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Molecular systematics and phylogenetics of Gracilariacean species from the Mediterranean Sea

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Abstract

Mediterranean reports of Gracilariaceae species, in particular those assigned to the *G. verrucosa* complex, were re-examined with the use of molecular tools, in order to verify their systematic position and better understand their distribution. Within this complex, we recognized four distinct taxa: *Gracilariopsis longissima*, *Gracilaria gracilis*, *Gracilaria longa* and a possible new species. The *rbc*L gene sequences, together with those of other terete Mediterranean entities, were included in a broad molecular phylogeny of the family. The reproductive characters of the studied taxa do not fit completely with published hypothesis on the generic and intrageneric relationships, suggesting that the anatomy of some subgroups should be better characterized.

Introduction

The order Gracilariales is well defined from both an anatomical (Fredericq & Hommersand, 1989a,b; Fredericq & Hommersand, 1990) and a molecular point of view (Freshwater et al., 1994; Saunders & Kraft, 1997; Harper & Saunders, 2001) within the Rhodophyta, but the intergeneric taxonomy had a somewhat more complex history (Bird et al., 1992; Bird et al., 1994; Bird, 1995; Bellorin et al., 2002). Recently, clarifying insights have come from systematic studies of the order that consider the ontogenesis of reproductive structures and *rbc*L gene phylogeny (Liao & Hommersand, 2003; Gurgel & Fredericq, 2004), nevertheless many problems remain at a lower taxonomic level, such as the *G. verrucosa*-complex. Populations previously referred to this taxon are now considered either *Gracilariopsis longissima* (S. Gmelin) Steentoft, Irvine et Farnham or *Gracilaria gracilis* (Stackhouse) Steentoft, Irvine et Farnham, or described as new species (Abbott, 1985; Zhang & Xia, 1985; Bird et al., 1986; Gargiulo et al., 1987; Steentoft et al., 1995). However, the name *Gracilaria verrucosa* (Hudson) Papenfuss, despite having been rejected (Irvine & Steentoft, 1995), is still used to identify economically important entities (Skiptsova, 2000; Imbs et al., 2001; Mancinelli & Rossi, 2001; Rath & Adhikary, 2002; Wang, 2002). Mediterranean reports of Gracilariacean species need re-examination after these systematic revisions. These taxa are frequently included in Mediterranean check-lists because of mere nomenclature changes rather than from actual verifications of specimens (Furnari et al., 2003). Other Mediterranean entities, e.g. *Gracilaria longa* Gargiulo, De Masi et Tripodi, share a similar morphology, making the segregation of these taxa difficult (Bird & Rice, 1990; Steentoft et al., 1995).

The aim of our study is to verify the systematic position and the Mediterranean distribution of some *G. verrucosa*-like populations, using *rbc*L gene sequence analysis. Moreover, sequence data set includes other terete Mediterranean species and those available for *Gracilaria* species from other geographical areas, in order to test how Mediterranean taxa match with proposed phylogenetic hypotheses.

Materials and methods

Sequence data generated for *rbc*L gene were submitted to GenBank and accession numbers together with

collection information are given in Table 1. DNA was isolated from freshly collected or dried thalli (both silica gel preserved or recovered from herbarium sheets) with a modified CTAB protocol (Doyle & Doyle, 1987). Ground material was incubated in 2× CTAB buffer (0.1 M Tris-HCl, 0.05 M Na_2EDTA, 1.4 M NaCl, 2% CTAB, 1% PVP, 0.5% (v/v) β-mercaptoethanol) for 120–180 min at room temperature under constant agitation. Polysaccharides were precipitated with incubation with 2.5 M K acetate on ice for 20 (Saunders, 1993). Nucleic acids were extracted three times with 1 volume of phenol-chlorophorm-isoamilic alcohol (25:24:1) and twice with 1 volume of chlorophorm-isoamilic alcohol (24:1), precipitated with isopropanol and redissolved in 0.01 M Tris-HCl-0.001 M EDTA pH 8.0 (Sambrook et al., 1989). If consistent amounts of RNA were detected a digestion with RNase was performed (Sambrook et al., 1989). Voucher specimens were preserved in 4% formalin in seawater, or dried in silica gel, or pressed as herbarium sheets and deposited in the Phycological Herbarium of the Department of Botanical Sciences of the University of Messina (MS). In order to prevent errors in sorting of samples, each DNA isolation was performed from a single individual, a fragment of which was kept as voucher formalin preserved and/or pressed for further inspections.

The *rbc*L gene was PCR amplified using primers listed in Freshwater and Rueness (1994). Sequencing reactions were performed by an external company (MWG Biotech AG, Ebersberg, Germany).

Nucleotide sequences were aligned by eye unambiguously due to the absence of insertion or deletion mutations. Additional published *rbc*L gene sequences from species of Gracilariaceae (Gurgel et al., 2003a; Gurgel & Fredericq, 2004) were added to the alignment. A data set of 52 *rbc*L gene sequences of Gracilariaceae was used for phylogenetic analyses. When more sequences were available for each species, just one was used, with the exception of *Gracilariopsis longissima* for which three sequences were used due to the higher sequence divergence and related taxonomic implications (see discussion). Three representatives, respectively from Halymeniales, Rhodymeniales and Plocamiales, were selected as outgroup taxa (Saunders & Kraft, 1997) (Table 1). The final alignment included 55 taxa of 1234 characters. When presented, sequence divergence is expressed as uncorrected nucleotide substitutions percentage.

All phylogenetic analyses were performed in PAUP* 4b10 for the Macintosh (Swofford, 2002). The model of sequence evolution was selected according to a hierarchical likelihood ratio test as implemented in Modeltest 3.06 (Posada & Crandall, 1998). The model selected (a general time reversible model with invariable sites and gamma distribution, GTR+I+G; Lanave et al., 1984), and associated parameters (base frequencies: $A = 0.3476$, $C = 0.1109$, $G = 0.1601$, $T = 0.3815$; substitution rate matrix: $A-C = 1.1547$, $A-G = 6.5514$, $A-T = 0.9262$, $C-G = 1.9585$, $C-T = 12.7219$, $G-T = 1.0000$; proportion of invariable sites = 0.5250, gamma parameter = 1.1055) were used in distance and maximum likelihood (ML) analyses.

Distance phylogenies were constructed with a neighbor joining (NJ) algorithm and with a heuristic search under the criterion of minimum evolution (ME), with 1000 random addition sequence replicates, holding 20 trees at each step, tree bisection and reconnection (TBR) as branch-swapping algorithm, saving all minimal trees (MulTrees). The steepest descent option in the branch swapping procedure was not used because of an unfixed bug in the current beta version of PAUP* (http://paup.csit.fsu.edu/problems.html).

Parsimony analysis was conducted under a heuristic search similarly to ME analysis. ML analysis was performed under a heuristic search, with 10 random addition sequence replicates, holding 1 tree at each step, with TBR branch-swapping algorithm and MulTrees option in effect. Distance and parsimony analyses were subjected to bootstrap re-samplings to estimate robustness of the internal nodes (Felsenstein, 1985), basing on 1000 replicates, but with 10 random addition sequence replicates, holding 1 tree at each step, in the heuristic searches. Bootstrap resampling was not performed on maximum likelihood analysis, due to computational limitations. In all analyses unrooted trees were constructed, and subsequently rooted with reference to the outgroup taxa.

Results

Among the 1234 bp analysed (positions 117–1350, 84.12% of the entire length of the gene), 449 were parsimony informative. Parsimony analyses resulted in 25 most parsimonious (MP) trees (tree length 2366, consistency index 0.3407, retention index = 0.5917), not shown. Distance analyses resulted in a NJ tree and a ME tree (ME score = 2.57491), not shown, similar to the MP trees. ML analysis resulted in a phylogenetic tree (ln likelihood = −12762.10785, topology recovered 9 times out of 10 replicates), presented in Figure 1.