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CRYPTIC DIVERSITY AND PHYLOGENETIC RELATIONSHIPS WITHIN THE *MASTOCARPUS PAPILLATUS* SPECIES COMPLEX (RHODOPHYTA, PHYLLOPHORACEAE)[1]

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***Mastocarpus papillatus* (C. Agardh) Kütz. is a common intertidal red alga occurring along the west coast of North America from Baja California to Alaska. Sequencing of both the chloroplast-encoded *rbc*L gene and the nuclear ribosomal internal transcribed spacer (ITS) regions of ~200 specimens from California to Alaska revealed that *M. papillatus* is actually a complex of at least five species. All five species have high bootstrap support in phylogenetic analyses of both genetic regions, and in the case of the ITS marker, the species also have distinctive patterns of indels. Three of the species are localized in the mid- to upper intertidal, whereas two of the species occur in the low intertidal. The species also have different geographic ranges that overlap in the Vancouver Island area of British Columbia. No distinctive, reliable morphological differences were observed among the species. Although a variety of names are available for species in the complex, it is not yet clear which name goes with which species. As part of the survey, I also sequenced other species of *Mastocarpus* in the northeast Pacific region, and I provide new distribution records for *M. jardinii* (J. Agardh) J. A. West and for a nonpapillate and probably undescribed species of *Mastocarpus*.**

Key index words: **cryptic species; *Mastocarpus*; North Pacific; nuclear ribosomal internal transcribed spacer; Phyllophoraceae; phylogeny; *rbc*L gene**

Abbreviation: **ITS, nuclear ribosomal internal transcribed spacer**

M. papillatus is a common species in intertidal communities in the northeast Pacific (Lindstrom and Foreman 1978, Dethier 1990). It occurs from Baja California, Mexico, to Alaska, according to Abbott and Hollenberg (1976, p. 525), who noted that it is ''probably the most common red alga on the Pacific Coast.''

The species has been well studied with regard to its taxonomy (Abbott 1972, Guiry et al. 1984), life history (West 1972, Polanshek and West 1975, 1977, Zupan and West 1988), and phylogenetic relationships (Fredericq and Lopez-Bautista 2002). Abbott (1972) subsumed at least three species into what was then called *Gigartina papillata* (C. Agardh) J. Agardh, noting that the species was highly variable morphologically and that the recognition of morphologically similar separate species could not be justified.

The nominate phase is the gametophyte, and it alternates with a crustose tetrasporophyte, previously identified as *Petrocelis franciscana* Setchell et N. L. Gardner (West 1972). Polanshek and West (1977) also observed an apomictic life history in which female gametophytes reproduced themselves without males or an alternate phase, and Zupan and West (1988) examined the geographic distribution of the two life history types in populations along the California coast and the Baja California coast of Mexico. Fredericq and Lopez-Bautista (2002) determined that *M. papillatus* belongs to a monophyletic *Mastocarpus* clade, which is closely related to several species of *Ahnfeltiopsis* [*A. leptophylla* (J. Agardh) P. C. Silva et T. C. DeCew and *A. paradoxa* (Suringar) Masuda] and to *Besa papillaeformis* Setchell.

The monophyly of the *Mastocarpus* clade and its intermediate position (not terminal, not basal) in the Phyllophoraceae as well as the widespread occurrence of *M. papillatus* in the northeast Pacific suggested that that species might be a suitable candidate for phylogeographic analysis in a region that has experienced repeated Pleistocene glaciation. The putative haploid nature of the upright thallus provided an additional incentive to study the species since it avoided the potential confounding effect of the heterozygous condition of a diploid organism on DNA analyses we might do. I was surprised to discover early in my endeavors that my collections of *M. papillatus* from this region contained very divergent ITS sequences, suggesting the existence of up to five species identified by this name. I then undertook more extensive and intensive geographic sampling to refute the hypothesis that five species were masquerading as *M. papillatus*. In this article, I describe and discuss the molecular diversity and the phylogenetic relationships within the *Mastocarpus* clade in the northeast Pacific.

MATERIALS AND METHODS

Between 30 and 50 mg of silica-gel-dried *Mastocarpus* from one thallus (specimens listed in Table S1, in the supplementary material, from sites shown in Fig. 1) were extracted using cetyl trimethyl ammonium bromide (CTAB) miniextraction, as described previously (Lindstrom and Fredericq 2003).

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To obtain complete nuclear ribosomal ITS sequences, I used forward primers that anneal in the 18S rRNA gene (either TW5 or ITS1) and reverse primers that anneal in the 28S rRNA gene (either JO6 or 28B—Lindstrom and Hanic 2005) and followed the PCR amplification protocol of Hughey et al. (2001). For chloroplast *rbc*L sequences, I used primers listed in and the amplification protocol described by Lindstrom and Fredericq (2003). PCR products were sequenced using ABI Applied Biosystems (Foster City, CA, USA) Big Dye Terminator V.3.1 cycle sequencing kit and protocol, with final steps carried out by the Nucleic Acid Protein Service Unit (University of British Columbia, Vancouver, BC, Canada).

Dr. Suzanne Fredericq provided unpublished ITS sequences for *A. leptophylla*, *M. jardinii* (CAL in analyses), and *M. papillatus* (BOD) from northern California, *A. paradoxa* and *M. yendoi* Masuda et T. Yoshida (as *M. mamillosus* [Goodenough et Woodward] Kützing) from Japan, and North Atlantic *M. stellatus* (Stackhouse) Guiry (NAT). Dr. Paul Gabrielson provided an unpublished *rbc*L sequence for an Alaskan *Mastocarpus* sp. (CPD) for use in the analyses. Drs. Wilson Freshwater and Max Hommersand shared unpublished *rbc*L sequences of *Mastocarpus* collections from California that provided expanded geographic records of taxa; these data were not used in analyses but are referred to in the Discussion. Published *rbc*L sequences for *A. leptophylla* (U21742—Fredericq and Ramírez 1996), *A. paradoxa* (AF388568—Fredericq et al. 2003), and *A. vermicularis* (C. Agardh) P. C. Silva et DeCew (AF388560—Fredericq et al. 2003) were used as outgroups in the analyses of *rbc*L sequences following the results of Fredericq and Lopez-Bautista (2002).

The sequence data were compiled and aligned using BioEdit (Hall 1999—alignments are available from the author upon request). Phylogenetic analyses were performed using the maximum-parsimony (MP), neighbor-joining (NJ), and maximum-likelihood (ML) algorithms of the computer program PAUP* 4.0b10 (Swofford 2002) as implemented by Lindstrom and Fredericq (2003). The appropriate model of evolution for likelihood analysis was determined by implementing the Modeltest program of Posada and Crandall (1998), which indicated that the most appropriate model for the *rbc*L data using the Akaike Information Criterion (AIC) was that of Tamura and Nei (1993), with unequal base frequencies but with substitution rates equal for transversions, a gamma distribution shape parameter, and the determined proportion of invariable sites. For the ITS data, the most appropriate model using AIC was that of Hasegawa et al. (1985), with unequal base frequencies but with substitution rates equal for transversions and for transitions (but unequal between transversions and transitions) and a gamma distribution shape parameter. Bootstrap proportions were determined based on 10,000 replicates for MP, 1,000 replicates for NJ, and 100 replicates for ML.

RESULTS

The *rbc*L gene was sequenced for the isolates listed in Table S1. Phylogenetic analyses of these sequences together with published sequences of *Ahnfeltiopsis* spp. showed that *Mastocarpus* specimens formed a monophyletic clade with 100% bootstrap support in all analyses (Fig. 2). Within the 1,347 bp alignment, there were 257 variable sites, among which 147 were parsimony informative. *M. jardinii* was the earliest-diverging taxon within the *Mastocarpus* clade. The remaining specimens

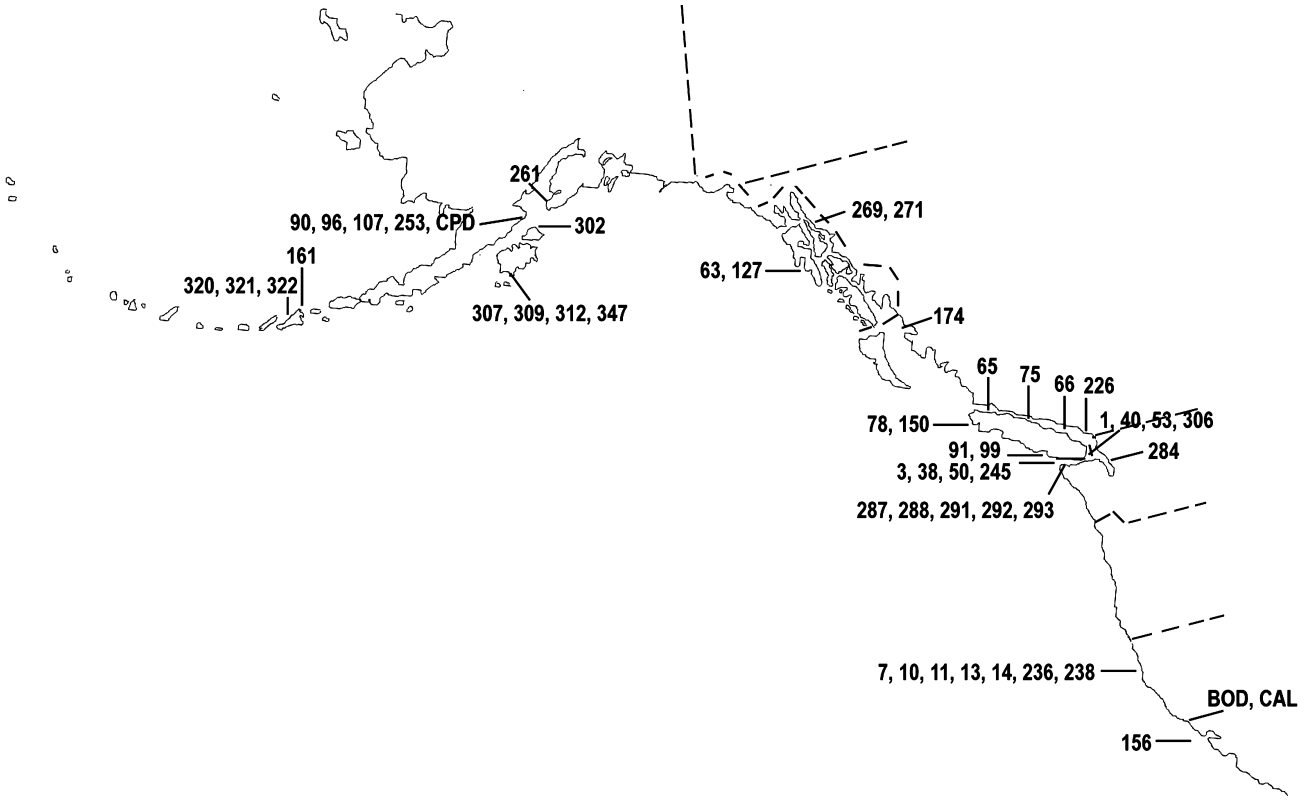

FIG. 1. Collection sites of specimens referred to in this study.

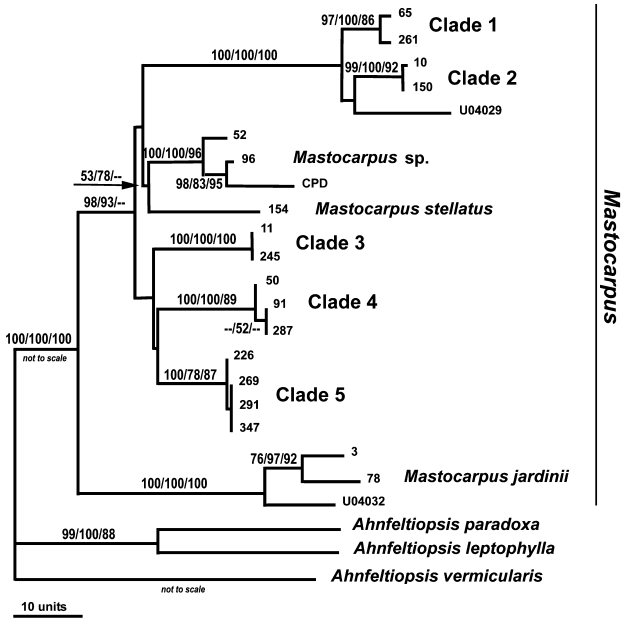

FIG. 2. Maximum-likelihood (ML) tree (−lnL = 4397.9493) for *rbc*L sequences. Numbers above branches (left to right) are bootstrap values >50% for maximum-parsimony (MP), neighbor-joining (NJ), and ML analyses.

occurred on a branch with strong bootstrap support in the MP and NJ analyses but no support in the ML analysis. These specimens fell into a number of strongly supported clades, but relationships among clades were not resolved. The sole exception was clades 1 and 2, which occurred together on a strongly supported clade. Specimens that had originally been identified in the field as *M. papillatus* are labeled in Figure 2 as clades 1–5. Also included in Figure 2 are an unidentified species of *Mastocarpus*, not readily confused with *M. papillatus* in the field because of its flat, narrow thallus and lack of papillae, and *M. stellatus* from the North Atlantic.

The ITS region was sequenced for more than 200 individuals of *Mastocarpus* from northern California to the eastern Aleutian Islands, Alaska, two individuals from northern Japan, and one individual from Norway. Thirty-three of these sequences, which covered the range of genetic variation within and between species, were used in the analyses described below together with the unpublished sequences listed above.

MP, NJ, and ML analyses produced similar topologies; the ML tree is shown in Figure 3, with bootstrap values for the analyses shown above branches that had support >50%. Since a major part of the variation in the sequences was the occurrence of indels, we also treated gaps as a fifth base in a parsimony analysis. In this analysis, deletions were reduced in size to reflect the probable number of events that led to the gap, based on differences in gap lengths among the sequences. Bootstrap values >50% for this analysis are shown below the branches.

As in the *rbc*L analyses, *Mastocarpus* again occurred as a monophyletic clade. *M. jardinii*, *M. stellatus*, *M. yendoi*, and the unidentified species of *Mastocarpus* mentioned above occurred on separate, strongly supported branches, as did clades 1–5 from the *rbc*L analyses. As in those analyses, clades 1 and 2 again occurred together on a strongly supported branch. Clade 3 was composed of two subclades, which appeared to be as divergent as clades 1 and 2 were from each other; however, there was little or no variation among specimens comprising the subclades. There was weak (MP and ML) to moderate (MP when gaps treated as fifth base) bootstrap support for a relationship between clade 3 and clades 1 and 2. Clade 4 occurred on a branch not clearly related to any other taxon. Clade 5 showed a close relationship to *M. jardinii* in all analyses, although bootstrap support was <50% when gaps were treated as a fifth base.

Analyses of concatenated *rbc*L and ITS sequences (not shown) produced results similar to the results from the separate analyses. The occurrence of indels caused the sequences in the different clades to vary in length from one another (Fig. 4). Whereas most of the variation in length was between clades, there was also some variation within clades. Within *Mastocarpus*, the overall pattern was one of decreasing sequence length from *M. jardinii* to clade 4 and increasing length from clade 4 to clade 1. Most differences were in the occurrence of small indels (1–3 bp), some of which were due to insertions of additional As or Ts in long sequences of those nucleotides, but there were larger indels of mixed nucleotides. One of the larger indels was a sequence of 11–13 nucleotides that occurred in *M. jardinii*, *M. stellatus*, *M. yendoi*, and *Mastocarpus* sp. but was absent in clades 1–5. *M. yendoi* had a 12 bp sequence that had 4–6 bp of homologous sequence in *M. jardinii* and *Mastocarpus* sp., but nothing in the remaining *Mastocarpus* taxa. Another large indel was a 7–8 bp sequence shared by all but clades 1, 2, and 3. Clades 1 and 2 had insertions of up to 7 bp found only in one or both of these clades.

Examination of voucher specimens revealed no clear-cut distinguishing morphological features separating the species that constitute clades 1–5 (see Fig. S1 in the supplementary material), with the possible exception of clade 4. Specimens in that clade have blades that are often distinctly constricted at some point along their length. The fact that some constriction can also be seen in specimens of other clades makes this character of questionable value. An anatomical examination of specimens to identify potentially distinguishing features was beyond the scope of this study.

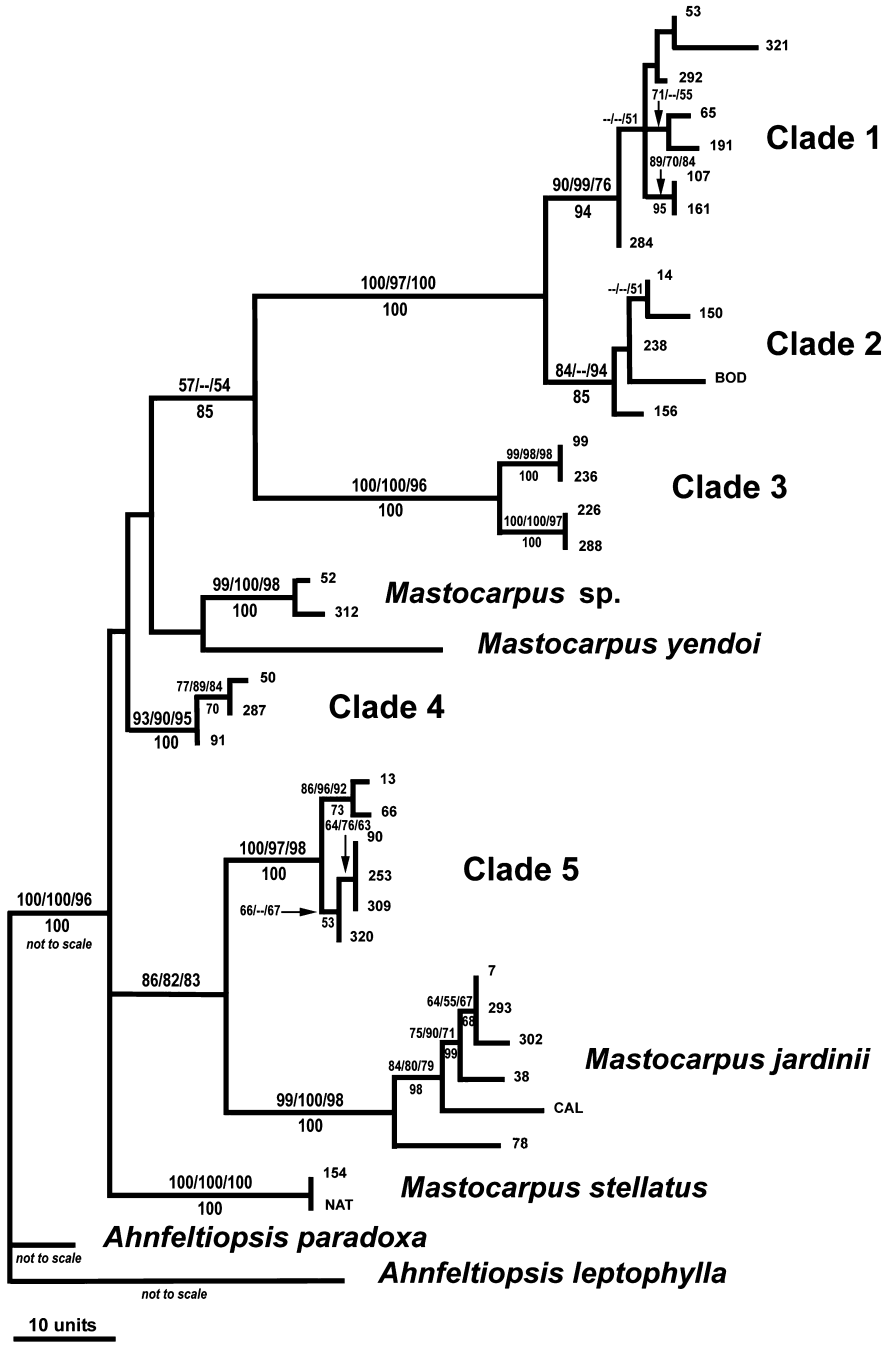

FIG. 3. Maximum-likelihood (ML) tree (−lnL = 3233.8240) for internal transcribed spacer (ITS) sequences. Numbers above branches (left to right) are bootstrap values for maximum-parsimony (MP), neighbor-joining (NJ), and ML analyses (only values >50% are shown); numbers below branches are bootstrap values for MP when gaps were treated as a fifth base.

DISCUSSION

The genus *Mastocarpus* was resurrected by Guiry et al. (1984) for species formerly placed in *Gigartina* but distinguished by canaliculate gametophytic thalli with female reproductive structures borne in papillae and by crustose tetrasporophytic thalli in which tetrasporangia were formed singly. They recognized four species from widely distributed locations: North Atlantic *M. stellatus*, northwest Pacific *M. pacificus* (Kjellman) Perestenko, northeast Pacific *M. jardinii*, and northeast Pacific *M. papillatus*. The occurrence of an additional Japanese species and one from Chile, both without names, was also mentioned. Fredericq and Lopez-Bautista (2002) confirmed that *Mastocarpus* spp. (*M. pacificus*, *M. papillatus*, and *M. stellatus*) occurred within a monophyletic clade related to *A. leptophylla*, *A. paradoxa*, and *Besa*

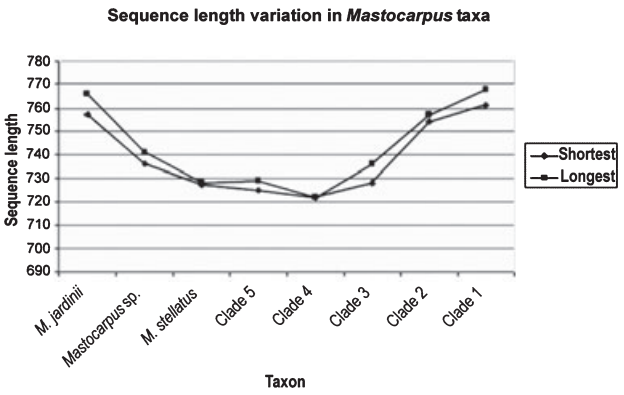

FIG. 4. Sequence length variation of the internal transcribed spacer (ITS) region in *Mastocarpus* species.

papillaeformis in the Phyllophoraceae. Our results confirm these studies and reveal additional diversity within the genus.

Both chloroplast-encoded *rbc*L and nuclear ribosomal ITS sequences provide markers that clearly identify individuals belonging to five clades that had previously been recognized as a single species, *M. papillatus*. All five clades have high bootstrap support for both genetic regions. Distinctions are based not just on nucleotide changes, but in the case of the ITS marker, also on distinctive patterns of indels. These observations indicate that the clades should be recognized as separate species.

Both genetic regions have been used in species-level taxonomy in the red algae. Cryptic species diversity was revealed by *rbc*L sequencing in *Gracilariopsis* (Gurgel et al. 2003) and *Grateloupia filicina* (De Clerck et al. 2005), among examples in the red algae. Bellorin et al. (2002) recognized as conspecific species of Gracilariaceae with ITS sequence divergences of 0.9% or less, and those with interspecific divergences exceeding 2% as distinct species. In a similar manner, Hughey et al. (2001) distinguished *Mazzaella oregona* from *M. phyllocarpa* using a 161 bp alignment of the ITS1. The two taxa varied at five positions, four of which represented indels and only one a base pair substitution.

In our analyses, the two most closely related clades, clades 1 and 2, differed by 2.4% (including indels) in the ITS region and 1.3% for the *rbc*L gene. These differences take into account only nucleotide positions that had fixed differences between the two clades so that even greater differences existed between individual specimens. A similar level of divergence (2.6%) was observed for the ITS region between the two subclades of clade 3, but there was no divergence in the *rbc*L gene between the two subclades. Moreover, the two subclades of clade 3 overlapped biogeographically, with both clades being collected at the same sites in northern California, Barkley Sound (BC), and the Strait of Juan de Fuca (BC). Only one of the subclades occurred in the Strait of Georgia and in northern British Columbia. The small number of specimens sequenced (12) that belonged to clade 3 limits the generalizations we can make about its biogeography. It is interesting, however, that one of the specimens (#226) had the ITS sequence of clade 3 but the *rbc*L sequence of clade 5. It was the only specimen to exhibit an indication of possible hybridization between clades.

Although these results indicate that *M. papillatus* is a complex of species that differ from one another at the molecular level, the species have hardly diverged morphologically (Fig. S1), so morphology cannot be used as a key feature to distinguish them. Nearly the entire range of morphologies, from narrow to broadly expanded blades and from unbranched to abundantly branched thalli, can be found within the two most widely distributed species, clades 1 and 5. Different habitat preferences and different biogeographic distributions (described below) provide some help in identifying which species occurs where without resorting to molecular methods. However, in many instances, to be sure which species is in hand, a molecular examination is required. Ludington et al. (2004, fig. 3) were also unable to distinguish morphologically the two species of the *M. papillatus* complex from central California that they identified using amplified fragment length polymorphisms.

Early workers recognized the morphological diversity within the genus in the North Pacific by creating a number of separate taxa, starting with *Sphaerococcus papillatus* (C. Agardh 1821—now *M. papillatus* [Kützing 1843, Guiry et al. 1984]) and including *Chondrus mamillosus* var. *ochotensis* (Ruprecht 1850—now *M. ochotensis* [Klochkova 1996]), *C. mamillosus* var. *sitchensis* (Ruprecht 1850—a combination in *Mastocarpus* has not yet been proposed), *C. mamillosus* var. *unalaschcensis* (Ruprecht 1850—now *M. unalaschcensis* [Klochkova 1996]), and *Gigartina pacifica* Kjellman (now *M. pacificus* [Perestenko 1980]). Additional names that may be available for some of the clades, but for which combinations in *Mastocarpus* have not been made, include *Gigartina cristata* (Setchell) Setchell et N. L. Gardner, *Gigartina dichotoma* N. L. Gardner, *Gigartina obovata* J. Agardh, *Gigartina sitchensis* (Ruprecht) Yendo, *Petrocelis franciscana* Setchell et N. L. Gardner, and *Petrocelis middendorfii* (Ruprecht) Kjellman. Which name(s) go with which clades will be the subject of future studies.

We do not know to which clade the name *M. papillatus* should be attached, nor do we know whether Abbott (1972) was correct in relegating *G. cristata* and *G. dichotoma* to synonyms of *M. papillatus*. Although we examined the ITS region in a number of collections from California, the type locality of all three taxa, the more extensive *rbc*L data of W. Freshwater and M. Hommersand indicate that members of clades 2, 3, and 5 and an unrecognized clade closely related to clade 2 are all

common on the coast of California. This finding suggests, as does the separation of clade 3 into two distinct subclades in our ITS analysis, that the northeast Pacific *M. papillatus* species complex may be even more speciose than the five clades recognized here.

We also do not know which species of the *M. papillatus* complex Polanshek and West (1975) cultured when they observed interfertility between Aleutian Islands and California isolates. Since clade 5 is the only species thus far recorded from both areas, it is possible that the crosses were between individuals belonging to that clade. However, the ability to cross and produce viable offspring is a condition that can extend beyond the speciation boundary (Coyne and Orr 2004), so it is possible that the crosses were between clades rather than within a clade. Thus, it is also possible that clades 1 and 2 were involved in the Aleutian × California crosses. These two clades appear to be the most closely related among the five clades we recognize, and they may have retained the ability to cross when cultured together despite the lack of genetic intermediates in field collections where their geographic distributions overlap (see below). Clade 1 is common in the Aleutians, whereas clade 2 is common in California.

This study does not address the genetic makeup of species in the *M. papillatus* complex as it relates to their life cycles. Some populations of the species complex exhibit both an alternation of heteromorphic generations and a direct type of life history (in which the female gametophyte recycles itself through carpospore production without benefit of fertilization), whereas other populations exhibit only an alternation of heteromorphic generations (Polanshek and West 1975, 1977, Zupan and West 1988). We suspect that the former variation occurs within a single species (W. Freshwater and M. Hommersand, unpublished *rbc*L sequence data). Ludington et al. (2004) also concluded that neither of the two non-interbreeding species they examined in the *M. papillatus* complex was genetically restricted to a direct type of reproduction because they observed field-collected tetrasporophytes, identified by their molecular signatures, for both species. Zuccarello et al. (2005) interpreted sequence differences between isolates of North Atlantic *Mastocarpus stellatus* to be sufficient to recognize separate species, but the plants with a direct life history had the plastid haplotype of one species and the mitochondrial haplotype of the other species. Divergence within *M. stellatus* suggestive of cryptic species diversity was also observed by Robba et al. (2006) using the mitochondrial cytochrome c oxidase subunit I (*cox1*) barcoding gene. Detailed studies combining life history characteristics and molecular sequences or signatures are required to clearly identify which species, if any, in the *M. papillatus* species complex have only an alternation of heteromorphic generations, which have both life cycle pathways, and which, if any, have only a direct life history.

The lack of bootstrap support for the backbone of the *Mastocarpus* phylogeny suggests that the genus may have experienced an episode of rapid speciation, or a radiation, recognized at the molecular level by the lack of a clear order to branching among a group of species, or their ancestors, which took part in the radiation. Late Cenozoic (Late Miocene to Pleistocene) radiations appear to be common among seaweeds and often involve at least one North Atlantic species and a number of North Pacific taxa (Lindstrom 2001). However, the same lack of support for branching order that suggests a radiation also makes it impossible for us to hypothesize at what point the genus entered the Atlantic from the Pacific (the presumed area of origin based on the restriction of the outgroups to the Pacific) and the possible movement of species between the eastern and western North Pacific, leading to speciation there as well. What the data do indicate is much more speciation activity in the northeast Pacific than in other regions. This region also experienced more glaciations during the Pleistocene than the northwest Pacific, and it is possible that Pleistocene glaciations acted like a species pump, increasing the rate of speciation due to periods of population isolation during glacial maxima. The idea of a species pump is supported in part by Weir and Schluter (2007), who observed a shorter time to divergence between sister species of New World birds and mammals at higher latitudes than in the tropics (but also a higher rate of extinction). The ability of *Mastocarpus* to survive and speciate during Pleistocene glaciations may reflect on the tenacity of its crustose phase, which allows it to withstand adverse conditions of the intertidal, including burial by sand and scouring by ice. Other recent studies have suggested that some taxa maintained their geographic ranges during Pleistocene glaciations in multiple cryptic refugia (Jacobs et al. 2004, Marko 2004, Wares and Cunningham 2005, Hickerson and Cunningham 2006). Earlier, Marko (1998) had suggested allopatric speciation might also occur during warm interglacials when populations are restricted to upwelling zones at more southerly sites.

Although differentiation does not extend to morphology, the species of the *M. papillatus* complex are distinguished by habitat and geographic distribution. Clades 1, 2, and 4 occur primarily in the mid- to high intertidal, whereas clades 3 and 5 occur mainly in the low intertidal. Figure 5 shows the current known geographic distributions of clades 1–5. Clades 1 and 2 differ primarily in their pattern of distribution, and this may reflect vicariant speciation (the geographic separation of previously interbreeding populations leading to divergence and ultimately to genetic isolation—Coyne and Orr 2004): clade 1 is known from northern Washington

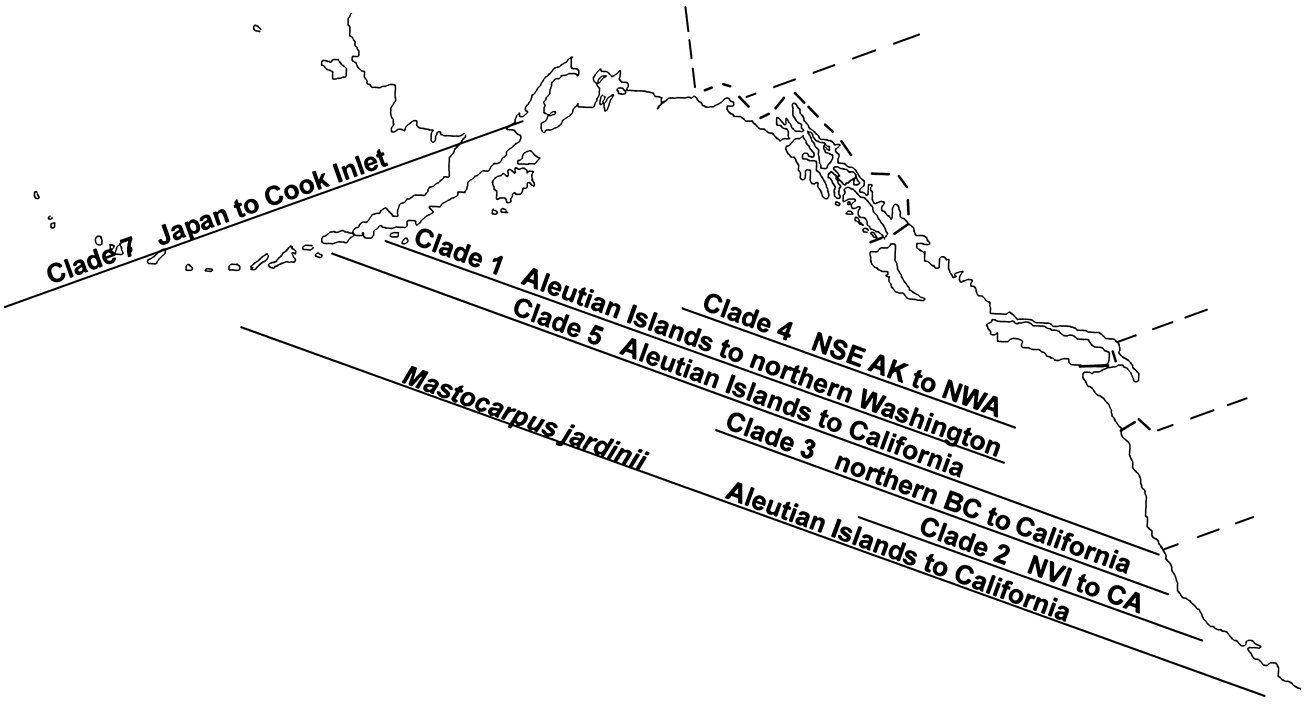

FIG. 5. Known geographic range of the species of *Mastocarpus* in the northeast Pacific.

(sample #284, Table S1) at least to the Aleutian Islands, Alaska (#321, GenBank DQ872477, UBC A85357), whereas clade 2 has been found on exposed coasts from California (#10, 14, 156, Table S1) to northern Vancouver Island (#150, Table S1) and at Clover (#1, GenBank EU186041, UBC A85272) and Harling Pts. (#40, GenBank DQ872479, UBC A85237), Victoria, British Columbia, in the eastern Strait of Juan de Fuca. Although these species ostensibly overlap in southern British Columbia, they have been found together only at Clover and Harling Pts., and no chimeric genotypes were detected at these sites. At its southern limit, clade 1 has thus far only been found on the east side of Vancouver Island (not on the exposed west coast), along both the north and south shores of the Strait of Juan de Fuca, in the San Juan Islands, and in Puget Sound.

Clade 3 has been collected from Southern California (Lindstrom, unpublished data) to northern British Columbia (#174, GenBank EU186047, UBC A85833). Clade 4 is known from northern Washington (#287, Table S1) to northern Southeast Alaska (#63, GenBank EU186048, UBC A85325), and clade 5 from central California (W. Freshwater and M. Hommersand, unpublished data) to the Aleutian Islands (#320, GenBank EU186055, UBC A85233). Additional collecting effort may expand the ranges of these taxa.

The present study also adds to the known distributions of two additional species of *Mastocarpus* (Fig. 5). We obtained sequences of *Mastocarpus* sp. from northern Japan (#52, Table S1) and from Alaska (from the eastern Aleutian Islands, #322, GenBank EU186061, UBC A85826; the Alaska Peninsula, #96; and Kodiak Island, #312; Table S1). *M. jardinii*, which had been recorded in the literature from San Luis Obispo County, California (Abbott and Hollenberg 1976, as *Gigartina agardhii* Setchell et N. L. Gardner), to northern British Columbia (Scagel et al. 1993), is now known to occur at least as far west as Adak Island, Aleutian Islands, Alaska (M. Lindeberg and S. Lindstrom, unpublished data). Among our collections, we found it in the Strait of Juan de Fuca as far east as Harling Pt. (#306, GenBank EU186058, UBC A85827) and in Johnstone Strait as far south as Rocky Bay (#75, GenBank EU186056, UBC A85342) on the east coast of Vancouver Island. In Alaska, we obtained specimens from Sitka Sound (#127, GenBank DQ872498, UBC A85225), Perevalnie Passage (#302, Table S1), and Kodiak Island (#307, GenBank EU186059, UBC A85354).

The treatment of gaps in sequence data can be problematic. When gaps provide an ambiguous alignment, they are often ignored, or they can be treated in a multistep procedure as described by Lutzoni et al. (2000). The gaps in our alignment were not ambiguous: we obtained the same topology when gaps were treated as missing data (MP, NJ, and ML analyses) or as a fifth base (MP analysis). Visual inspection of the alignment showed that most gaps were due to the insertion or deletion of a fixed sequence of 1–6 nucleotides. Even some larger gaps appeared to have occurred as a single event, as noted above. Therefore, we did not implement the

multistep treatment of Lutzoni et al. Rather, we coded each putative event as a single gap and analyzed each gap as a fifth base. This step did not change the topology of the phylogeny, but it did strengthen most of the branches in bootstrap analysis as seen in Figure 3.

Divergences among species of *Mastocarpus* suggest that the genus is relatively young, although not as young as some of the green genera we have studied. Van Oppen (1995) estimated a divergence of 13–14 Ma for the clade containing *Acrosiphonia*, *Spongomorpha*, *Urospora,* and *Ulothrix*, and we have observed variation in 93 of 585 bp positions (15.9%) in the ITS for 11 species in this group (S. Lindstrom and L. A. Hanic, unpublished). This is close to the value of 157 of 854 bp positions (18.4%) in the ITS for the 35 isolates of *Mastocarpus* included in the present study (however, these values do not take indels into account). Thus, it seems reasonable to assume that *Mastocarpus* is also mid- to late Miocene in age. The late Miocene to Pliocene period is recognized as a time of genetic diversification in the northeast Pacific due to peaks in coastal upwelling and existence of geographic barriers (Jacobs et al. 2004). The occurrence of a transition/transversion ratio >2.0 in *Mastocarpus* also suggests a relatively recent divergence (Bakker et al. 1995).

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Supplementary Material

The following supplementary material is available for this article:

Fig. S1. Morphologies of some sequenced specimens belonging to clades 1–5 (*Mastocarpus papillatus* species complex) and clade 7 (*Mastocarpus* sp.).

Table S1. Sources of *Mastocarpus* specimens for which the *rbc*L gene and/or internal transcribed spacer (ITS) region were sequenced. All specimens were collected by the first author, except as noted.

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