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Molecular characterisation and development of rapid molecular methods to identify species of Gracilariaceae from the Atlantic coast of Morocco

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ABSTRACT

In Gracilariaceae, species identification is traditionally based on gross morphology; therefore the taxonomic status of terete individuals remains frequently problematic due to the lack of diagnostic characters to identify specimens. Different morphospecies have been recorded along the Atlantic coast of Morocco; however, no clear diagnostic characters were available to discriminate between terete species. Rapid molecular techniques have been developed recently to resolve many taxonomic problems and to re-assess the global diversity and biogeography in algae. In this study, molecular markers were used as DNA barcoding to characterise species. The sequence of the Rubisco spacer allowed identification of six species of Gracilariaceae: *Gracilaria gracilis*, *Gracilaria dura*, *Gracilaria conferta*, *Gracilaria vermiculophylla*, *Gracilaria multipartita* and *Gracilariopsis longissima*. In order to identify species with certainty, two simple and rapid methods based on the amplification of rDNA ITS and PCR-RFLP of the large subunit of the Rubisco were developed.

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1. Introduction

Marine red algae of the genus *Gracilaria* are a major agarophyte resource in the world and are cultivated for the phycocolloid industry or for integrated mariculture (Troell et al., 2003). The wide phenotypic variability of some terete species (Santelices and Valera, 1993) and the lack of diagnostic characters in the Gracilariaceae (Bird, 1995) makes the systematic study of this group problematic and some species are consequently often misidentified (Byrne et al., 2002; Cohen et al., 2004). Taxonomy of this family is however fundamental to a better knowledge of the biology, biochemistry, and utilization of these algae (Bird, 1995). Indeed, closely related species of *Gracilaria* may present different agar yields and gel quality (Craigie, 1990), consequently precise species identification is crucial to perform high-quality agar extraction.

In Gracilariaceae, species identification is traditionally based on gross morphology and reproductive characters (Bird, 1995). In the absence of reproductive structure, identification of terete specimens is almost impossible because of the lack of clear morphological diagnostic characters.

In the past two decades, molecular techniques have been used to resolve many taxonomic problems and to re-assess the global diversity and biogeography of the genus Gracilaria (Wattier et al., 1997; Bellorin et al., 2002; Gurgel et al., 2004). DNA nucleotide sequence analyses of different DNA regions have been used to infer phylogenetic relationships at the species level: chloroplast-encoded *rbc*L gene (Gurgel and Fredericq, 2004; Gurgel et al., 2004; Gargiulo et al., 2006), *rbc*L–*rbc*S intergenic spacer region (i.e. Rubisco spacer; Destombe and Douglas, 1991; Goff et al., 1994; Byrne et al., 2002; Iyer et al., 2005; Terada and Shimada, 2005; Rueness, 2005), the mitochondrial *cox2*–*cox3* spacer (Cohen et al., 2004; Terada and Shimada, 2005), the *cox1* gene (Robba et al., 2006) and the nuclear internal transcribed spacers of the ribosomal cistron (i.e. ITS; Goff et al., 1994; Bellorin et al., 2002). Sequence analyses of mitochondrial *cox2*–*3* spacer and of plastid Rubisco spacer allowed the identification of cryptic species in Australia (Byrne et al., 2002) and in Chile and New Zealand (Cohen et al., 2004). Different molecular approaches were also attempted to delineate species based on ITS-RFLP analyses (Candia et al., 1999) or on RFLP on plastid DNA (González et al., 1996), however, one of the most effective, inexpensive and simplest methods was based on ITSs length variation detected by PCR (Wattier et al., 1997).

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The identification of the Gracilariaceae along the Moroccan coast remains problematic. Recently, the checklist of the seaweeds of the Moroccan coasts reported the existence of at least eight morphospecies (Benhissoune et al., 2002). However, only five morphospecies were regularly reported along the Atlantic region from Casablanca to El Jadida (Mazagan) (Dangeard, 1949; Gayral, 1958). These species comprise four terete species: *Gracilaria gracilis* (Stackhouse) Steentoft, Irvine et Farnham as *Gracilaria confervoides* (Linnaeus) Greville, *Gracilaria conferta* (Schousboe ex Montagne) Montagne, *Gracilaria dura* (C. Agardh) J. Agardh and *Gracilaria bursa-pastoris* (Gmelin) Silva as *Gracilaria compressa* (C. Agardh) Greville as well as one flattened species: *Gracilaria multipartita* (Clemente) Harvey. The other species were far less frequent and were reported more sporadically as *Gracilaria armata* (C. Agardh) Greville, *Gracilaria cervicornis* (Turner) J. Agardh, *Gracilaria vermiculata* P. Dangeard or *Gracilariopsis longissima* (Stackhouse) Irvine, Steentoft and Farnham. This last species was previously confused with *G. gracilis* as *G. verrucosa* (Hudson) Papenfuss (Steentoft et al., 1995) and therefore Moroccan records require re-examination (Benhissoune et al., 2002).

The aims of the present study are (i) to clarify the taxonomic identities of widespread gracilarioid species sampled along the Atlantic coast of Morocco in the region of El Jadida using the Rubisco spacer region sequences and (ii) to develop easy and rapid molecular tools using ITSs length variation and *rbc*L-RFPL, as DNA barcodes to identify these species.

2. Materials and methods

2.1. Sampling

Sixty-two samples (Table 1) were collected from December 1999 to December 2002 in the region of El Jadida. Putative species were identified using gross morphological characteristic and ecological environment according to the description made by Dangeard (1949) and Gayral (1958). The two flattened samples MGR1 and SGM18 were unambiguously attributed to the morphospecies *G. multipartita* whereas only eight of 60 terete samples were clearly identified, according to their gross morphology and/or their clear-cut ecological characteristics. The two morphologically indistinguishable terete species, *G. gracilis* and *Gp. longissima*, were identified only by their respective habitat (rocky versus muddy shore). These height terete samples, as well as the MGR1 sample of *G. multipartita*, were chosen for the Rubisco spacer sequencing (Table 1). Three additional samples of *G. multipartita* and *G. bursa-pastoris* from France (St Lunaire) and Tunisia (Djerba) were selected to complete the analyses. Field samples were dried in silica gel until DNA extraction.

The other 52 ambiguous terete individuals sampled in the region of El Jadida were used to test the new species delineation procedures developed in this study.

2.2. DNA extraction, Rubisco spacer PCR reactions

DNA extraction was performed according to the method used by Cohen et al. (2004). Twelve of the samples listed in Table 1 were sequenced for the Rubisco spacer using the primers $rbcF_1$ and $rbcR_2M_2$ (Zuccarello et al., 1999). PCR amplifications for Rubisco spacer region were carried out in 30 μl reaction volume, from approximately 5–10 ng of template DNA, using a PTC200^{TM} thermocycler (MJ Research). Reaction mixture contained 1× Abgene PCR buffer, 200 μM each dNTP, 50 pmol each of the rbcF1 and $rbcR_2M_2$ primers, 2 mM $MgCl_2$ and 1 U Thermoprime Plus Taq polymerase (Abgene). PCR reaction was composed of: an initial denaturation at 94 °C for 4 min followed by 5 cycles of 30 s at 94 °C, 30 s at 45 °C, 30 s at 72 °C, followed by 25 cycles of 30 s at 94 °C, 30 s at 55 °C, 30 s at 72 °C and a final elongation step of 5 min at 72 °C. Sequences of Rubisco spacer amplified fragments were obtained with an ABI 3100 Sequencer (Applied Biosystem, Foster City, CA).

2.3. Rubisco spacer sequences analysis

The sequences were aligned and sequence data were deposited in GenBank (DQ984677–DQ984688, see Table 1). Twenty-two additional Rubisco spacer sequences of members of the Gracilariaceae were retrieved from Genbank. We compiled all the alignment of partial Rubisco spacer sequences: 271 base pairs including gaps, except for the sequence DQ984682 of *G. conferta* that was only 215 base pairs including gaps. Sequence alignment is available in Genbank as a PopSet information. The sequence polymorphism and nucleotide diversity were estimated with DNAsp software (Rozas et al., 2003). All phylogenetic analyses were performed with PAUP* v.4.0.10 (Swofford, 2002). Phylogenetic trees were generated by maximum likelihood (ML) and maximum parsimony (MP) methods. The comparison of these methods allows assessing the reliability of the inferred topology. All gaps were treated as missing data. For ML analysis appropriate model was selected using unweighted AIC in Modeltest 3.7 (Posada and Crandall, 1998). MP trees were inferred from heuristic searches of random sequence addition using only the phylogenetically informative characters, unordered, under the Fitch criterion of equal weights for all substitutions (Fitch, 1971), tree bisection reconnection, saving multiple trees (MULTREES). Ten random addition sequence replications were performed per bootstrap replicate and one hundred trees were kept at each step. Support for all nodes for all trees was assessed by bootstrap analysis on the data set using 1000 replicates, as implemented in PAUP* v.4.0.10, and the sequence of *Gracilariopsis funicularis* was used as outgroup.

2.4. ITSs amplification length polymorphism

The operon of nuclear ribosomal genes includes two internal transcribed spacers: (ITS1) between the 18S and 5.8S rDNA and (ITS2) between the 5.8S and 25S rDNA. ITS size variation method is efficient in distinguishing species of the Gracilariaceae family (Wattier et al., 1997) and the length of ITSs is already known for two of our species of interest *G. gracilis* and *Gp. longissima* (Goff et al., 1994). ITS size variations were determined first for the 12 individuals sequenced for the Rubisco spacer, and then for all the 53 other sampled individuals. For PCR reaction, we used the same reaction mixture than for Rubisco spacer PCR and the program was composed of: an initial denaturation at 94 °C for 4 min followed by 35 cycles of 30 s at 94 °C, 30 s at 55 °C, 90 s at 72 °C and a final elongation step of 5 min at 72 °C. Two PCR were made for each individual: the primers TW81 and CD12 were used to amplify the ITS1 region (Wattier et al., 1997) and the newly designed primers ITS2-F (5′-TAACAAGGTTTCCGTAGGTG-3′) and ITS2-R (5′-GGTC-RTCTTGTCTGATTTGAG-3′), to amplify the ITS1-ITS2 region (i.e. ITS1-5.8S rDNA-ITS2). Size variations of ITS were resolved on 2% agarose gel electrophoresis, stained with ethidium bromide, Superladder-Mid 1 kit (ABgene) was used as size marker.

2.5. RFLP's on amplified fragments of Rubisco

A fragment of 1397 bp of the large subunit of the ribulose 1,5-bisphosphate carboxylase/oxygenase gene (*rbc*L) was amplified for *G. gracilis* and *G. dura* (individual MGR3 and MGR177A, respectively, Table 1) using the same PCR program and reaction mixture as for the Rubisco spacer but using the newly defined primers: *rbc*L-RFLP-F (5′-CWAAAATGGGATATTGGGAT-3′) and *rbc*L-RFLP-R

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