

Molecular survey of *Codium* species diversity in southern Madagascar

Heroen VERBRUGGEN* & Joana F. COSTA

School of BioSciences, University of Melbourne, Victoria 3010, Australia

Abstract – We present a molecular survey of the species in the green algal genus *Codium* that were collected as part of the *Atimo Vatae* expedition to southern Madagascar. Based on clustering analysis of partial *tufA* and *rbcL* sequences, we recognize 11-12 species-level clusters in this area. Through a combination of morphological identifications and DNA comparisons, these clusters are identified as *C. mozambiquense*, *C. spongiosum*, *C. lucasii* subsp. *capense*, *C. duthieae*, *C. decortiatum*, *C. prostratum*, *C. dworkense*, *C. taylorii*, *C. arenicola* and *C. cf. cicatrix*, and a new ball-shaped species. We present a phylogenetic tree inferred from a concatenated alignment with *tufA*, *rbcL* and *rps3-rpl16* to show the placement of these species in the broader context of the genus and to analyse the biogeographic affinities of the southern Madagascan *Codium* flora. We conclude that the Madagascan flora shares elements with temperate South Africa and contains tropical Indo-Pacific elements. It also has endemic elements, some clearly at the species level, some possibly representing isolated populations of more widely-ranging species.

Biogeography / Bryopsidales / *Codium* / marine green algae / molecular systematics / Madagascar

INTRODUCTION

Madagascar is a large island in the western Indian Ocean, nearly 500 km east from Mozambique (SE Africa). It is part of the group of (sub-)continents that resulted from the breakup of Gondwana during the Mesozoic. The block consisting of the Indian subcontinent and Madagascar separated from Gondwana roughly 130 million years ago and started drifting northwards. Madagascar and India separated about 88 million years ago (Smith *et al.*, 1994). As a consequence of its long isolation, a peculiar flora and fauna with very high endemism has evolved on the island. While the marine fauna and flora of the island do not show the high levels of endemism seen in their terrestrial counterparts, endemics are known for most groups of marine organisms (Goodman & Benstead, 2005). Madagascar, which ranges from about 12 to 26 degrees latitude, is very rich in marine ecosystems, including tropical coral reefs in the north and temperate-water rocky reefs in the south.

In 2010, the *Atimo Vatae* expedition in the *La Planète Revisitée* series was mounted to study the biota of the temperate water marine ecosystems along the extreme South coast of the island (<http://laplaneterevisitee.org/en/87/accueil>). This colder water region stretching from Androka in the west to slightly east of Taolagnaro (Fort-Dauphin) is known for its extensive algal growth and relatively

* Correspondence and reprints: heroen.verbruggen@gmail.com

high marine endemism (UNESCO 2012). Over a period of approximately two months (late April to mid June 2010), 2470 seaweed collections were made by seven collectors. The collections are housed in PC.

The green algal genus *Codium* (Codiaceae, Bryopsidales) is siphonous in structure, i.e. individuals consist of a single giant tubular cell that branches to form a more complex morphology. In *Codium*, the siphons form a distinct medulla consisting of a network of thin siphons and a cortex of inflated siphon endings called utricles. *Codium* features several types of external morphologies: mat-forming, spherical and branched. In the latter category, there are species that are entirely upright and species that have a sprawling habit, attaching to the substrate at various points and often showing fusions between branches that come into contact.

There is very little published work on the genus *Codium* in Madagascar. Schmidt (1923) studied some collections made by Voeltzkow, which included the mat-forming species *C. arabicum* and *C. acuminatum*, the latter being described as a new species. In his unpublished thesis, Farghaly (1980) reported *C. arabicum*, *C. capitatum*, *C. cicatrix*, *C. dwarkense* and an invalidly published new species *C. madagascariensis*. Coppejans collected several *Codium* species in Tuléar and Tolanaro in August 2002, with samples in GENT and partial results described in an unpublished thesis (Douterlungne, 2003). This expedition added the species *C. prostratum*, *C. extricatum*, *C. geppiorum*, *C. repens* and an unidentified species to the list.

In the surrounding western Indian Ocean, more work has been done. This includes monographs of the *Codium* species of South Africa (Silva, 1959a) the tropical East African coast (Van den heede & Coppejans, 1996), the Arabian Sea (Børgesen, 1946; Nizamuddin, 1997; Nizamuddin, 1999a; Nizamuddin, 1999b; Nizamuddin, 2000; Nizamuddin & Gul, 2005; Nizamuddin, 2001; Espinel-Velasco, 2005) as well as studies of marine flora of some islands of the Mascarene ridge: Mauritius (Børgesen, 1940; Børgesen, 1948; Børgesen, 1949; Børgesen 1953), Réunion (Payri, 1985) and Rodrigues (Coppejans *et al.*, 2004).

The aim of this study was to assess the species-level biodiversity among *Codium* samples collected during the *Atimo Vatae* expedition of 2010. Our approach consists of using partial *rbcL* and *tufA* sequences (DNA barcodes) to assess species boundaries and studying the relationships among species from Madagascar and other parts of the world using a multi-gene phylogeny. We discuss the biogeography of the species based on these results.

MATERIALS AND METHODS

The samples used in this study were collected from 20 localities on the South coast of Madagascar (Table 1, Fig. 1), and samples are lodged in PC. We also provide the locality data as a Google Earth KMZ file at FigShare (<http://dx.doi.org/10.6084/m9.figshare.1290723>). Specimens from South Africa and the East African coast (Tanzania) lodged in GENT and MELU were studied for comparison.

Morphological observations were made on pressed specimens housed at PC. Anatomical details were observed with a Leica MZ8 stereomicroscope and a Leica DM750 compound microscope equipped with a Canon EOS 600D digital

Table 1. Specimens studied here, with Genbank accession numbers of their sequences and localities. Complete alignments used for species delimitation and phylogenetic analyses are available from FigShare, as is a Google Earth KML file with the localities (<http://dx.doi.org/10.6084/m9.figshare.1290723>)

<i>Species-level cluster</i>	<i>Sample</i>	<i>rbcL</i>	<i>tufA</i>	<i>Locality</i>	<i>Latitude</i>	<i>Longitude</i>	<i>Site code</i>
<i>C_arenicola</i>	MAD2023		KP685943	Tuléar: Pointe Barrow	-25.30000	44.30167	2
<i>C_arenicola</i>	MAD0695		KP685925	Anosy: Vieux port Fort Dauphin	-25.02500	47.00000	16
<i>C_no.ID.58</i>	MAD1962	KP686031	KP685941	Beloha: Cap Sainte Marie	-25.55333	45.11000	7
<i>C_no.ID.58</i>	MAD1945		KP685939	Beloha: Cap Sainte Marie	-25.56500	45.11500	8
<i>C_no.ID.58</i>	MAD1635	KP686026	KP685935	Anosy: Baie des Galions	-25.16000	46.74333	10
<i>C_cicatrix.1</i>	MAD1797	KP686029	KP685938	Beloha: Lavanono	-25.47167	44.92667	5
<i>C_cicatrix.2</i>	MAD0852	KP686021	KP685928	Beloha: Ambatobe	-25.45667	44.95667	6
<i>C_cicatrix.2</i>	MAD0853	KP686022	KP685929	Beloha: Ambatobe	-25.45667	44.95667	6
<i>C_decoriticatum</i>	MAD0450	KP686018	KP685923	Anosy: Cap Ranavalona: Bevava	-25.07333	46.95500	12
<i>C_decoriticatum</i>	MAD2134		KP685944	Anosy: Sainte Luce	-24.76500	47.21333	20
<i>C_duthieae.3</i>	MAD0336	KP686016	KP685920	Anosy: Mahanoro	-24.96833	47.10167	18
<i>C_duthieae.3</i>	MAD0337	KP686017	KP685921	Anosy: Mahanoro	-24.96833	47.10167	18
<i>C_dwarkense.1</i>	MAD0256	KP686013	KP685916	Anosy: Cap Antsirabe	-25.04667	46.99833	14
<i>C_dwarkense.1</i>	MAD0638	KP686019	KP685924	Anosy: Flacourt	-25.02833	47.00167	15
<i>C_dwarkense.1</i>	MAD0290	KP686014	KP685918	Anosy: phare d'Evatra	-24.97833	47.09833	17
<i>C_dwarkense.1</i>	MAD0311	KP686015	KP685919	Anosy: phare d'Evatra	-24.97833	47.09833	17
<i>C_dwarkense.1</i>	MAD0138	KP686011	KP685912	Anosy: Mahanoro	-24.96833	47.10167	18
<i>C_dwarkense.1</i>	MAD0164	KP686012	KP685913	Anosy: Mahanoro	-24.96833	47.10167	18
<i>C_dwarkense.1</i>	MAD2460		KP685945	Anosy: Sainte Luce	-24.77000	47.20667	19
<i>C_lucasii.cap.1</i>	MAD1178	KP686023	KP685931	Beloha: Cap Malainpioka	-25.35833	44.83333	3
<i>C_lucasii.cap.1</i>	MAD1946	KP686030	KP685940	Beloha: Cap Sainte Marie	-25.56500	45.11500	8
<i>C_lucasii.cap.1</i>	MAD1636	KP686027	KP685936	Anosy: Baie des Galions	-25.16000	46.74333	10
<i>C_lucasii.cap.1</i>	MAD1748	KP686028	KP685937	Anosy: Baie des Galions	-25.14667	46.75000	11
<i>C_lucasii.cap.1</i>	MAD0696	KP686020	KP685926	Anosy: Vieux port Fort Dauphin	-25.02500	47.00000	16
<i>C_mozambiquense</i>	MAD0182		KP685915	Anosy: Mahanoro	-24.96833	47.10167	18
<i>C_prostratum</i>	MAD2010		KP685942	Tuléar: Lagon de Baravo	-25.20333	44.32167	1
<i>C_prostratum</i>	MAD1174		KP685930	Beloha: Cap Malainpioka	-25.35833	44.83333	3
<i>C_spongiosum</i>	MAD1252		KP685932	Beloha: Lavanono	-25.42000	44.93833	4
<i>C_spongiosum</i>	MAD1253		KP685933	Beloha: Lavanono	-25.42000	44.93833	4
<i>C_spongiosum</i>	MAD1254	KP686024	KP685934	Beloha: Lavanono	-25.42000	44.93833	4
<i>C_taylorii</i>	MAD0753		KP685927	Anosy: Andavaka	-25.20000	46.63333	9
<i>C_taylorii</i>	MAD1634	KP686025		Anosy: Baie des Galions	-25.16000	46.74333	10
<i>C_taylorii</i>	MAD0431		KP685922	Anosy: Cap Ranavalona: pointe Ehoala	-25.07500	46.96167	13
<i>C_taylorii</i>	MAD0259		KP685917	Anosy: Cap Antsirabe	-25.04667	46.99833	14
<i>C_taylorii</i>	MAD0177		KP685914	Anosy: Mahanoro	-24.96833	47.10167	18



Fig. 1. Sampling sites for *Codium* specimens during the *Atimo Vatae* expedition along the South coast of Madagascar and included in this study. We also provide the locality data as a Google Earth KMZ file at FigShare (<http://dx.doi.org/10.6084/m9.figshare.1290723>).

camera. Morphological identifications were mainly based on monographs from adjacent regions (Van den heede & Coppejans, 1996; Silva, 1959a) and comparisons with other species known from the western Indian Ocean. When we present dimensions of structures, these represent multiple measurements of the structure and are given in the following format: e.g. 105–165–195 μm , where the first value is the 20-percentile of measurements, the underlined value is the median, and the third value is the 80-percentile. We do not list extreme values.

Total DNA was isolated following a CTAB protocol modified from Doyle & Doyle (1987). Amplifications of *tufA* and *rbcL* markers were performed in a total reaction volume of 25 μL consisting of 1X MyTaq buffer, 0.4 μM of each primer, 0.625 units of MyTaqTM (Bioline) and 1 μL of template DNA. Specific primers and PCR profiles are described elsewhere for both *tufA* (Famà *et al.*, 2002) and *rbcL* (Shimada *et al.*, 2004; Verbruggen *et al.*, 2007) fragments. The *rps3-rpl16* region was amplified following Provan *et al.* (2004).

Amplicons were sequenced by Macrogen (Seoul, Korea) and added to the existing dataset derived from the work of Verbruggen *et al.* (2007) with additional data published in recent papers (Verbruggen *et al.*, 2012; Oliveira-Carvalho *et al.*, 2012). Genbank accession numbers for *rbcL* and *tufA* sequences

of Madagascan samples are found in Table 1. Other sequences generated from South African and Tanzanian material for comparison as well as sequences generated for the multi-marker phylogeny are available on Genbank under accession numbers KP685748-KP686051.

Species boundaries were assessed by generating distance trees (UPGMA and NJ) of the *tufA* and *rbcL* sequence alignments. The clustering was done with MEGA 6.06 using 100 bootstrap replicates, the maximum composite likelihood method, with transitions and transversions included, a gamma-distribution to accommodate for rate differences across sites, and the pairwise deletion option set for missing data. Based on these trees, we identified tight clusters of sequences separated from other such clusters by longer branches (i.e. higher sequence divergence).

Once the species boundaries had been defined, we built a multi-gene phylogeny to infer the relationships between species more reliably. Sequences of *rbcL*, *tufA* and *rps3-rpl16* were gathered from specimens representative of each species and concatenated into a single alignment in which each species is represented by a single concatenated sequence. The intergenic spacer in the *rps3-rpl16* region was removed prior to concatenation. Model selection with Partitioned Model Tester (Verbruggen, 2010) using the Bayesian information criterion suggested analysing the concatenated alignment with a partitioned model in which genes are separate and codon positions 1+2 are separate from positions 3, in other words 6 partitions in total (3 genes \times 2 codon position categories), and unlinked GTR + Γ_4 models of sequence evolution for each partition. Maximum likelihood analysis was carried out with RAxML 8.1.15 using this partitioned model and 500 normal bootstrap replicates (Stamatakis, 2014).

Data, supplementary information, and high-resolution figures are also available from FigShare (<http://dx.doi.org/10.6084/m9.figshare.1290723>).

RESULTS AND DISCUSSION

Species diversity in southern Madagascar

Our DNA-based survey of species diversity indicated that our 35 sequenced samples belong to 11 or 12 species (Supplement S1, Table 1). The clustering trees generally showed clear-cut separation between clusters of sequences separated from each other by longer branches with high bootstrap support, suggesting that these clusters are separate species, and we will refer to them as "species-level clusters". As previously found by Saunders & Kucera (2010), *tufA* had higher levels of divergence and showed clearer separation of species than *rbcL*, and we have started using this gene as the primary DNA barcode for *Codium* instead of *rbcL* exon 1 (Verbruggen *et al.*, 2007). Nevertheless, the results obtained from *tufA* and *rbcL* lead to identical conclusions regarding species limits among Madagascan samples (Supplement S1). Species-level clusters are indicated in our work with a reference to their identity, for example *C_taylorii* indicates a species-level cluster that matches morphologically with *Codium taylorii*. If cryptic diversity is found within morphologically defined species, the different species-level clusters that correspond to the same morphological species name are indicated with a number

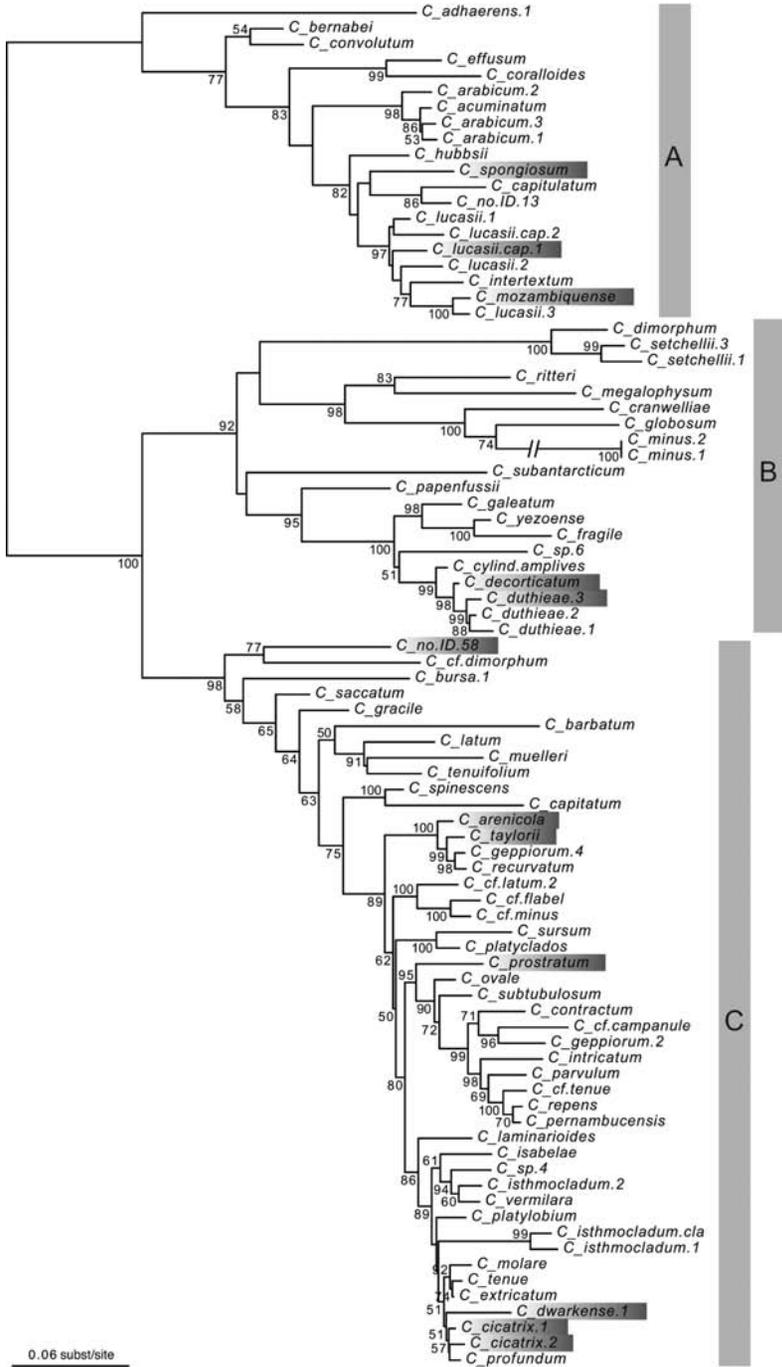


Fig. 2. Maximum likelihood phylogenetic tree of *Codium* species based on a concatenated alignment of *rbcL*, *tufA* and *rps3-rpl16*. Species present in Madagascar are indicated in grey. Values at nodes indicate bootstrap support (only shown if > 50).

following the name. For example, *C_duthieae.1*, *C_duthieae.2* and *C_duthieae.3* are three species-level clusters that are to be interpreted as cryptic species within the morphological definition of *Codium duthieae*.

The species present in Madagascar are also highlighted in the multi-marker phylogenetic tree (Fig. 2), and because this tree is more reader-friendly than the clustering trees in Supplement S1, we will use it as a guide to run through the species identifications. As in previously published phylogenies, the tree consists of three major lineages indicated as A, B & C following Verbruggen *et al.* (2007).

Of the mat-forming *Codium* species in clade A, there are three among our Madagascan collections: *C. spongiosum*, *C. lucasii* subsp. *capense* and *C. mozambiquense*.

Codium spongiosum was found in just one locality, Lavanono. The thallus and utricle morphology of the Madagascan material matches well with descriptions of South African material (Silva, 1959a). The DNA sequences cluster with those of the same species from other locations (Supplement S1).

Codium lucasii subsp. *capense* (species-level cluster *C_lucasii.cap.1*) was found along almost the entire coast (3-18, Fig. 1). It closely resembles South African material with the same name in having utricles in large clusters and often somewhat constricted below the apex. The utricle diameter of Madagascan material (95–120–145 µm) is somewhat larger than that given for South African material (60–105 µm; Silva, 1959a). *Codium lucasii* is a complex of many species-level clusters that need to be recognized as separate species. Besides the species discussed here, there are the species-level clusters *C_lucasii.1* from Australia, the type locality of the species, *C_lucasii.2* from Japan, *C_lucasii.3* from East Africa, and *C_lucasii.cap.2* from the Arabian Sea. It will take a more focused study of this complex to characterize the morphological features of the different species-level clusters and sort out the nomenclature in order to propose an appropriate name for each of them.

Codium mozambiquense was represented by only a single specimen from Mahanoro. Morphologically, the specimen matches the description of the species by Silva (1959a), with utricles in clusters, measuring 95–100–118 µm in diameter and on average 590 µm in length. Some of the utricles had a zone of hair scars between 75 µm and 150 µm below the utricle tip, while other utricles were completely devoid of scars. The DNA sequence of the sample clusters with sequences of *C. mozambiquense* from South Africa.

There are two species from clade B, both of which are in the *Codium decorticutum* complex (Verbruggen *et al.*, 2007) characterized by large branched thalli that are flattened at the branch points and have very large utricles.

Codium decorticutum was represented by two specimens: a juvenile one (MAD2134) and a larger one (MAD0450). The herbarium sheet of the latter contains many similar specimens, but we consider only the one that was subsampled for DNA work (indicated on the sheet, Fig. 3). Utricles of the larger specimen measured 265–280–330 µm in diameter and 975–1030–1115 µm in length, with a length over diameter ratio of 3.1–3.6–4.0. These measurements match well with the material described from the Atlantic Ocean (Silva, 1959b). The juvenile sample had much smaller utricles (D: 175–200–220 µm, L: 440–480–545) and would not be readily recognized as *C. decorticutum* without the DNA sequence. In external appearance, neither of the samples resembles typical *C. decorticutum*. Unlike typical *C. decorticutum*, which easily reaches one metre in length, our plants are small. The larger specimen (MAD0450) is only about 5 cm tall. The plant branches in widely spaced, unequal dichotomies, and it

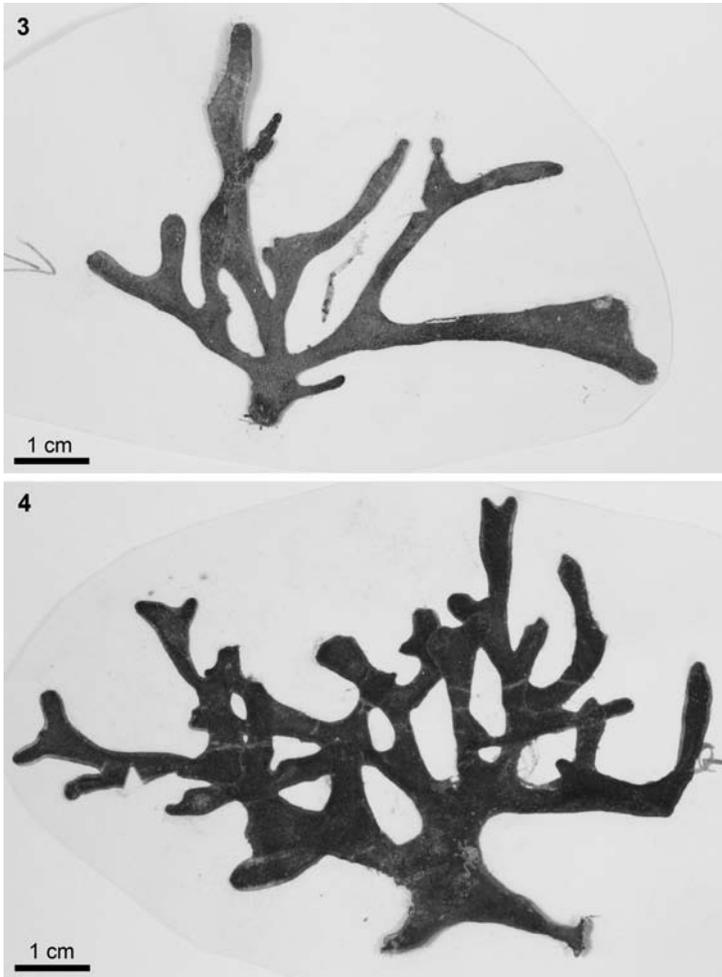
appears to be compressed throughout (Fig. 3). Typical *C. decorticutum* has cylindrical branches flattened only at the dichotomies and the branching is more equal and at smaller angles (Silva, 1959b). The identification of these plants as *C. decorticutum* would have been problematic without the DNA sequences. The DNA sequences we used for comparison are from specimens collected throughout the Eastern and Western Atlantic Ocean, where this species is well known.

Codium duthieae (species-level cluster *C_duthieae.3*) was represented by two samples from Mahanoro. The samples match the morphological description of the species. Utricles measured 530–550–570 µm in diameter and 950–1050–1150 µm in length, with a length over diameter ratio of 1.8–1.9–2.4. One of the plants (MAD0336) is 8 cm high, with cylindrical branches of about 5 mm wide that flatten at the dichotomy. The other plant (MAD0337) appears to be a stub of an older plant that died back, and it is covered in small new growth, something that is observed occasionally in *Codium* species (pers. obs.). The species-level cluster *C_duthieae.3* is one of the members of a complex of species that also contains *C_cylindricum*, *C_decorticutum*, *C_duthieae.1* and *C_duthieae.2*. The morphology of these species-level clusters overlaps strongly, and it is difficult to distinguish between them based on morphology alone (e.g., Van den heede & Coppejans, 1996).

Finally, clade C is represented by six or seven species in South Madagascar. These include a ball-forming species (probably new species) and several branched species, some of them sprawling and some of them upright (*C. arenicola*, *C. taylorii*, *C. taylorii*, *C. prostratum*, *C. dwarkense*, *C. cicatrix*).

The species-level cluster *C_no.ID.58* is a ball- or cushion-forming species that, to our knowledge, is not known to science. Three collections of this species were made, two in Cap Sainte Marie on the south-west coast and one in Baie des Galions on south-east coast. The DNA sequences of the specimens were identical to one another and did not cluster closely with those of any of our collections from elsewhere. We aim to describe this species and provide a detailed comparison with other ball-shaped species in a separate paper.

Codium arenicola was collected from two sites more than 200 km apart from each other: Pointe Barrow and Vieux Port Fort Dauphin. Our identification of this species is based primarily on a match of its DNA sequences with those of specimens from the Pacific Ocean that are a close match to the description of *Codium arenicola* (type locality Indonesia). Morphologically, the Madagascan samples are up to 8 cm tall, with branching at wide angles and somewhat unequal (Fig. 4). The utricles are 220–240–280 µm in diameter and 520–580–700 µm long (ratio of 1.9–2.5–3.1) and bear gametangia ca. 245 µm long and 95 µm in diameter. The flattened to slightly domed utricle tips bear numerous papillae around their edges. A morphological comparison to the species from South Africa (Silva, 1959a) would suggest this species is closest to *C. cicatrix*, but the external morphology of that species is more equally dichotomous. The external morphology resembles the Philippine species *C. bartlettii* and *C. papillatum*, two very similar species with widely spaced branching and papillae along the utricle edges (Tseng & Gilbert, 1942). Børgesen (1949) reported *C. bartlettii* from nearby Mauritius. The Madagascan material does not perfectly match these species, as its utricles are generally shorter than the 700–900 µm listed for typical utricles of the Philippine material (Tseng & Gilbert, 1942). The material also matches reasonably well with *C. arenicola*, a species that is not well known because it was validated only recently (Chacana & Silva, 2014). This species has branched thalli with compressed to cylindrical branches and swollen utricles 230–350 µm in



Figs 3-4. Flattened branched *Codium* species. **3.** *Codium decorticatum*, specimen MAD0450. **4.** *Codium arenicola*, specimen MAD0695.

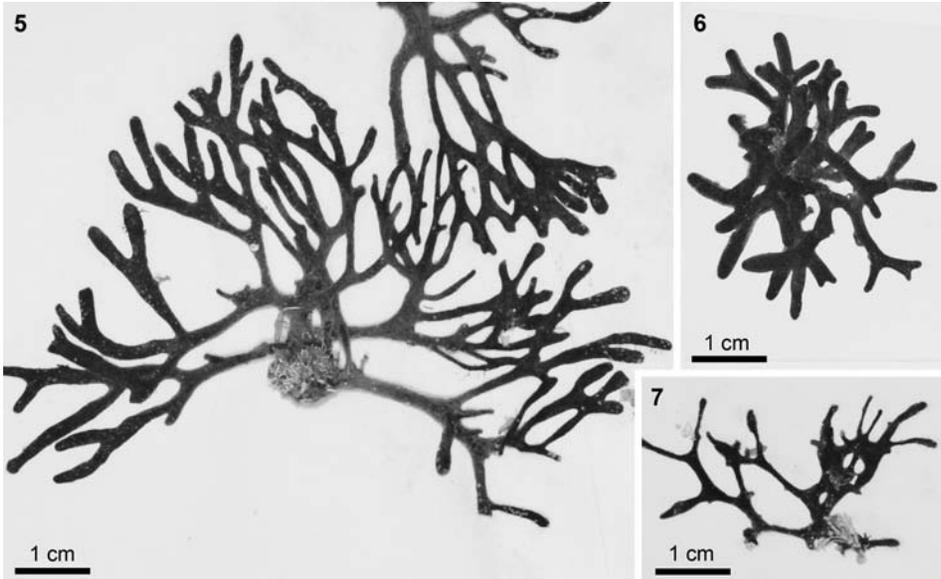
diameter and 550–600 μm long with many hair scars or papillae close to the utricle tip (Skelton *et al.*, 2007, Chacana *et al.*, 2003, Chacana & Silva, 2014). Because of the morphological and molecular match with *C. arenicola* samples from the Pacific Ocean and the slight morphological differences with *C. cicatrix* and *C. bartlettii*, we chose to name this species-level cluster *C. arenicola*.

Codium taylorii was found in five sites along the southeast coast (9, 10, 13 and 14 in Fig.1). The material had the typical irregular branching pattern and flattened branches throughout the thallus. Utricles were 135–150–185 μm in diameter and 570–620–645 long (ratio of 3.5–4.2–4.6), which is similar in diameter but shorter than utricles of Tanzanian and Caribbean material (Van den heede & Coppejans, 1996; Silva, 1959b). However, the DNA sequence data and external appearance of the species leaves no doubt about its identification.

Codium prostratum is a sprawling species with thick anastomosing branches. There are two collections among those studied. Their morphology corresponds well with South African and tropical East African material in all aspects (Silva, 1959a, Van den heede & Coppejans, 1996). The DNA sequences of Madagascan material were identical to those from South African and Tanzanian specimens.

Codium dwarkense (species-level cluster *C_dwarkense.1*) was found along the southeast coast (14-20, Fig. 1). The DNA sequences of the seven sequenced Madagascan specimens are all identical and cluster with sequences of other samples of this species from elsewhere in the Indian Ocean. This material is fairly consistent anatomically, with cylindrical, sometimes slightly capitate utricles of 135–165–185 μm in diameter and 390–470–520 μm long (ratio 2.4–2.8–3.8). Utricles have a rounded tip and some hairs or scars are present just below the rounding of the tip. The habit of the samples ranges from spread-out sprawling plants with small clusters of rhizoids forming secondary holdfasts (Fig. 7), small upright plants without secondary holdfasts (Fig. 6), to larger upright plants with irregularly dichotomous branching (Fig. 5). Because of this, upright specimens key out as *C. dwarkense* while sprawling samples are resolved as *C. geppiorum* using a tropical east African key (Van den heede & Coppejans, 1996). These same authors noted that branches of Tanzanian *C. dwarkense* occasionally have the tendency to reattach to the substrate. Our DNA data suggest that the species is highly morphologically variable, including specimens that are a good match to the sprawling species *C. geppiorum* to samples with the upright habit of typical *C. dwarkense*. In our previous work (Verbruggen *et al.*, 2007; Verbruggen *et al.*, 2012; Oliveira-Carvalho *et al.*, 2012), we had only sprawling samples of this species-level cluster hence we assigned it the name *C. geppiorum.1*. Based on our new insights, we have changed its name to *C_dwarkense.1*. This observation also has implications for the interpretation of previous *C. geppiorum* records for the region. At this stage we cannot say whether these records all belong to the species being discussed here, or whether genuine *C. geppiorum* also occurs in the area. To further complicate matters, *C. geppiorum* is a complex of cryptic species (Verbruggen *et al.*, 2007), so specimens with a morphological match to *C. geppiorum* could belong to any one of a range of species-level clusters.

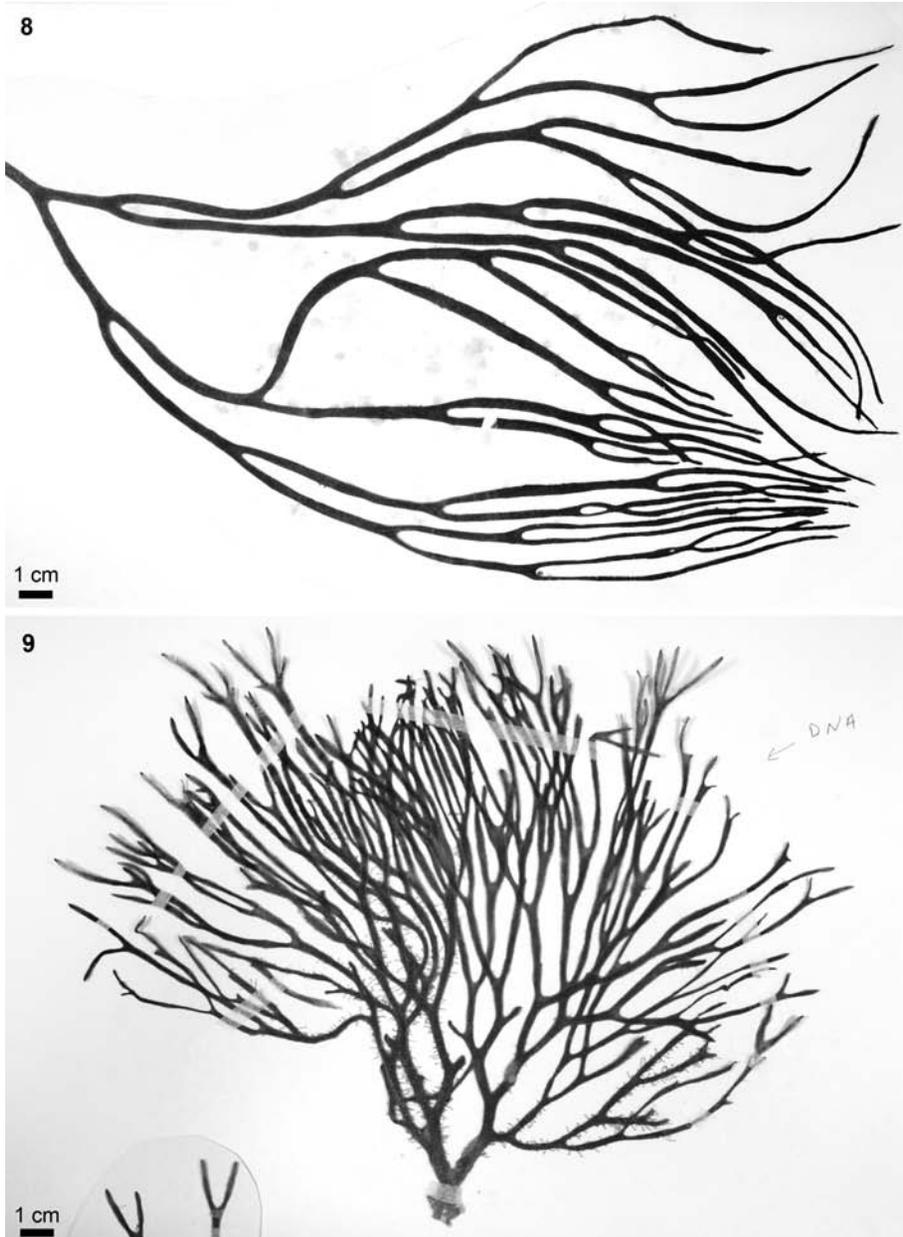
Finally, there are the species-level clusters *C_cicatrix.1* and *C_cicatrix.2*. They are very close in their morphology and DNA sequences, and we are uncertain whether they are one or two species. Samples from both clusters form large plants of 10–30 cm tall (Figs 8-9) with utricles ranging widely in size (160–230–275 μm diameter, 350–540–700 μm long, ratio 1.6–2.3–2.7). Both have flattened utricle tips with hair scars and papillae around the utricle tip, and apparently also on the utricle tip, although the presence of these scars and papillae is variable. This morphological information would suggest there is only a single species among our collections. The DNA data rather suggest that two species are present. Based on UPGMA trees showing the similarity between sequences, the specimens from Madagascar fall in two lineages indicated *C_cicatrix.1* and *C_cicatrix.2* (Fig. 10). For *tufA*, the sequence of MAD1797 (*C_cicatrix.1*) is closer to some Caribbean samples of *Codium profundum* than it is to the other lineage *C_cicatrix.2*. For *rbcL*, where we have also sequenced material from Tanzania, the sequences of *C_cicatrix.1* and *C_cicatrix.2* are also dissimilar and there is a third lineage with the Tanzanian material (TZ0111, TZ0239). The divergences between *C_cicatrix.1* and *C_cicatrix.2* (uncorrected p-distances of 0.7% for *rbcL* and 1.3% for *tufA*) are similar to that of closely related



Figs 5-7. Morphological variability in *Codium dwarkense*. **5.** Upright plant, specimen MAD0638. **6.** Small non-sprawling plant, specimen MAD0164. **7.** Sprawling plant, specimen MAD0311.

but clearly different species elsewhere in *Codium* or in other genera of Bryopsidales. For instance, in *Rhipilia*, clearly distinct but closely related species differed by 0.6-0.8% for *rbcL* and 1.1-1.4% for *tufA* (Verbruggen & Schils 2012). In *Codium*, distances between cryptic entities in *C. duthieae* (Verbruggen *et al.*, 2007) are 0.7–0.8% for *rbcL* and 0.7–2.2% for *tufA*. Another useful point of comparison is the divergence between *C. tenue* and *C. extricatum* in Fig. 10, which is similar in magnitude than the divergence between the *C. cicatrix*.1/2 lineages. At this stage of our knowledge, it is difficult to judge what the most appropriate decision about species boundaries in this group is. It will take multi-marker approaches and morphological observations on many more samples to arrive at sound conclusions.

We feel that we should also comment on the identity we have assigned to these clusters, i.e. *C. cicatrix*.1 & 2. Based on thallus form and utricle features, these collections are intermediate between *C. cicatrix* and *C. extricatum*. Silva (1959a), who described both species, did not specifically discuss the differences between these similar species, and his key relies on overlapping ranges of utricle shapes and dimensions to distinguish between them. In tropical East African collections, Van den heede & Coppejans (1996) also noted that there was a morphological grey zone between *C. cicatrix* and *C. extricatum*. Considering that our sequences of Tanzanian material also fall in the complex consisting of *C. cicatrix*.1,2&3, it seems likely that Van den heede & Coppejans (1996) were looking at material similar to ours. We have decided to apply the name *cicatrix* to our two lineages for two reasons. First, the samples have some broad cuneate utricles with papillae, features that Van den heede & Coppejans (1996) stress as typical for *C. cicatrix*. Second, there is a lineage containing South African samples matching with the description of *C. extricatum* very well (Silva, 1959a), and this lineage is clearly separated from our Madagascan lineages



Figs 8-9. The morpho-species *Codium cicatrix*. **8.** A member of species-level cluster *C_cicatrix.2*, specimen MAD0853. **9.** A member of species-level cluster *C_cicatrix.1*, specimen MAD1797.

(Figs 2 and 10). Based on these two indications, and once again stressing the need for more detailed study of this complex, it seemed appropriate to apply the name *cicatrix* to both lineages.

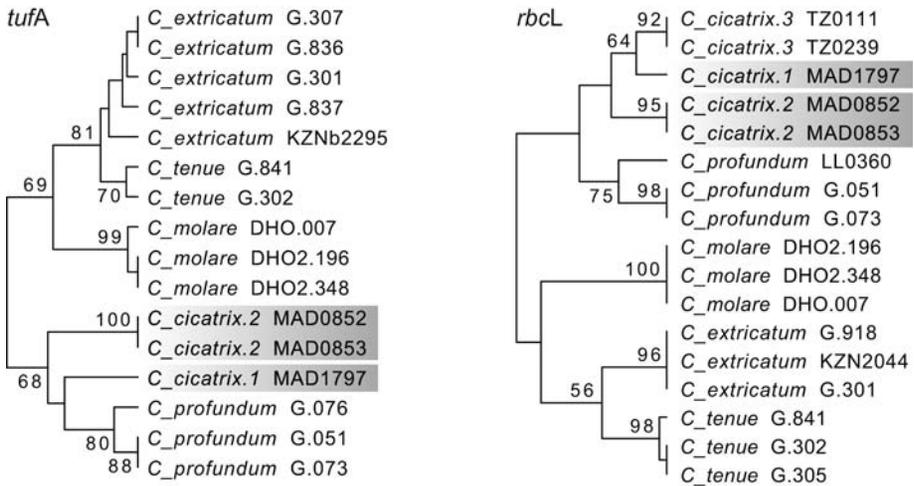


Fig. 10. UPGMA clustering trees of *tufA* and *rbcL* sequences illustrating sequence divergence between species-level clusters *C_cicatrix.1*, *C_cicatrix.2* and related entities. These trees are not to be interpreted as phylogenies; they simply depict sequence similarity. Values at nodes are bootstrap values and are only shown if > 50.

Reports not confirmed by our survey

A number of species previously reported for Madagascar (or nearby) were not among the samples we have analysed here. The mat-forming species *Codium arabicum* was reported from the coral reefs near Tuléar (Farghaly, 1980) and *C. acuminatum* from Tamatave Reef (Schmidt, 1923). The former species probably does not occur in the colder waters along the South coast. The latter, which was originally described from Toamasina further North along the Madagascar East coast, is also known from subtropical East Africa (Inhaca in Mozambique, Sodwana in South Africa) and may have been missed in our survey.

Three branched, sprawling species reported from the area were not found among our collections. *Codium vaughanii* was not reported from Madagascar but is known to occur on many surrounding islands as well as the East African coast. However, this species is now considered to be a synonym of *C. prostratum* (Silva *et al.*, 1996), which was recorded among our samples. Douterlungne (2003) reports *C. repens* from Anakao near Tuléar (specimen HEC15183, GENT). This area is much more tropical than the South coast, which may be the reason we have not found this species among our samples. A third species reported from Madagascar but not explicitly listed above is *C. geppiorum*. However, specimens matching this morphology are present in the *C. dwarkense* species-level cluster. Whether previous records of *C. geppiorum* actually belong to *C. dwarkense* or whether *C. geppiorum* does in fact occur further North in Madagascar is difficult to establish without further study.

There is one upright species with flattened branches that was reported from Mauritius (Børgesen, 1949) but not among the species we listed above: *C. bartlettii*. This species was described from Puerto Galera in the Philippines and is characterized by unequal branching of the thallus and wide branching angles (Tseng & Gilbert, 1942). In external appearance, our *C. decorticatum* plants are a

close match to *C. bartlettii* and the utricle dimensions are also comparable. Similarly, the samples we have identified as *C. arenicola* show the sort of wide unequal branching seen in *C. bartlettii*, and once again the utricle size ranges are compatible. This raises the possibility that records of *C. bartlettii* from the western Indian Ocean are *C. arenicola* or small plants of *C. decorticutum*. However, we have not studied material from Mauritius nor do we have DNA sequences of *C. bartlettii* plants from the Philippines, so this hypothesis will need to be tested in more detail in the future.

Five upright branched species with cylindrical branches have been reported from Madagascar but are not listed among our records. *Codium tomentosum* is reported from Madagascar but this species name has been applied to almost any dichotomously branched species with cylindrical branches. In reality, *C. tomentosum* is now thought to be restricted to Europe and North Africa (Silva *et al.*, 1996), a hypothesis receiving further support by the absence of DNA sequences matching *C. tomentosum* from outside Europe and North Africa in our large and globally sampled DNA datasets. *Codium extricatum* is listed for the coral reefs of Tuléar (Douterlungne, 2003) and the colder waters of Taolagnaro (Farghaly, 1980). Either we have missed this species in our survey or the earlier collections identified as *C. extricatum* belong to our *C_cicatrix.1/2* complex. As mentioned above, samples of this complex resemble *C. extricatum* in their external morphology and anatomy. The true *C. extricatum* from South Africa is a clearly separated lineage from *C_cicatrix.1* and *C_cicatrix.2* in our phylogeny. *Codium capitatum* has been reported for Taolagnaro (Farghaly, 1980). This species from temperate South Africa would be hard to mistake for any of the species present in our survey based on morphology or DNA signature, so it is likely that it has not been found during this expedition. Finally, *Codium madagascariense*, an invalid name (Silva *et al.*, 1996), was described by Farghaly (1980) based on a single specimen from the proximity of Tuléar (as *C. madagascariensis*). This upright species with fairly thick branches does not match any of our collections closely.

Biogeographic considerations

From a biogeographic perspective, the flora shows similarities to surrounding areas in the western Indian Ocean but also shows some elements currently only known from Madagascar. Several of the species are common and have a wide tropical distribution. *Codium taylorii* is found in the tropics and subtropics in both the Atlantic and Indo-Pacific basins. *Codium dwarkense* is widespread in the Indian Ocean. *Codium arenicola* is Indo-Pacific. The species *C. spongiosum* is known from subtropical regions around the world (e.g. Brazil, Hawaii, Australia, Japan). In the area, it is known from South Africa, Rodrigues and Mauritius.

There are several links with the East African coast. *Codium decorticutum* is mostly a subtropical Atlantic species but extends into the Indian Ocean along the South African coast. Most records of this species in the Indo-Pacific are likely to be misidentifications, as there appear to be no reliable morphological features distinguishing this species and those of the *C. duthieae* complex (Van den heede & Coppejans, 1996). Our molecular data suggest that *C. decorticutum* only occurs in the SE corner of the Indian Ocean (South Africa and Madagascar), and that other records in the Indian Ocean are more likely to be *C. duthieae*. *Codium prostratum* is a species distributed along the East African coast from South Africa to Kenya and has also been reported from the islands of Mauritius, Réunion

and Rodrigues, so its presence in Madagascar is unsurprising. The species *C. lucasii.cap.1* is shared with South Africa, but sequences from Madagascar differ by a few base pairs from those of South Africa, indicating that populations from these areas are unlikely to be connected by gene flow. A similar situation is seen in *C. mozambiquense* and *C. dwarkense*.

In addition to these species illustrating the link with the regional East African seaweed flora, there are a number of links with other areas. The *Codium duthieae* complex is interesting from a biogeographic perspective (Verbruggen *et al.*, 2007). It consists of three sibling species, each of which occupies a remote warm-temperate region at opposite ends of the Indian Ocean (*C. duthieae.1* in South Africa, *C. duthieae.2* in southern Australia, and *C. duthieae.3* in the Arabian Sea). Based on geographic proximity, one would expect to find the South African species *C. duthieae.1* in Madagascar, or a new entity closely related to it. Instead, we found two samples of *C. duthieae.3*, the species previously known only from the Arabian Sea (Yemen, Oman, Iran, Pakistan, and into NW India). At present we cannot explain this disjunct distribution, but judging from the lack of variation among *tufA* and *rbcL* sequences it must be a rather recent phenomenon. Whether the species evolved in Madagascar and migrated north, or vice versa, cannot be derived from our data.

The species *C. arenicola* was already mentioned as an example of a widespread tropical species. However, one of the Madagascan samples (MAD2023) is identical in sequence to a sample from Sri Lanka and is separated from the other representatives of the species. Our limited sampling of this separate lineage does not allow for solid conclusions, but this might indicate another recent link between the northern Indian Ocean and Madagascar.

C_no.ID.58 is currently known only from Madagascar and may be endemic. The species-level clusters *C. cicatrix.1* and *C. cicatrix.2* are also only known from Madagascar, but it would be premature to call them endemic species because species delimitation is problematic in this area of the tree and the surrounding areas need to be sampled more intensively.

It is clear that the limited sampling of this study is unlikely to comprehensively cover the *Codium* flora of the entire length of the southern Madagascan coastline, so undoubtedly this treatise is incomplete. Nonetheless, this study of the samples collected during the *Atimo Vatae* expedition has expanded our knowledge of the flora, bringing the species count to 12 or 13 species for the South coast (our 11-12 plus the record of *C. capitatum* but not that of *C. extricatum*), and to 17-18 for the entire island of Madagascar (up from 10; count includes *C. madagascariense* and *C. geppiorum*). The species count of 11-12 for the temperate coast is lower than that for other temperate water areas in or adjacent to the Indian Ocean, such as South Africa (19 species) and southern Australia (17 species). The expedition has also led to several insights regarding the systematics and biogeography of western Indian Ocean *Codium* species. Of special mention are the discovery of the new ball-shaped species, the insights into the wide morphological range of *C. dwarkense* as well as the distinction between *C. cicatrix* and *C. extricatum*. From a biogeographic perspective, the island appears to be a mix of common tropical and subtropical species with several western Indian Ocean species and some unique elements that may turn out to be endemics. While there are endemics at the species level, most Madagascan *Codium* endemism is situated at a lower level: the Madagascan populations of many *Codium* species with a wider range form DNA clusters separate from those of other localities, suggesting that the Madagascan populations do not mix with those from elsewhere.

Acknowledgements. We thank Bruno de Reviere, Florence Rousseau, Line Le Gall, Rob Anderson and Eric Coppejans for collecting the samples we report on. We also thank Rob Anderson and John Bolton for sending material of South African species and Klaas Pauly, Lennert Tyberghein and Katrien Van Nieuwenhuyze for help in the field in Tanzania. We thank Julien Brisset for help with collections and Sofie D'hondt for generating some of the DNA data. We thank Rob Anderson and John Huisman for their constructive reviews of this manuscript. Materials used in this study were collected during the *Atimo Vatae* expedition to South Madagascar (Principal Investigator, Philippe Bouchet), part of a cluster of Mozambique-Madagascar expeditions funded by the Total Foundation, Prince Albert II of Monaco Foundation, and Stavros Niarchos Foundation under "Our Planet Reviewed", a joint initiative of Muséum National d'Histoire Naturelle (MNHN) and Pro Natura International (PNI) in partnership with Institut d'Haliéutique et des Sciences Marines, University of Toliara (I.H.SM) and the Madagascar bureau of Wildlife Conservation Society (WCS). The Institut de Recherche pour le Développement (IRD) deployed its research catamaran *Antéa*. Funding during the preparation of this manuscript was provided by the Australian Research Council (FT110100585 to HV), the Australian Biological Resources Study (RFL213-08 to HV) and the University of Melbourne (MIRS/MIFRS to JFC).

REFERENCES

- BØRGESEN F., 1940 — Some marine algae from Mauritius. I. Chlorophyceae. *Kongelige Danske videnskabernes selskab, Biologiske meddelelser* 15 (4): 1-81.
- BØRGESEN F., 1946 — Remarks on some Codiums from the Arabian Sea. *Journal of the Indian botanical society*: 1-8.
- BØRGESEN F., 1948 — Some marine algae from Mauritius. Additional lists to the Chlorophyceae and Phaeophyceae. *Kongelige Danske videnskabernes selskab, Biologiske meddelelser* 20 (12): 1-55.
- BØRGESEN F., 1949 — Some marine algae from Mauritius. Additions to the parts previously published. *Kongelige Danske videnskabernes selskab, Biologiske meddelelser* 21 (5): 1-48.
- BØRGESEN F., 1953 — Some marine algae from Mauritius. Additions to the parts previously published. V. *Kongelige Danske videnskabernes selskab, Biologiske meddelelser* 21 (9): 1-62.
- CHACANA M., SILVA P.C., PEDROCHE F.F. & GIL-RODRIGUEZ M.C., 2003 — *Codium profundum* y *C. guineense*: nuevas citas para las islas Canarias y observaciones en *C. elisabethae* (Chlorophycota). *Vieraea* 31: 267-280.
- CHACANA M.E. & SILVA P.C., 2014 — Validation of the name *Codium arenicola* M.E. Chacana & P.C. Silva. *Nova Hedwigia* 98: 247-251.
- COPPEJANS E., LELIAERT F., VERBRUGGEN H., DE CLERCK O., SCHILS T., DE VRIESE T. & MARIE D., 2004 — The marine green and brown algae of Rodrigues (Mauritius, Indian Ocean). *Journal of natural history* 38: 2959-3019.
- DOUTERLUNGNE D., 2003 — *De mariene zeevieren (Chlorophyta, Phaeophyta, Rhodophyta) van de regio Tuléar, zuid-west kust Madagascar: floristiek en biogeografie*. Ghent, Ghent University, Biological Sciences.
- DOYLE J.J. & DOYLE J.L., 1987 — A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical bulletin* 19: 11-15.
- ESPINEL-VELASCO A.N., 2005 — Phylogeography of Arabian *Codium* species and their relation to other Indo-Pacific taxa. Ghent, Ghent University, Phycology Research Group, 34 p.
- FAMÀ P., WYSOR B., KOOISTRA W. & ZUCCARELLO G.C., 2002 — Molecular phylogeny of the genus *Caulerpa* (Caulerpales, Chlorophyta) inferred from chloroplast *tufA* gene. *Journal of phycology* 38: 1040-1050.
- FARGHALY M.E.-S., 1980 — Algues benthiques de la Mer Rouge et du bassin occidental de l'Océan Indien. Etude taxonomique et essai de répartition, notamment des Udotécées. In *Sciences*. Montpellier, Université des Sciences et Techniques du Languedoc.
- GOODMAN S.M. & BENSTEAD J.P., 2005 — Updated estimates of biotic diversity and endemism for Madagascar. *Oryx* 39: 73-77.
- NIZAMUDDIN M., 1997 — A new species of the genus *Codium* Stackh., from the coast of Pakistan. *Pakistan journal of botany* 29: 179-184.

- NIZAMUDDIN M., 1999a — *Codium saifullahii* Nizam., sp nov from the coast of Pakistan. *Pakistan journal of botany* 31: 215-218.
- NIZAMUDDIN M., 1999b — An unusual flat species of *Codium* (Codiales-Chlorophyta) from the coast of Pakistan. *Pakistan journal of botany* 31: 383-386.
- NIZAMUDDIN M., 2000 — A new cylindrical species of *Codium* Stackhouse from the coast of Karachi, Pakistan. *Pakistan journal of botany* 32: 235-238.
- NIZAMUDDIN M., 2001 — Genus *Codium* Stackhouse from northern coast of the Arabian Sea (Pakistan). *Pakistan journal of marine biology* 7: 147-232.
- NIZAMUDDIN M. & GUL S., 2005 — Occurrence of *Codium prostratum* Levering, 1938 (non-PC Silva, 1959) from northern Arabian sea coast (Pakistan). *Pakistan journal of botany* 37: 725-727.
- OLIVEIRA-CARVALHO M.F., OLIVEIRA M.C., BARRETO PEREIRA S.M. & VERBRUGGEN H., 2012 — Phylogenetic analysis of *Codium* species from Brazil, with the description of the new species *C. pernambucensis* (Bryopsidales, Chlorophyta). *European journal of phycology* 47: 355-365.
- PAYRI C.E., 1985 — Contribution to the knowledge of the marine benthic flora of La Réunion Island (Mascareignes Archipelago, Indian Ocean). *Proceedings of the Fifth International Coral Reef Congress, Tahiti* 6: 638-640.
- PROVAN J., MURPHY S. & MAGGS C.A., 2004 — Universal plastid primers for Chlorophyta and Rhodophyta. *European journal of phycology* 39: 43-50.
- SAUNDERS G.W. & KUCERA H., 2010 — An evaluation of *rbcL*, *tufA*, *UPA*, *LSU* and *ITS* as DNA barcode markers for the marine green macroalgae. *Cryptogamie, Algologie* 31: 487-528.
- SCHMIDT O.C., 1923 — *Beiträge zur Kenntnis der Gattung Codium Stackh.* Stuttgart, E. Schweizerbart (*Bibliotheca botanica* 91), 67 p.
- SHIMADA S., HIRAOKA M., SERISAWA Y. & HORIGUCHI T., 2004 — Phylogenetic studies in the genus *Codium* (Chlorophyta) from Japan. *Japanese journal of phycology* 52: S35-S39.
- SILVA P.C., 1959a — The genus *Codium* (Chlorophyta) in South Africa. *Journal of South African botany* 25: 101-165.
- SILVA P.C., 1959b — *Codium* (Chlorophyta) in the tropical western Atlantic. *Nova Hedwigia* 1: 497-536.
- SILVA P.C., BASSON P.W. & MOE R.L., 1996 — *Catalogue of the benthic marine algae of the Indian Ocean*. Berkeley, University of California Publications in Botany. p.
- SKELTON P.A., SOUTH G.R. & CRAMER J., 2007 — The benthic marine algae of the Samoan Archipelago, South Pacific, with emphasis on the Apia district. *Nova Hedwigia* 132: 1-350.
- SMITH A.G., SMITH D.G. & FUNNELL B.M., 1994 — *Atlas of Mesozoic and Cenozoic coastlines*. Cambridge, Cambridge University Press. 99 p.
- STAMATAKIS A., 2014 — RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* 30: 1312-1313.
- TSENG C.K. & GILBERT W.J., 1942 — On new algae of the genus *Codium* from the South China Sea. *Journal of the Washington academy of sciences* 32: 291-296.
- UNESCO 2012 — Southern Madagascar: the “deep south”. http://www.vliz.be/projects/marineworldheritage/sites/1.6_%20Madagascar%20S.php?item=The%20Indian%20Ocean
- VAN DEN HEEDE C. & COPPEJANS E., 1996 — The genus *Codium* (Chlorophyta, Codiales) from Kenya, Tanzania (Zanzibar) and the Seychelles. *Nova Hedwigia* 62: 389-417.
- VERBRUGGEN H., LELIAERT F., MAGGS C.A., SHIMADA S., SCHILS T., PROVAN J., BOOTH D., MURPHY S., DE CLERCK O., LITTLER D.S., LITTLER M.M. & COPPEJANS E., 2007 — Species boundaries and phylogenetic relationships within the green algal genus *Codium* (Bryopsidales) based on plastid DNA sequences. *Molecular phylogenetics and evolution* 44: 240-254.
- VERBRUGGEN H., 2010 — PMT: Partitioned Model Tester, v.1.01.
- VERBRUGGEN H., PAULY K. & DE CLERCK O., 2012 — The new species *Codium recurvatum* from Tanzania. *European journal of phycology* 47: 216-222.
- VERBRUGGEN H. & SCHILS T., 2012 — *Rhipilia coppejansii*, a new coral reef-associated species from Guam (Bryopsidales, Chlorophyta). *Journal of phycology* 48: 1090-1098.

