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DIVERSITY OF *HALIMEDA* (BRYOPSIDALES, CHLOROPHYTA) IN NEW CALEDONIA: A COMBINED MORPHOLOGICAL AND MOLECULAR STUDY[1]

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Halimeda* is a genus of calcified and segmented green macroalgae in the order Bryopsidales. In New Caledonia, the genus is abundant and represents an important part of the reef flora. Previous studies recorded 19 species that were identified using morphological criteria. The aim of this work was to reassess the diversity of the genus in New Caledonia using morpho-anatomical examinations and molecular analyses of the plastid *tuf*A and *rbc*L genes. Our results suggest the occurrence of 22 species. Three of these are reported for the first time from New Caledonia: *Halimeda kanaloana*, *H. xishaensis*, and an entity resembling *H. stuposa*. DNA analyses revealed that the species *H. fragilis* exhibits cryptic or pseudocryptic diversity in New Caledonia. We also show less conclusive evidence for cryptic species within *H. taenicola

Key index words: **biodiversity; coral reefs; *Halimeda*; New Caledonia; *rbc*L; taxonomic revision; *tuf*A**

***Abbreviations*: *tuf*A, elongation factor TU; *rbc*L, RUBISCO large subunit**

The genus *Halimeda* Lamouroux (Bryopsidales, Chlorophyta) is a well-studied group of calcareous green macroalgae. It occurs worldwide in tropical and subtropical marine regions but also in the Mediterranean Sea (Hillis-Colinvaux 1980, Kooistra et al. 2002). It is a conspicuous component of coral reef ecosystems known for its biomass and species richness, and occurs from shallow habitats down to 150 m deep on the reef slope (Hillis-Colinvaux 1985, Littler et al. 1986). *Halimeda* species are composed of calcareous segments connected to each other by noncalcified medullar siphons at the nodal joint. The internal structure of segments is composed of a network of medullar siphons ramifying into a cortex and terminating in inflated peripheral utricles. The outermost utricles adhere to each other forming a continuous membrane that envelops the internal space of the segment in which calcium carbonate precipitates as aragonite (Hillis-Colinvaux 1980). They are important primary producers, providing food and habitat to several coastal species of invertebrates and parrotfishes (Hillis-Colinvaux 1980, Hay et al. 1994). *Halimeda* is regarded as an important structural component contributing to most of the biogenic carbonate of reefs from the Cenozoic to the present (Payri 1988, 1995, Taylor et al. 2009). The life history of *Halimeda* includes periodical sexual reproduction during which the entire cytoplasm turns into reproductive cells and the thallus dies, this is called holocarpic reproduction (Meinesz 1980). The calcified segments then break down and crumble, contributing to sand formation. They also reproduce clonally by elongating subterranean rhizoids and fragmentation (Walters et al. 2002). Species display a diversity of forms and various habits colonizing sandy and rocky habitats. Some species feature high intraspecific morphological variability, like *Halimeda heteromorpha* N'Yeurt which displays trilobed to reniform segment forms and has either a small bulbous attachment structure anchoring it in sand or a small cushion of filaments fixing it to rock. Because of such high levels polymorphism, identification of *Halimeda* species can be difficult.

After its description by Lamouroux (1812), the genus was subdivided in four species groups based on external morphological criteria (*Tunae*, *Pseudo-opuntia*, *Opuntia*, and *Rhipsalis*) by J. G. Agardh (1887: 80–89). The analyses of nodal structure and utricles initiated by Askenasy (1888) and expanded

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by Barton (1901), Hillis (1959) and Hillis-Colinvaux (1980), led to the recognition of five lineages: *Rhipsalis* (eight species), *Opuntia* (seven species), *Halimeda* (11 species), *Micronesicae* (three species), and *Crypticae* (one species). In the molecular phylogenetic studies of Kooistra et al. (2002) and Verbruggen and Kooistra (2004), the classification was further revised, including the reinstatement of section *Pseudo-opuntia* and the merger of *Crypticae* with *Micronesicae*. In addition, *Halimeda melanesica* Valet, which was classified in *Micronesicae* by Hillis-Colinvaux (1980), was transferred to *Rhipsalis*. Kooistra et al. (2002) further identified the presence of cryptic diversity in the genus. During the last decade, Verbruggen and coworkers carried out multigene phylogenetic studies to further improve our understanding of species delimitation and evolutionary relationships in *Halimeda* (Verbruggen and Kooistra 2004, Verbruggen et al. 2005a,b, 2006, 2007, 2009a, b). Although the section-level lineages have been well characterized, many taxonomical ambiguities remain at the species level. The number of formally recognized species is 44 (Guiry and Guiry 2011), although molecular tools indicate at least 52 evolutionarily significant units, some of which are probably cryptic or pseudocryptic new species in need of description (Verbruggen et al. 2009b).

In New Caledonia, *Halimeda* is common to very abundant in many coralgal reef ecosystems. Most of the sandy or muddy areas of lagoons and back reefs are colonized by species with bulbous holdfasts that form extensive meadows that are sometimes mixed with seagrasses or *Caulerpa*. These meadows have an important ecological function because they represent one of the three major lagoon habitats structured by macrophytes together with seagrass and *Sargassum* beds (Garrigue 1995). Various species form thick sprawling mats or large draperies on hard substrata of reef flats, coral pinnacles and the fore-reef slope. *Halimeda* contributes significantly to the reef biogenic carbonate and represents one of the dominant bioclastic constituents of lagoon sediments (Chevillon 1996). The importance of the genus as a structural component of Cenozoic reefs makes it a good indicator in sedimentary archives and, along with coralline red algae, it is used to reconstruct paleoenvironment and climatic histories (Massieux 1976, Payri and Cabioch 2004). When Valet (1966, 1968) published his taxonomical work on Chlorophyta from New Caledonia, he identified eight species including the new species *Halimeda melanesica* Valet. Since then, several ecological and floristic studies have been conducted (Garrigue and Tsuda 1988, Garrigue 1995) and during the past decade, important new collections have been made in a wide variety of locations around New Caledonia. The numerous specimens collected were identified to currently recognized taxa based mostly on morphological and anatomical analyses. The most comprehensive inventory to date lists 19 species (Payri 2007). However, the material available has been insufficiently studied in many instances and the diversity may have been underestimated. Furthermore, very little molecular systematic work has been done to verify species identification, and only 12 sequences belonging to five species have been generated from New Caledonian material (Kooistra et al. 2002, Verbruggen et al. 2005a,c, 2009a).

Considering the importance of *Halimeda* in the actual coralgal system and paleoecology of New Caledonia, it is clear that the available collections deserve to be completely reevaluated. Hence, in this study we aim to revise the species diversity of *Halimeda* in New Caledonia by characterizing and comparing the morphology and anatomy of New Caledonian morphotypes and evaluating variation in the plastid DNA markers *tuf*A and *rbc*L. Our strategy consists of defining morphotypes in the collections based on morpho-anatomical analyses and subsequently sequencing the *tuf*A DNA barcodes of a majority of the specimens to evaluate species boundaries, infer the validity of morphotypes and assess the diversity of the genus in the study area.

MATERIALS AND METHODS

Site and specimens collections. New Caledonia is located in the Southwest Pacific Ocean, between 18° and 23° S and 164° and 167° E, about 1,500 km east of Australia (Fig. 1). It is composed of a main island named "Grande Terre" and several groups of smaller islands, coral reefs, and lagoons. The New Caledonian lagoon represents a total surface area of 24,000 km², which makes it one of the biggest in the world (Andréfouët et al. 2004, 2009). It is surrounded by a continuous 1,600 km long barrier reef, located from 10 to 70 km away from the Grande Terre coast, and expanding from the Entrecasteaux reefs to the northwest and the Isle of Pines to the southeast. The isolated Chesterfield-Bellona reefs are situated toward the west, half way toward the Australian coast, and the Loyalty Islands are located to the east of the main island.

A total of 155 New Caledonian samples pressed as herbarium vouchers and kept in the IRD-NOU phycological collection at IRD (Institut de Recherche pour le Développement) in Noumea were considered for morphological analyses. They were collected mainly by SCUBA from the intertidal to 60 m depth. All specimens were morphologically characterized and a selection of them was used for DNA analyses from ethanol or silica gel preserved thallus pieces.

The New Caledonian sampling sites included the southwest lagoon of Grande Terre (casual collections between 2004 and 2010), Isle of Pines (BIODIP, November 2005), the Loyalty Islands (BSM-Loyauté, March–April, 2005), La Côte Oubliée (CORALCAL1, March 2007); the Chesterfield—Bellona-Bampton area (CORALCAL2, July 2008), and Le Grand Lagon Nord (CORALCAL3, February 2009).

Additional collections from the Maldives (Baa Atoll, 2008), French Polynesia (Moorea, 2007, Biocode Moorea Project) as well as samples logged in the Ghent University algal herbarium from Jamaica, Costa Rica, Philippines, Kenya, Indonesia, Maldives, Palau, Australia, Tanzania, Nicaragua, Hawaii, French Polynesia, Thailand, Fiji, Micronesia, Wallis, Chagos, and Colombia were included in the analyses.

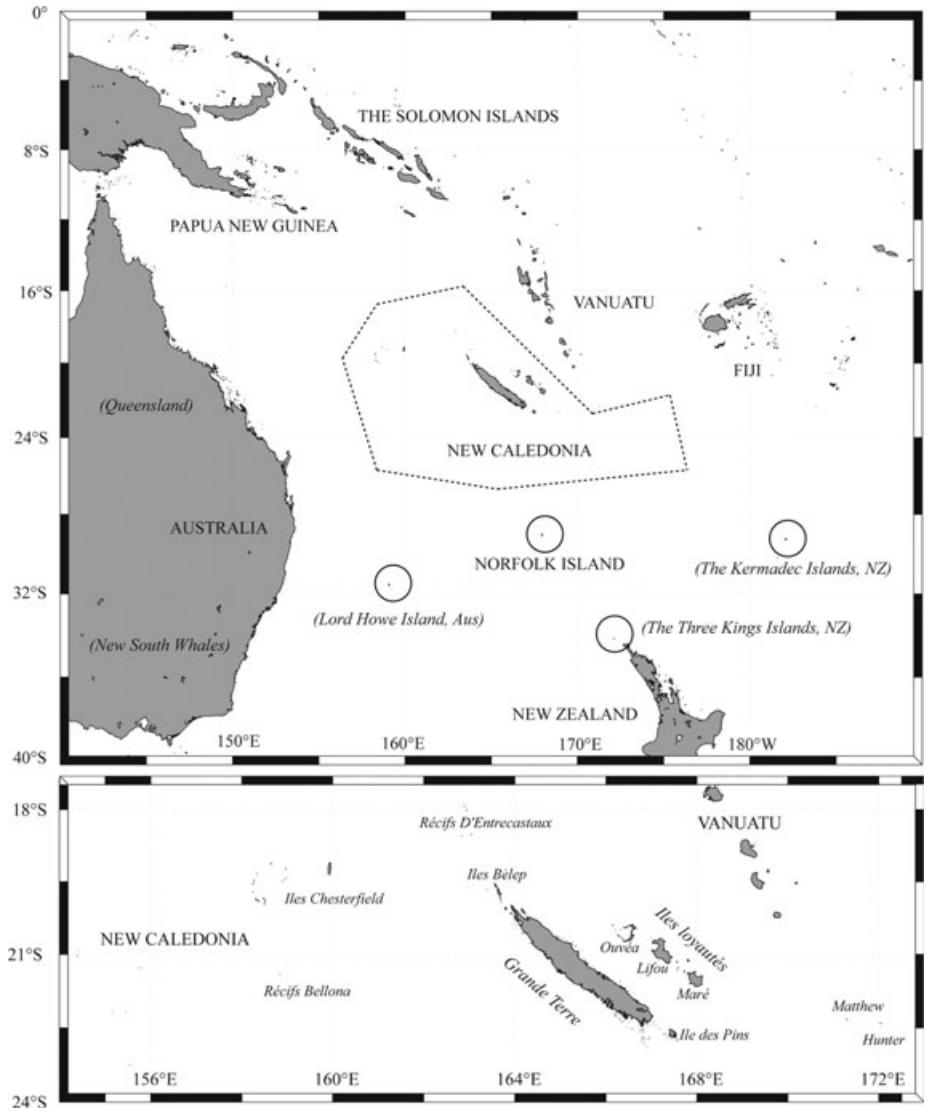

FIG. 1. Geographic position of New Caledonia in the South West Pacific (A) and map of the New Caledonian islands and reefs (B).

Morphology and anatomy. The morphological features of the samples were recorded following protocols described by Hillis-Colinvaux (1980) and Verbruggen et al. (2005a,b). We registered both macroscopic criteria such as holdfast type, shape of the thallus or segment form as well as anatomical characters of the nodes and the utricles, which were observed with stereo and light microscope after dissection and decalcification using HCl 10%. Utricles from noncalcified apical segments and basal segments were excluded from the analyses as recommended by Verbruggen et al. (2005d).

Specimens were subsequently grouped into morphotypes (groups of similar morphology) according to morphological analyses of the criteria listed above. All the morphological and anatomical characters were evaluated simultaneously for all the specimens to place them into their corresponding lineages, according to Verbruggen and Kooistra (2004). Within each lineage, the specimens were separated into different morphotypes looking for morphological and anatomical discontinuities in the arrangement and size ranges of the segments, medullar filaments, utricles, and nodal fusions. Finally, the morphotype boundaries often, but not always correspond to species delimitation. Subsequent DNA analyses were used to confirm or reject species placement within these groups.

DNA extraction and PCR. A total of 141 samples preserved in ethanol, 10 fresh samples and 60 dried samples from the herbarium were processed. Samples preserved in ethanol were air dried for 12 h prior to DNA extraction. A few segments or part of one segment (~0.5 cm long) were crushed in liquid nitrogen and total DNA was extracted using the DNeasy Plant minikit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. PCR was carried out in a total volume of 25 μL containing 2 μL of DNA extract, 0.2 μM of forward and reverse primers (Table S1, see Supporting Information), 0.2 mM of each dNTP, 2.5 μL of reaction buffer 10X (including 1.5 mM of $MgCl_2$), and 1.25 U of Taq polymerase (Jump Start Red Taq, Sigma). PCR parameters included an initial denaturation phase at 94°C for 2 min, 40 (*tuf*A) or 35 (*rbc*L) cycles of 1 min at 94°C, 1 min at annealing temperature (Table S1), 1 min at 72°C, and a final extension of 5 min at 72°C.

DNA sequencing. The majority of samples were sequenced in both directions with the PCR primers by Macrogen

(Macrogen Inc., Seoul, Korea) using the BigDye TM terminator method. The other samples were purified on column MinElute (Qiagen) and sequenced with an ABI Prism BigDye Terminator v.3.1 cycle sequencing kit (Applied Biosystems, Carlsbad, CA, USA) at the Plateforme du Vivant (IRD, Nouméa, New Caledonia). The 20 μL reaction volume contained 0.8 μL of ready reaction premix, 4 μL of BigDye sequencing buffer, 4 μM of each primer, and 10 ng of purified DNA. The sequencing reaction included a denaturation phase at 96°C for 1 min, 60 cycles of 15 s at 98°C, 10 s at 50°C, and 4 min at 60°C. The sequencing products were purified on Cephadex G50. Automated sequencing was performed on an ABI Prism 310 Genetic Analyzer (Applied Biosystems).

Sequence alignment and phylogenetic analyses. Sequences were aligned manually using the BioEdit sequence alignment editor (Hall 1999). A total of 124 *tuf*A and *rbc*L sequences of *Halimeda* were downloaded from GenBank (Hanyuda et al. 2000, Verbruggen et al. 2005a,b,c,d, 2006, 2007, 2009b, Lam and Zechman 2006, Curtis et al. 2008, Cocquyt et al. 2009) and added to our sequence alignments.

Our phylogenetic analyses consisted of two parts. First, a global phylogeny of the genus was generated using a concatenated dataset of *rbc*L and *tuf*A. The purpose of this analysis was to show the major groupings in the genus and their relationships. Second, we performed analyses of only *tuf*A sequences for the five major groupings observed in the first step. These analyses were performed to yield information about species delimitation and relationships within the major groupings of the genus.

For the first analysis, *tuf*A and *rbc*L were concatenated when both were available for a sample. Phylogenies were inferred with neighbor joining (NJ), maximum parsimony (MP), and maximum likelihood (ML) in MEGA4 (NJ, MP; Tamura et al. 2007) or PHYML (ML; Guindon and Gascuel 2003). For ML analyses, the model of nucleotide substitution was estimated using Findmodel (available at http://www.hiv.lanl.gov/content/sequence/findmodel/findmodel.html). Support for nodes was assessed by the bootstrap method (Felsenstein 1985) using 1,000 replicates for NJ and MP analyses and 100 replicates for ML analyses. Nodes were considered as weak below 70%, moderately supported between 71% and 84%, and strongly supported above 85%. A sequence of *Avrainvillea rawsonii* was used as outgroup for the first analysis (Verbruggen et al. 2006). For the lineage-specific analyses of *tuf*A, a sequence from a related lineage was used as outgroup. The resulting lineage-specific haplotype trees were used to delimit species with a method previously shown to yield accurate species boundaries in integrated morphological-molecular studies of *Halimeda* (e.g., Verbruggen et al. 2005a,b). The method consists of identifying well-supported clades that are preceded by a relatively long branch and have comparably low sequence diversity within. Alignments and trees are available through Treebase (http://www.treebase.org).

RESULTS

Morphology. Morphological investigation of the 187 New Caledonian samples led to the recognition of 20 morphotypes (Table 1) into which each individual from New Caledonia could be unambiguously assigned. These morphotypes, designated A–T matched the descriptions of (A) *H. cylindracea*, (B) *H. borneensis*, (C) *H. macroloba*, (D) *H. fragilis*, (E) *H. micronesica*, (F) *H. lacunalis*, (G) *H. discoidea*, (H) *H. macrophysa*, (I) *H. magnidisca*, (J) *H. taenicola*, (K) *H. gracilis*, (L) *H. minima*, (M) *H. opuntia*, (N) *H. distorta*, (O) *H. heteromorpha*, (P) *H. kanaloana*, (Q) *H. xishaensis*, (R) *H. velasquezii*, (S) *H. gigas*, and (T) *H. melanesica*, respectively (Table S2 in the Supporting Information). Morphological characters of the morphotypes are summarized in Table 1.

Morphotypes A, B, C, P could easily be distinguished from the others by the presence of pores at the nodal fusion and a well-developed bulbous holdfast for anchorage in sandy substrata. They could be distinguished from one another by their segment form. Morphotype A showed cylindrical segments, simple or trilobed whereas morphotype B presented flat trilobed segments and morphotype C had large reniform segments. Morphotype P presented basal cylindrical segments similar to morphotype A, but its segments were flat and trilobed. Morphotype T also presented pores at the nodal fusion but lacked well-developed holdfasts.

Morphotypes D and E were distinguished by the absence of nodal fusion and by the separation of the utricles after decalcification. Morphotype D had a basal segment with many daughter segments in all directions (at 360° all around) whereas morphotype E showed a basal "handlike" segment with numerous branching segments in its upper part (oriented about 180°). Both had a single holdfast.

Morphotypes F, G, H, I, J, Q, and S featured erect thalli with a single holdfast and complete nodal fusion of two or three siphons. Morphotype F could be distinguished by rounded, oval, or cuneate segments and utricles with a diameter <50 μm in surface view. Morphotype G showed high polymorphism, but constant and distinctive anatomical criteria with inflated secondary utricles generally exceeding 100 μm in diameter. Morphotype H has a thallus smaller than morphotype G and utricles visible to the bare eye and featuring a characteristic honeycomb aspect before decalcification. Morphotype I had basal segments forming a long and thin stipe with a well-developed bulbous holdfast. Morphotype J could be distinguished from the other morphotypes by thick cuneate, cylindrical, or oval segments and a single rhizoidal holdfast; the anatomical studies pointed out an inflated tertiary layer of utricles with a width over 110 μm.

Morphotype Q was very similar to morphotype G for the external morphology but occasionally featured distinctive undulate segments. It had very large peripheral utricles and relatively small secondary utricles compared to morphotype G (peripheral utricle length: 80–160 μm, width: 60–93 μm; secondary utricles length: 100–200 μm, width: 60–135 μm).

Morphotype S presented flat and glossy segments, most of which were reniform or oval, to 31 mm long and 42 mm broad. While the external morphology is reminiscent of morphotype G, it could be easily distinguished by its internal anatomy because it possessed larger peripheral utricles (peripheral utricle length: 92–187 μm, width: 130–240 μm).

TABLE 1. Summary of the morphology for morphotypes recognized in this study.

	Holdfast type	General shape	Basal region	Segments of top half of thallus	Nodal siphons	Number of utricles layers	Cortical utricles dimensions	Utricles after decalcification	Utricles form in surface view	Number of specimens studied	Identification
A	Bulbous	Erect	Pseudo-stipe of barrel-shaped segments with a single ramification	Cylindrical or trilobed; L: 1–9 mm; W: 1–8 mm	Single unit with pores	3–5	L1 = 20–80 μm; W1 = 10–36 μm; L2 = 10–70 μm; W2 = 10–44 μm	Detached	Rounded or polygonal	7	*Halimeda cylindracea*
B	Bulbous	Erect	Well developed, broad fan-shaped segment bearing multiple daughter segments	Flat, trilobed to discoid; L: 4–11 mm; W: 4–14 mm	Single unit with pores	3 or more	L1 = 40–80 μm; W1 = 20–48 μm; L2 = 28–88 μm; W2 = 24–48 μm	Attached	Polygonal	10	*H. borneensis*
C	Bulbous	Erect	Broad pseudo-stipe with a single ramification	Flat, discoid to reniform; L: 8–20 mm; W: 14–30 mm	Single unit with pores	3–4	L1 = 56–120 μm; W1 = 34–60 μm; L2 = 60–93 μm; W2 = 34–54 μm	Attached	Rounded or polygonal	10	*H. macroloba*
D	Single tight rhizoidal mat	Compact, bushy	Funnel-shaped basal segment bearing many daughter segments	Flat, reniform; L: 3–8 mm; W: 5–13 mm	Siphons go through node unfused	2–3	L1 = 40–88 μm; W1 = 20–40 μm; L2 = 14–50 μm; W2 = 12–32 μm	Detached	Rounded	6	*H. fragilis*
E	Single tight rhizoidal mat	Bushy; dense branching near base	Fan-shaped basal segment bearing many daughter segments	Flat, reniform to wedge-shaped and trilobed; L: 3–8 mm; W: 5–14 mm	Siphons go through node unfused	2–3	L1 = 20–88 μm; W1 = 16–44 μm; L2 = 12–100 μm; W2 = 8–40 μm	Detached but remaining in groups	Rounded	7	*H. micronesica*
F	Single tight rhizoidal mat	Erect	Broad, barrel-shaped segments	Flat, rounded-oval, some cuneate; L: 3–9 mm; W: 3–12 mm	Complete fusion of 2–3 siphons	3	L1 = 48–120 μm; W1 = 10–48 μm; L2 = 20–88 μm; W2 = 20–32 μm	Attached	Polygonal	6	*H. lacunalis*
G	Single tight rhizoidal mat or bulbous	Erect, very polymorphic	Variable, often broad and small basal segment	Flat, highly polymorphic (discoid, oval, reniform, rarely cylindrical to wedge-shaped); L: up to 29 mm; W: up to 33 mm	Complete fusion of 2–3 siphons	2, rarely 3	L1 = 49–160 μm; W1 = 16–48 μm; L2 = 80–240 μm; W2 = 72–176 μm	Attached	Polygonal	11	*H. discoidea*
H	Single tight rhizoidal mat	Small, erect	Single small basal segment	Flat, discoid to reniform; L: 15 mm; W: 24 mm	Complete fusion of 2–3 siphons	2	L1 = 80–120 μm; W1 = 40 μm; L2 = 96–160 μm; W2 = 56–80 μm	Attached	Polygonal	2	*H. macrophysa*
I	Bulbous	Erect	Basal segments often long and thin, stalk-like	Flat, broad, discoid; L: 22–25 mm; W: up to 22–26 mm	Complete fusion of 2–3 siphons	2	L1 = 100–150 μm; W1 = 30–65 μm	Attached	Polygonal	1	*H. magnidisca*

TABLE 1. Continued

	Holdfast type	General shape	Basal region	Segments of top half of thallus	Nodal siphons	Number of utricles layers	Cortical utricles dimensions	Utricles after decalcification	Utricles form in surface view	Number of specimens studied	Identification
J	Single tight rhizoidal mat	Erect	Basal segments often broad and square, forming a stipe	Thick flattened, broad cuneate to oval; L: 2–16 mm; W: 2–19 mm	Complete fusion of 2–3 siphons	3	L1 = 40–88 μm; W1 = 24–48 μm; L2 = 68–184 μm; W2 = 28–100 μm	Attached	Polygonal	10	*H. taenicola*
K	Multiple dispersed rhizoidal holdfasts	Sprawling, sparse ramifications	Segments somewhat flattened with 4 or more daughter segments	Flat, trilobed, broad ovoid to narrow cuneate; L: 2–6 mm; W: 1–6 mm	Complete fusion of 2 (rarely 3) siphons	2	L1 = 40–88 μm; W1 = 20–32 μm; L2 = 60–300 μm; W2 = 24–60 μm	Attached	Polygonal, sometimes rounded	7	*H. gracilis*
L	Single tight rhizoidal mat	Erect or pendent, curtain like; branching in single plane	Basal segment trilobed, generally bearing 3 daughter segments	Flat, reniform or oval; L: 3–4 mm; W: 4–7 mm	Short fusion of 2 or rarely 3 siphons	1–2	L1 = 20–40 μm; W1 = 14–40 μm; L2 = 24–96 μm; W2 = 12–48 μm	Attached	Polygonal	7	*H. minima*
M	Multiple dispersed rhizoidal holdfasts	Sprawling, forming compact, bushy mats	Basal segment could not be identified	Flat, reniform, sometimes ribbed; L: 3–7 mm; W: 4–8 mm	Short fusion of 2 or rarely 3 siphons	1–3	L1 = 12–16 μm; W1: 15–19 μm; L2 = 12–37 μm; W2 = 9–12 μm	Attached	Polygonal, sometimes rounded	3	*H.opuntia*
N	Multiple dispersed rhizoidal holdfasts	Sprawling mats	Basal segment could not be identified	Flat, reniform to trilobed, often keeled; L = 4–8 mm; W = 6–20 mm	Short fusion of 2–5 siphons	3	L1 = 30–53 μm; W1 = 21–37 μm; L2 = 19–109 μm; W2 = 16–31 μm	Attached	Polygonal	8	*H. distorta*
O	Single tight rhizoidal mat	Erect, bushy	Small and broad segments, sometimes forming stipe	Flat, trilobed to reniform; L = 3–8 mm; W = 1–10 mm	Single unit or groups without pores	2–3	L1 = 31–94 μm; W1 = 22–47 μm; L2 = 47–125 μm; W2 = 25–40 μm	Attached	Polygonal	4	*H. heteromorpha*
P	Bulbous	Erect	Barrel-shaped segments forming stipe	Flat, broad trilobed to flabellate; L: 12 mm; W: 15–20 mm	Single unit with pores	3	L1 = 67–113 μm; W1 = 47–73 μm; L2 = 47–87 μm; W2 = 33–66 μm	Attached	Polygonal	2	*H. kanaloana*
Q	Single tight rhizoidal mat	Erect	Subcylindrical basal segment	Flat but often undulate, discoid to reniform; L: 4–20 mm; W: 5–32 mm	Complete fusion of 2–3 siphons	1–2	L1 = 80–160 μm; W1 = 60–93 μm; L2 = 100–200 μm; W2 = 60–135 μm	Attached	Polygonal	7	*H. xishaensis*
R	Single tight rhizoidal mat	Erect or pendent	Subterete or compressed basal segment	Flat, broad oval to reniform; L: 2–7 mm; W: 3–10 mm	Short fusion of 2–3 siphons	2	L1 = 24–30 μm; W1 = 14–16 μm; L2 = 16–60 μm; W2 = 16–20 μm	Adhering slightly or separated	Rounded or polygonal	2	*H. velasquezii*

TABLE 1. Continued

	Holdfast type	General shape	Basal region	Segments of top half of thallus	Nodal siphons	Number of utricles layers	Cortical utricles dimensions	Utricles after decalcification	Utricles form in surface view	Number of specimens studied	Identification
S	Single tight rhizoidal mat	Erect or pendent	Single small basal segment	Flat, discoid to reniform; L: 25–31 mm; W: 40–42 mm	Complete fusion of 2–3 siphons	2, rarely 3	L1 = 130–240 μm; W1 = 96–150 μm; L2 = 117 μm; W2 = 128 μm	Attached	Polygonal	2	*H. gigas*
T	Single tight rhizoidal mat	Erect	Multiple segments larger and broader than the upper ones	Flat, cuneate, trilobed; L: 3–5 mm; W: 4–5 mm	Single unit with small pores	3	L1 = 40–70 μm; W1 = 40–56 μm; L2 = 44–92 μm; W2 = 32–52 μm	Attached	Polygonal	3	*H. melanesica*

L = length, W = width. Digits following these abbreviations indicate: 1 = primary utricle, 2 = secondary utricle.

Morphotype K presented a complete nodal fusion of pairs of siphons and could be distinguished by a spreading, sprawling or bushy thallus, multiple attachment points, numerous ramifications and long and thin branches with trilobed and cylindrical segments.

Morphotypes L, M, N, and R shown identical nodal structures with partial fusion of two or three siphons (rarely more) for a short distance. Morphotype L had an erect or pendent curtain-like thallus with a single attachment to the substrata, branching in one plane except sometimes at first segments. Morphotype R was similar to morphotype L but in surface view its peripheral utricles were narrower than those of morphotype L (14–16 μm for R vs. 14–40 μm for L). Morphotype M was characterized by a bushy and compact thallus with multiple attachment points to the substrata, small reniform segments heavily calcified sometimes ribbed and brittle. Morphotype N possessed a sprawling thallus with prostrate and erect portions, with multiple fixation points and reniform or trilobed segments of variable size and organized in perpendicular planes. It can be distinguished from morphotype M by bigger and twisted segments.

Morphotype O had a partial nodal fusion forming a single unit with very rare pores (small and not clearly visible) and 3–4 utricle layers, a polymorphous thallus, a basal part sometimes forming a pseudostalk, holdfast with matted rhizoids adapted to hard substrata. Segments were small, mostly trilobed but occasionally reniform.

DNA analyses. A total of 223 *tuf*A and 52 partial *rbc*L sequences, including data downloaded from GenBank (109 and 15, respectively), were aligned. New sequences were submitted to GenBank; accessions are listed in Table S2. The *tuf*A alignment was 857 bp, including gaps. The partial *rbc*L sequences (first 689 bp) were obtained with the primer pair *rbcL*1-*rbcL*3 (Table S1). Other primer pairs did not provide consistent results and were thus not analyzed further. NJ, MP, and ML analyses produced similar tree topologies for both markers analyzed either separately or in combination. A summary of the tree resulting from the ML analysis applied to the concatenated (*tuf*A + *rbc*L) sequence alignment is shown in Figure 2. While the backbone of the tree did not receive acceptable support, five lineages representing the five sections of Verbruggen and Kooistra (2004) were consistently recovered: *Rhipsalis* (lineage L1), *Micronesicae* (L2), *Halimeda* (L3), *Pseudo-opuntia* (L4), and *Opuntia* (L5). The *tuf*A gene, for which the biggest dataset was available (see Table S2), was analyzed separately for each section to get a more detailed picture of species boundaries within these lineages.

Lineage 1 (Rhipsalis). Morphotypes A, B, C, O, P, and T grouped within section *Rhipsalis* with GenBank sequences available for *H. heteromorpha*, *H. macroloba*, *H. kanaloana*, *H. incrassata*, *H. melanesica*,

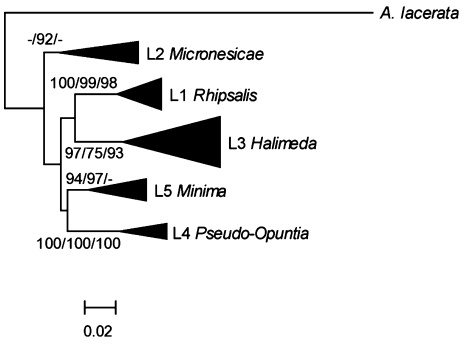

FIG. 2. Overall structure of the maximum likelihood (ML) phylogenetic tree obtained for the genus *Halimeda* based on concatened partial *tuf*A + *rbc*L sequences. Bootstrap proportion values (only BP > 70%) are indicated for ML/neighbor joining/maximum parsimony analyses. Triangles represent sequences grouping per clade. Outgroup: *Avrainvillea lacerata* (FJ432651).

H. borneensis, *H. cylindracea*, *H. monile*, and *H. simulans* (Fig. 3). The section was subdivided into five moderately to strongly supported subclades (73% $\leq$ BP $\leq$ 100%) denoted (i)–(v). The sequences of *H. borneensis* grouped together (v) but with two distinctive subgroups from French Polynesia and New Caledonia.

Sequences of each morphotype grouped together and our identifications of these morphotypes matched with the species names on the downloaded GenBank sequences. One exception is specimen (IRD 5311, see arrow on Fig. 3), which we assigned to morphotype A (*H. cylindracea*), but formed a lineage clearly separated from other species. While the sequence grouped with GenBank sequences of *H. monile*, *H. simulans*, and *H. incrassata*, the genetic distinctness of this sequence suggests that it represents a separate species. A closer morphological analysis indicated similarities with *H. stuposa*.

Lineage 2 (Micronesicae). Sequences available for morphotypes D and E grouped within section *Micronesicae* with GenBank sequences for *H. micronesica*, *H. fragilis*, *H. cryptica*, and *H. pygmaea* (Fig. 4). Morphotype E from the Maldives strongly grouped (BP > 99%, Fig. 4) with *H. micronesica*. Specimens initially ascribed to morphotype D (*H. fragilis*) appeared to be polyphyletic and grouped in three well-supported subgroups (BP > 99%, Fig. 4). One specimen from Chesterfield grouped with sequences for *H. fragilis* from Guam and the Northern Mariana Islands (noted *H. fragilis* 1), whereas the three specimens from the Maldives formed a distinct clade with other sequences for *H. fragilis* from Philippines, Tanzania, and Maldives (noted *H. fragilis* 2). Two other specimens formed a distinct lineage (*H. fragilis* 3) sister to *H. pygmaea* (Fig. 4).

Lineage 3 (Halimeda). Morphotypes G, I, Q, H, J, F, and S grouped within section *Halimeda*, which included GenBank sequences for *H. discoidea*, *H. gigas*, *H. magnidisca*, *H. xishaensis*, *H. cuneata*, *H. macrophysa*, *H. taenicola*, *H. lacunalis*, *H. tuna*, *H. hummii*, and *H. scabra* (Fig. 5). This lineage was subdivided into 10 moderately to strongly supported clades (78% < BP < 100%, Fig. 5). Overall, the morphotype assignments of our samples were consistent with the identified GenBank sequences with which the samples clustered. However, small deviations did occur. Morphotype G (*H. discoidea*) is polyphyletic, with widely divergent clades containing Indo-Pacific and Atlantic/Caribbean specimens (see also Kooistra et al. 2002, Verbruggen et al. 2005b, 2009b). Specimens in morphotype J (*H. taenicola*) formed a moderately to well-supported clade (82% $\leq$ BP $\leq$ 90%), but was subdivided into two divergent subgroups, one with individuals from French Polynesia and the Maldives, and the second with specimens from Chesterfield.

Lineage 4 (Pseudo-opuntia). Only sequences available for morphotype K (*H. gracilis*) grouped within the *Pseudo-opuntia* lineage with GenBank sequences available for *H. gracilis* and *H. lacrimosa* (Fig. 6). Morphotype K sequences formed a moderately supported clade (72% $\leq$ BP $\leq$ 81%, Fig. 6) with *H. gracilis*. As can be seen in Figure 6, *H. gracilis* is a complex consisting of at least four genetically distinct species (see also Verbruggen et al. 2009b). Our specimens from New Caledonia and Maldives all grouped in clade "*H. gracilis* 1," which is the clade also containing material from Sri Lanka, the type locality. Interestingly, the *H. gracilis* 1 lineage exhibits quite divergent subclades, hinting toward the possibility that additional cryptic diversity is present.

Lineage 5 (Opuntia). Sequences available for morphotypes L, M, N, and R grouped within the *Opuntia* lineage with GenBank sequences for *H. renschii*, *H. minima*, *H. distorta*, *H. opuntia*, *H. velasquezii*, *H. copiosa*, and *H. goreauii* (Fig. 7). As anticipated on morphological grounds, morphotype M grouped with sequences of *H. opuntia* (75% $\leq$ BP $\leq$ 97%, Fig. 7). *H. distorta* appeared polyphyletic with two clades noted *H. distorta* 1 and *H. distorta* 2 weakly to strongly supported (BP = 94% and BP = 76%, respectively). Sequences obtained for morphotype N grouped within the subgroup *H. distorta* 2. Morphotype R grouped strongly with *H. velasquezii* sequences (BP = 93%, Fig. 6). Sequences for *H. minima* and *H. renschii* formed a large clade (BP > 89%, Fig. 6) including a subgroup for *H. renschii* (BP = 78%).

Morphotype L was found in the *H. minima*/*H. renschii* group within two subclades: sequences from the Maldives grouped moderately with *H. minima* 1 from Australia and Tanzania (BP = 72%) and sequences from New Caledonia grouped strongly with *H. minima* 4 from the Philippines and Fiji. Three unidentified samples from Northern Mariana Islands and Fiji (*H. no ID*) were strongly grouped together (BP = 100%) and distinctive from other species.

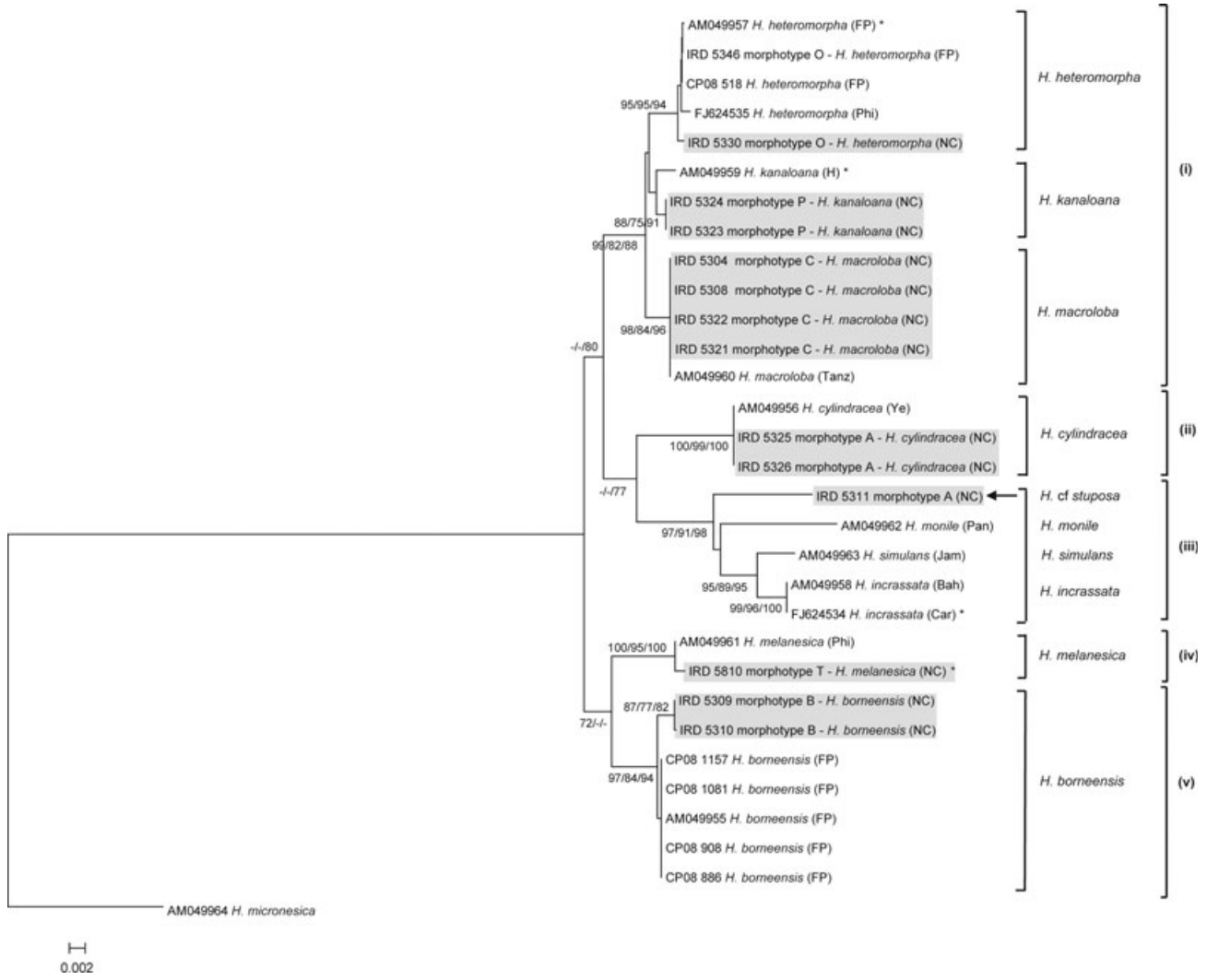

FIG. 3. Neighbor joining (NJ) analysis for section *Rhipsalis* (L1) based on *tuf*A sequence alignment. Bootstrap values (BP > 70%) are indicated for NJ/maximum parsimony/maximum likelihood analyses. Outgroup: *Halimeda micronesica*. Localities are noted as follows, FP: French Polynesia; Phi: Philippines; NC: New Caledonia; Tanz: Tanzania; H: Hawaii; Ye: Yemen; Pan: Panama; Jam: Jamaica; Bah: Bahamas; and Car: Caribbean. Asterisks indicate type localities and the arrow points to an unexpected grouping and boxes highlight New Caledonian samples.

DISCUSSION

Our primary aim was to characterize the species diversity of the genus *Halimeda* in New Caledonia using a combination of molecular tools and morphological investigations. Besides addressing this goal, our results have implications for our knowledge of the taxonomy and global diversity of the genus and warrant reflecting upon the suitability of conventional morphological identification criteria and methods for delimiting species in the genus.

Approaches toward species delimitation. Species delimitation is one of the principal goals of systematic biology. Classically, species were delimited based on morphological differences between perceived entities. Since the early 1990s, DNA sequencing has made it increasingly clear that species boundaries based on perceived morphological differences have not always reflected biological reality. They have shown that morphologically defined entities can encompass various genetic species (cryptic diversity) and that DNA clusters at the species level can be morphologically diverse (e.g., Zuccarello and West 2003, Leliaert et al. 2009). As a consequence of the morphological plasticity of algae and the fact that morphological species delimitation can be deceptive, DNA sequences are very useful complements to morphological data in defining species boundaries.

In *Halimeda*, various approaches have been taken toward species delimitation. All work predating the DNA sequencing era has used external morphological and anatomical features to define species boundaries. More recently, Verbruggen et al. (2005a, 2006) started by defining species boundaries based

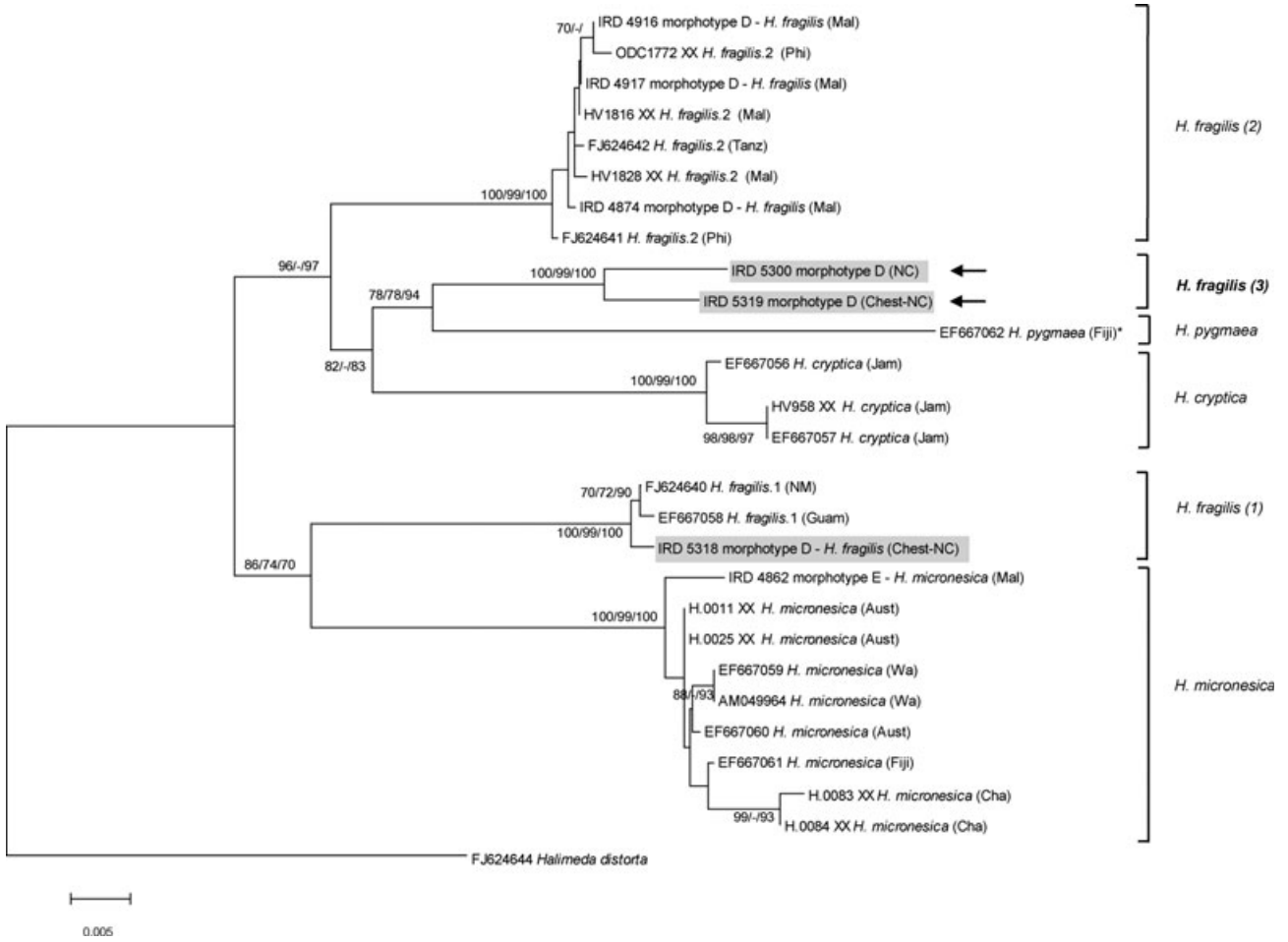

FIG. 4. Neighbor joining (NJ) analysis of section *Micronesicae* (L2) based on *tufA* sequence alignment. Bootstrap values (BP > 70%) are indicated for NJ/maximum parsimony/maximum likelihood analyses. Outgroup: *Halimeda distorta*. Localities are noted as follows, Mal: Maldives; Phi: Philippines; Tanz: Tanzania; NC: Grande terre, New Caledonia; Chest-NC: Chesterfield, New Caledonia; Jam: Jamaica; NM: North Marianna; Aust: Australia; Wa: Wallis; and Cha: Chagos. Asterisks indicate type localities, arrows point at unexpected groupings and boxes highlight New Caledonian samples.

on sets of DNA barcodes and subsequently looked for morphological characters coinciding with the DNA-based species boundaries. In this study, we tried an intermediate approach. We defined morphotypes based on analyses of our external morphological and anatomical datasets and subsequently sequenced the samples and verified whether the morphotypes corresponded to DNA clusters. Although this was true in most cases, we were unable to capture the full species diversity in our morphotypes—some of them showed cryptic diversity at the DNA level.

The recognition of species-level clades in this study followed previous work on *Halimeda*, in which species limits are defined manually by inspecting haplotype trees to identify well-supported clades that are preceded by a relatively long branch and have comparably low sequence diversity within. We acknowledge that this method is to some degree subjective, in that different workers may disagree on what constitutes a "well-supported clade preceded by a relatively long branch and comparably low sequence diversity within." This is where the taxonomist's judgment comes in, which is based on the joint interpretation of the haplotype tree and the morphological information. In recent years, more objective methods have been developed to pinpoint species boundaries in DNA sequence datasets (Pons et al. 2006, Leliaert et al. 2009, Monaghan et al. 2009). These methods, and the general mixed Yule-coalescence approach in particular, correspond well to the method we have used here. It relies on differences between slower above-species tree branching (speciation model) and faster within-species tree branching (haplotype coalescence), which is what is more intuitively assessed with the method we use.

Over the years, several DNA markers have been used for questions near the species level in *Halimeda*. The early work by Kooistra used 18S nrDNA sequences (Hillis et al. 1998, Kooistra et al. 1999), which turned out to be too conservative for fine-grained work and were replaced with the ITS region

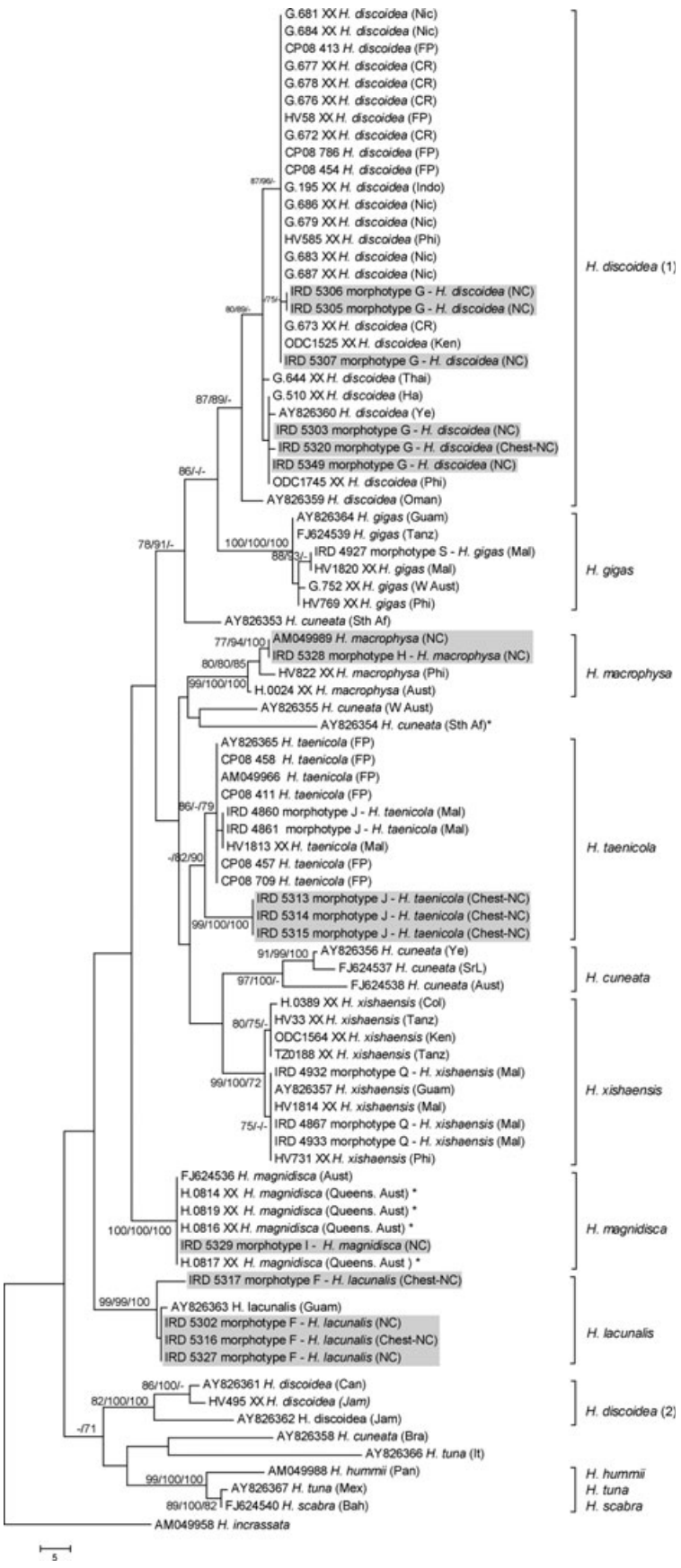

FIG. 5. Maximum parsimony (MP) analysis of section *Halimeda* (L3) based on *tufA* sequence alignment. Bootstrap values (BP > 70%) are indicated for MP/neighbor joining/maximum likelihood analyses. Outgroup: *Halimeda incrassata*. Localities are noted as follows, Nic: Nicaragua; FP: French Polynesia; Mal: Maldives; Chest-NC: Chesterfield; New Caledonia; NC: New Caledonia; Ye: Yemen; SrL: Sri Lanka; Aust W: Western Australia; Sth Af: South Africa; Tanz: Tanzania; Bre: Brasil; It: Italia; Can: Canaries; Jam: Jamaica; Pan: Panama; Mex: Mexico; Bah: Bahamas; Thai: Thailand; Phi: Philippines; and CR: Costa Rica. Asterisks indicate type localities and boxes highlight New Caledonian samples.

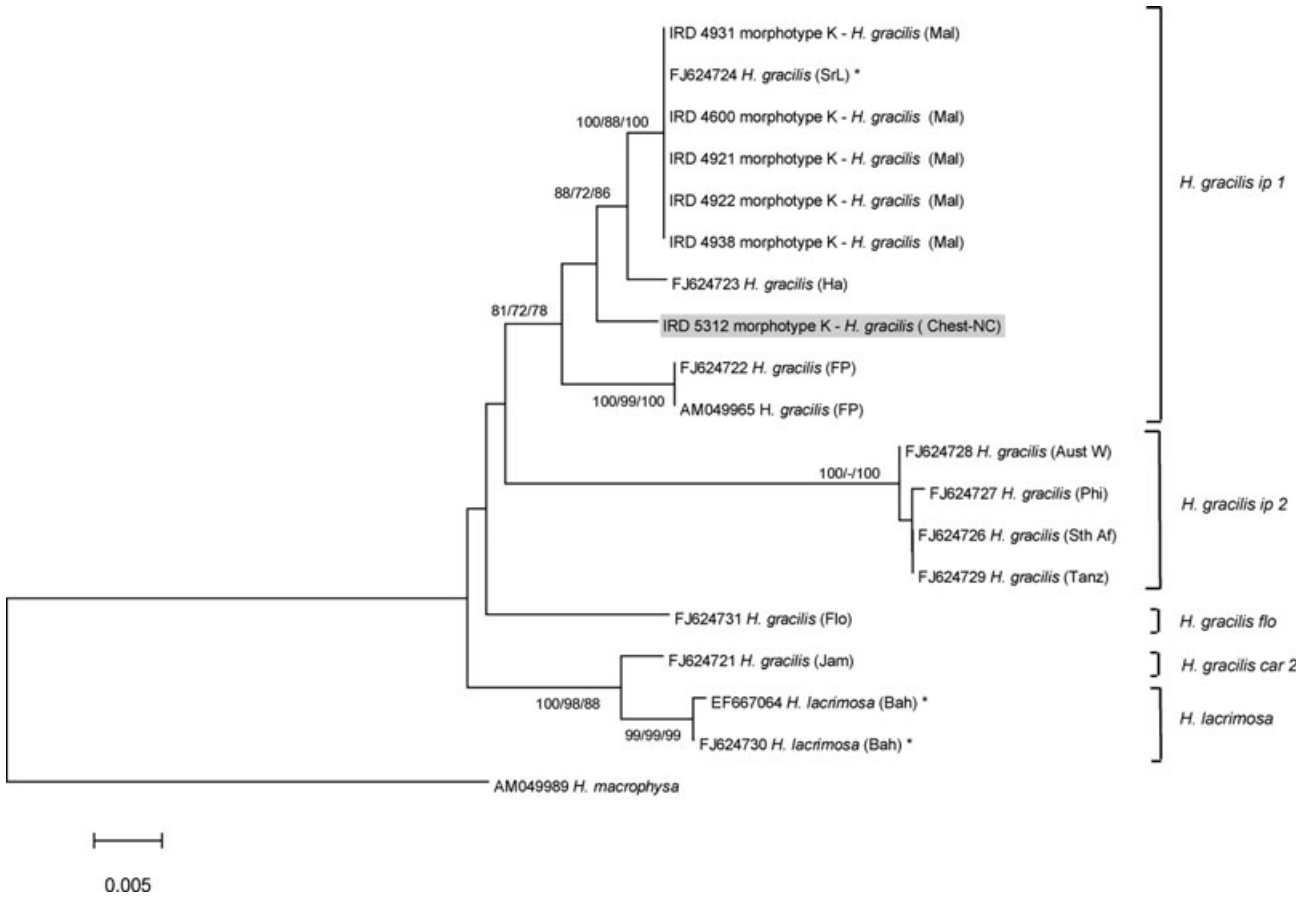

FIG. 6. Neighbor joining (NJ) analysis of section *Pseudo-opuntia* (L4) based on *tufA* sequence alignments. Bootstrap values (BP > 70%) are indicated for NJ/maximum parsimony/maximum likelihood analyses. Outgroup: *Halimeda macrophysa*. Localities are noted as follows: Mal: Maldives; SrL: Sri Lanka; Ha: Hawaii; Chest-NC: Chesterfield, New Caledonia; PF: French Polynesia; Aust W: West Australia; Phi: Philippines; Sth Af: South Africa; Tanz: Tanzania; Flo: Florida; Jam: Jamaica; and Bah: Bahamas. Asterisks indicate type localities and the box highlight New Caledonian sample.

(Kooistra et al. 2002, Verbruggen et al. 2005a,b, 2006). Slightly later, the plastid marker UCP7 (Provan et al. 2004), comprising partial *rps*19 and *rps*3 sequences and the spacer between them, was introduced for species delimitation in sections *Rhipsalis* and *Halimeda* (Verbruggen et al. 2005a,b, 2006, 2009b). Recently, the plastid *tuf*A gene was proposed as the DNA barcode of choice for the Ulvophyceae (Saunders and Kucera 2010). This marker had already been used for phylogenetic purposes in the genus *Halimeda* (Verbruggen et al. 2005a,b,c,d, 2006, 2007, 2009b) and for species delimitation in two of its sections (*Pseudo-opuntia* and *Opuntia*: Verbruggen et al. 2009b). In this study, the use of the *tuf*A barcode is extended to all sections of the genus. It clearly follows from our results that the gene has potential as a barcoding marker as it shows enough resolution to distinguish between species-level clades, shows the presence of cryptic diversity within morphotypes (e.g., *H. fragilis*), and indicates the presence of new species and/or species not currently present in the DNA databases (e.g., *H. cf. stuposa*, *H. no ID*).

Morphological identification criteria. Among the nine morphological characters analyzed in this study, many appeared to show overlap between species. For example, it can be difficult to discriminate between *H. discoidea,* and *H. xishaensis* using only thallus and segment form. However, the same two characters allow perfect discrimination between other species (e.g., *H. cylindracea* vs. *H. macroloba*). Bulbous holdfasts were found to occur in sections *Rhipsalis* (L1; except for *H. melanesica*) as well as *Halimeda* (L3), which was interpreted by Verbruggen and Kooistra (2004) to be a response to a specific adaptation to growth in sandy or muddy substrata. Verbruggen et al. (2005d) noted that some deviant segments should not be included in morphological analyses because they can mislead species assignment. Following their conclusion that basal, apical, and noncalcified segments may not be representative of the average morphology of a species, these segment types were excluded from this study. It is clear that external morphology may be used for a primary classification of individuals into morphological groups, but this enables species identification

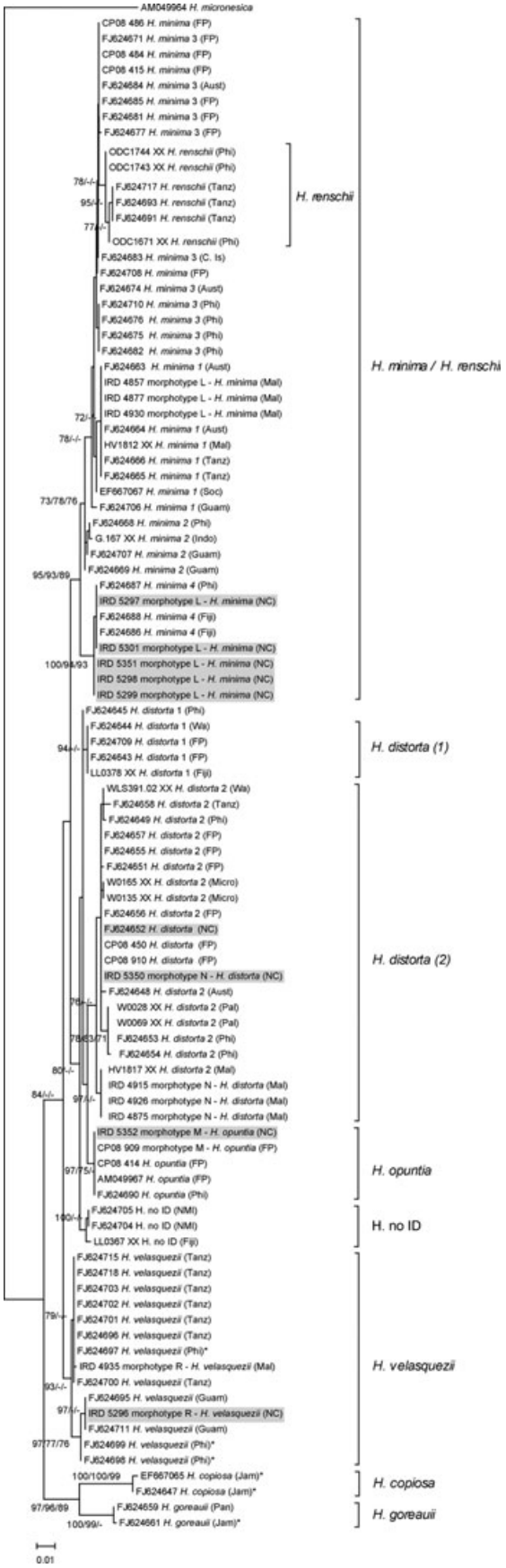

FIG. 7. Maximum likelihood (ML) analysis of section *Opuntia* (L5) based on *tufA* sequence alignments. Bootstrap values (BP > 70%) are indicated for ML/neighbor joining/maximum parsimony analyses. Outgroup: *Halimeda micronesica*. Localities are noted as follows, FP: French Polynesia; Aust: Australia; Phi: Philippines; Tanz: Tanzania; C. Isl: Cook Islands; Mal: Maldives; Soc: Socotra; NC: New Caledonia; Wa: Wallis; Micro: Micronesia; Pal: Palau; NMI: North Marianna Island; Jam: Jamaica; and Pan: Panama. Asterisks indicate type localities and boxes highlight New Caledonian samples.

only in rare cases. Anatomical characters appear more reliable to identify species. Features like the nodal fusion type and the size and form of utricles show more variation among species (Verbruggen et al. 2005a), and therefore represent the main characters for morphological identification. However, these features show overlap between species, too. A combination of external and anatomical features obviously extends the range of distinctions that can be made and is the preferred strategy for morphological discrimination between species. Even though a well-contemplated combination of external morphological and anatomical characters can provide a powerful source of information for the identification of a majority of *Halimeda* species, it is certainly possible that they will not permit distinction between the most similar of cryptic species pairs.

In this study, which defined morphotypes prior to DNA analyses, morphological and anatomical identifications were consistent with molecular results in a majority of cases, except for morphotypes A, D, and L, which were present in multiple genetic clusters. A hybrid identification approach based on morpho-anatomical features for more distinctive species and DNA tools for the cryptic species complexes may thus be reliable, although an inherent risk of such an approach is that it may leave cryptic diversity within morphologically distinctive taxa undetected.

Taxonomical implications. As mentioned above, there was generally a clear distinction between sequence clusters at the species level and 14 of 20 morphotypes grouped unambiguously with sequences previously identified to the same species: morphotype B = *H. borneensis*, C = *H. macroloba*, E = *H. micronesica*, F = *H. lacunalis*, H = *H. macrophysa*, I = *H. magnidisca*, J = *H. taenicola*, M = *H. opuntia*, O = *H. heteromorpha*, P = *H. kanaloana*, Q = *H. xishaensis*, R = *H. velasquezii*, S = *H. gigas*, and T = *H. melanesica*. Clearly, these species can be incorporated unambiguously in the list of New Caledonian *Halimeda* species.

Conversely, for 6 of the 20 morphotypes, the situation was more complex due to cryptic or pseudocryptic diversity. We distinguish between two different scenarios. The first is that within New Caledonia, specimens from one morphotype belong to different cryptic entities. This is the case for morphotypes A = *H. cylindracea* (2 entities) and

D = *H. fragilis* (2 entities). The second scenario is that the morphospecies in question is known to contain cryptic diversity, but the New Caledonian samples belong to only one of the constituent cryptic entities. This is the case for morphotypes G = *H. discoidea* (two entities), K = *H. gracilis* (four entities), L = *H. minima* (four entities), and N = *H. distorta* (two entities). We will now discuss each of these six problematic cases and their taxonomic consequences in more detail.

Morphotype A, which was thought to correspond to *H. cylindracea*, appeared polyphyletic in our phylogenetic analysis. As could be expected the majority of the samples of this morphotype matched Genbank sequences belonging to *H. cylindracea*. However, a single sequence attributed to morphotype A (IRD 5311) formed a separate lineage related to *H. incrassata*, *H. simulans*, and *H. monile* in subgroup (iii; Fig. 3). A closer morphological analysis demonstrated similarities of this specimen to *H. stuposa*. First and considering the identification key to *Halimeda* species of section *Rhipsalis* (Verbruggen et al. 2005a), some details can help us to differentiate the specimen from *H. cylindracea*: it has a basal zone (basal segments up to the holdfast and underneath the first ramifications) of 1.6 cm long, segments thicker than *H. cylindracea* (in average, 1.5–2 mm vs. <1.5 mm for *H. cylindracea*) and its segments are not cylindrical throughout the thallus but most of them were trilobed. Secondly, it possessed segments always <12.5 mm wide (most in the 2–6 mm range), and its peripheral utricles averaged 35.8 μm in width and 58.8 μm in length, whereas the width of its secondary utricles equaled 28 μm in width with supranodal siphons shorter than 300 μm (208 μm). In fact, following Verbruggen et al. (2005a), our specimen presented all the criteria to be identified as *H. stuposa*, but since no DNA sequence data are available for confirmed samples of *H. stuposa* for verification, we converged on "*H.* cf. *stuposa*" as the identification for this clade.

Morphotype D (Lineage 2), identified as *H. fragilis*, showed clear evidence for cryptic diversity. The New Caledonian samples were only present in two of these (*H. fragilis* 1 and *H. fragilis* 3), but our samples from the Maldives, Tanzania, and the Philippines formed a third cluster (*H. fragilis* 2). Closer examination did not reveal obvious morphological differences between the clusters.

The morphospecies *H. discoidea* (lineage 3) appeared polyphyletic with two distinct and unrelated clusters. The first (*H. discoidea* 1) contained all sequences from Indo-Pacific material, including all our New Caledonian samples belonging to morphotype G. The second cluster comprised all sequences from Atlantic specimens (*H. discoidea* 2). The genetic distinctness of Atlantic and Indo-Pacific specimens belonging to the morphospecies has been discussed in detail in the literature (Kooistra et al. 1999, Verbruggen et al. 2005b, 2009b).

The polyphyly of *H. gracilis* initially noted by Kooistra et al. (2002) has later been confirmed with better taxon sampling (Verbruggen et al. 2009b, this study). It is now clear that this morphospecies is a complex of four species (Fig. 6), two from the Indo-Pacific region (*H. gracilis* ip 1 & ip 2), and two from the Atlantic Ocean (*H. gracilis* flo & car 2). Our specimens from New Caledonia and the Maldives (morphotype K) all grouped within *H. gracilis* ip 1, which includes the specimen from the type locality and can safely be considered as the true *H. gracilis*. The other groups, which are not the focus of this study as they do not occur in New Caledonia, should be subjected to more intensive morphological investigation in an attempt to identify diagnostic characters.

Our molecular data resolved *Halimeda minima* into four clusters, one of which (*H. minima* 4) is present in New Caledonia. The polyphyly of this species was initially noted by Kooistra et al. (2002), who distinguished three distinct groups, while Verbruggen et al. (2009b) also recognized the fourth cluster noted here. The new data presented here show that while two of the four clusters are clearly distinct (*H. minima* 2 and *H. minima* 4), sequences of *H. minima* 1, *H. minima* 3, and *H. renschii* are very closely related and do not always form clear-cut clusters, indicating that the taxonomy of the species *H. minima* and *H. renschii* needs to be reconsidered. The principal morphological criterion used to separate *H. minima* from *H. renschii* is that the holdfast region of the latter consists of a region at least 1 cm high in which the basal segments are covered in rhizoids and sand particles of several segments, whereas the former has a simple holdfast at the basal segment (Hillis-Colinvaux 1980). However, it is known that holdfasts can show within-species variation depending on habitat (Verbruggen et al. 2006). As the *H. renschii*—*minima* 1—*minima* 3 complex does not appear to be present in New Caledonia, we will not treat this any further, but this group needs a more critical evaluation.

Our analyses resolved *H. distorta* in two groups (cf. Verbruggen et al. 2009b). Although the cluster noted *H. distorta* 1 appears to be restricted to the Pacific, *H. distorta* 2 grouped specimens from the wider Indo-Pacific area, including our specimens from New Caledonia (morphotype N). Kooistra and Verbruggen (2005) merged *H. hederacea* with *H. distorta* based on the lack of separation of these taxa in ITS nrDNA sequences, and it is not clear how the two clusters we find within *H. distorta* relate to these older names. Additional work is clearly needed to sort out the taxonomy of this complex.

We wish to make a final taxonomic note about *H. taenicola*. Even though this species is recovered as monophyletic in our study, it is composed of two distinct clusters one of which has samples only from the Chesterfield reef area. We have not been able to find marked anatomical or morphological

differences between these two subgroups, although our specimens from the Maldives did look bushier than specimens from Chesterfield. However, this character may not be valuable to discriminate the two subgroups because the analysis is based on few specimens and the general shape of the thallus may be strongly influenced by the environment.

Diversity of Halimeda in New Caledonia. This study identified 22 species of *Halimeda* in New Caledonia, representing all five sections of the genus. Eighteen species were confirmed using a combination of DNA analyses and morphological investigation, that is, *H. borneensis*, *H. cylindracea*, *H. discoidea*, *H. distorta*, *H. fragilis* 1, *H. fragilis* 3, *H. gracilis*, *H. heteromorpha*, *H. kanaloana*, *H. lacunalis*, *H. macroloba*, *H. macrophysa*, *H. magnidisca*, *H. minima*, *H.melanesica*, *H. opuntia*, *H. taenicola*, and *H. velasquezii*. Some morphospecies were shown to harbor cryptic diversity. In one case, two cryptic species were present in New Caledonia (*H. fragilis* 1 and *H. fragilis* 3). In four other cases, the species in question was shown to contain several cryptic species but only one of these cryptic species was present in New Caledonia. This was the case for *H. discoidea* (only the Indo-Pacific entity in New Caledonia), *H. gracilis* (only entity no. 1 in New Caledonia), *H. minima* (only entity no. 4 in New Caledonia), and *H. distorta* (only entity no. 2 in New Caledonia).

Three other species were identified based on morphological criteria only: *H. micronesica*, *H. xishaensis*, and *H. gigas*. Regrettably, we were unable to generate DNA data for these species. However, because these species represent clear-cut morphotypes that have been shown to form distinct DNA clusters (Verbruggen et al. 2005b,c, this study) with no reports of cryptic diversity, a high degree of confidence can be attached to these records.

We also identified a DNA cluster (*H. cf stuposa*) that forms a distinct lineage in molecular analyses and thus represents a proper species. Although the specimen in question bears resemblance to *H. stuposa*, this identification is not certain as the morphology does not match exactly and no sequences of typical *H. stuposa* samples are available.

The report of the species *H. kanaloana* for New Caledonia represents a major extension of the known distribution range of this species, as this species was previously known only from Hawaii and possibly Japan (Ryu Kyu Islands). The New Caledonian specimens had previously been identified as *H. incrassata* (Garrigue 1985), which is morphologically close to *H. kanaloana* in various aspects, but can be distinguished on the basis of six features including the dimensions of subperipheral and peripheral utricles, and the distance between the base of the nodal fusions and the first siphon ramification above the node (Verbruggen et al. 2006). They also differ in their distribution: *H. incrassata* is restricted to the Atlantic Ocean while *H. kanaloana* is a Pacific Ocean species (Verbruggen et al. 2006, this study). Similarly, the recognition of *H. xishaensis* in New Caledonia marks a major extension of the known range of this species because, to our knowledge, it was previously only recorded from the Xi Sha or Paracel Islands in the South China Sea (Dong and Tseng 1980). Finally, we obtained here the first sequence of *H. melanesica* from its type locality (Lifou, New Caledonia), which will facilitate accurate molecular identification of specimens of this species from other parts of the world.

We acknowledge the IRD divers team and boat pilots for their contribution to this work through kind and careful assistance. Thanks also to all collectors who have contributed to the study: G. Lasne (BIOCENOSE), E. Fontan, O. Cornubert, and D. Ponton. Thanks to C. Fauvelot for providing samples from French Polynesia and Antoine de N'Yeurt for help with identification of specimens collected in Moorea. Thanks to Coppejans E., Dargent O., Tyberghein L., Leliaert F., Diaz R., Galanza C., Huisman J., De Clerck O., Schils T., Payo D. A., and Pauly K. for collecting Ghent samples. We sincerely thank C. Majorel (Plateforme du vivant) and S. D'hondt (Ghent University) for assisting with the molecular work. HV is a postdoctoral fellow of the Research Foundation—Flanders. LD is a PhD fellow of the Province Sud- New Caledonia.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web site:

Table S1. Oligonucleotide primers used for amplification and sequencing with detail of sequences, annealing temperature, and previous publication.

Table S2. List of *Halimeda* specimens and sequences used in this study.