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The Gracilariaceae Germplasm Bank of the University of São Paulo, Brazil—a DNA barcoding approach

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Abstract The University of São Paulo Gracilariaceae Germplasm Bank has 50 strains collected mostly in Brazil, but also elsewhere in the world. This bank has been used as a source of material for research developed locally and abroad. With over 200 species, some of which have high economic value, the family Gracilariaceae has been extensively studied. Nonetheless, taxonomic problems still persist by the existence of cryptic species, phenotypic plasticity, and broad geographic distribution. In the case of algae kept in culture for long periods of time, the identification is even more problematic as a consequence of considerable morphological modification. Thus, the use of molecular markers has been shown to be an efficient tool to elucidate taxonomic issues in the group. In this work, we sequenced the 5′-end of the *cox*1 gene for 41 strains and the universal plastid amplicon (UPA) plastid region for 45 strains, covering all 50 strains in the bank. In addition, the *rbc*L for representatives of the *cox*1/UPA clusters was sequenced for 14 strains. The original species identification based on morphology was compared with the molecular data obtained in this work, resulting in the identification of 13 different species. Our analyses indicate that *cox*1 and UPA are suitable markers for the delineation of species of Gracilariales in the germplasm bank. The addition of DNA barcode tags to the samples in the Gracilariaceae germplasm bank and the molecular identification of the species will make this bank even more useful for future research as the species can be easily traced and confirmed.

Keywords *cox*1 · DNA barcoding · Germplasm bank · Gracilariales · *rbc*L · UPA

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Introduction

The family Gracilariaceae is widely distributed on tropical and temperate marine coasts of the world. The main genera in the family are *Gracilaria* Greville with around 167 species and *Gracilariopsis* E.Y. Dawson with 20 species (Algaebase, Guiry and Guiry 2010). These seaweeds have considerable economic importance as the main global source of agar (Oliveira et al. 2000), which is largely used by the pharmaceutical and food industries (Oliveira and Plastino 1994). The quantity and quality of agar vary among species of Gracilariales; therefore, a precise identification may be very important (Macchiavello et al. 1999; Skriptsova and Nabivailo 2009).

In spite of the great effort applied to understand the biology of the group, precise taxonomic identification is limited by phenotypic plasticity, the occurrence of cryptic species, and the absence of male and cystocarpic reproductive structures (e.g., Fredericq and Hommersand 1989; Gurgel et al. 2003, 2004; Oliveira 1984). Many alternative approaches to conventional morphological analysis have been attempted (cf. Oliveira and Plastino 1994; Plastino and Oliveira 1996), including, for example, hybridization (Plastino and Oliveira 1988, 1990). To accomplish that, E.C. Oliveira and E.M. Plastino established the Germplasm Bank of the Laboratory of Marine Algae of the University of São Paulo (LAM-USP) for crossing experiments (Plastino et al. 1995; Plastino and Oliveira 1988, 1990, 1996, 1997, 2000). The germplasm bank currently has

50 strains of Gracilariaceae in culture, some of which have been kept in vitro for more than 30 years. These strains were mostly collected in Brazil, but also elsewhere in the world (Lourenço and Vieira 2004).

This bank has been used as a source of material for several investigations, contributing to the knowledge of different aspects of this economically important group of algae. The life history of some species has been completed in vitro: *Gracilaria birdiae* (Costa and Plastino 2001); *Gracilaria caudata* and *Gracilaria cornea* (Oliveira and Plastino 1984); *Gracilaria chilensis* (Plastino and Oliveira 1984); *Gracilaria domingensis* (Guimarães et al. 1999); and *Gracilaria tenuistipitata* (Barufi et al. 2010). Physiological aspects related to growth rates (Ferreira et al. 2006; Plastino et al. 1998, 2004; Ursi et al. 2008; Ursi and Plastino 2001; Yokoya and Oliveira 1992a, b, 1993), pigment characterization (Barufi et al. 2010; Costa and Plastino 2011; Guimarães et al. 2003; Plastino et al. 2004), photosynthetic and respiratory characterization (Ursi et al. 2003), enzymatic activity (Chow et al. 2004, 2007; Chow and Oliveira 2008; Collén et al. 2003; Lopes et al. 1997, 2002; Rossa et al. 2002), and polysaccharide content (Guimarães et al. 2007) have been studied using strains from the germplasm bank. Furthermore, some strains have been used in color inheritance studies (Costa and Plastino 2001, 2011; Guimarães et al. 2003; Plastino et al. 1999, 2004), ultrastructure characterization (Bouzon et al. 2000, 2011; Guimarães and Plastino 1999; Plastino and Costa 1999, 2001), axenic tissue cultures (Ramlov et al. 2009; Yokoya 2000), phylogeny and systematic studies (Bellorin et al. 2002; Bird and Oliveira 1986), and gene sequencing and expression studies (Falcão et al. 2008, 2010; Hagopian et al. 2002, 2004; Nyvall et al. 2011).

However, once in culture, strains of *Gracilaria* and *Gracilariopsis* may change their morphology, usually remaining infertile, thus making species identification very difficult, if not impossible. Therefore, it is necessary to implement a more direct approach, based on the use of molecular markers, for the identification and tracking of species in the bank.

Different molecular markers have been used for Gracilariales, such as the nuclear gene coding for the small subunit of ribosomal RNA, the internal transcribed spacers of ribosomal genes, and the *rbc*L gene coding for RUBICO large subunit (Bellorin et al. 2002, 2008; Bhattacharya et al. 1990; Goff et al. 1994; Gurgel et al. 1999). These markers proved to be suitable for species identification and phylogenetic analysis within the group, but they are of relatively large size, requiring some effort for amplification and sequencing with the need for several internal primers, which results in additional cost in both time and resources.

On the other hand, the technique of DNA barcoding is a fast, practical, and uniform system based on polymerase chain reaction (PCR) amplification of relatively short (~400–700 bp) DNA fragments that can be fully sequenced with the same two primers used in PCR (Savolainen et al. 2005). Hebert et al. (2003) proposed the use of the 5′-end of the mitochondrial gene *cox*1 coding for cytochrome oxidase 1 to facilitate the rapid identification of specimens and as a powerful ally in understanding biodiversity. Given the difficulties that exist in species identification in several red algae, Saunders (2005) proposed and developed primers for the use of *cox*1 for DNA barcoding in this group of organisms. Another region that has been proposed by Sherwood and Presting (2007) as a DNA barcode for photosynthetic organisms is the universal plastid amplicon (UPA), which is part of the chloroplast gene coding for the large ribosomal RNA (23SRNAr).

In this work, we first sequenced the 5′-end of *cox*1 and the UPA region of Gracilariaceae kept in culture in the LAM-USP Germplasm Bank. These sequences were compared and grouped. Moreover, chloroplast DNA sequences for *rbc*L were obtained for each of the different groups. In this way, the original species identification based on morphology was compared with the molecular data obtained in this work, leading to the identification of 13 different species in the bank.

Materials and methods

Samples were collected from several locations (Table 1) and transported to the laboratory. The unialgal cultures were established from apical segments or spores. As soon as the algae were brought to the lab, a careful process for the removal of contaminants using brushes under stereoscopic microscope was performed. Successive cleanups were performed at 2–4 days, with the algae kept in sterile seawater without nutrients (Plastino and Oliveira 1990). Once isolated, cultures were maintained in modified von Stosch (Ursi and Plastino 2001) enriched seawater, diluted to 50 % with sterile seawater (32 psu). The cultures were kept at 25±1°C under 30 μmol photons m^{-2} s^{-1} photosynthetically active radiation (PAR) provided by 40-W daylight fluorescent tubes on a 14-h light/10-h dark cycle. The medium was renewed monthly.

Before DNA extraction, the apical fragments of each sample were transferred to Erlenmeyer flasks with 50 mL of enriched seawater for 2 weeks. Cultures were maintained under 150 μmol photons m^{-2} s^{-1} PAR and aerated for 30 min h^{-1}. The medium was renewed weekly. After this period, the algae were rapidly rinsed in fresh water, blotted dry, frozen in liquid nitrogen, and stored at −70°C.

DNA extraction, PCR amplification, and sequencing

DNA was extracted from approximately 30 mg of frozen samples by grinding in liquid nitrogen and using the method

described by Bellorin et al. (2002). The mitochondrial *cox*1 was amplified and sequenced using the synthetic primers GazF1 and GazR1 described by Saunders (2005). The plastidial UPA was amplified and sequenced using the primers p23Sv_f1 and p23Sv_r1 described by Sherwood and Presting (2007). The plastidial *rbc*L was amplified with the primers FrbcL and RbcS and sequenced with the addition of internal primers described by Freshwater et al. (1994). PCR amplification, purification, and sequencing are described in Milstein et al. (2011).

Molecular analyses

The consensus *Gracilaria* and *Gracilariopsis cox*1, UPA, and *rbc*L sequences were each aligned using ClustalW within BioEdit (Hall 1999) together with sequences of the same markers available from the GenBank. For *rbc*L, the sequences of *Curdiea racovitzia* Hariot and *Melanthalia abscissa* (Turner) J.D. Hooker and Harvey were used as outgroups. The following matrices were assembled: 57 sequences (41 sequences generated in this work and 16 obtained from databanks) and 664 positions for *cox*1; 56 sequences (45 sequences generated in this work and 11 obtained from databanks) and 370 positions for UPA; and 78 sequences (14 sequences generated in this work and 64 obtained from databanks) and 1,393 positions for *rbcL*. For all three markers, positions corresponding to amplification primers were excluded.

For *cox*1 and UPA only, a neighbor-joining (NJ) analysis using PAUP* 4.0b10 (Swofford 2002) with 2,000 replicates of bootstrap was performed to visualize the species groups. For *rbc*L, an evolution model was selected using MrModeltest 2.2 (Posada and Crandall 1998), and phylogenetic analyses were inferred by: (1) NJ with 2,000 replicates of bootstrap; (2) maximum parsimony heuristic search, using starting trees obtained by stepwise addition, with random sequence addition (ten replicates) using tree bisection–reconnection branch-swapping algorithm and with 2,000 replicates of bootstrap, using the PAUP* 4.0b10 (Swofford 2002); and (3) Bayesian analysis with two runs of four chains and with 4,000,000 generations sampled every 100 (initial 100,000 generations were discarded as burn in) using MrBayes (v3.1.2) (Huelsenbeck and Roanquist 2001).

Results

The germplasm bank has a total of 50 samples of Gracilariaceae; out of which 33 originated from Brazil and 17 from abroad (Table 1). Sequences for the 5′-end of *cox*1 were obtained for 41 samples and presented 16 unique sequences that grouped into nine clusters representing different species (Fig. 1). Sequences for the UPA plastid region were obtained for 45 samples and presented 13 unique sequences that grouped into clusters representing different species (Fig. 2).

In our experience, the UPA region was easier to amplify and sequence compared to *cox*1 based on the number of PCR and sequencing reactions needed to obtain each consensus sequence. For 45 UPA sequences, 47 PCR and 106 sequencing reactions were performed, whereas to obtain 41 *cox*1 sequences, 76 PCR and 284 sequencing reactions were performed. As a consequence, around 2.5-fold more reagents were needed to obtain *cox*1 sequences than to obtain a similar number of UPA sequences.

The *cox*1 and UPA sequences obtained for the germplasm bank samples, plus the few available sequences in the GenBank, were clustered using NJ (Figs. 1 and 2). In both analyses, the genera *Gracilaria* and *Gracilariopsis* were segregated. For the *cox*1 analysis, four groups were formed for *Gracilariopsis*: one containing all the *Gp. tenuifrons* from several locations of Brazil and Venezuela with an intraspecific variation of 0–0.3 %; one for *Gp. longissima* from the eastern North Atlantic; one for *Gp. lemaneiformis* from Ecuador; and one for *Gp. andersonii* from Canada (Fig. 1). For the UPA, the same clusters were formed, with the exception of *Gp. andersonii*, which was not included in the analyses by the lack of UPA sequences, and with the addition of the only UPA sequence of *Gp. mclachlanii* (Fig. 2).

Analyses of *cox*1 and UPA sequences of *Gracilaria* sequences from the germplasm bank grouped in clusters or isolated branches represent six and nine lineages, respectively (Figs. 1 and 2). *G. caudata* samples from northeastern Brazil (CE/BR) formed a sister cluster to a sample from southeastern Brazil (SP/BR), albeit at a distance of some 3,000 km from each other, with a *cox*1 intraspecific divergence of 6 bp (0.8 %) between them (Fig. 1). For UPA, a *G. caudata* cluster was also formed, but without intraspecific variation (Fig. 2). Samples of *Gracilaria cornea* cultivated in Israel (originally collected in the Caribbean), Brazil, and Mexico formed a cluster in both the *cox*1 and UPA analyses with a *cox*1 intraspecific variation of 0–5 bp (0–0.8 %). For *G. birdiae*, only a UPA sequence was obtained, showing the close relationship of this species to *G. cornea* (only 0.3 % divergence). Samples of *Gracilaria gracilis* from Argentina, Portugal, Namibia, Brazil, and Norway grouped in both the *cox*1analysis, with an intraspecific variation of 0–2 bp (0–0.5 %), and the UPA analysis without intraspecific variation. Samples of *G. isabellana* from Brazil grouped both in the *cox*1 and UPA analyses without intraspecific variation. The other samples, *G. domingensis*, *G. tenuistipitata*, *G.* sp. BG0057, and *G. rangiferina*, did not group with significant support to other species in either the *cox*1 or UPA analyses.

The *rbc*L was sequenced for 14 samples selected from one or more representatives of each of the *cox*1 and/or UPA clusters (Table 1): *G. caudata*, *G. cornea*, *G. domingensis*, *G. gracilis*,

Table 1 Strains maintained in culture in the Gracilariaceae Germplasm Bank at the University of São Paulo

Species	Original identification	Bank #	Collection site	Collector	Date	SPF	Life cycle phase	*cox*1 (664 bp)	*rbc*L (1,393 bp)	UPA (370 bp)
Gracilaria caudata J.Agardh	*Gracilaria caudata*	BG0028	Ubatuba, SP, Brazil	E. Plastino	25 April 1985	–	Female gametophyte	JQ843317[a]		JQ952643
		BG0058	Guajiru, Trairi, CE, Brazil	E. Oliveira	16 September 1989	55510	Male gametophyte	JQ935789	JQ843355	JQ952644
		BG0059	Guajiru,Trairi, CE, Brazil	E. Oliveira	16 September 1989	55511	Female gametophyte	JQ843318		JQ952645
		BG0060	Guajiru, Trairi, CE, Brazil	E. Oliveira	16 September 1989	55512	Tetrasporophyte	JQ843319	JQ843356	JQ952646
	Gracilaria "verrucosa"	BG0081	Ubatuba, SP, Brazil		July 1993	52029	Female gametophyte			JQ907427
	Gracilaria caudata	BG0087	Praia do Meireles, Fortaleza, CE, Brazil	E. Plastino	28 February 1992	55807	Tetrasporophyte			JQ952642
		BG0088	Praia do Meireles, Fortaleza, CE, Brazil	E. Plastino	28 February 1992	55807	Male gametophyte	JQ843320		JQ952647
		BG0091	Praia do Pacheco, Fortaleza, CE, Brazil	E. Plastino	26 February 1992	55813	Tetrasporophyte	JQ935790		JQ952648
	Gracilaria cornea	BG0055	Guajiru, Trairi, CE, Brazil	E. Oliveira	16 September 1989	54946	Tetrasporophyte	JQ843324		JQ907432
		BG0067	Israel	A. Critchley	September 1992	–	Infertile plant	JQ843322		JQ907429
Gracilaria cornea J. Agardh	*Gracilaria cornea*	BG0112	Guajiru, Trairi, CE, Brazil	E. Plastino	February 1992	55662	Tetrasporophyte	JQ843321		JQ907428
		BG0113	Guajiru, Trairi, CE, Brazil	E. Plastino	February 1992	55662	Tetrasporophyte	JQ935791		JQ952650
	Gracilaria sp.	BG0115	Guajiru, Trairi, CE, Brazil	E. Plastino	February 1992	55662	Tetrasporophyte	JQ843323	JQ843357	JQ952649
	Gracilaria birdiae Plastino & E.C. Oliveira	BG0140	Rio do Fogo, RN, Brazil	M. Amaral		–	Tetrasporophyte			JQ907430
Gracilaria domingensis (Kützing) Sonder ex Dickie	*Gracilaria mammillaris* (Montagne) M.A. Howe	BG0005	Ubatuba, SP, Brazil	E. Oliveira	28 October 1981	26055	Male gametophyte		JQ843358	
	Gracilaria cervicornis	BG0007	São Sebastião, SP, Brazil	E. Plastino	11 December 1982	26774	Male gametophyte	JQ843329	JQ843359	JQ907438
	Gracilaria "verrucosa"	BG0033	Puerto Madryn, Argentina	E. Oliveira	29 March 1986	27985	Tetrasporophyte	JQ935792		JQ952651
	Gracilaria "verrucosa"	BG0045	Puerto Madryn, Argentina	E. Oliveira	24 March 1986	27985	Tetrasporophyte	JQ935793		JQ952652
Gracilaria gracilis (Stackhouse) M. Steentoft, L.M. Irvine & W.F. Farnham	*Gracilariopsis lemaneiformis*	BG0061	Liideritz, Namibia	A. Critchley	Decmebr19 1989	–	Infertile plant	JQ843331		JQ907440
	Gracilariopsis tenuifrons	BG0084	Pajuçara, AL, Brazil	E. Plastino	03 March 1994	55734	Tetrasporophyte	JQ843332	JQ843360	JQ907442
	Gracilaria verrucosa	BG0094	Norway	E. Oliveira	1982	–	Male gametophyte			JQ907441
		BG0095	Norway	E. Oliveira	1982		Tetrasporophyte	JQ843333		
Gracilaria lacinulata (M. Vahl) M.A. Howe	*Gracilaria* sp.	BG0036	Cabo Frio, RJ, Brazil	E. Plastino	06 November 1987	52189	Tetrasporophyte	JQ843335		JQ907448
		BG0037	Cabo Frio, RJ, Brazil	E. Plastino	06 November 1987	52190	Male gametophyte	JQ843338	JQ843361	JQ907454
		BG0038	Cabo Frio, RJ, Brazil	E. Plastino	06 November 1987	52191	Female gametophyte	JQ843337		JQ907453
		BG0048	Cabo Frio, RJ, Brazil	E. Plastino	06 November 1987	52191	Tetrasporophyte	JQ935794		JQ952653
Gracilaria rangiferina (kützing) Piccone	*Gracilaria* sp.	BG0092	Guajiru, Trairi, CE, Brazil	E. Plastino	08 April 1993	56055	Tetrasporophyte		JQ843362	JQ907456
Gracilaria tenuistipitata C.F.Chang & B.M. Xia	*Gracilaria tenuistipitata*	BG0062	Haikou, Hainan Island, China	E.Oliveira			Gametophyte		AY673996	AY673996
Gracilaria sp.	*Gracilaria domingensis*	BG0057	Guajiru, Trairi, CE, Brazil	E. Oliveira	16 September 1989	54948	Tetrasporophyte			JQ907443
Gracilariopsis lemaneiformis (Bory de Saint-Vicent) E.Y. Dawson, Acleto & Foldvik	*Gracilaria* "esmeralda"	BG0078	Ecuador	E. Oliveira		–		JQ843342		JQ907465
	Gracilaria "martin"	BG0079	Ecuador (tank)	E. Oliveira		–		JQ843343	JQ843363	JQ907466
Gracilariopsis mclachlanii Buryo, Bellorin & M.C. Oliveira	*Gracilaria* sp.	BG0072	Zanzibar, Tanzania	E. Oliveira	June 1992	–	Infertile plant		JQ843365	JQ907469
Gracilariopsis longissima (S.G. Gmelin) M. Steentoft, L.M. Irvine & W.F. Farnham	*Gracilaria* sp.	BG0051	England			–	Male gametophyte	JQ843345		JQ907468
		BG0052	England			–	Female gametophyte	JQ843344	JQ843364	JQ907467

Table 1 (continued)

Species	Original identification	Bank #	Collection site	Collector	Date	SPF	Life cycle phase	*cox*1 (664 bp)	*rbc*L (1,393 bp)	UPA (370 bp)
Gracilariopsis tenuifrons (C.J. Bird & E.C. Oliveira) Fredericq & Hommersand	*Gracilariopsis tenuifrons*	BG0039	Cabo Frio, RJ, Brazil	E. Plastino	06 November 1987	52192	Female gametophyte	JQ935796		JQ952655
		BG0040	Cabo Frio, RJ, Brazil	E. Plastino	06 November 1987	52192	Male gametophyte	JQ843354		JQ952663
		BG0042	Arraya, La Peña, Venezuela	M. Aponte	30 March 1989	52027	Tetrasporophyte	JQ843353		JQ952662
		BG0043	Arraya, La Peña, Venezuela	M. Aponte	30 March 1989	52027	Tetrasporophyte	JQ935800	JQ843368	JQ952661
		BG0044	Arraya, La Peña, Venezuela	M. Aponte	30 March 1989	–	Tetrasporophyte	JQ843351		JQ907472
	Gracilaria sp.	BG0047	Valença, BA, Brazil	E. Oliveira	16 December 1987	27958	Tetrasporophyte	JQ935795		JQ952654
	Gracilaria caudata	BG0050	Punta Banda, Baja California, México	E. Oliveira	07 June 1989	54484	Male gametophyte	JQ935798	JQ843366	JQ952659
	Gracilariopsis tenuifrons	BG0054	Arraya, La Peña, Venezuela	M. Aponte	30 March 1989	–	Male gametophyte	JQ843346		JQ907470
		BG0064	Itanhaém, SP, Brazil		11 May 1990	55323	Tetrasporophyte	JQ843349		JQ952657
		BG0073	Lagoa de Mundau, AL, Brazil	E. Plastino	21 January 1993	55700	Infertile plant	JQ935799	JQ843367	JQ952660
		BG0073a	Lagoa de Mundau, AL, Brazil	E. Plastino	21 January 1993	55700	Infertile plant	JQ843347		JQ952656
		BG0073b	Lagoa de Mundau, AL, Brazil	E. Plastino	21 January 1993	55700	Infertile plant	JQ843348		
		BG0082	Ubatuba, SP, Brazil	E. Plastino	July 1993	56086	Male gametophyte	JQ935801		
		BG0083	Ubatuba, SP, Brazil	E. Plastino	July 1993	56087	Female gametophyte	JQ843350		
		BG0085	Pajuçara, Al, Brazil	E. Plastino	03 March 1994	55734	Female gametophyte	JQ935797		JQ952658
		BG0086	Mundaú, AL, Brazil	E. Plastino	03 March 1994	55734	Female gametophyte			JQ907471

[a] GenBank accession numbers

Gp. isabellana, *G. rangiferina*, *Gp. tenuifrons*, *Gp. lemaneiformis*, *Gp. longissima*, and *Gp. mclachlanii*. The *rbc*L for *G. tenuistipitata* was previously sequenced for the same strain used in this work and was available from GenBank. The phylogenetic analyses for these *rbc*L sequences with others from the Genbank are shown in Fig. 3. By using two other Gracilariaceae genera, *Curdiea* and *Melanthalia*, as outgroups, the species of the genus *Gracilariopsis* formed a monophyletic assemblage highly supported in all analyses, but *Gracilaria* was monophyletic only in the Bayesian analysis (0.89 a posteriori probability). The sequences obtained from the samples in the germplasm bank clustered with other available sequences from the same species obtained in the GenBank. The germplasm bank strains of *Gp. tenuifrons* from Brazil, Mexico, and Venezuela grouped with a *Gp. tenuifrons* from Guadaloupe in the Caribbean. The sample of *Gp. longissima* from England grouped with another sample from the same species from Italy. The sample of *Gp. lemaneiformis* from Ecuador grouped with another one from Peru. The sample of *Gp. mclachanii* grouped with a previously sequenced sample from Tanzania.

The *Gracilaria* species formed different clades with varying support. A basal clade was formed only in the Bayesian analyses joining *G. vermiculophylla*, *G. chilensis*, and *G. tenuistipitata*. The following groupings were observed: (1) Strains of *G. caudata* from Brazil grouped with *G. caudata* from FL, USA; (2) *G. cornea* from Brazil grouped with one strain from Venezuela; (3) *G. domingensis* from Brazil formed a clade; (4) *G. isabellana* from Brazil grouped with a sample from Venezuela (as *G. lacinulata* in GenBank); and (5) *G. tenuistipitata* from China grouped with a sample attributed to the same species from India.

Discussion

The LAM-USP Germplasm Bank has been a very useful resource for several studies on Gracilariales. Successful unialgal isolation is a key step in setting up cultures. Once isolated, samples of *Gracilaria* and *Gracilariopsis* can be maintained in vitro for a long time at relatively low cost and with little labor, as the medium only needs to be replaced once a month by the requirement of low irradiance. For experimental purposes, apices are progressively cultivated in higher irradiance and nutrients. Depending on the treatment, the species can be successfully propagated in 1 month.

Only rarely is it possible to identify *Gracilaria* species without the presence of cystocarps and male reproductive structures. Identification based on gross morphology and vegetative anatomy is generally subjective and cumbersome because of high morphological plasticity, which explains the frequent misidentifications and extensive synonymy in this

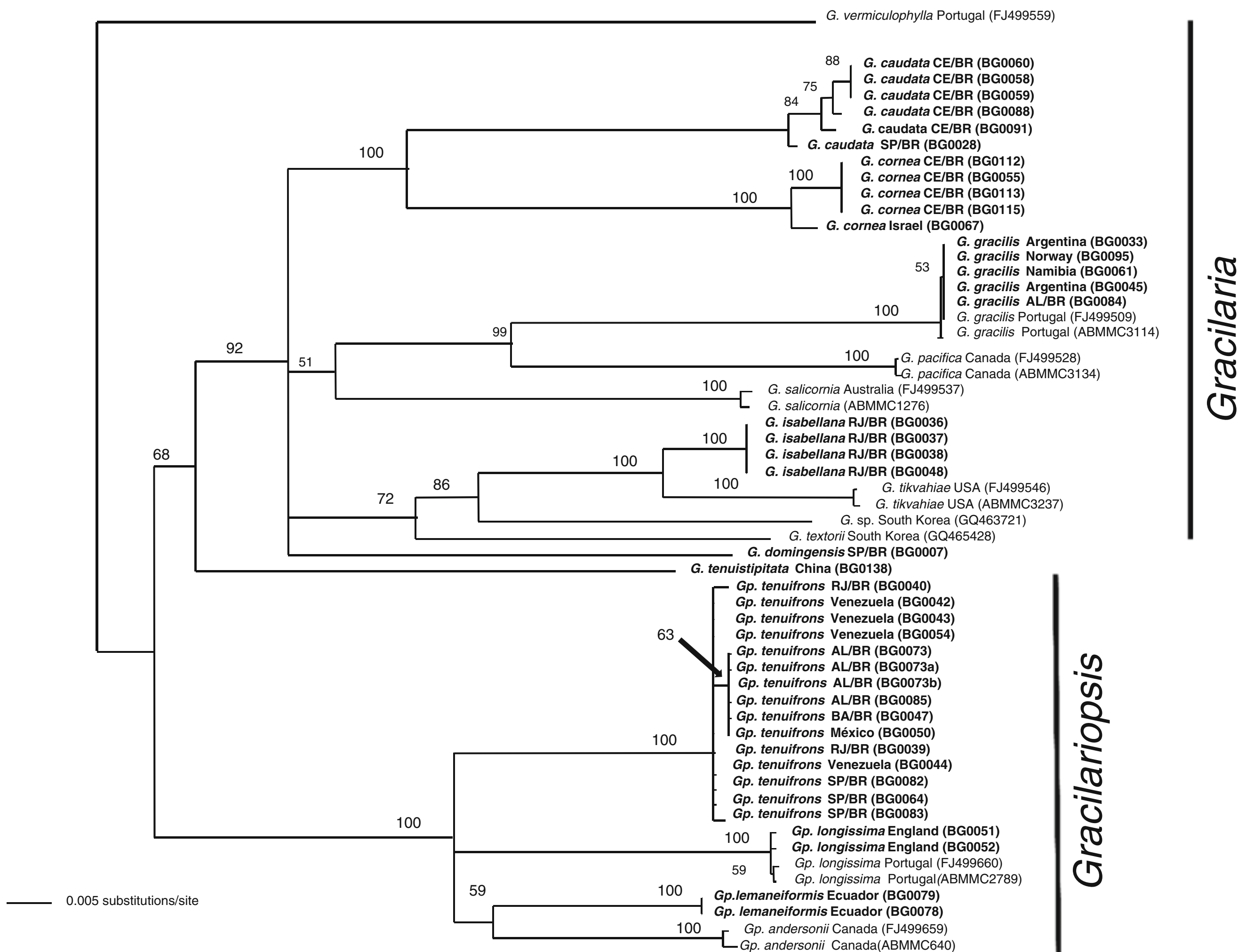


Fig. 1 Neighbor-joining phylogram for the *cox*1 region showing the grouping of the Gracilariaceae sequenced in this study (*in bold*) and available from databanks (GenBank and BOLD). Strain numbers are *in brackets* (see Table 1 for information on each strain). Bootstrap support values for 2,000 replicates are indicated on branches

group. Most species have no economic value, but for the few that do, this confusion in nomenclature has practical consequences (Bellorin et al. 2002, 2004; Saunders 2009).

Although *Gracilariopsis* has a much smaller biodiversity, species identification is even more difficult in the genus. With the sole exception of *Gracilariopsis silvana* Gurgel, which is flattened, all *Gracilariopsis* taxa are terete and stringy, looking very much alike. Therefore, in this genus, species are separated mostly based on geographical distribution, rather than on morphology and anatomy. This seemed adequate until some papers showed that some species of Gracilariales have a broad distribution and may be invasive (Bellorin et al. 2004; Saunders 2009).

Consequently, in addition to its inherent academic value, the use of short molecular tags for species identification is also demanded by industry, as a matter of economic exigency. Furthermore, molecular tags can be quite useful for field studies and to pinpoint the occurrence of invasive species. For instance, Saunders (2009) identified the invasive species *G. vermiculophylla* in Canadian waters using the 5′ region of *cox*1 in routine DNA barcoding of Gracilariales.

Based on the work of several investigators, the data obtained so far indicate that a significant amount of intraspecific variation (~1 %) for *cox*1 may occur in some in *Gracilaria* species. For example, intraspecific variation for *cox*1 found for the *Gracilaria* and *Gracilariopsis* species in this work was, in some cases, higher (up to 0.8 %) than that found by Saunders (2005)(1 or 2 bp ~0.2 %) for several genera of Rhodophyta. Yang et al. (2007) used *cox*1 (1,245 bp) to evaluate intraspecific variation in *G. vermiculophylla* from Asia and found a pairwise divergence up to 11 bp (0.9 %). Similar to the results of Yang et al. (2007), *G. caudata* from the southeast coast diverged by 0.8 % from strains of the same species from the northeastern coast of Brazil, and *G. cornea* cultivated in Israel (but originally collected in the Caribbean region, Alvaro Israel personal com.) diverged by 0.8 % from strains of the same species from Brazil.

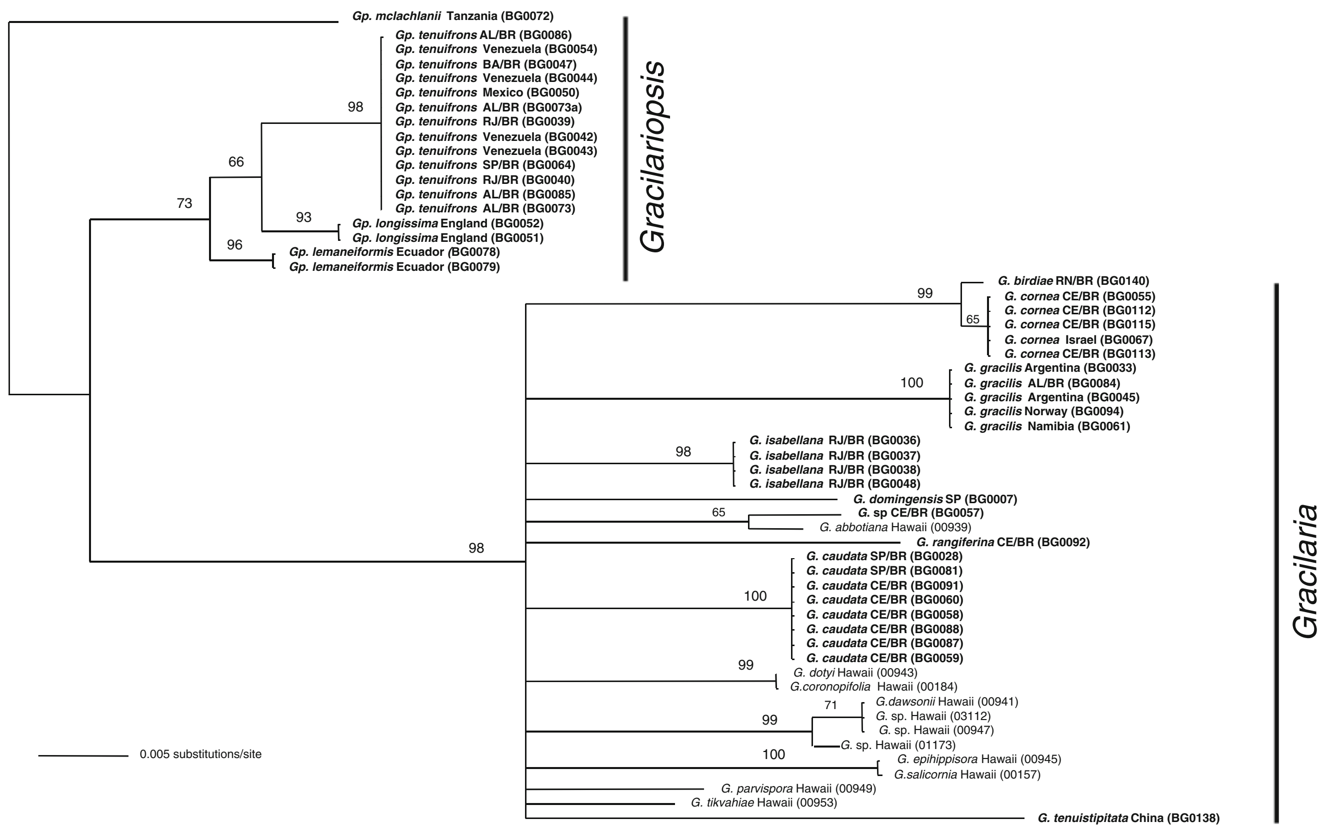


Fig. 2 Neighbor-joining phylogram for the UPA region showing the grouping of the Gracilariaceae sequenced in this study (*in bold*) and available from the Hawaiian Algal Database. Strain numbers are *in brackets* (see Table 1 for information on each strain). Bootstrap support values for 2,000 replicates are indicated on branches

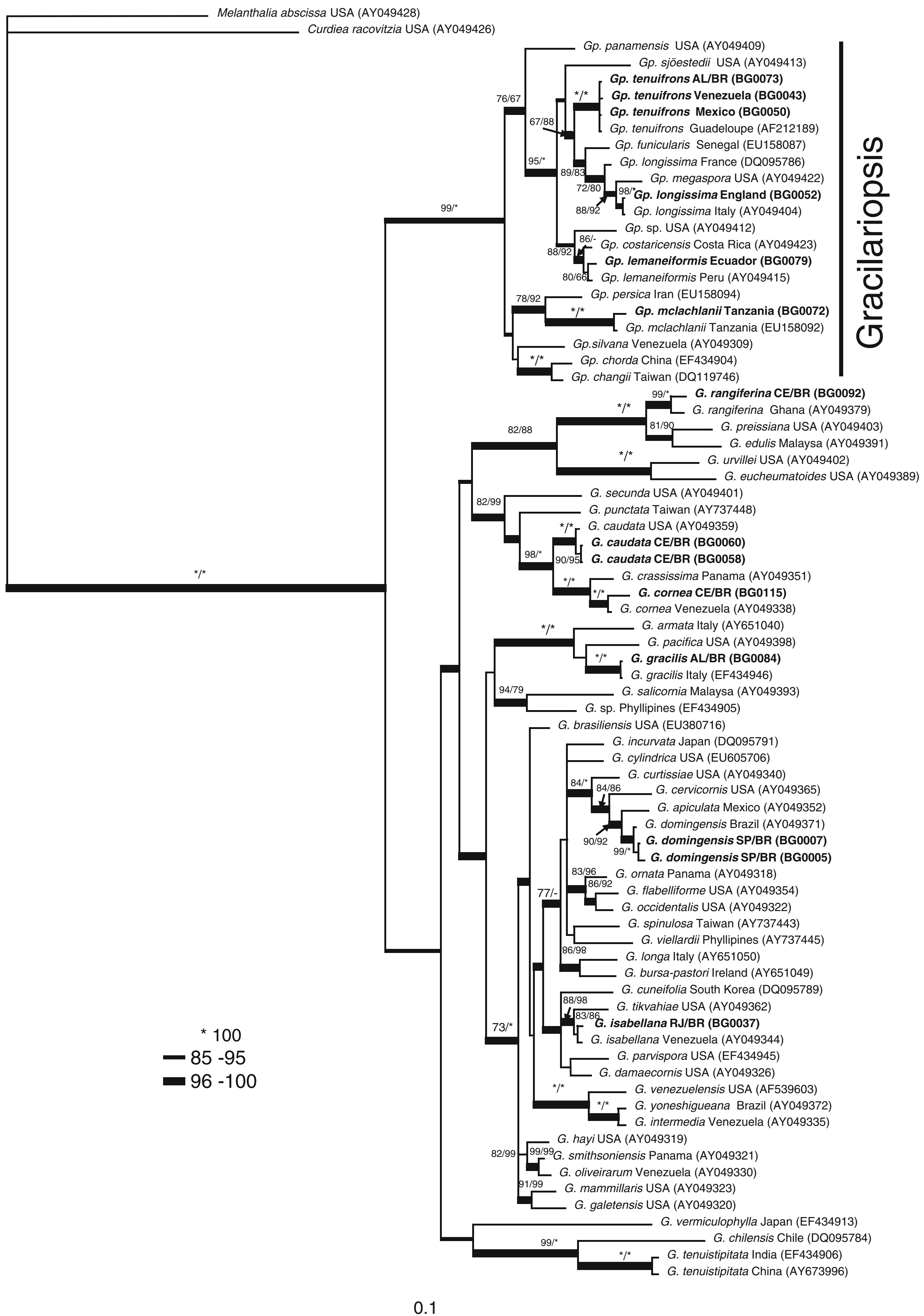
The interspecific variation for *cox*1 found for *Gracilariopsis* (5.8–6.5 %; 38–43 bp) was similar to the one found by Saunders (2005), but for *Gracilaria* (9.1–13.7 %; 60–91 bp), the values were higher. For this region, Saunders (2005) found that interspecific variation in several genera of Rhodophyta was around 30–40 bp, with some exceptions. For *cox*1, Yang et al. (2007) found that the interspecific nucleotide difference was also high among different species of Gracilariales (>41 bp, 3.2–16.1 % of 1,245 bp). Thus, the use of *cox*1 seems to be adequate for DNA barcoding of species in the Gracilariales, as previously demonstrated in various red algae (Robba et al. 2006; Saunders 2005, 2009).

As expected, the UPA sequences were more conserved and showed less interspecific and no intraspecific variation, and as in *cox*1 (Sherwood et al. 2010), interspecific variation for UPA was relatively higher for *Gracilaria* species (2.2–5.2 %; 8–19 bp) than that observed for *Gracilariopsis* (0.8–3.7 %; 3–14 bp). Nonetheless, the interspecific variation found for UPA was enough to separate the species in the germplasm bank. Considering that UPA is easier to amplify and sequence than *cox*1, UPA is a reliable molecular marker that can be used as a routine tag for the addition and tracking of strains in culture collections.

Relatively few sequences of *cox*1 and UPA for Gracilariales species are to be found in the databanks. Therefore, to help in species identification, *rbc*L was sequenced for one or more representatives of each of the *cox*1 and/or UPA clusters, as there are *rbc*L sequences for several species of Gracilariales available in the GenBank. The use of *rbc*L confirmed the previous identification of most samples or helped in the identification of those that were not given a species name when first included in the germplasm bank (Table 1).

In a few cases, the molecular marker results did not corroborate the original morphological identification. For example, based on the molecular markers, BG0007, originally identified as *G. cervicornis*, and BG0005, originally identified as *G. mammillaris*, both correspond to *G. domingensis*, while BG0050, originally identified as *G. caudata* (collected in Mexico), was identified as *Gp. tenuifrons* based on molecular markers. These discrepancies indicate a possible mislabeling during the manipulation of the isolates along the 20 years of

Fig. 3 Consensus tree derived from Bayesian analyses of *rbc*L sequences obtained in this study (*in bold*) and available from Genbank (*accession numbers in brackets*). Thickness of the branches indicates Bayesian a posteriori probabilities. Bootstrap supports (2,000) replicates which are shown on the branches as follows: maximum parsimony/neighbor-joining ▶


Melanthalia abscissa USA (AY049428)
Curdiea racovitzia USA (AY049426)
Gp. panamensis USA (AY049409)
Gp. sjöestedii USA (AY049413)
Gp. tenuifrons AL/BR (BG0073)
Gp. tenuifrons Venezuela (BG0043)
Gp. tenuifrons Mexico (BG0050)
Gp. tenuifrons Guadeloupe (AF212189)
Gp. funicularis Senegal (EU158087)
Gp. longissima France (DQ095786)
Gp. megaspora USA (AY049422)
Gp. longissima England (BG0052)
Gp. longissima Italy (AY049404)
Gp. sp. USA (AY049412)
Gp. costaricensis Costa Rica (AY049423)
Gp. lemaneiformis Ecuador (BG0079)
Gp. lemaneiformis Peru (AY049415)
Gp. persica Iran (EU158094)
Gp. mclachlanii Tanzania (BG0072)
Gp. mclachlanii Tanzania (EU158092)
Gp.silvana Venezuela (AY049309)
Gp. chorda China (EF434904)
Gp. changii Taiwan (DQ119746)
Gracilariopsis
G. rangiferina CE/BR (BG0092)
G. rangiferina Ghana (AY049379)
G. preissiana USA (AY049403)
G. edulis Malaysa (AY049391)
G. urvillei USA (AY049402)
G. eucheumatoides USA (AY049389)
G. secunda USA (AY049401)
G. punctata Taiwan (AY737448)
G. caudata USA (AY049359)
G. caudata CE/BR (BG0060)
G. caudata CE/BR (BG0058)
G. crassissima Panama (AY049351)
G. cornea CE/BR (BG0115)
G. cornea Venezuela (AY049338)
G. armata Italy (AY651040)
G. pacifica USA (AY049398)
G. gracilis AL/BR (BG0084)
G. gracilis Italy (EF434946)
G. salicornia Malaysa (AY049393)
G. sp. Phyllipines (EF434905)
G. brasiliensis USA (EU380716)
G. incurvata Japan (DQ095791)
G. cylindrica USA (EU605706)
G. curtissiae USA (AY049340)
G. cervicornis USA (AY049365)
G. apiculata Mexico (AY049352)
G. domingensis Brazil (AY049371)
G. domingensis SP/BR (BG0007)
G. domingensis SP/BR (BG0005)
G. ornata Panama (AY049318)
G. flabelliforme USA (AY049354)
G. occidentalis USA (AY049322)
G. spinulosa Taiwan (AY737443)
G. viellardii Phyllipines (AY737445)
G. longa Italy (AY651050)
G. bursa-pastori Ireland (AY651049)
G. cuneifolia South Korea (DQ095789)
G. tikvahiae USA (AY049362)
G. isabellana RJ/BR (BG0037)
G. isabellana Venezuela (AY049344)
G. parvispora USA (EF434945)
G. damaecornis USA (AY049326)
G. venezuelensis USA (AF539603)
G. yoneshigueana Brazil (AY049372)
G. intermedia Venezuela (AY049335)
G. hayi USA (AY049319)
G. smithsoniensis Panama (AY049321)
G. oliveirarum Venezuela (AY049330)
G. mammillaris USA (AY049323)
G. galetensis USA (AY049320)
G. vermiculophylla Japan (EF434913)
G. chilensis Chile (DQ095784)
G. tenuistipitata India (EF434906)
G. tenuistipitata China (AY673996)
Gracilaria
* 100
85 -95
96 -100
0.1

media and vial changes. This is further supported by the fact that *G. domingensis* is not found on the coast of São Paulo State (both BG0007 and BG0005 were originally collected from the São Paulo coast). *Gp. tenuifrons* has not been cited to Mexico, thus reinforcing the idea that some mistake was made with the labeling of specimens in the laboratory. Besides, the original algae have a *verrucosa*-type spermatangia distribution, which is different from *Gp. tenuifrons* that presents a *chorda*-type. These results highlight the importance of routinely using molecular markers to identify species kept in the germplasm bank.

Conclusions

Implementing the use of molecular markers for strains contained in the germplasm bank allowed us to define the existence of 13 different species in the bank. Unpublished sequences for *cox*1 and UPA were generated for 7 and 12 species of Gracilariales, respectively. Both *cox*1 and UPA were suitable DNA barcode markers to help track species of Gracilariaceae kept in culture in the germplasm bank, although UPA demanded considerably less effort and material for amplification. On the other hand, *cox*1 presents, in some cases, a low level of intraspecific variation and could be used to track individual strains of different populations of the same species, which can be also useful for the purpose of germplasm bank management. The addition of the DNA barcode tag to the samples in the Gracilariaceae germplasm bank and the molecular identification of the species will make this bank even more useful for future research as the species can be easily traced and confirmed.

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References

Barufi JB, Oliveira EC, Plastino EM, Oliveira MC (2010) Life history, morphological variability and growth rates of the life phases of *Gracilaria tenuistipitata* (Rhodophyta: Gracilariales) in vitro. Sci Mar 74:297–303

Bellorin AM, Oliveira MC, Oliveira EC (2002) Phylogeny and systematics of the marine algal family Gracilariaceae (Gracilariales, Rhodophyta) based on SSU DNAr and ITS sequences of Atlantic and Pacific species. J Phycol 38:551–563

Bellorin AM, Oliveira MC, Oliveira EC (2004) *Gracilaria vermiculophylla*: a western Pacific species of Gracilariaceae (Rhodophyta) first recorded from the eastern Pacific. Phycol Res 52:69–79

Bellorin AM, Buriyo A, Sohrabipour J, Oliveira MC, Oliveira EC (2008) *Gracilariopsis mclachlanii* sp. Nov. and *Gracilariopsis persica* sp. Nov. of the Gracilariaceae (Gracilariales, Rhodophyceae) from the Indian Ocean. J Phycol 44:1022–1032

Bhattacharya D, Elwood HJ, Goff LJ, Sogin ML (1990) Phylogeny of *Gracilaria lemaneiformis* (Rhodophyta) based on sequence analysis of its small ribosomal RNA coding region. J Phycol 26:181–186

Bird CJ, Oliveira EC (1986) *Gracilaria tenuifrons* sp. Nov. (Gigartinales, Rhodophyta) a specie from the tropical western Atlantic with superficial spermatangia. Phycologia 25:313–320

Bouzon ZL, Miguens F, Oliveira EC (2000) Male gametogenesis in the red algae *Gracilaria* and *Gracilariopsis* (Rhodophyta, Gracilariales). Cryptogam Algol 21:33–47

Bouzon ZL, Schmidt EC, Almeida AC, Yokoya NS, Oliveira MC, Chow FF (2011) Cytochemical characterization and ultrastructural organization in calluses of the agarophyte *Gracilariopsis tenuifrons* (Gracilariales, Rhodophyta). Micron 42:80–86

Chow FF, Oliveira MC (2008) Rapid and slow modulation of nitrate reductase activity in the red macroalga *Gracilaria chilensis* (Gracilariales, Rhodophyta): influence of different nitrogen sources. J Appl Phycol 20:325–332

Chow FF, Oliveira MC, Pedérsen M (2004) In vitro assay and light regulation of nitrate reductase in the red alga *Gracilaria chilensis*. J Plant Physiol 161:769–776

Chow FF, Capociama V, Faria R, Oliveira MC (2007) Characterization of nitrate reductase activity in vitro in the red seaweed *Gracilaria caudata* J. Agardh (Rhodophyta, Gracilariales). Rev Bras Bot 30:123–129

Collén J, Pinto E, Pedersén M, Colepicolo P (2003) Induction of oxidative stress in the red macroalga *Gracilaria tenuistipitata* by pollutant metals. Arch Environ Contam Toxicol 45:337–342

Costa VL, Plastino EM (2001) Histórico de vida de espécimes selvagens e variantes cromáticas de *Gracilaria* sp. (Gracilariales, Rhodophyta) in laboratory. Rev Bras Bot 24(suppl):491–500

Costa VL, Plastino EM (2011) Color inheritance and pigment characterization of red (wild-type), greenish-brown, and green strains of *Gracilaria birdiae* (Gracilariales, Rhodophyta). J Appl Phycol 23:599–605

Falcão VDR, Tonon AP, Oliveira MC, Colepicolo P (2008) RNA Isolation method for polysaccharide rich algae: agar producing *Gracilaria tenuistipitata* (Rhodophyta). J Appl Phycol 20:9–12

Falcão VDR, Oliveira MC, Colepicolo P (2010) Molecular characterization of nitrate reductase gene and its expression in the marine red alga *Gracilaria tenuistipitata* (Rhodophyta). J Appl Phycol 22:613–622

Ferreira LB, Barufi JB, Plastino EM (2006) Growth of red and green strains of the agarophyte tropical *Gracilaria cornea* (Gracilariales, Rhodophyta) in laboratory. Rev Bras Bot 29:185–190

Fredericq S, Hommersand MH (1989) Proposal of the Gracilariales ord. nov. (Rhodophyta) based on an analysis of the reproductive development of *Gracilaria verrucosa*. J Phycol 25:213–227

Freshwater DW, Fredericq S, Butler BS, Hommersand MH, Chase MW (1994) A gene phylogeny of the red algae (Rhodophyta) based on plastid *rbc*L. Proc Natl Acad Sci USA 91:7281–7285

Goff LJ, Moon DA, Coleman AW (1994) Molecular delineation of species and species relationship in the red algal agarophytes *Gracilariopsis* and *Gracilaria* (Gracilariales). J Phycol 30:521–537

Guimarães M, Plastino EM (1999) Plastid organization of color variants of the red macroalga *Gracilaria domingensis* (Gracilariales). Acta Microsc 8(Sup. C):795–796

Guimarães M, Plastino EM, Oliveira EC (1999) Life-history, reproduction, and growth of *Gracilaria domingensis* (Gracilariales, Rhodophyta) from Brazil. Bot Mar 42:481–486

Guimarães M, Plastino EM, Destombe C (2003) Green mutant frequency in natural populations of *Gracilaria domingensis* (Gracilariales, Rhodophyta) from Brazil. Eur J Phycol 38:165–169

Guimarães M, Viana AG, Duarte MER, Ascêncio SD, Plastino EM, Noseda MD (2007) Low-molecular-mass carbohydrates and soluble polysaccharides of green and red morphs of

Gracilaria domingensis (Gracilariales, Rhodophyta). Bot Mar 50:314–317

Guiry MD, Guiry GM (2010) Algaebase. World-wide electronic publication, National University of Ireland, Galway. http://www.algaebase.org. Accessed 24 Aug 2011

Gurgel CFD, Fredericq S, Norris JN (1999) Characterization and biogeographic affinities of the red algal genus, *Gracilaria* (Gracilariales), in the Gulf of México. J Phycol 35(suppl):13

Gurgel CFD, Liao LM, Fredericq S, Hommersand MH (2003) Systematyics of *Gracilariopsis* (Gracilariales, Rhodophyta) based on *rbc*L sequence analyses and mophological evidence. J Phycol 39:154–171

Gurgel CFD, Fredericq S, Norris JN (2004) *Gracilaria apiculata* and *G. flabelliformis* (Gracilariaceae, Rhodophyta): restoring old names for common tropical western Atlantic species, including the recognition of three new subspecies, and a replacement name for "*G. lacinulata*". Cryptogam Algol 25:367–396

Hagopian JC, Nyvall P, Oliveira MC (2002) Purification of plastid DNA from an enriched rhodoplast fraction of the red alga *Gracilaria tenuistipitata*. Plant Mol Biol Rep 20:406

Hagopian JC, Reis M, Kitajima JP, Bhattacharya D, Oliveira MC (2004) Comparative analysis of the complete plastid genome sequence of the red alga *Gracilaria tenuistipitata* var. *liui* provides insights on the evolution of rhodoplasts and their relationship to other plastids. J Mol Evol 59:464–477

Hall TA (1999) BioEdit: a user-friendly biological alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser 41:95–98

Hebert PDN, Cywinska A, Ball SL, de Waard JR (2003) Biological identifications through DNA barcodes. Proc R Soc Lond B 270:313–321

Huelsenbeck JP, Ronquist F (2001) MRBAYES: Bayesian inference of phylogeny. Bioinformatics 17:754–755

Lopes PF, Oliveira MC, Colepicolo P (1997) Diurnal fluctuation of nitrate reductase activity in the marine red alga *Gracilaria tenuistipitata* (Rhodophyta). J Phycol 33:225–231

Lopes PF, Oliveira MC, Colepicolo P (2002) Characterization and daily variation of nitrate reductase in *Gracilaria tenuistipitata* (Rhodophyta). Biochem Biophys Res Commun 295:50–54

Lourenço SO, Vieira AAH (2004) Culture collections of microalgae in Brazil: progress and constraints. Nova Hedwigia 79:149–173

Machiavello J, Saito R, Garófalo G, Oliveira EC (1999) A comparative analysis of agarans from commercial species of *Gracilaria* (Gracilariales, Rhodophyta) grown in vitro. Hydrobiologia 399:105–108

Milstein D, Medeiros AS, Oliveira EC, Oliveira MC (2011) Will a DNA barcoding approach be useful to identify *Porphyra* species (Bangiales, Rhodophyta)? A case study with Brazilian taxa. J Appl Phycol. doi:10.1007/s10811-011-9702-3

Nyvall P, Collén J, Reis MS, Pedersén M, Setubal JC, Varani AM, Colepicolo P, Oliveira MC (2011) Analysis of expressed sequence tags from the agarophyte *Gracilaria tenuistipitata* (Rhodophyta). J Appl Phycol. doi:10.1007/s10811-011-9681-4

Oliveira EC (1984) Taxonomic criteria in the genus *Gracilaria* Greville (Rhodophyta): an experience with the Western Atlantic species. Hydrobiologia 116/117:55–58

Oliveira EC, Plastino EM (1984) The life-history of *Gracilaria* (Rhodophyta) from Brazil. J Phycol 32:203–208

Oliveira EC, Plastino EM (1994) Gracilariaceae. In: Akatsuka I (ed) Biology of economic algae. SSB Academic Publishing, The Hague, pp 185–226

Oliveira EC, Alveal K, Anderson RJ (2000) Mariculture of the agar-producing gracilarioid red algae. J Phycol 8:345–377

Plastino EM, Costa VL (1999) Ultrastructure of vegetative branches of the red macroalga *Gracilaria* sp. (Gracilariales). Acta Microsc 8 (suppl):793–794

Plastino EM, Costa VL (2001) Anomalous plastids in a light green strain of the red macroalga *Gracilaria* sp. (Gracilariales). Acta Microsc 3(Sup. C):315–316

Plastino EM, Oliveira EC (1988) Sterelity barriers among species of *Gracilaria* (Rhodophyta, Gigartinales) from the São Paulo littoral, Brazil. Brit Phycol J 23:267–271

Plastino EM, Oliveira EC (1990) Crossing experiments as an aid to the taxonomic recognition of the agarophyte *Gracilaria*. In: Oliveira EC, Kautsky N (eds) Cultivation of seaweeds in Latin America. University of São Paulo, São Paulo, pp 127–133

Plastino EM, Oliveira EC (1996) Approaches to the identification of terete Brazilian Gracilariaceae. Hydrobiologia 326/327:145–148

Plastino EM, Oliveira EC (1997) *Gracilaria caudata* J. Agardh (Gracilariales, Rhodophyta)—restoring an old name from a common Western Atlantic alga. Phycologia 36:225–232

Plastino EM, Oliveira EC (2000) *Gracilaria birdiae* (Gracilariales, Rhodophyta) a new specie from the tropical South American Atlantic with terete frond and deep spermatangial conceptacles. Phycologia 41:389–396

Plastino EM, Paula EJ, Oliveira EC (1995) Técnicas de hibridación en macroalgas marinas. In: Alveal K, Ferrario ME, Sar E (eds) Manual de Métodos Ficológicos. Universidad de Concepción, Concepción, pp 479–487

Plastino EM, Ursi S, Heimbecker AMC (1998) Efeito da temperatura e salinidade no crescimento de *Gracilariopsis tenuifrons* (Gracilariales, Rhodophyta). In: Paula EJ, Cordeiro-Marino M, Pupo Santos D, Plastino EM, Fujii M, Yokoya N (eds) IV Congresso Latino Americano de Ficologia, II Reunião Ibero-Americana de Ficologia e VII Reunião Brasileira de Ficologia. Sociedade Brasileira de Ficologia, São Paulo, pp 359–369

Plastino EM, Guimarães M, Matioli SR, Oliveira EC (1999) Codominant inheritance of polymorphic color variants of *Gracilaria domingensis* (Gracilariales, Rhodophyta). Genet Mol Biol 22:105–108

Plastino EM, Ursi S, Fujii MT (2004) Color inheritance, pigment characterization, and growth of a rare light green strain of *Gracilaria birdiae* (Gracilariales, Rhodophyta). Phycol Res 52:45–52

Posada D, Crandall KA (1998) Modeltest: testing the model of DNA substitution. Bioinformatics 14:817–818

Ramlov F, Plastino EM, Yokoya NS (2009) Efeitos do ágar no crescimento de explantes e na formação de calos em morfos pigmentares de *Gracilaria domingensis* (Kutzing) Sonder ex Dickie (Gracilariales, Rhodophyta). Rev Bras Bot 32:605–614

Robba L, Russell SJ, Barker GL, Brodie J (2006) Assessing the use of the mitochondrial *cox*1 marker for use in DNA barcoding of red algae (Rhodophyta). Am J Bot 93:1101–1108

Rossa MM, Oliveira MC, Okamoto OK, Lopes PF, Colepicolo P (2002) The effect of visible light effect on superoxide dismutase (SOD) activity in the red alga *Gracilariopsis tenuifrons* (Gracilariales, Rhodophyta). J Appl Phycol 14:151–157

Saunders GW (2005) Applying DNA barcoding to red macroalgae: a preliminary appraisal holds promise for future applications. Phil Trans R Soc 360:1879–1888

Saunders GW (2009) Routine DNA barcoding of Canadian Gracilariales (Rhodophyta) reveals the invasive species *Gracilaria vermiculophylla* in British Columbia. Mol Ecol Resour 9:140–150

Savolainen V, Cowan RS, Vogler AP, Roderick GK, Lane R (2005) Taxonomy, DNA, and the barcode of life. Phil Trans R Soc 360:1805–1811

Sherwood AR, Presting GG (2007) Universal primers amplify a 23S rDNA plastid marker in eukaryotic algae and cyanobacteria. J Phycol 43:605–608

Sherwood AR, Kurihara A, Conklin KY, Sauvage T, Presting GG (2010) The Hawaiian Rhodophyta Biodiversity Survey (2006–2010): a summary of principal findings. Plant Biol 10:258

Skriptsova AV, Nabivailo YV (2009) Comparison of three gracilarioids: growth rate, agar content and quality. J Appl Phycol 21:443–450

Swofford DL (2002) PAUP* phylogenetic analysis using parsimony (* and other methods), version 4. Sinauer, Sunderland

Ursi S, Plastino EM (2001) Crescimento in vitro de linhagens de coloração vermelha e verde clara de *Gracilaria* sp. (Gracilariales, Rhodophyta) em dois meios de cultura: análise de diferentes estádios reprodutivos. Rev Bras Bot 24:587–594

Ursi S, Pedérsen M, Plastino EM, Snoeijs P (2003) Intraspecific variation of photosynthesis, respiration and photoprotective carotenois in *Gracilaria birdiae* (Gracilariales: Rhodophyta). Mar Biol 142:997–1007

Ursi S, Guimarães M, Plastino EM (2008) Deleterious effect of TRIS buffer on growth rates and pigment contents of *Gracilaria birdiae* Plastino & E.C. Oliveira (Gracilariales, Rhodophyta). Acta Bot Bras 22:891–896

Yang EC, Kim MS, Geraldino PJL, Sahoo D, Shin J, Boo SM (2007) Mitochondril *cox*1 and plastid *rbc*L genes of *Gracilaria vermiculophylla* (Gracilariaceae, Rhodophyta). J Appl Phycol 20:161–168

Yokoya NS (2000) Apical callus formation and plant regeneration controlled by plant growth regulators on axenic culture of the red alga *Gracilariopsis tenuifrons* (Gracilariales, Rhodophyta). Phycol Res 48:133–142

Yokoya NS, Oliveira EC (1992a) Effects of salinity on the growth rate, morphology and water content of some Brazilian red algae of economic importance. Cienc Mar 18:49–64

Yokoya NS, Oliveira EC (1992b) Geographic distribution and growth responses to temperature variation of some South American red algae of economic importance. J Appl Phycol 4:339–345

Yokoya NS, Oliveira EC (1993) Effects of temperature and salinity on spore germination and sporeling development of South American agarophytes. Phycol Res 41:283–293