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PHYLOGENY OF GRACILARIACEAE (RHODOPHYTA): EVIDENCE FROM PLASTID AND MITOCHONDRIAL NUCLEOTIDE SEQUENCES[1]

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Gracilariaceae are mostly pantropical red algae and include ~230 species in seven genera. Infrafamilial classification of the group has long been based on reproductive characters, but previous phylogenies have shown that traditionally circumscribed groups are not monophyletic. We performed phylogenetic analyses using two plastid (universal plastid amplicon and *rbc*L) and one mitochondrial (*cox*1) loci from a greatly expanded number of taxa to better assess generic relationships and understand patterns of character distributions. Our analyses produce the most well-supported phylogeny of the family to date, and indicate that key characteristics of spermatangia and cystocarp type do not delineate genera as commonly suggested. Our results further indicate that *Hydropuntia* is not monophyletic. Given their morphological overlap with closely related members of *Gracilaria*, we propose that *Hydropuntia* be synonymized with the former. Our results additionally expand the known ranges of several Gracilariaceae species to include Brazil. Lastly, we demonstrate that the recently described *Gracilaria yoneshigueana* should be synonymized as *G. domingensis* based on morphological and molecular characters. These results demonstrate the

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utility of DNA barcoding for understanding poorly known and fragmentary materials of cryptic red algae.

***Key index words: Gracilaria*; *Gracilariopsis*; *Hydropuntia*; systematics; taxonomy**

***Abbreviations*: BP, Bootstrap percentage; *cox*1, gene encoding cytochrome oxidase I (COI); dNTP, triphosphate desoxirribonucleotides; *G.*, *Gracilaria*; *Gp.*, *Gracilariopsis*; *H.*, *Hydropuntia*; ITS, internal transcribed spacer; MCMC, Markov Chain Monte Carlo; ISS, index of substitution saturation; ML, maximum likelihood; PP, (Bayesian) Posterior probability; *rbc*L, gene encoding the large subunit of RuBisCo (ribulose biphosphate carboxilase oxigenase); SSU, small subunit; UPA, universal plastid amplicon**

The red algal family Gracilariaceae includes ~230 species in seven genera (Guiry and Guiry 2014). Most species in the family are benthic, and nearly all are free-living with the exception of parasitic species in the genera *Congracilaria* and *Gracilariophila*. The family is largely pantropical, but also occurs in temperate and boreal regions. The two genera *Curdiea* and *Melanthalia* are represented predominately in the southern hemisphere with many species endemic to Oceania (Guiry and Guiry 2014). Members of the group are commercially important and include several species of *Gracilaria*, which are widely cultivated for the production of agar (Oliveira et al. 2000). In addition, some species are of particular pharmacological interest for molecules that have the potential for treating conditions such as cancer, AIDS, general inflammation, arthritis, as well as viral, bacterial, and fungal infections (Almeida et al. 2011).

The monophyly of Gracilariaceae is well-supported by molecular data (Gurgel and Fredericq 2004). In addition, its members possess numerous shared morphological and anatomical characters, particularly aspects of their archegonia (Fredericq and Hommersand 1989). Species and genera within the family have similarly been delimited based on reproductive characters, especially the distribution of spermatangia and cystocarp structure (Fredericq and Hommersand 1990). According to Yamamoto (1984), the spermatangia of Gracilariaceae can be distributed in superficial layers or in conceptacles. The manner in which the spermatangia are distributed into conceptacles are diverse and include three main types: textorii in which spermatangia are borne in shallow crypts, verrucosa in which spermatangia are in single, deep crypts, and henriquesiana in which spermatangia are in deep, confluent crypts. The first two, textorii and verrucosa, have long been a defining characteristic of the largest genus in the family, *Gracilaria*. *Hydropuntia* Montagne (1842; valid name for *Polycavernosa* C. F. Chang et B. M. Xia, see Wynne 1989) possesses only the henriquesiana type. Important diagnostic characteristics of the cystocarp for species delimitation include: the presence and location of nutrient tubular cells, which connect to the gonimoblasts and pericarp; presence of small, cytoplasm-rich cell layers (inner pericarp) located at the base of the carposporophyte; and the presence of a primary or secondary fusion cell. The consensus is that cystocarps in *Gracilariopsis* do not possess nutrient tubular cells (Fredericq and Hommersand 1990, Liao and Hommersand 2003), however, the relationship between cystocarp characters and their utility for circumscribing other genera remains unclear.

The use of spermatangia and cystocarp type to define the generic taxonomy of Gracilariaceae has been problematic. Wynne (1989) transferred some species of *Gracilaria* to *Hydropuntia* based on these features. Abbott et al. (1991), however, observed that these traits might not be discrete. Verrucosa and henriquesiana type spermatangia, for example, have been observed in the same thalli of some species, suggesting that the verrucosa type may be an early developmental stage of the henriquesiana type. Abbott et al. (1991) similarly observed overlapping features of cystocarp anatomy. For example, they identified that the lower tubular cells thought to define *Hydropuntia* are also present in some species of *Gracilaria*. Based on the overlapping nature of these characteristics, Abbott et al. (1991) recommended a broader circumscription for these species by proposing that all *Hydropuntia* be transferred to *Gracilaria*. This was investigated using molecular phylogenetics by Bird et al. (1992, 1994) who identified that *Hydropuntia* was monophyletic. They, in turn, proposed that *Hydropuntia* be recognized as a subgenus of *Gracilaria*. Tseng and Xia (1999) subsequently conducted a thorough morphological investigation of the group and accepted this proposal. The defining features for this subgenus included confluent spermatangial conceptacles and tubular nutritive cells restricted to the base of the cystocarp. Later, however, Bellorin et al. (2002) conducted a phylogenetic analysis using ITS and SSUrDNA, which included increased taxon sampling, and identified that *Hydropuntia* was not monophyletic. Species within this genus were variously related to other species of *Gracilaria*.

In an attempt to further resolve this conundrum, Gurgel and Fredericq (2004) used plastid *rbc*L across a dense taxonomic sampling to assess generic limits within the family. Their analyses identified three well-supported subclades: *Curdiea* + *Melanthalia*, *Gracilariopsis*, and *Gracilaria* sensu lato (s.l.). Based on their phylogenetic results, they proposed recognizing three genera within their *Gracilaria* s.l. clade: *Hydropuntia, Gracilaria* sensu stricto (s.s.), and a third genus, which was unnamed. Relationships within the *Gracilaria* s.l. clade received varying support. *Hydropuntia* and the unnamed new genus were each well-supported, *Gracilaria* s.s., which includes

the vast majority of the species in the family, was poorly supported, and relationships between these three subclades were unclear. This lack of resolution complicates our understanding of major clades within Gracilariaceae, and limits our understanding of the utility of spermatangial and cystocarp characters to delineate major clades within the family.

Here, we improved the phylogenetic study of Gurgel and Fredericq (2004) by greatly expanding taxon sampling and increasing the number of genetic loci. Our newly included samples are mostly from South America, and especially from Brazil. Brazil includes a remarkably high diversity of Gracilariaceae, with 24 species (Lyra 2014), most of which remain unsampled for molecular study. We also expanded our sampling to include three molecular markers: the plastid regions *rbc*L and universal plastid amplicon (the UPA), and mitochondrial *cox*1. *rbc*L has been used successfully to infer phylogenetic relationships within and among red algae, including Gracilariaceae (e.g., Gurgel and Fredericq 2004); UPA has been proposed by Sherwood and Presting (2007) as a DNA barcode for photosynthetic organisms; *cox*1 is a well-established barcoding locus for red algae (Saunders 2005, 2008, Robba et al. 2006, Hajibabaei et al. 2007, Clarkston and Saunders 2010). The latter two regions have not commonly been used in broad phylogenetic studies such as ours. In addition, we DNA barcoded numerous distinct populations within several species, especially for those that are highly variable in morphology. Our analyses provide the best resolved phylogeny of Gracilariaceae to date, which will greatly facilitate a revised generic delimitation, and shed new insights into species ranges and species delimitation within the family.

MATERIALS AND METHODS

Taxon sampling and species identification. We collected 94 specimens along the coast of Brazil (Table S1 in the Supporting Information), supplementing existing data in GenBank (Table S2 in the Supporting Information). Samples from the field were transported to the laboratory, cleaned, and sorted carefully under a stereomicroscope (Leica® Zoom 2000; Heerbrugg, Switzerland). Three to five thalli fragments were preserved in silica gel desiccant for subsequent DNA extraction. Materials for morphological observations were preserved in 4% formalin in seawater. Voucher specimens were deposited in the herbaria Alexandre Leal Costa Bahia (ALCB) of the Universidade Federal da Bahia and in the Universidade de São Paulo (SPF), Brazil. Species identification was accomplished by searching relevant literature and by consulting general herbaria collections (especially ALCB, SPF) and type species deposited at the New York Botanical Garden (*Gracilaria intermedia* - NY00900180; *G. cuneata* - NY00900179; and *G. ornata* - NY00900183), the British Museum (*G. cervicornis* - BM000936191; *G. foliifera* - BM001039032; *G. cornea* - BM001038942; *G. crassissima* - BM000936163; and *G. secunda* - BM000936179), the Farlow Herbarium of Harvard University (*G. blodgettii*), and the United States National Herbaria (*G. flabelliformis* subsp. *aionana* – US208953; *G. flabelliformis* subsp. *simplex* – US208954).

Molecular methods. Total DNA was extracted from ~20 to 40 mg of samples by maceration in liquid nitrogen using the procedure outlined by Faugeron et al. (2001), or alternatively using the Chelex method described by Cohen et al. (2004). Molecular markers were PCR amplified under the following conditions: 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.2 μM each primer, 5 ng of genomic DNA, and 1.25 U of Taq DNA polymerase (Invitrogen, São Paulo, SP, Brazil, or Qiagen, Valencia, CA, USA) for a total volume of 25 μL. The amplification and sequencing reaction for obtaining *cox*1 used the primers GazF1 and GazR1 (Saunders 2005); *rbc*L used primers FrbcL and RrbcS (Freshwater et al. 1994) plus the additional internal primers R753 and F492 (Freshwater and Rueness 1994); and UPA used primers p23Sv_f1 and p23Sv_r1 (Sherwood and Presting 2007). PCR was performed with an initial denaturation step at 94°C for 10 min, followed by 35 cycles of 30 s at 90°C, 30 s at 50°C (variations of 47°C and 55°C for *rbc*L and UPA, respectively), and 2 min at 72°C, with a final 10 min extension cycle at 72°C. PCR products were sequenced in both directions using dye terminators and sequencing protocols at GeneWiz (Cambridge, MA, USA, http://www.genewiz.com/).

We used the red algal species *Gelidium crinale* (Hare ex Turner) Gaillon and *Rhodymenia pseudopalmata* (J. V. Lamouroux) P. C. Silva for rooting purposes, which have been shown to be closely related to the Gracilariaceae (Verbruggen and Theriot 2008).

Phylogenetic analyses and alternative topology testing. Electropherograms were assembled and edited using Geneious v6.0.6 (Drummond et al. 2011). Phylogenetic relationships were estimated using both maximum likelihood (ML) and Bayesian methods. The ML analyses were conducted using RAxML v7.2.8 (Stamatakis 2006) with a single GTRGAMMA model. The best-scoring ML tree and 200 bootstrap trees were obtained using the rapid hill-climbing algorithm (Stamatakis et al. 2008). The Bayesian analyses were implemented using the parallel version of BayesPhylogenies v2.0 (Pagel and Meade 2004) with a reversible-jump implementation of the mixture model as described by Venditti et al. (2008). This approach allows the fitting of multiple models of sequence evolution to each character in an alignment without a priori partitioning. Two independent Markov chain Monte Carlo (MCMC) analyses were performed, and the consistency of stationary-phase likelihood values and estimated parameter values was determined using Tracer v1.5. We ran each MCMC analysis for 10 million generations, with trees and parameters sampled every 1,000 generations. Bayesian posterior probabilities were determined by building a 50% majority-rule consensus tree from two MCMC analyses after discarding the initial 20% burn-in generations.

Nucleotide substitution saturation was measured using an entropy-based index of substitution saturation (I_{SS}; Xia et al. 2003) as implemented in DAMBE (Xia and Xie 2001). I_{SS} was estimated for each gene with gaps treated as unknown states. The incongruence length difference (ILD) test (Farris et al. 1995) was performed using PAUP* v 4.0b10 to test the presence of conflicts between genes.

The concatenated matrix (Fig. 1) was generated by combining the *cox*1, *rbc*L, and UPA data. It includes taxa that have been sequenced for all the three gene regions, except for *Gracilaria chilensis* C. J. Bird, McLachlan & E. C. Oliveira and *G. tenuistipitata* C. F. Chang & B. M. Xia, in which sequences were acquired only for *cox*1 and *rbc*L. Alternative topology tests were performed in an ML framework using the approximately unbiased (AU) test (Shimodaira 2002). We tested the monophyly of the genus *Hydropuntia*, which in our combined analysis and in our taxon dense analysis using *rbc*L, was demonstrated to be nonmonophyletic. In this case,

Hydropuntia was enforced to be monophyletic in the topology inferred from our combined data set. The constrained searches were conducted using RAxML as described above and tested against the optimal unconstrained ML trees using scaleboot v0.3-3 (Shimodaira 2008).

RESULTS

Sequences/matrices. We generated 45, 53, and 64 new Gracilariaceae sequences for *rbc*L, *cox*1, and UPA, respectively. For each gene, a multiple alignment was generated, excluding PCR priming sites and ragged ends, as follows: *cox*1 (Fig. S1 in the Supporting Information) included 96 sequences (53 new and 43 published) and 616 base pairs (bp) of which 242 bp were variable and 187 bp were parsimony informative in the ingroup; UPA (Fig. S2 in the Supporting Information) included 111 sequences (64 new and 47 published) and 300 bp of which 44 bp were variable and 34 bp of were parsimony informative in the ingroup; and *rbc*L (Fig. 2, A and B) included 137 sequences (45 new and 92 published) and 1,071 bp of which 339 bp were variable and 268 bp were parsimony informative. All new *cox*1, UPA, and *rbc*L sequences, and specimens' data were deposited in the Barcode of Life Data Systems (http://www.boldsystems.org/) and GenBank (Table S1). The combined data matrix (Fig. 1) included 31 sequences (16 new and 15 published) with 1,984 bp.

We estimated I_{SS} for each gene. The critical I_{SS} value was estimated assuming a symmetrical ($I_{SS.C1}$) or pectinate ($I_{SS.C2}$) topology, and the *P*-value was inferred using the two-tailed *t*-test. As I_{SS} approaches 1, or if I_{SS} is not smaller than the critical I_{SS} value, then sequences are determined to exhibit substantial saturation (Xia et al. 2003). Our saturation analyses demonstrate that for the three genes included in our concatenation analyses, only *cox*1 shows evidence of saturation if the true topology is pectinate (Table 1). The individual gene trees from *cox*1, UPA, and *rbc*L were not topologically in conflict with each other at >80% bootstrap support (data for *cox*1 and UPA not shown). Additionally, the ILD test suggested that there were no significant differences between the individual data sets ($P = 0.15$). Thus, we combined all the three genes

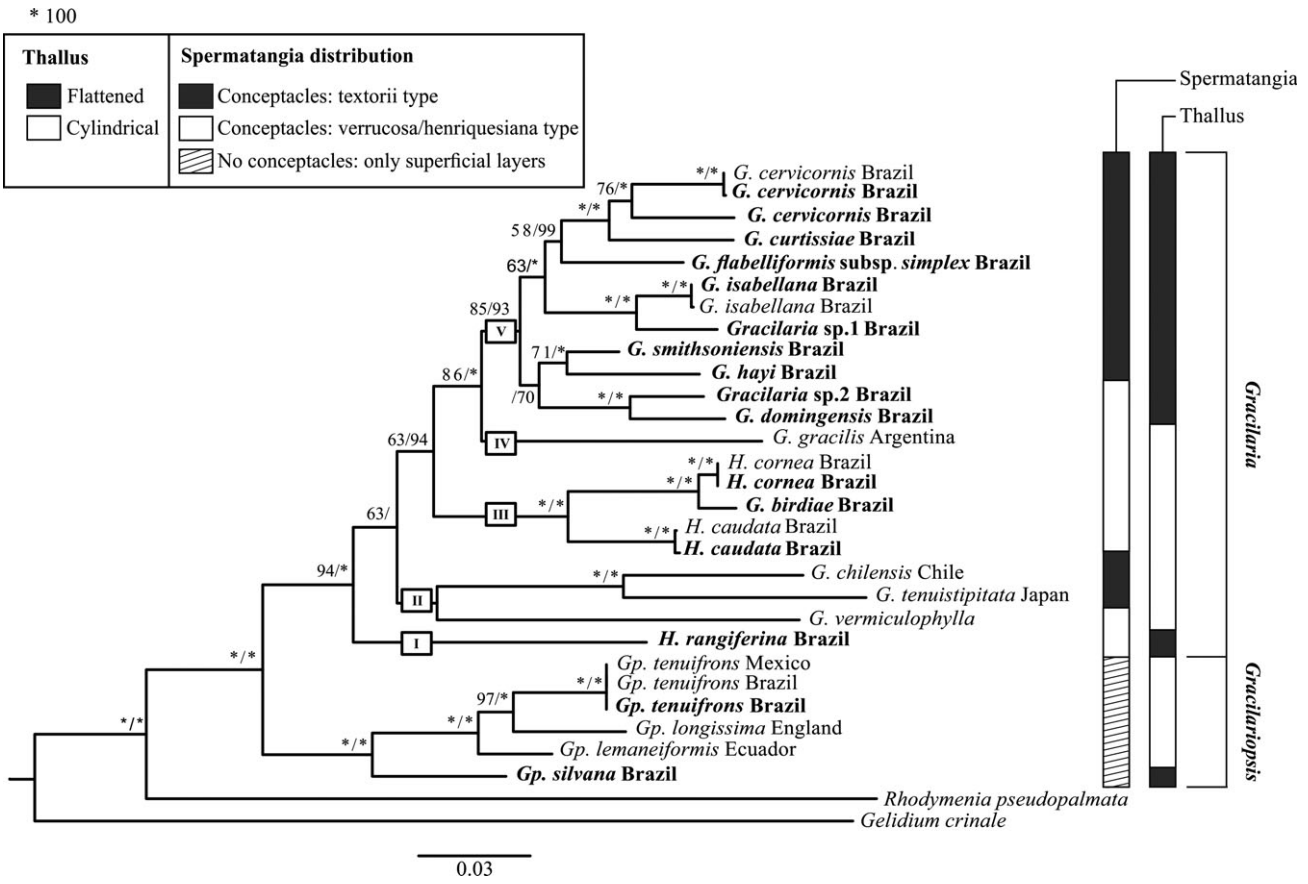

FIG. 1. The optimal maximum likelihood (ML) topology based on the combined three-gene (*rbc*L, UPA, and *cox*1), reduced taxon data set (ntax = 24, missing data = 0.5%). Support values >50 are indicated. Values above branches are ML bootstrap values (left) and Bayesian posterior probabilities converted to percentages (right). An asterisk indicates that the node was supported at 100. Roman numbers, I–V, identify *Gracilaria* subclades discussed in the main text. New sequences produced in this study are in boldface. Data are otherwise from GenBank. Spermatangia and thallus type mapped to the right of the phylogeny.

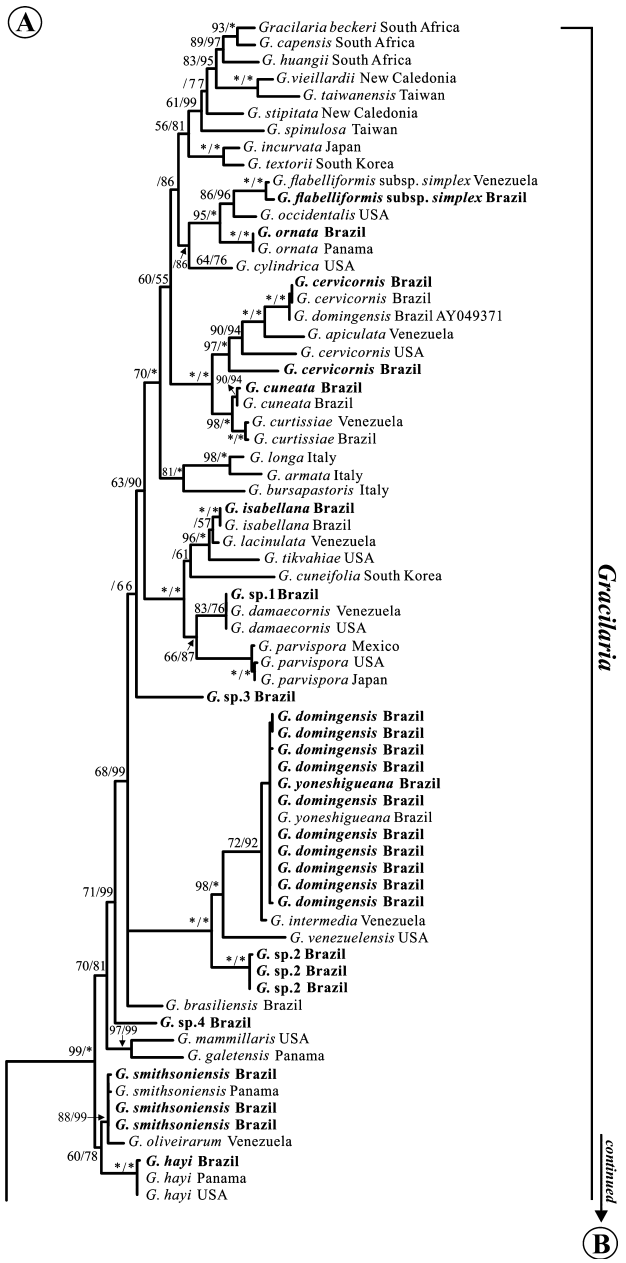

FIG. 2. The optimal maximum likelihood (ML) topology based on the *rbc*L data set (ntax = 83, missing data = 0%). Support values >50 are indicated. Values above branches are ML bootstrap values (left) and Bayesian posterior probabilities converted to percentages (right). An asterisk indicates that the node was supported at 100. New sequences produced in this study are in boldface. Data are otherwise from GenBank.

and analyzed them simultaneously (Fig. 1). For our combined analysis, we reduced most species to a single taxon to ensure maximal gene sampling density. We additionally present the single gene tree using *rbc*L (Fig. 2) because it includes the densest taxon sampling and is the most resolved of the three individual gene trees. Spermatangia and thallus characters illustrated on our combined data set in Figure 1 do not differ substantially from trends discerned from the phylogeny generated from *rbc*L alone.

Phylogenetic results. In our combined three-gene analysis, Gracilariaceae forms a well-supported clade (100 bootstrap percentage [BP] and 1.0 posterior probability [PP]) with three major well-supported subclades: *Curdiea/Melanthalia*, *Gracilariopsis*, and *Gracilaria* (Fig. 1). *Hydropuntia* is not monophyletic and comprises two distinct lineages. We found that constraining *Hydropuntia* to be monophyletic was a significantly worse fit to the optimally resolved phylogeny inferred using the combined data ($P = 0.028$, AU test).

Nineteen taxa were identified among our sampling effort in Brazil based on a combination of morphology and molecular data, 17 of *Gracilaria* and 2 of *Gracilariopsis*: *Gracilaria birdiae* Plastino & E. C. Oliveira; *G. caudata* J. Agardh; *G. cervicornis* (Turner) J. Agardh; *G. cornea* J. Agardh; *G. cuneata* Areschoug; *G. curtissiae* J. Agardh; *G. domingensis* (Kützing) Sonder ex Dickie; *G. flabelliformis* subsp. *simplex* Gurgel, Fredericq & J. N.Norris; *G. hayi* Gurgel, Fredericq & J. N.Norris; *G. isabellana* Gurgel, Fredericq & J. N. Norris; *G. ornata* J. E. Areschoug; *G. rangiferina* (Kützing) Piccone; *G. smithsoniensis* Gurgel, Fredericq & J. N. Norris in Abbott & McDermid; *Gracilaria* sp. 1, *Gracilaria* sp.2; *Gracilaria* sp.3; *Gracilaria* sp.4; *Gp. silvana* C. F. D.Gurgel, S. Fredericq & J. N. Norris; *Gp. tenuifrons* (C. J. Bird & E. C. Oliveira) Fredericq & Hommersand.

DISCUSSION

Our phylogeny is the largest and most well-resolved for Gracilariaceae to date. We identified three, large well-supported subclades within the family, which were also identified by Gurgel and Fredericq (2004). One, including the genera *Curdiea* plus *Melanthalia*, a second including all *Gracilariopsis* species, and a third, which we refer to as *Gracilaria* s.l. (Fig. 2). *Gracilaria* s.l. includes the species currently recognized in *Hydropuntia*, plus the unnamed new genus proposed by Gurgel and Fredericq (2004). These results allow us to draw several conclusions about past taxonomic considerations. In the relevant subsections below, we focus on well-supported clades inferred from our combined analysis (Fig. 1) that exhibit more clearly defined morphological tendencies. On the basis of our results, we propose that six, not seven, genera be recognized in the family: *Congracilaria*, *Curdiea*, *Gracilaria* (including *Hydropuntia*, and the unnamed genus sensu Gurgel and Fredericq 2004), *Gracilariophila*, *Gracilariopsis*, and *Melanthalia*. These analyses further indicate that contrary to some assertions (Saunders 2005), short barcoding loci may be useful for phylogenetic inference. *cox*1 produces a reasonably well-supported phylogeny for the family, which is congruent to the plastid phylogeny, especially from *rbc*L. Finally, our

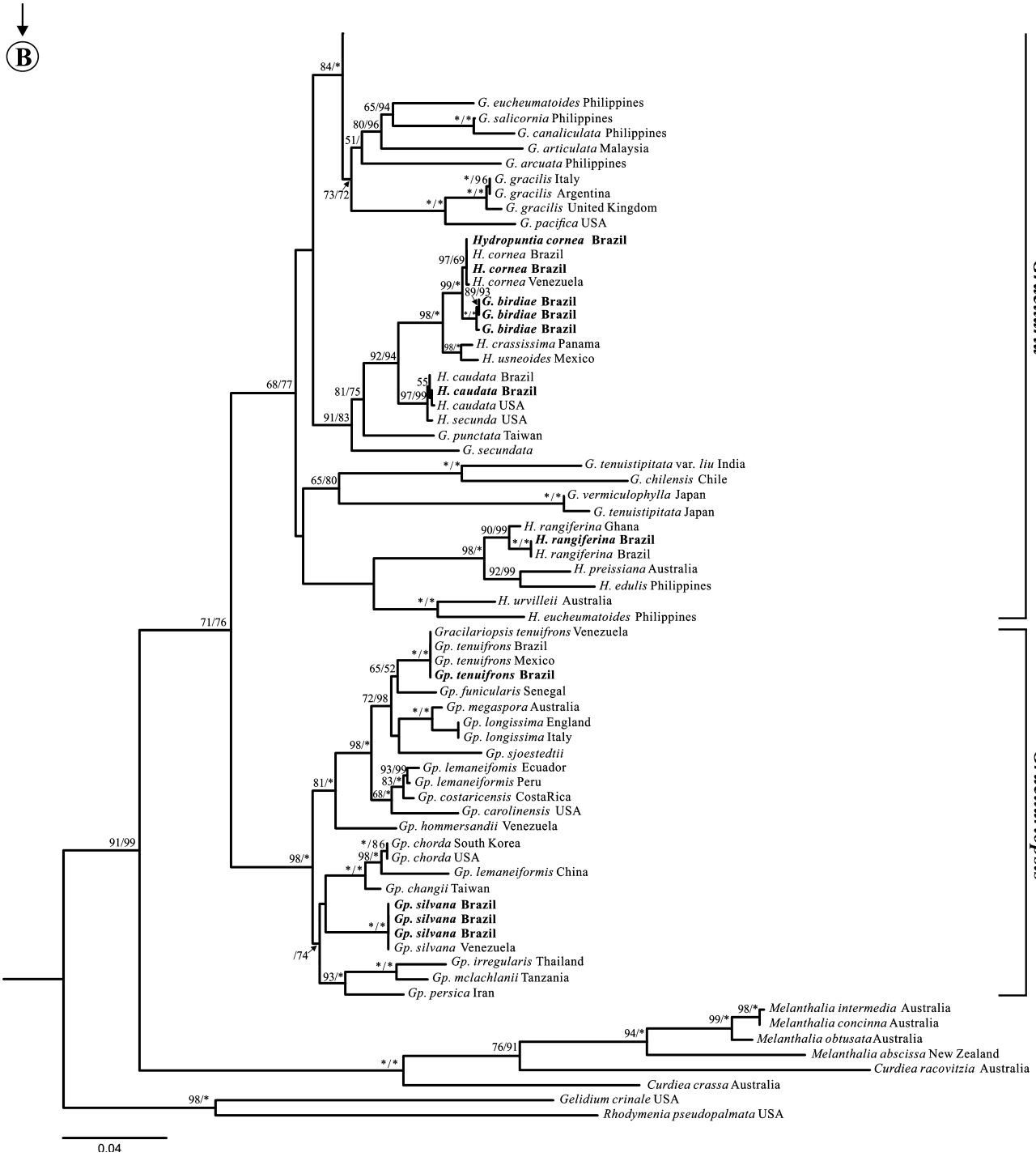

FIG. 2. (continued)

TABLE 1. Estimated I_{SS} for each gene. The critical I_{SS} value was estimated assuming a symmetrical ($I_{SS.C1}$) or pectinate ($I_{SS.C2}$) topology, and the *P*-value was inferred using the two-tailed *t*-test.

	I_{SS}	$I_{SS.C1}$	*T*-value	*df*	*P*-value	$I_{SS.C2}$	*T*-value	*df*	*P*-value
*cox*1	0.5349	0.7318	8.2753	245	0.0000	0.4460	3.7392	245	0.0002
*rbc*L	0.3483	0.7669	24.569	411	0.0000	0.5044	9.1022	411	0.0000
UPA	0.3393	0.6566	6.5164	66	0.0000	0.3997	1.2400	66	0.2194

I_{SS}, index of substitution saturation; *df*, degrees of freedom; UPA, universal plastid amplicon.

densely sampled *rbc*L phylogeny (Fig. 2) sheds insights on species range expansions and species delimitations within the family.

Gracilaria *expanded to include* Hydropuntia*,* Gracilaria s.s.*, and the previously unnamed new genus sensu Gurgel and Fredericq.* One of our key findings is that *Hydropuntia* is not monophyletic (Fig. 1). This was previously demonstrated by Bellorin et al. (2002), but our analysis includes enhanced taxon sampling and phylogenetic resolution. Here, species in the genus are placed in two separate clades: a well-supported (100 BP and 1.0 PP) core *Hydropuntia* clade (III, Fig. 1), which includes most members of the genus, and a lone species, *Gracilaria rangiferina* (clade I, Fig. 1; traditionally placed in *Hydropuntia*), which is well-supported (94 BP and 1.0 PP) as sister to the rest of *Gracilaria* (Fig. 1). The latter placement is particularly relevant because *G. rangiferina* forms a clade in our expanded *rbc*L (Fig. 2) analysis with the type species of the genus, *Hydropuntia urvillei* Montagne. With the exception of *G. rangiferina*, species in this clade possess cylindrical thalli (vs. flat in *G. rangiferina*) and all species have henriquesiana type spermatangia. Finally, our inference of the nonmonophyly of *Hydropuntia* is robust: a monophyletic *Hydropuntia* is strongly rejected (AU test, $P = 0.028$).

Gurgel and Fredericq (2004) identified a weakly supported *Hydropuntia* clade, which they proposed to maintain as distinct from *Gracilaria* on morphological grounds. They argued that species of *Hydropuntia* possess cystocarps composed of gonimoblast cells in short chains terminating in apical carposporangia that are arranged radially, and nutritive tubular cells connecting the gonimoblast to the base of the cystocarp. For *Gracilaria* s.s., in contrast, they indicated that species possess cystocarps composed of gonimoblasts and carposporangia arranged in longer chains that are dichotomously or irregularly branching, and nutritive tubular cells connecting the gonimoblast to the outerpericarp. In addition, Gurgel and Fredericq used spermatangia type to segregate these two genera. They argued that *Gracilaria* s.s. includes species with only verrucosa and textorri spermatangia. In contrast, *Hydropuntia* species possess only the henriquesiana type.

The morphological characters differentiating *Hydropuntia* from *Gracilaria* tend to overlap between species, which calls into question the separation of *Hydropuntia* sensu Gurgel and Fredericq. As previous authors have observed (Bellorin 2002, Nunes 2005), some *Hydropuntia* species possess nutritive tubular cells connecting the gonimoblast to the outer pericarp (e.g., *H. caudata* (J. Agardh) Gurgel & Fredericq), and some *Gracilaria* species possess nutritive tubular cells connecting the gonimoblast to the base of the cystocarp (e.g., *G. domingensis*). In the case of spermatangia*,* Oliveira et al. (1983) determined that the type specimen of *Gracilaria domingensis* (MEL44407) possessed the verrucosa type spermatangia. However, in subsequent morphological analyses by others (Abbott et al. 1991, Bellorin et al. 2002), species of *Gracilaria* were determined to exhibit both verrucosa and henriquesiana type spermatangia, including *G. domingensis* (Kützing) Sonder ex Dickie. This indicates that spermatangia type does not readily distinguish *Hydropuntia* from *Gracilaria* s.s., as circumscribed by Gurgel and Fredericq. Moreover, Abbott et al. (1991) identified that at least in some cases, these spermatangia types are found together on a single specimen, and thus likely represent different stages in a developmental series. In these cases, the verrucosa type was hypothesized to represent the early developmental stage, and the henriquesiana type the later stage. On the basis of these results, we propose that the genus *Gracilaria* be expanded to include *Hydropuntia*. The combination of spermatangia and cystocarp morphology that have traditionally been used to define these genera overlap in some cases, thus motivating our broad taxonomic concept for the genus.

Gurgel and Fredericq (2004) also found a well-supported clade (98 BP and 0.99 PP) within *Gracilaria* s.l., which they identified as a new, but unnamed, genus. The species in this group have a Pacific distribution and are characterized by their terete and slender thallus, with a gradual transition from the medullary to the cortical region. These characteristics are shared with at least some *Gracilaria* species (e.g., *G. caudata* and *G. birdiae*). Their argument for maintaining these species as a separate genus was based on their phylogeny, and unspecified differences in reproductive characters. Abbott et al. (1991), Bellorin (2002), and Terada and Yamamoto (2002), however, indicated that species in this clade exhibit a broad and diverse array of spermatangia types. Species of this unnamed genus are represented in our phylogeny by *G. chilensis, G. tenuistipitata*, and *Gracilaria vermiculophylla* (Ohmi) Papenfuss (clade II, Fig. 1). Here, *G. chilensis* and *G. tenuistipitata* have textorii type spermatangia; *G. vermiculophylla* (Ohmi) Papenfuss, in contrast, has verrucosa type spermatangia (Terada and Yamamoto 2002). Cystocarp features are also not unique to species in this clade. They share the presence of nutrient tubular cells that are restricted to the cystocarp floor with other species of *Hydropuntia* (Liao and Hommersand 2003). Thus, on the basis of this information, we propose that in addition to expanding *Gracilaria* to include members of *Hydropuntia*, we propose the same approach to members of the unnamed genus sensu Gurgel and Fredericq (2004).

Finally, although we identified several additional well-supported subclades of *Gracilaria*, these clades are not readily diagnosed by geography or morphology. Of these, three clades are worth more detailed discussion. The well-supported clade (100 BP and 1.0 PP) including the species *Gracilaria cornea* (as *Hydropuntia cornea*), *G. birdiae,* and *G. caudata* (as

H. caudata; clade III, Fig. 1) are diagnosed by their cylindrical thalli and verrucosa or henriquesiana type spermatangia. These species are mostly distributed around the Atlantic, except *G. cornea,* which also occurs in the Indian Ocean on the coast of Tanzania. Many of these features, however, that characterize this clade are likely symplesiomorphic because they are also found in the more distantly related clade *G. gracilis* (IV, Fig. 1), which has a wide distribution including the Atlantic, Pacific, and Indian Oceans. This latter species is in turn well-supported (86 BP and 1.0 PP) as sister to a clade (V, Fig. 1) of species with exclusively flattened thalli and mostly textorii type spermatangia (cf. *G. domingensis* and *Gracilaria* sp.2 nov.). Further phylogenetic sampling, including additional genes and taxa, plus more detailed information on morphology is necessary before further taxonomic re-circumscriptions can be proposed within *Gracilaria*.

Gracilariopsis. *Gracilariopsis* is an easily characterized genus and forms a strongly supported clade (98 BP and 1.0 PP in the *rbc*L tree, and 100 BP and 1.0 PP in the combined analyses). The genus is diagnosed by species with spermatangia distributed in superficial layers, and cystocarps that lack nutrient tubular cells.

Species determination with barcoding. DNA barcoding has been shown to be a potentially powerful tool for identifying cryptic new species (e.g., Hebert et al. 2004). In our analyses, we identify four potentially new species of Gracilariacae from Brazil. These species are labeled in our figures as *Gracilaria* sp.1, *Gracilaria* sp.2, *Gracilaria* sp.3, and *Gracilaria* sp.4. They are diagnosed based on *rbc*L sequences, which are 2.8%–5.8% divergent from their closest relatives. Additionally, these specimens are morphologically distinct from other known species of *Gracilaria* and form separate clades in our molecular analysis (Figs. 1 and 2). These new species will be described and diagnosed in an upcoming publication (Lyra et al. unpublished) and will include additional samples to describe all of the relevant reproductive stages.

For one of these new species, we identified discrepancies between our identifications and the identified material available in GenBank. The specimens we assigned to *Gracilaria* sp.1 in our phylogeny (Fig. 2) were identical to GenBank accessions (AY049326 and AY049327) previously identified as *Gracilaria damaecornis* Agardh by Gurgel and Fredericq (2004). Their specimens of *G. damaecornis*, however, lacked a description of the spermatangia, which is important for species identification. Our barcoded samples from members of the clade that included the specimens identified as *Gracilaria damaecornis* by Gurgel and Fredericq were taken from diverse localities and possess flattened thalli and textorii type spermatangia. If these samples were *G. damaecornis*, in contrast, they should possess a terete or subterete thallus with verrucosa type spermatangia (Bellorin 2002). Thus, given this distribution of character states and our phylogenetic findings, we think it is unlikely that the accessions identified by Gurgel and Fredericq (2004) are *G. damaecornis*. At the moment, it is not possible to easily relate members of this clade to any described species of Gracilariaceae, and we thus think it represents an undescribed species (*Gracilaria* sp.1.).

Another situation that deserves attention is the species *Gracilaria yoneshigueana* Gurgel, Fredericq & J. N. Norris. This species was previously described using molecular data from a juvenile specimen (Gurgel et al. 2004; US204329). Our phylogeny (Fig. 2), which includes the Gurgel et al. (2004) Genbank accession of *G. yoneshigueana* (AY049372), places this species in a clade with moderate to high support (Fig. 2; 72 BP and 0.92 PP) with other accessions we barcoded as *Gracilaria domingensis*. In contrast, Gurgel et al. inferred that this new species was instead distantly related to *G. domingensis*. However, from our analysis, it appears that their reference specimen of *G. domingensis* (AY049371) was incorrectly identified. Our analysis instead indicates that this misidentified specimen is a well-supported (100 BP and 1.0 PP) member of one of the two *G. cervicornis* clades we identified (Fig. 2). A morphological assessment of our specimens identified as *G. cervicornis* verify this claim: all specimens we examined, representing both subclades of *G. cervicornis*, possess textorii type, and not verrucosa/henriquesiana type, spermatangia like those of *G. domingensis* (Fig. 1). We additionally confirmed this key difference by examining the type material of *G. cervicornis* (BM000936191), and the protologue and type (the latter by one of us, E.C.O.; MEL44407) of *G. domingensis*. Under these circumstances, we have doubt about the taxonomic status of the newly described species *G. yoneshiguena*. Given the close phylogenetic associations and lack of obvious morphological differences between *G. yoneshigueana* and *G. domingensis*, we propose that *G. yoneshigueana* be recognized as a synonym of *G. domingensis*.

Our DNA barcoding also helped to better access Gracilariaceae species distribution. Using morphological and anatomical analysis, Nunes (2005) identified 19 species of Gracilariales in Bahia, Brazil. We identified that eight species identified by Nunes were misidentified, indicating the limitation of using only morphology in the taxonomy of this group. Additionally, we recorded two new occurrences for Brazil, *Gracilaria hayi* and *Gracilariopsis silvana*. *G. hayi* is morphologically very similar to *G. cuneata*, and *Gp. silvana* is the only *Gracilariopsis* species with a flattened thallus described to date. The later species is likely to be more common than thought because it is often misidentified as *G. cervicornis*. Along these lines, other species that are difficult to distinguish on morphological grounds are likely to benefit from barcoding. *G. birdiae*, for example, is thought to be morphologically indistinguishable from *G. caudata* (Costa et al.

2012). In our analyses, however, these species are easily distinguished from each on molecular grounds. Individuals of each species (Fig. 1 and 2) from diverse populations form reciprocally well-supported clades (100 BP and 1.0 PP for each species). Furthermore, *cox*1 additionally indicates that the divergence within each subclades is at least 9.9%, suggesting the possibility of cryptic species within these species. Our material of *G. birdiae* is more closely related to *G. cornea*, a well-supported clade (100 BP and 0.1 PP, Fig. 1) whose divergence from *G. birdiae* can be as low as 2.1% (*cox*1).

Conclusions. Our analyses demonstrate the utility of the barcoding loci *cox*1 and UPA for species delimitation and for broader phylogenetic analyses. When analyzed simultaneously with *rbc*L, they increased resolution and support, resulting in the largest and most well-resolved phylogeny of Gracilariales to date. Our results demonstrate that *Hydropuntia* is nonmonophyletic, and based on morphological and anatomical grounds should be transferred to *Gracilaria*. We identified four potentially new species of Gracilariaceae from Brazil, and recorded two new occurrences for Brazil, *Gracilaria hayi* and *Gracilariopsis silvana*. Molecular and morphological results showed *G. yoneshigueana* cannot be distinguished from *G. dominguensis*, and should be synonymized accordingly. *G. cervicornis* is not monophyletic and represents at least two species, whose limits demand additional morphological studies. Our combined morphological, anatomical, and molecular study has allowed us to better understand the phylogeny and taxonomy of Gracilariaceae, and to contribute to the identity of hidden diversity in a difficult clade from a poorly sampled tropical area.

TAXONOMIC CHANGES

Gracilaria caudata J. Agardh, 1852, Sp. Gen. Ord. Alg. 2(2), p. 598.

Homotypic synonym: *Ceramianthemum caudatum* (J. Agardh) Kuntze, 1891; *Hydropuntia caudata* (J. Agardh) Gurgel and Fredericq 2004.

Gracilaria cornea J. Agardh, 1852, Sp. Gen. Ord. Alg. 2(2), p. 598.

Homotypic synonym: *Ceramianthemum corneum* (J. Agardh) Kuntze, 1891; *Hydropuntia cornea* (J. Agardh) M. J. Wynne 1989.

Gracilaria corymbiata (N. Rodríguez de Rios) E. K. Ganesan, 1990, A catalog of benthic marine algae and seagrasses of Venezuela, p. 237.

Basionym: *Polycavernosa corymbiata* N. Rodríguez de Rios, 1986, (38), p. 23.

Homotypic synonym: *Hydropuntia corymbiata* (N. Rodríguez de Rios) M. J. Wynne 1989.

Gracilaria crassissima (P. L. Crouan & H. M. Crouan) P. L. Crouan & H. M. Crouan, 1866, Essai de classification des algues de la Guadeloupe, p. 46.

Basionym: *Plocaria crassissima* P. L. Crouan & H. M. Crouan, 1865, Essai Alg. Guadeloupe, p. 20.

Homotypic synonyms: *Polycavernosa crassissima* (P. L. Crouan & H. M. Crouan) Fredericq & J. N. Norris, 1985; *Hydropuntia crassissima* (P. L. Crouan & H. M. Crouan) M. J. Wynne 1989.

Heterotypic synonym: *Gracilaria horizontalis* F. S. Collins & Hervey, 1917.

Gracilaria crouaniorum G. M. Lyra, J. M. C. Nunes & C. C. Davis, ***nom. nov.***

Replaced synonym: *Gracilaria secunda* P. L. Crouan & H. M. Crouan, Essai de classification des algues de la Guadeloupe, 1865, p. 19. (non *G. secunda* (C. Agardh) Zanardini, 1840).

Homotypic synonym: *Hydropuntia secunda* Gurgel and Fredericq 2004.

Gracilaria edulis (S. G. Gmelin) P. C. Silva, 1952, A review of nomenclatural conservation in the algae from the point of view of the type method, p. 293.

Basionym: *Fucus edulis* S. G. Gmelin, 1768, p. 113.

Homotypic synonyms: *Fucus lichenoides* var. *edulis* (Gmelin) Turner, 1808; *Fucus lichenastrum* var. *edulis* (Gmelin) Poiret, 1817; *Hydropuntia edulis* (S. G. Gmelin) Gurgel and Fredericq 2004; *Fucus coralloides* Poiret, 1808 (nom. superfl. & illegit.); *Sphaerococcus lichenoides* var. *tenuis* C.Agardh, 1822 (nom. superfl. & illegit.); *Alga coralloides* Rumphius 1750 (invalid).

Heterotypic synonymy: *Fucus lichenoides* Turner, 1808. *(non* S. G. Gmelin 1768); *Gigartina lichenoides* J. V. Lamouroux, 1813.; *Sphaerococcus lichenoides* (J. V. Lamouroux) C.Agardh, 1817; *Fucus lichenastrum* Poiret, 1817; *Gracilaria lichenoides* (J. V. Lamouroux) Greville, 1830; *Plocaria lichenoides* (J. V. Lamouroux) J.Agardh, 1847; *Ceramianthemum lichenoides* (J. V. Lamouroux) Kuntze, 1891; *Sphaerococcus vieillardii* Kützing, 1863; *Sphaerococcus lemania* Kützing, 1868; *Gracilaria lichenoides* f. *lemania* (Kützing) V.May, 1948; *Sphaerococcus setaceus* Kützing, 1868, illegit (non J. Agardh ex Frauenfeld 1854); *Sphaerococcus spinescens* Kützing, 1868; *Gracilaria spinescens* (Kützing) J.Agardh, 1876; *Ceramianthemum spinescens* (Kützing) Kuntze, 1891; *Polycavernosa fastigiata* C. F. Chang & B. M. Xia,1963; *Hydropuntia fastigiata* (Chang & B. M. Xia) M. J. Wynne 1989; *Gracilaria taenioides* J.Agardh, 1852; *Ceramianthemum taenioides* (J. Agardh) Kuntze, 1891; *Gracilaria lichenoides* f. *taenioides* (J. Agardh) V. May, 1948; *Gracilaria bifaria* J. Agardh, 1901.

Gracilaria eucheumatoides Harvey, 1860, Characters of new algae, chiefly from Japan and adjacent regions, collected by Charles Wright in the North Pacific Exploring Expedition under Captain James Rodgers, p. 331.

Homotypic synonym: *Ceramianthemum eucheumatoides* (Harvey) Kuntze 1891; *Hydropuntia eucheumatoides* (Harvey) Gurgel and Fredericq 2004.

Gracilaria multifurcata Børgesen, 1953, Some marine algae from Mauritius. Additions to the parts previously published, p. 42.

Homotypic synonyms: *Polycavernosa multifurcata* (Børgesen) Chang & B. Xia, 1963; *Hydropuntia multifurcata* (Børgesen) M. J. Wynne 1989.

Gracilaria perplexa K. Byrne & Zuccarello, 2002, *Gracilaria* species (Gracilariaceae, Rhodophyta) from southeastern Australia, including a new species, *Gracilaria perplexa* sp. nov.: Morphology, molecular relationships, and agar content, p. 302.

Homotypic synonym: *Hydropuntia perplexa* (K. Byrne & Zuccarello) Conklin, O_Doherty & A. R. Sherwood, 2014.

Gracilaria preissiana (Sonder) Womersley, 1976, Studies on southern Australian taxa of Solieriaceae, Rhabdoniaceae, and Rhodophyllidaceae (Rhodophyta), p. 109.

Basionym: *Rhodymenia preissiana* Sonder, 1845, p. 56.

Homotypic synonyms: *Rhodophyllis preissiana* (Sonder) Kützing 1849; *Calliblepharis preissiana* (Sonder) Harvey 1859; *Hydropuntia preissiana* (Sonder) Gurgel and Fredericq 2004.

Heterotypic synonyms: *Calliblepharis pannosa* Harvey 1855; *Gracilaria pannosa* (Harvey) J. Agardh 1885; *Ciliaria pannosa* (Harvey) Kuntze 1891.

Gracilaria rangiferina (Kützing) Piccone, 1886, Alghe del viaggio di circumnavigazione della Vettor Pisani, p. 71.

Basionym: *Sphaerococcus rangiferinus* Kützing, 1849, Sp. Alg., p. 779.

Homotypic synonyms: *Hydropuntia rangiferina* (Kützing) Gurgel and Fredericq 2004.

Heterotypic synonyms: *Gracilaria dentata* J. Agardh1852; *Ceramianthemum dentatum* (J. Agardh) O. Kuntze 1891; *Polycavernosa dentata* (J. Agardh) Lawson & John, 1982; *Hydropuntia dentata* (J. Agardh) Wynne 1989, *Gracilaria henriquesiana* Hariot, 1908; *Polycavernosa henriquesiana* (Hariot) Chang et Xia, 1968; *Hydropuntia henriquesiana* (Hariot) Wynne 1989.

Gracilaria tsudae (I. A. Abbott & I.Meneses) I. A. Abbott, 1991, *Gracilaria mixta*, sp. nov. and other western Pacific species of the genus (Rhodophyta: Gracilariaceae), p. 223.

Basionym: *Polycavernosa tsudae* I. A. Abbot & I. Meneses, 1987, p. 195.

Homotypic synonyms: *Hydropuntia tsudae* (I. A. Abbott & I. Meneses) M. J. Wynne 1989.

Gracilaria urvillei (Montagne) I. A. Abbott, 1991, *Gracilaria mixta*, sp. nov. and other western Pacific species of the genus (Rhodophyta: Gracilariaceae), p. 23.

Basionym: *Hydropuntia urvillei* Montagne, 1842, p. 7.

Homotypic synonym: *Hydropuntia urvillei* Montagne, 1842.

Gracilaria usneoides (C. Agardh) J. Agardh, 1852, Species genera et ordines algarum, seu descriptiones succinctae specierum, generum et ordinum, quibus algarum regnum constituitur, 2(2), p. 595.

Basionym: *Sphaerococcus usneoides* C. Agardh, 1822, p. 333.

Homotypic synonyms: *Laurencia usneoides* (C. Agardh) Kützing 1849; *Ceramianthemum usneoides* (C. Agardh) Kuntze 1891; *Hydropuntia usneoides* (C. Agardh) Gurgel and Fredericq 2004.

Gracilaria xiae-abbottii G. M. Lyra, J. M. C. Nunes & C. C. Davis, ***nom. nov.***

Replaced synonym: *Polycavernosa divergens* Xia & Abbott, 1987, (26), p. 409.

Synonym: *Hydropuntia divergens* (B. M. Xia & I. A. Abbott) M. J. Wynne 1989.

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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:

Figure S1. The optimal maximum likelihood (ML) topology based on the *cox*1 data set (ntax = 37, missing data = 0.5%).

Figure S2. The optimal maximum likelihood (ML) topology based on the UPA data set (ntax = 32, missing data = 0%).

Table S1. Taxa sequenced in this study with voucher information, and GenBank accession numbers.

Table S2. Published taxa included in the analysis, with locality information, reference publication, and GenBank accession numbers.