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GENETIC PATTERNS IN THE CALCIFIED TROPICAL SEAWEEDS *HALIMEDA OPUNTIA*, *H. DISTORTA*, *H. HEDERACEA*, AND *H. MINIMA* (BRYOPSIDALES, CHLOROPHYTA) PROVIDE INSIGHTS IN SPECIES BOUNDARIES AND INTEROCEANIC DISPERSAL[1]

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The section *Opuntia* within the green seaweed genus *Halimeda* includes sprawling and pendant thalli composed of strongly calcified segments. Within this section, identification of Atlantic material is straightforward, but Indo-Pacific material is often difficult to key out. This is particularly true for specimens resembling *H. opuntia*, *H. distorta*, and *H. hederacea*; many specimens do not fit any type or are morphologically intermediate. The goals of the present study are to define morphologically and genetically distinct groups among such specimens and to assess phylogeographic patterns within these groups. Specimens were collected throughout the geographical and morphological range. Sequences of *H. minima* and *H. gracilis* were included as outgroups. Two morphological groups were discerned within the ingroup; the first fit *H. opuntia*, whereas most specimens in the second group, referred to as the *distorta–hederacea* complex, did not fit any species description unambiguously. The latter were subdivided into two subgroups corresponding more or less to *H. hederacea* and *H. distorta*, yet intermediates between these morphs existed. A phylogeny inferred from partial nuclear rDNA sequences showed one lineage with *H. opuntia* and a second one containing the *distorta–hederacea* complex, thus corroborating the two major morphological groups. The *distorta–hederacea* complex contained two clades that show only partial correspondence with the morphological subgroups. Therefore, *H. hederacea* is synonymized with *H. distorta*. Phylogeographic structure within *H. opuntia* indicated that this species dispersed from the Indo-Pacific into the Atlantic. Fossil records of the species also show occurrence at Pacific sites throughout the last 10^5 years and a sudden appearance in the Caribbean and Bahamas during the last millennium.

Key index words: **calcareous algae; cryptic species; cognate; dispersal; *Halimeda*; *H. distorta*, *H. hederacea*, *H. opuntia*; introduced species; ITS; morphology; phylogeny; phylogeography; SSU rDNA; taxonomy**

Abbreviations: **ITS, internal transcribed spacer; ML, maximum likelihood; MP, maximum parsimony; SSU, small subunit**

The seaweed genus *Halimeda* (Bryopsidales, Chlorophyta) is easily recognized by its calcified and segmented habit (Hillis-Colinvaux 1980). Yet identification at the species level is, at times, more demanding (Hillis-Colinvaux 1980), especially within section *Opuntia* (Verbruggen and Kooistra 2004). This section currently includes nine described species: western Atlantic *H. copiosa* Goreau & Graham and *H. goreauii* Taylor; Indo-western Pacific *H. distorta* (Yamada) Colinvaux, *H. hederacea* Colinvaux, *H. howensis* Noble & Kraft, *H. minima* (Taylor) Colinvaux, *H. renschii* Hauck, and *H. velasquezii* Taylor; and pantropical *H. opuntia* (Linnaeus) Lamouroux (Hillis-Colinvaux 1980, Kraft 2000, Bandeira-Pedrosa et al. 2003).

Our first goal is defining species boundaries within a particularly problematic group of specimens within the section *Opuntia*, namely, specimens resembling *H. opuntia*, *H. distorta*, and *H. hederacea* (Barton 1901, Yamada 1941, 1944, Colinvaux 1968, 1969, Hillis-Colinvaux 1980). The group is monophyletic according to phylogenies in Kooistra et al. (2002) and Verbruggen and Kooistra (2004), but it is unclear whether the species are monophyletic. Type specimens and species descriptions in this group often poorly reflect the morphological diversity encountered and many intermediate morphologies occur.

Morphological plasticity could present one reason for identification problems. *Halimeda opuntia*, for example, forms brittle networks composed of almost tripartite

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segments in shaded sheltered lagoons and compact cushions of reniform segments on wave-exposed rocky surfaces (Littler and Littler 2000, p. 406, personal observations). Plasticity can obscure species boundaries, resulting in description of oddities on the fringes of the plasticity range as new species. Unjust lumping is also possible because discrimination between species may fail if shared morphological trends along environmental gradients obscure species-specific differences. Moreover, genetic, biological, and morphological boundaries between some species may become fuzzy through hybridization. Recent work in the related genus *Caulerpa* (Famà et al. 2000, Durand et al. 2002, de Senerpont Domis et al. 2003) and in several coral genera (Veron 1995, Van Oppen 2001) also shows considerable intraspecific genetic diversity and ill-defined species boundaries.

Our second goal is to address phylogeographic patterns and in particular dispersal directionality within the aforementioned group. Kooistra et al. (2002) revealed phylogeographic structure within the genus *Halimeda*, but their sample coverage did not permit addressing intraspecific patterns. Their data suggest interoceanic dispersal within *H. opuntia*, but more specimens are needed to confirm directionality and to assess whether it has been a unique event or happened multiple times independently.

To address these issues, we collected specimens across the morphological diversity and distribution range of the group and sorted them based on shared morphological traits. A phylogeny was inferred from a region of their nuclear rDNA sequence also used in Kooistra et al. (2002). Obtained morphological groups were then compared with clades in the phylogeny to define monophyletic taxa and identify their boundaries. Collection sites of the specimens were used to construct an area phylogram to identify dispersal events and to determine their directionality. Morphological information from specimens belonging to *H. minima* was included to ascertain morphological distinctness of this species from the group in the focus of this study. Sequences of *H. minima* and *H. gracilis* Harvey ex J. Agardh were included as nearby and more distant outgroups, respectively. The latter species belongs to section *Pseudo-opuntia*, the sister of section *Opuntia* (Verbruggen and Kooistra 2004).

MATERIALS AND METHODS

Collections of specimens and their preservation were carried out as described in Kooistra et al. (2002). The specimens in Table 1, which are lodged in the Ghent University Herbarium, were assigned to groups on the basis of their gross morphology. When possible, these groups were given species names by comparison with descriptions and illustrations in Barton (1901), Taylor (1950, 1960), Colinvaux (1968, 1969), and Hillis-Colinvaux (1980).

The DNA purification, PCR amplification, and sequencing of the target region were as described in Kooistra et al. (2002). The region comprised the small subunit (SSU, from approximately position 500 onward), an insert in the SSU (Hillis et al. 1998, Durand et al. 2002), the internal transcribed spacer regions (ITS1, ITS2), and the 5.8S rDNA. Alignment was done by eye using Sequence Alignment Editor Se-Al, version 2.0a11 (http://evolve.zoo.ox.ac.uk/). The alignment is available at the TreeBASE server (http://www.treebase.org) under study accession number S1208, matrix accession number M2087.

Phylogenetic analyses of the alignment were carried out using PAUP*, version 4.0.b10 (Swofford 2002). Hierarchical likelihood ratio tests (alpha = 0.01) were performed on a sequence data set including all *Halimeda* rDNA sequences available to us on 1 January 2004 using Modeltest v3.06 (Posada and Crandall 1998) to find the most appropriate model and settings for maximum likelihood (ML) analyses. The ML analysis was carried out under the heuristic search option and tree bisection/reconnection-branch swapping and was constrained using aforementioned Modeltest parameter settings. The ML-bootstrap analysis (1000 replicates) was carried out using the fast stepwise addition option and other settings as with the heuristic search in ML analysis.

The ML-bootstrap analyses were carried out first among the sequences within the *H. opuntia* clade only and among those within the *distorta–hederacea* complex only. Then, two sequences were selected from the *H. opuntia* clade and two from the *distorta–hederacea* clade, each on both sides of the basal dichotomies of these clades in the ML tree. All other sequences in the *H. opuntia* clade and the *distorta–hederacea* clade were then deleted before a global bootstrap analysis.

Maximum parsimony (MP) trees were generated under the heuristic search procedure with the tree bisection/reconnection-branch swapping algorithm. Ambiguities were treated as polymorphisms and gaps as missing data. Branches were collapsed if their minimum length was zero. The resulting trees were filtered to retain only the shortest ones. MP-bootstrap values were obtained using 1000 replicates under the same settings as with MP analysis but with maximum number of trees retained per cycle (set MaxTrees) limited to 200.

RESULTS

Morphological observations. The specimens in Table 1 could be divided into three major morphological groups. In the first group, thalli consisted of small segments; the basal ones were thick and tripartite and the upper segments were thin and flat, most often broad ellipsoidal to ovate (Fig. 1a) but sometimes broad trilobed (Fig. 1b). Segments were often markedly ribbed; the ribs resulted from bundles of medullary siphons connecting the segment's basal node with the nodes along the segments upper rim. Thalli attached at their basal segment only (Fig. 1, a and b), pending from the side of rocks. This morphology conforms to the descriptions of *H. minima* in Taylor (1950), Colinvaux (1968), and Hillis-Colinvaux (1980).

Specimens in the second group possessed thalli of which the upper segments have a reniform outline and a clearly lobed outer edge (Fig. 1d). In most cases, segments were markedly ribbed. The three to five ribs ran from the segments' base to the nodes along the distal perimeter. The nodes were clearly visible and often protruded above the segment perimeter. The segment surface appeared dull and felt rough. Considerable plasticity was observed in segment shape. Segments from cushion-shaped specimens encountered on wave-affected reef crests were reniform (Fig. 1, d and e), whereas those in the brittle networks

TABLE 1. Taxa used in analyses with voucher number of specimen, collection site, and the GenBank accession code of the obtained sequences.

Taxon	Specimen number	GENT accession	Region	Geographical location	GenBank
Halimeda hederacea	**99–045**[a]	H.0475	WP	Townsville, Queensland, Australia	**AF407269**
H. cf. hederacea	97–059	H.0008	CP	Honounou, Hawaii	AF525647
	98–143	H.0509	WP	Lapu Lapu, Mactan I., Cebu, Philippines	**AF525652**
	99–005	H.0287	CP	Tahiti, Fr. Polynesia	AF525653
	L0238135	Not applicable	WP	E. of Melolo, Sumba, 09'54'S 120'42.5'E, Indonesia	AF525648
H. distorta	WLS060-02	WLS060-02	CP	Wallis Island (France)	AY649375
H. cf. distorta	98–111	H.0023	WP	Lizard Island, Queensland, Australia	AF525642
	98–121[a]	H.0522	WP	Lizard Island, Queensland, Australia	AF407268
	98-152	H.0507	WP	Lapu Lapu, Mactan I., Cebu, Philippines	AF525651
	99–007	H.0288	CP	Tuamotu, Rangiroa Atoll, Fr. Polynesia	AF525643
	99–011	H.0291	CP	Tuamotu, Rangiroa Atoll, Fr. Polynesia	AF525646
	99–012	H.0292	CP	Tuamotu, Rangiroa Atoll, Fr. Polynesia	AF525644
	99-013	H.0293	CP	Tuamotu, Rangiroa Atoll, Fr. Polynesia (*minima*-like)	AF525645
	99–147	H.0276	WP	New Caledonia	AF525640
	99–151	H.0280	WP	New Caledonia	**AF525641**
	HV275	HV275	CP	Tuamotu, Rangiroa Atoll, Fr. Polynesia	AY649374
	WLS422-02	WLS422-02	CP	Wallis Island (France)	AY649377
H. distorta–hederacea	98–135	H.0526	WP	Lapu Lapu, Mactan I., Cebu, Philippines	AF525649
	98–142	H.0483	WP	Lapu Lapu, Mactan I., Cebu, Philippines	AF525650
H. gracilis	97–076	No voucher	WA	Cayo Zapatilla, Panama	AF525608
	97–089	H.0367	WA	I. Escudo de Veraguas, Panama	AF525607
	98–093	H.0405	WA	I. Grande, Panama	**AF525609**
	99–109[a]	H.0259	WA	Galeta I., Panama	AF407259
	HEC-11839[a]	HEC11839	IO	Beruwala, Sri Lanka	**AF407257**
H. minima	98–128A	H.0336	WP	Lizard I., Queensland, Australia	AF525619
	99–025[a]	SOC251	IO	Rhiy di-Quatanhin, SW-tip, Socotra, Jemen	**AF407264**
	99–026[a]	SOC384	IO	Bidolih, Nogid, S-coast, Socotra, Jemen	**AF407263**
	99–075	PH526	WP	Santa Cruz I., Zamboanga City, Mindanao, Philippines	**AF525618**
	99–089	H.0381	WP	Bile Bay, Guam	AF525620
	99–093	H.0380	WP	Apra Harbor (–25 m), Guam	**AF525622**
	99–098[a]	H.0382	WP	Apra Harbor (–25 m), Guam (fragil, lax specimen)	**AF407265**
	WLS169-02	WLS169-02	CP	Wallis Island (France)	AY649379
	WLS193-02	WLS193-02	CP	Wallis Island (France)	AY649378
H. opuntia	95–Guam1	H.0618	WP	Agat Bay, Guam	AF525630
	95–hon-07	H.0616	WA	I. Roatan, Honduras	AF525628
	95–sa-2	H.0566	WA	I. San Andres, Colombia	AF525623
	96-io-002	H.0534	IO	Chagos Islands	AF525627
	98-096	H.0485	WA	I. Grande, Panama	AF525624
	98–106	H.0519	WP	Lizard I., Queensland, Australia	AF525632
	98–114[b]	H.0332	WP	Lizard I., Queensland, Australia	AF525615
	98–119	H.0481	WP	Lizard I., Queensland, Australia	AF525636
	98–126	H.0525	WP	Lizard I., Queensland, Australia	AF525635
	98–144	H.0523	WP	Lapu Lapu, Mactan I., Cebu, Philippines	AF525637
	98–189	H.0262	WA	Tamardane, Brazil	**AF525639**
	98–192	H.0527	WA	Key Largo, Florida	AF525626
	99–017	H.0289	CP	Tuamotu lagoon, Rangiroa, Fr. Polynesia	AF525631
	99–044[a]	H.0484	WP	One Tree I. Near Townsville, Australia	AF407267
	99–047	H.0489	WP	Townsville, Queensland, Australia	AF525634
	99–096	H.0482	WP	Apra Harbor, Guam	AF525638
	99–131	HEC12583	IO	Zanzibar, Tanzania	**AF525629**
	99–185	H.0506	WA	Cayo Nancy, Bocas del Toro, Panama (brittle mesh)	AF525625
	HV450	HV450	WA	Discovery Bay, Jamaica	AY649373
	HV61	HV61	CP	Moorea, Fr. Polynesia	AY649380
	WLS090-02	WLS090-02	CP	Wallis Island (France)	AY649376

An "A" behind the voucher code indicates that the sample contained several specimens from which more than one has been sequenced. Sequences indicated in bold have been included in the global ML analysis. Vouchers with an L number are located in the Leiden herbarium. All other specimens have been lodged in the Ghent University Herbarium, Belgium (GENT). CP, central Pacific; EA, eastern Atlantic; EP, eastern Pacific; I, island; IO, Indian Ocean; MED, Mediterranean Sea; WA, western Atlantic; WP, western Pacific.

[a]Also used in Kooistra et al. (2002).

[b]ITS2 of this specimen could not be amplified.

found in shaded lagoonal environments possessed an almost tripartite outline (Fig. 1c). This morphology conforms to the descriptions of *H. opuntia* in the literature (Barton 1901, Taylor 1950, 1960, Hillis-Colinvaux 1980).

Specimens in the third group possessed segments of different morphology at the base and the apex of the thallus (Fig. 2, a–j). Near the base, segments were often tripartite (Fig. 2, g–j). These segments consisted basically of three bundles of medullary siphons and their

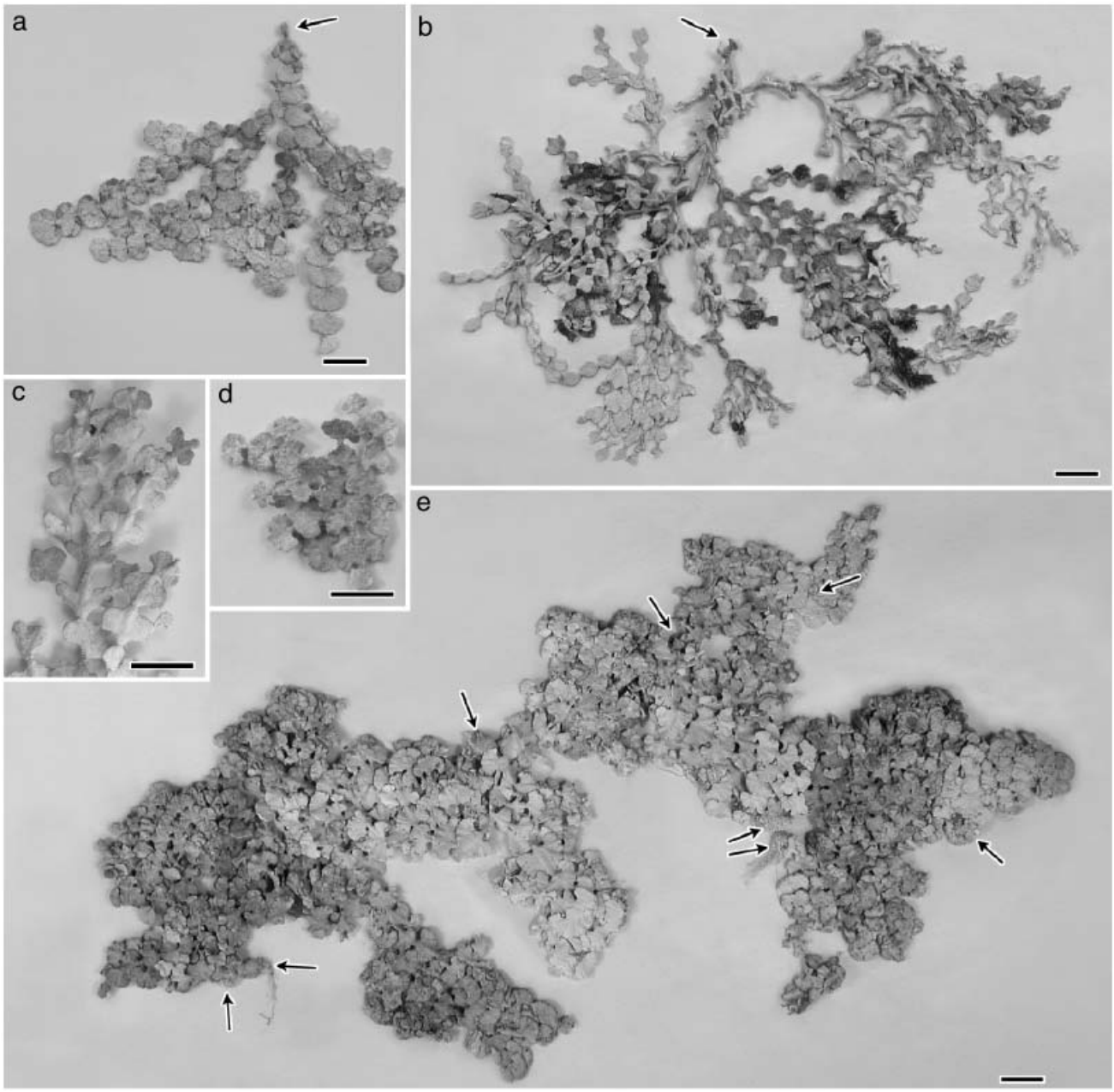

FIG. 1. *Halimeda minima* and *Halimeda opuntia*. Arrows indicate holdfasts. Scale bars, 1 cm. (a–b) *Halimeda minima*. (a) Habit of a plant with broad ovate segments, HV746. (b) Habit of a plant with relatively narrow segments, HV765. (c–e) *Halimeda opuntia*. (c) Tripartite segments of a plant growing in the shade, SEY735. (d) Reniform segments of a plant growing in sunny conditions, PH163. (e) Habit of a plant growing in sunny conditions, HV6.

surrounding cortex, each proceeding from the segment base to the daughter segments or uncorticated pits. Segments higher up the thallus were flat to contorted, often in one and the same thallus. They usually showed a clear ribbing, and their nodes were not elevated markedly above the segment perimeter. The outline of the upper segments was highly variable and led us to define morphological subgroups with "varieties" in each. These different morphologies were quite distinct, but a few intermediates were observed. Thalli were sprawling (Fig. 2, a, f, and i), and rhizoid tufts for secondary attachment were usually present throughout the thallus (Fig. 2e). As a reference, the third group is cited as the *distorta–hederacea* complex.

The first subgroup (Fig. 2, a–e) included stiff sprawling thalli forming mats about 10–15 cm across. In all specimens, segments of the middle and distal regions were relatively large; those near the base were smaller, thick, and tripartite. The segment surface appeared shiny and felt smooth in some dried specimens. This subgroup is referred to as *H. hederacea*. Two varieties were present within this subgroup.

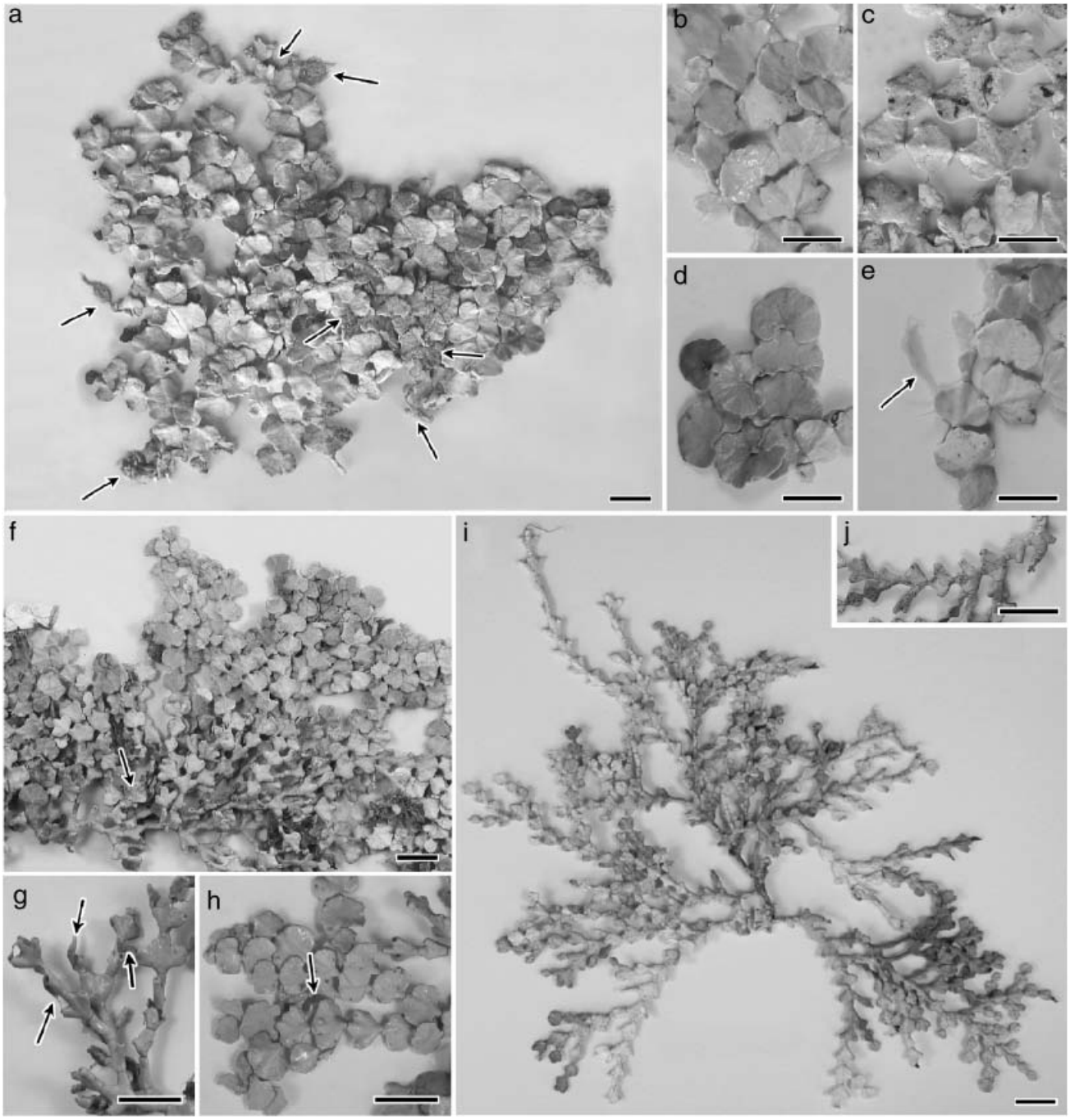

FIG. 2. *Halimeda distorta–hederacea* complex. Arrows indicate holdfasts. Scale bars, 1 cm. (a–e) *Halimeda* cf. *hederacea* (morphs 1a and 1b). (a) Thallus habit (morph 1a), HV199. (b) Segment morphology (morph 1a), HV600. (c) Segment morphology (morph 1a), HV572. (d) Segment morphology (morph 1b), HV127. (e) Detail of a tuft of holdfast rhizoids, HV600. (f–j) *Halimeda* cf. *distorta* (morphs 2b and 2c). (f) Part of a chimerical thallus (morph 2b), HV767. (g) Segment morphology of proximal segments (morph 2b), HV767. (h) Segment morphology of distal segments (morph 2b), HV767. (i) Thallus habit (morph 2c), HV275b. (j) Segment morphology (morph 2c), HV275b.

(1a) Distal segments large, hederifoliate, broader than high, relatively thick, usually ribbed, and, at times, keeled (Fig. 2, a–c). These thalli are conform *H. opuntia* forma *hederacea* as described in Barton (1901).

(1b) Distal segments large, reniform, broader than high, relatively thin, flat (not keeled), and usually not ribbed (Fig. 2d). This morph is not described in the *Halimeda* monographs.

The second subgroup included lax sprawling thalli with relatively small upper segments. Segments near the base were thick and tripartite. The segment surface was dull and coarse in dried specimens. This subgroup is referred to as *H. distorta* and contained three varieties.

(2a) Distal segments small, about as broad as high, relatively thin, distorted. This morph is depicted in Hillis-Colinvaux' (1980) description of *H. distorta*. It is not depicted here.

(2b) Distal segments relatively small, broad ovate to reniform, broader than high, flat, usually ribbed (Fig. 2, f and h). This morph is not depicted in the *Halimeda* monographs unless maybe in figure 20 in Barton (1901, as *H. opuntia* forma *triloba*).

(2c) Distal segments generally as those of the basal region: tripartite over trilobed to rhomboidal, relatively small, flat to slightly distorted (Fig. 2, i and j). This morph is depicted in Taylor (1950) as *H. opuntia* forma *elongata* (plate 41).

The specimens used for molecular phylogenetic analyses have been assigned to the above-mentioned subgroups and varieties (Fig. 3a). Note that the specimens depicted in Figures 1 and 2 do not always correspond to sequenced specimens (Table 1).

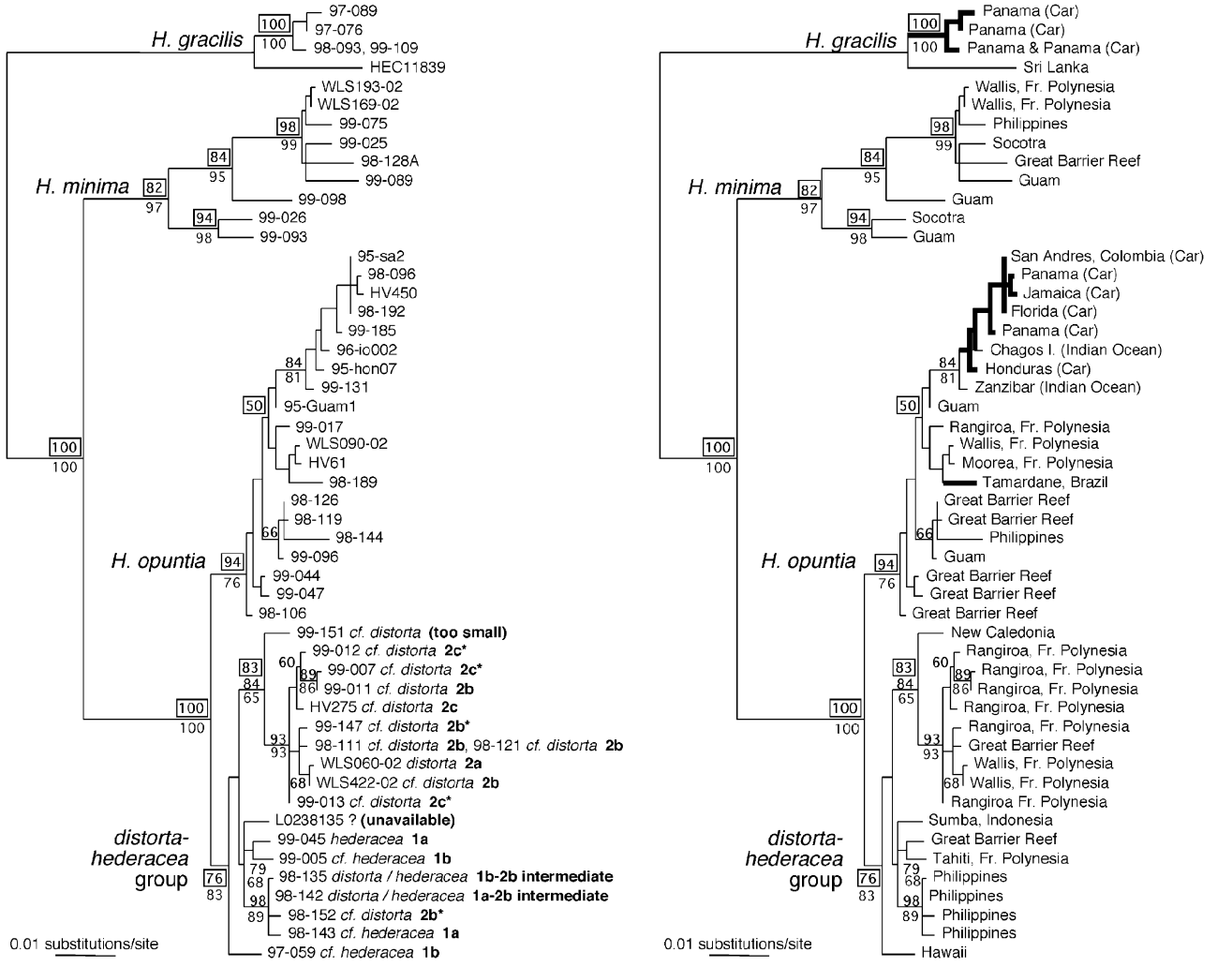

FIG. 3. (a) Maximum likelihood tree inferred from partial nuclear rDNA cistron data of *Halimeda minima*, *H. opuntia*, the *distorta–hederacea* group, and *H. gracilis*. The latter have been treated as outgroup (see Table 2). Car, Caribbean; Fr., French. Bootstrap values greater than or equal to 50% are indicated to the left of the clade. The ML and MP bootstrap values are indicated above and below branches, respectively. The ML bootstrap values not encased in a box have been generated by analyses of sequences within the *H. opuntia* clade or within the *distorta–hederacea* clade; values encased in a box have been generated in the global ML analysis (see text). Specimen numbers are explained in Table 1. Identification of specimens within the *distorta–hederacea* complex into morphological subgroups is indicated behind the specimen numbers (meaning of 1a–2c, see text). An asterisk denotes that only a few segments were available for study. The topology is available from TreeBASE under the same accession number as the alignment. (b) Area phylogram representation of a. Collection regions are indicated at end nodes. For more detailed information on collection sites, see Table 1. Car, Caribbean; Fr., French. The most parsimonious explanation for the occurrence of Atlantic specimens has been marked as thickened branches.

This is because the latter were often clippings, too small to give a complete image of the thallus morphology as seen in Figures 1 and 2.

Sequence analyses and phylogenies. The alignment of the 53 sequences was straightforward; only 24 of 1758 positions needed introduction of gaps in some of the sequences, and no ambiguous alignment possibilities were encountered. One hundred forty-nine positions were parsimony informative and were located predominantly in the ITS regions. Virtually none of the sequences obtained from conspecific specimens were identical, not even if the specimens originated from proximal sites. The least rejected sequence evolution model resulting from the hierarchical likelihood ratio test was a general time reversible one with estimated values for the following parameters: base frequencies: A = 0.206, C = 0.271, G = 0.305, T = 0.218; substitution rates: A ↔ C = 1.211, A ↔ G = 1.890, A ↔ T = 1.524, C ↔ G = 0.653, C ↔ T = 3.525 relative to G ↔ T = 1.000; proportion of invariable sites = 0.550; gamma shape parameter = 0.455. An ML tree inferred from all 54 sequences is shown in Figure 3a. The tree is 619 steps long (rescaled consistency index = 0.689, homoplasy index = 0.456; −ln L = 5073.4327). MP analysis resulted in 205 equally MP trees of which the 66 shortest were 628 steps long (rescaled consistency index = 0.699; homoplasy index = 0.463; −ln L between 5427.6007 and 5438.4730; MP trees not shown). These MP trees were nine steps longer than the ML tree, but the topology was essentially the same as that of the ML tree.

The ML tree shows a well-supported (94%) clade with all sequences from specimens with a morphology conforming that of *H. opuntia*. The *H. opuntia* clade revealed considerable intraspecific variation, but little phylogenetic structure was recovered among the sequences; only two internal clades obtained any support above the 50% threshold.

All sequences from specimens belonging to the *distorta–hederacea* complex grouped in a clade with 76% ML-bootstrap support as nearest sister to the *H. opuntia* clade. Sequence variation within the *distorta–hederacea* clade was comparable with that within the *H. opuntia* clade as illustrated by similar overall branch lengths in the two clades, but phylogenetic structure was better resolved in the former; several internal clades obtained support. Sequences from lax thalli with small segments (morphologies 2a–2c) were all but one recovered in the first major clade of the *distorta–hederacea* complex (Fig. 3a), whereas those obtained from stiffer thalli with relatively large segments (morphologies 1a–1b) were found at the basal polytomy of the complex (Fig. 3a). Phylogenetic separation of morphs was incomplete: Specimens 98–135 and 98–142 showed an intermediate morphology, and specimen 98–152 with its lax thallus and small segments was recovered among the stiff thalli with large segments in the lower polytomy. The previously described further division (indicated with a, b, or c) within the two major morphological groups did not reveal clear grouping in the phylogeny.

TABLE 2. Distribution of ambiguities over the alignment.

Species	*Halimeda opuntia*	*Halimeda distorta complex*	*Halimeda minima*	Total
Ambiguity				
R (AG)	21	8	1	30
Y (CT)	51	20	8	79
S (CG)	2	4	0	6
W (AT)	9	8	0	17
K (GT)	3	1	0	4
M (AC)	8	11	1	20
N	11	1	3	15
Total	105	53	13	171
Sequences	23	18	13	54
No. positions showing ambiguities in:				
SSU	1	2	1	
Insert	5	1	2	
ITS1	6	8	3	
5.8S	0	0	0	
ITS2	27	19	2	

Sequences of *H. minima* were recovered in a clade as sister to the clade with *H. opuntia* and the *distorta–hederacea* complex. Differences among sequences in this clade were more pronounced than within the *H. opuntia* clade or within the *distorta–hederacea* complex. In fact, three genetically distinct groups separated by long branches and obtaining high bootstrap support were discernible within the *H. minima* clade.

Table 2 lists the distribution of ambiguities over the sequences of *H. minima*, *H. opuntia*, and the *distorta–hederacea* complex. Although about the same number of sequences has been included for *H. opuntia* and the *distorta–hederacea* complex, the former showed about twice as many ambiguities than the latter. Y was the most frequent ambiguity encountered across all sequences, followed by R, M, and W. Table 2 also lists the distribution of ambiguities over the different sequence regions; ambiguities were encountered mainly in the ITS2 region of the *distorta–hederacea* complex and *H. opuntia*; they were far less frequent in the ITS2 of *H. minima*, and they were rare in the other sequence regions. Ambiguities were encountered at positions in the alignment where the remaining sequences belonging to the same species contained either one or the other base of which the particular ambiguity was composed. As an example, positions showing ambiguity Y in some *H. opuntia* sequences contained C or T in the remaining sequences of this species.

Phylogeography. In the area phylogram in Figure 3b, sample sites (Table 1) have been indicated at the end nodes of the tree in Figure 3a to infer the most parsimonious phylogeographic explanation of their distribution. All sequences of *H. minima* and the *distorta–hederacea* complex were obtained from Indo-Pacific specimens. Within the *distorta–hederacea* complex, Philippine specimens grouped together and so did many—but not all—French Polynesian ones. Sequences of *H. opuntia* showed a basal Pacific grade

with a well-supported (84%ML, 81%MP) clade with specimens from the Caribbean and Indian Ocean. Although the Caribbean sequences form a grade with one from the Indian Ocean among them, monophyly for the Caribbean sequences is not rejected because of the total lack of bootstrap support within this clade. The sequence of a Brazilian specimen grouped with Pacific neighbors and not with the Caribbean ones, though monophyly for Atlantic sequences is not strictly rejected because all clades separating the well-supported clade with Caribbean sequences from the Brazilian one did not obtain sufficient support.

DISCUSSION

Classical versus genealogical interpretation of species boundaries. Our major morphological subdivision of the specimens corresponds with the phylogenetic one; all sequences of the specimens belonging to our *H. opuntia* morph form a clade and so do those in the *distorta–hederacea* complex and those in the *H. minima* morphological group. Nonetheless, conflict occurs between the classical and phylogenetic interpretation of species in the studied group for two reasons. First, well-supported lineages exists within the *H. minima* clade and the *distorta–hederacea* complex, suggesting the existence of genetically separated populations or even biologically distinct taxa therein. Second, species as perceived and delineated by classical taxonomy (Hillis-Colinvaux 1980) appear to conflict with our phylogenetic clades and morphological groups.

Specimens assignable to *H. minima* are genetically and morphologically distinct from the group of specimens assignable to *H. opuntia* and the *H. distorta–hederacea* complex. Results support those in Verbruggen and Kooistra (2004) that *H. minima* consists of at least two genetically distinct lineages, that variation among the sequences within the clades is comparable to that within other *Halimeda* species, and that each lineage seems to be widely distributed. Therefore, we believe that there exist at least two biologically distinct species in *H. minima*. The *H. minima* group is morphologically diverse, and morphological patterns within this group should be addressed with a more sizable set of specimens, including *H. howensis*, *H. renschii*, and *H. velasquezii*.

Halimeda opuntia constitutes a monophyletic taxon. The tightly packed cushions composed of reniform segments encountered on wave-exposed sites and brittle networks of tripartite segments found in mangles (see illustrations in Littler and Littler 2000 and Fig. 1, c–e) do not show any grouping in the tree and probably result entirely from plasticity. Moreover, if cushions found on exposed reef flats are maintained in shaded aquaria, they sprout brittle thalli like those encountered in mangles. Similar morphological plasticity under influence of environmental factors has been demonstrated in *H. cylindracea* Decaisne (Gilmartin 1960).

Hillis-Colinvaux (1980) merged all forms described in *H. opuntia* under a single species, and at first sight our results prove her right. Nonetheless, Hillis-Colinvaux' interpretation of *H. opuntia* may have been too broad. *Halimeda distorta* (our morph 2a) is described very narrowly in her monographs, whereas our morphs 2b and 2c do not seem to be represented in her work. The segments of these morphs are often tripartite like those of *H. opuntia* growing in low light conditions. Furthermore, distal segments of morph 2b resemble those of typical *H. opuntia* cushions. Hillis-Colinvaux (1980) stressed that very few specimens of *H. distorta* were at her disposal. Given the fact that our morphs 2b and 2c are relatively common in Pacific atolls, a region well studied by Hillis-Colinvaux, we suspect that she was misguided by the resemblance between both species and included our morphs 2b and 2c in *H. opuntia*.

In the *distorta–hederacea* complex, morphotypes seem to correlate with evolutionary history because the clade, including lax specimens with relatively small segments (including *H. cf. distorta*), is distinct from the remaining grade with stiff specimens and large segments (including *H. cf. hederacea*). The specimens resembling the types of *H. distorta* (WLS060-02) and *H. hederacea* (98–143) are recovered in the clade and the basal grade, respectively, but not all specimens in the clade resemble the type of *H. distorta* and neither do all those in the basal group resemble that of *H. hederacea*. Apparently, the type specimens are just morphs within one or two morphologically more broadly defined species inside the *distorta–hederacea* complex. The absence of any monophyly among the observed varieties suggests that these are part of plasticity ranges like those observed in *H. opuntia*; in fact, many in-between cases were noted already.

The taxonomic history of the entity *H. hederacea* has been a dynamic one. Barton (1901) recognized *H. opuntia* forma *hederacea* as a distinct form within *H. opuntia*. Colinvaux (1968) recognized it as *H. hederacea*, but merged it with Atlantic *H. copiosa* one year thereafter (Colinvaux 1969). Recently, Kooistra et al. (2002) demonstrated that Atlantic and Indo-Pacific *H. copiosa* are cognates, distantly related entities that have converged upon comparable thallus habit and anatomy; therefore, they proposed to reestablish *H. hederacea* provisionally. The taxonomic history of *Halimeda distorta* is somewhat more straightforward. First, Yamada (1941, 1944) described it as a form of *H. incrassata* (Ellis) Lamouroux, and subsequently Colinvaux (1968) elevated this form to the species level.

The absence of a tight correlation between morphology and phylogeny in the *H. distorta–hederacea* complex calls for a re-taxonomization of the group. Even though morphotypes correlate with clades and grades, the relatively low support of the different subgroupings and the existence of specimens showing intermediate morphologies leave us with a lack of evidence to sustain the distinction between *H. hederacea* and *H. distorta*. Therefore, we propose the

provisional merger of both species. Because the formae *H. incrassata* forma *distorta* and *H. opuntia* forma *hederacea* were elevated to the specific rank in the same study, either name can be used. We are of the opinion that *distorta* is the more appropriate one for two reasons. First, the epithet *distorta* means distorted, indicating that segments are not entirely flat. This is the case in a majority of our specimens, either in the basal or the distal part. Only a small part of the specimens has ivy leaf-like segments, making the *hederacea* epithet less appropriate. Second, the denomination *hederacea* could bring about confusion with *H. copiosa*. The latter species occurs exclusively in the Atlantic Ocean, whereas the *hederacea–distorta* complex is restricted to the Indo-Pacific. Yet the species *H. hederacea* was described by Colinvaux (1968) based on a combination of Indo-Pacific and Atlantic specimens. The Atlantic specimens belong to *H. copiosa* sensu Kooistra et al. (2002). In our proposal, *H. copiosa* refers to Atlantic specimens corresponding to the description in Goreau and Graham (1967) and *H. distorta* refers to all Indo-Pacific specimens corresponding to our morphs 1a, 1b, 2a, 2b, and 2c and their intermediates (Fig. 2, a–j).

Discordances between classical and genealogical perceptions of species need to be stressed. Although for *H. opuntia* both perceptions seem to correspond well, classical *H. minima* appears to be under-taxonomized, whereas the *H. distorta–hederacea* group either has been over-taxonomized or has been taxonomized using the wrong morphological characters. The existence of several genetically distinct taxa within morphologically perceived species has also been observed in other *Halimeda* lineages (Kooistra et al. 2002). All these cases represented genetically distinct Atlantic and Indo-Pacific taxa that had converged upon one another morphologically, possibly because they grow in highly similar environments. Cryptic and pseudocryptic species have been noted also in other green algal groups (Angeler et al. 1999, Coleman 2001, Durand et al. 2002, O'Kelly et al. 2004).

Additional information on species boundaries could be acquired by studying reproductive events and anatomical characters. In a group of closely related *Halimeda* species (Atlantic *H. incrassata*, *H. monile* (Ellis et Solander) Lamouroux, and *H. simulans* Howe), the timing of concerted spawning differed between species (Clifton 1997, Clifton and Clifton 1999). A similar approach combined with interfertility studies could provide insights into the nature of subgroups uncovered in the present study. Furthermore, studies analyzing both morphometric and DNA sequence data (Verbruggen et al. 2005) can reveal which morphological characters can be used to diagnose different species and their internal clades.

Intraspecific and intraindividual sequence variation. If the well-supported clades within the *H. distorta–hederacea* complex and within *H. minima* represent biologically distinct species, then considerable sequence variation still abounds within each of these species. Such high intraspecific variation is also encountered within monophyletic morphologically defined species in other *Halimeda* lineages (Verbruggen and Kooistra 2004, unpublished data) and within their distant bryopsidalean relative *Caulerpa racemosa* (Forsskål) C. Agardh (Famà et al. 2000, Durand et al. 2002). Possible explanations for such high sequence variation are a high mutation rate, poorly performing concerted evolution (Dover 1982, Arnheim 1983, Jorgensen and Cluster 1988), ancestral polymorphism in arising species (Durand et al. 2002), and hybridization and polyploidization events (Scholin et al. 1995, Wendel et al. 1995).

Lineage-specific high mutation rates on the rDNA sequences could explain the high intraspecific sequence variation observed in both *Halimeda* and *Caulerpa* (Famà et al. 2000, Durand et al. 2002, Verbruggen and Kooistra 2004, this study) as well as a highly elevated substitution rate in nuclear SSU rDNA sequences of Bryopsidalean algae in general (Zechman et al. 1999). If elevated rates affect the SSU, then the same may be true for the far less conserved ITS sequences.

Concerted evolution generally performs poorly if organisms reproduce exclusively clonally or if the rDNA sequences occur scattered throughout the genome. *Halimeda* species, and particularly members of the section *Opuntia*, can grow clonally for extended periods (Hillis-Colinvaux 1980, Walters et al. 2002), but generally populations also experience frequent reproductive events during which a large part of the biomass is shed as gametes (Hillis-Colinvaux 1980, Drew and Abel 1988, Clifton and Clifton 1999). Given such frequent and massive sexual reproduction, one would expect rapid reshuffling and homogenization of all copies along a chain of rDNA sequences (Hillis and Dixon 1991).

Hybridization events can explain the observed intraindividual sequence variation (Table 2) and the apparent lack of intraspecific phylogenetic structure among sequences within *Halimeda*. Hybridization may have been more extensive in *H. opuntia* than in the *H. distorta–hederacea* complex because despite comparable sequence variation (as illustrated by similar branch lengths in the *H. opuntia* clade and the clade with the *distorta* complex), phylogenetic resolution is worse among the sequences of *H. opuntia*, and these sequences show a markedly higher number of intraindividual polymorphisms (Table 2). Similarly, high polymorphism and ill-defined species boundaries have been assigned to hybridization in corals (Van Oppen et al. 2001).

Phylogeographic structure within the distorta–hederacea and opuntia clades. Although the results of this study support the distinctness of the *H. opuntia* clade from the one with *H. distorta–hederacea* complex, it has not improved phylogeographic resolution within these two clades. Both the huge distribution ranges and the apparent paucity of phylogeographic patterning within the clades of *H. opuntia* and the *H. distorta–hederacea* complex suggest that *Halimeda*

disperses over large distances and that dispersion is apparently ongoing and frequent. Mature *Halimeda* thalli are unlikely long-distance travelers because they are calcified and therefore sink rapidly if they become dislodged (Walters et al. 2002). Yet it might be the propagules and the juvenile uncalcified thalli (Meinesz 1980) that do most of the traveling (Kooistra et al. 2002).

Interoceanic dispersal. Our data suggest that *H. opuntia* is of Indo-Pacific origin because its nearest neighbor taxa (the *distorta–hederacea* complex and *H. minima*) are all strictly Indo-Pacific. Moreover, within the *H. opuntia* clade in Figure 3b, all sequences from Caribbean specimens and two from Indian Ocean specimens form a well-supported clade within a grade of Pacific sequences. *Halimeda opuntia* appears to have settled the Brazilian coast independently from the region comprising the Caribbean and Bahamas because the sequence of the Brazilian specimen is distinct from those of Caribbean specimens and appears in a different part of the phylogeny. However, bootstrap support for the various clades in the *H. opuntia* clade is insufficient to confirm independent dispersal. The data suggest that the Caribbean founders arrived from the Indian Ocean because sequences of specimens from that region are recovered in the basal part of the Indo-Caribbean clade. Yet, this inference is weak because only two specimens from the Indian Ocean were included.

Halimeda opuntia may have arrived in the Caribbean, Bahamas, and Brazil as a hitchhiker in the fouling biota on ship hulls. It is now a common constituent of western Atlantic vegetations, forming nascent biohermal structures in lagoons and dense sprawls over leeward reef slopes (Hillis-Colinvaux 1980). The very first collection of *H. opuntia*, from Jamaica, at the close of the 17th century (Sloane 1707) proves its presence in the Caribbean three centuries ago. Yet interoceanic shipping commenced about another two centuries earlier, at the end of the 15th century. The possibility of an arrival in the western Atlantic before the closure of the Panama Isthmus at about 3.1 Ma B.P. (Coates and Obando 1996) is unlikely because that event could be linked to the separation of Atlantic and Indo-Pacific clades deeper down in the *Halimeda* phylogeny (Kooistra et al. 2002), and one of these Indo-Pacific clades gave rise to *H. opuntia*.

Evidence from the fossil stratigraphy also suggests recent settlement of *H. opuntia* in the Caribbean and Bahamas. Calcified segments of this species accumulate between 1 and 2 m per millennium (Drew and Abel 1985, Hudson 1985, Freile 2004). At Pacific undisturbed sites, extensive deposits of segments belonging to *H. opuntia* and its sister, *H. distorta* (Finckh 1904, Drew and Abel 1985), must have accrued over several 100,000 years. Instead, layers of *H. opuntia* segments at Atlantic locations (Hudson 1985, Andersen and Boardman 1989) are no more than 1 m thick, suggesting occurrence for a millennium at the most. As an example, modern day channel sands of Pigeon Creek (San Salvador, Bahamas) consist of up to 50% *Halimeda* segments with a considerable portion of those represented by *H. opuntia* (Mitchell 1986). In contrast, a nearby and ecologically comparable Sangamon interglacial unit contains only small amounts of *Halimeda* segments, but there is no trace of *H. opuntia* (Thalman and Teeter 1983).

The notion that extant *H. opuntia* is not a western Atlantic native is relevant for paleontologists and ecologists alike. Undisturbed layers of fossil segments may allow more precise dating of the arrival as well as reconstruction of how local biota adapted to the newcomer over a period spanning several centuries. Generally, species are considered to be nonindigenous if scientific records exist from the times predating their arrival. Unfortunately, however, human meddling with the global marine biodiversity dates further back than keen scientific interest in this biodiversity and "important constituents of the local seaweed flora" may well be historic invaders.

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