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Evolution and phylogeography of *Halimeda* section *Halimeda* (Bryopsidales, Chlorophyta)

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Abstract

Nuclear ribosomal and plastid DNA sequences of specimens belonging to section *Halimeda* of the pantropical green seaweed genus *Halimeda* show that the group under scrutiny contains many more genetically delineable species than those recognized by classical taxonomy. Discordances between phylograms inferred from nuclear and plastid DNA sequences suggest that reticulate evolution has been involved in speciation within the clade. Nonetheless, our data do not allow ruling out certain alternative explanations for the discordances. Several pseudo-cryptic species are restricted to the margins of the generic distribution range. In a clade of *H. cuneata* sibling species from widely separated subtropical localities in the Indian Ocean, the South African sibling branches off first, leaving the Arabian and West Australian species as closest relatives. We hypothesize that geographic isolation of the siblings may have taken place following Pleistocene or Pliocene periods of climatic cooling during which subtropical species occupied larger distribution ranges. A more basal separation of Atlantic, Indo-Pacific, and Mediterranean species indicates vicariance. The alternative events that could have caused this vicariance are discussed.

Keywords: Biogeography; Chlorophyta; Evolution; *Halimeda*; Incomplete lineage sorting; Indian ocean; Marine speciation; Phylogeny; Reticulate evolution

1. Introduction

In the tropical marine realm, patterns and processes of speciation are seldom obvious. A striking contradiction in this context is that while marine populations are presumed to be more open than their terrestrial counterparts as a consequence of genetic remixing brought about by ocean currents, many species show large genetic differences between geographically separated populations (e.g., Duke et al., 1998; Lessios et al., 2003; McMillan and Palumbi, 1995), sometimes to such a degree that geographic entities deserve species status (e.g., De Clerck et al., 2005; Muss et al., 2001; Pakker et al., 1996). Additionally, several marine species have been shown to contain cryptic or pseudo-cryptic diversity unlinked with geography (Knowlton, 1993).

Marine macroalgae abound in almost all coastal habitats. Despite their high diversity and abundance, the patterns of their evolution and processes involved in their speciation have not yet been intensively studied. The green algal genus *Halimeda*, the focus of this paper, is among the better studied (Kooistra et al., 2002; Kooistra and Verbruggen, 2005; Verbruggen et al., 2005c; Verbruggen and Kooistra, 2004).

Halimeda is a common inhabitant of tropical and warm-temperate marine environments and a prominent

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primary producer, source of food and habitat, and carbonate sand producer (Hillis-Colinvaux, 1980). The algal body of *Halimeda* is composed of flattened, green, calcified segments interconnected by uncalcified nodes (Hillis-Colinvaux, 1980; Lamouroux, 1812). From the anatomical point of view, the entire algal body consists of a single, tubular cell that branches to form an organized network of siphons (Barton, 1901; Hillis-Colinvaux, 1980). In the medulla, siphons run in the axial direction and ramify sparsely. In the cortex, siphon ramification is denser. The short, cortical siphon branches are inflated and called utricles. Sexual reproduction occurs periodically (Drew and Abel, 1988) and gametes are released into the water column during mass spawning events (Clifton, 1997). Sympatric species have been shown to spawn in slightly different timeframes (Clifton, 1997; Clifton and Clifton, 1999). Reproduction is followed by death of the alga, after which the calcified segments disconnect and contribute to the sediment (Freile et al., 1995; Meinesz, 1980). These segments endure in the fossil record and often make up the bulk of the carbonate structure of tropical reefs (Bassoullet et al., 1983; Hillis-Colinvaux, 1986).

Kooistra et al. (2002) examined the phylogeny, biogeography and historical ecology of the genus on the basis of partial nuclear ribosomal cistron sequences (partial 18S, ITS1, 5.8S and ITS2) of 28 out of the 33 species recognized at that time. Sequences grouped in five clear-cut clades, which subsequently formed the foundation of a new sectional classification (Verbruggen and Kooistra, 2004). Kooistra et al. (2002) also showed that certain clades within the genus were characterized by ecological properties such as growth on unconsolidated substrates and in sheltered localities. Finally, it was shown that each of the five lineages featured distinct Atlantic and Indo-Pacific subgroups, indicating that vicariance has been at play.

The focus of this study is on section *Halimeda* of the genus, within which eleven morphological species are currently recognized. The majority of these species are epilithic and occur in wave-affected habitats (Verbruggen and Kooistra, 2004). Section *Halimeda* is of special interest within the genus for a variety of reasons. Firstly, former studies (Kooistra et al., 2002; Verbruggen and Kooistra, 2004) indicated that *H. tuna* and *H. discoidea* were non-monophyletic, raising taxonomic interest in the section. Secondly, the section was sparsely sampled in these former studies: most species were represented by only one or a few individuals, and especially the subtropical regions in which the genus occurs were undersampled. Thirdly, the marker used in the studies of Kooistra et al. (2002) and Verbruggen and Kooistra (2004) was unable to completely resolve the relationships between species. Fourthly, the fact that section *Halimeda* is the most widely distributed section of the genus, occurring also in the Mediterranean Sea, Eastern Atlantic Islands, the Pacific coasts of tropical America, and ranging to higher latitudes than other sections, makes it of special biogeographic interest. Lastly, the section features two species whose phylogeographic patterns could provide insight in the evolution of Indian Ocean algae. It concerns *H. cuneata*, which has a disjunct distribution in the subtropical basins of the Indian Ocean (SE Africa, SW Australia, Arabian Sea) and Indo-Pacific *H. discoidea* (sensu Kooistra et al., 2002), which is present in tropical waters of the Indo-Pacific and has a small population in Oman (Jupp, 2002).

Special attention goes to the species *H. cuneata*, *H. discoidea*, and *H. tuna*. The former species (Figs. 1A–N) is defined morphologically by a stalk zone between subsequent segments and peripheral utricles adhering laterally over approximately half their length (Hillis-Colinvaux, 1980). The stalk zone can take the form of a stretch of uncorticated medullar filaments (Figs. 1B, F, H, and N) or a corticated cushion segment (Fig. 1D). Segments are wedge-shaped (Figs. 1A, C, E, I, and M) or discoid (Figs. 1G and K). Two forms of *Halimeda cuneata* (forma *undulata* and forma *digitata*) described by Barton (1901) do not possess a stalk zone (Figs. 1J and L) and are recognized as forms within *H. cuneata* by some (e.g., Littler and Littler, 2003) or synonymized with other species by other taxonomists (e.g., Hillis-Colinvaux, 1980). *Halimeda discoidea* (Figs. 1O–Q) is characterized by discoid to kidney-shaped segments and large subperipheral utricles bearing multiple peripheral utricles (Hillis-Colinvaux, 1980). *Halimeda tuna* (Figs. 1R and S) can be recognized by relatively small wedge-shaped to discoid segments and rather small subperipheral utricles typically bearing three peripheral utricles (Hillis-Colinvaux, 1980). Hillis-Colinvaux (1980) lists *H. discoidea* and *H. tuna* as pantropical; the latter also occurs in the Mediterranean Sea. *Halimeda cuneata* has a disjunct distribution in the subtropical parts of the Indian Ocean, with populations in Australia, SE Africa, SW Madagascar and the Arabian Sea (Hillis-Colinvaux, 1980) and has recently been reported from Brazil (Bandeira-Pedrosa et al., 2004). The *H. cuneata* forms *undulata* and *digitata* occur in the tropical Indo-Pacific.

The goals of this study are (1) to elucidate the phylogenetic history of *Halimeda* section *Halimeda* using plastid DNA markers (*tuf*A, *rps*19–*rps*3, and *rpl*5–*rps*8–*inf*A) and nuclear ribosomal sequences (partial 18S, ITS1, 5.8S, and ITS2), (2) to evaluate the phylogenetic status of *Halimeda cuneata*, *H. discoidea*, and *H. tuna* using molecular tools and specimens covering more of the morphological variability and distribution ranges than was the case in former studies, and (3) to examine biogeographic patterns within the section as a whole and phylogeographic patterns within *H. discoidea* and *H. cuneata*.

2. Materials and methods

Taxa of *Halimeda* section *Halimeda* were collected throughout most of their distribution ranges. Vouchers

Fig. 1. Illustration of morphological variation in the species *Halimeda cuneata* (A–N), *H. discoidea* (O–Q) and *H. tuna* (R, S). (A, B) *H. cuneata* 1. (C, D) *H. cuneata* 2. (E, F) *H. cuneata* 3. (G, H) *H. cuneata* 4. (I, J) *H. cuneata* f. *digitata*. (K, L) *H. cuneata* f. *undulata*. (M, N) *H. cuneata* 7. (O) *H. discoidea* 1. (P) *H. discoidea* 2. (Q) *H. discoidea* 3. (R) *H. tuna* 1. (S) *H. tuna* 2.

were deposited in the Ghent University Herbarium (GENT). Identifications followed Hillis-Colinvaux (1980), Ballantine (1982) and Noble (1986) and, for *H. cuneata* forms, Barton (1901) and Littler and Littler (2003). We were unable to obtain specimens suitable for DNA analysis of three species of section *Halimeda* (*H. gigas*, *H. scabra*, and *H. xishaensis*). *Halimeda gigas* was represented in the studies of Kooistra et al. (2002) and Verbruggen and Kooistra (2004), but these specimens belong to *H. cuneata* f. *undulata*, an entity not recognized in the monographic work these authors used for their identifications (Hillis-Colinvaux, 1980). Extraction of DNA followed Kooistra et al. (2002). The nuclear ITS1–5.8S–ITS2 region was amplified according to Kooistra et al. (2002) and plastid partial *rps*19–*rps*3 (UCP7) according to Provan et al. (2004). Amplified products were sequenced with an ABI Prism 3100 automated sequencer (Applied Biosystems, Foster City, CA) and submitted to Genbank (accession numbers in Appendix 1). For a subset of specimens, additional sequences were obtained (plastid partial *tuf*A: Famà et al. (2002); plastid *rpl*5–*rps*8–*inf*A [UCP3]: Provan et al. (2004); nuclear 18S rDNA: Kooistra et al. (2002)).

Partial *rps*19–*rps*3 and *tuf*A DNA sequences were aligned on the basis of a blueprint created by alignment of these regions' amino acid sequences using ClustalW 1.82 at the EBI (European Bioinformatics Institute) server, with default settings. Alignment of the *rpl*5–*rps*8–

*inf*A plastid region was more complex. Open reading frames were assessed using ORF Finder at the NCBI (National Center for Biotechnology Information) server. The initiation codon of *rps*8 was situated within the *rpl*5 gene in all ingroup sequences, so that a few (4–17) bases were used for both genes. The region of overlap was duplicated for alignment. Between *rps*8 and *inf*A, a small (5–32 bp) spacer was present. Amino acid sequences corresponding to the different genes were aligned using ClustalW 1.82 at the EBI server, with default settings. The obtained amino acid alignment was used as a blueprint for DNA sequence alignment. The *rps*8–*inf*A spacer was excluded from the alignment. After alignment, sequence blocks of all plastid regions (*tuf*A, *rps*19–*rps*3, and *rpl*5–*rps*8–*inf*A) were concatenated. Nuclear ribosomal DNA sequences were aligned by eye. Alignments are available from the first author upon request and from Treebase (http://www.treebase.org/treebase/).

Neighbor joining analysis was applied to the *rps*19–*rps*3 and ITS1–5.8S–ITS2 alignments to aid identification of groups of specimens at the species level. The NJ analyses were carried out in PAUP* 4.0b10 (Swofford, 2003), using the ML distance measure. Likelihood parameter settings were determined using Modeltest 3.5 (Posada and Crandall, 1998). NJ bootstrapping analysis (500 replicates) was carried out. Maximum parsimony (MP) and maximum likelihood (ML) analyses were carried out using the same software. The search options were: starting trees obtained by stepwise random sequence addition for MP analysis and a NJ starting tree for ML analysis, TBR branch swapping, maximum 10^3 rearrangements for ML and 10^6 per addition-sequence replicate for MP, and 25 addition-sequence replicates for MP. Bootstrapping was not carried out under the MP and ML criteria. The combined plastid and 18S–ITS1–5.8S–ITS2 sequence alignments were subjected to heuristic ML analysis in PAUP* 4.0b10, with base-substitution models determined by Modeltest 3.5. Settings were as mentioned above for NJ analysis. Heuristic ML bootstrapping (100 replicates) was carried out with five addition-sequence replicates per bootstrap replicate. Bayesian posterior probabilities to indicate statistical support for interior branches were calculated using MrBayes v3.0B4 (Ronquist and Huelsenbeck, 2003). The different genes and regions (plastid *tuf*A, *rps*19, *rps*3, *rpl*5, *rps*8, and *inf*A; and nuclear 18S, ITS1, 5.8S, and ITS2) were subjected to MrModeltest 2.0 (Nylander, 2004) independently and optimal substitution models were specified to MrBayes for each gene separately (Ronquist and Huelsenbeck, 2003). Analyses were run with four Markov chains for 10^6 generations, with a tree saved every 100th generation. The first 1000 trees were discarded as burn-in. Identical ML and BI analyses were carried out on an alignment of combined plastid sequences after exclusion of all positions showing gaps in the ingroup. A single *H. gracilis* sequence was used as outgroup in all above analyses (Appendix 1). *Halimeda gracilis* belongs to section *Pseudo-opuntia* within the so-called opuntioid lineage, which is the sister clade of section *Halimeda* (Verbruggen and Kooistra, 2004). Sequences of *H. gracilis* could be readily aligned with those of the other species in the study. Sequences of Indo-Pacific *H. discoidea* were subjected to heuristic ML analysis in PAUP* 4.0b10 and Bayesian analysis in MrBayes v3.0B4, with base-substitution models determined by MrModeltest 2.0. All settings for ML and BI were as mentioned above, but no outgroup was specified.

Significance of length differences between trees obtained by the analyses described above and user-defined trees was assessed using the Shimodaira–Hasegawa (SH) test (Shimodaira and Hasegawa, 1999; Goldman et al., 2000) implemented in PAUP* 4.0b10, with resampling-estimated log-likelihood (RELL) optimization and 1000 bootstrap replicates.

3. Results

3.1. DNA sequence data

Information on length, variability and base composition of the molecular markers can be found in Table 1. Different species exhibited markedly divergent ITS1–5.8S–ITS2 and partial *rps*19–*rps*3 sequences. The species *H. cuneata*, *H. discoidea*, and *H. tuna* each comprised two or more genotypic groups. Sequences within such genotypic groups differed in only one or a few positions (or not at all) while sequences among genotypic groups differed more substantially (Fig. 2). Modeltest suggested different models of base substitution among regions (Table 1).

3.2. Pseudo-cryptic species diversity

The phylograms resulting from NJ analysis of partial *rps*19–*rps*3 and ITS1–5.8S–ITS2 sequences are presented in Figs. 3 and 4, respectively. The specimens clustered in a number of dense clades (boxed in figures) with little or no internal structure. These clades corresponded to genotypic groups recognized in the sequence alignments. Branches leading to different genotypic clusters were of variable length and obtained high support (bootstrap proportion generally > 90 and often 100). The ML and MP topologies (available through TreeBase) were highly similar to the NJ trees, showing dense clusters of specimens identical to those recognized in the NJ trees and branch lengths highly comparable to those of the NJ trees. All clades receiving bootstrap support in the NJ tree were present in the obtained ML tree and the majority rule consensus tree of all obtained equally MP trees.

Table 1
Length, variability, base composition, and selected substitution models of the molecular markers[a]

	*rps*19–*rps*3[c]	ITS1–5.8S–ITS2[c]	18S– ITS1–5.8S–ITS2[d]	*tuf*A	*rpl*5–*rps*8–*inf*A
Sequence length[b]	549–603	443–476	1671–1700	858	623–677
Alignment length	660	528	1756	858	735
Constant positions	385	350	1515	589	425
Variable positions	275	178	241	249	310
Parsimony informative positions	210	145	119	153	153
A	40.37%	22.83%	20.85%	36.89%	31.24%
C	14.89%	31.05%	27.38%	11.39%	17.21%
G	14.62%	28.95%	31.44%	20.55%	17.65%
T	30.12%	17.17%	20.33%	31.17%	33.90%
Selected substitution model	TVM + G	GTR + I + G	18S: GTR + I + G ITS1: K80 + G 5.8S: K80 + I + G ITS2: HKY + G	GTR + I + G	*rpl*5: GTR + G *rps*8: GTR + G *inf*A: JC + G

[a] The selected model for the concatenated plastid data set as a whole was GTR+I+G.
[b] Ingroup sequences only.
[c] Statistics of the data set with all specimens; Modeltest ran without distinction between regions.
[d] The selected model for the 18S–ITS1–5.8S–ITS2 data set as a whole was GTR+I+G.

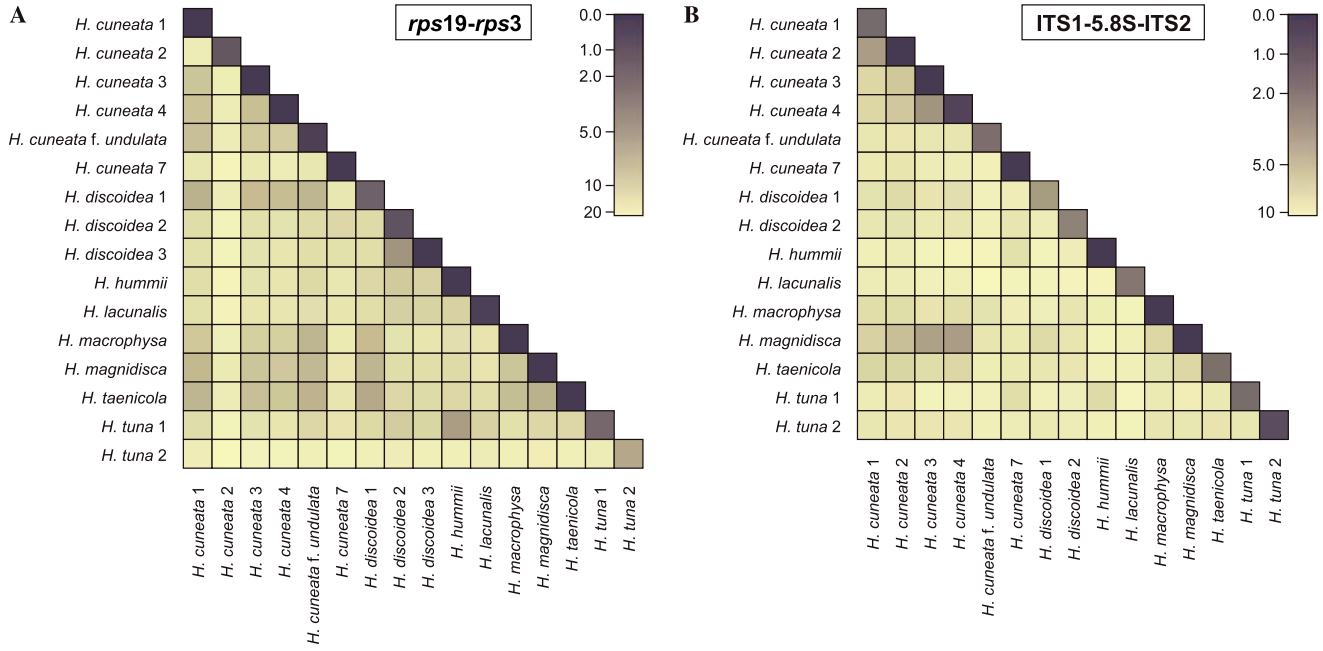

Fig. 2. Pairwise uncorrected distances of *rps*19–*rps*3 (A) and ITS1–5.8S–ITS2 (B) sequence data (in percent divergence).

These patterns of similarity were observed for the plastid and nuclear data sets.

Several species turned out to comprise two or more genotypic clusters. *Halimeda tuna* sequences separated in two distinct clades and *H. discoidea* consisted of three divergent genotypic clusters. Within the genetic diversity of *H. cuneata*, seven distinct clusters were disclosed. Sequences of *H. cuneata* f. *digitata* were recovered within the *H. discoidea* 1 clade. Numbering of genotypic clusters within morphologically perceived species was artificial; it was based on the clusters' sequence of occurrence in Fig. 3, not on morphological hypotheses. Sequence divergence values between specimens from different genotypic clusters boxed in Figs. 3 and 4 were considerably larger than values between species from the same genotypic cluster (Fig. 2). Uncorrected *p*-distances were 0.4% ± 0.8% within genotypic clusters and 10% ± 4% between genotypic clusters for the *rps*3–*rps*19 alignment and 0.5% ± 0.6% within genotypic clusters and 8% ± 2% between genotypic clusters for the ITS1–5.8S–ITS2 alignment (weighted average ± standard deviation; see also Fig. 2). *Halimeda tuna* 2 showed relatively high within-genotypic cluster DNA sequence divergence of the *rps*3–*rps*19 region (3%) but not of the ITS1–5.8S–ITS2 region (0.3% ± 0.2%). The opposite is true for *H. discoidea* 1, showing a relatively high within-genotypic cluster

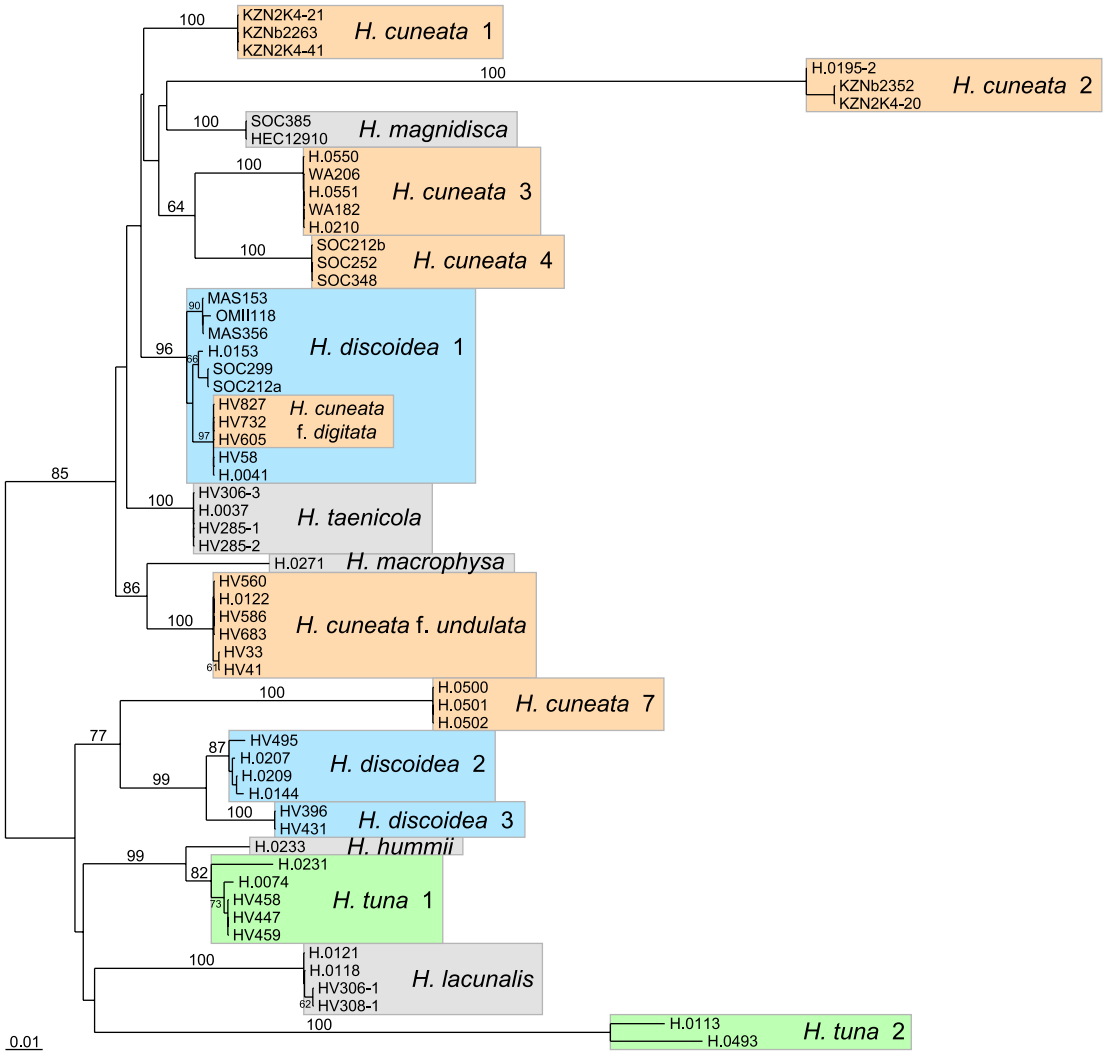

Fig. 3. Neighbor joining phylogram inferred from *rps*19–*rps*3 sequences of 60 *Halimeda* specimens (score = 1.13797). The outgroup was removed from the tree. Bootstrap proportions exceeding 50% are indicated at branches.

divergence for the ITS1–5.8S–ITS2 region (2% ± 2%) but not for the *rps*3–*rps*19 region (0.7% ± 0.5%). The high ITS1–5.8S–ITS2 within-genotypic cluster sequence divergence in *H. discoidea* 1 was attributable to the subdivision of this cluster into two divergent sister clades that was also present but showed much less divergence in the *rps*3–*rps*19 data (Figs. 4 and 5). In a few cases, sequence divergence between different genotypic clusters was relatively small. This was the case for *H. discoidea* 2–3 and *H. hummii*–*H. tuna* 1 for the *rps*3–*rps*19 data and *H. cuneata* 1–2 and the species triplet *H. magnidisca*–*H. cuneata* 3–*H. cuneata* 4 for the ITS1–5.8S–ITS2 data (Fig. 2). Here again, relatively large between-cluster divergences in one alignment did not necessarily correspond to comparably large divergences in the other alignment.

Identifications of specimens using former monographic works are presented in Appendix 1 and Figs. 3 and 4. Re-examination of the external morphology of the specimens used in the phylogenetic reconstructions revealed certain morphological differences between genotypic clusters identified in Figs. 3 and 4. Specimens of *H. cuneata* 1 possessed thick, relatively narrow, cuneate segments (Fig. 1A) and a long, decorticate stalk zone (Fig. 1B). The more delicate specimens of *H. cuneata* 2 stood apart in that they possessed fully corticated cushion segments (Fig. 1D) and relatively broader segments (Fig. 1C). Our specimens of *H. cuneata* 3 are characterized by relatively large segments (Fig. 1E) and a long, decorticate stalk zone (Fig. 1F). Discoid segments (Fig. 1G) and a relatively short decorticate stalk zone (Fig. 1H) were found in *H. cuneata* 4. The specimens belonging to forms *digitata* and *undulata* of *H. cuneata* lacked a stalk zone (Figs. 1J and L) and were further characterized by large digitate (Fig. 1I) and reniform, undulate (Fig. 1K) segments, respectively. The small specimens of *H. cuneata* 7 were composed of small, cuneate to discoid segments (Fig. 1M) and short, decorticate stalk zones which were not present between all segments. No apparent macro-morphological differences were found between specimens of *H. discoidea* 1 and 2 (Figs. 1O and P). Specimens belonging to *H. discoidea* 3 had larger,

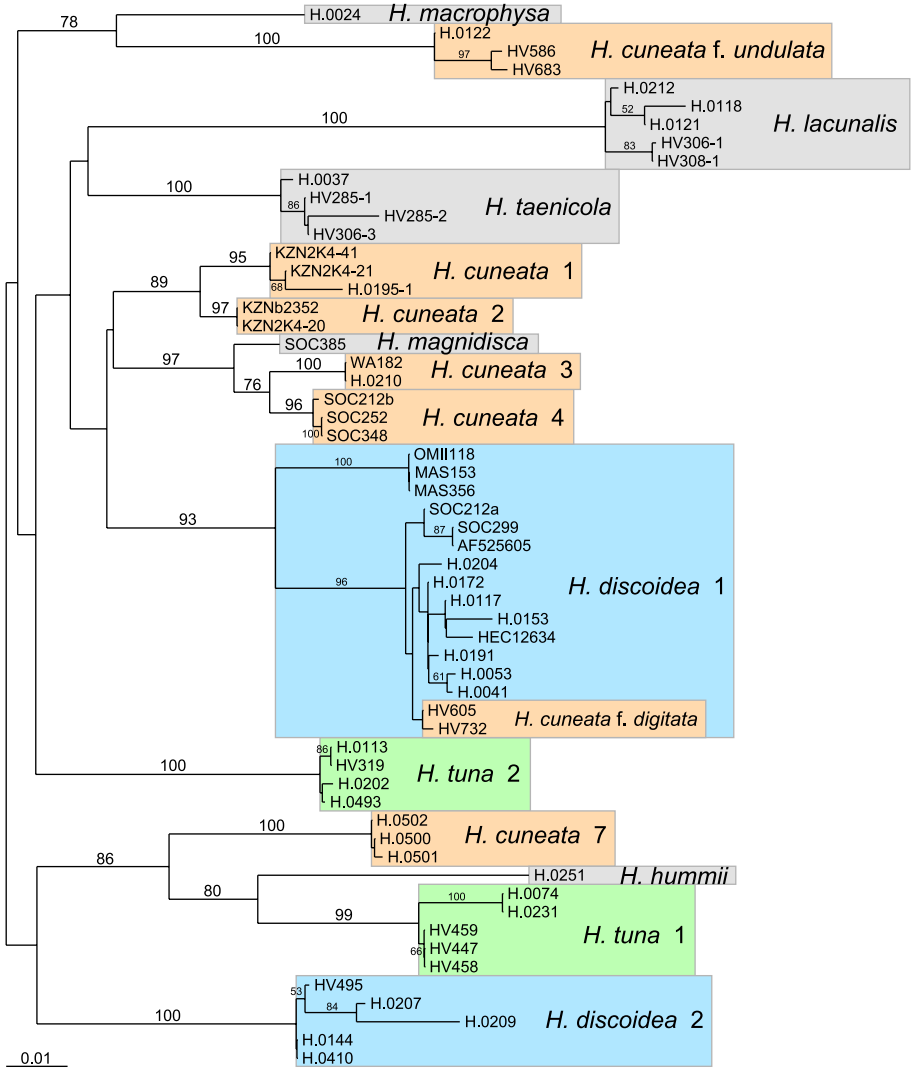

Fig. 4. Neighbor joining phylogram inferred from ITS1–5.8S–ITS2 sequences of 59 *Halimeda* specimens (score = 0.90205). The outgroup was removed from the tree. Bootstrap proportions exceeding 50% are indicated at branches.

flatter, less pigmented segments (Fig. 1Q) than specimens belonging to both other *H. discoidea* genotypic clusters. Segments of *H. tuna* 1 were often cuneate (Fig. 1R), whereas those of the majority of segments of specimens belonging to *H. tuna* 2 were discoid to reniform (Fig. 1S).

Genotypic clusters within morphologically perceived species generally accorded with geographic origin of the samples. Of the two *H. tuna* clades, one contained specimens collected in the Caribbean Sea (*H. tuna* 1) and the second contained specimens collected in the Mediterranean Sea (*H. tuna* 2). Specimens belonging to *H. cuneata* 1 and 2 were all collected in SE Africa; those of *H. cuneata* 3 came from SW Australia, and specimens of *H. cuneata* 4 originated from the Arabian Sea (Socotra). Specimens of *H. cuneata* f. *undulata* were collected from several places in the Indo-Pacific, and specimens belonging to *H. cuneata* 7 came from Brazilian populations. Within *H. discoidea*, genotypic cluster 1 contained specimens from throughout the Indo-Pacific. Specimens of the second genotypic cluster were collected throughout the Atlantic, and specimens of genotypic cluster 3 originated from the Caribbean Sea.

The phylogeographic structure of *H. discoidea* 1 (including *H. cuneata* f. *digitata*) is presented in Fig. 5. Sequences of the ITS1–5.8S–ITS2 region formed three main groups (Fig. 5A). The first, most widely ranging group contained specimens from the tropical parts of the Indian and Pacific Oceans. The second and third group contained two specimens from Socotra and three specimens from Oman, respectively. The *rps*19–*rps*3 tree (Fig. 5B) contained less sequences but also showed distinctness of Omanese and Socotran sequences. Philippine and French Polynesian sequences formed a clade widely divergent from the other sequences. The Hawaiian specimen, which was contained in the tropical group of the ITS1–5.8S–ITS2 tree, was not contained in the clade with the Philippine and French Polynesian specimens in the *rps*19–*rps*3 phylogram.

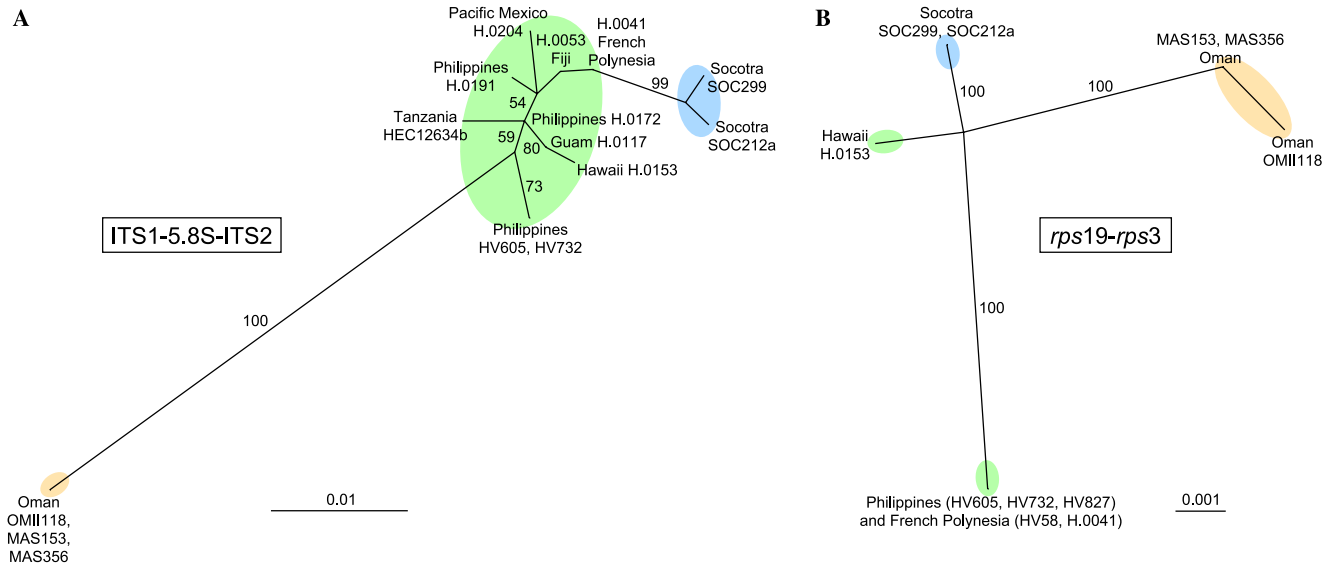

Fig. 5. Internal phylogeny of *H. discoidea* 1. (A) Single ML tree inferred from ITS1–5.8S–ITS2 sequences ($-\ln L = 817.15664$). (B) Single ML tree inferred from *rps*19–*rps*3 sequences ($-\ln L = 795.24718$). Bayesian posterior probabilities exceeding 50% are indicated at branches.

3.3. Phylogenies, topological discordances and historical biogeography

Fig. 6 shows the ML phylograms inferred from concatenated plastid sequences (Fig. 6A) and 18S–ITS1–5.8S–ITS2 sequences (Fig. 6B). Analysis of individual cpDNA markers (not shown) yielded topologies highly similar to the concatenated plastid phylogram. Clade support in such single-marker trees was low, only the basal split and some species pairs receiving support. Analyses of a concatenated nrDNA + cpDNA alignment were not attempted because of obvious discordances between the nrDNA and cpDNA trees, which are discussed below. Exclusion of gap sites from the concatenated plastid data did not influence topology and had little impact on support, short branches generally obtaining slightly less support. Likewise, alternative outgroup species (*H. micronesica*, *H. macroloba*, and *H. opuntia*) did not impact ingroup relationships. Sequences grouped in two major clades in both phylograms. Whereas the upper clade was strictly Indo-Pacific, the lower clade contained a Mediterranean species, an Indo-Pacific species, and all Atlantic–Caribbean species. The lower clade consisted of 4 lineages among which relationships remained unresolved. *Halimeda hummii* and *H. tuna* formed a well-supported group in both phylograms, as did *H. discoidea* 2 and 3. *Halimeda cuneata* 7 was recovered as closest sister to the *H. discoidea* 2–3 group in the plastid tree but as closest sister of the *H. hummii–tuna* clade in the nuclear phylogram.

Within the upper, Indo-Pacific lineage, phylogenetic structure was well-resolved in the plastid phylogram (Fig. 6A). There were three main species clusters. The first comprised *H. cuneata* 1, *H. magnidisca*, and *H. discoidea* 1; the second comprised three subtropical *H. cuneata* haplotypes (2, 3, and 4 – in gray box); and the third comprised *H. taenicola*, *H. cuneata* f. *undulata* and *H. macrophysa*. In the clade comprising the three subtropical *H. cuneata* entities (gray box), the SE African entity (2) branched off first, leaving the SW Australian (3) and Arabian (4) entities as closest sisters. In the nuclear tree (Fig. 6B), the Indo-Pacific lineage was relatively poorly resolved, and structured differently than in the plastid phylogram. *Halimeda cuneata* 1 and 2 clustered, and so did *H. cuneata* 3 and 4 together with *H. magnidisca*. As was the case in the plastid tree, *H. cuneata* f. *undulata* and *H. macrophysa* were sisters. The placement of *H. cuneata* 2 and *H. magnidisca* differed between the nuclear and plastid trees.

Topological discordances among trees were tested for significance using the Shimodaira–Hasegawa test. In two of six cases, clades obtained by ML analysis of one data set were rejected by the other data set (Fig. 7). Additionally, for the strictly subtropical *H. cuneata* clade in the plastid tree, the alternative topology with the Australian entity taking a basal position and the SE African and Arabian entities as closest sisters was tested against the original topology. This alternative was significantly worse than the original tree (length difference = 14.14948; $p = 0.017$).

4. Discussion

The obtained molecular phylogenetic data broach several issues about the evolutionary history of *Halimeda* section *Halimeda*. First, the *rps*19–*rps*3 and ITS1–5.8S–ITS2 phylograms show that the group under scrutiny contains many more genetically delineable species than those recognized by classical taxonomy. Sec-

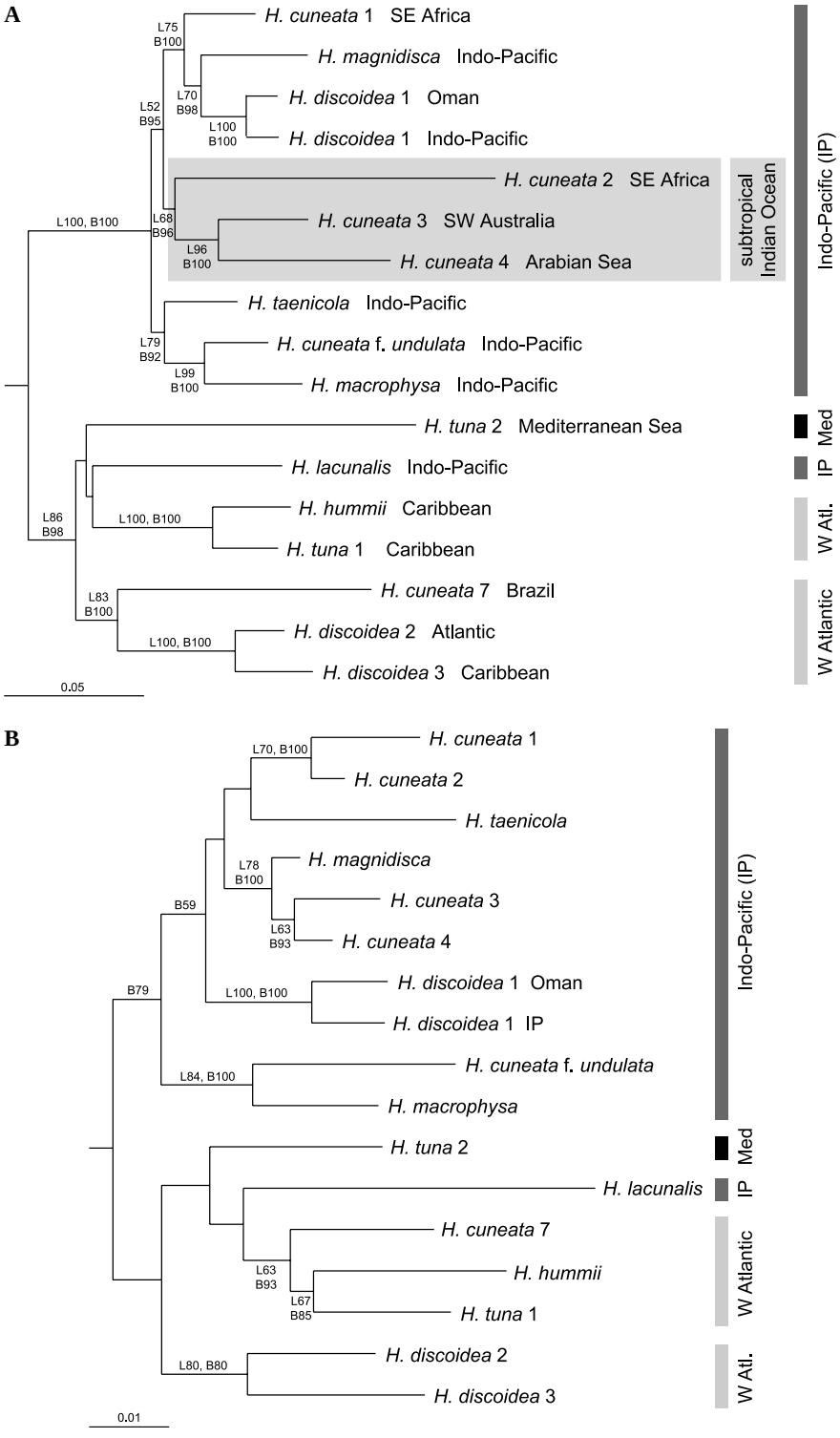

Fig. 6. ML phylograms inferred from plastid and nuclear ribosomal DNA sequences. (A) ML tree of concatenated plastid data of 17 *Halimeda* taxa. $-\ln L = 11579.11$. (B) ML tree inferred from nuclear ribosomal DNA data of 17 *Halimeda* taxa. $-\ln L = 5113.61$. Outgroup was pruned from the trees. ML bootstrap proportions (L…) and Bayesian posterior probabilities (B…) for clades (expressed in percentage) that exceeded 50 are indicated at the appropriate branches. Scalebars are in substitutions per site.

ond, the topological discordances between nuclear and plastid phylograms are suggestive of reticulate speciation. Third, the fact that some cryptic species are restricted to the margins of the generic distribution range alludes to the importance of peripatric isolation. Fourth, the separation of Indo-Pacific from Atlantic species in the phylograms confirms the idea of vicariance. Finally, the topology of *H. cuneata* reveals infor-

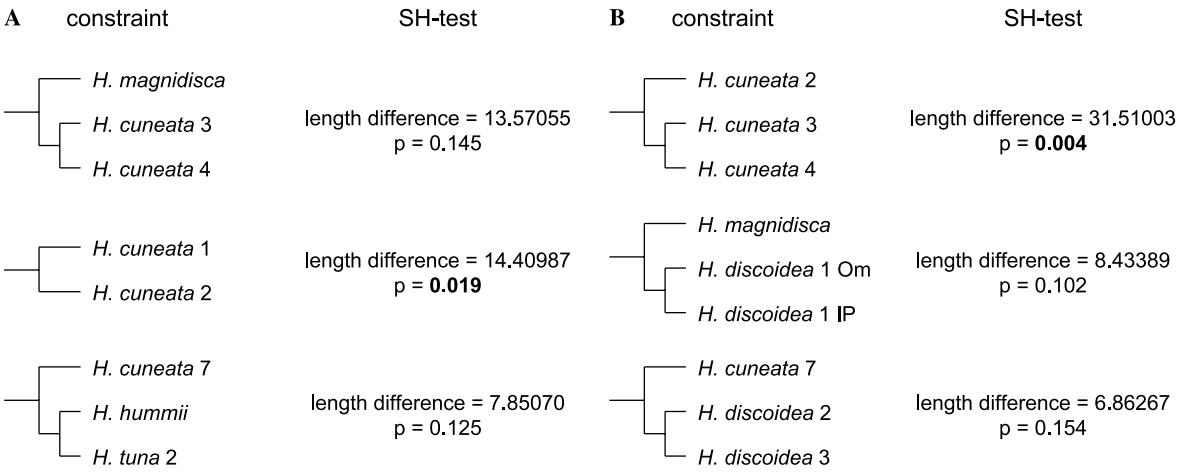

Fig. 7. Discordances between chloroplast and nuclear markers: Shimodaira–Hasegawa test results. (A) Shows species relationships suggested by the 18S–ITS1–5.8S–ITS2 data (Fig. 6B) that did not conform to the plastid topology (Fig. 6A). These relationships were used as constraints for ML analysis of the concatenated plastid data. The significance of length difference between such constrained trees and the original plastid topology of Fig. 6A were tested using the SH-test. Length differences and levels of significance are listed. Similarly, clades species relationships suggested by the plastid data set were tested against nuclear data (B).

mation about the historical biogeography of subtropical locations in the Indian Ocean. In what follows, these five topics will be addressed in more detail.

4.1. Pseudo-cryptic species

A number of observations entice regarding the genotypic clusters identified in Figs. 3 and 4 as species. Firstly, the genotypic clusters are widely divergent from one another and show relatively little internal sequence divergence (Fig. 2). Secondly, plastid and nuclear DNA sequences data reveal identical genotypic clusters (Figs. 3 and 4). Thirdly, traditional species appear non-monophyletic. Lastly, there are indications that genotypic clusters can be recognized morphologically. The genotypic clusters conform to the genealogical as well as the genotypic cluster species concept (Baum and Donoghue, 1995; Mallet, 1995). Moreover, similar genotypic clusters in *Halimeda* section *Rhipsalis* comply with the biological species concept (Kenneth Clifton, pers. comm.; see also Verbruggen et al., 2005c). In the latter section, modification of morphological species definitions after detailed morphometric studies allows application of the morphological species concept as well (Verbruggen et al., 2005c,d).

Assuming that each densely packed genotypic cluster in Figs. 3 and 4 constitutes a species implies the existence of sixteen rather than eight species in the examined group. Kooistra et al. (2002) and Verbruggen and Kooistra (2004) first revealed the existence of widely divergent genealogical species within morphological *Halimeda* species. In their analyses, Atlantic and Indo-Pacific specimens of certain species were recovered in different clades. This inter-oceanic cryptic diversity is corroborated by our data. Additionally, we reveal the existence of a second level of formerly unrecognized diversity in *H. discoidea* and *H. cuneata*, situated within ocean basins. A similar pattern was found previously for *H. minima* (Kooistra et al., 2002; Kooistra and Verbruggen, 2005) and Indo-Pacific *H. incrassata* (Verbruggen et al., 2005c,d).

Our findings show that traditional, morphology-based taxonomical practices have not provided sufficient resolution to detect differences between certain genotypic cluster species. Yet morphological differences between populations which now turn out to be distinct genealogical species were noted before but considered insufficient for recognition as separate species. For example, small morphological differences between *H. tuna* 1 and 2 have been reported but the species was not split because of high levels of intra-regional morphological plasticity (Hillis, 1959; Hillis-Colinvaux, 1980). Similarly, Bandeira-Pedrosa et al. (2004) considered the option that Brazilian *H. cuneata* (our cluster 7) evolved independently from the Indo-Pacific diversity of this species but nevertheless refrained from describing it as a new species. Our sequence data show that this Brazilian species evolved within the Atlantic clade and is not closely related to any of the Indo-Pacific *H. cuneata*-species, demonstrating yet another case of convergent evolution in the Atlantic and Indo-Pacific ocean basins. The macro-morphological observations on the specimens used in our phylogenetic analyses indicate that morphological differences are present between at least some of the genotypic cluster species comprised within the morphological species *H. cuneata*, *H. discoidea* and *H. tuna*. However, the sample size and depth of morphological observations of the present study are insufficient to allow accurate species delineation (Verbruggen et al., 2005c).

The fact that morphological differences were observed between the pseudo-cryptic species uncovered by our molecular phylogenetic study argues that the inaccuracy of current species delineations has resulted from taxonomic conservatism. Verbruggen et al. (2005c), in their molecular phylogenetic and morphometric study of *Halimeda* section *Rhipsalis*, showed that the morphological species *H. incrassata* comprised three genotypic cluster species. These genotypic cluster species did not form recognizable groups in PCA ordinations of morphometric data, witnessing their strong similarity and, in a sense, justifying the conservatism of taxonomists who did not have molecular data at their disposal. Morphological differences between the three species became apparent after the morphometric data was subjected to discriminant analysis using the three genotypic cluster species as a priori groups. We expect a similar situation for *Halimeda* section *Halimeda*. The combined action of molecular and morphometric tools has been successful at defining morphological boundaries between pseudo-cryptic species in several Bryopsidalean green algae (de Senerpont-Domis et al., 2003; Verbruggen et al., 2005a,b,c,d).

4.2. Topological discordances: possible reticulate speciation

Reticulate speciation causes the genome of the daughter species to contain traces from both parental species. The nuclear DNA of the daughter species will be a mixture of the genomes of both parent taxa. Nuclear ribosomal DNA, however, will in many cases be homogenized by interlocus concerted evolution during the next couple of generations (Liao, 1999; Small et al., 2004). Plastid inheritance is clonal, and in normal circumstances only one parent contributes the entire plastid genome. As a consequence of these different modes of nrDNA and plastid DNA inheritance, reticulate evolutionary events (ancient hybridization) can cause topological differences among trees inferred from nrDNA and plastid DNA sequences. In contrast to higher plants, where reticulate evolution is well-studied (Linder and Rieseberg, 2004), the roles of hybrid speciation and introgression in green algal diversification have not been thoroughly assessed. However, karyological (Kapraun, 1993, 1994; Kapraun and Buratti, 1998), and molecular (Durand et al., 2002) studies suggest that they may be at play. Artificial hybrids between the green microalgae *Eudorina* and *Pleodorina*, which have been kept in culture for decades and are capable of reproduction, have been studied (Coleman, 2002, and references therein). However, such hybrids are not known to occur in green algae in the wild.

Of the topological discordances between nuclear and plastid phylograms in the present study, only the position of *H. cuneata* 2 was truly conflicting according to Shimodaira–Hasegawa tests. The fact that *Halimeda* is a broadcast spawner (Clifton, 1997) could promote hybridization between sympatric species in the absence of intrinsic reproductive barriers. In the *H. incrassata–monile–simulans* species group, gametes are released in species-specific time intervals, which is suggestive of hybridization avoidance (Clifton, 1997; Clifton and Clifton, 1999; Kooistra et al., 2002). Despite these arguments in favor of reticulate evolution, other possibilities cannot be completely excluded. Incomplete lineage sorting, the complexity of ITS sequence alignment, and amplification and sequencing of paralogous sequences or pseudogenes are all known to limit the phylogenetic utility of rDNA–ITS sequence data (Alvarez and Wendel, 2003), and are valid and non-refutable alternative explanations for the observed pattern. The limited data at hand do not allow us to unequivocally single out the causes of topological discordances (Holder et al., 2001). In order to discern between the possibilities, karyological, genomic, and single-copy nuclear sequence data could be utilized (Hegarty and Hiscock, 2005).

4.3. Geographic speciation modes

The current distribution ranges of genotypic cluster species and the phylogenetic relationships between them suggest that examples of several geographic modes of speciation have been involved in the diversification of *Halimeda* section *Halimeda*. Although certain conditions have to be met to be able to reconstruct geographic modes of speciation from current distribution ranges and phylogenetic relationships (e.g., assumption that distribution ranges of ancestral species can be inferred from distribution ranges of its extant daughter species; Barraclough and Vogler, 2000; Losos and Glor, 2003), a few scenarios can be drawn from our data that can be used as starting hypotheses for subsequent studies of geographic modes of speciation at the population level (Losos and Glor, 2003).

The pseudo-cryptic species contained within *H. cuneata*, *H. discoidea* and *H. tuna* often appear to occupy non-overlapping distribution ranges. The most marked examples are *H. tuna*, of which the pseudo-cryptic species in the Mediterranean Sea and Atlantic Ocean are distinct, and *H. cuneata*, which has isolated pseudo-cryptic species in Brazil, SE Africa, the Arabian Sea, and Western Australia. Cryptic endemicity, the partitioning of cryptic species among geographical locations, is fairly common in marine algae (e.g., De Clerck et al., 2005; Gurgel et al., 2003; Pakker et al., 1996) and sedentary marine animals (e.g., Carlin et al., 2003; Muss et al., 2001).

Under the assumption that the distribution ranges of the genotypic clusters mentioned above are non-overlapping, allopatric speciation appears to be the most common mechanism of species formation within the section

under study. An interesting case in the data set represents the position of Omanese *H. discoidea* 1 specimens. The genotypic cluster formed by Omanese specimens is highly divergent from the large haplotype cluster of tropical Indo-Pacific specimens (Fig. 5). The Arabian Sea, which separates the Omanese population from the tropical ones is characterized by seasonal upwelling of cold, nutrient-rich water causing a pseudo-high-latitude effect with associated cold-water seaweed community (Schils, 2002; Sheppard et al., 1992) from which *H. discoidea* appears to be absent. It is possible that the small Omanese population, which seems to be restricted to a stretch of coastline of a few hundreds of kilometers only mildly influenced by upwelling, represents a peripatric founder population which has diverged strongly from the tropical population by genetic drift.

Halimeda discoidea 2 and 3 are sympatric in much of the Caribbean basin (HV, unpublished results). Whereas specimens from genotypic cluster 2 are most commonly found in shallow bays and lagoons, our specimens from genotypic cluster 3 all originated from deeper waters (15–50 m) along outer reef slopes, suggesting that these species originated sympatrically by habitat shift. *Halimeda cuneata* 1 and 2 are also sympatric but do not show obvious ecological differences.

Interestingly, in cases of cryptic species diversity within the Indo-Pacific and within the Atlantic Ocean, cryptic species with restricted distribution ranges are confined to the edges of the generic distribution range, often in regions characterized by colder temperatures (Arabian Sea, SE Africa, SW Australia). The distribution range of marine green algae is known to be strongly governed by temperature (van den Hoek, 1982). Species intolerant of tropical temperatures may thus show anti-tropical populations, and genetic isolation of such populations may be promoted through the absence of suitable stepping stones in the tropics and the small average dispersal distances of most marine algae (cf. Kinlan and Gaines, 2003).

4.4. Global biogeography

The basal division of sequences in an Indo-Pacific and a (mainly) Atlantic clade conforms to the results of previous studies (Kooistra et al., 1999; Kooistra et al., 2002; Verbruggen and Kooistra, 2004; Verbruggen et al., 2005d). Marine dispersal of tropical marine organisms between the Atlantic and Indo-Pacific ocean basins is currently prohibited by the North–South orientation of the African and American continents. A number of vicariance events are commonly invoked to explain Atlantic–Indo-Pacific sister relationships. The earliest event is the widening of the central Atlantic Ocean (Jurassic – Smith et al., 1994). The second is the closure of the Tethys Sea in the Middle East (Miocene – Rögl and Steininger, 1984). The third event, situated in the Pliocene, is the rise of the Central American Isthmus (Coates and Obando, 1996). The fourth and most recent barrier between the Atlantic and Indo-Pacific oceans for tropical organisms was the intensification of the Benguela upwelling in South Africa (late Pliocene – Marlow et al., 2000). Kooistra et al. (2002), in their molecular phylogenetic study of *Halimeda*, favored the Pliocene rise of the Central American Isthmus as the vicariance event causing basal splits in the five sections of the genus.

The first scenario is not supported by the fossil record: there have been no reports of *Halimeda* in the Caribbean before the Miocene except for one (Beckmann and Beckmann, 1966; cited after Bassoullet et al., 1983), the age of which was questioned by Bassoullet et al. (1983). Similarly, the third and fourth scenarios can be considered unlikely because the occurrence of such recent events relatively deep in the phylogeny disaccord with the presence of extant species in Plio- and Miocene deposits (Bassoullet et al., 1983; Dragastan et al., 2002). However, given the potential of *Halimeda* species to converge onto similar morphologies (Kooistra et al., 2002; Verbruggen et al., 2005c), reports of extant species from the fossil record could be erroneous due to iterative convergent evolution, which was hypothesized by Kooistra et al. (2002) but argued against by Dragastan et al. (2003). A solution could lie in the application of detailed morphometric studies on extant species and their putative fossil representatives (Verbruggen et al., 2005a). Such morphometric taxonomic studies have been successful at discriminating between extant species, even between some that converged onto similar morphologies (Verbruggen et al., 2005c).

Besides the fossil record, the evolutionary position of Mediterranean *H. tuna* 2 can contribute to the discussion. The relatively basal position of this species in the lower clade suggests that it is a paleo-endemic from the time that the Mediterranean Sea was formed rather than a recent invader from the Atlantic. This would imply that the vicariance event that caused the split in our trees was associated with the closure of the Tethys Sea in the Middle East. This scenario has two flaws. Firstly, a more derived position of Mediterranean *H. tuna* may be concealed by extinction. Secondly, it requires assuming that the lineage that give rise to *H. tuna* survived the Messinian crisis (Duggen et al., 2003), during which the Mediterranean Sea almost completely dried up, possibly by taking refuge in residual basins (Bianchi and Morri, 2000; Myers, 1996; Stanley, 1990).

In conclusion, the second scenario, in which the basal split in our trees results from the closure of the Tethys Sea in the Middle East in the Miocene, is best supported by the fossil record and the position of Mediterranean *H. tuna* 2. Vicariance in the Middle East has

been invoked to explain vicariance in several other marine taxa of Tethyan origin (e.g., Barber and Bellwood, 2005; Ellison et al., 1999; Streelman et al., 2002). For what *Halimeda* is concerned, the hypothesis of Miocene vicariance should be further examined using detailed morphometric re-examination of fossils and application of a molecular clock to our sequence data. Molecular clocks have not yet been calibrated for Bryopsidalean algae.

4.5. Biogeography of the subtropical Indian Ocean

The subtropical regions of the Indian Ocean (Arabian Sea, SE Africa, SW Australia) foster rich seaweed floras and high endemism (Bolton et al., 2004; Phillips, 2001; Schils, 2002). Moreover, biogeographic links between these regions have been described on the basis of shared taxa (Joosten and van den Hoek, 1986; Norris and Aken, 1984; Schils and Coppejans, 2003). Hommersand (1986) put forward three evolutionary-biogeographic scenarios that could explain the affinities between these distant floras. First, convergent evolution as a response to similar environments could cause the pattern. Second, common taxa could represent relics of a continuous distribution along the Cretaceous coast of Gondwanaland that was fragmented by the northward migration of Africa, Australia, and the Indian subcontinent. Third, the links could have come about through dispersal of species from their origin in SW Australia to SE Africa and the Arabian Sea through the Indian Ocean during periods of global cooling in the latter half of the Cenozoic.

Interpretation of the origin and topology of the subtropical *H. cuneata* 2–4 clade of Fig. 6A may provide additional information on the subtropical floristic similarities. That this clade is not monophyletic in the nuclear tree (Fig. 6B) should not hinder biogeographic interpretation. If the discordances were caused by reticulate speciation, biogeographic inference is a matter of reconciling information from both trees, taking into account that both parental species of species with discordant positions must have been sympatric. Alternatively, if the discordances between our trees were caused by incomplete lineage sorting, rDNA paralogues–pseudogenes or ITS alignment errors, one would expect the plastid tree to provide the more accurate representation of evolutionary history because (1) the clonal inheritance of plastid DNA shows faster coalescence through a smaller effective population size (Small et al., 2004), (2) no paralogues or pseudogenes are known for plastid genes in the Ulvophycean algae, and (3) alignment of plastid genes is unequivocal.

Morphological convergence, the first possible cause put forward by Hommersand (1986), is not an issue for biogeographic interpretation within the *H. cuneata* 2–4 species group because this group is monophyletic (with relatively high support) in the plastid phylogram. Neither does *H. cuneata* 2–4 fit the second scenario because it would imply that the 18S region of *Halimeda* evolves much slower than that of other green algal lineages (e.g., Olsen et al., 1994) while in reality *Halimeda* and its allies show higher mutation rates (Kooistra et al., 2002; Zechman et al., 1999). Furthermore, because the fossil record shows very little species diversification until the Late Cretaceous – Paleocene (Hillis, 2001), it is against all probability that a derived clade such as *H. cuneata* 2–4 would date back to the Early Cretaceous coasts of Gondwanaland.

A more recent scenario seems to fit our data and the fossil record better. Transequatorial divergence dating back to the Plio-Pleistocene has been corroborated by molecular clock analyses for antitropical fish, echinoderm and mollusk species (Burridge, 2002; Hilbish et al., 2000; Waters and Roy, 2003). The migrational directionality from SW Australia towards SE Africa (via the Arabian Sea) that was hypothesized by Hommersand (1986) does not correspond with the branching order in the plastid tree (Fig. 6A). An alternative hypothesis explaining the observed pattern is that the focal *H. cuneata* species were derived from a parental population that originated from a tropical ancestor at low latitude during a Plio-Pleistocene period of global cooling and was distributed throughout the Indian Ocean. Since the time of divergence, the populations have been isolated in relatively small geographic areas in the subtropics, allowing them to diverge. This hypothesis seems fit for a predominantly tropical genus like *Halimeda*, whereas the hypothesis of migration of species across low latitudes as proposed by Hommersand (1986) would be more fit for the predominantly temperate red algae that he focused on.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2005.06.015.

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