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# RAPD differentiation of *Grateloupia lanceola* and the invasive *Grateloupia turuturu* (Gigartinales, Rhodophyta) in the Iberian Peninsula

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*Grateloupia* is a genus of red algae in which many species are notoriously difficult to define; this situation greatly complicates the assessment of their geographical ranges. A member of this genus, *G. lanceola,* is typical of warm-temperate seas but retains some highly localized populations at higher latitudes on the northwestern Iberian Peninsula (Galicia). Nevertheless, the presence of these northern populations has been largely overlooked; instead, they have been regarded as, or suspected to be, another case in the expansion of the adventive congeneric *Grateloupia turuturu* along European waters. In this study, we have examined the genetic similarity between specimens of *G. lanceola* and *G. turuturu* from Galicia using randomly amplified polymorphic DNA (RAPD) markers. The large genetic distance between species detected provides molecular-based support for the occurrence of *G. lanceola* in the northwestern Iberian Peninsula as a separate species from the invasive *G. turuturu,* corroborating earlier morphological and ecological observations. Also, *G. lanceola* specimens from Galicia were genetically similar to those from their type locality in the southern Iberian Peninsula, confirming their conspecific character. Our results imply that the first records of invasive *G. turuturu* in northwestern waters of Spain were those from the early 1990s; contrary to most references in the literature, previous older records from the early 1980s belonged to the congeneric *G. lanceola.*

KEY WORDS: *Grateloupia lanceola, Grateloupia turuturu,* Iberian Peninsula, invasive seaweeds, RAPD markers

## INTRODUCTION

The foliose species of the genus *Grateloupia* are a taxonomically challenging group of red algae, as many exhibit extensive morphological plasticity whereas distinctive taxonomical characters are usually scarce; predictably, the group has suffered several reorganizations (Ardré & Gayral 1961; Dawson *et al.* 1964; Gavio & Fredericq 2002). *Grateloupia lanceola* (J. Agardh) J. Agardh *emend.* Ardré & Gayral is a member of this group originally described from the southwestern Iberian Peninsula and northwest Africa (Agardh 1841). Typical of warm-temperate waters, this species has been reported widely along West Africa from Morocco and Senegal to Angola (Dangeard 1949; Gayral 1958; Ardré & Gayral 1961; Lawson & John 1982). However, its apparent range extends farther north: in the early 1980s *G. lanceola* was first reported from the northwestern Iberian Peninsula (Galicia), albeit restricted to small populations located within or at the immediate vicinity of a single bay (Bahía de La Coruña; Pérez-Cirera *et al.* 1989). Unfortunately, these records from Galicia have gone largely unnoticed, overlooked by a combination of the aforementioned taxonomic uncertainties that surround foliose *Grateloupia* (Ardré & Gayral 1961; Dawson *et al.* 1964) and the fact that another morphologically similar representative of this group, *Grateloupia turuturu* Yamada (as *G. doryphora,* see Gavio & Fredericq 2002), is a successful invasive seaweed on European Atlantic coasts (Farnham & Irvine 1973; Cabioch *et al.* 1997; Stegenga & Otten 1997; Maggs & Stegenga 1999) where it is expanding its range (Simon *et al.* 2001). Hence, the presence of the warm-temperate *G. lanceola* in the northwestern Iberian Peninsula waters has been interpreted either as another episode in the introduction of *G. turuturu* into European coasts (Cabioch *et al.* 1997; Marston & Villalard-Bohnsack 2000; Simon *et al.* 2001) or, more cautiously, referred to as *Grateloupia* sp., suggesting uncertainties regarding its identity and whether or not these populations were introduced (Marston & Villalard-Bohnsack 2002).

The invasive *G. turuturu* (as *G. doryphora*) was first recorded from the northwestern Iberian Peninsula in the early 1990s (ICES 1992). Thereafter, it has exhibited a distinctive range-expanding behavior (Bárbara & Cremades, unpublished observations) that sharply contrasts with the confined populations of *G. lanceola* from this region (Bárbara & Cremades 2004). The co-ocurrence of these two foliose *Grateloupia* species in the region offered a new opportunity to elucidate the uncertainty that has shadowed the presence of *G. lanceola* in northwestern Iberian Peninsula waters. In this study we used randomly amplified polymorphic DNA (RAPD) markers to compare these two foliose *Grateloupia* species currently found in northwestern Iberian Peninsula waters; our aim was to provide molecular-based evidence in support of our former morphology-based identification of *G. lanceola* as a species not to be confused with the invasive *G. turuturu.* Moreover, specimens of *G. lanceola* from Galicia were compared with individuals from the southern Iberian Peninsula area where this species was originally described back in the mid-nineteenth century (Agardh 1841) to reaffirm their conspecific character.

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## MATERIAL AND METHODS

On the basis of morphological features (in Bárbara & Cremades 2004), several populations of the two species of *Grateloupia* were identified in northwestern Iberian Peninsula waters. *Grateloupia lanceola* was reported from two sites, Isla Redonda (site code GR) and Santa Cristina (GC), both in the Bahía de La Coruña area; *G. turuturu* was reported from three sites: Isla Redonda (GR), Point Batuda (GB) and Tragove (GT) (Fig. 1); GR is, to our knowledge, the only location where both species occur in sympatry in Galicia. Additional specimens of *G. lanceola* were collected from two locations on the Mediterranean coast of the southern Iberian Peninsula: Point Araña (site code MA) and Point Misericordia (MM). Field-collected tetrasporophytes were transported in plastic bags inside a cool box to the laboratory; only healthy individuals devoid of epiphytes were retained for analyses. In the laboratory, algal samples were washed under running tap water and rinsed with distilled water. Washed samples were blotted dry on a piece of clean paper, desiccated in silica gel and kept at 10°C until analysis. Total genomic DNA extraction followed that described by Doyle and Doyle (1987) with minor modifications for DNA miniprep. Silica-gel–dried tissue (~1 $cm^2$) was ground with liquid nitrogen, placed in 2× CTAB isolation buffer (2% w/v hexadecyl-trimethylammonium bromide [CTAB], 1.4 M NaCl, 100 mM Tris-HCl pH 8.0, 0.2% v/v β-mercaptoethanol, 20 mM EDTA) with K-proteinase and left for 1–2 h at 55°C. The homogenate was then incubated 1 h at 37°C with 0.5 mg/ml A-RNase. DNA was then extracted twice with PCIA (phenol:chloroform:isoamyl alcohol, 25:24:1) and once with CIA (chloroform:isoamyl alcohol, 24:1), and precipitated with ice-cold isopropanol and 3 M sodium acetate. The pellet was washed with 70% ethanol (1 h), air-dried, and resuspended in 100 µl Tris-EDTA. DNA concentration was spectrophotometrically quantified (Hewlett-Packard UV 8453, Palo Alto, CA, USA) while quality was assessed by 1% agarose gel electrophoresis.

Initially, 13 decanucleotides of arbitrary sequence (primers Q3, Q6, Q7–Q15, Q17 and Q18: Operon Technologies Inc. Alameda, CA, USA) were used to prime the RAPD-PCR reactions for five specimens of each species collected from site GR. The study was then extended to the remaining five locations when RAPD patterns were obtained for two specimens per location using 10 primers, selected among those initially employed with individuals from GR, which yielded easily interpretable patterns (primers Q7–Q15 and Q18). DNA amplifications were performed in 25-µl volumes containing 40 ng template DNA, 25 pmol of primer, one bead from the RAPD Analysis Beads kit (Amersham Biosciences, Uppsala, Sweden) and Milli-Q water (Millipore, Billerica, MA, USA). Reactions were carried out in a DNA Thermal Cycler (Bio-Rad, Hercules, CA, USA) with an initial denaturing step of 5 min at 95°C, followed by 45 cycles of 1 min at 95°C, 1 min at 37°C, 2 min at 72°C, and a final extension step for 5 min at 72°C. Reproducibility was assessed by running duplicates of randomly selected amplifications on different days. Negative controls omitted template DNA and positive controls included DNA from strands C1a and BL21(DE3) of *Escherichia coli* and primer 2 (5′-d[GTTTCGCTCC]-3′) (Amersham Biosciences). Amplification products (12.5 µl) were resolved in a 1.5% TBE agarose gel, stained with ethidium bromide and visualized and photographed under UV light. Molecular weights were estimated by reference to a 100 base-pair ladder.

Presence/absence of bands was visually scored. Only clear and bright amplification products were retained for analysis. Pair-wise similarities were calculated for all combinations of individuals using the Nei and Li similarity coefficient (Nei & Li 1979): $S = 2a/((a + b) + (a + c))$, where a is the number of bands shared by both individuals, b is the number of bands displayed only by individual A and c is the number of bands displayed only by individual B. The pair-wise similarity matrix was used to construct an unweighted pair-group cluster (UPGMA). Genetic distance was estimated as 1-S.

## RESULTS

Five *G. lanceola* and five *G. turuturu* specimens were used in an earlier phase of this study when plants were collected from the only site in northwestern Spain where both species are sympatric (site GR). For these 10 individuals, 13 RAPD primers generated 155 fragments of which 114 were polymorphic. Banding patterns of the two species were clearly distinguishable, 60 of the 114 polymorphic bands were specific for *G. lanceola* specimens (i.e. displayed by the five individuals of this species) while 48 were specific for *G. turuturu* specimens, resulting in RAPD genetic distances ranging from 0.569 to 0.582. The five specimens of each species had almost identical banding patterns: only 2 of the 155 possible bands were polymorphic among *G. lanceola* individuals, 3 were polymorphic among the *G. turuturu* individuals, and 1 locus was polymorphic for individuals of both species. Consequently, within-population RAPD genetic distance ranged from 0.000 to 0.010 for *G. lanceola* and from 0.000 to 0.017 for *G. turuturu.*

When the study was extended to other locations from the northwestern and southern Iberian Peninsula, 76 bands were scored for the 14 *Grateloupia* specimens using 10 RAPD primers. Only one fragment was monomorphic across the whole set. RAPD patterns clearly separated both species: 49 of the 76 bands were species-specific (i.e. displayed by all the individuals of one species), resulting in RAPD genetic distances between species ranging from 0.784 to 0.920 (Table 1). Banding patterns of specimens from the same species but different locations were very similar: genetic distance varied from 0.079 to 0.162 for *G. turuturu* specimens and from 0.027 to 0.176 for *G. lanceola* specimens. The latter range includes distances across regions, as no increase in the genetic distance was detected when individuals of *G. lanceola* from the northwestern Iberian Peninsula were compared with those from the southern Iberian Peninsula. The genetic distance between specimens from the same location was slightly lower, ranging from 0.028 to 0.081 for *G. lanceola* and from 0.013 to 0.041 for *G. turuturu.* In the UPGMA tree (Fig. 2), each species forms a distinct cluster; the average pair-wise genetic distance between the two clusters is 0.849. In comparison, genetic distances within each cluster were below 0.150; the specimens tended to group according to their location of origin.

## DISCUSSION

The large genetic variation detected between taxa supports our morphological differentiation of two foliose *Grateloupia* species

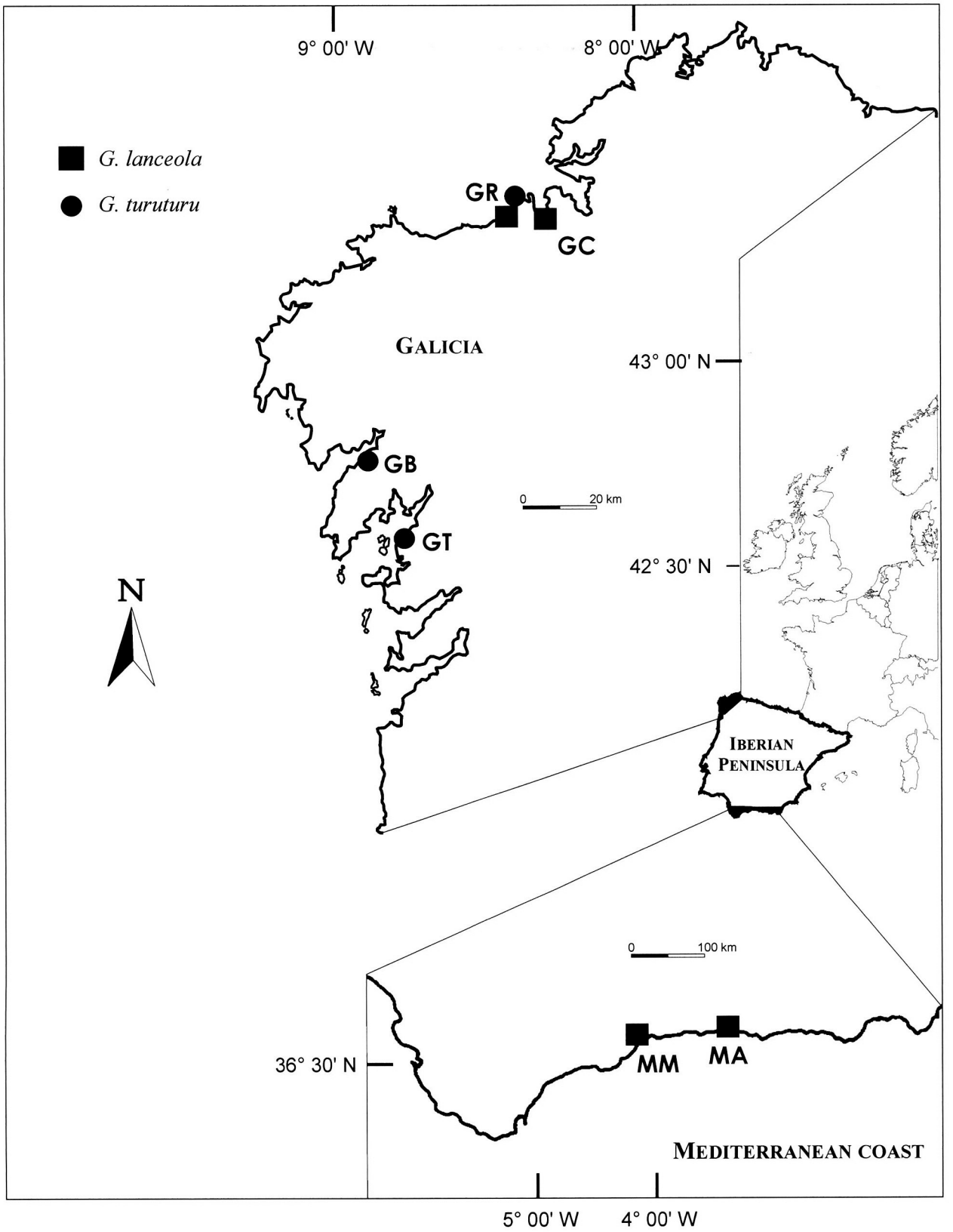


**Fig. 1.** Sampling locations of specimens of *Grateloupia* in the northwestern and southern Iberian Peninsula.

**Table 1.** Pair-wise Nei and Li's genetic distances between *G. lanceola* and *G. turuturu* individuals from Iberian Peninsula locations based on 76 RAPD loci.

| | *G. turuturu* | | | | | | *G. lanceola* | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *GB #1* | *GB #2* | *GR #1* | *GR #2* | *GT #1* | *GT #2* | *GR #1* | *GR #2* | *GC #1* | *GC #2* | *MA #1* | *MA #2* | *MM #1* | *MM #2* |
| *G. turuturu* | | | | | | | | | | | | | | |
| *GB #1* | — | | | | | | | | | | | | | |
| *GB #2* | 0.013 | — | | | | | | | | | | | | |
| *GR #1* | 0.128 | 0.114 | — | | | | | | | | | | | |
| *GR #2* | 0.132 | 0.143 | 0.026 | — | | | | | | | | | | |
| *GT #1* | 0.093 | 0.105 | 0.147 | 0.151 | — | | | | | | | | | |
| *GT #2* | 0.079 | 0.091 | 0.158 | 0.162 | 0.041 | — | | | | | | | | |
| *G. lanceola* | | | | | | | | | | | | | | |
| *GR #1* | 0.808 | 0.784 | 0.836 | 0.859 | 0.857 | 0.831 | — | | | | | | | |
| *GR #2* | 0.859 | 0.861 | 0.887 | 0.884 | 0.912 | 0.884 | 0.061 | — | | | | | | |
| *GC #1* | 0.813 | 0.816 | 0.867 | 0.863 | 0.833 | 0.836 | 0.143 | 0.118 | — | | | | | |
| *GC #2* | 0.787 | 0.789 | 0.867 | 0.863 | 0.806 | 0.808 | 0.143 | 0.176 | 0.056 | — | | | | |
| *MA #1* | 0.863 | 0.865 | 0.890 | 0.887 | 0.886 | 0.859 | 0.088 | 0.061 | 0.086 | 0.143 | — | | | |
| *MA #2* | 0.792 | 0.795 | 0.870 | 0.867 | 0.838 | 0.787 | 0.083 | 0.114 | 0.108 | 0.108 | 0.56 | — | | |
| *MM #1* | 0.813 | 0.816 | 0.893 | 0.890 | 0.861 | 0.808 | 0.114 | 0.118 | 0.139 | 0.139 | 0.057 | 0.054 | — | |
| *MM #2* | 0.840 | 0.842 | 0.920 | 0.918 | 0.889 | 0.836 | 0.114 | 0.118 | 0.111 | 0.111 | 0.057 | 0.027 | 0.028 | — |

in the northwestern Iberian Peninsula. It is similar to or even higher than the genetic distances reported in previous studies in which RAPD markers have successfully differentiated between congeneric macroalgae species (Patwary *et al.* 1993; Meneses 1996; Marston & Villalard-Bohnsack 2002). Thus, our results support the claim that the confined populations of a foliose *Grateloupia* located in the Bahía de La Coruña area (sites GR and GC) at least since the early 1980s (Pérez-Cirera *et al.* 1989) must not be seen as another incursion of *G. turuturu* into European waters (Bárbara & Cremades 2004).

Indeed, our results agree with some of the findings from a previous comparison of foliose *Grateloupia* from geographically diverse populations: six alien populations of *G. turuturu* and five populations referred to as *Grateloupia* sp. (Marston & Villalard-Bohnsack 2002). The herbarium specimens in the latter group were morphologically indistinguishable from those identified as *G. turuturu* (see Marston & Villalard-Bohnsack 2000) and, interestingly, included individuals from a location that coincides with our site GC (see Table 1, collection site 7, Bahía de La Coruña, Spain, in Marston & Villalard-Bohnsack 2002). Collected in 1997, those individuals most certainly belonged to our *G. lanceola* taxon as the latter has been known from this bay since the early 1980s (Pérez-Cirera *et al.* 1989),

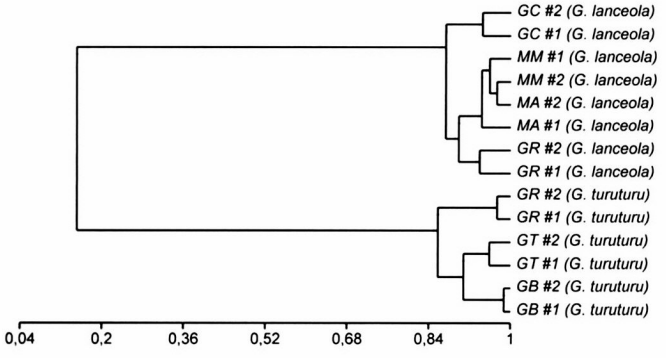


**Fig. 2.** UPGMA dendrogram of RAPD pair-wise Nei and Li similarity coefficient for six *G. turuturu* and eight *G. lanceola* individuals from northwestern and southern Iberian Peninsula.

while *G. turuturu* was first reported there only in 1999 (Herbarium SANT-*Algae* 13665, Universidad de Santiago de Compostela). Actually, the RAPD genetic distance between the *G. turuturu* and the *Grateloupia* sp. individuals, Galicia specimens included, estimated by Marston & Villalard-Bohnsack (2002), varied from 0.80 to 0.94 for 78 RAPD loci; this range is very similar to our estimates of interspecies genetic distance from the extended study for a comparable number of RAPD bands. Marston and Villalard-Bohnsack (2002) provided further evidence on the character of the *Grateloupia* sp. from Galicia as a species different from *G. turuturu* by comparing the nuclear internal transcribed spacer and mitochondrial cox2-cox3 intergenic spacer sequences in a reduced number of individuals. The level of genetic divergence between both taxa fall within the range of sequence divergence between species estimated in previous studies of red algae.

In the specific case of *G. lanceola*, our study has gone one step further, showing that the specimens from Galicia are genetically similar to topotype material from the southern Iberian Peninsula. The genetic distance between specimens from the northwestern and southern Iberian Peninsula ranged from 0.061 to 0.143, and it was mostly indistinguishable from the distance found when individuals from each Galicia location were compared with one another. This low genetic variation fits well with previous comparisons of conspecific individuals of foliose *Grateloupia* from geographically distant locations. Thus, RAPD genetic distances among *G. turuturu* specimens from both sides of the North Atlantic Ocean also ranged from 0.09 to 0.33 (Marston & Villalard-Bohnsack 2002). Topotype material is commonly employed in species identification, even with molecular techniques (e.g. Gavio & Fredericq 2002). Yet, it might still be argued that collecting specimens from the area where a species was described more than 150 yr ago does not guarantee that they actually belong to the same species. However, former morphological work (Pérez-Cirera *et al.* 1989) has shown that Galicia specimens of *G. lanceola* perfectly fit the original description of this species provided by Agardh (1841) and by Ardré & Gayral (1961). Furthermore, they are morphologically identical not only to present-day topotype

material from the southern Iberian Peninsula but also to herbarium specimens collected from that region as early as 1948, well before the invasive *G. turuturu* was first reported for European waters. Hence, the present study provides independent molecular-based support to earlier morphological work which anticipated the conspecific nature of the foliose *Grateloupia* currently found in the northwestern and southern Iberian Peninsula. Altogether, it represents strong evidence in favor of the identification of the foliose *Grateloupia* from the Bahía de La Coruña as *G. lanceola.*

The foliose *Grateloupia* species form a difficult group that has undergone several taxonomic revisions (e.g. Ardré & Gayral 1961; Dawson *et al.* 1964). Only recently have DNA analyses been used to evaluate their relationships (Wang *et al.* 2000; Kawaguchi *et al.* 2001; Gavio & Fredericq 2002; Marston & Villalard-Bohnsack 2002). Those studies have frequently relied on sequence analysis, which undeniably outperforms RAPD markers when pursuing phylogenetic relationships. DNA regions commonly employed in phylogenetic studies usually yield little base pair variation within species but show more extensive variation between species. In contrast, RAPDs show a much higher variation between individuals to the point that they might seem more appropriate for intraspecific population studies (Coleman 1996). As shown in this and previous studies (Patwary *et al.* 1993; Marston & Villalard-Bohnsack 2002), when individuals from different species are compared, their RAPD banding patterns can be so diverse as to largely preclude assessing reliable relationships. Yet, the RAPD method can still provide a simpler and more affordable alternative to sequence comparison whenever the aim is, as in the present study, just to discriminate between species rather than try to establish their phylogenetic relationships. Indeed, RAPDs have been formerly recommended for discriminating between difficult species in the Rhodophyta (Patwary *et al.* 1993). This recommendation has been scarcely followed. This study represents an example of the usefulness of RAPD markers as a simple tool to separate morphologically similar species of macroalgae.

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