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RHIPILIA COPPEJANSII, A NEW CORAL REEF-ASSOCIATED SPECIES FROM GUAM (BRYOPSIDALES, CHLOROPHYTA)[1]

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The new species *Rhipilia coppejansii* is described from Guam. This species, which has the external appearance of a *Chlorodesmis* species, features tenacula upon microscopical examination, a diagnostic character of *Rhipilia*. This unique morphology, along with the *tuf*A and *rbc*L data presented herein, set this species apart from others in the respective genera. Phylogenetic analyses show that the taxon is nested within the Rhipiliaceae. We discuss the diversity and possible adaptation of morphological types in the Udoteaceae and Rhipiliaceae.

Key index words: **Bryopsidales; *Chlorodesmis*; DNA barcodes; morphology; *rbc*L; *Rhipilia*; taxonomy; *tuf*A**

The Bryopsidales is an order of green seaweeds primarily found in tropical marine ecosystems. Its representatives are acellular, that is, they lack cross-walls and construct complex thalli from a single giant tubular cell (Vroom and Smith 2003b, Cocquyt et al. 2010). In most species, this cell is branched and, depending on the arrangement of the branches, species can have various levels of anatomical complexity. The tubular branches are called siphons, and the Bryopsidales are commonly known as siphonous algae. Despite their bizarre anatomy, siphonous algae are among the most common and ecologically dominant groups of seaweeds found in tropical marine habitats (Hillis-Colinvaux 1986, Vroom and Smith 2003a). Besides the calcified, reef-forming genera *Halimeda* and *Udotea*, they also comprise several noncalcified taxa, some of which are also dominant (e.g., *Caulerpa*), but the majority of which are less conspicuous inhabitants of the reef slope. The focus of this study will be on the Udoteaceae and the Rhipiliaceae, two related bryopsidalean families (Verbruggen et al. 2009a).

The species of the Udoteaceae cover a wide spectrum of morphologies and the great majority of them are calcified. Members of the genus *Udotea* have multiaxial stipes and fan- or funnel-shaped blades (Littler and Littler 1990b). *Rhipidosiphon* is structurally similar, but has a much simpler uniaxial stipe and a single-layered blade (Littler and Littler 1990a, Coppejans et al. 2011). *Penicillus* and *Rhipocephalus* both consist of a stipe subtending a cap. Whereas, in *Penicillus*, the cap has a brush-like structure, that of *Rhipocephalus* consists of numerous imbricated blades along a central stalk (Littler and Littler 2000). In addition to these rather complex thallus architectures, the Udoteaceae also contain the genus *Chlorodesmis*. As its name suggests (chloro = green, desma = bundle), individuals consist of a simple tuft of branched green siphons. This feature, as well as the fact that the genus is noncalcified, sets *Chlorodesmis* apart from the more complex genera. Many genera of the Udoteaceae, including *Chlorodesmis*, are abundant in shallow coral reefs and lagoons (Littler and Littler 2000).

The Rhipiliaceae are much less conspicuous and abundant. They consist of three genera of noncalcified taxa that have rather small thalli, and mainly occur in deeper parts of the coral reef slope. *Rhipilia* species generally form bladelets that consists of a fan of longitudinally oriented siphons interconnected by small side branches that form tenacula and adhere to adjacent siphons, creating a meshwork that forms the blade (Millar and Kraft 2001). *Rhipiliopsis* species also form bladelets, but adjacent siphons adhere to one another by means of lateral adhesion papillae instead of side branches (Kraft 1986). *Rhipiliella* is morphologically similar to *Rhipiliopsis*, but has deciduous blades along a stalk (Kraft 1986).

During recent collection campaigns in the Piti bomb holes reef in Guam (western Pacific), an entity with a fascinating morphology was discovered. In its general appearance and ecology, it resembles the udoteacean genus *Chlorodesmis*, as it forms green tufts at the base of coral colonies. However, upon microscopical examination, tenacula reminiscent of the Rhipiliaceae were observed in the plants. The

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goal of this study was to provide a detailed morphological description of this entity, and evaluate its taxonomic status and phylogenetic affinities with DNA sequence analyses.

MATERIAL AND METHODS

Morphological examination. Freshly collected specimens were observed using Nikon AZ-100 and 80i microscopes and images were taken using Nikon DS-Fi1 digital cameras. Series of images were stacked to achieve sharpness across the object plane. Crystals were examined using differential interference contrast microscopy.

Molecular phylogenetics. DNA was extracted from three specimens of the entity in question (GH0003055 = PITI044, GH0011082 = G.451, GH0011084 = G.453) as well as two samples of *Rhipilia pusilla* (Womersley) Ducker from South Australia (A88532, G.091), two samples of *R. orientalis* A. Gepp, and E.S. Gepp from Australia (AD-A88500, AD-A88388), two samples of *R. nigrescens* Coppejans and Prud'homme van Reine from Australia (H.0847, H.0864), and a sample of *Chlorodesmis fastigiata* (C. Agardh) Ducker from Guam (GH0003035 = IPAN0014). A complete list of species authorities used in this article can be found in Table 1. The plastid genes *tuf*A and *rbc*L were amplified and sequenced following previously described procedures (Verbruggen et al. 2009a), and the new sequences were submitted to Genbank JQ082480–JQ082497. After preliminary analyses had shown the various sequences of the Guam entity to cluster closely together, a single *tuf*A sequence and a single *rbc*L sequence were retained to represent the entity in further analyses. The other species were also represented by a single sequence per species. The sequences were then added to the alignments of a previously published dataset (Verbruggen et al. 2009b). The *tuf*A and *rbc*L alignments were analyzed separately and collectively using maximum likelihood (ML) and Bayesian inference (BI). The Bayesian Information Criterion was used to select a suitable partitioning strategy and model of sequence evolution. This procedure was carried out using Partitioned Model Tester 1.01 (Verbruggen 2010), and resulted in a three-partition strategy (first, second, and third codon positions across genes) with uncoupled GTR+Γ_4 models for each partition. ML trees were inferred from 100 randomized MP trees using RAxML 7.2.6 (Stamatakis 2006), and confidence was assessed using 500 bootstrap replicates. Bayesian inference used MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003). Two independent runs, each consisting of four incrementally heated chains and using default priors were run for 2 million generations. Convergence was assessed and a suitable burn-in of 0.2 million generations determined using Tracer (Rambaut and Drummond 2009). All alignments and the files used for analysis are available from the first author's website (http://www.phycoweb.net) and TreeBase (http://www.treebase.org).

TABLE 1. Author names of species cited in text, following Guiry and Guiry (2011).

Species	Author
Chlorodesmis baculifera	(J. Agardh) Ducker
Chlorodesmis caespitosa	J. Agardh
Chlorodesmis dotyi	Trono
Chlorodesmis fastigiata	(C. Agardh) Ducker
Chlorodesmis haterumana	Tanaka and Itono
Chlorodesmis hildebrandtii	A. Gepp and E.S. Gepp
Chlorodesmis papenfussii	Ducker
Chlorodesmis pusilla[a]	Womersley
Chlorodesmis sinensis	C.K. Tseng and M.L. Dong
Pseudochlorodesmis abbreviata	(Gilbert) Abbott and Huisman
Pseudochlorodesmis furcellata	(Zanardini) Børgesen
Rhipidosiphon javensis	Montagne
Rhipilia coppejansii	Schils and Verbruggen
Rhipilia fungiformis	A.B. Joly and Ugadim
Rhipilia geppii	W.R. Taylor
Rhipilia nigrescens	Coppejans and Prud'homme van Reine
Rhipilia orientalis	A. Gepp and E.S. Gepp
Rhipilia penicilloides	A.D.R. N'Yeurt and D.W. Keats
Rhipilia pusilla	(Womersley) Ducker
Rhipilia tomentosa	Kützing

[a] Now known as *Rhipilia pusilla* (Womersley) Ducker.

RESULTS AND DISCUSSION

Morphological observations. The dark green thallus of the entity from Guam consists of densely and irregularly aggregated siphons growing in tufts and reaches up to 8 cm in height (Fig. 1). Thalli do not have stipes and were attached to limestone substrates by means of rhizoids (5–22 μm diam.) that develop proximally from the siphons (Fig. 2) and contain chloroplasts. Annular constrictions have not been observed at the proximal end of the siphons, but slight irregular inflations can be observed in a region where a multitude of rhizoids are initiated.

Siphons are loose, but sparsely interconnected using tenacula. Four types of tenacular attachments were observed within a single thallus: (i) hook-shaped tenacula (Fig. 3A), (ii) tapering and bent siphon tips (Fig. 3B), (iii) discoid tenacula (Fig. 3C), and (iv) tenacula with 2–4 fingers (prongs, Fig. 3D–H). Tenacula have been observed on primary siphons and lateral branches. Siphons are cylindrical and measure 50–105 μm in diameter (78 ± 13 μm). Branching is variable (Fig. 4A), with irregular branching (lateral branches; Fig. 4B) as well as dichotomous ramifications (Fig. 4C), and trifurcations (Fig. 2A) occurring in the same thallus. The distance between successive ramifications varies from a few μm to over a cm. Siphons are inflated just below the ramifications (90–130 μm diam.), and strongly constricted above (18–36 μm in diam. Fig. 4C). The constrictions lack annulations of cell-wall thickenings (Fig. 5A). Siphon constrictions that were not associated with any type of branching were occasionally observed in the studied specimens. Crystals corresponding to type 1b crystalline cell inclusions as defined by Leliaert and Coppejans (2004) were found. In contrast to the broad hexagonal and diamond shaped type 1b crystals depicted by Leliaert and Coppejans (2004), *R. coppejansii* has prism-shaped crystals with a regular hexagonal base (Fig. 5B, arrowheads). The Udoteaceae and Rhipiliaceae families are heteroplastic, and both chloroplasts and amyloplasts were seen in the investigated specimens. Amyloplasts (4–7 μm diam.) were especially abundant in the region surrounding the constrictions (Fig. 5C). Chloroplasts were numerous

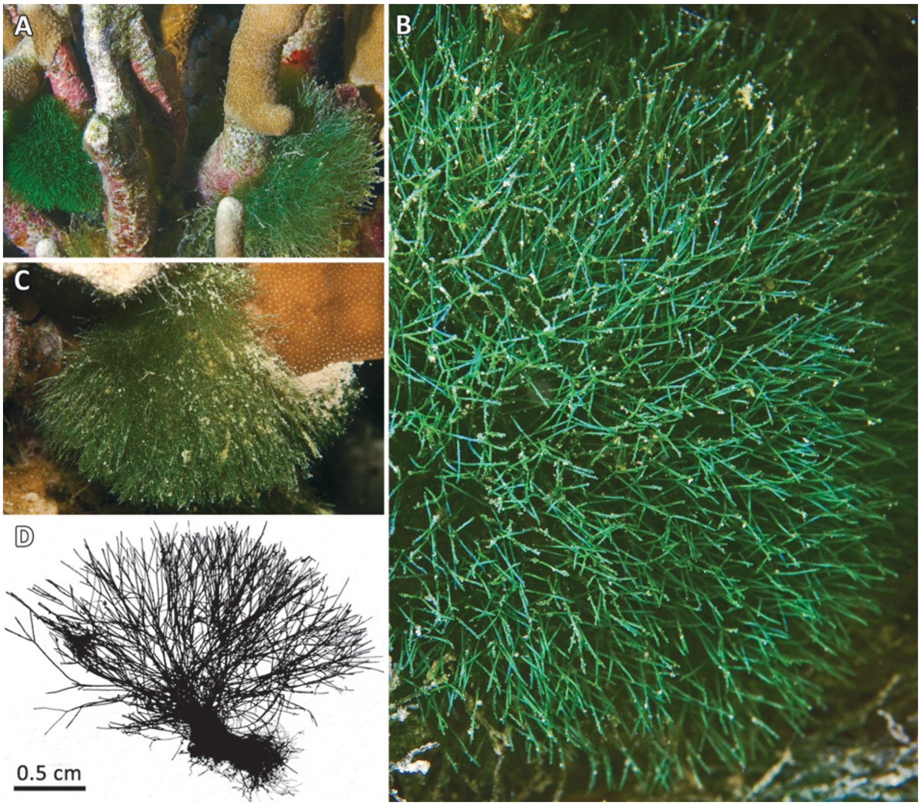

FIG. 1. Habit of *Rhipilia coppejansii*. (A) Upright growth of *R. coppejansii* in between branches of the coral *Porites cylindrica*. (B) *In situ* close-up of the alga, showing the predominant dichotomous branching pattern. (C) *R. coppejansii* hanging underneath plates of the coral *Porites rus*. (D) Complete thallus displaying the rhizoidal mass from which the upright siphons develop.

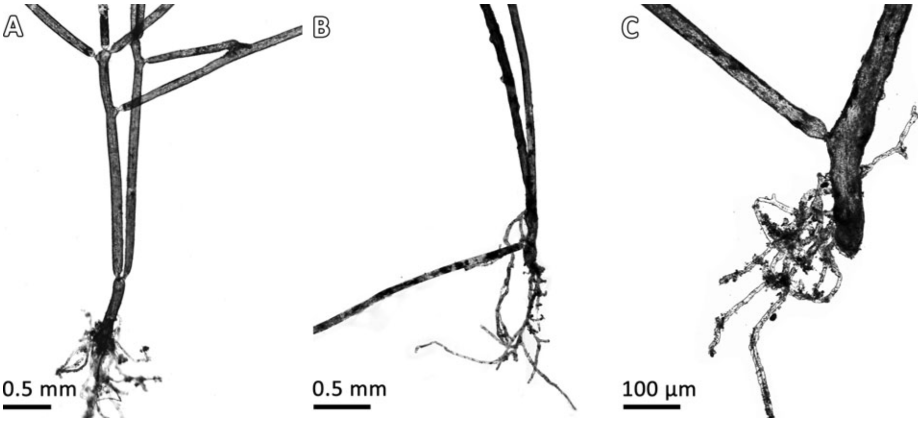

FIG. 2. Basal part of *Rhipilia coppejansii* showing rhizoids.

throughout the thallus (Fig. 5D), and two shapes were discerned: whereas parietal chloroplasts were generally subspherical in shape (1.5–4 µm diam., Fig. 5F), central chloroplasts were elongate-ovoid-to-spindle-shaped (2–6 µm long, Fig. 5E) and moved throughout the thallus via cytoplasmic streaming.

DNA observations. Molecular phylogenetic analysis of the final DNA sequence alignment, which consisted of 39 taxa and 2,175 characters (*rbc*L = 1320, *tuf*A = 855), yielded the ML tree in Figure 6A. The entity from Guam, indicated in the black box, is firmly recovered in the Rhipiliaceae, nested in a strongly supported clade with *R. tomentosa* and *R. nigrescens*. As was shown in previous studies (Verbruggen et al. 2009a,b), the genus *Rhipilia* is nonmonophyletic, with *Rhipiliopsis profunda* and *Pseudochlorodesmis* strain HV1204 branching from within a group of *Rhipilia* species. The two *Chlorodesmis* strains included in the analysis were recovered in the Udoteaceae (gray box). The overall structure of

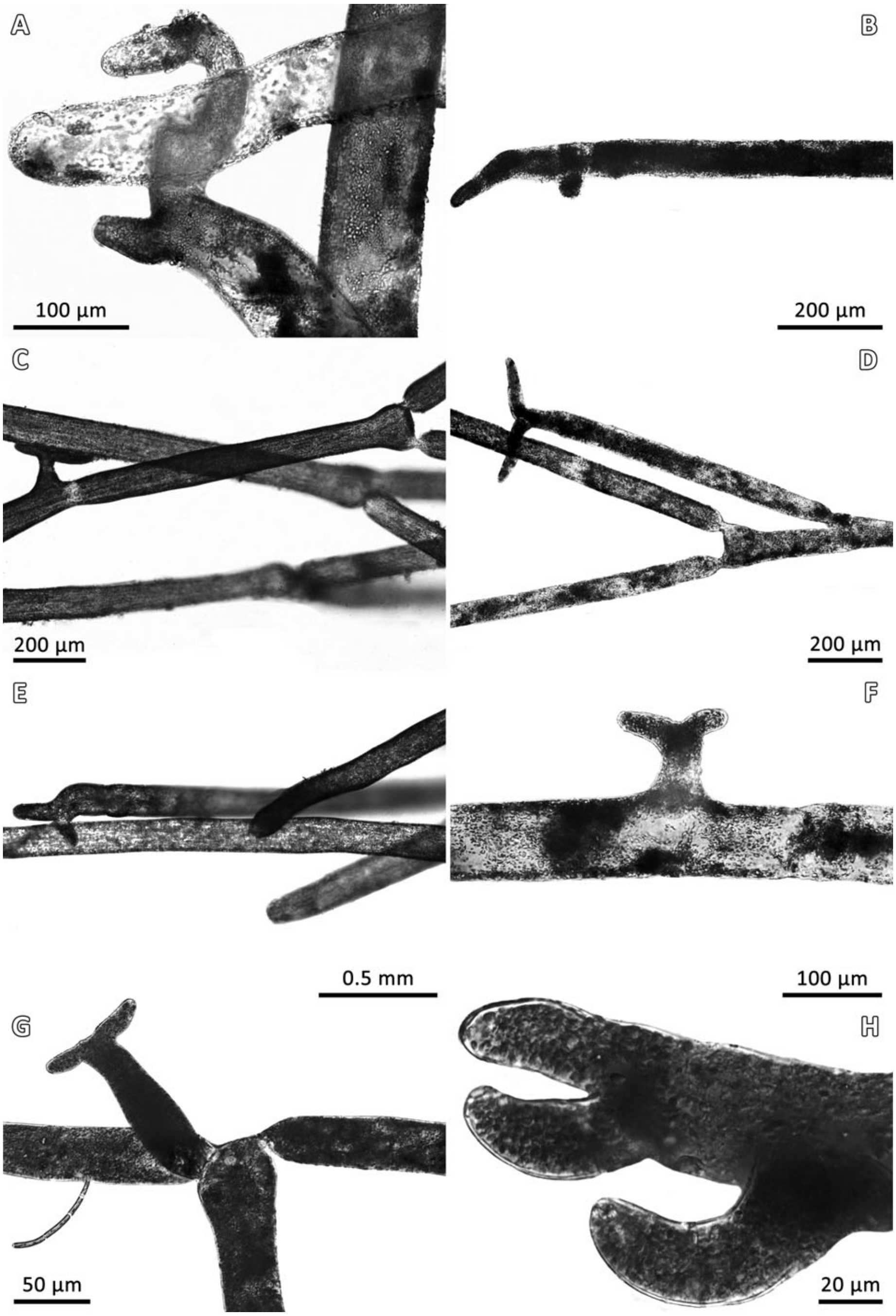

FIG. 3. Tenacula of *Rhipilia coppejansii*. (A) Hook-shaped tenaculum. (B) Modified branch tip. (C) Disk-like tenaculum connecting two siphons. (D) Two-pronged tenaculum on lateral branch. (E) Two-pronged tenaculum and unmodified siphon tip connecting siphons. (F) Two-pronged tenaculum laterally initiated from a siphon. (G) Two-pronged tenaculum on a stunted branch of a trifurcation. (H) Three-pronged tenaculum.

the tree corresponds to that presented in Verbruggen et al. (2009b) from which the dataset was taken. Although the earliest divergences and most divergences within families are relatively well-resolved, the relationships among the five families of core Halimedineae remains poorly resolved. However, this does not constitute a problem for the interpretation of the position of the focal taxa of this study. A UPGMA analysis of the *rbc*L sequences of multiple specimens per species of the entity from Guam along with sequences of the closely related *R. nigrescens* and *R. orientalis* (Fig. 6B) shows clear divergence between the three entities, even though genetic distances between them are small (0.6%–0.8% for *rbc*L, 1.1%–1.4% for *tuf*A). These results confirm the higher interspecific divergence of *tuf*A sequences

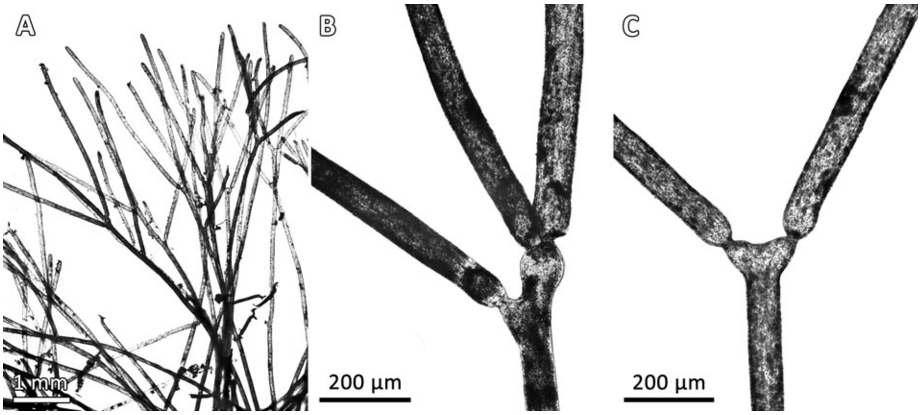

FIG. 4. Branching pattern of *Rhipilia coppejansii*. (A) Di- and tritochomous branching. (B) Dichotomous branching and lateral branch development, showing subdichotomous inflated siphons. (C) Dichotomous branching displaying subdichotomous inflated siphons and supradichotomous constrictions.

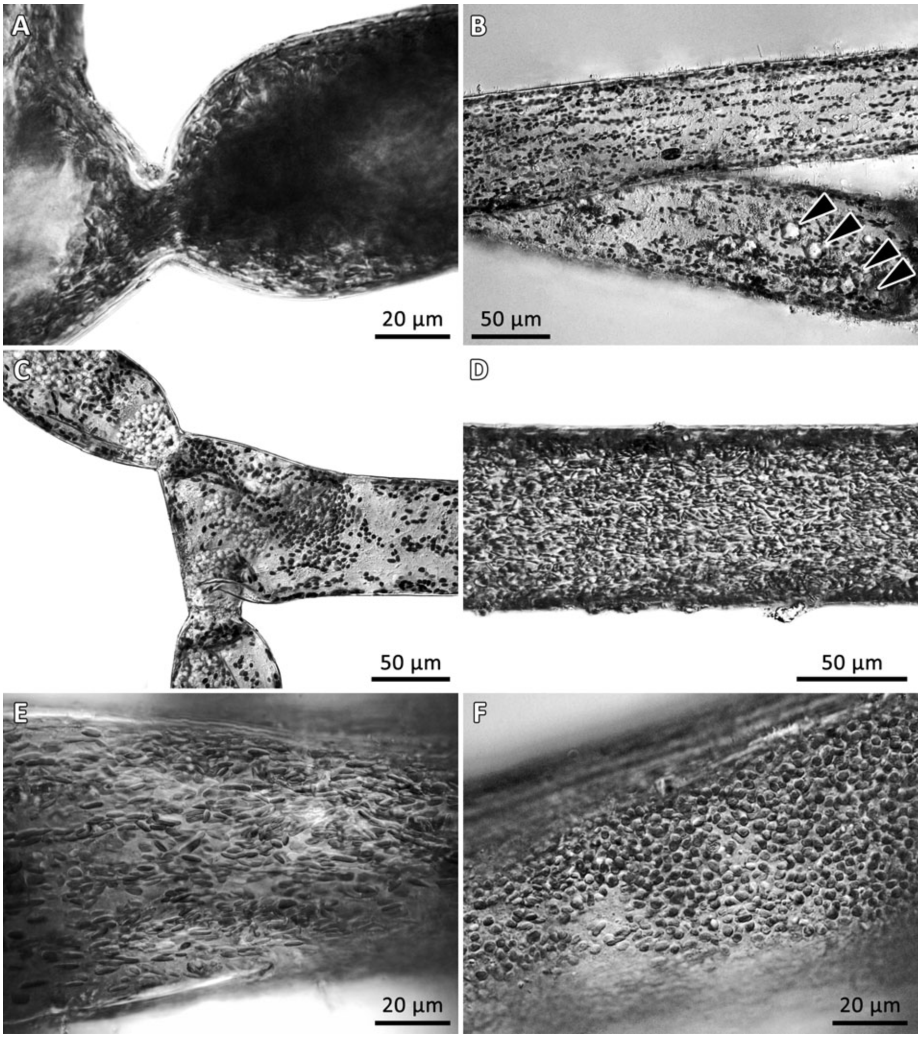

FIG. 5. Anatomical features of *Rhipilia coppejansii*. (A) Close-up of a constriction displaying the absence of cell-wall thickenings. (B) Siphon with regular hexagonal crystals (arrowheads). (C) Aggregation of amyloplasts (white) near cell constrictions. (D) Siphons contain large numbers of chloroplasts that mask amyloplasts and crystalline cell inclusions. (E) Elongated to spindle-shaped chloroplasts dispersed throughout the siphons. (F) Subspherical parietal chloroplasts.

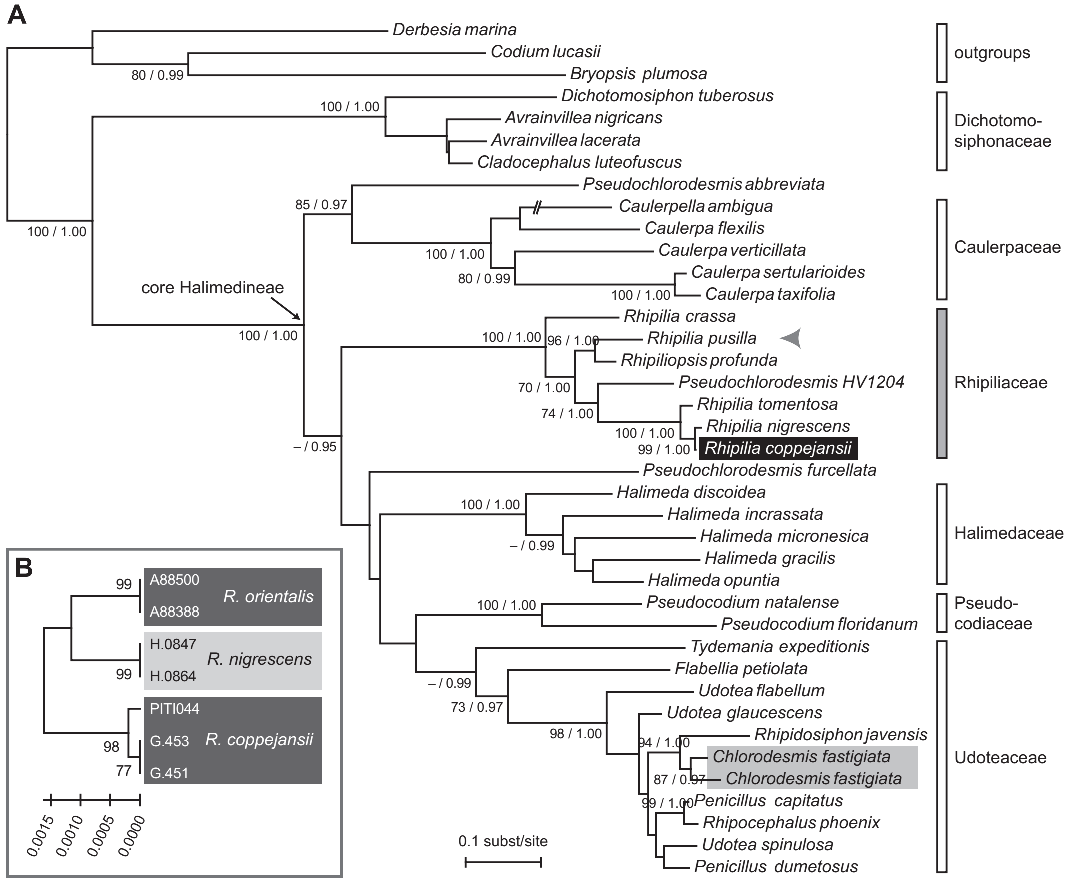

FIG. 6. Molecular phylogenies. (A) Maximum likelihood tree of 39 Halimedineae taxa showing the position of *Rhipilia coppejansii*, the entity from Guam, within the Rhipiliaceae. (B) UPGMA dendrogram of *rbc*L haplotype distances, showing the distinctness between the closely related species *R. orientalis*, *R. nigrescens* and *R. coppejansii*.

as compared to *rbc*L sequences (Saunders and Kucera 2010), making it the better candidate for DNA barcoding in the Bryopsidales.

Taxonomic treatment. It follows from the morphological and molecular results that the entity from Guam should be described as a new species in the genus *Rhipilia*.

Rhipilia coppejansii Schils et Verbruggen, sp. nov.

Latin diagnosis: A speciebus in genere Chlorodesmis tenaculis dispersis et typi-II crystallis in cellulis differt. A speciebus in genere Rhipilia habitu et thalli statura simili Chlorodesmis generi differt. A speciebus in familia Rhipiliaceae et in familia Udoteaceae sequentiis geneticis tufA differt.

English diagnosis: Differs from species of the genus *Chlorodesmis* in having dispersed tenacula and type 2 crystalline cell inclusions. Differs from other *Rhipilia* species in its *Chlorodesmis*-like habit and thallus size. The *tuf*A DNA barcode of the species is distinct from those of other species in the Rhipiliaceae and Udoteaceae and is available for comparison on Genbank.

Holotype: GH0011082 = G.451; Jun 14, 2009; coll. T. Schils; 3 m depth; Piti Bomb Holes, Guam (13.472118°N, 144.703204°E). Deposited in GENT.

Isotype: GH0011084 = G.453. Deposited in GUAM.

Paratypes: GH0011434 deposited in US. GH0011435 deposited in BISH. GH0011436 deposited in AD. GH0011438 deposited in GUAM. Paratype collection information: Oct 20, 2010; coll. T. Schils; 3 m depth; Piti Bomb Holes, Guam (13.472118°N, 144.703204°E).

Etymology: Named in honor of our former PhD supervisor Eric Coppejans, an inspiring mentor with a keen interest in coral reef-associated algae and Bryopsidales in particular. He has published extensively on green algae, including a study describing a new species of *Rhipilia* (Coppejans and Prud'homme van Reine 1989, 1990).

Distribution: So far only known from Guam. Besides the type locality, the species has also been recorded during monitoring surveys in Apra Harbor (13.447638°N, 144.627361°E; 4 m depth; Schils et al. 2011).

Habitat: The new species is locally abundant in between ''fingers'' of the corals *Porites cilindrica* and *P. rus* at shallow depths on sheltered reefs (2–5 m depth). The network of rhizoids forms a mat over coral rubble and living coral colonies. *Rhipilia coppejansii* appears to cope well with high turbidity levels as suspended fine sediment is regularly observed at the type locality and the alga not only grows among coral, but also on limestone substrate covered by fine silt deposits.

Comparison to other taxa. The only other *Rhipilia* species with a filamentous tuft-like morphology is *Rhipilia pusilla* from Kangaroo Island, southern Australia (Womersley 1955, Ducker 1966). *Rhipilia coppejansii* differs from *R. pusilla* in having larger thalli (1.5 vs. 8 cm high), thicker siphons, longer tenacular siphons, tenacula that do not only occur near the thallus base, consistent presence of constrictions at ramifications, the lack of cell-wall thickenings at constrictions, and the absence of cell-wall undulations of siphons. The species also have distinctive *tuf*A and *rbc*L sequences (Fig. 6A, *R. pusilla* is indicated with arrowhead). Finally, they differ in their known distribution range (tropical vs. temperate; western Pacific vs. southern Australia) and habitat (shallow coral reef systems vs. tide pools). It is interesting to note that *Rhipilia pusilla* was initially described as *Chlorodesmis pusilla* based on its filamentous, tuft-like habit (Womersley 1955), and it was subsequently transferred to *Rhipilia* based on the presence of tenacula (Ducker 1966). This transfer is supported by the position of *R. pusilla* in our molecular phylogeny.

As it is possible that the new *Rhipilia* species has previously been misidentified as *Chlorodesmis*, a common genus of coral reef algae, we will also provide some comparison with *Chlorodesmis* species from the region. Three species of *Chlorodesmis* have been reported for Guam: *C. caespitosa*, *C. fastigiata*, and *C. hildebrandtii* (Lobban and Tsuda 2003). A fourth species, *C. dotyi*, is known from Micronesia, but has thus far only been reported for Mokil Atoll (Pohnpei). As mentioned previously, the tenacula of *R. coppejansii* are a diagnostic feature that readily distinguishes the species from all currently accepted *Chlorodesmis* species. Furthermore, unlike many *Chlorodesmis* species, the filaments are perfectly cylindrical and not torulose toward the base. The siphon diameter of *R. coppejansii* is larger than that of *C. haterumana*, but smaller than the size ranges listed for most other *Chlorodesmis* species (Ducker 1969, Yoshida 1998), although it does fall within the size range of *C. dotyi* and *C. fastigiata* siphons (Trono 1971). Besides these features, the acicular crystalline cell inclusions of crystal-containing *Chlorodesmis caespitosa*, *C. fastigiata*, and *C. haterumana* (Ducker et al. 1965) correspond to type 2 crystals *sensu* Leliaert and Coppejans (2004), whereas those of *R. coppejansii* are of type 1b. The symmetrical constrictions above siphon forkings of *R. coppejansii* differ from the asymmetrical constrictions in *C. fastigiata* and *C. papenfussii* (Ducker 1969, Coppejans et al. 2001). *Rhipilia coppejansii* also lacks the characteristic bulbous or elongate stipe of *C. baculifera* and *C. papenfussii* (Ducker 1969).

Morphological diversity and adaptation. The recovery of a *Chlorodesmis*-like morphology in the Rhipiliaceae warrants a brief discussion of morphological diversity across the Halimedineae. It was previously shown that *Pseudochlorodesmis*, a genus of diminutive species consisting of a siphon that branches only a few times (if at all), consists of a para- or polyphyletic assemblage of early-branching lineages (Verbruggen et al. 2009b), which can also be seen in Figure 6A. One strain (*P. abbreviata*) is sister to the Caulerpaceae, a second species (*P. furcellata*) is sister to the clade comprising Halimedaceae, Pseudocodiaceae, and Udoteaceae, and the third strain (HV1204) is recovered within the Rhipiliaceae. The exact position of some of the strains (e.g., *P. furcellata*) differs somewhat from the previous analysis, probably as a consequence of different models of sequence evolution and taxon sampling used in this study, but their early-branching nature is confirmed. The fact that the *Pseudochlorodesmis* strains branch off early in the core Halimedineae may be indicative that such simple siphons represent the ancestral morphology of the group, although our current knowledge is too fragmentary to draw sound conclusions on the matter.

The currently recognized families of the core Halimedineae (Caulerpaceae, Rhipiliaceae, Halimedaceae, Pseudocodiaceae, Udoteaceae) largely consist of species with more complex thalli (Gepp and Gepp 1911, Vroom et al. 1998, Littler and Littler 2000, Verbruggen et al. 2009a). Within at least two of these families with more complex morphologies, the Udoteaceae and Rhipiliaceae, reductions to simpler morphologies have occurred independently.

The Udoteaceae comprise the widest range of morphological types, including the simple stalked blades of the genus *Rhipidosiphon*, the more complex stalked blades of *Flabellia* and *Udotea* several of which are corticated, the brush-like morphology of the genus *Penicillus*, the stalked caps of *Rhipocephalus* consisting of layered blades, resembling an artichoke, and the remarkably simpler tufts of siphons typical of *Chlorodesmis*. A study of the evolution of morphological types in the Udoteaceae overturned the traditional notion that simple morphologies like that of *Chlorodesmis* were primitive, instead showing that the earliest-branching lineages featured relatively complex corticated blades and that the simple *Chlorodesmis* morphologies evolved from more complex forms by reduction or neoteny (Kooistra 2002). Interestingly, this study also showed that this reduction to a simple form did not occur just once, but at least two times independently, as *Chlorodesmis caespitosa* was recovered within a clade of *Penicillus* species, whereas *Chlorodesmis fastigiata* was shown to be nested in a lineage with diverse morphologies (*Udotea*, *Rhipocephalus* and *Penicillus*). The two *Chlorodesmis fastigiata* sequences included in this study do form a single clade, sister to *Rhipidosiphon javensis*, but our taxon sampling in the Udoteaceae in general and *Chlorodesmis* in particular is insufficient to make meaningful contributions to this topic.

As for the Rhipiliaceae, at first sight one would think that they are a fairly homogeneous assemblage consisting of blade-like thalli built up by interlinked siphons. However, the recent discoveries, including the *Chlorodesmis*-like morphology described herein and the *Pseudochlorodesmis* specimen recently recovered within the Rhipiliaceae (Verbruggen et al. 2009b) falsify this notion. Although it is difficult to come to general conclusions about whether simpler morphologies are primitive or derived based on the limited set of taxa in our analysis, we suspect that at least in case of *R. coppejansii*, the simple morphology is derived, as it is nested within more typical *Rhipilia* forms (*R. tomentosa*, *R. nigrescens*, *R. orientalis*). It is worth noting that within the Rhipiliaceae, a few species have also evolved more complex thalli. Unlike the typical bladelets, *R. fungiformis*, *R. geppii*, and *R. tomentosa* have developed thick, sponge-like thalli (Littler and Littler 2000, Millar and Kraft 2001). *Rhipilia penicilloides* is another exception that forms spongy stalks bearing brush-like heads (N'Yeurt and Keats 1997), not unlike the udoteacean genus *Penicillus*, but noncalcified.

It is plausible that the divergent morphology of *R. coppejansii* among *Rhipilia* species reflects an adaptive trait. Typically, *Rhipilia* species occur in relatively deep habitats (>5 m), with their fan-shaped or peltate thalli oriented perpendicular to the incoming light. The integrity of the blade and maintenance of its orientation are facilitated by the ample tenacula that increase the rigidity of these species' thalli. The shallow-water species *R. pusilla* has a tuft-like morphology with few tenacula, increasing the flexibility of its thallus and reducing drag in wave-swept environments. The other tuft-like species *R. coppejansii* mostly grows in between or underneath dense coral formations. In this habitat, the flexibility conveyed by the loose siphons permits growth in small spaces, and orienting the siphons toward the light. We want to note that the adaptive scenarios formulated herein, are solely based on correlations between field conditions and morphological observations. Although such correlations can lead to the formulation of interesting hypotheses, they need to be subjected to ecological experimentation and biophysical modeling before robust conclusions can be drawn.

Based on the diversity of secondary metabolites in the Udoteaceae that serve as feeding deterrents (Amsler 2008), and the observation that *R. coppejansii* grows in great abundance at a marine protected area near a fish feeding station, it is to be expected that the alga contains natural products similar to the cytotoxic compounds that have been isolated for *Chlorodesmis* species and other Bryopsidales (Wells and Barrow 1979, Paul and Fenical 1985).

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