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Red alga *Grateloupia imbricata* (Halymeniaceae), a species introduced into the Canary Islands

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SUMMARY

Specimens of *Grateloupia* from Gran Canaria in the Canary Islands were used to molecularly ascertain which of the species has been used in physiological and bio-technological experiments. The *rbc*L sequence analysis revealed that four out of five analyzed specimens (i.e. those commonly collected for physiological research) formed a monophyletic clade with *G. imbricata* from Korea, Japan, and China, and were quite different from any other species of the genus. Another sample, which was associated with cage nets used for fish aquaculture, was grouped with *G. lanceolata* from Japan, though it appears too early as yet to identify it as such. This is, thus, proof of a new introduction of a marine macroalga, since *G. imbricata* is an Asian species, native to Japan and Korea, in the Canary Islands. The role of international shipping in the introduction of the species is discussed.

Key words: *Grateloupia imbricata*, introduced species, *rbc*L, Rhodophyta, the Canary Islands.

INTRODUCTION

Extensive physiological and bio-technological research has been produced, using specimens of *Grateloupia* collected and cultured in the Canary Islands in the Atlantic (e.g. Robaina *et al*. 1990a,b; García-Jiménez *et al*. 1996, 1998, 2007; Rodrigo & Robaina 1997; Marián *et al*. 2000; Sacramento *et al*. 2004, 2007). Most of the experiments were carried out with samples assigned to *Grateloupia doryphora* (Montagne) Howe.

Grateloupia is the largest genus of the Halymeniaceae, containing around 50 species, which are sometimes difficult to define due to their morphological variation and lack of clear-cut morphological characters. This situation is further complicated with the morphological variations associated with the colonization of new environments, since *Grateloupia* species have been reported as among the major invasive genera in the marine ecosystem (Inderjit *et al*. 2006). For example, controversy existed about the occurrence of *G. doryphora* in the European Ocean area, since it was difficult to sustain the contention of the probable route by which *G. doryphora* was introduced from the south-east Pacific to the European coasts. Eventually, Gavio and Fredericq (2002) pointed out that *Grateloupia turuturu* Yamada was the correct name of the specimens from the Atlantic, identified under the name *G. doryphora*. This work by Gavio & Fredericq in 2002 spurred our research team into action, since the *Grateloupia 'doryphora'* used for many physiological experiments in the Canary Islands, might require the corroboration of its actual taxonomic assignation.

Molecular markers have helped to assign and re-instate problematic algal species. The large subunit of the Rubisco (*rbc*L) has been very commonly used for identification and phylogeny at the various levels of taxonomic ranks of red algae (Freshwater *et al.* 1994; Kawaguchi *et al.* 2001; Yang & Boo 2004; De Clerck *et al.* 2005). In this work, *rbc*L DNA sequence data were used to taxonomically assign *Grateloupia* specimens used in physiological and bio-technological experiments.

MATERIALS AND METHODS

The descriptions of habitat, location and date of collection of the specimens used in this study are given in Table 1 and Figure 1. Voucher specimens are deposited in the Biology Department, University of Las Palmas de Gran Canaria Herbarium (BCM, sheets 1591, 1648, and 6100), in the Canary Islands, Spain, and at Chungnam National University Herbarium (CNUK, sheets C40121 and C40122), Daejeon, Korea.

DNA samples were extracted from approximately 5 mg of dried thalli, ground in liquid nitrogen with Invisorb Spin Plant Mini Kit (Invitek, Berlin-Buch, Germany), according to the manufacturer's instructions, or extracted from approximately 100 mg of tissue

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Table 1. Description of samples used in this study (details in Fig. 1)

Sample name, Location (collection date)	Habitat	GenBank accession number
San Cristobal, Las Palmas de Gran Canaria, Gran Canaria, Spain (May 2007)	Samples inhabiting rocky pools in the upper intertidal zone. Specimens of up to 7 cm high dichotomous ramification and irregular proliferations along the margins (Fig. 1C)	EU024817
Melenara, Telde, Gran Canaria, Spain (May 2007)	Samples inhabiting cracks near intertidal lower limits. Specimens small (1–2 cm high) and dichotomus branched	EU024818
Jinamar-1 Las Palmas de Gran Canaria, Gran Canaria, Spain (May 2006)	Samples inhabiting rocky pools in the upper, intertidal zone. Specimens of up to 7 cm high, dichotomous ramification and irregular proliferations along the margins very similar to Cristobal samples.	EU157960
Jinamar-2 (dichotomous), Las Palmas de Gran Canaria, Gran Canaria, Spain (May 2006)	Samples inhabiting cracks near intertidal lower limits. Specimens small (1–2 cm) and dichotomously branched similar to Melenara samples.	EU157959
Jaulas, Melenara, Telde, Gran Canaria, Spain (May 2007)	Samples inhabiting attached to the nets used in cages for fish aquaculture located 100 meters in front of Melenara population (EU024818). Specimens that may reach 20 cm high flattened, gelatinous thalli of dichotomous ramification and irregular proliferations along the margins	EU024819
Korea-21 Cheju Island, Korea (April, 2007)	Attached on rocks at lower intertidal zone. Specimen with dichotomous branches, with a size of approx. 4 cm (Fig. 1D)	EU152267
Korea-22 Cheju Island, Korea (April, 2007)	Attached on rocks at lower intertidal zone. Specimen with dichotomous branches, with a size of approx 3 cm	EU152268

following a standard Hexadecyltrimethylammonium bromide (CTAB) procedure Murray and Thompson (1980). The plastid *rbc*L region was amplified and sequenced using primers F7–R753 and F645–RrbcS (Freshwater & Rueness 1994; Lin *et al*. 2001; Gavio & Fredericq 2002). The polymerase chain reaction (PCR) was carried out using a total volume of 25 µL, containing 0.5 U *TaKaRa Ex Taq* DNA polymerase (TaKaRa Shuzo Co., Shiga, Japan), 2.5 mM of each dNTP, 2.5 µL of the 10X *Ex Taq* Buffer (Mg^{2+} free), 2 mM MgCl2, 10 pmol of each primer and 1–10 ng template DNA. The PCR reaction was carried out with an initial de-naturation at 94°C for 5–10 min, followed by 35 cycles of amplification; 94°C for 30 s, 50°C for 30 s, and 72°C for 2 min or 98°C for 10 s, 55°C for 5 s, and 72°C for 30 s, with a final extension at 72°C for 10 min. The PCR products were purified with the High Pure PCR Product Purification Kit (Roche Diagnostics Gmbh, Mannheim, Germany) before direct sequencing. The sequences of the forward and reverse strands were determined for all taxa, using an ABI PRISM 377 DNA Sequencer (Applied Biosystems, Foster City, CA, USA) at the Research Centres of Chungnam National University, Daejeon, Korea and the University of Las Palmas de Gran Canaria. The electropherogram output for each specimen was edited using the program Sequence Navigator v. 1.0.1 (Applied Biosystems). The alignment of the *rbc*L sequences was based on the alignment of the inferred amino-acid sequence, and then was refined visually.

A total of 1006 base pairs (bp) *rbc*L were aligned for 52 taxa, including published sequences from GenBank (38 sequences): *Grateloupia imbricata* Holmes (AB061376, AB055479, AB061377, AB038607, AF299252), *Grateloupia lanceolata* (Okamura) Kawaguichi (AY775398, AY775385), *Grateloupia elliptica* Holmes (AB055476), *Grateloupia americana* S. Kawaguchi & H.W. Wang (AF48814, AY772037), *Grateloupia livida* (Harvey) Yamada (AF488815), *Grateloupia asiatica* S. Kawaguchi & H. W. Wang (AY775395), *Grateloupia acuminata* Holmes (AB055480), *Grateloupia schmitziana* (Okamura) S. Kawaguchi & H.W. Wang (AB061398), *Grateloupia divaricata* Okamura (AY178764), *Grateloupia turuturu* Yamada (AF488820, AJ868495, AY775386), *Grateloupia sparsa* (Okamura) Chiang (AB055474), *Grateloupia subpectinata* Holmes (AB114208, AB114213, AY775392), *Grateloupia phuquocensis* Tanaka & Pham-Hoàng Hô (AY772022), *Grateloupia longifolia* Kylin (AY772034), *Grateloupia belangeri* (Bory) De Clerck, Gavio, Fredericq, Cocquyt & Coppejans (AY772035), *Grateloupia capensis* O. De Clerck (AJ868466), *Grateloupia dichotoma* J. Agardh (AY772031, AF488823), *Grateloupia somalensis* Hauck (AY772021), *Grateloupia stipitata* J. Agardh (AF488816), *Grateloupia minima* P.L. Crouan & H.M. Crouan (AJ868471), *Grateloupia doryphora* (Montagne) M.A. Howe (AF488817), *Grateloupia carnosa* Yamada & Segawa (AB038608), *Grateloupia filicina* (J.V. Lamouroux) C. Agardh (AY772029), *Grateloupia hawaiiana* E.Y. Dawson *(AY772030), Grateloupia filiformis* Kützing (AF488822)*, Grateloupia ramossissima* Okamura (AF488811) and *Grateloupia catenata* Yendo (AB0386161) and *Pachymenia cornea* (Kützing) Chiang (U21588), *Pachymenia crassa* Lindauer (U21598),

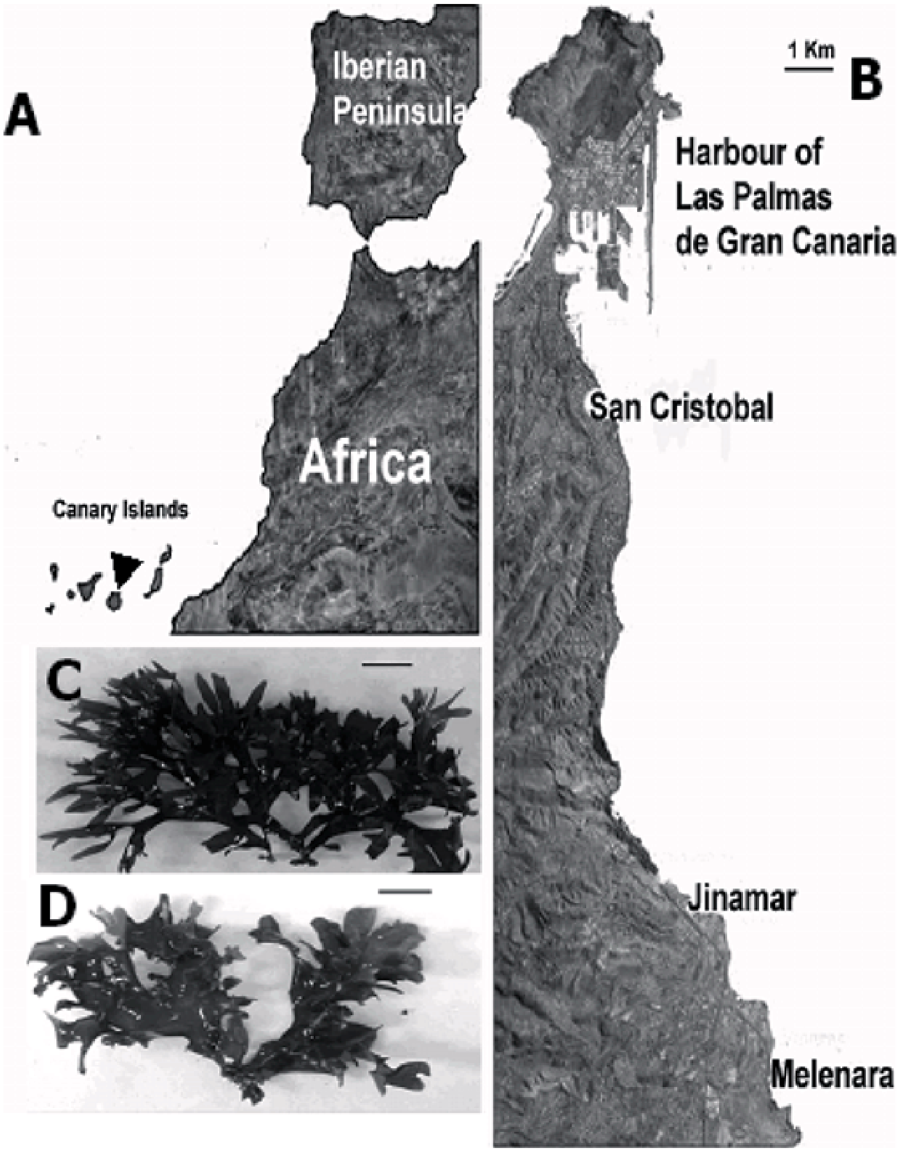

Fig. 1. Map, collection sites and habit of *Grateloupia imbricata*. (A) Map of the Canary Islands and Gran Canaria (arrowhead). (B) Collection sites along the northeast coast of Gran Canaria near the harbor of Las Palmas de Gran Canaria. (C) Habit of San Cristobal specimen from Gran Canaria. (D) Specimen from Korea. Bar, 1 cm.

Aeodes orbitosa (Suhr) F. Schmitz (U21599), *Halymenia dilatata* Zanardini (AB038604), *Halymenia floresia* (J. Agardh) Womersley & J.A. Lewis (AB038603), *Polyopes constrictus* (Turner) J. Agardh (AB055468) and *Polyopes polyideoides* Okamura (AB055469) were used as outgroups.

Maximum likelihood (ML) analyses were carried out using PAUP* 4.0b.10 (Swofford 2002). The model of sequence evolution was chosen, based on the results of the successive approximation method (Sullivan *et al*. 2005). The general time reversible (GTR) model with four-classed gamma distribution for rate heterogeneity (Γ) and proportion of invariable site (I) were used for evolution model parameter estimation. Tree likelihoods were estimated using a heuristic search with 100 random-addition-sequence replicates, and tree bisection–reconnection (TBR) branch swapping. To test the stability of nodes, bootstrap analyses were carried out with 500 replicates. Maximum parsimony (MP) analyses were conducted using PAUP*. All heuristic searches were carried out with 1000 replicates, using random-addition of taxa, keeping only the best tree, and holding 10 trees at each step, using TBR branch swapping, collapsing zero-length branches, and using MULTREES. Bootstrap support values were calculated using 1000 replicates with the following options selected: heuristic search; TBR branch swapping; collapse of zero-length branches; and random-sequence-addition with one replicate.

RESULTS

Seven sequences newly determined in the present study were deposited in the GenBank (Table 1). Variable sites were 346 positions (34.4%), and 269

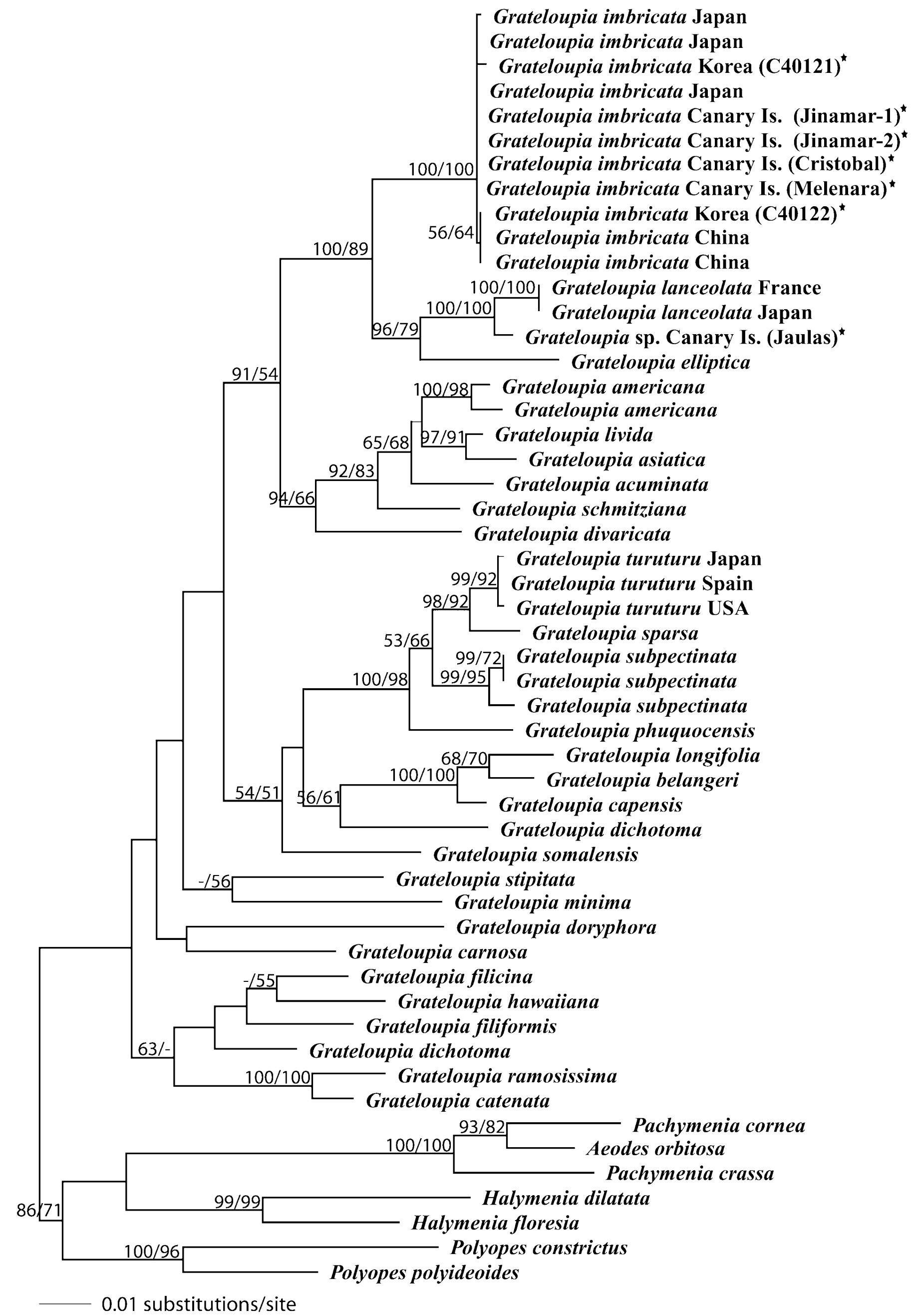

Fig. 2. Maximum likelihood (ML) tree of *Grateloupia imbricata* and *Grateloupia* sp. inferred from *rbc*L sequence calculated using the general time reversible (GTR) + Γ + I model of evolution ($-\ln L = 6808.6188$; substitution rate matrix $R_{AC} = 0.531$, $R_{AG} = 4.730$, $R_{AT} = 0.776$, $R_{CG} = 1.189$, $R_{CT} = 10.207$, $R_{GT} = 1$; base frequencies $\pi_A = 0.318$, $\pi_C = 0.146$, $\pi_G = 0.212$, $\pi_T = 0.323$; shape parameter $(\alpha) = 0.2870$; proportion of invariable site $(I) = 0.2143$). Star indicates sequences analyzed in the present study. Numerals above branch refer to ML and maximum parsimony (MP) bootstrap values. Bars represent nodes with support values <50%.

positions (26.7%) were parsimoniously informative in the sequences used in this study.

The *rbc*L analysis revealed that four out of five specimens (i.e. those from the coasts of San Cristobal, Jinamar and Melenara) formed a monophyletic clade with *Grateloupia imbricata* from Korea, Japan and China, and were quite different from other species of the genus (Fig. 2). The fifth specimen, collected from and associated with cage nets, was grouped with *G. lanceolata* from Japan; however, it appears too early as yet to identify it as such, due to the fact that a single specimen was used, which proved to have a sister group relationship to *G. lanceolata* (Fig. 2).

DISCUSSION

Since Haroun *et al*. (2002) reported the presence of *Grateloupia doryphora, G. dichotoma*, and *G. filicina* in the Canary Islands, our results provide evidence of the existence of two new records: *Grateloupia* sp. until further confirmation as *G. lanceolata,* and *G. imbricata.* The results obtained also give support for the misidentification of *G. imbricata* from at least these three populations, which have been identified as *G. doryphora,* at San Cristobal, Jinamar or Melenara, and used in biotechnological and physiological research.

Grateloupia imbricata, as is *G. lanceolata*, is an Asian species, native to Japan and Korea (Verlaque *et al*. 2005). Therefore, its presence in the Canary Islands provides evidence of its introduction as an alien species. To our knowledge, this is the first report on the introduction of the Asian *G. imbricata* in the Atlantic. It should thus be added to the list of introduced species of red algae from the northwest Pacific Ocean to the Atlantic Ocean region (Verlaque *et al*. 2005; Inderjit *et al*. 2006).

The introduction of marine species is a growing concern, and macroalgae constitute a significant component in the so-called Non-Indigenous Marine Species (NIMS, Schaffelke *et al*. 2006). International harbors are considered to be the main foci of introduction and the initial establishment of invading species (Ruiz & Hewitt 2002). The Canary Islands are volcanic oceanic islands (27°37′ to 29°25′-N; 13°20′ to 18°10′-W), located northwest of the African continent. This advantageous geographic position makes it a trade and communication hub between Europe, America and Asia, and most specifically with Korea, Japan and China, due to the large and important harbors on the islands that have supported conspicuous trade and fishery activities over the last 50 years (i.e. the Japan Tuna fisheries based in the harbor of Las Palmas de Gran Canaria). These facts point to international shipping as the plausible vector for the introduction (i.e. ballast waters, hull fouling. Mineur *et al*. 2007).

Following the criteria depicted in Inderjit *et al*. (2006), the introduced species of the genus *Grateloupia* may become invasive, in the sense that ecologic and/or economically harmful effects may follow an introduction. Recent examples that have shown this have been *G. turuturu* (as *G. doryphora*) along the Rhode Island coasts (Harlin & Villalard-Bohnsack 2001) and Brittany (Simon *et al*. 2001). In the case of the *G. imbricata* in Gran Canaria, still small populations are to be encountered at a few locations along the northeast coast through to the Southern city coastline, very close to the harbor of Las Palmas de Gran Canaria, which is the probable point of introduction (Fig. 1). Its spread around the Canary Islands and to other European or Atlantic coasts needs to be monitored. In conclusion, two new records of the *Grateloupia* species are reported for the Canary Islands; *Grateloupia* sp. (possibly *G. lanceolata*), and *G. imbricata,* a native Asiatic species, which has most probably been introduced into the islands through shipping.

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