITS-2* sequences of *S. swartzii* collected by Low (2015) in Singapore are recovered in the same clade as *S. swartzii* collected by Mattio and Payri (2009) in New Caledonia, lending support to its identity.

Within the section *Ilicifoliae*, our analyses recover a strongly supported monophyletic group represented by *Sargassum* sp. as a sister group to *S. ilicifolium* (Fig. 3). Seven specimens collected from this study in Singapore are nested within the *Sargassum* sp. clade and these specimens represent a newly recorded taxon in Singapore. Based on our results and those of Mattio and Payri (2009), supplemented by morphological observations by the latter, we suggest that this clade potentially represents a new species and not simply a variant of *S. ilicifolium*. Nevertheless, considering the large number of unstudied species from the Indo-Pacific region, a more comprehensive taxonomic reassessment of *Sargassum* species in this region is crucial before establishing any new taxon (Mattio & Payri 2009).

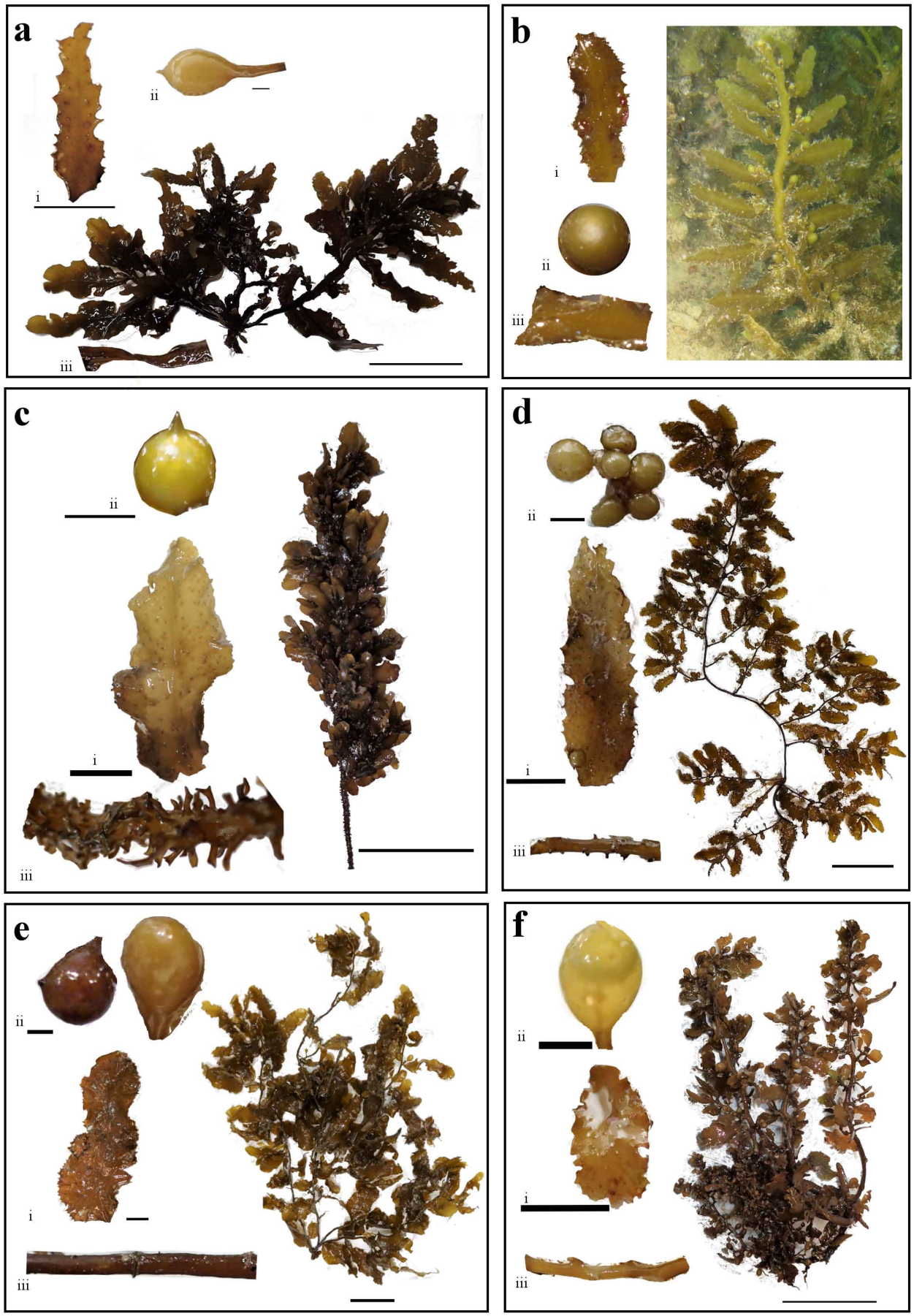

FIGURE 2. Thallus with i, leaf; ii, vesicle; and iii, stem morphology of Singapore taxa: **a** *Sargassum aquifolium*, **b** *S. swartzii*, **c** *S. polycystum*, **d** *S.* cf. *granuliferum*, **e** *S. ilicifolium*, **f** *S.* sp. Scale bar: thallus = 5 cm; leaves = 1 cm; vesicles = 2 mm.

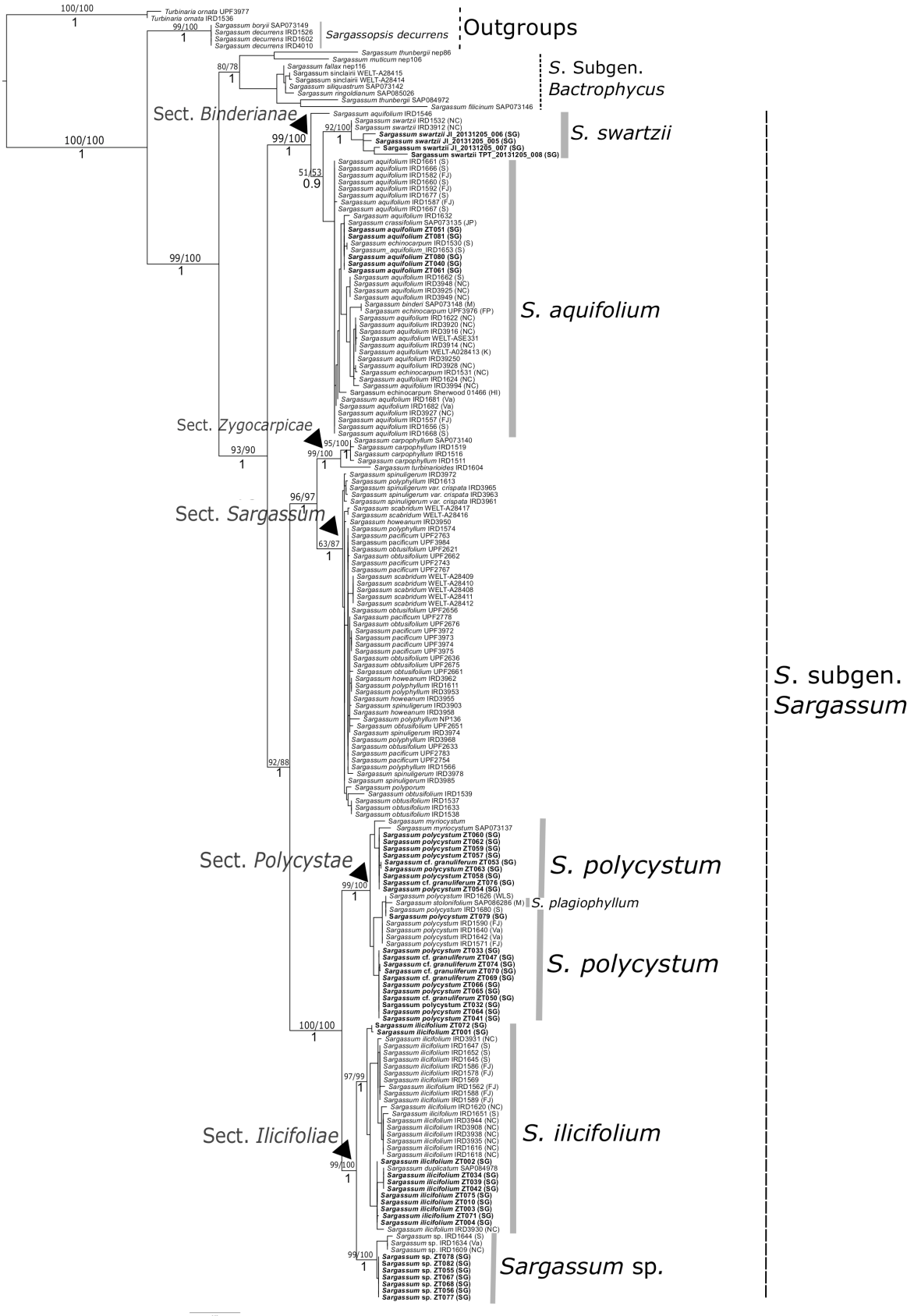

FIGURE 3. Phylogenetic reconstruction of *Sargassum* (Phaeophyceae) with *Turbinaria* as outgroup, based on a maximum likelihood (ML) analysis of the concatenated *ITS-2* + *rbc*LS + *cox*3 alignment. Bootstrap proportions indicated for maximum parsimony (MP)/ maximum likelihood (ML) when >0.5 and posterior probabilities for Bayesian inference (BI) when >0.8 for nodes at the species level and above. Sequences from Singapore are in bold and herbarium accession numbers are indicated. Sections within the subgenus *Sargassum* (*Sargassum*) are indicated at their nodes.

Low sequence variation among taxa in the section *Sargassum* has been hypothesized to be due to a rapid and recent geographic expansion (Mattio & Payri 2009). Indeed, our analyses show that there is an absence of monophyletic species in the section (Fig. 3), suggesting that dispersal and introgression between populations could have led to the limited genetic differentiation among morphologically disparate species (Mattio & Payri 2009). This hypothesis needs to be tested with more data from fresh collections. Even though none of the specimens collected in this study belong in the section *Sargassum,* one member—*Sargassum obtusifolium*—has been recorded near Singapore waters (Phang *et al.* 2016). Targeted collections in the under-sampled Southeast Asian region would be helpful in resolving species in the section *Sargassum*.

In conclusion, by integrating the present work and a recent study by Low (2015), we have recovered a total of six species in Singapore, comprising five of the 41 species historically recorded in the literature, and a newly recorded putative taxon. These morphological and molecular analyses provide insights into Singapore's *Sargassum* diversity valuable for both ongoing ecological studies and upcoming regional phylogenetic analyses.

Acknowledgements

We would like to thank members of the Reef Ecology Laboratory, National University of Singapore, especially Nicholas W. L. Yap and Y. C. Tay, for helping with field and lab work, Jenny Fong for assisting with specimen collection, James E. M. Stach in the School of Biology at Newcastle University for access to laboratory facilities and equipment, as well as National Parks Board Singapore for granting the research permit NP/RP16-156. We are grateful to Darren C. J. Yeo and two anonymous reviewers for comments that helped improve the manuscript.

Compliance with Ethical Standards

Funding: This research is supported by the National Research Foundation, Prime Minister's Office, Singapore under its Marine Science R&D Programme (MSRDP-P03; R-154-000-A25-281).

Conflict of interest: The authors declare that they have no conflict of interest.

Ethical approval: This article does not contain any studies with animals performed by any of the authors.

Sampling and field studies: All necessary permits for sampling and observational field studies have been obtained by the authors from the competent authorities and are mentioned in the acknowledgements.

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