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Sequencing of the *rbcl* Marker Reveals the Nonnative Red Alga *Grateloupia taiwanensis* (Halymeniaceae, Rhodophyta) in Alabama

MICHAEL S. DEPRIEST AND JUAN M. LÓPEZ-BAUTISTA

Mobile Bay, AL has been the site for the introduction of several terrestrial and freshwater invasive species, including red imported fire ants (*Solenopsis invicta*) and spike-topped apple snails (*Pomacea bridgesii*). The Gulf of Mexico has also been invaded by several marine animal species, such as zebra mussels (*Dreissena polymorpha*). To date, no invasive marine macroalga has been reported in the Mobile Bay area. However, recent collections of an unusual species of *Grateloupia* (Halymeniaceae, Rhodophyta) in Alabama indicate that an introduction has been made. On the basis of phylogenetic analysis of the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (*rbcl*) marker, the species has been identified as *Grateloupia taiwanensis* S.M. Lin & H.Y. Liang. This is the first report of *G. taiwanensis* outside its native range.

Grateloupia C. Agardh is a genus of benthic marine red algae (Rhodophyta), currently containing around 90 species (Guiry and Guiry, 2012). It is the largest genus in the family Halymeniaceae. Species of this genus occur throughout the world in warm temperate to tropical marine waters. Several *Grateloupia* species are known in the Gulf of Mexico, specifically *Grateloupia gibbesii* Harvey and *Grateloupia pterocladina* (M.J. Wynne) S. Kawaguchi & H.W. Wang, as well as many reports of unidentified *Grateloupia* species (see Fredericq et al., 2009). Wynne (2011) listed a total of 11 species in the western Atlantic, including *G. gibbesii*, *G. pterocladina*, and *Grateloupia filicina* (J.V. Lamouroux) C. Agardh. Wynne, however, remarked that past identifications of *G. filicina* in the western Atlantic, including the Gulf of Mexico, are doubtful due to the results of De Clerck et al. (2005b), which indicated that true *G. filicina* may be restricted to the Mediterranean Sea and Macaronesia. This suggests that tropical collections of *G. filicina* actually belong to a different species.

The genus *Grateloupia* is known for having simple morphologies that make distinguishing species difficult, and DNA sequencing has been instrumental in generic and species-level circumscriptions (e.g., Wang et al., 2001; De Clerck et al., 2005b; Lin et al., 2008). Specimens previously identified as *G. filicina*, frequently reported throughout the world, actually show an unexpectedly high amount of genetic diversity and are therefore morphologically static. As a result of *G. filicina* being demonstrated to be polyphyletic, several new species have been split from the group (e.g., Kawaguchi et al., 2001; De Clerck et al., 2005b). Even in the past few years, the genus

has gained many new species, including *Grateloupia huangiae* S.-M. Lin & H.-Y. Liang, *Grateloupia dalianensis* H.W. Wang & D. Zhao, and *Grateloupia yinggehaisensis* H.W. Wang & R.X. Luan. The publications in which these species are described (Lin and Liang, 2011; Zhao et al., 2012) include molecular phylogenetic analyses to more clearly delineate these taxa. Additional taxonomic work is definitely necessary to continue to resolve systematic inconsistencies and to account for unexpected, newly discovered diversity in the genus *Grateloupia*.

In addition to these taxonomic concerns, it is important to consider that *Grateloupia* contains species that are known to be aggressively invasive, most notably *Grateloupia turuturu* Yamada. *Grateloupia turuturu*, along with several other *Grateloupia* species, has been introduced in Italy (Cecere et al., 2011), New Zealand (D'Archino et al., 2007), Great Britain (Farnham and Irvine, 1973), France (Cabioch et al., 1997; Verlaque, 2001; Verlaque et al., 2005; Figueroa et al., 2007), and the Atlantic coast of the United States (Villalard-Bohnsack and Harlin, 1997; Gavio and Fredericq, 2002; Marston and Villalard-Bohnsack, 2002). Due to the difficulty and cost of stopping an invasive marine algal species—for example, the 2000 accidental introduction of the green alga *Caulerpa taxifolia* (M. Vahl) C. Agardh in California (Anderson, 2005)—efforts to prevent species introductions or to detect the presence of a potential species are imperative for conservation of native diversity. Recent collections of *Grateloupia* made by the authors on the Alabama coast have included specimens that could not be morphologically identified according to known taxa from the area. The

current paper presents the identification of a previously unknown nonnative species of *Grateloupia* from the Alabama Gulf Coast using large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (*rbcl*) sequence analysis, demonstrates its position within *Grateloupia* using phylogenetics, and suggests hypotheses regarding the possible causes and circumstances of its colonization.

MATERIALS AND METHODS

Twenty-one samples of *Grateloupia* of unknown species and four samples of *G. gibbesii* were collected from the locations listed in Table 1. Individuals were found growing in the intertidal or higher subtidal zone on rocks or cast ashore. Upon collection, a small portion of thallus was taken from each individual and desiccated in a plastic bag with silica gel for later molecular analysis. The remainder of each individual was vouchered on a herbarium sheet; specimens were deposited in The University of Alabama Herbarium. DNA extraction of the desiccated samples was performed using the DNEasy Plant Mini Kit (Qiagen, Valencia, CA). The manufacturer's recommendations were followed until the final elutions, which were performed with deionized water preheated to 65°C instead of the elution buffer.

The *rbcl* marker, widely used for red algae in both species identification (e.g., Saunders, 2009) and phylogenetics (e.g., De Clerck et al., 2005a), was amplified for all specimens. Polymerase chain reaction (PCR) followed the methods of Rindi et al. (2009). Primer sequences were provided by G. W. Saunders (University of New Brunswick, Fredericton, Canada, pers. comm.) after standard primers failed to amplify. Procedures for agarose gel electrophoresis, cleaning, quantification of DNA, and capillary sequencing were carried out according to Rindi et al. (2009). Sequences were assembled using Geneious Pro v5.1.7 (Drummond et al., 2010) and added to a database of published *rbcl* sequences from GenBank <<http://www.ncbi.nlm.nih.gov/genbank/>> for 18 *Grateloupia* samples selected as an accurate representation of genetic diversity in the genus (Table 1). The species *Yonagunia formosana* (Okamura) Kawaguchi & Masuda was selected as the outgroup after Lin and Liang (2011). Sequences were aligned using MUSCLE sequence alignment (Edgar, 2004) in Geneious. After alignment, sequences were manually checked for accuracy and truncated to uniform length to avoid including "missing" data due to incomplete and partial published sequences. Other than trimming, no adjustments were made

to the alignment. Pair-wise distances between sequences were calculated in Geneious when applicable.

Parameters for maximum likelihood (ML) and Bayesian inference (BI) were determined using jModelTest 2.1 (Guindon and Gascuel, 2003; Posada, 2008). ML analysis was executed in GARLI v2.0 (Zwickl, 2006) with 500 bootstrap replicates, starting from a random tree. Bootstrap confidence values were obtained via Consense (Felsenstein, 2005) on the CIPRES Science Gateway (Miller et al., 2010). Values obtained from Consense were converted to a percentage value and rounded down. BI was executed in MrBayes v3.1.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). The final tree was obtained in the NEXUS file format, rooted with *Y. formosana*, and processed in FigTree v1.3.1 <<http://tree.bio.ed.ac.uk/software/figtree/>> and Adobe Illustrator CS3 (Adobe Systems Incorporated, San Jose, CA) for publication.

RESULTS

After alignment, the *rbcl* data set consisted of 20 taxa with 1,195 base pairs each. All 21 sequences of *Grateloupia* from Alabama were identical, and all four sequences of *G. gibbesii* were identical; therefore, only one sequence was included in the alignment for each taxon. The alignment contained no gaps, reflecting an accurate alignment and the absence of insertions and deletions in *rbcl* of red algae. The TrN + G model was selected by ModelTest. Nucleotide frequencies, substitution rates, and gamma shape parameter were estimated by GARLI. The resulting phylogram, with bootstrap confidence values, is given in Figure 1. The alignment and tree produced in this analysis are available in TreeBASE: <<http://purl.org/phylo/treebase/phylows/study/TB2:S13391>>.

DISCUSSION

The current study presents samples from an unidentified *Grateloupia* population in Alabama. The *rbcl* sequences for the Alabama *Grateloupia* samples are identical to each other and nearly identical (<0.1% divergent) to *G. taiwanensis* S.-M. Lin & H.Y. Liang in Lin et al. (2008). Near-complete similarity indicates that these specimens are conspecific. The unidentified *Grateloupia* in Alabama is therefore determined to be *G. taiwanensis*. This assertion is supported by the sequence divergences among *G. taiwanensis* and some of its most closely related taxa (>3.1% divergent from *G. huangiae* and relatives) in this

TABLE 1. List of species used in this study, with collection information and references.

Species	Locality	Collection data	GenBank accession	Reference
<i>Grateloupia americana</i> S. Kawaguchi & H.W. Wang	Whale Park, near Sitka (Baranof Island), AK	S. Lindstrom, 21 April 2000	AF488814	Gavio and Fredericq (2002)
<i>Grateloupia belangeri</i> (Bory) De Clerck, Gavio, Fredericq, Cocquyt & Coppejans	Yzerfontein, Western Cape Province, South Africa	O. De Clerck, 24 Nov. 1999	AY772035	De Clerck et al. (2005a)
<i>Grateloupia capensis</i> O. De Clerck	Kommeġje, Cape Peninsula, South Africa	O. De Clerck, 1 June 2003	AJ868466	De Clerck et al. (2005b)
<i>Grateloupia doryphora</i> (Montagne) M.A. Howe	Playa de San Francisco, Bahía de Ancón, Ancón, Lima, Peru	P. Carbajal, 15 Sep. 2001	AF488817	Gavio and Fredericq (2002)
<i>Grateloupia elliptica</i> Holmes	Oryuzako, Miyazaki Prefecture, Japan (cultured material)	21 Dec. 1997	AB038605	Wang et al. (2000)
<i>Grateloupia filicina</i> (Lamouroux) C. Agardh	Cala Aiguafreda, Begur, Province of Girona, Spain	L. Lavelli, 1 May 2002	AJ868474	De Clerck et al. (2005b)
<i>Grateloupia gibbesii</i> Harvey	Charleston Harbor, Charleston, SC	M.S. DePriest, 22 June 2012	JX645160	This study
<i>Grateloupia huangiae</i> S.-M. Lin & H.-Y. Liang	Makang, Taipei County, N.E. Taiwan	S.-M. Lin, 30 April 2002	HME590410	Lin & Liang (2011)
<i>Grateloupia longifolia</i> Kytlin	Yzerfontein, Western Cape Province, South Africa	O. De Clerck, 2 June 2003	AY772023	De Clerck et al. (2005a)
<i>Grateloupia minima</i> P.L. Crouan & H.M. Crouan	Yzerfontein, Western Cape Province, South Africa	O. De Clerck, 2 June 2003	AJ868487	De Clerck et al. (2005b)
<i>Grateloupia phuquocensis</i> Tanaka & Pham-Hoàng Hô	Kaalawai, Oahu, HI	O. De Clerck, 26 April 2003	AY772022	De Clerck et al. (2005a)
<i>Grateloupia somalensis</i> Hauck	Plage de Monseigneur, Fort Dauphin, Madagascar	E. Coppejans, 31 Aug. 2002	AY772021	De Clerck et al. (2005a)
<i>Grateloupia sparsa</i> (Okamura) Chiang	Ōhara, Chiba Prefecture, Japan	9 April 2000	AB055473	Kawaguchi et al. (2001)
<i>Grateloupia subpectinata</i> Holmes	Irago-misaki, Asumi Peninsula, Aichi Prefecture, Japan	3 July 2000	AB114213	Faye et al. (2004)
<i>Grateloupia taiwanensis</i> S.-M. Lin & H.-Y. Liang	Sail Rock, Kenting National Park, S. Taiwan	S.-M. Lin, 2 Oct. 2002	EU292742	Lin et al. (2008)
<i>Grateloupia turuturu</i> Yamada	Onabama, Iwaki, Fukushima Prefecture, Japan	16 Sep. 1999	AB055475	Kawaguchi et al. (2001)
<i>Grateloupia turuturu</i>	The Kench, Hayling Island, Langstone Harbour, Hampshire, UK	R.L. Fletcher, 28 March 2002	AY100002	Gavio and Fredericq (2002)
<i>Grateloupia turuturu</i>	Lagoon of Venice, Venice, Italy	A. Sfriso	FN821950	Cecere et al. (2011)
<i>Grateloupia</i> sp.	Fort Morgan, AL	J.M. López-Bautista, 5 May 2004	JX645159	This study

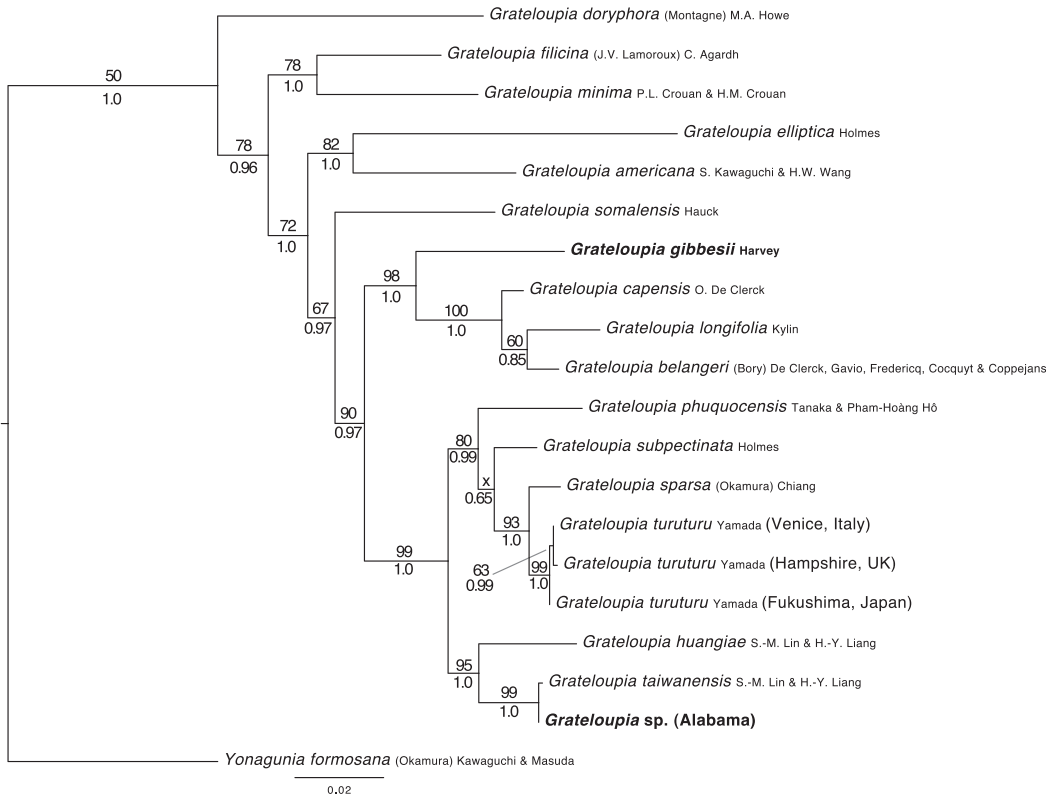


Fig. 1. Maximum likelihood (ML) phylogram of the *rbcL* marker of selected species of *Grateloupia*, using the TrN + G model. Numbers above branches indicate ML bootstrap confidence values (500 replicates). Numbers below branches indicate Bayesian posterior probabilities. “X” indicates support <50%. Scale bar = substitutions per site.

analysis and previously published phylogenies. *Grateloupia taiwanensis* has not been previously reported from the Gulf of Mexico (Fredericq et al., 2009) or the western Atlantic (Wynne, 2011). Before the current study, the distribution of *G. taiwanensis* was known to include only Taiwan (Lin et al., 2008). Therefore, we consider *G. taiwanensis* to be a nonnative species in the Gulf of Mexico. We also consider the introduction of *G. taiwanensis* to be recent; this is supported by the lack of previous reports of *Grateloupia* specimens from the Gulf of Mexico with the morphological features typical of *G. taiwanensis* (large size and proliferous blades, see Figure 2, which make it very conspicuous in intertidal habitats) and the previous experience of the authors collecting in the Gulf of Mexico.

On the basis of its *rbcL* sequence, *G. gibbesii* does not appear to be conspecific with any species in this analysis or with any species with a published *rbcL* sequence in GenBank. The samples collected of this species are very close (~5 km) to the type locality of this alga, Sullivan’s Island, SC (Harvey, 1853). Therefore,

we conclude that our identification of this alga is correct and that *G. gibbesii* represents a unique evolutionary lineage. Before the current study’s publication, the authors became aware of the possibility that the unknown *Grateloupia* found in Alabama might not be *G. taiwanensis* but *G. gibbesii* because this species had already been known in the Gulf of Mexico and sequence data had not been generated for it. However, because these two species show a sequence divergence of 6.8% in *rbcL*, this is not the case. Future collections and sequencing of *G. gibbesii* from the Gulf of Mexico are needed to confirm its presence.

The phylogeny reconstructed in the current study shows that *G. taiwanensis* is closely related to other taxa known primarily from the Pacific Ocean. Of the species included in the analysis, *G. huangiae* was described most recently (Lin and Liang, 2011) and is found in Taiwan. *Grateloupia sparsa* is widely distributed in the Asian Pacific, along with *G. turuturu*. However, *G. turuturu* is found throughout the world as an invasive species. None of these species has been found



Fig. 2. Herbarium sample of *Grateloupia taiwanensis* from Alabama.

in the Gulf of Mexico, and of these, only *G. turuturu* is known outside the Pacific Ocean. On the basis of these distributions, it appears likely that the most recent common ancestor of this group occurred in Asia and that *G. taiwanensis* was introduced to Alabama from Taiwan, rather than vice versa. This is concordant with the pattern of introductions of species of *Grateloupia* from Asia, most notably *G. turuturu* but also including several other species (Verlaque et al., 2005).

Apart from the current study, which reports *G. taiwanensis* for the first time from Alabama, the extent of the occurrence of *G. taiwanensis* in the Gulf of Mexico is currently unknown. Additional collections are being made to *Grateloupia taiwanensis* to determine their current expansion and any possible detrimental effects this introduction might have on marine communities.

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