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DEFENSE EVOLUTION IN THE GRACILARIACEAE (RHODOPHYTA): SUBSTRATE-REGULATED OXIDATION OF AGAR OLIGOSACCHARIDES IS MORE ANCIENT THAN THE OLIGOAGAR-ACTIVATED OXIDATIVE BURST[1]

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Combined phylogenetic, physiological, and biochemical approaches revealed that differences in defense-related responses among 17 species belonging to the Gracilariaceae were consistent with their evolutionary history. An oxidative burst response resulting from activation of NADPH oxidase was always observed in two of the subgenera of *Gracilaria* sensu lato (*Gracilaria*, *Hydropuntia*), but not in *Gracilariopsis* and in species related to *Gracilaria chilensis* ("*chilensis*" clade). On the other hand, all species examined except *Gracilaria tenuistipitata* var. *liui* and *Gracilariopsis longissima* responded with up-regulation of agar oligosaccharide oxidase to an challenge with agar oligosaccharides. As indicated by pharmacological experiments conducted with *Gracilaria chilensis* and *Gracilaria* sp. "*dura*," the up-regulation of agar oligosaccharide oxidase involved an NAD(P)H-dependent signaling pathway, but not kinase activity. By contrast, the activation of NADPH oxidase requires protein phosphorylation. Both responses are therefore independent, and the agar oligosaccharide-activated oxidative burst evolved after the capacity to oxidize agar oligosaccharide, probably providing additional defensive capacity to the most recently differentiated clades of Gracilariaceae. As demonstrated with *Gracilaria gracilis*, *Gracilaria dura*, and *Gracilariopsis longissima*, the different responses to agar oligosaccharides allow for a fast and nondestructive distinction among different clades of gracilarioids that are morphologically convergent. Based upon sequences of the chloroplast-encoded *rbc*L gene, this study suggests that at least some of the samples from NW America recorded as *Gs. lemanaeiformis* are probably *Gs. chorda*. Moreover, previous records of *Gracilaria conferta* from Israel are shown to be based upon misidentification of *Gracilaria* sp. "*dura*," a species that belongs to the *Hydropuntia* subgenus.

***Key index words:* *Gracilaria*; *Gracilariopsis*; *Hydropuntia*; innate immunity; oxidative burst**

***Abbreviations:* DPI, diphenylene iodonium; ITS, internal transcribed spacer; *rbc*L, RUBISCO LSU; ROS, reactive oxygen species**

Among brown macroalgae, the capacity to respond to alginate degradation products with an oxidative burst is a universal trait of the order Laminariales

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(Küpper et al. 2002). Similarly, major clades within vascular plants are capable of sensing specific cell wall matrix oligosaccharide defense elicitors (Nürnberger et al. 2004, Kaku et al. 2006). The resistance of some *Gracilaria* species against opportunistic pathogens and epiphytes has also been demonstrated to be mediated by agar oligosaccharides that are generated during enzymatic attacks upon the host agar cell wall matrix. For example, in material from Israel that has so far been recognized as *G. conferta* (see Table S1 in the supplementary materials for taxonomic authors), agar oligosaccharides activated a defense response against agar-degrading bacteria (Weinberger and Friedlander 2000) that was correlated with an oxidative burst—a fast and transient production of reactive oxygen species (ROS) (Weinberger et al. 1999). The production of ROS was probably catalyzed by NADPH oxidase since it was (i) located at the plasma membrane and (ii) sensitive to nanomolar concentrations of diphenylene iodonium (DPI), a specific inhibitor of NADH- and NADPH-dependent enzymes (Weinberger et al. 2005). A repeated activation of NADPH oxidase was only possible after a refractory time of several hours, probably due to an involvement of protein phosphorylation events in the signal transmission between receptor protein and NADPH oxidase. A role of protein kinase(s) in the activation of the oxidative burst response was also indicated by the fact that Staurosporine—a specific inhibitor of such kinases—fully prevented the release of H_2O_2 (Weinberger et al. 2005).

Another species, *G. chilensis*, also responded with an increased production of H_2O_2 when oligosaccharides from agar were added to the medium. However, the source of H_2O_2 was located in the cell wall and was shown to be insensitive to DPI and Staurosporine. Moreover, it could be repeatedly activated at any time. Further investigation revealed that in *G. chilensis* the H_2O_2 production was catalyzed by an agar oligosaccharide oxidase, acting specifically on agar oligosaccharides with a free reducing end (Weinberger et al. 2005). High expression of agar oligosaccharide oxidoreductase in *G. chilensis* was correlated with increased resistance toward *Acrochaetium* sp., a red algal filamentous epiphyte. Moreover, treatment of *G. chilensis* with agar oligosaccharide resulted in increased expression of agar oligosaccharide oxidoreductase within 24 h (in preparation). Therefore, this species apparently has a detection system for either agar oligosaccharides or their oxidation products that activate transcription factors, although NADPH oxidase is not activated.

The surprisingly different responses to agar oligosaccharides of *G. conferta* from Israel and of *G. chilensis* obviously invited a comparative study of additional members of the Gracilariaceae, with the aim of searching for common defensive traits among genera and subgenera. This approach required a careful taxonomic identification of the specimens that were studied. Certain Gracilariaceae can be easily identified based on their typical compressed thallus morphology, but the precise taxonomic identification of sterile cylindrical specimens is a difficult task. For example, *G. gracilis*, *G. dura*, and *Gs. longissima*, three species with converging morphology that often form mixed stands in the NE Atlantic, are difficult to distinguish based upon morphological characteristics (Steentoft et al. 1995, Destombe et al. 2010). Molecular methods are therefore required to solve taxonomic problems within the Gracilariaceae. Sequencing of the chloroplast-encoded *rbc*L gene has been demonstrated to provide optimal resolution at the species level (Gurgel et al. 2003, 2004, Gurgel and Fredericq 2004, Gargiulo et al. 2006). In addition, methods based on the analysis of rDNA internal transcribed spacer (ITS) size variation (Wattier et al. 1997) and on restriction maps (RFLP) of amplified DNA (Goff and Coleman 1988, Scholfield et al. 1991, Candia et al. 1999, Guillemin et al. 2008) allow for relatively fast resolution of limited numbers of species.

In this study, we surveyed the physiological response of different species of Gracilariaceae to agar oligosaccharide, using a DNA-barcoding approach to identify species. The principle of this method is to use short DNA sequences as a tool to identify and assign individuals to known taxonomic entities. This approach allowed us to investigate the evolution of defense responses in the Gracilariaceae, comparing response patterns among phylogenetically different clades of this group.

MATERIALS AND METHODS

Materials. Sixty different strains of Gracilariaceae were used in this study, and 31 are presented in Table S1. Of them, nine have been deposited for future reference in a public culture collection (CCAP, Oban, UK). The remaining 29 samples were collected in two sites close to Roscoff in Brittany, France (Le Theven [48°42′ N, 4°2′ W] and Ile de Batz [48°44′ N, 3°59′ W]) and consisted of 14 *Gs. longissima,* five *G. dura,* and 10 *G. gracilis.* They were only used for a control experiment (see below) and therefore are not included in Table S1. To complement our study on the evolution of the defense responses mediated by agar oligosaccharides in Gracilariaceae, *Dasya baillouviana* (Ceramiales) was added as an outgroup (Table S1).

Three different strains of Gracilariaceae were obtained from M. Pedersén as unialgal fragments (Table S1; Nos. 18, 25, and 26) and grown for several months in the laboratory to generate the biomass needed. They were incubated in aerated SFC medium (Correa et al. 1988) at 15°C and at a photon flux density of 45 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (in the case of *G. tenuistipitata* var. *liui,* medium was 70% seawater and 30% tap water). *G. chilensis* (Table S1, No. 24) was also obtained as a unialgal fragment and grown up in a tank as described by Weinberger et al. (2005). Four nonunialgal strains, originating from tank cultivation, and one individual from a sea-based cultivation facility, as well as all specimens from natural populations, were obtained with a sufficient size to conduct the experiments. After collection, the thalli were transported within 60 h to the laboratory. Prior to experimentation, they were incubated for

at least 1 week in seawater to give them time to recover from transportation stress. Specimens from France were incubated in tanks with aeration and flow-through seawater at sea surface temperature. Day length was 12 h, and the photon flux density was 45 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Other specimens from temperate environments were incubated under the same light condition, but at 16°C and in aerated flasks containing 5 L of seawater (reduced salinity [16 PSU] in the case of *D. baillouviana*), which was changed every other day. Specimens from the Caribbean, the Mediterranean, and China were incubated in the same medium, but at room temperature (22°C–26°C) and exposed to indirect sunlight.

Species identification. Only a few specimens were identified unambiguously based upon their typical morphology. These were *G. bursa-pastoris* and *G. multipartita*. Because of the lack of diagnostic morphological characters in terete species of the Gracilariaceae (Bird 1995), all terete samples were identified using molecular diagnostic markers except *G. tikvahiae*, *G. chilensis*, and *G. conferta* (see Table S1). *G. tikvahiae* was collected from a natural population that had been previously identified as *G. tikvahiae* based upon sequencing of the *rbc*L gene (Gurgel and Fredericq 2004). Similarly, *G. chilensis* from Chile was collected from a farm population that had been previously confirmed as *G. chilensis* based upon sequencing of RUBISCO spacer, ITS1 region, and COX2-3 spacer (Cohen et al. 2004). Moreover, *G. conferta* was collected from a natural population in Morocco that had been previously identified based upon sequencing of RUBISCO spacer (Guillemin et al. 2008). Twenty-three samples were identified based upon sequencing of the *rbc*L gene and alignment with known sequences already deposited in GenBank. Only GenBank sequences of >98.5% of similitude with our *rbc*L sequence were considered as effective to typify our specimens. The primers and methods used to amplify the *rbc*L gene were described by Guillemin et al. (2008). *G. vermiculophylla* from El Jadida, Morocco (Table S1, No. 20), like the remaining 29 specimens not described in Table S1, was identified based upon their different RFLP patterns of the ITS and *rbc*L gene according to the method developed by Guillemin et al. (2008). The two remaining strains used in this study, *G. tenuistipitata* var. *liui* and *G.* sp. ''*pseudochilensis*,'' were already characterized by molecular methods (Cohen et al. 2004, Hagopian et al. 2004). The sample of *G.* sp. ''*pseudochilensis*'' corresponds to an undescribed sibling species of *G. chilensis* from New Zealand (Cohen et al. 2004).

Phylogenetic analysis. Phylogeny reconstructions were based on an *rbc*L coding sequence of 648 bp, and all sequences noted in Table S1 were used except DQ241581 for *G. secunda*, where only 404 bp were available. To complete the sequences of Table S1, the sequences of 11 Gracilariaceae species were retrieved from GenBank that represent all the major evolutionary lineages within Gracilariaceae as defined by Gurgel and Fredericq (2004): *Gs. tenuifrons* (AY049418), *G. edulis* (AY049387), *G. eucheumatoides* (AY049389), *G. arcuata* (AY049383), *G. salicornia* (AY049385), *G. tikvahiae* (AY049434 and AY049362), *G. venezuelensis* (AF539603), *G. intermedia* (AY049336), *G. hayi* (AY049315), *G. mammillaris* (AY049323), and *G. bursa-pastoris* (AY049375 and AY651032). Six species were used as outgroup: *Dasya baillouviana* (AF083373), *Epymenia obtusa* (AF385647), *Rhodymenia pseudopalmata* (AY168656), *Pachymenia carnosa* (AF385640), *Grateloupia doryphora* (AF488817), and *Grateloupia imbricata* (EU024817). In total, 56 sequences were used. The alignment was performed using the program Clustal (under default conditions), integrated with MEGA 3.1 (Kumar et al. 1993), and the result is available in FASTA format in the online supplementary material (see Appendix S1). As the sequence used is coding, a data partition using codon position was used. The best-fit models were selected for each of the three codon positions by the Akaike information criterion (AIC) tests implemented in the Treefinder program (Jobb et al. 2004) and their characteristics are summarized in the online supplementary material (see Appendix S2). Phylogenetic relationships were inferred with a mixed model in a maximum-likelihood framework by using Treefinder, version January 2008 (Jobb et al. 2004), and support for the nodes was assessed with 1,000 bootstrap pseudoreplicates.

To investigate the evolution of defense responses in the Gracilariaceae, phylogenetic relationships between the 18 species used (17 Gracilariaceae and *Dasya baillouviana*, see Table S1) were studied by exploring the underlying phylogenetic information from partially overlapping data sets. We performed a heuristic search of supertree space using the most similar supertree method criterion proposed in Clann (Creevey and McInerney 2004). Three other data sets (Cohen et al. 2004, Gurgel and Fredericq 2004, Guillemin et al. 2008) were used to complement this study.

Elicitation experiments. All chemicals and solvents used were from Sigma (St. Quentin, France) and Merck (Darmstadt, Germany). Short-time incubations for elicitation were conducted in petri dishes on a shaker, using autoclaved seawater as medium. Algal fresh weight density was generally 50 $mg \cdot mL^{-1}$, and the temperature during incubation was either 16°C or room temperature, depending on the temperature during the preceding incubation (see above). Two replicates of each individual were incubated in the presence of 5 μM DPI, which was added to the medium 30 min prior to elicitation from a stock solution prepared with DMSO. Two additional replicates were incubated with DMSO only. H_2O_2 in the medium of all replicates was quantified in intervals of 1 min as luminol-dependent luminescence, using a Lumat LB 9507 luminometer (EG&G Berthold, Bad Wildbad, Germany). The analytical procedure of the luminol/ferricyanide assay was described in detail by Weinberger et al. (2005).

Agar oligosaccharide at 300 μM was added to all four replicates (with and without DPI) when the level of H_2O_2 in the medium was stable. For the production of agar oligosaccharide, β-agarase from *Zobellia galactanivorans* was produced and used to degrade agarose (Lot no. GC530223; Eurogentec, Seraing, Belgium) as described earlier (Allouch et al. 2003). The saccharide was added from a stock solution containing 10 $mg \cdot mL^{-1}$, which was stored at −20°C. Based on its content of reducing saccharides, the molarity of the stock solution was 15 mM (Weinberger et al. 2005). After the addition of agar oligosaccharides to the algal medium, H_2O_2 concentrations were quantified in all replicates for 10 more minutes. After this time period, replicates with DPI were discarded. Replicates without DPI were thoroughly rinsed and transferred into clean petri dishes containing autoclaved seawater. Within 30 min after the first challenge with agar oligosaccharides, they were challenged again, to test whether they were in a refractory state. After the second challenge with agar oligosaccharides, thalli were shock frozen in liquid nitrogen and stored at −80°C.

H_2O_2 releases observed with the 31 gracilarioids listed in Table S1 were tested for significant ($P = 0.05$) differences among subgenera. For this purpose, the data were Box–Cox transformed and analyzed by analysis of variance (ANOVA) and Tukey's test, using the software package Statistica 7.0 (StatSoft, Tulsa, OK, USA). Fulfillment of the assumptions of homogeneity of variance and normal data distribution was tested with the Levine test ($P = 0.05$) and the Shapiro–Wilks test ($P = 0.05$), respectively.

Native PAGE of agar oligosaccharide oxidase. Algal samples were ground in liquid nitrogen and incubated at 4°C for 60 min in 1 $mL \cdot g^{-1}$ of extraction buffer (50 mM TRIS–HCl, pH 9.5, 500 mM potassium chloride, and 10 mM β-mercaptoethanol). Protein extracts were assayed for protein concentrations (Bradford 1976) and separated (50 μg of total protein per lane) by PAGE (Laemmli 1970) on 12% acrylamide gels, using

a buffer system free of sodiumdodecylsulphate. For staining of agar oligosaccharide oxidase, the gels were incubated for 3.5 h on a shaker at room temperature in phosphate buffer (0.1 M, pH 7.6) containing 0.16 mM phenazine methosulfate (PMS) and 0.24 mM 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Agar oligosaccharide substrate was added to the mixture at 10 mM. Additional stainings were conducted without this substrate to allow for distinction of bands that represent agar oligosaccharide oxidase and other bands.

RESULTS

Identification. Specimens were identified without difficulties either based upon their morphology or their genetic markers. Most of the *rbc*L sequences obtained were completely or nearly identical with sequences that have been published earlier (95.6% of the specimens analyzed showed a sequence similarity >99.8%, Table S1). Only in the case of *G. conferta* from Morocco did the *rbc*L gene display a relatively high dissimilarity of 1.3% with the closest related specimen that has so far been analyzed, *Gracilaria* sp. from Italy (Table S1). However, RFLP patterns of its ITS region and *rbc*L gene match perfectly with the size expected for *G. conferta* as published by Guillemin et al. (2008). Overall, our study included 17 confirmed species of Gracilariales, which comprised two different species of *Gracilariopsis* (*Gs. longissima*, *Gs. chorda*), as well as 15 species of *Gracilaria* and one species of Ceramiales, *D. baillouviana* (Table S1).

Our phylogenetic analysis is congruent with the previous result obtained by Gurgel and Fredericq (2004), showing a clear distinction between the two clades of *Gracilariopsis* and *Gracilaria* sensu lato. In

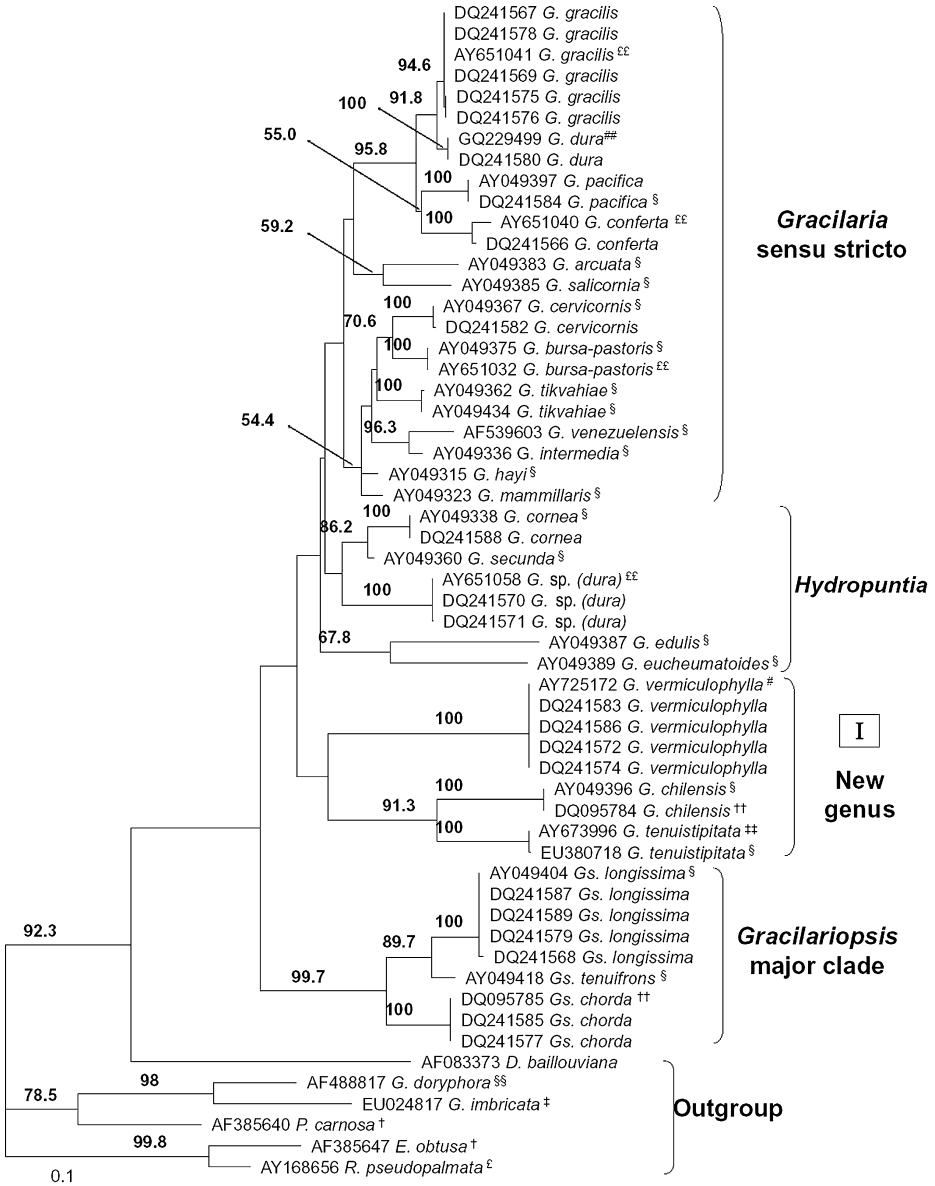

FIG. 1. Maximum-likelihood (ML) tree of score −4,731.45 inferred with a mixed model (Treefinder program, Jobb et al. 2004) using *rbc*L coding sequences of 648 bp with data partition using codon position. Twenty-nine species are represented, including 23 Gracilariaceae. The representation of the major evolutionary lineages within Gracilariaceae is defined according to Gurgel et al. (2004). Supports for the nodes (>50%) were assessed with 1,000 bootstrap pseudoreplicates and are noted above the nodes. The publications associated with the GenBank downloaded sequence are noted as symbols: £B. Gavio and S. Fredericq (unpublished), ‡Garcia-Jimenez et al. (2008), ‡‡Hagopian et al. (2004), †Hommersand and Fredericq (2003), ††Kim et al. (2006), §Gurgel and Fredericq (2004), §§Gavio and Fredericq (2002), ££Gargiulo et al. (2006), #Rueness (2005), ##Destombe et al. (2010).

the *Gracilaria* sensu lato clade, three subgroups were also identified: a subgroup including the four species of the first ''subgenus'' proposed by Gurgel and Fredericq (2004) (*G. chilensis*, *G.* sp. ''*pseudochilensis*,'' *G. vermiculophylla*, *G. tenuistipitata* var. *liui*), a subgroup constituted by three species of the second subgenus (''*Hydropuntia*''; *G. cornea*, *G. secunda*, and *G.* sp. ''*dura*''), and a subgroup including the eight species of the third subgenus (''*Gracilaria* sensu stricto''; *G. bursa-pastoris*, *G. multipartita*, *G. tikvahiae*, *G. cervicornis*, *G. conferta*, *G. gracilis*, *G. dura*, and *G. pacifica*) (Fig. 1).

Responses to agar oligosaccharides. A release of H_2O_2 was detected after addition of agar oligosaccharides into the medium of all tested gracilarioid strains, but not with *D. baillouviana* (Fig. 2). The intensity of this release, however, varied greatly. Particularly high rates of 100 nmol $\cdot g^{-1} \cdot min^{-1}$ or more were observed in all members of *Gracilaria* sensu stricto. By contrast, members of the *G. chilensis* clade and *Gracilariopsis* released <100 nmol $H_2O_2 \cdot g^{-1} \cdot min^{-1}$, and ANOVA revealed that their responses were significantly different from that of *Gracilaria* sensu stricto (Table 1). Members of the *Hydropuntia* clade behaved similarly to *Gracilaria* sensu stricto, with the exception of *G. cornea*.

In members of *Gracilaria* sensu stricto and *Hydropuntia*, a repeated challenge with agar oligosaccharide after 30 min generally resulted in a mean H_2O_2 release reduction by 50% or more as compared to the first response (Fig. 2, Table 1). By contrast, in members of the *G. chilensis* clade and of *Gracilariopsis*, the mean response after a repeated challenge always exceeded 50% of the first response, and in many cases, it even exceeded the first response (Fig. 2). ANOVA and Tukey's test revealed a significant difference among these two latter and the two former clades (Table 1).

Irrespective of the presence or absence of DPI in the medium during challenge with agar oligosaccharides, the release of H_2O_2 by members of the *G. chilensis* clade and *Gracilariopsis* was always in the same order of magnitude (Fig. 2, Table 1). The two subclusters differed from *Gracilaria* sensu stricto and *Hydropuntia* where a complete or nearly complete inhibition of H_2O_2 release was observed. In the cases of *G.* sp. ''*dura*'' from Israel and *G. cornea*, H_2O_2 was not released when DPI was applied, but

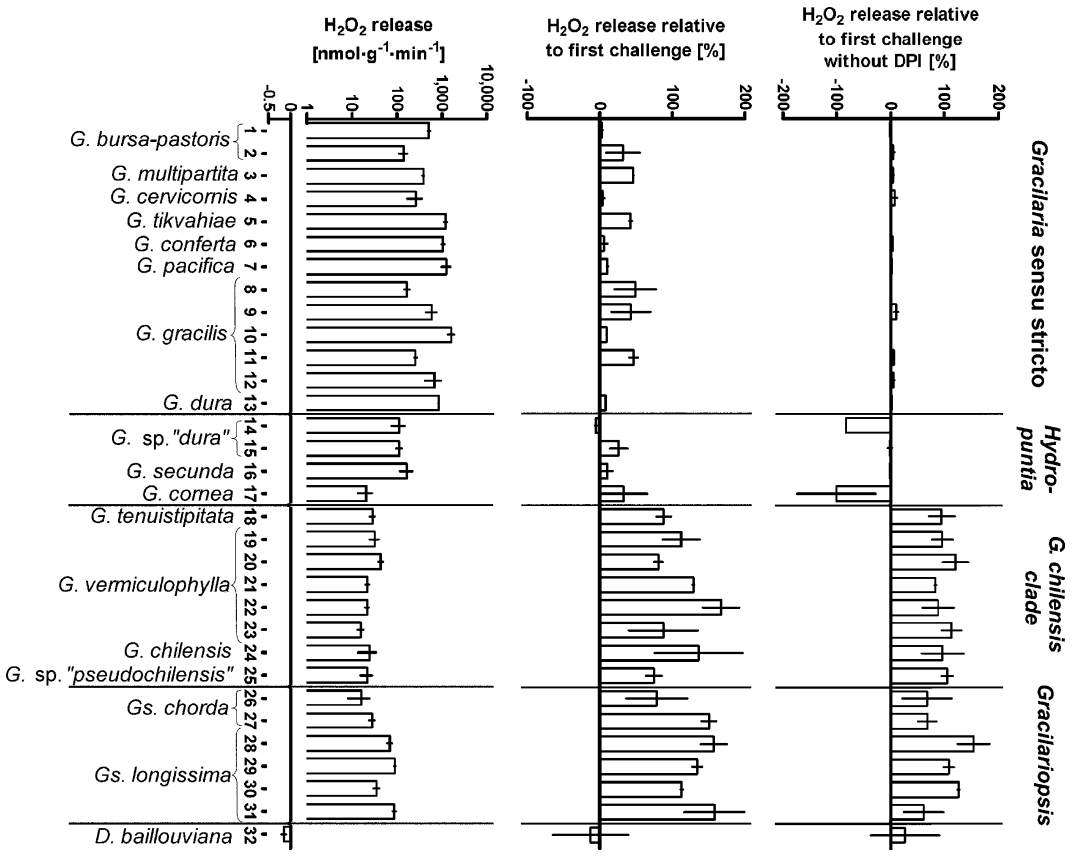

FIG. 2. Release of H_2O_2 after challenge of 31 gracilarioid algal strains and *Dasya baillouviana* with agar oligosaccharide. The release was quantified after a first challenge (bottom), after a second challenge in fresh medium 30 min later (center), and after a first challenge in medium containing 5 μM diphenylene iodonium (DPI) (top). Median +/− range, $n = 2$.

TABLE 1. H_2O_2 release by different clades of gracilarioids (mean ± 95% CI) after a first challenge with agar oligosaccharides, after a second challenge 30 min later, and after a first challenge in the presence of diphenylene iodonium (DPI) at 5 nM.

	n	I. After first challenge (nmol · g^{-1} · min^{-1})	II. After second challenge (% of I)	III. After first challenge in the presence of DPI (% of I)
Gracilaria sensu stricto	13	692.0 ± 273.4^{a}	23.2 ± 11.6^{A}	$3.3 \pm 1.8^{\alpha}$
Hydropuntia	4	103.5 ± 84.8^{b}	16.1 ± 23.5^{A}	$-46.7 \pm 71.9^{\beta}$
G. chilensis clade	8	26.2 ± 6.8^{c}	109.6 ± 25.5^{B}	$100.2 \pm 9.8^{\gamma}$
Gracilariopsis	6	$53.9 \pm 30.2^{b,c}$	132.1 ± 30.0^{B}	$97.9 \pm 36.3^{\gamma}$

Within each column, different letters (column I: a, b, c; column II: A, B; column III: α, β, γ) indicate data that are significantly different (one-way analysis of variance [ANOVA], $P = 0.05$, and Tukey's test, $P = 0.05$).

TABLE 2. Minima and maxima of H_2O_2 release by *Gracilaria gracilis*, *Gracilaria dura*, and *Gracilariopsis longissima* after a first challenge with agar oligosaccharides, after a second challenge 30 min later, and after a first challenge in the presence of diphenylene iodonium (DPI) at 5 nM.

	H_2O_2 release by		
	G. gracilis ($n = 10$)	*G. dura* ($n = 5$)	*Gs. longissima* ($n = 14$)
A. First challenge (nmol · g^{-1} · min^{-1})	105.0–1,135.8	309.6–1,090.6	1.0–50.7
B. Second challenge (% of A.)	−0.5–24.2	0.4–38.2	38.8–198.9
C. First challenge in the presence of DPI (% of A)	−8.4–11.6	−0.9–6.5	58.4–155.1

the concentration of H_2O_2 in the medium even decreased.

To test the suitability of H_2O_2 release in response to agar oligosaccharides as a trait for differentiating *G. gracilis* and *G. dura* from *Gs. longissima*, 29 specimens belonging to either of these species were collected from mixed stands. Their response to agar oligosccharides was quantified, and, afterward, they were identified by means of RFLP of ITS1. After a first challenge, all 15 individuals belonging to *G. gracilis* and *G. dura* released >100 nmol · g^{-1} · min^{-1}, while all of the 14 individuals belonging to *Gs. longissima* released markedly less (Table 2). After a second challenge, no *G. gracilis* or *G. dura* released >38.5% of the H_2O_2 released after the first challenge, while no *Gs. longissima* released less. An even clearer difference was detected when the H_2O_2 release response in the presence of DPI was compared (Table 2).

Native PAGE revealed that proteins showing agar oligosaccharide oxidase activity were present in nearly all examined taxa (Fig. 3). Only in *G. tenuistipitata* var. *liui* and *Gs. longissima* no such activity was detected. Treatment with agar oligosaccharide generally resulted in stronger or even in de novo expression of isoforms of this enzyme 24 h later. Electrophoretic banding of agar oligosaccharide oxidase differed from species to species, following no defined pattern. Three isoforms were detected in *G. gracilis*, and two isoforms in *G. pacifica*, *G. secunda*, and *G. vermiculophylla*, while the remaining taxa only expressed one detectable isoform.

The expression of agar oligosaccharide oxidase after treatment of *G.* sp. ''*dura*'' and *G. chilensis* with agar oligosaccharides was reduced—although in both species not fully prevented—when NADH- or NADPH-dependent enzymes were inhibited with DPI at 10 μM (Fig. 4). By contrast, an inhibition of kinases with 100 μM Staurosporine did not affect the expression of agar oligosaccharide oxidase in these species.

DISCUSSION

The taxonomic treatment of our material provides some new information about the distribution and phylogenetic status of certain gracilarioid seaweeds. For instance, the presence of *Gs. chorda* in the North American west coast has, to our knowledge, not been demonstrated yet. Instead, records of *Gs. lemanaeiformis* exist for Bamfield and other places in the NE Pacific (Scagel et al. 1993, Goff et al. 1994). *Gs. lemanaeiformis*, however, is nowadays considered endemic to Peru (Gurgel et al. 2003), and it is possible that at least some of the records from NW America are due to confusion with *Gs. chorda*. This view is supported by the fact that a specimen from China with the provisional name ''*Gs. lemanaeiformis*'' also turned out to be *Gs. chorda* in our study. On the other hand, *Gs. chorda* was not detected in a recent study of gracilarioids from British Columbia (Saunders 2009), and as our material of *Gs. chorda* was obtained from a strain collection, the possibility of confusion of strains in the past cannot be fully ruled out.

Our results also clarify the phylogenetic status of *G. conferta*. Of two very distinct specimens with the provisional name ''*G. conferta*,'' one was collected in Morocco, 400 km south of the type locality at Cap Spartel. Morphologically, this material fully

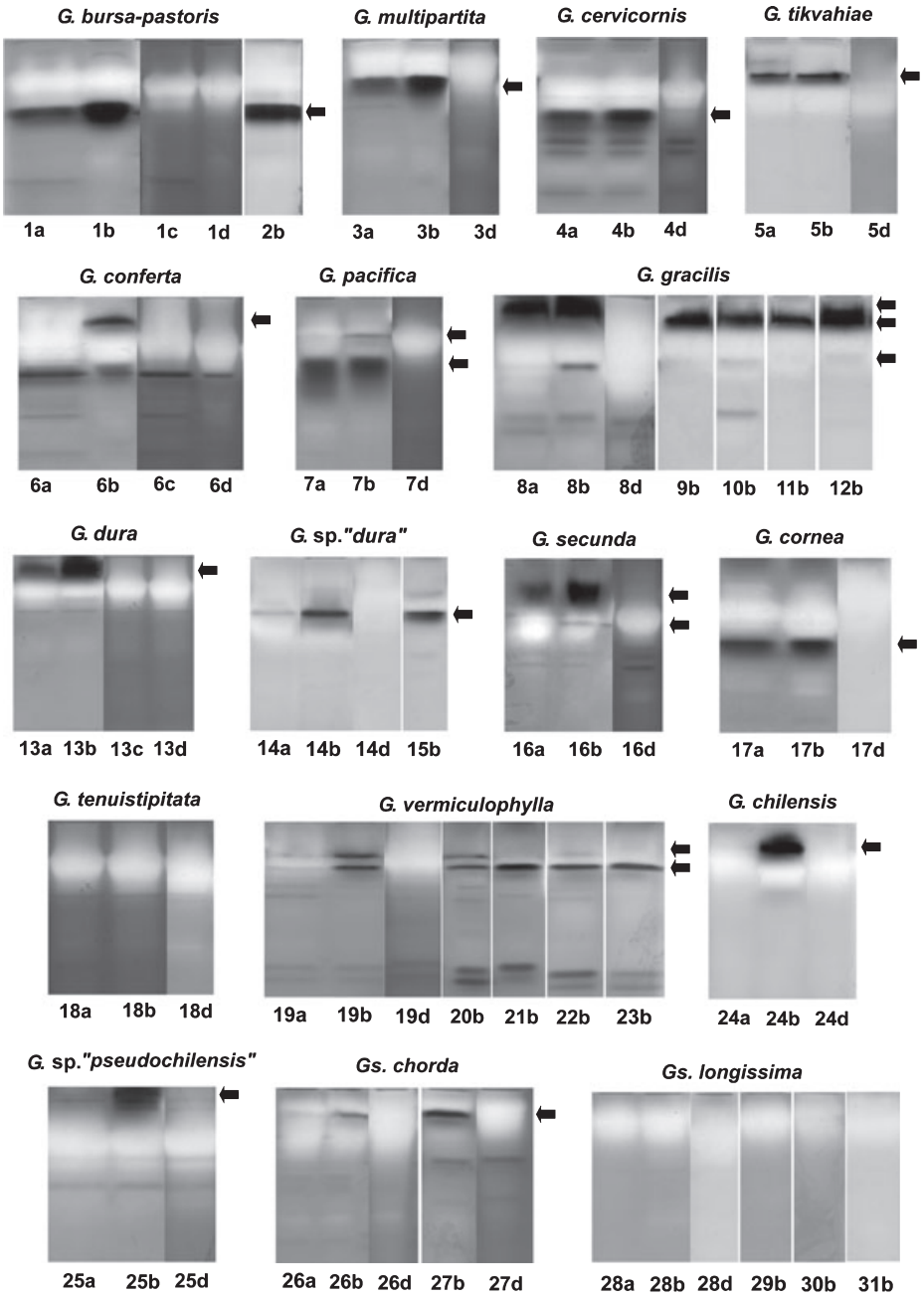

FIG. 3. Expression of agar oligosaccharide oxidoreductase in 31 gracilarioid strains (1–31) before (a, c) and 24 h after (b, d) challenge with agar oligosaccharide. Crude protein (50 μg per lane) was separated by electrophoresis under nondenaturating conditions. The gels were developed in the presence (a, b) and absence (c, d) of agar oligosaccharide. Arrows indicate bands that only appeared in the presence of agar oligosaccharide and therefore represent agar oligosaccharide oxidoreductase.

corresponded to the type material (PC – Herb. Thuret) and to descriptions of *G. conferta* published by Gargiulo et al. (1992). It seems, therefore, that this specimen actually can be considered as *G. conferta*. Its *rbc*L sequence, which had not been previously published, indicates that it belongs to *Gracilaria* sensu stricto, with *G. pacifica* and *G. gracilis* as close relatives. This result is in agreement with previously published sequence data of all three species for the RUBISCO spacer (Guillemin et al. 2008). In contrast with the material from Morocco, the material obtained as ''*G. conferta*'' from Israel was morphologically clearly different from the type material. Based upon its *rbc*L sequence, this second entity was demonstrated to belong to the subgenus *Hydropuntia* and to be conspecific with the sample from Italy described as ''*G. dura*'' by Gargiulo et al. (1992, 2006). Interestingly, material of *G. dura* from Morocco was demonstrated in an earlier study to be related to *Gracilaria* sensu stricto rather than to *Hydropuntia* when its RUBISCO spacer and *rbc*L were examined (Guillemin et al. 2008, Destombe et al. 2010), suggesting that at least two species are currently confused under the name *G. dura*: the first one recognized as a sister species of *G. gracilis* (referred as *G. dura* in this article), and the second one related to the genus *Hydropuntia* (referred as *G.* sp. ''*dura*'' hereafter).

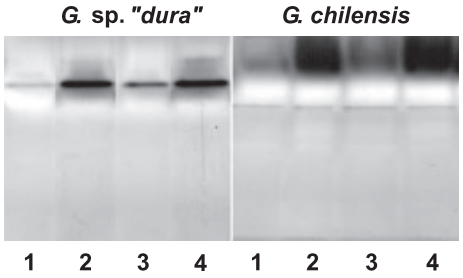

FIG. 4. Expression of agar oligosaccharide oxidoreductase in *Gracilaria* sp. ''*dura*'' and *Gracilaria chilensis*, 24 h after incubation without (1) or with (2–4) agar oligosaccharide. In treatments 3 and 4, 10 μM diphenylene iodonium (DPI) and 100 μM Staurosporine were present before and during the challenge, respectively. Crude protein (50 μg per lane) was separated by electrophoresis under nondenaturating conditions, and the gels were developed in the presence of agar oligosaccharide.

All studies conducted so far with *G. conferta* from Israel—including ours—dealt with the same species that has been identified in this report as *G.* sp. ''*dura*'' (M. Friedlander, personal communication). This cultivated material originated from an initial field sample, which was apparently misidentified as *G. conferta*. As a consequence, there is now little evidence left for the presence of *G. conferta* in the Mediterranean east of Sicily and the Adriatic Sea (Gargiulo et al. 1992).

All gracilarioids examined in our study released H_2O_2 after challenge with agar oligosaccharides. However, only species belonging to *Gracilaria* sensu stricto and *Hydropuntia* behaved as previously described for *G.* sp. ''*dura*'' from Israel. Members of these two clades were largely incapable of H_2O_2 release when they were either exposed to relatively low concentrations of the NADPH-oxidase inhibitor DPI or in a refractory state after previous challenge with agar oligosaccharides. This was not the case with species belonging to the *G. chilensis* clade or *Gracilariopsis*, all of which behaved as previously described for *G. chilensis*, releasing H_2O_2 repeatedly after challenge with agar oligosaccharides, even when DPI was present. Involvement of protein kinase and NADPH oxidase in the production of H_2O_2—the classical oxidative burst response—seems therefore to be limited to *Gracilaria* sensu stricto and *Hydropuntia*, while alternative mechanisms are apparently activated in the other two clades. Agar oligosaccharide oxidase activity may account for such alternative H_2O_2 production in most cases. Nearly all gracilarioids examined in our study expressed proteins displaying agar oligosaccharide oxidase activity, which indicates that oxidation of agar oligosaccharide may be widely distributed in the Gracilariaceae. However, in spite of the finding that *G. tenuistipitata* var. *liui* and *Gs. longissima* appeared to lack agar oligosaccharide oxidase, they responded consistently with NADPH-oxidase-independent H_2O_2 release when challenged with agar oligosaccharides. Therefore, either the extraction of the enzyme or its assays was flawed, or a third and yet unidentified mechanism of H_2O_2 production might have evolved in these species.

Altogether, the responses of gracilarioids to agar oligosaccharides (Fig. 5) seem to be consistent with the phylogenetic relationships inferred from *rbc*L sequences by Gurgel and Fredericq (2004). In particular, the establishment of the *G. chilensis* clade as an independent genus is supported by the incapacity of this group for an oxidative burst in response to agar oligosaccharides. This incapacity is also characteristic of *Gracilariopsis* and may be used as a taxonomic trait to discriminate both groups from *Gracilaria* and *Hydropuntia*. For example, our results obtained with

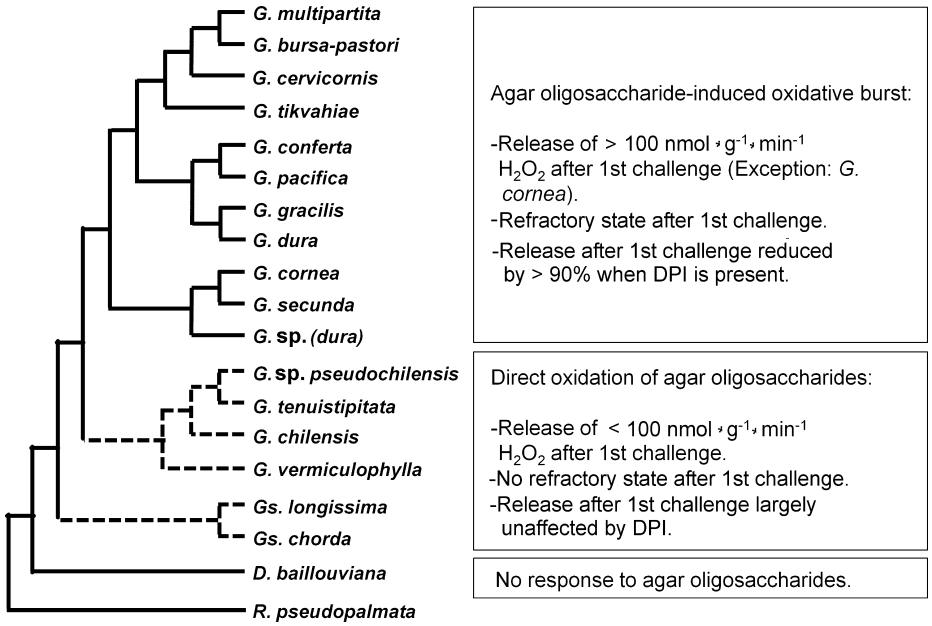

FIG. 5. Best topology representing the phylogenetic relationships between the 18 species studied (17 Gracilariaceae and *Dasya baillouviana*, see Table S1 in the supplementary material) determined by heuristic search of supertree space using the Most Similar Supertree Method (dfit) criterion (Clann program, Creevey and McInerney 2004). Besides this study, three partially overlapping data sets were used: Guillemin et al. (2008), Cohen et al. (2004), and Gurgel and Fredericq (2004). *D., Dasya; R., Rhodymenia.*

G. gracilis, *G. dura*, and *Gs. lemaneiformis* show that even small specimens may now be reliably separated in a fast and nondestructive way by quantifying their H_2O_2 release in response to agar oligosaccharides.

In our study, specimens belonging to *Hydropuntia* (namely, *G. cornea*) were often characterized by relatively low rates of H_2O_2 release. This was possibly due to a particularly strong antioxidant capacity, as indicated by the fact that *G. cornea* and *G.* sp. ''*dura*'' from Israel scavenged H_2O_2 at high rates when H_2O_2 production was inhibited by DPI. However, H_2O_2 release rates as low as in *Hydropuntia* were also observed in two individuals belonging to *G. gracilis* and *G. bursa-pastoris*. A clear-cut distinction of *Hydropuntia* and *Gracilaria* based upon the response to agar oligosaccharides is therefore not possible. An evaluation of the antioxidant systems of *Hydropuntia* and *Gracilaria* as a taxonomic trait was not the subject of our study and would require further investigation.

The fact that an oxidative burst response was absent in *Gracilariopsis* and in the *G. chilensis* subgenus leads to the question of whether these more ancestrally differentiated clades are capable of NADPH-oxidase expression. The available information on NADPH-oxidase expression in red algae is very limited (Hervé et al. 2005), but a DNA homolog of the NADPH-oxidase gene gp91phox has been detected in *Gracilaria lemanaeiformis* from Zhanshan Bay/Qingdao/China (GenBank accession number gb|BI544175.1, no publication linked). Material of *G. lemaneiformis* from the same origin was also examined in our study and identified as *Gracilariopsis chorda* (Table S1). At least one member of *Gracilariopsis* seems therefore to be capable of NADPH-oxidase expression. Nonetheless, NADPH-oxidase activation following exposure to agar oligosaccharides is apparently a derived character within the Gracilariaceae and thus, evolutionary, a relatively newly acquired response. It probably evolved with the acquisition of membrane-bound specific receptors for agar oligosaccharides or of a new signaling pathway that interlinked these receptors and NADPH oxidase.

In our study, species belonging to all four subclades responded to treatments with agar oligosaccharides with up-regulation of agar oligosaccharide oxidase. Thus, a universal presence of receptors for agar oligosaccharides in the Gracilariaceae cannot be entirely ruled out. On the other hand, our study provides preliminary evidence that different signaling pathways must be involved in the up-regulation of agar oligosaccharide oxidase and in the activation of NADPH oxidase: in *G.* sp. ''*dura*,'' the oxidative burst response is known to be sensitive to Staurosporine, but application of this kinase inhibitor had no effect on the expression of agar oligosaccharide oxidase in the same species and in *G. chilensis*. Apparently, protein phosphorylation is required for the activation of NADPH oxidase, but not for up-regulation of agar oligosaccharide oxidoreductase.

Nonetheless, the upregulation of agar oligosaccharide oxidoreductase involves a defined cellular signaling pathway, since it could be inhibited with DPI. Interestingly, several studies with vascular plants (Delledonne et al. 2001) and some recent studies with algae have demonstrated a function for NO radicals in defense signaling. These are typically generated by NO synthase, a DPI-sensitive enzyme, and it would be interesting to investigate in the future whether this pathway is also activated in the Gracilariaceae during exposure to agar oligosaccharides, mediating the expression of agar oligosaccharide oxidase.

Obviously, the oxidative burst may provide new defensive functions, but they are still under discussion. A direct role of NADPH-oxidase products as defense metabolites or as limiting substrates of the biosynthesis of defense metabolites is one of those functions. In *G.* sp. ''*dura*,'' significant amounts of agar-degrading microorganisms were eliminated or repelled within 15 min of exposure to nanomolar concentrations of agar oligosaccharides (Weinberger and Friedlander 2000, Weinberger et al. 2001). Such an effect could not be activated in *G. chilensis*, even when oligosaccharide concentrations 100 times higher were applied (Weinberger et al. 2005). Therefore, the oxidative burst seems to relate to eliminating or repelling associated microorganisms. This effect may not only result from direct cytotoxicity of the NADPH-oxidase products such as H_2O_2 but also from the resulting activation of vanadium haloperoxidase and increased release of halogenated hydrocarbons (Weinberger et al. 2008). Furthermore, it has been suggested that ROS generated by NADPH oxidase during the oxidative burst may play a role as second messengers in defense-related signaling cascades (Laloi et al. 2004, Van Breusegem and Dat 2006). Possibly, several independent transduction pathways are activated upon agar oligosaccharide detection in *Gracilaria* sensu stricto and *Hydropuntia*, as observed for the oligoguluronate-induced oxidative burst in the brown algal kelp *Laminaria digitata* (Cosse et al. 2009).

In conclusion, our study shows that the oxidation of agar oligosaccharides is nearly universal in gracilarioids. After such an oxidation, an NAD(P)H-dependent signaling pathway may induce expression of agar oligosaccharide oxidase. Sensing of agar oligosaccharides via a membrane-bound receptor and concomitant activation of an NADPH oxidase is restricted to the subgenera *Hydropuntia* and *Gracilaria* senso stricto. At present, however, it remains an open question whether both responses share common downstream signaling pathways. Our data also show that defensive traits may allow us to distinguish species and clades of the Gracilariaceae that cannot be identified based upon morphological characters. However, the capacity of the most ancestral genera

of the Gracilariaceae (*Melanthalia* and *Curdiaea*) and of other agarophytes (i.e., *Gelidium*) for the agar oligosaccharide-induced oxidative burst and the expression of agar oligosaccharide oxidase still remain to be examined.

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Supplementary Material

The following supplementary material is available for this article:

Table S1. List of species studied: identification, collection information, and GenBank accession numbers. Species identification was based either on morphology or molecular markers (*rbc*L or ITS1). For each *rbc*L sequence, percent of similarity with the closest sequence in GenBank is noted—the number of base pairs on which was made the comparison between the two sequences is given in parentheses.

Appendix S1. Fifty-six aligned *rbc*L coding sequences of 648 bp, representing 29 species and including 23 Gracilariaceae.

Appendix S2. Best-fit models selected for each of the three codon positions by the AIC tests implemented in TREEFINDER (Jobb et al. 2004).

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