

PHYLOGEOGRAPHY OF *GRACILARIA TIKVAHIAE* (GRACILARIACEAE, RHODOPHYTA):
A STUDY OF GENETIC DISCONTINUITY IN A CONTINUOUSLY DISTRIBUTED
SPECIES BASED ON MOLECULAR EVIDENCE¹

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Gracilaria tikvahiae, a highly morphologically variable red alga, is one of the most common species of Gracilariaceae inhabiting Atlantic estuarine environments and the Intracoastal Waterway of eastern North America. Populations of *G. tikvahiae* at the extremes of their geographic range (Canada and southern Mexico) are subjected to very different environmental regimes. In this study, we used two types of genetic markers, the chloroplast-encoded *rbcL* and the nuclear internal transcribed spacer (ITS) region, to examine the genetic variability within *G. tikvahiae*, for inferring the taxonomic and phylogenetic relationships between geographically isolated populations, and to discuss its distributional information in a phylogeographic framework. Based on *rbcL* and ITS phylogenies, specimens from populations collected at the extreme distributional ranges reported for *G. tikvahiae* are indeed part of the same species; however, *rbcL*- but not ITS-based phylogenies detected phylogenetic structure among the ten *G. tikvahiae* different haplotypes found in this study. The four distinct *rbcL* lineages were identified as 1) a Canadian–northeast U.S. lineage, 2) a southeast Florida lineage, 3) an eastern Gulf of Mexico lineage, and 4) a western Gulf of Mexico lineage. We found no evidence for the occurrence of *G. tikvahiae* in the Caribbean Sea. Observed phylogeographic patterns match patterns of genetic structures reported for marine animal taxa with continuous and quasicontinuous geographic distribution along the same geographic ranges.

Key index words: biogeography; *Gracilaria tikvahiae*; Gracilariaceae; ITS; phylogeography; *rbcL*; rDNA; Rhodophyta

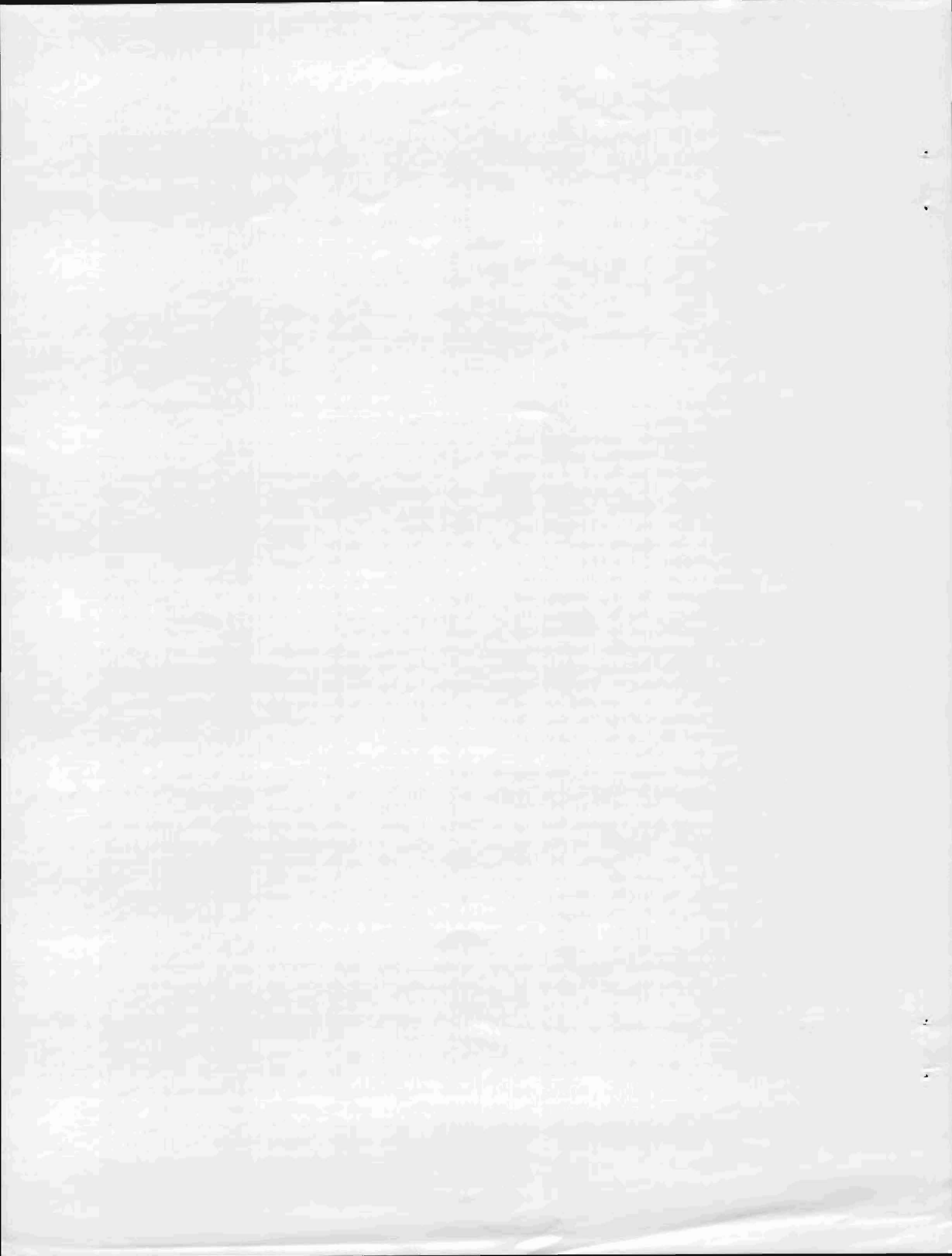
Abbreviations: indels, nucleotide insertions and deletions events; ITS, internal transcribed spacer; ML, maximum likelihood; MP, maximum parsimony;

rbcL, large subunit of the RUBISCO gene; SPN, statistical parsimony network

Gracilaria tikvahiae is one of the most common of seven species of Gracilariaceae (Gracilariales) currently recognized in the eastern North American benthic algal flora. It is a eurythermal and euryhaline red alga (tolerance range, 8–60 psu) (Bird and McLachlan 1986) typical of bays, inlets, and estuarine environments (Bird et al. 1979, McLachlan 1979). Morphological variation within this species ranges from terete to completely flat thalli, and this phenotypic variation resulted in much taxonomic confusion. The extent to which taxa have been misidentified with this species is still unknown. Specimens of *G. tikvahiae* that exhibit somewhat similar external morphologies have been reported under different names throughout the northwestern Atlantic as “*G. verrucosa*” or “*G. foliifera* var. *angustissima*” (Taylor 1957, 1960, Ganesan 1989, Schneider and Searles 1991, Bellorin et al. 2002). Because of the morphological diversity and taxonomic confusion and the range of the geographic distribution of *G. tikvahiae*, especially its southernmost limit, the number of cryptic species passing under this name is still unresolved. Several locales have been proposed as the southern limit of this species: the mid-Atlantic region of the Florida Peninsula (i.e. Palm Beach County; McLachlan 1979), the southern Gulf of Mexico (Edwards 1970), the southern Caribbean (Littler and Littler 2000), and Brazil (Schneider and Searles 1991, p. 326, Oliveira-Filho 1977, as “*G. foliifera*”). However, Ganesan (1989) noted that because of the presence of morphological characters that are scarce or ambiguous, records of this species for the Caribbean and South America need to be considered with caution and require critical examination. Outside the western Atlantic, *G. tikvahiae* has been recorded as an invasive species in Hawaii (Abbott 1999).

At the distributional extremes of well-known populations of *G. tikvahiae*, specimens are subjected to different temperatures and photoregimes. For instance, in subboreal Canada *G. tikvahiae* overwinters under ice

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sheets (McLachlan 1979), whereas in Mexico the species experiences warm tropical temperatures year around. The distinct environmental conditions inhabited by northern and southern populations of *G. tikvahiae* raise the question whether these populations are part of the same species or present enough genetic difference to place them in distinct taxa. For some warm-temperate estuarine species, the southern tip of the Florida Peninsula corresponds to a place of genetic discontinuity isolating the Gulf of Mexico populations from those of the Atlantic and promoting speciation, possibly due to historical (and maybe present) barriers to gene flow (Collin 2001, Gold et al. 2002). Marine ecosystems at the southern tip of the Florida Peninsula and the Florida Keys facing the Atlantic Ocean and the Caribbean Sea are distinct and characterized by a marine flora that is different from that of the warm-temperate Carolinian environments (Humm 1969, Searles 1984). The effect of historical factors (e.g. Pleistocene glaciations) and the Florida Peninsula working as a biogeographic barrier on near-shore marine organisms has been studied and confirmed using molecular techniques (Avisé 2000). Molecular studies have also revealed the presence of other genetic discontinuities along the eastern coast of the Florida Peninsula that influence the distribution of not only marine (Reeb and Avisé 1990, Adamkewicz and Harasewych 1996, Collin 2001) but also terrestrial species (Rising and Avisé 1993).

Molecular DNA sequence data have been used to study taxonomic and geographic relationships in red algae for which morphological characters are scarce and/or ambiguous (Brodie et al. 1998, Broom et al. 1999, Zuccarello et al. 2000, McIvor et al. 2001, Zuccarello and West 2002, Lindstrom and Fredericq 2003). However, few intraspecific molecular studies exist that explore the genetic distribution and discontinuity in the economically important order Gracilariiales (Goff and Coleman 1988, Candia et al. 1999). In this study we used two types of genetic markers to test whether the tropical populations of *G. tikvahiae* (from Mexico) pertain to the same species as temperate populations from Canada, to investigate the biogeography of this species, and to investigate whether morphologically different specimens are also genetically distinct. The markers used were the chloroplast-encoded *rbcL* gene and the nuclear internal transcribed spacer (ITS) regions 1 (ITS1) and 2 (ITS2) and the intervening 5.8S rDNA region.

MATERIALS AND METHODS

Sampling. Twenty specimens of *G. tikvahiae* were obtained from 17 distinct locations (Table 1, Fig. 1). Collections were made from specimens growing attached in the shallow subtidal or from drifting mats in estuarine ecosystems such as salt marshes, lagoons, protected bays, intracoastal waterways, jetties, and mangroves, regardless of sex or life-cycle stage and based on opportunity. Only thalli 12 cm or longer were considered for both molecular and morphological analyses, and the range of morphological variation reported for

G. tikvahiae was compiled. For molecular work, thalli were silica gel dried or liquid preserved in 70% isopropyl alcohol. Voucher specimens were liquid preserved in 5% formalin/seawater, kept and stored in silica gel pressed and/or pressed on herbarium sheets, and then deposited in the Herbarium of the University of Louisiana at Lafayette (LAF) and the Algal Collection of the U.S. National Herbarium, Smithsonian Institution (US). Habits of the recently collected specimens were compared with collections of *G. tikvahiae* deposited in the two mentioned herbaria.

DNA extractions, PCR amplifications, and sequencing. DNA samples were prepared using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) from fresh, silica gel dried, or 70% isopropyl alcohol fixed specimens. The *rbcL* gene was amplified using the primer combinations *rbcL*start-R753, F57-R753, F577-R1381, and F993-RbcSstart and sequenced with primers *rbcL*start, F7, F57, F492, F577, F753, F993, R753, R1105, R1381, and RbcSstart (Freshwater and Rueness 1994). PCR amplifications of the ITS1, 5.8S rDNA, and ITS2 regions were tested using all ITS primers listed in Lindstrom et al. (1996) and Goff et al. (1994). PCR primer combinations that produced the best results and therefore adopted to amplify the ITS data in this study were CI-Red5.8R and Red5.8F-AB28. ITS sequencing primers used were CI, ITS1, ITS2, ITS3 (Lindstrom et al. 1996), TW81, Red5.8F, Red5.8R, ITS2-800+, ITS2-700+, and ITS2-1100- (Goff et al. 1994). Protocols for double-stranded PCR amplification and automated DNA sequencing used for both genetic markers are identical to those given in Lin et al. (2001).

Phylogenetic analyses. The generated sequence data were compiled and aligned with Sequencher (Gene Codes Corp., Ann Arbor, MI, USA) and MacClade 4.0 (Maddison and Maddison 2000) and exported for phylogenetic analysis. The *rbcL* alignment included two outgroup sequences, *G. aff. damacornis* and *G. lacimulata* (Table 1), selected because of their closest phylogenetic relationship with *G. tikvahiae* (Gurgel and Fredericq 2004). The first 100 bp of the 1467-bp *rbcL* sequences were removed from the analyses due to missing data at this region on the alignment. The flanking regions of ITS1 and ITS2 corresponding to the 3' end of the small subunit rDNA and the 5' end of the large subunit rDNA genes were removed from the analyses, restricting the data set to the ITS1, 5.8S rDNA, and ITS2 regions. ITS phylogenetic analyses were performed unrooted because sequence divergence between different but closely related species of *Gracilaria* did not provide reliable alignments. Sequence information for *rbcL* and ITS markers and GenBank accession numbers are listed in Table 1. Alignments were deposited in TreeBase (study accession # = S967).

Maximum parsimony (MP) and maximum likelihood (ML) analyses as implemented in PAUP* v4.0 beta 10 (Swofford 2001) were conducted for both *rbcL* and ITS data sets. Parsimony trees were obtained under the Fitch criterion of equal weights for all substitutions (Fitch 1971). MP analyses were inferred based on a two-step approach. First, we conducted heuristic searches of 5000 random replications, holding 20 trees at each step, TBR swapping algorithm with the MULTREES and STEEPEST DESCENT options, excluding uninformative characters. Second, most parsimonious trees saved on the first analysis were swapped to completion in a second step MP analysis. ML trees for both data sets were inferred based on a heuristic search of 1000 random replications, holding 10 trees at each step, TBR swapping algorithm with MULTREES and STEEPEST DESCENT options. The optimal models of sequence evolution to fit the data alignments were estimated by hierarchical likelihood ratio tests performed by Modeltest v3.06 with alpha = 0.01 (Posada and Crandall 1998). The optimal model used for the *rbcL* data set was the

TABLE 1. List of species studied: identification, collection information, *Gracilaria tikvahiae* location numbers on the map of Figure 1, *rbcL*, ITS1, 5.8S, ITS2 GenBank accession numbers, and fraction sequenced.

Species of <i>Gracilaria</i>	Specimen collection data	<i>G. tikvahiae</i> isolate number	GenBank accession no., % sequenced <i>rbcL</i> haplotype names, ITS sequence identifications	
			<i>rbcL</i> haplotype names	ITS sequence identifications
<i>G. aff. damaecornis</i> J. Agardh	Harbor Branch Oceanographic Institution north jetty, Indian River, Fort Pierce, Florida, USA, 27°32'00" N × 80°20'39" W, coll. C.F.D. Gurgel, 13 July 1998		AY049326 (100%)	—
<i>G. lacimulata</i> (Vahl) Howe	La Encrucijada, Peninsula Paraguana, Falcón State, Venezuela; coll. C.F.D. Gurgel, J.E. Conde, C. Carmona; #FG-19, 13 July 1999		AY049344 (97.1%)	—
<i>G. tikvahiae</i> McLachlan	Canada: specimen #1, Morret pond, Pomquet Harbor, Antigonish Co., Nova Scotia, coll. C.J. Bird; #FG-99, 3 July 1999	1	AY049434 (97.0%) h6	AY049449 (100%) g1
	Canada: specimen #2, Morret Pond, Pomquet Harbor, Antigonish Co., Nova Scotia, 45°39'N × 61°50'W, coll. C. J. Bird, #FG-101, 03 July 1999	2	AY426750 (97.4%) h7	AY049448 (100%) g1
	Rhode Island: Charleston breachway, USA, coll. C.F.D. Gurgel, #FG-159, 11 August 2000	3	AY049443 (97.5%) h7	AY049459 (100%) g2
	New Jersey: Lakona beach, Barnegat Bay, USA, coll. C.T. Frankovich, #FG-109, 05 April 1999	4	AY049436 (98.7%) h7	AY049458 (100%) g3
	Delaware: Rehoboth Bay, Dewey Beach, USA, coll. C.F.D. Gurgel, #FG-146, 12 August 2000	5	AY049442 (97.4%) rh7	—
	North Carolina: North Masonboro inlet jetty, Wrightsville Beach, New Hanover Co., USA, coll. W.D. Freshwater, #FG-102, 28 May 1998	6	AY049435 (94.1%) h7	AY049457 (89.0%) g4
	SE Florida: Sebastian Inlet, Vero Beach Co., USA, 27°51'20"N × 80°27'06"W, coll. C.F.D. Gurgel, 10 October 1998	7	AY049432 (99%) rh8	AY049451 (99.4%) g5
	SE Florida: Indian River, close to the McLarty Treasure Museum, N. Hutchinson Is., USA, 27°50'12"N × 80°26'11"W, coll. C.F.D. Gurgel, 10 October 1998	8	AY049446 (95.2%) h7	AY049450 (100%) g6
	SE Florida: Harbor Branch Oceanographic Institution north jetty, Indian River, Fort Pierce, St. Lucie Co., USA, 27°32'00" N × 80°20'39" W, coll. C.F.D. Gurgel, 13 July 1998	9	AY049447 (95.6%) h9	AY049452 (96.7%) g7
	SE Florida: Cultured <i>Gracilaria</i> strain, variety brown, Harbor Branch Oceanographic Institution, Fort Pierce, St. Lucie Co., USA, leg. M. D. Hanisak, vii.1998	10	AY049362 (95.4%) h10	AY049463 (100%) g8
	W Florida: Tampa Bay, specimen #1—drift form, close to the mouth of Cockroach Bay, USA, coll. C. J. Dawes, #FG-94, 26 October 1999	11	AY049349 (98.4%) h5	AY049461 (100%) g9
	W Florida: Tampa Bay, specimen #2—lax form, close to the mouth of Cockroach Bay, USA, coll. C. J. Dawes, #FG-95, 26 October 1999	12	AY426749 (98.4%) h5	AY049462 (100%) g10
	W Florida: Tampa Bay, north side of 7192 Gandy Blvd., USA, coll. C. Aregood, #FG-143, 29 November 1999	13	AY049441 (97.8%) h5	AY049460 (100%) g11
	W Florida: Tampa Bay, drifting in a mangrove channel, south side of Gandy Blvd., USA, coll. C. Aregood, #FG-144, 29 November 1999	14	AY049440 (98.6%) h5	—
	Mississippi: Gulf Island National Seashore, Horn Island-Petit Bois, USA,	15	—	AY049453 (100%) g12

Table 1. (continued)

Species of <i>Gracilaria</i>	Specimen collection data	<i>G. tikvahiae</i> isolate number	GenBank accession no., % sequenced <i>rbcl</i> haplotype names, ITS sequence identifications	
			<i>rbcl</i> haplotype names	ITS sequence identifications
	coll. Carter, #FG-141, 06 June 2000 Texas: specimen #1 cylindrical, Redfish Bay, Port Aransas, USA, coll. S. Fredericq & C.F.D. Gurgel, 18 May 1998	16	AY049445 (93.7%) h1	—
	Texas: specimen #2 flat, Redfish Bay, Port Aransas, USA, coll. S. Fredericq & C.F.D. Gurgel, 18 May 1998	17	AY049444 (96.2%) h2	—
	Mexico: Alvarado Lagoon, Alvarado, Puerta Prieta area, 18° 47.80' N × 95° 48.83' W, coll. C.F.D. Gurgel, 15 February 1998,	18	AY049433 (98.4%) h1	AY049454 (100%) g8
	Mexico: San Agustín, Santana City, Vera Cruz State, 19° 55.23' N × 96° 31.85' W, coll. C.F.D. Gurgel, 9 February 1998	19	AY049437 (99.1%) h3	AY049456 (100%) g14
	Mexico: Mangrove, Paraiso town, Porto Ceibas, 18° 25.98' N × 93° 09.85' W, coll. C.F.D. Gurgel, 14 February 1998	20	AY049438 (98.4%) h4	AY049455 (100%) g13

HKY85+I+G (Hasegawa-Kashino-Yano model [Hasegawa et al. 1985] with invariable sites and gamma distribution) and the GTR+I+G for the ITS data set. The parameters estimated for the *rbcl* data set were as follows: nucleotide frequencies

A = 0.3095; C = 0.1601; G = 0.2154; T = 0.3150; substitution model $\mu/\nu = 1.9201$; proportion of invariable sites = 0.8290, and a gamma distribution with shape parameter = 0.8115. The parameters estimated for the ITS data set were as follows:

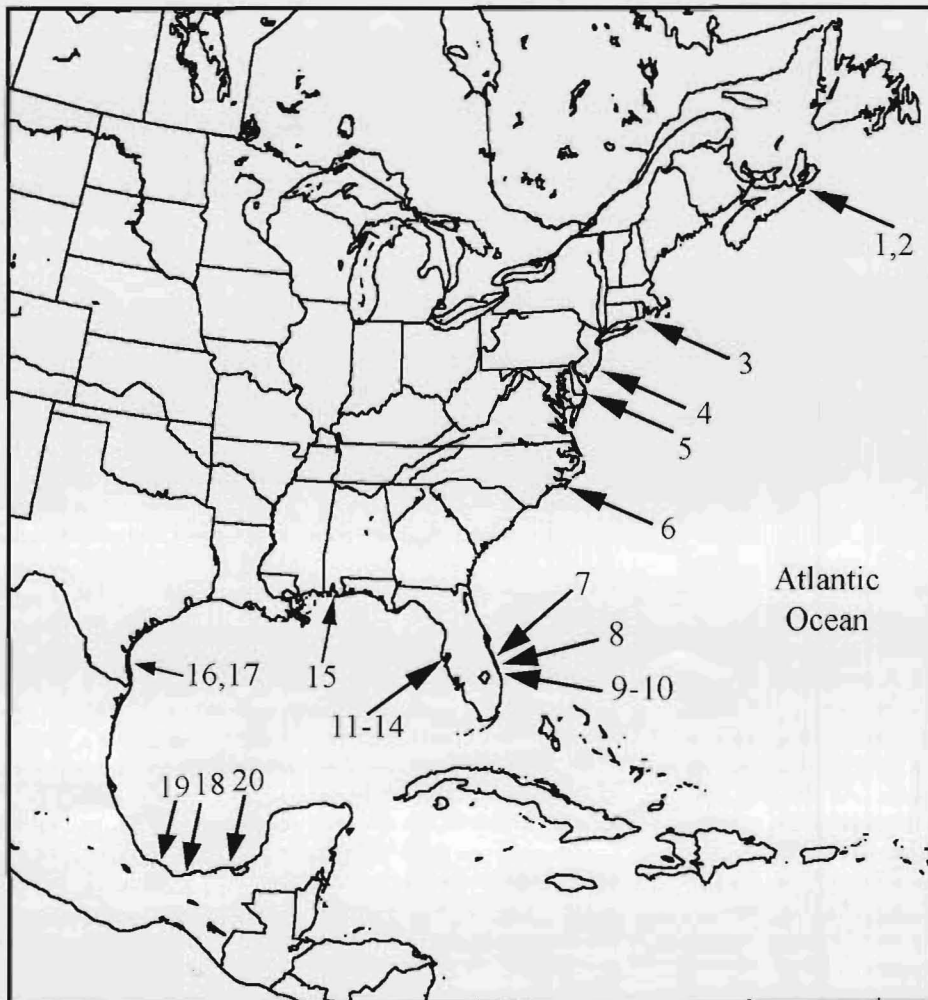


FIG. 1. Map showing distribution of *Gracilaria tikvahiae* collections. Arrows point to regions from where specimens were collected. Numbers coincide with location numbers in Table 1. The geographic distribution range of *G. tikvahiae* extends from Canada down to the Yucatan Peninsula, Mexico.

nucleotide frequencies A = 0.2655; C = 0.2006; G = 0.2299; T = 0.3040; substitution model: A-C = 1.8194, A-G = 8.4437, A-T = 1.9219, C-G = 1.9327, C-T = 18.8526, G-T = 1.0; proportion invariable sites = 0.5508, and gamma distribution with shape parameter = 1.4584. Nonparametric bootstrap values for nodes in MP and ML phylograms were calculated based on 3000 and 1000 resamplings, respectively.

Phylogenetic relationships among *rbcL* haplotypes and ITS genotypes were also estimated using the statistical parsimony procedure of Templeton et al. (1992) developed specifically to reconstruct within-species gene networks. In this case, the term parsimony refers to the minimum number of differences separating two individual sequences rather than a global minimum tree length based on shared derived characters. The statistical parsimony procedure was performed using the computer package TCS version 1.13 (Clement et al. 2000). DNA sequences with pairwise absolute distance values equal to zero were considered the same and were also collapsed into haplotypes (*rbcL*) or genotypes (ITS) by TCS.

Pairwise sequence divergence values cited in this study are based on uncorrected *p* distances, which is equivalent to the number of segregating sites per nucleotide site. Pairwise *p* distance values were calculated by PAUP*.

RESULTS

rbcL data set. In the 1367-bp *rbcL* alignment, including the two outgroup sequences, 60 bp vary and 22 bp were parsimony informative. When the two outgroup sequences were excluded from the alignment, there were 12 variable and four parsimony-informative sites. Ten haplotypes (h1–h10) were obtained among the 19 *G. tikvahiae* sequenced samples. A compressed *rbcL* alignment including details of substitution type and codon position are given in Figure 2. The overall genetic diversity among *rbcL* haplotypes ranged between 0.073% and 0.512%. Geographically, most distant haplotypes (Canada vs. Mexico) presented a smaller *rbcL* pairwise genetic divergence that ranged from 0.073% (h1 vs. h7) to 0.293% (h6 vs. h3) when compared with samples from geographically closer localities. The haplotype from a specimen cultured at the Harbor Branch Oceanographic Institution, h10, was ranked the most divergent in pairwise comparisons, with sequence distances ranging from 0.219% (h10 vs. h8–h9) to 0.512% (h10 vs. h3).

The two-step MP *rbcL* phylogenetic analysis resulted in a single most parsimonious tree, and all *G. tikvahiae* *rbcL* sequences formed a monophyletic group (Fig. 3). The ML analysis resulted in three trees of similar topology and same likelihood score ($-\ln = 1841.36448$; data not shown). Internal nodes in the *G. tikvahiae* clade that were not resolved in the MP tree were also not resolved in the ML result. *rbcL* phylograms recognized four genetically and geographically distinct lineages: 1) a western Gulf of Mexico lineage, 2) an eastern Gulf of Mexico lineage, 3) a southeastern Florida lineage, and 4) an eastern Canada–northern U.S. lineage (Fig. 3). In all *rbcL* phylograms, eastern Gulf of Mexico haplotypes always appeared at the most basal position, whereas haplotypes from the western Gulf of Mexico and southeastern Florida appeared as two derived line-

h1	CGACGCT <u>T</u> ACT <u>T</u>
h2	CGACGCT <u>T</u> ACT <u>G</u>
h3	CGACGCTGCCT <u>T</u>
h4	CGACGCTTATT <u>T</u>
h5	TAACGCTTACT <u>T</u>
h6	CAACACTTACT <u>T</u>
h7	CAACGCTTACT <u>T</u>
h8	CAGCGCTTACT <u>T</u>
h9	CAGTGCTTAC <u>T</u>
h10	CAGTGTATACT <u>T</u>

FIG. 2. *Gracilaria tikvahiae* compressed alignment of 10 *rbcL* haplotypes (only the variable sites are shown). Haplotype names coincide with those cited in Table 1 (h1–h10). Substitutions at first and second codon positions marked as underlined and bold-faced characters, respectively.

ages. Further phylogenetic relationships among *rbcL* lineages are still not clear and received low bootstrap support. Haplotypes were not shared among the four geographic locations, with the exception of h7 that was collected in southeast Florida but pertains to the Canadian lineage (Figs. 2 and 3).

The *rbcL* statistical parsimony network (SPN) does not exhibit a single star-shaped topology but instead comprises four distinct internal nodes composed of three actual haplotypes (h1, h7, and h8) and a fourth hypothetical haplotype (Fig. 4A). These four internal nodes are connected to the other seven derived sampled haplotypes. Haplotypes from the western Gulf of Mexico lineage (h1–h4) form a separate group characterized by a G at position 585 that distinguishes them clearly from the remaining haplotypes that have a synapomorphic A at this position. The eastern Gulf of Mexico haplotype (h5) is recognized in the *rbcL* SPN by a T at position 381 (Fig. 4A).

ITS data set. The original PCR amplified fragment consisted of 1235 bp. After removing the flanking regions of the ITS1 and ITS2 that correspond to the 3' end of the small subunit rDNA (= 120 bp) and the 5' end of the large subunit rDNA genes (= 28 bp), the ITS sequence data set included in the analyses was 1087 bp long. The length of the ITS1 region ranged from 292 to 295 bp, of the 5.8S rDNA from 209 to 214 bp, and the ITS2 region from 577 to 578 bp. ITS regions were completely sequenced with the exception of three samples that had missing data in the 5.8S region, not exceeding 11% of the entire number of sequenced bp (Table 1, % sequenced). No different ITS genotypes were observed within the same specimen, and only one ITS genotype was obtained from each isolate with the protocols implemented in this study. The ITS alignment (no outgroups) presented 13 distinct indels composed of one to two nucleotides in length. When indels were considered as a fifth character state, there were 43 variable and nine

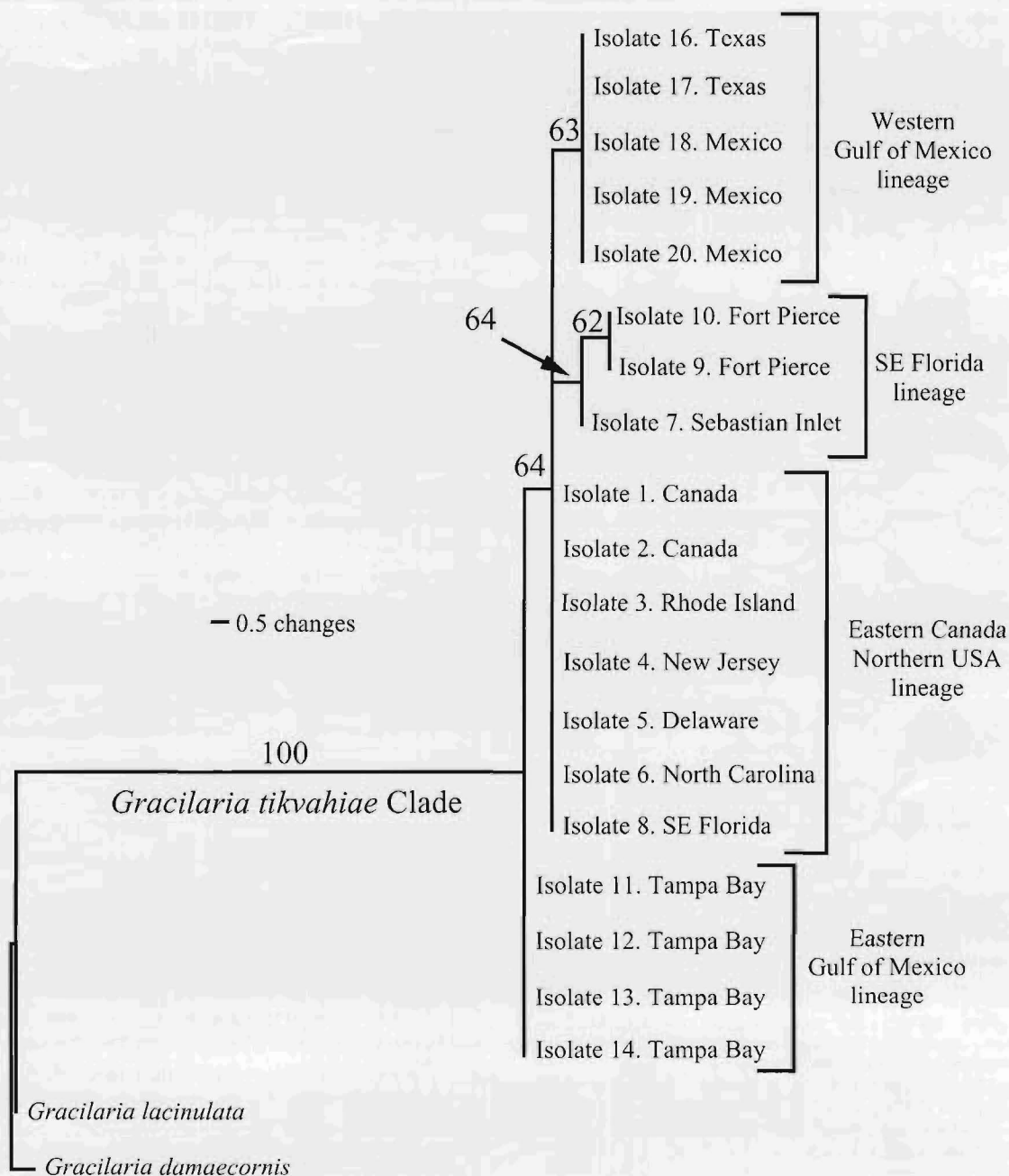


FIG. 3. Single most parsimonious tree based on a data set of 19 *Gracilaria tikvahiae* *rbcL* sequences. Tree length = 23 steps, consistency index = 0.956, retention index = 0.967. The numbers above the branches represent nonparametric bootstrap values (50% or higher) based on 3000 replicates.

parsimony-informative nucleotide sites (Fig. 5). The presence of indels occurred in all distinct sequences and ranged between 0.09% and 1.2% of the total number of base pairs in the alignment. When indels were not considered as a fifth character state, there were 30 variable and seven parsimony-informative nucleotide sites. Fourteen distinct ITS genotypes were found among the 16 samples (g1–g14), and a compressed alignment is given in Figure 5. The overall sequence divergence among ITS genotypes

ranged from 0.09% to 1.47% (considering indels as a fifth character state). The most divergent genotype was g12 from Mississippi, with pairwise genetic distances ranging from 0.83% (g12 vs. g7) to 1.47% (e.g. g12 vs. g9), followed by g2 from Rhode Island (0.46%–1.29%).

The MP analyses of the ITS data produced three most parsimonious trees, with and without considering gaps as a fifth character state. A consensus from any of the two sets of three most parsimonious trees did not

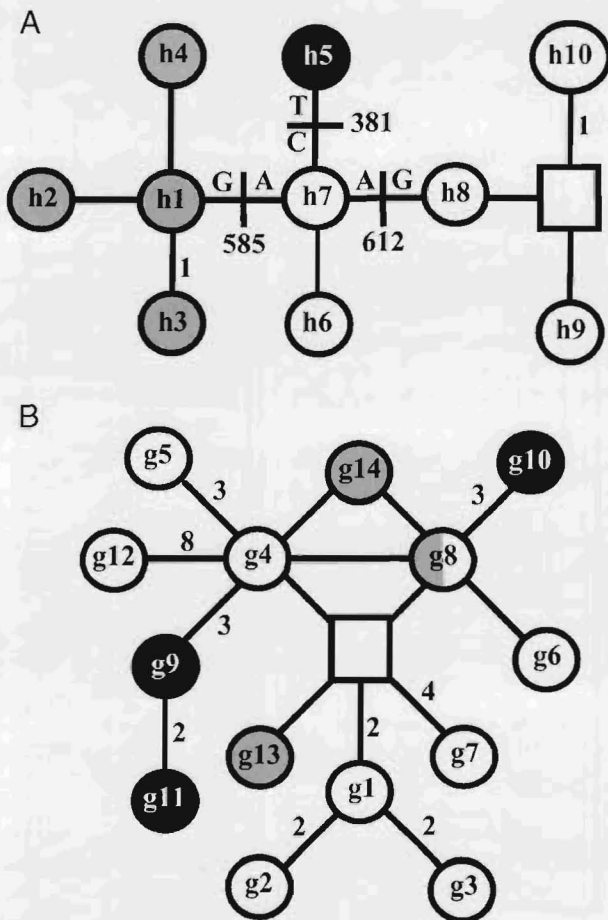


FIG. 4. Main network of relationships for *Gracilaria tikvahiae* DNA sequences based on the statistical parsimony method of Templeton et al. (1992) and considering indels as a fifth character state. Lines indicate the mutational connections. All branches are one change long (= parsimony based evolutionary steps that are equal to the number of substitutions separating two connected sequences) unless annotated as numbers next to each branch. Circles correspond to actual sampled sequences identified by their names (quoted inside circles). Squares correspond to hypothetical DNA sequences. *rbcL* haplotype (h1-h10) and ITS genotype (g1-g14) names coincide with those mentioned in Table 1 and Figures 2-6, respectively. Black, gray, and white circles correspond, respectively, to a Tampa Bay, a western Gulf of Mexico, and an Atlantic origin for the sequences obtained. (A) Statistical parsimony network based on 10 *rbcL* haplotypes. (B) Statistical parsimony network based on 14 ITS1-5.8S-ITS2 nuclear rDNA genotypes. Genotype g8 was sampled in both the western Gulf of Mexico and the Atlantic.

produce the same topology as found in the *rbcL* analyses, but both recognized two clades with low bootstrap support (data not shown). One clade was composed of north Atlantic specimens (from Canada, Rhode Island, and New Jersey, bootstrap values = 62%), and the second was composed of the Tampa Bay specimens (bootstrap values = 65%). The ML analysis of the ITS data set also resulted in three phylograms with equal likelihoods ($-\ln = 1780.76885$). A consensus of these three ML trees had the same topology as the two consensus

trees obtained in the ITS MP analyses and similar values of bootstrap support (Fig. 6). The ITS SPN presented a netlike topology with one internal hypothetical sequence (Fig. 4B). No phylogeographic structure was found in the ITS SPN. Genotypes g4, g8, and the hypothetical genotype (square) were the most internal sequences in the network and presented the highest number of connections to the remaining genotypes: six, five, and six branches, respectively. These three sequences, together with g14, comprised the center of the ITS phylogenetic network. The genotype from Mississippi, g12, was identified as the most divergent from its closest sampled genotype (= nine evolutionary steps or eight intermediate states).

For both datasets (*rbcL* and ITS) and regardless of the traditional method of phylogenetic analysis used (MP or ML), specimens from Canada, Rhode Island, New Jersey, and Tampa Bay, Florida were always found in a distinct clade (Figs. 3 and 6). Morphological comparisons within and among *G. tikvahiae* populations included in this study displayed high levels of intra- and interpopulation phenotypic variation (Fig. 7) with completely flat to completely cylindrical specimens, often inhabiting the same location. No morphological synapomorphy could be distinguished for any particular lineage that otherwise could define a particular intraspecific taxon. However, thalli of drifting specimens among the Mexican populations were typically flatter, thinner, narrower, and more densely branched (Fig. 7, B and C), a phenomenon hitherto only observed in Mexican collections.

DISCUSSION

Even with a relatively low number of phylogenetically informative characters and a small sample size, a dramatic result was the striking similarity in the pattern of cpDNA divergence in *G. tikvahiae* to that of other previously reported marine and nonmarine species (Avice 2000). The geographic location of the genetic disjunction separating different *G. tikvahiae* phylogroups cannot be precisely determined with our limited sampling, and in addition, regions of genetic disjunctions along the Florida Peninsula differ among distinct taxa. A more robust sampling along the entire geographic distribution of *G. tikvahiae* is crucial to further test the hypothesis of genetic disjunction between different populations. However, the four *rbcL* phylogenetic lineages found in this study (Fig. 3) are in agreement with some specific marine phylogeographic studies and are suggestive of three regions where the disjunction of *G. tikvahiae* haplotypes might occur: 1) northeastern Florida, 2) the southern tip of the Florida Peninsula, and 3) some place in northern Gulf of Mexico.

Northeastern Florida. A separation between the southeastern Florida and northeastern U.S. haplotype groups has also been observed for the marine oyster *Crassostrea virginica* (Reeb and Avice 1990); the horseshoe crab *Limulus polyphemus* (Riska 1981,

g1 TCATTTCTGAAAAGTT - GGTGTATGG - CGGTTTACGAAAGGTC
 g2 TCATTTCTGAAATGTT - GGTGTATGG - AGGTTTACGTAAGGTC
 g3 TCATTTCTGAAAAGTT - GGTGTATGGCCGGTTTACGACAAGTC
 g4 TCAT - - CTGAAA?????TGGGTATGG - CGGTTTACGA?????
 g5 TAAT - - CTGAAAAG - T - GGGGCATGG - CGGTTTACTAAAGGTC
 g6 TCAT - - CTGAAAAGTT - GCGGTATGG - CGGTTTACGAATGGTC
 g7 - CAT - - C - - A - - A?TT - GGGGTATGG - CGGTTTACGAAAGGTC
 g8 TCAT - - CTGAAAAGTT - GGGGTATGG - CGGTTTACGAATGGTC
 g9 TCAT - - CTGAAAAGTT - GGGGTATGG - CGACTTATGAATGTTG
 g10 TCAT - - CTG - AAAGTT - GGGGT - AGG - CGGCTTACGAATGGTC
 g11 TCAT - - CTGAAAAGTTGGGGGTATAG - CGACTTATGAAAGTTG
 g12 TCGA - - ATGAAAATTT - GGGCTATGG - CAGTCCGCGAAAGGCC
 g13 TCAT - - CTGAAAAGTT - GGGGTATGC - CGGTTTACGAAAGGTC
 g14 TCAT - - CTGAAAAGT - - GGGGTATGG - CGGTTTACGAATGGTC

FIG. 5. *Gracilaria tikvahiae* compressed alignment of 14 ITS1-5.8S-ITS2 nuclear rDNA genotypes (only variable sites are shown, considering indels as a fifth character state). Genotype names (g1-g14) coincide with those cited in Table 1 and Figures 4 and 6, respectively. -, missing base (indel); ?, missing data (not sequenced).

Saunders et al. 1986); marine toadfishes, *Opsanus tau* and *O. beta* (Avisé et al. 1987); and the seaside sparrow *Ammodramus marilimus* (Avisé and Nelson 1989). The geographic isolation of haplotypes observed between northern and southern coastal marine populations along the eastern coast of Florida is usually not abrupt, and haplotypes with higher frequencies in one population may be found with lower frequencies in other geographically close but distinct popu-

lations (lack of complete genetic isolation). However, as samples are drawn far from the proposed genetic disjunction locations, the genetic distance between populations tend to increase sharply (Fischer 1960, Reeb and Avisé 1990, Avisé 1992, 1994, Collin 2001).

Southern tip of the Florida Peninsula. On the basis of DNA sequence analysis, divergence between Atlantic and Gulf of Mexico haplotype groups have also been documented for sea anemones (McCommas 1982),

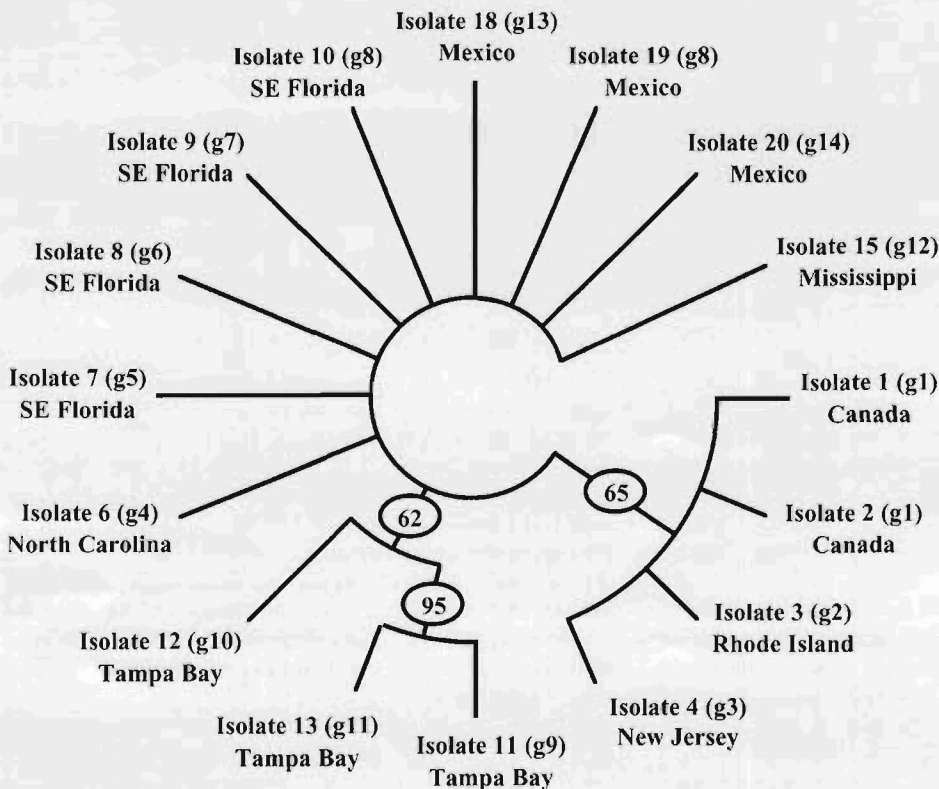


FIG. 6. Strict consensus of three ML trees with equal likelihood scores ($-\ln = 1870.76885$) based on a data set composed of 16 *Gracilaria tikvahiae* ITS rDNA sequences. The numbers inside circles on the branches represent nonparametric bootstrap values (50% or higher) based on 1000 replicates.

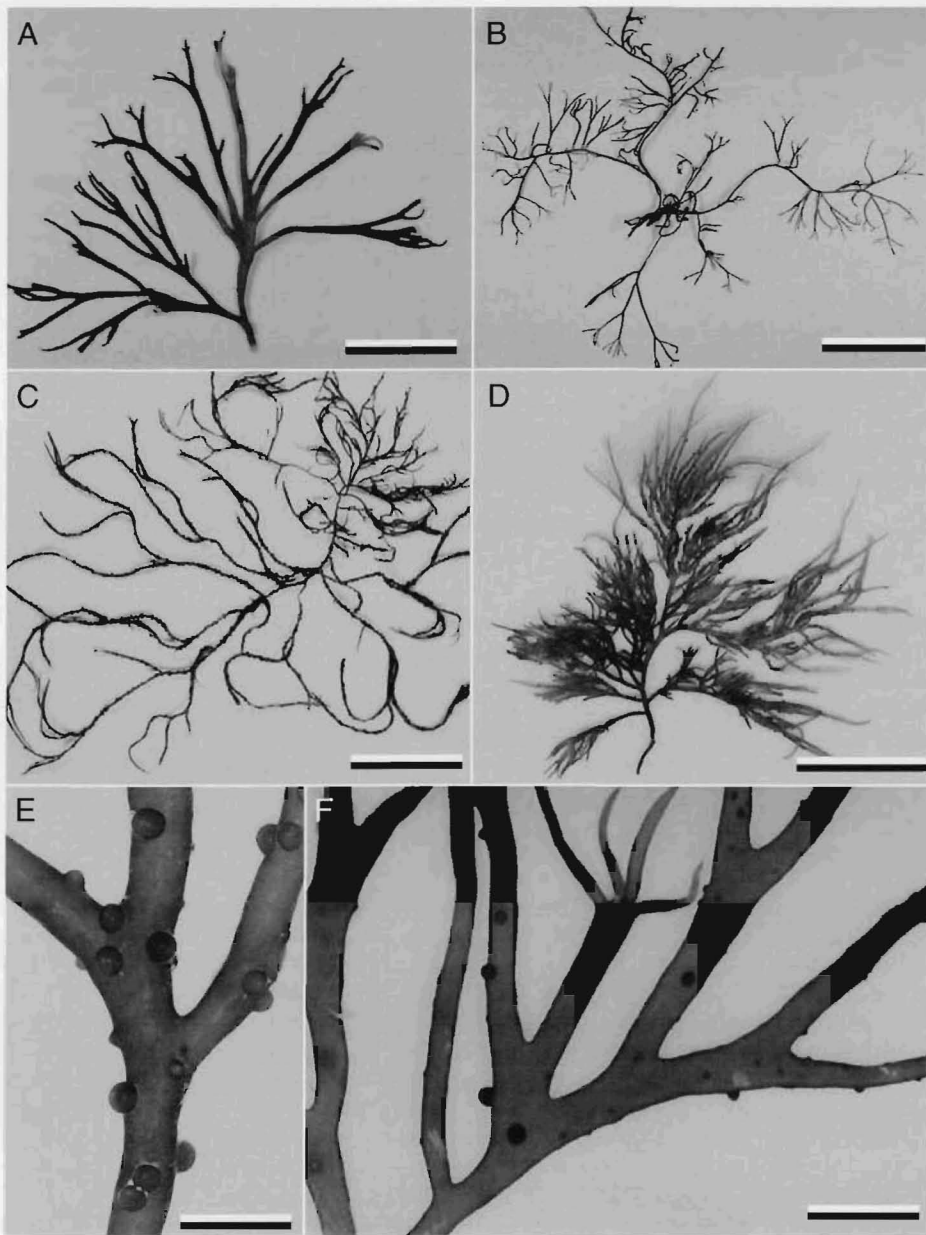


FIG. 7. *Gracilaria tikvahiae* phenotypic variation. (A) Specimen from Paraiso, Porto Ceibas, Mexico. Scale bar, 2 cm. (B) Specimen from San Agustin lagoon, Santana, Vera Cruz State, Mexico. Scale bar, 4 cm. (C) Specimen from Port Aransas, Texas, USA. Scale bar, 6 cm. (D) Specimen from Morret pond, Pomquet Harbor, Antigonish Co., Nova Scotia, Canada. Scale bar, 4 cm. (E-F) Cystocarpic specimens collected growing attached and co-occurring in Sebastian Inlet, eastern Florida, USA. (E) Cylindrical specimen. Scale bar, 0.5 cm. (F) Flat specimen. Scale bar, 1 cm.

mussels (Sarver et al. 1992), marsh and stone crabs (Bert and Harrison 1988, Felder and Staton 1994), mosquito fish (Wooten et al. 1988), and the black sea bass (Bowen and Avise 1990). Contemporary genetic disjunctions often persist across the southern tip of the Florida Peninsula, a region known as a geographic barrier to common estuarine species (Felder and Staton 1994).

Northern Gulf of Mexico. *rbcL* results suggest the existence of a unique eastern Gulf of Mexico lineage. If this is the case, places for possible genetic discontinuities in continuously and quasicontinuously distributed benthic species along the northern Gulf of Mexico include the mouth of the Mississippi River (Adamkewicz and Harasewych 1996) and/or the

Chenier Plain, a large marsh/estuarine system, between southeastern Louisiana and the northeastern coast of Texas. Very few hard substrata for intertidal and subtidal species are located in the later inshore region, which is characterized by shallow muddy substrata (Staton and Felder 1995). Despite several attempts to obtain molecular data from specimens from the northern Gulf of Mexico, efforts were unsuccessful. In the northern coast of the Gulf of Mexico, *G. tikvahiae* populations are most likely patchy, opportunistic, and transient (Kapraun 1974, as *G. foliifera* var. *angustissima*).

The application of *rbcL* DNA sequences to identify sources of marine macroalgal species invasions has been successfully applied (McIvor et al. 2001). If re-

gionally endemic, the observed pattern of geographic distribution for some *rbcL* haplotypes may help to identify the geographic origin of introduced *G. tikvahiae* populations in nonnative habitats, such as Hawaii (Abbott 1999, p. 217).

Coalescence theory predicts that the most common haplotypes in a gene pool will tend to be the oldest, and most of these old (ancestral) haplotypes will be interior nodes of the haplotype tree (Crandall 1996, p. 118). Despite the low sampling size in this study that drastically biases the frequencies of the sampled sequences, *rbcL* SPN suggests that the most likely ancestor for the *rbcL* haplotypes in the data set is h7 with an Atlantic distribution, and that h1 might be the ancestor sequence for the western Gulf of Mexico haplotypes sampled. Haplotypes from the western Gulf of Mexico form a distinct branch in the *rbcL* SPN with a topology suggestive of a local genetic diversification.

The unlike topology of the ITS SPN presented no phylogeographic structure and did not support the occurrence of any local radiation event besides the branch composed exclusively of northern Atlantic genotypes (g1, g2, and g3). The ITS SPN suggests two independent origins for the three genotypes found in Tampa Bay (from g4 and g8), whereas western Gulf of Mexico and Atlantic genotypes present a more complex phylogenetic history. The higher levels of connectivity among the four most internal nodes and thus the *a priori* oldest genotypes in the data set (g4, g8, g14, and the a hypothetical genotype) suggest a reticulate pattern of evolution (= recombination). The ITS SPN displayed more phylogenetic resolution than the MP and the ML results that were composed mainly of unresolved nodes. This result agrees with published theoretical and empirical evidence that the SPN procedure developed by Templeton et al. (1992) greatly outperforms traditional nonparametric bootstrapping with MP when the number of nucleotide substitutions is small and the number of shared positions is large, as is the case of this and most other intraspecific level studies (Crandall 1994).

Based on our results, we conclude that 1) *G. tikvahiae* has evolved from a common ancestry, 2) geographically distant populations of *G. tikvahiae* (e.g. Mexico vs. Canada) inhabiting ecologically distinct environments (e.g. tropical vs. temperate ecosystems) encompass a same species, 3) the intraspecific genetic variation found among *rbcL* and ITS DNA sequences is not expressed in habit shape (cylindrical vs. flat phenotypes), and 4) there appears to be phylogenetic groups within *G. tikvahiae* that, so far, roughly correspond to phylogeographic patterns reported for other marine organisms along the Gulf of Mexico and the eastern North American coast.

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