Phylogenetic ecology
of
octocoral - gastropod associations

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Phylogenetic ecology of octocoral - gastropod associations

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Aan opa Ger,
voor het delen van zijn liefde voor beestjes en de natuur

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Introduction and thesis outline

> "*There are some four million different kinds of animals and plants in the world. Four million different solutions to the problems of staying alive. This is the story of how a few of them came to be as they are.*"
> Sir David Attenborough – Life on Earth (1979)

Background

Symbiosis is amongst one of the most important evolutionary drivers on coral reefs. Corals cannot survive without dinoflagellates (zooxanthellae) living in their tissue because the dinoflagellates provide essential nutrients for the coral to survive and visually impaired alpheid shrimps dig out burrows and let goby fish live in the same burrow with them because the goby acts as a guard for the shrimp while it is digging the burrow. These are just two examples of many more, how species-specific interactions have evolved on coral reefs and how species have adapted. For this thesis the interactions between soft corals (Octocorallia) and marine snails of the family Ovulidae are studied. Soft corals form an important component of coral reefs because they are a host for many species such as: shrimps, brittle stars, copepods, nudibranchs, worms, fish, snails etc. Without octocorals the number of primarily marine invertebrates might be far less on coral reefs. Among the symbiotic snails are also the snails belonging to the family Ovulidae. These snails roam on the octocorals and in most cases perfectly blend in to the surrounding octocoral branches and polyps by using camouflage and mimicry.

Together these species groups are used as model taxa for research on the evolution of species associations in the marine environment and on the speciation and adaptation processes involved. Even though the octocorals and ovulid snails live in close association with each other, they both have their own evolutionary background and history of research therefore both species groups will be briefly introduced, separately, in the next two sections.

A brief introduction to Octocorallia

Octocorallia are an important component of coral reef ecosystems. Many species depend on Octocorallia as a host for unlimited food or as a refuge against potential predation. With almost 3,200 Octocorallia species described (Appeltans *et al*., 2012; Ofwegen, 2015), their abundance in shallow and deep, and tropical and polar waters houses a magnitude of other marine species such as shrimps, snails, echinoderms, copepods, fish and worms (Whitley 1970; Mase 1989; Humes, 1990; Goh *et al*., 1999; Buhl-Mortensen and Mortensen, 2004; Neves *et al*., 2007; Reijnen *et al*., 2010; Dias *et al*., 2015;). Their contribution to the marine biodiversity in the oceans is therefore of great importance. Soft corals are like stony corals, also reef builders but to a lesser extent than the stony corals do (Jeng *et al*., 2011). In addition, Gabay *et al*., (2014) showed that some species of octocoral are less susceptible to ocean acidification; octocorals have a significant advantage over the less resilient scleractinians as host species. In addition, octocorals contain secondary metabolites that can be new sources for anti-biotics or anti-cancer medicines (Pawlik, 2012) and serve as a nursery for commercially important fish species (Poulos *et al*., 2013).

The research on the ecological value of soft corals is not on par with other sessile invertebrates such as scleractinians. This is probably the result of difficulties in the identification of species which can be challenging and time consuming. Identification of Octocorallia is primarily based on the habitus and the minute calcareous skeletal parts referred to as sclerites. Each

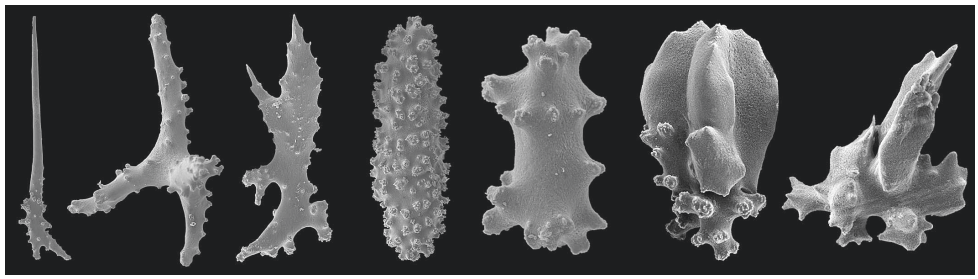


Fig. 1. Different types and shapes of sclerites found in Octocorallia photographed by Scanning Electron Microscopy (SEM). Images not to scale.

individual polyp and the tissue between the polyps, called coenenchyme, contain different types of sclerites. These sclerites showcase a plethora of shapes and forms, which are used to separate species and genera (Fig. 1).

The importance of these characteristic sclerite shapes was not recognised until the late 1800s and early 1900s, mostly because microscopes were not advanced enough at the time. As a result species-specific sclerites were not or only poorly depicted and species descriptions were quite general and could fit most other species described in that specific genus or family. This hampers present-day taxonomic/systematic research on Octocorallia. Molecular approaches (McFadden *et al*., 2006, 2014; Bilewitch *et al*., 2014; Reijnen *et al*., 2014; Zapata *et al*., 2015) help in the identification of species and integrate these results with the morphological features to study the complex relationships between species.

The Octocorallia are subdivided in three different orders (Alcyonacea, Helioporacea (Blue Corals a.o), Pennatulacea (Sea Pens)). Each of these orders is retrieved as a monophyletic group but within the largest order, the Alcyonacea, none of the described suborders were retrieved as monophyletic clades (McFadden *et al*., 2006). The systematics and taxonomy of the Alcyonacea remain inconclusive until today and can probably only be elucidated with large sampling effort of species across different oceanic bodies including the application of new molecular techniques such as RAD sequencing (Herrera and Shank, 2015) and full genome analyses (Zapata *et al*., 2015). Until then, the traditional morphological characters will remain amongst the most important features in identifying Octocorallia.

A brief introduction to Ovulidae

Linnaeus (1758) was the first to binomially treat an ovulid gastropod in his *Systema Naturae* (*Bulla ovum*). Currently this species is known as *Ovula ovum* or as the 'common egg cowry'. At the time the family Ovulidae was not yet erected and ovulid species (n=5) were placed in the genus *Bulla*. Fleming erected the family 'Ovuladae' in 1822, which was later changed in Ovulidae. Fleming did not provide an extensive description for the family, his remarks were: "Both extremities of the aperture canaliculated. The inhabitants of all the genera *Ovula*, *Calpurna*, and *Volva*, are unknown. The last genus includes the *Bulla patula* of Pennant." As said, the soft tissue for all ovulid species was unknown and they had only little shells at hand. For the Cypraeidae described seven years earlier by Rafinesque, 1815, however, Fleming mentions that species have a large mantle that can cover the shell. It is not exactly clear when soft tissue be-

came available for research, but Weinkauff (1881) compared Cypraeidae shells with those belonging to the Ovulidae and concluded that, based on the smoothness of the outer layer of the shell, and a line on the shell where both sides of the mantle meet, the Ovulidae must have a large mantle covering the shell like Cypraeidae. This is indeed one of the similarities between these two families, but it is not unique for these families and can, for example, be found in species belonging to the genera *Bulla*, *Lamellaria*, and *Trivia*.

The biggest shell morphological difference between the two is that Ovulidae lack dentation on the ventrum (ventral, axial side of aperture; and have a columellar depression instead) whereas all Cypraeidae species have dentation on both sides of the shell opening. Yet there are exceptions within the Ovulidae and some species have developed a dentated funiculum on the ventral, adapical side of the shell.

One other character that all Ovulidae have in common is that they are all associated with Anthozoa, with the largest number of species associated with Octocorallia. The first notions of association with Octocorallia were made in the late 1800s. Weinkauff (1881) mentions *Melithaea ochracea* as a host for *Ovula sempieri* (=*Prosimnia semperi*) and *Ovula spelta* (=*Neosimnia spelta*) was described as "*auf Korallen lebend*". *Neosimnia spelta* was also found by Schiaparelli *et al.* (2005), who observed different colour varieties of ovulid mantles when species were found on different hosts. Nonetheless, their molecular phylogeny showed that these morphological differences in the mantle pattern did not prove to represent different species. The

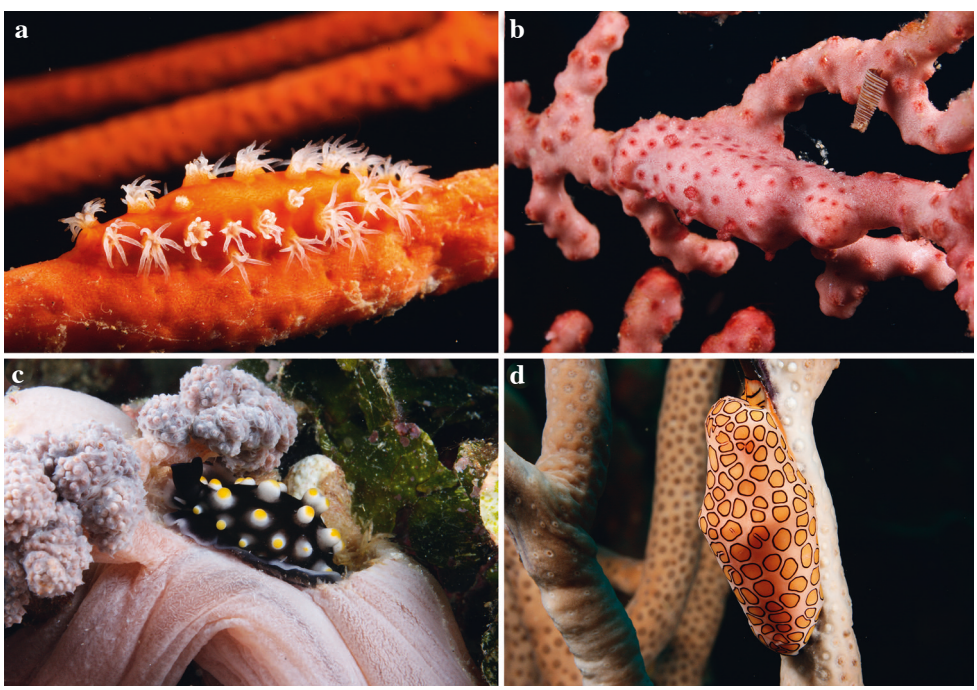


Fig. 2. a) Mimicked polyps on the mantle of an *Hiatavolva* sp. roaming on the branch of a Ellisellid b) *Prosimnia piriei* perfectly camouflaged on the branch of an *Euplexaura* sp. c) A juvenile *Ovula ovum* mimicking the habitus of a phyllidiid nudibranch d) One of the best known ovulids, *Cyphoma gibbosum*, with an aposematic mantle colouration and pattern.

mantles covering the shell in Ovulidae are to a certain degree variable in colour and are in most cases specially adapted to resemble the host coral the snails live on (camouflage) sometimes even mimicking host structures such as polyps (mimicry) or can be very unambiguous, advertising noxious properties (aposematism) (Fig. 2).

It is not exactly clear how ovulids obtain their colour but alimentary homochromy is thought to be one of the possibilities. In alimentary homochromy species sequester the pigments in their mantle by feeding on their host. This phenomenon is supported by data on European ovulid species, which shows that a species found on differently coloured hosts also showed similar differences in mantle colouration (Salvini-Plawen, 1972). Conversely, aposematic species are brightly coloured and do not match their host species, but probably use their colouration to advertise toxic properties (Rosenberg, 1992). The most common and popular aposematic species is *Cyphoma gibbosum* or better known under its vernacular name 'Flamingo tongue' which is a gregarious feeder on Caribbean octocorals (Gerhart, 1986).

Because soft tissue for ovulid species was not available for research purposes for a long time, only shell morphological features were used to systematically arrange the species in the Ovulidae. The first overview of ovulid species was commenced by Kiener (1843) and followed by Sowerby (1848), Reeve (1865), Weinkauff (1881), Tryon (1885), Schilder (1932) and Cate (1973). Each author discussed and rearranged the ovulid species according to their own studies. It was not until Schilder (1932), who had more specimens at hand by studying museum collections, that the first more modern attempt on re-classifying the family Ovulidae was conducted. The 'modern' classification attempt was later continued by Cate (1973) who was the first one to taxonomically and systematically deal with more than 100 nominal species and also provided data and images of type species. The most recent monograph on the Ovulidae deals with over 200 nominal species (Lorenz and Fehse, 2009) and also includes type localities and images of type species.

Where in the 19th century the locations for Ovulidae species often were not specified or not known at all, we now know that Ovulidae occur in all oceans except for the arctic seas. One ovulid species (*Simnia patula*) can even survive shallow cold-water environments and has been recorded from Norway (Høisæter *et al.*, 2011), which is, together with the Orkney Islands, probably the most northerly distribution for an ovulid. This is also the only ovulid species that can be found in Dutch waters (Schrieken *et al.*, 2011).

The majority of ovulid species is found on tropical coral reefs with their highest biodiversity in the "Coral Triangle". Most species occur on shallow coral reefs but some species have extended their depth distribution even below the mesophotic zone exceeding 1000 meter of depth (Lorenz and Fehse, 2009).

Molecular data on ovulids was first published by Meyer (2004) and followed by Schiaparelli *et al.* (2005). The close affiliation between Cypraeidae and Ovulidae was confirmed with the help of phylogenetic analyses by both authors. Yet, Meyer only analysed seventeen ovulids whereas Schiaparelli *et al.* (2005) reconstructed the first Ovulidae phylogeny based on 16S mtDNA sequences obtained from 33 nominal species. The authors also mapped shell morphological features on the phylogeny reconstruction and looked at the host species distribution of Ovulidae. Based on these molecular results some subfamilies were not retrieved as monophyletic entities and associations within nominal subfamilies remained problematic. The comparison with shell morphology showed that there is not much similarity in shell shape between closely affiliated clades of ovulid species. The differences in shell morphology, intrinsic asso-

ciations with host species, perfect matching of colours and ornamentations between host and snails, or the occurrences of aposematic behaviour in the snails raises the questions how the obligate associations between Octocorallia and Ovulidae have originated. Due to the close ties between these species groups and the differences in morphology and appearances in the snails they lent themselves to be model organisms to study coevolution and the development of defence strategies in the marine environment.

Thesis outline

This thesis is built upon two pillars: the Octocorallia and the Ovulidae. To unravel the relationships and evolutionary history between these species, multidisciplinary approaches were used to study the relationships between and within both species groups. Molecular data, phylogenetic approaches, molecular dating, morphological analyses, bioactivity studies and SEM photography have all been used to shed light on the relationships within and between the Octocorallia and Ovulidae. What the chapters unifies are the locations where the research has been conducted. This thesis is divided in three sections that each more or less correspond with a geographically specified oceanic basin: the Atlantic (Chapter 1 to 3) and the Indo-Pacific (Chapter 4 to 6). The only exception is Chapter 7, which combines most of the data from the previous chapters and therefore has a global approach.

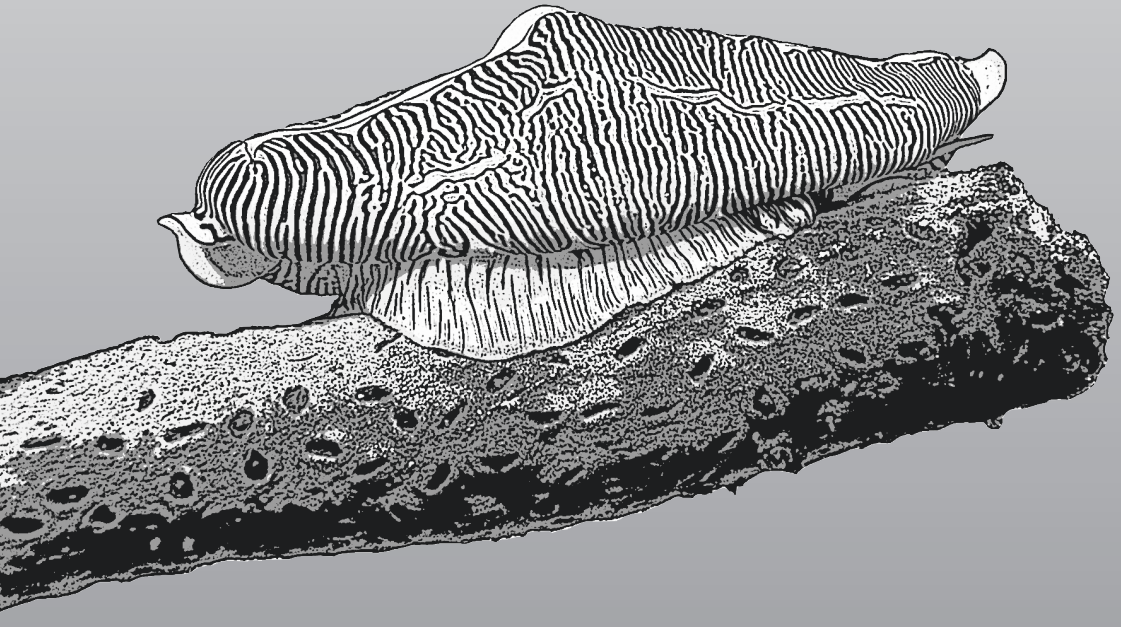
The first chapter in the 'Atlantic section' (**Chapter 1)** identifies the associations between Caribbean ovulids and octocorals and provides a provisional phylogeny reconstruction of Atlantic Ovulidae. However, in **Chapter 2**, a phylogeny reconstruction on a larger dataset with more specimens and additional molecular markers shows that synonymies are expected within Atlantic *Cyphoma*. In **Chapter 3** a more experimental approach is used to identify the bioactivity of the host corals of Ovulidae but also Cryptochiridae, which are solely associated with stony corals. With the help of a luminotester and light emitting *Aliivibrio fisheri* bacteria the bioactivity (EC_{50} values) of large numbers of Caribbean corals are assessed and plotted, together with host-symbiont data, onto phylogeny reconstructions of the respective host corals to assess if bioactivity of the hosts species is a possible driver in symbiont speciation.

In the second section, the chapters focus on octocoral and ovulid representatives from 'The Indo-Pacific'. **Chapter 4** investigates the morphological diversity and host preferences of species within the Ovulidae genus *Crenavolva*. One of these *Crenavolva* species is considered rare and confined to deep water. However there are only minor morphological differences when this species is compared to a congener from shallower depths. The systematic and taxonomic whereabouts of these *Crenavolva* species are assessed with a phylogeny reconstruction. In the next chapter, **Chapter 5**, the traditional subfamily division of the family Ovulidae is re-assessed with a large-scale molecular dataset. In particular species of the subfamily Aclyvolvinae have been troublesome in their identification. To shed light on the species identification, a four marker (mtDNA and nDNA) molecular approach, in combination with morphological analysis of the shell shapes of Aclyvolvinae species, is used. Systematic changes of genera and species in the subfamily Aclyvolvinae are proposed accordingly. In **Chapter 6** a similar approach to chapter 5 is used, on one of the most speciose gorgonian families and important hosts for Ovulidae, the Melithaeidae. The generic subdivision in the Melithaeidae has been questioned for decades by researchers. Morphological features are considered inconclusive and troublesome. To explicate on the generic divisions a combination of four molecular marker is used in combination with Scanning Electron Microscope (SEM) photographs of the morphological features of the sequenced specimens used as well as 44 type specimens of Melithaeidae species acting as a

backbone for the molecular data. Since the type specimen of the type species for the family Melithaeidae is considered lost, a new type specimen for the type species of the family is assigned. By revising the genera in the family Melithaeidae six secondary homomyns are resolved. The junior secondary homonyms are provided with new species epithets.

The final chapter, **Chapter 7**, combines all data for the Ovulidae and the Octocorallia collected from various locations in the Caribbean, Mediterranean, Red Sea and Indo-West Pacific. First the species associations are plotted in a tanglegram identifying all the relations between Octocorallia as a host and Ovulidae as symbionts. Coevolutionary events between Octocorallia and Ovulidae are tested with cophylogenetic software and to determine the evolutionary speciation rates of both species groups molecular dating is used with the help of fossil records and sequence divergence estimates.

The Atlantic



Chapter 1

Host specificity and phylogenetic relationships among Atlantic Ovulidae (Mollusca: Gastropoda)

Bastian T. Reijnen, Bert W. Hoeksema, Edmund Gittenberger

Contributions to Zoology (2010) 79: 69-78

Abstract

Ovulid gastropods and their octocoral hosts were collected along the leeward coast of Curaçao, Netherlands Antilles. New molecular data of Caribbean and a single Atlantic species were combined with comparable data of Indo-Pacific Ovulidae and a single East-Pacific species from GenBank. Based on two DNA markers, viz. COI and 16S, the phylogenetic relationships among all ovulid species of which these data are available are reconstructed. The provisional results suggest a dichotomy between the Atlantic and the Indo-Pacific taxa. Fully grown *Simnialena uniplicata* closely resembles juvenile *Cyphoma gibbosum* conchologically. *Cymbovula acicularis* and *C. bahamaensis* might be synonyms. The assignments of Caribbean host species for *Cyphoma gibbosum*, *C. signatum*, *Cymbovula acicularis* and *Simnialena uniplicata* are revised.

Introduction

Ovulid snails are obligate associates of Cnidaria. As far as known, most occur associated with octocorals (Anthozoa: Octocorallia: Alcyonacea), but in both the Caribbean and the Indo-Pacific some ovulid species feed on antipatharians (Anthozoa: Hexacorallia: Antipatharia) (Tazioli *et al*., 2007). The species of *Pedicularia* Swainson, 1840, that have been classified with the Ovulidae for a long time (Goud and Hoeksema, 2001), live on stylasterid corals (Hydrozoa: Athecatae: Filefera). *Pedicularia* differs from the undisputed ovulids in radula morphology (Simone, 2004); it is now classified in the separate family Pediculariidae (Fehse, 2007; Lorenz and Fehse, 2009). Thirty-seven species of Ovulidae Fleming, 1822, are known from the Caribbean and Atlantic area (Lorenz and Fehse, 2009). The dominant genus in the Caribbean is *Cyphoma* Röding, 1798, with 14 species of which *Cyphoma gibbosum* (Linnaeus, 1758) is the most common. Due to the low number of ovulid species in the Caribbean and the well-known diversity of Octocorallia (Bayer, 1961) parasite/host relationships are most easily studied here. It has been hypothesized that colour patterns and texture of ovulid mantles may either mislead potential predators by mimicking its host's branches and polyps, or that its colour acts as a warning of unpalatability (aposematic species) (Rosenberg, 1992; Schiaparelli *et al*., 2005). Snails closely resembling coral branches and polyps of their host may be camouflaged in such a way that they are almost undetectable for predators, such as shown by the Caribbean *Cymbovula acicularis* (Lamarck, 1810), which uses its mantle colour and protrusions to mimic the branches and polyps of its gorgonian host (Fig. 1d). As a first step to a better understanding of the associations between alcyonaceans and ovulids, Yamamoto (1972) and Schiaparelli *et al*. (2005) studied the snails and their

Fig. 1. Species in situ. a) *Cyphoma gibbosum* b) *C. signatum* c) *C. gibbosum* (juvenile) d) *Cymbovula acicularis* e) *Simnialena uniplicata* (juvenile).

hosts in the Indo-Pacific. In the present paper we primarily deal with the Ovulidae and their octocoral hosts of the Caribbean island of Curaçao. Our new data on species associations are used to investigate whether the gastropod species should be considered generalists or specialists. Based on of the molecular data from this study and GenBank, a provisional molecular phylogeny reconstruction of Ovulidae is presented.

Material and methods

In April - June 2005, both ovulid snails and their octocoral hosts were sampled using SCUBA diving at 32 localities off the leeward western coast of Curaçao, Netherlands Antilles. Observations on the windward eastern shore were hampered by exposure to

Table 1. Newly sequenced species with GenBank accession numbers and sequence data obtained from GenBank, referring to (S) Schiaparelli *et al*. (2005) and (M) Meyer (2003).

Species	16S accession number	CO-I accession number
Adamantia florida (Kuroda, 1958)	AY 161396 (M)	AY 161629 (M)
Calpurnus lacteus (Lamarck, 1810)	AY 161398 (M)	AY 161631 (M)
Calpurnus verrucosus (Linnaeus, 1758)	AY 161397 (M)	AY 161630 (M)
Crenavolva cf. *rosewateri* Cate, 1973	AY 161394 (M)	AY 161627 (M)
Crenavolva tokuoi Azuma, 1989	AY 161390 (M)	AY 161623 (M)
Cyphoma gibbosum Linnaeus, 1758	AY 161400 (M)	AY 161633 (M)
Cyphoma gibbosum Linnaeus, 1758	GU 363427	GU 363439
Cyphoma gibbosum Linnaeus, 1758	GU 363428	GU 363440
Cyphoma gibbosum Linnaeus, 1758	GU 363429	GU 363441
Cyphoma gibbosum Linnaeus, 1758	GU 363430	GU 363442
Cyphoma gibbosum Linnaeus, 1758	GU 363431	GU 363443
Cyphoma gibbosum Linnaeus, 1758	GU 363432	GU 363444
Cyphoma gibbosum Linnaeus, 1758	GU 363433	GU 363445
Cymbovula acicularis (Lamarck, 1810)	GU 363434	GU 363446
Cymbovula acicularis (Lamarck, 1810)	GU 363436	GU 363448
Cymbovula acicularis (Lamarck, 1810)	GU 363437	GU 363449
Cypraea tigris (Linnaeus, 1758)	AY 161489 (M)	AY 161722 (M)
Dentiovula takeoi Cate & Azuma, 1973	AY 534354 (M)	AY 534431 (M)
Jenneria pustulata Lightfoot, 1786	AY 161402 (M)	AY 161635 (M)
Neosimnia arcuata (Reeve, 1865)	AY 161401 (M)	AY 161634 (M)
Ovula ovum (Linnaeus, 1758)	AY 161399 (M)	AY 161632 (M)
Phenacovolva tokioi (Cate, 1973)	AY 161393 (M)	AY 161626 (M)
Phenacovolva weaveri Cate, 1973	AJ 868565 (S)	AY 161628 (M)
Primovula concinna Adams & Reeve, 1848	AY 534353 (M)	AY 534430 (M)
Prionovolva brevis Sowerby I, 1828	AY 161391 (M)	AY 161624 (M)
Prosimmia semperi (Weinkauff, 1881)	AJ 868548 (S)	AY 534432 (M)
Simnia patula (Pennant, 1777)	GU 363438	GU 363450
Simnialena uniplicata (Sowerby 2nd, 1848)	GU 363435	GU 363447
Volva volva (Linnaeus, 1758)	AY 534352 (M)	AY 534429 (M)

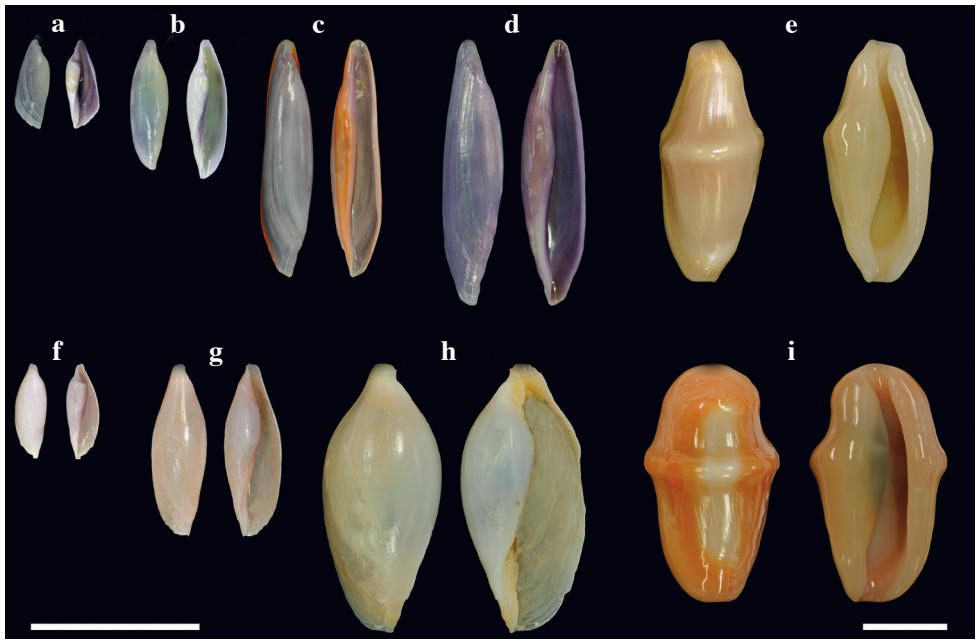


Fig. 2. Shells in dorsal and ventral view. a-d) *Cymbovula acicularis* (juvenile and adult); e) *Cyphoma signatum*; f-g) *Simnialena uniplicata* (juvenile); h) *Simnia patula*; i) *Cyphoma gibbosum*. Scale bars 1 cm (left, for a-d and f-h; right, for e and i).

the open sea. All snails and hosts were photographed in situ and collected by hand, and subsequently stored as vouchers in the collection of NCB Naturalis. Three species of the Atlantic ovulid *Simnia patula* (Pennant, 1777), dredged from the Dutch North Sea bottom by fishermen, were added as additional samples for the phylogeny reconstruction. For the Ovulidae the nomenclature accepted by the 'Checklist of European Marine Mollusca' (CLEMAM, 2008) and Cate (1973) was followed, except for *Neosimnia aequalis* sensu stricto (not Sowerby II, 1832), which is referred to as *N. arcuata* (Reeve, 1865), in accordance with Lorenz and Fehse (2009). The status of the former nominal taxon will remain uncertain as long as no valid lectotype selection has taken place, but this cannot affect the use of the latter name.

Molecular analyses

For molecular phylogeny reconstructions, tissue samples from the foot of the snails were used to extract DNA with the E.Z.N.A. Mollusc DNA Kit (Omega Biotek). The primer sets published by Meyer (2003) and Schiaparelli *et al*. (2005) were used to amplify the mtDNA markers COI and 16S marker, respectively. The PCR reaction mixtures were composed after Gittenberger *et al*. (2006). For 16S, 0.005 ml MilliQ was replaced by an equal volume of Qsolution (Qiagen). The annealing temperature used for 16S was set at 52°, whereas for COI a ramp was used, starting at 40° and ending at 44°, increasing with 0.1° s^{-1}. Sequencing was performed on a MegaBace 1000, 96 capillary

Table 2. Host-species associations between Ovulidae and Octocorallia.

Octocoral taxa		*Cyphoma gibbosum*	*Cyphoma signatum*	*Cymbovula acicularis*	*Simnialena uniplicata*
Briareidae					
Briareum	*asbestinum* (Pallas, 1766)	3	-	-	-
Gorgoniidae					
Gorgonia	*flabellum* Linnaeus, 1758	3	-	2	2
	mariae Bayer, 1961	-	-	1	-
	ventalina Linnaeus, 1758	5	-	12	2
Pseudopterogorgia	*acerosa* (Pallas, 1766)	4	-	5	-
	americana (Gmelin, 1791)	11	-	-	-
	bipinnata (Verrill, 1864)	2	-	7	-
	rigida (Bielschowsky, 1929)	2	-	-	-
Pterogorgia	*citrina* (Esper, 1792)	1	-	-	-
Plexauridae					
Eunicea	*calyculata* (Ellis & Sollander, 1786)	3	-	-	-
	clavigera Bayer, 1961	4	-	-	-
	knightyi Bayer, 1961	2	-	-	-
	succinea (Pallas, 1766)	1		-	-
	tourneforti Milne Edwards & Haime, 1857	6	-	-	-
Muricia	*muricata* (Pallas, 1766)	2	-	-	-
Plexaura	*flexuosa* Lamouroux, 1821	7		-	-
	homomalla (Esper, 1792)	1	-	-	-
Plexaurella	*dichotoma* (Esper, 1791)	3	1	-	-
	grisea Kunze, 1916	2	-	-	-
	nutans (Duchassaing & Michelotti, 1860)	1	-	-	-
Pseudoplexaura	*porosa* (Houttuyn, 1772)	6	-	-	-
Unidentified		3	-	-	-

sequencer at Leiden University, and on an Automatic Sequencer 3730xl by Macrogen, Korea. The raw sequence data were assembled and edited using Sequencher 4.2 (Gene Codes Corporation®) and aligned with ClustalX. The sequences in the COI dataset were all checked for stop codons. All sequences were referenced against GenBank (National Center for Biotechnology Information, NCBI) to ensure that non-targeted DNA had not been sequenced. For the phylogeny reconstruction, 17 GenBank sequences for both 16S and COI (Table 1), representing the same species, were combined with sequences of the Atlantic species.

For the single individual of *Cyphoma signatum*, COI could not be amplified. Therefore, this species was excluded from the dataset. The final alignment consisted of 28 sequences containing 961 base pairs. Newly sequenced species are deposited in

Table 3. Overview of collected Octocorallia that were not found to be parasitized by Ovulidae.

Anthothelidae	*Erythropodium caribaeorum* (Duchassaing & Michelotti, 1860)
Ellisellidae	*Ellisella barbadensis* (Duchassaing & Michelotti, 1864) *Ellisella elongata* (Pallas, 1766)
Plexauridae	*Eunicea calyculata coronata* Bayer, 1961 *Eunicea fusca* (Duchassaing & Michelotti, 1860) *Eunicea laciniata* Duchassaing & Michelotti, 1860 *Eunicea mammosa* Lamouroux, 1816 *Eunicea succinea plantaginea* (Lamarck, 1815) *Eunicea* sp. A *Muricea atlantica* (Kükenthal, 1919) *Muricea laxa* Verrill, 1864 *Plexaura nina* Bayer & Deichman, 1958 *Plexaura* sp. A *Pseudoplexaura flagellosa* (Houttuyn, 1772) *Pseudoplexaura* sp. A *Pseudoplexaura* sp. B *Pseudoplexaura* sp. C
Gorgoniidae	*Pseudopterogorgia* sp. A *Pseudopterogorgia* sp. B *Pterogorgia guadelupensis* Duchassaing & Michelin, 1846

GenBank under accession numbers GU363427 – GU363450. Within 16S highly variable regions exist, formed by either insertions or deletions. Aligning this region proved to be very difficult and the unalignable region, consisting of 75 base pairs in length (position 166 till 241), was deleted. To check whether both datasets could be combined, an incongruence length difference test (ILD-test) was performed. This test resulted in a P-value (P = 1.00) allowing the data combination. To determine the optimal evolutionary models the combined molecular dataset was subjected to Modeltest (Posada and Crandall, 1998). This resulted in the Generalised Time Reversible evolutionary model + invariable sites + gamma (GTR I+G). The obtained parameters were used to run a maximum likelihood search in PAUP* 4.0b (Swofford, 2003). No fewer than a 100 bootstrap replicates were used to evaluate the robustness of the nodes. The search was carried out with tree bisection-reconnection (TBR). Gaps were in all cases treated as missing character and not as a fifth character state. The selected outgroup sequence was that of the cypraeid *Cypraea tigris* (Linnaeus, 1758).

Results

Species and associations

A total of 104 samples of ovulids was collected, representing viz. *Cyphoma gibbosum* (Linnaeus, 1758), *Cyphoma signatum* Pilsbry and McGinty, 1939, *Simnialena uniplicata*

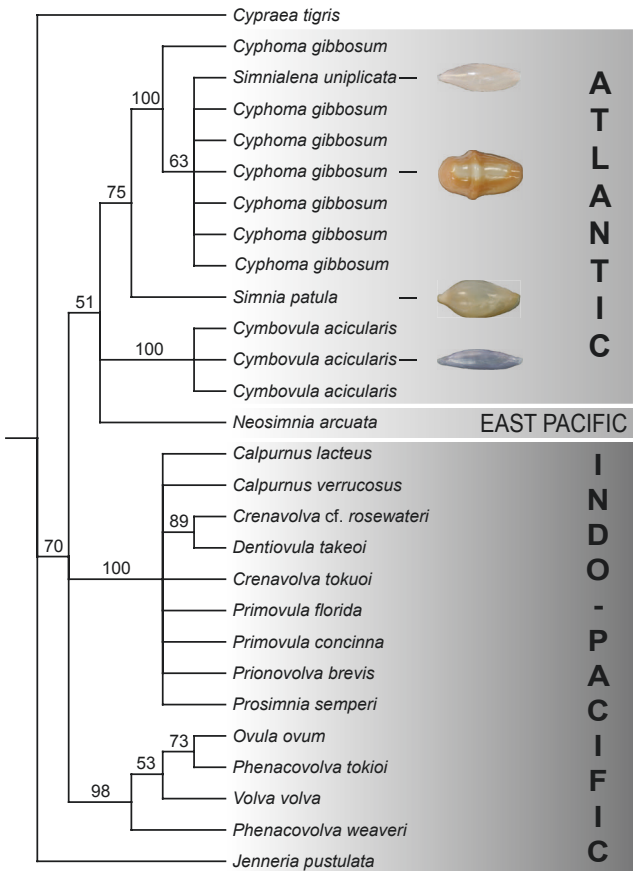


Fig. 3. Maximum likelihood analysis for combined Atlantic and Indo-Pacific Ovulidae based on 16S and CO-I data. The values above the branches represent bootstrap values (100 replicates).

(Sowerby II, 1848) and *Cymbovula acicularis* (Lamarck, 1810) (Fig. 2, including specimen resembling the so-called *C. bahamaensis*).

The 72 snails of *Cyphoma gibbosum* that could be studied were found with 21 alcyonacean species, representing nine genera. The 27 individuals of *Cymbovula acicularis* were found in association with five gorgoniid species, belonging to two genera. *Simnialena uniplicata* occurred with two congeneric host species, but since only four individual snails were found, it would be premature to derive any conclusions about host specificity. Unfortunately, only a single specimen of *Cyphoma signatum* could be studied, which was associated with *Plexaurella dichotoma* (Esper, 1791). This gorgonian species was also mentioned by Botero (1990), who additionally reported the congeneric *P. nutans* as a host for *C. signatum*. Due to the poverty of its records, the host preferences of this ovulid remain largely unknown. From a total of 46 octocoral species recorded at Curaçao, 26 (57%) were found to be occasionally parasitised by one or more ovulid species (Table 2).

Not all encountered Octocorallia species were found associated with ovulids. Additionally, a list was composed (Table 3) of encountered Octocorallia without associated ovulids.

Phylogeny reconstruction
Cyphoma gibbosum is a common Caribbean species that is easily recognized by its colour pattern and morphology. To exclude possible sibling species occurring on, for example, different hosts or at other localities at Curaçao, several individuals from different Octocorallia species and from different localities along the coast were sequenced. Based on the molecular data no sibling species occurrence was detected. In Fig. 3 the results of the combined dataset subjected to a maximum likelihood analysis (ML) with bootstrap values is presented.

The phylogeny reconstruction indicates that there is a separation between the Atlantic and the Indo-Pacific clade (moderately supported, bootstrap value 70), although the East-Pacific species *Neosimnia arcuata* clusters with the Atlantic clade (poorly supported, bootstrap value 51). *Simnialena uniplicata* (Fig. 2f-g), *Neosimnia arcuata* and *Cymbovula acicularis* (Fig. 2a-d) are characterized conchologically by long and slender shells, but in the cladogram *S. uniplicata* does not appear as sister species to either *N. arcuata* or *C. acicularis*. Instead, it forms a highly supported (bootstrap value 100) clade with the *Cyphoma* group. The individual sequence of the ovulid specimen resembling *Cymbovula bahamaensis* (Fig. 2c) forms a highly supported clade (bootstrap value 100) with the included *C. acicularis* species.

Discussion

Associations
Reported host preferences of *Cyphoma gibbosum* (Bertsch, 1984; Lasker *et al*., 1988; Botero, 1990; Nowlis, 1993; Chiaponne *et al*., 2003) are partly confirmed and supplemented with new observations of associations of this species, showing once more that *C. gibbosum* is a generalist parasite (Table 2). *Cymbovula acicularis* turned out to be another generalist. It remains unclear why 43% of the encountered alcyonacean species did not appear as hosts for ovulids at the time of our fieldwork (Table 3). It is known that Octocorallia may produce secondary metabolites as protection against predation (Ciereszko and Schneider, 1987; Chiaponne *et al*., 2003), but the effect of this defence strategy on ovulids is still largely unexplored. Other factors, such as the nutritional value of the corals (O'Neal and Pawlik, 2002) and the unpalatability of sclerites (Alstyne and Paul, 1992), may also influence host choices. An overview of natural products produced by West Indian gorgonian octocorals reveals that many types of secondary metabolites are found. The largest class of metabolites encountered in Caribbean alcyonaceans are diterpenoids, followed by the sesquiterpenes (Rodriguez, 1995). For some of these compounds that are obtained from alcyonaceans, such as *Erythropodium caribaeorum*, feeding experiments were performed, resulting in the observation that coral extracts are deterrent to fish. Also, crude extracts from the gorgonian *Gorgonia ventalina*, containing terpenoids, were used in feeding experiments with *C. gibbosum*. As a result, *C. gibbosum* consumed only 49% of an artificial diet containing terpenoids (Alstyne and Paul, 1992). The sclerites of the gorgonian species that we found as hosts for *Simnialena uniplicata* and *Cymbovula acicularis* turned out to be relatively small. The sclerites of *Gorgonia* spp. and *Pseudopterogorgia* spp. have average sizes of 0.10 mm

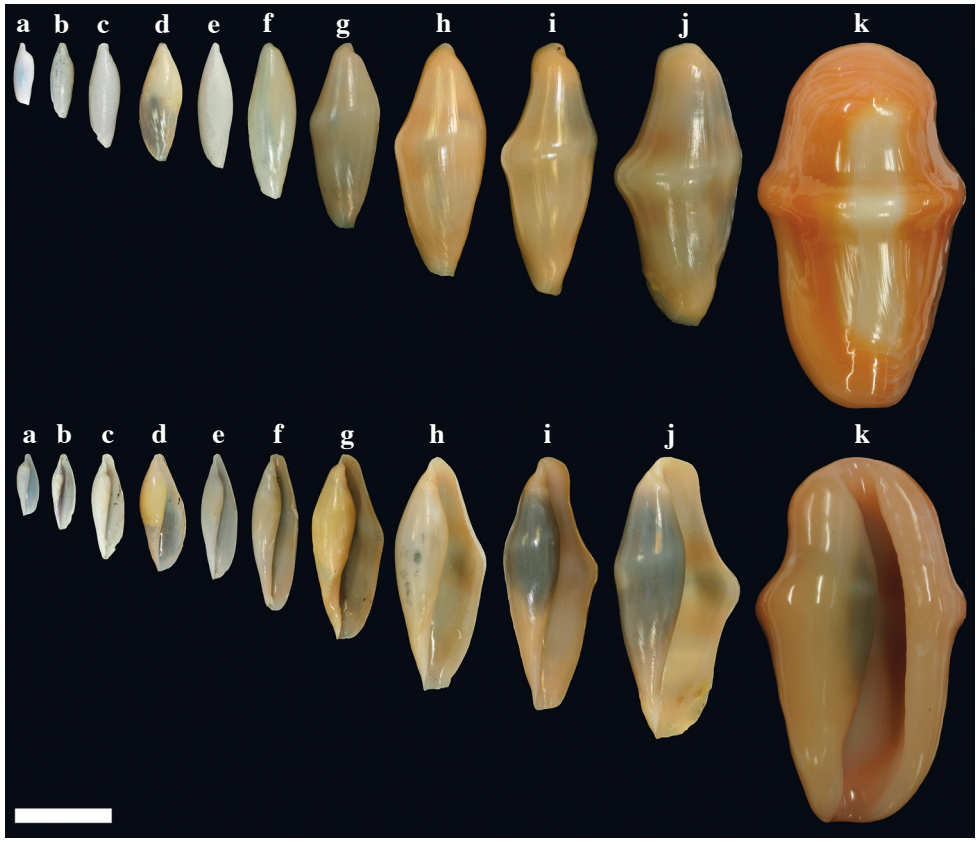


Fig. 4. Shells in dorsal and ventral view. *Simnialena uniplicata* (d) compared to a series of shells of *Cyphoma gibbosum* (a-c, e-k) in different developmental stages. Scale bar 1 cm.

and 0.10-0.15 mm, respectively, whereas 0.30-1.00 mm is common for other Octocorallia species, like *Eunicea* and *Pseudoplexaura* species (Bayer, 1961). For *Cyphoma gibbosum*, however, the sclerites' size seems to be irrelevant for host selection, since this species is found on corals containing either small or large sclerites. By analysing faecal pellets which all contained sclerites, we conclude that *C. gibbosum* at least ingests sclerites instead of eating around them.

Paedomorphosis

In the phylogeny reconstruction, *Simnia patula* is the sister group of the combined *Simnialena uniplicata* and *Cyphoma* species group. This is surprising because, at first sight, fully grown individuals of *Cyphoma* species clearly differ from *S. uniplicata* in shell morphology. However, when shells of *S. uniplicata* are compared to a series of shells of *C. gibbosum* in various growth-stages (Fig. 4), it turns out that *S. uniplicata* closely resembles juvenile *C. gibbosum* (as well as juvenile ovulids in general).

Both species lack a clear funiculum (narrow ridge of callus at the ventral side of the shell close to the aperture) and have rounded, tapering ends, character states that are absent in fully-grown *C. gibbosum*. Identification of juvenile ovulid shells is difficult, if not impossible, due to a lack of diagnostic shell characters. However, some specimens that were collected alive showed a mantle colour pattern diagnostic for *C. gibbosum*, viz. bright orange spots, encircled with a black line at a whitish background (Fig. 1). *Cymbovula acicularis* had a nearly transparent mantle, sometimes with white protuberances. In *Simnialena uniplicata* the mantle is entirely black, whereas in *Cyphoma signatum* it had a distinct yellow/black fingerprint pattern. This leads to the conclusion that the mantle colour and pattern may be diagnostic in Ovulidae (Mase, 1989) and that this character can be used to distinguish fully grown *Simnialena uniplicata* from juvenile *C. gibbosum*. Therefore, we hypothesize that *S. uniplicata* exemplifies paedomorphosis.

Systematics, biogeography and nomenclature

According to the principles of phylogenetic systematics, the species referred to as *Simnialena uniplicata* should be called *Cyphoma uniplicata* (Fig. 3). Meanwhile, the status of the nominal genus *Simnialena* Cate, 1973, with its insufficiently known type species *Simnialena marferula* Cate, 1973, remains unclear. According to Lorenz and Fehse (2009: 105), '*S. marferula* is a close relative of *S. uniplicata*'. This conclusion, on which we cannot elaborate here, is based on similarities in shell morphology. The DNA sequences of specimens belonging to *Cymbovula acicularis* and specimens that agree with the description of *C. bahamaensis* (Figs 2c-d) are almost identical. As a consequence, these nominal taxa should most probably be considered synonyms, as has also been suggested by Lorenz and Fehse (2009) based on morphological data. There is a moderately strong supported dichotomy between the Atlantic and the Indo-Pacific taxa, with *Neosimnia arcuata* from the East-Pacific having an aberrant, but poorly supported, position in the cladogram, where it clusters with the Atlantic taxa. Together, these species represent the Simniinae Schilder, 1927. Furthermore, two undisputed clades were found among the Indo-Pacific taxa, supporting the occurrence of the subfamilies Prionovolvinae Fehse, 2007, and Ovulinae Fleming, 1822, respectively. In order to get a better understanding of the phylogeny and parasite/host associations of the Atlantic Ovulidae, additional shells and DNA material are needed. DNA obtained from other ovulids occurring in the Atlantic area (e.g. *Cyphoma macumba* Petuch, 1979; *C. versicolor* Fehse, 2003; *C. mcgintyi* Pilsbry, 1939) may elucidate the taxonomical position of the genus *Cyphoma* as a monophyletic group. However, several ovulid species are rare and generally only their shells are found, which hampers further investigations.

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Chapter 2

Coat of many colours - DNA reveals polymorphism of mantle patterns and colouration in Caribbean *Cyphoma* Röding, 1798

Bastian T. Reijnen and Sancia E.T. van der Meij

Abstract

The associated fauna of corals form a major part of the biodiversity on reefs, and many taxa live in close association with a host organism. In the Caribbean the enigmatic gastropod genus *Cyphoma* is commonly observed, where it lives in association with a range of gorgonian hosts. Each species in the genus *Cyphoma* has a unique, characteristic mantle pattern and colouration, which easily separates the nominal species. Because of its abundance and recognisability *C. gibbosum* has been used as a model organism in several studies concerning allelochemicals, reef degradation, and physical defence mechanisms. Molecular analyses based on four molecular markers (COI, 16S, H3 and 28S) on four *Cyphoma* species (*C. gibbosum*, *C. mcgintyi*, *C. signatum* and an undescribed black morph) from three localities throughout the Caribbean show that they actually represent morphological varieties of a single, genetically homogeneous species. As a result *C. mcgintyi* and *C. signatum* are synonymised with the type species *C. gibbosum*. The striking morphological differences in mantle pattern and colouration are hypothesised to be the effect of an evolutionary process. Three possible scenarios for the observed morphological variety are discussed: rapid diversification, supergenes or discontinuous variation.

Introduction

Biodiversity on reefs is dominated by highly diverse invertebrate taxa that are understudied and incompletely described (Reaka-Kudla, 1997). Many of these taxa live in association with corals on which they rely for food, habitat and/or settlement cues. Arthropods are the most numerous associated taxa on [stony] corals, followed by molluscs (Stella *et al*., 2011). Goh *et al*. (1999) reported on 30 species (belonging to 17 families) living in association with gorgonians in Singapore. For the molluscs, the gorgonian associated fauna included bivalves (e.g. *Pteria*), nudibranchs (e.g. *Phyllodesmium*, *Tritonia*), and gastropods (Ovulidae). The widespread family Ovulidae occurs in all temperate and tropical oceans and all but one species (*Volva volva* (Linnaeus, 1758)) live intrinsically associated on Octocorallia and Antipatharia (Cate, 1973; Lorenz and Fehse, 2009). Ovulid snails roam the branches of their host corals and feed on the polyps and tissue. This feeding behaviour can leave big feeding scars on their hosts (Gerhart, 1990). Ovulids have a mantle, which can cover their entire shell; the different colours, patterns and appendices provide camouflage for their host or, reversely, advertise their toxicity with conspicuous, aposematic mantle patterns and colourations (Rosenberg, 1992).

The best-known Atlantic ovulid species *Cyphoma gibbosum* (Linnaeus, 1758) is better known under its vernacular name 'Flamingo tongue'. These large, brightly coloured snails can easily be detected on various gorgonian species throughout the Caribbean (Simone, 2004; Lorenz and Fehse, 2009; Reijnen *et al*., 2010; Humann *et al*., 2013). *Cyphoma gibbosum* is an easy to recognize species that can locally occur in high densities (Chiappone *et al*., 2013), and is therefore often used as a model organism. It has been used in studies dealing with allelochemicals and physical defence systems (Van Alstyne and Paul, 1992; Vrolijk and Targett, 1992; Wahlen *et al*., 2010), studies on their association with fungal diseases in Caribbean gorgonians (Rypien and Baker, 2009) and research on reef degradation and predation (Gerhart, 1990; Burkepile and Hay, 2007; Evans *et al*., 2013). The appeal of *C. gibbosum* as a model organism or "marine lab rat" is not shared by the other nominal *Cyphoma* species such as *C. signatum* Pilsbry and McGinty, 1939 or *C. mcgintyi* Pilsbry, 1939.

The genus *Cyphoma* has 14 extant species recognised by Lorenz and Fehse (2009) and 13-15 extant species according to Rosenberg (2015). Most *Cyphoma* species are considered to be relatively rare (Lorenz and Fehse 2009) and as a result there are fewer studies on these nominal *Cyphoma* species. Botero (1990), Ruesink and Harvell (1990), and Reijnen *et al*. (2010) studied the host species of *C. signatum*, whereas Ghiselin and

Fig. 1. Sequenced nominal *Cyphoma* species showing their different mantle patterns and colour varieties. a) *Cyphoma gibbosum* on *Pseudoplexaura* sp. b) *C. gibbosum* on *Pseudoplexaura* sp. c) *C. gibbosum* with atypical mantle pattern (only dots around mantle edges) on *Briareum asbestinum* d) *C.* cf. *allenae* on an *Antillogorgia americana* e) *C. signatum* on *Plexaurella dichotoma* f) Juvenile *C. signatum* on *Gorgonia ventalina* g) *Cyphoma* sp. (black morph) on *Eunicea tourneforti* h) *C. mcgintyi* from Florida, USA. Photos: a-g) B.T. Reijnen, all from Curaçao; h) Florida Museum of Natural History. ▶


a
b
c
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Wilson (1966) studied the anatomy, natural history and reproduction of this species. Apart from the before mentioned studies there are no records of *Cyphoma* species, other than *C. gibbosum*, in scientific literature. The most recent addition to the genus is *Cyphoma eludens* Lorenz and Braun, 2015. This species was described from St. Helena and the Canary Islands in the Atlantic. Two *Cyphoma* species are not found in the Atlantic Ocean but instead have an East Pacific distribution, namely *C. emarginata* (Sowerby I, 1830) and *C. arturi* Fehse, 2006. All other *Cyphoma* occur in the Atlantic on shallow reefs (intertidal) and in deep water (1200 m), from Florida to southern Brazil, and from the Caribbean to the Canary Islands and St. Helena (Lorenz and Fehse, 2009; Humann *et al*., 2013).

The majority of the *Cyphoma* species can easily be identified *in situ* with the help of the characteristic patterns and colouration of their mantle, which are considered species specific in Ovulidae (Mase, 1989; Fig. 1). In contrast, interpretation of the morphological anatomical features in *Cyphoma* can be troublesome (Simone, 2004). This is supported by Lorenz and Fehse (2009: 90) who state: "Some of the species of this genus are difficult to identify with only the shells at hand" and "Most species are characterized mainly by the colour pattern of the mantle lobes and the foot". For example, *C. signatum* and *C. mcgintyi* can easily be differentiated based on their respective colour patterns (fingerprint pattern vs. black dots), but based on just shell morphological features these species can hardly be distinguished. There are also exceptions where shell morphological features are clear in separating species, for example between *C. gibbosum* and *C. signatum*. The differences in shell outline (oval vs. rhomboid) and shell colour (often orange in *C. gibbosum*) easily separate the two (Fig. 1).

Besides the typical species-specific mantle patterns some unusual *Cyphoma* morphotypes have been recorded (e.g. Lorenz and Fehse, 2009: A202-204; Humann *et al*., 2013: 175). Because of their unusual appearance and apparent rarity these morphotypes have not yet been identified to species level, or formally described as separate species, and so far remain mysterious members of the genus *Cyphoma*. Contrasting features between nominal species identifications and morphological features such as colour patterns are not uncommon. Examples are not just from the marine realm, but also from terrestrial and aquatic environments e.g. *Caridina* fresh water shrimps and *Tylomelania* fresh water snails (Rintelen *et al*., 2004, 2007), *Heliconius* butterflies (Joron *et al*., 2011) and *Cepea* land snails (Richards *et al*., 2013).

To investigate the morphological difference in shell shape, mantle patterns and colouration in *Cyphoma* spp. more closely, we re-used data obtained for a previous study on *Cyphoma* (Reijnen *et al*., 2010) and supplemented this dataset with an additional 26 specimens belonging to three ovulid species and one unidentified morphotype. Two additional markers were supplemented to the previous data set. Here we show the results of phylogenetic analyses based on four molecular markers (COI, 16S mtDNA; 28S and H3 nDNA) tested on three nominal *Cyphoma* species and one undescribed colour variety (Fig. 2g), as well as three temperate Atlantic representatives of the subfamily Simniinae (*Cymbovula acicularis*, *Neosimnia spelta*, *Simnia patula*).

Fig. 2. Dorsal and ventral views of the shells from nominal species in this study. a) *Cyphoma signatum* (RMNH.Mol.100828) b) *C. mcgintyi* (UF.446893a) c) *C. gibbosum* (UF.446879) d) *C. mcgintyi* (UF.446893b; juvenile) e) *C.* sp. (black morph) (RMNH.Mol.337800).

Material and methods

Collecting

Cyphoma specimens and their host corals were collected during fieldwork at the leeward side of Curaçao (Dutch Caribbean) in 2005 and 2013, and from St. Eustatius in 2015 (Fig. 3).

When possible, *in situ* photographs were made to document the mantle patterns and colouration. Subsamples were taken from the host corals for their identification based on sclerite morphology. All specimens were preserved in 80% ethanol and deposited in

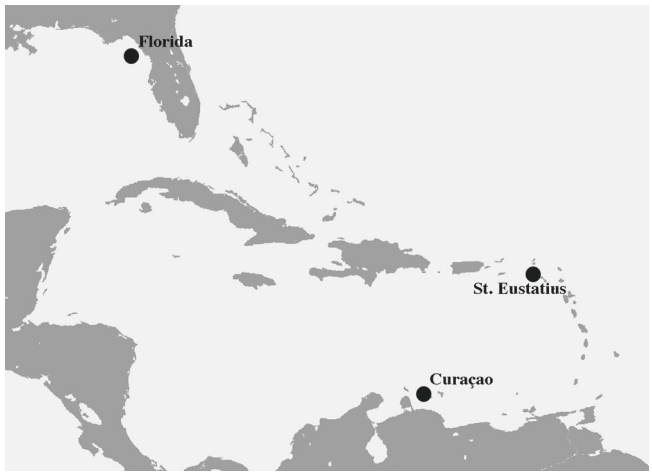

Fig. 3. Sites from which the *Cyphoma* spp. and other ovulids were collected in the Caribbean.

the mollusc collection of Naturalis Biodiversty Center, Leiden, The Netherlands (collection coded as RMNH.Mol). Three samples of *Cyphoma mcgintyi* and one additional sample of *C. gibbosum* were obtained from the Florida Museum of Natural History (FLMH; Suppl. Mat. 1). Identifications of the snails were based on Kaicher (1991), Fehse (2003), Lorenz and Fehse (2009) and Humann *et al*., (2013), their octocoral hosts were identified with the help of Bayer (1961).

Molecular analyses

Soft tissue from the foot or mantle was used for DNA extractions. Samples were either extracted individually with the DNAeasy Blood & Tissue kit, or as a part of the 'barcoding initiative' at Naturalis Biodiversity Center with the Machery-Nagel DNA extraction kit on a KingFisher Flex extraction robot. Extraction was performed according to the respective protocols, except for the lysis times, which were performed overnight (approx. 17 h.) and the final elution volume that was decreased to 100 μL and 150 μL respectively. Before PCR amplification, extracts were diluted 100 to 300 times to lower the ratio of inhibitors versus DNA. Each PCR reaction contained 2.5 μl CoralLoad PCR buffer, 0.5 μl dNTP's, 1.0 μl for each primer (Table 2), 0.3 μl Taq polymerase, 18.7 μl PCR water and 1.0 μl template. For the 28S marker, 5 μl of PCR water was replaced with 5.0 μl Q-solution. Each PCR program consisted of initial denaturation for 3 min at 95° C, followed by 39 cycles of 10 sec 95° C, specific annealing temperature (Table 1) for 1 min, with an extension of 1 min. A final extension of 10 min was used as a final step in the PCR programme. PCR amplification was performed on a C1000 Touch Thermal Cycler (Bio-RAD). Sequencing of the PCR products was performed at either Macrogen Europe or at BaseClear on an ABI Automated Sequencer 3730xl capillary sequencer. Sequences were edited in Sequencher 4.10.1.

All novel sequences are uploaded to GenBank, accession numbers: KT372440 – KT372515. Additional sequences of Caribbean ovulids (Reijnen *et al*., 2010) were downloaded from GenBank (Suppl. Mat. 1) and aligned on the GUIDANCE server

Table 1. Primer information of the markers used in this study, including annealing temperatures, sequenced regions and fragment sizes.

Primer names	Primer sequence	Region	Annealing T	Fragment size (bp)	Reference
H3F	ATGGCTCGTACCAAGCAGACVGC	Histone H3 (nuclear)	50	~ 380	Colgan *et al*., 2000
H3R	ATATCCTTRGGCATRATRGTGAC	Histone H3 (nuclear)	50	~ 380	Colgan *et al*., 2000
LSU5	TAGGTCGACCCGCTGAAYTTAAGCA	28S (nuclear)	50	~ 800	Littlewood *et al*., 2000
LSU 800rc	GACTCCTTGGTCCGTGTTTC	28S (nuclear)	50	~ 800	Reijnen, subm.
16Sar	CGCCTGTTTATCAAAAACAT	16S (mitochondrial)	52	~ 540	Palumbi *et al*., 1996
16Sbr	CCGGTCTGAACTCAGATCACGT	16S (mitochondrial)	52	~ 540	Palumbi *et al*., 1996
LCO-1490	GGTCAACAAATCATAAAGATATTGG	COI chondrial)	50	~ 660	Folmer *et al*., 1994
HCO-2198	TAAACTTCAGGGTGACCAAAAATCA	COI (mitochondrial)	50	~ 660	Folmer *et al*., 1994

Table 2. Genetic variation (%) of Atlantic Ovulidae between and within nominal *Cyphoma* species groups.

Between groups (no. of specimens)	1	2	3	4	6	7	8	9	Within groups	
1. *Cyphoma gibbosum* (n=19)									*Cyphoma gibbosum*	0.1
2. *Cyphoma* sp. (n=2)	0.1								*Cyphoma* sp.	0.1
3. *Cyphoma signatum* (n=6)	0.2	0.2							*Cyphoma signatum*	0.2
4. *Cyphoma. mcgintyi* (n=3)	0.3	0.4	0.3						*Cyphoma mcgintyi*	0.2
5. *Simnialena uniplicata* (n=1)	0.3	0.4	0.2	0.6					*Simnialena uniplicata*	-
6. *Cymbovula acicularis* (n=12)	6.2	6.6	6.7	7.1	7.8				*Cymbovula acicularis*	0.2
7. *Neosimnia spelta* (n=1)	6.5	7.1	6.7	7.7	7.3	7.4			*Neosimnia spelta*	-
8. *Simnia patula* (n=1)	7.7	8.7	8.1	9.5	8.9	9.2	7.6		*Simnia patula*	-
9. Outgroup (n=1)	9.3	9.6	10.3	10.1	11.6	10.2	12.0	12.7	Outgroup	-

(Penn *et al*., 2010) using the MAFFT algorithm (alignment score: 0.792612). Gene regions that could not be amplified for certain specimens were replaced by N's in the final alignment.

The final alignment contains 46 specimens and the concatenated dataset is 2,355 base pairs in length including insertions and/or deletions. The Indo-Pacific species *Ovula ovum* (Linnaeus, 1758) was selected as outgroup. The dataset of the individual markers were subjected to the model-testing algorithm in jModeltest and MEGA6 (Tamura *et al*., 2013), and were based on the uncorrected Akaike Information Criterion (AIC). Bayesian analyses were performed in MrBayes 3.2.0 (Ronquist and Huelsenbeck, 2003) and were run for 4,000,000 generations with six chains. Data was sampled every 100 generations. The final split frequency between the two independent runs was < 0.01. Garli2.0 (Zwickl, 2006) was used to determine the phylogenetic relationships based on the maximum likelihood approach. The phylogeny was reproduced using 1,000 bootstrap iterations. Species delimitation was tested with an Automatic Barcode Gap Discovery tool (ABGD; Puillandre *et al*., 2011). Default settings were used and analysis was performed with the Jukes-Cantor (JC69) algorithm. Genetic distances were calculated in MEGA6.

Results

The phylogram (Fig. 4) shows three groups containing from top to bottom: 1) *Cyphoma* spp. including *Cyphoma* sp. (RMNH.Mol.100770) which was formerly identified as *Simnialena uniplicata* (Reijnen *et al*., 2010), 2) *Cymbovula acicularis*, 3) *Neosimnia spelta* and *Simnia patula*. All groups are well supported by the Bayesian and maximum-likelihood analysis analyses. Phylogenetic relationships between *Cymbovula acicularis* and the group containing *Neosimnia spelta* and *Simnia patula* have low support values (57/87). Within the clade containing the nominal *Cyphoma* species there is no clustering observed concordant with the respective species identifications (*C. gibbosum*, *C. signatum*, *C. mcgintyi*, *Cyphoma* sp. black morph). There is however a small cluster of specimens that is highly supported (95/85), which contains all three *C. mcgintyi* specimens and one representative of *C. signatum*, but the branch lengths are short.

In the alignment only five nucleotide sites out of 2,347 positions support the grouping of these four specimens. One of these nucleotide sites is within the non-coding 16S region, while the other four are situated in the coding COI region. Each of these four nucleotide sites are on the third codon position of the protein translation and hence do not change the translation of the protein coding alignment when compared with the other *Cyphoma* spp. All other nominal *Cyphoma* species are distributed randomly throughout the group and do not show phylogenetic affinities based on morphological characters.

To investigate the observed random positioning of the nominal *Cyphoma* species in more detail, the genetic distances between and within the nominal *Cyphoma* species were calculated (Table 2). Genetic distance values within species were almost as low as between *Cyphoma* species (within range: 0.1-0.2%; between range: 0.1-0.4%). When distances values were calculated between *Cyphoma* spp. and *Cymbovula acicularis*,

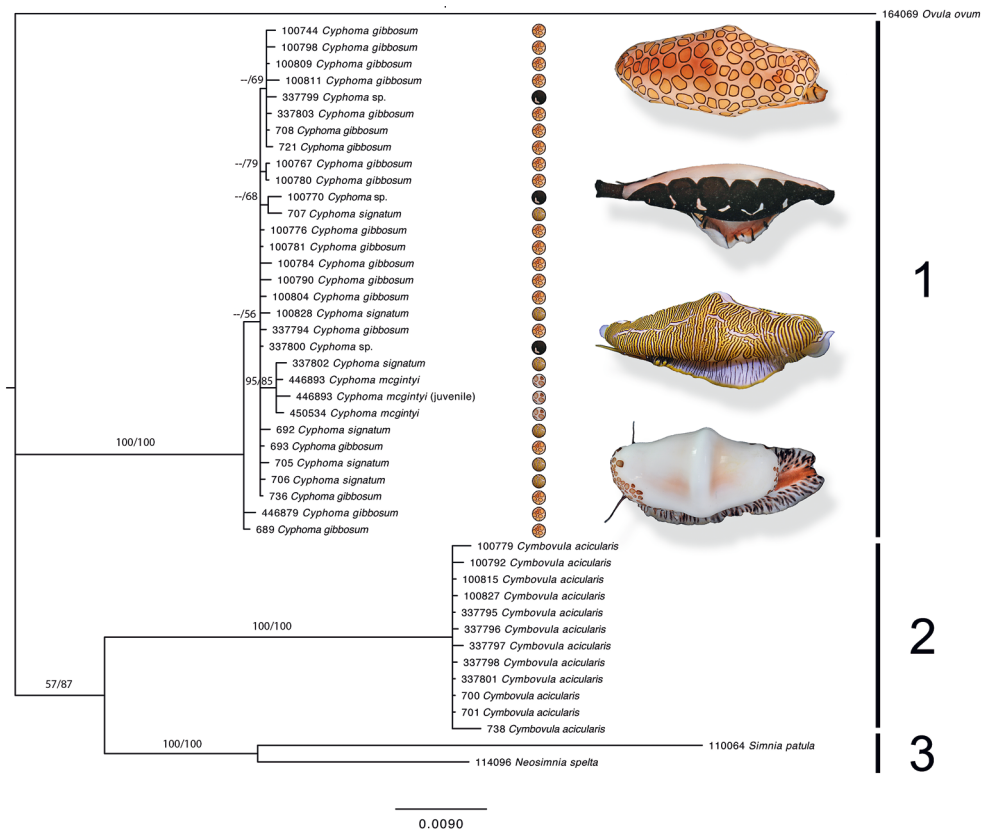

Fig. 4. Phylogram based on the Bayesian consensus tree showing Bayesian inference support values (left) and Maximum likelihood bootstrap support values (right). For the *Cyphoma* species their respective characteristic mantle patterns are depicted per specimen including photographs of the live animals (not to scale).

Simnia patula or *Neosimnia spelta* genetic distance values were notably higher (0.1-0.4% between *Cyphoma* spp. vs. 6.5-8.1% between *Cyphoma* spp. or *Cymbovula acicularis*, *S. patula* and *N. spelta*).

To test whether the phenotypic differences in colours and patterns comprise different nominal *Cyphoma* species, an ABGD species delimitation analysis was performed. This test resulted in five groupings: 1) *Cyphoma gibbosum*, *C. mcgintyi*, *C. signatum*, *Cyphoma* sp. black morph, *Simnialena uniplicata* 2) *Cymbovula acicularis* 3) *Neosimnia spelta* 4) *Simnia patula* 5) *Ovula ovum*. The ABGD results are therefore congruent with the results from the phylogenetic analyses and do not differentiate the nominal *Cyphoma* species (with their unique mantle patterns and colouration) in separate species groups.

The earlier identification of *Simnialena uniplicata* in Reijnen *et al.*, (2010) most likely constitutes a misidentification. In the phylogenetic analyses *S. uniplicata* clusters with all other *Cyphoma* species, without significant genetic difference between these

specimens. Clear diagnostic characters are missing, because of the juvenile state of this particular specimen. Based on the photograph in Reijnen *et al*. (2010: Fig. 1e), a similar phenotype as *Cyphoma* sp. black morph is apparent and therefore this specimen is now identified as such.

Discussion

Mantle colouration and mantle patterns in Ovulidae were long thought to be diagnostic species characters and were used as such by several authors (Mase, 1989; Reijnen *et al*., 2010; Lorenz and Fehse, 2012; Lorenz and Brown, 2015). Mase (1989) did not only look at the shell and mantle, but also patterns and colours on the foot, antenna and siphon of Japanese ovulids. The very distinct and recognisable mantle patterns such as the yellow/black fingerprint pattern in *C. signatum* and the orange coloured dots in *C. gibbosum* have so far been used to separate these species. Nevertheless, the morphological characters (mantle patterns, mantle colouration, shell morphological features) in *Cyphoma* do not correspond with the observed genetic results. The genetic distance values between nominal *Cyphoma* species (Table 2) are comparable with the genetic variation found in Indo-Pacific *Crenavolva* (Reijnen, 2015). In that specific case *Crenavola chiapponii* was synonymised with *C. aureola* based on genetic data and morphological similarity. The discrepancy between the morphological data and the molecular results in this study are difficult to reconcile. However, various scenarios can explain the findings presented here. Possible hypotheses for example are: rapid diversification, supergenes or discontinuous variation.

In a scenario of rapid divergence, trophic specialisation is frequently a key feature that characterises sister species (Vailant *et al*., 2011). Such trophic specialisation is not known in the *C. gibbosum* "complex". Nominal *Cyphoma gibbosum* is a generalist predator that has been found associated with at least 21 different host species from at least nine different genera (Reijnen *et al*., 2010). Morphotypes resembling nominal *Cyphoma signatum* are uncommon on most reefs and as a result ecological data is rare for this species. Most specimens are found on the genus *Plexaurella*, yet a juvenile resembling *C. signatum* was observed on *Gorgonia ventalina* (Fig. 1f) suggesting that *C. signatum* is a less species-specific feeder on octocorals than previously assumed.

The second scenario is that the phenotypic diversity in *Cyphoma gibbosum* is regulated by a supergene. A supergene consists of multiple strongly linked loci that determine the phenotype, without difference in the studied molecular markers (this study 28S, 16S, H3, COI) (Joron *et al*., 2006, 2011). The typical *Cyphoma gibbosum* orange-spotted-morph would be the general phenotype and rare phenotypes, such as the yellow fingerprint pattern in *C. signatum*, the less common morph (Cook, 2005). In case of the shell morphological features it is more difficult to reconcile the data. Reijnen (2015) showed that in Ovulidae minor shell morphological characters used for separating nominal species should be considered a morphological variety within a single species. The presence of different morphotypes in a species is not unique within the family Ovulidae. Schiaparelli *et al*. (2005) recognised different morphotypes for one Atlantic/

Mediterranean and four Indo-Pacific species (*Neosimnia spelta*, *Pellasimnia brunneiterma*, *Dentiovula dorsuosa*, *Diminovula punctata* and *Habuprionovolva aenigma*, respectively) and could not discriminate between the morphs based on 16S molecular data.

A third hypothesis is that *Cyphoma gibbosum* is "caught in speciation" which is reflected by the discontinuous variation in morphology, but (not yet) in the studied genes. This hypothesis is supported by the idea that "phenotype precedes genotype" is a common mode of evolution (Palmer, 2004). A similar case was observed in the shrimp species *Conchodytes meleagrinae* (Fransen and Reijnen, 2013). Shrimp specimens from different bivalve hosts showed very dissimilar colour patterns and were thought to be distinct species. Molecular analyses showed that based on their genetic barcodes these species could not be distinguished from each other. It was therefore hypothesised that this species recently speciated.

It is unclear which of the proposed hypotheses is the most parsimonious and fits the observed results for the genus *Cyphoma* the best. What should be considered is that morphological features in the Ovulidae are probably more plastic than previously thought (Schiaparelli *et al*., 2005; Reijnen, 2015). The closely related cowrie family Cypraeidae shows a contrasting pattern where multiple cryptic lineages have been uncovered by genetics (Meyer, 2003; Moretzsohn, 2014), as is the case in many other taxa (Bickford *et al*., 2007). Reports of distinct morphospecies attributed to a single, genetically homogeneous species are available, but far less common. Several reports are however available, for example: polychaetes (Willette *et al*., 2015, and references therein), sea stars (Harley *et al*., 2006), and caridean shrimps (Bauer, 2004).

Taxonomic account

Resulting from the molecular results and species delimitation analysis as presented before, *Cyphoma signatum* and *C. mcgintyi* need to be synonymised with *Cyphoma gibbosum*. The synonymy of this species is as follows:

Family Ovulidae Fleming, 1822
Genus *Cyphoma* Röding, 1798
Cyphoma gibbosum (Linnaeus, 1758)

Bulla gibbosa Linnaeus, 1758: 726
? *Cyphoma dorsatum* Röding, 1798: 21
? *Ovula pharetra* G. Perry, 1811: pl. 53, fig. 2
? *Ovula rostrata* Mörch, 1877: 53
? *Cyphoma precursor* Dall, 1897
Cyphoma alleneae Cate, 1973: 67-68, figs 151, 151c
Cyphoma signata Pilsbry & McGintyi, 1939: 3, pl. 1, fig. 1, 1a, 2, 2a, 9, 10
Cyphoma mcgintyi Pilsbry, 1939: 108
? *Cyphoma macumba* Petuch, 1979: 515-517, figs. 1c-d, 2b-c
Simnialena uniplicata. — Reijnen *et al*., 2010: fig. 1e, 2f-g

Remarks: Ghiselin and Wilson (1966) already mentioned that there are no striking morphological differences between *C. gibbosum* and *C. signatum* when it comes to the external morphology and mantle cavity. The radular morphology of *C. gibbosum* and other Atlantic ovulids was studied by Bandel (1984) and Simone (2004) and both concluded that radular morphology does not differ significantly between ovulid species. Reid (2000) warns about using radular morphology as a morphological character, because of ecophenotypic plasticity, convergence and intraspecific variation.

Simone (2004: 88) included *C. allenae* in the synonymy of *C. gibbosum*, albeit without further discussion. Additionally, he discussed the taxonomy and systematics of other *Cyphoma* species such as *C. intermedium*, *C. macumba* and *C. signatum*. According to Rosenberg (1996, in Simone 2004) and Simone (2004) *C. macumba* is a possible synonym of *C. signatum*. Simone (2004) investigated the type species and did not observe clear morphological differences based on the shells alone. Nevertheless, Lorenz and Fehse (2009) separated the species based on their mantle features and a minor shell morphological feature (callus-denticles on the outer labrum). Here we provisionally follow Simone's (2004) suggestion that *C. macumba* is a synonym of *C. signatum*, and hence *C. gibbosum*. Rosenberg (2009) includes the following synonymies of *C. gibbosum*: *Cyphoma dorsatum* Röding, 1798, *Ovula pharetra* G. Perry, 1811, *Ovula rostrata* Mörch, 1877, and *Cyphoma precursor* Dall, 1897. We provisionally included these synonymies here as well.

Simone (2004) also investigated the anatomy of *Cyphoma intermedium* (Sowerby I, 1828). The discussion of this species starts with the statement that this species has a highly variable shell and that it closely resembles some more recently described *Cyphoma* species (*C. kathiewayae*, *C. guerrini*, *C. macumba*, *C. rosenbergi*). According to Simone these species all fit in the range of the two most extreme phenotypes of *C. intermedium* and are therefore likely synonyms of the latter.

Variability of morphological characters, in combination with molecular data, should be taken into account in future research on Ovulidae. Unnecessary profusion of species names and other taxonomical problems can be avoided by assessing both morphological and molecular data. It is very likely that more synonymies, rather than species, need to be uncovered in the taxonomy of the Ovulidae.

Acknowledgements

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Suppl. Mat. 1. Species information and GenBank accession codes including references, an asterisk marks GenBank accession numbers which were previously misidentified in Reijnen *et al*., 2010.

Species	Code	Location (locality code)	Longitude / Latitude	Depth (m)
Cymbovula acicularis (Lamarck, 1810)	RMNH.Mol. 100779	Curaçao, Barank'i Karanito, CUR.15	12°02'13.5"N 68°48'14.2"W	25
Cymbovula acicularis (Lamarck, 1810)	RMNH.Mol.100792	Curaçao, Daaibooi, CUR.19	12°12'42.9"N 69°05'0°5.4"W	6
Cymbovula acicularis (Lamarck, 1810)	RMNH.Mol.100815	Curaçao, Caracasbaai, CUR.17	12°04'25.8"N 68°51'46.5"W	17
Cymbovula acicularis (Lamarck, 1810)	RMNH.Mol.337795	Curaçao, Blauwbaai, CAO.04	12°08'05.7"N 68°59'03.5"W	10
Cymbovula acicularis (Lamarck, 1810)	RMNH.Mol.337796	Curaçao, St. Marie, CAO.06	12°11'53.0"N 69°04'45.1"W	14
Cymbovula acicularis (Lamarck, 1810)	RMNH.Mol.337801	Curaçao, Marie Pampoen, CAO.21	12°05'26.7"N 68°54'17.8"W	10
Cymbovula acicularis (Lamarck, 1810)	RMNH.5004206	St. Eustatius, The Blocks, EUX015	17°27'50.9"N 62°59'06.8"W	-
Cymbovula acicularis (Lamarck, 1810)	RMNH.5004207	St. Eustatius, The Blocks, EUX015	17°27'50.9"N 62°59'06.8"W	-
Cymbovula acicularis (Lamarck, 1810)	RMNH.5004208	St. Eustatius, Blind Shoal, EUX039	17°30'37.1"N 63°00'27.1"W	-
Cyphoma gibbosum (Linnaeus, 1758)	RMNH.Mol.100767	Curaçao, Blauwbaai, CUR.10	12°22'29.9"N 68°59'27.7"W	8
Cyphoma gibbosum (Linnaeus, 1758)	RMNH.Mol.100776	Curaçao, Playa Jeremy, CUR.12	12°19'44.1"N 69°09'00.2"W	17
Cyphoma gibbosum (Linnaeus, 1758)	RMNH.Mol.100780	Curaçao, Barank'i Karanito, CUR.15	12°02'13.5"N 68°48'14.2"W	10
Cyphoma gibbosum (Linnaeus, 1758)	RMNH.Mol.100781	Curaçao, Barank'i Karanito, CUR.15	12°02'13.5"N 68°48'14.2"W	9
Cyphoma gibbosum (Linnaeus, 1758)	RMNH.Mol.100804	Curaçao, Sint Michielsbaai, CUR.21	12°08'50.9"N 68°59'56.6"W	14
Cyphoma gibbosum (Linnaeus, 1758)	RMNH.Mol.100809	Curaçao, Superior Producer, CUR.22	12°05'21.5"N 68°56'35.5"W	18
Cyphoma gibbosum (Linnaeus, 1758)	RMNH.Mol.100811	Curaçao, Superior Producer, CUR.22	12°05'21.5"N 68°56'35.5"W	5
Cyphoma gibbosum (Linnaeus, 1758)	RMNH.Mol.337794	Curaçao, Waterfabriek I, CAO.02	12°06'31.0"N 68°57'01.2"W	-
Cyphoma gibbosum (Linnaeus, 1758)	UF.446879	USA, Florida, N of St. Petersburg	28°35'55.7"N 84°15'41.4"W	27
Cyphoma gibbosum (Linnaeus, 1758)	RMNH.5004209	St. Eustatius, Aquarium, EUX012	17°30'22.6"N 63°00'22.0"W	-
Cyphoma gibbosum (Linnaeus, 1758)	RMNH.5004210	St. Eustatius, Aquarium, EUX012	17°30'22.6"N 63°00'22.0"W	-
Cyphoma gibbosum (Linnaeus, 1758)	RMNH.5004211	St. Eustatius, Blairs Reef, EUX019	17°28'13.6"N 62°59'30.2"W	-
Cyphoma gibbosum (Linnaeus, 1758)	RMNH.5004212	St. Eustatius, Twelve Guns, EUX029	17°28'12.8"N 62°58'58.7"W	-
Cyphoma gibbosum (Linnaeus, 1758)	RMNH.5004213	St. Eustatius, Blue Bead Hole II, EUX037	17°28'37.4"N 62°59'29.6"W	-
Cyphoma mcgintyi (Pilsbry, 1939)	UF.446893a	USA, Florida, N of St. Petersburg	28°32'16.1"N 84°16'21.7"W	26

Host organism	COI	16S	28S	H3	Reference
Antillogorgia acerosa (Pallas, 1766)	GU363447 *	GU363434	-	KT372494	Reijnen *et al*., 2010; this publication
Gorgonia ventalina (Linnaeus, 1758)	GU363448	GU363436	KT372474	KT372497	Reijnen *et al*., 2010; this publication
Antillogorgia bipinnata (Verrill, 1864)	GU363449	GU363437	-	KT372501	Reijnen *et al*., 2010; this publication
Gorgonia ventalina (Linnaeus, 1758)	KT372445	KT372460	KT372481	KT372507	this publication
Antillogorgia acerosa (Pallas, 1766)	KT372446	KT372461	KT372482	KT372508	this publication
Antillogorgia acerosa (Pallas, 1766)	KT372449	KT372464	KT372485	KT372511	this publication
-	KX360175	KX360216	KX360199	KX360187	this publication
-	KX360176	KX360217	KX360200	KX360188	this publication
Antillogorgia	KX360177	KX360218	KX360202	KX360189	this publication
Antillogorgia acerosa (Pallas, 1766)	KT372440	-	KT372470	KT372491	this publication
Briareum asbestinum (Pallas, 1766)	-	KT372455	KT372471	KT372493	this publication
Gorgonia ventalina (Linnaeus, 1758)	GU363440	GU363428	KT372472	KT372495	Reijnen *et al*., 2010; this publication
Plexaurella nutans (Duchassaing & Michelotti, 1860)	GU363444	GU363432	KT372473	KT372496	Reijnen *et al*., 2010; this publication
Pseudoplexaura porosa (Houttuyn, 1772)	GU363443	GU363431	KT372475	KT372498	Reijnen *et al*., 2010; this publication
Antillogorgia americana (Gmelin, 1791)	GU363446 *	GU363433	KT372476	KT372499	Reijnen *et al*., 2010; this publication
Muricea muricata (Pallas, 1766)	GU363441	GU363429	KT372477	KT372500	Reijnen *et al*., 2010; this publication
Eunicea sp.	KT337794	KT372459	KT372480	KT372506	this publication
unknown	KT372451	KT372466	KT372487	KT372513	this publication
-	KX360170	KX360209	KX360192	KX360180	this publication
-	-	KX360211	KX360194	KX360181	this publication
Plexaura nina/homomalla	KX360172	KX360213	KX360196	KX360183	this publication
-	-	KX360206	-	KX360179	this publication
-	KX360171	KX360212	KX360195	KX360182	this publication
unknown	KT372452	KT372467	KT372488	KT372514	this publication

Suppl. Mat. 1. Cont.

Species	Code	Location (locality code)	Longitude / Latitude	Depth (m)
Cyphoma mcgintyi (Pilsbry, 1939)	UF.446893b	USA, Florida, N of St. Petersburg	28°32'16.1"N 84°16'21.7"W	26
Cyphoma mcgintyi (Pilsbry, 1939)	UF.450534	USA, Florida, NNW of St. Petersburg, S of Big Bend area	28°39'04.0"N 84°23'03.8"W	26-30
Cyphoma signatum Pilsbry & McGinty, 1939	RMNH.Mol.100828	Curaçao, Marie Pampoen /Carpile, CUR.05	12°05'42.1"N 68°54'43.0"W	5
Cyphoma signatum Pilsbry & McGinty, 1939	RMNH.Mol.337802	Curaçao, Marie Pampoen, CAO.21	12°05'26.7"N 68°54'17.8"W	8
Cyphoma signatum Pilsbry & McGinty, 1939	RMNH.5004214	St. Eustatius, Aquarium, EUX012	17°30'22.6"N 63°00'22.0"W	-
Cyphoma signatum Pilsbry & McGinty, 1939	RMNH.5004215	St. Eustatius, Shark Reef, EUX018	17°30'37.1"N 63°00'27.1"W	-
Cyphoma signatum Pilsbry & McGinty, 1939	RMNH.5004216	St. Eustatius, Blairs Reef, EUX019	17°28'13.6"N 62°59'30.2"W	
Cyphoma signatum Pilsbry & McGinty, 1939	RMNH.5004217	St. Eustatius, Shark Reef, EUX018	17°30'37.1"N 63°00'27.1"W	-
Cyphoma sp.	RMNH.Mol.337799	Curaçao, Holiday Beach, CAO.11	12°06'34.1"N 68°56'49.3"W	4
Cyphoma sp.	RMNH.Mol.337800	Curaçao, Holiday Beach, CAO.11	12°06'34.1"N 68°56'49.3"W	4
Neosimnia spelta (Linnaeus, 1758)	RMNH.Mol.114096	Spain, Begur, Aigua Blava	41°56'08,0"N 03°13'04,9"E	<15
Ovula ovum (Linnaeus, 1758)	RMNH.Mol.164069	Malaysia, Borneo, Kapalai Island, SEM.10	04°13'04,8"N 118°40'20,1"E	3
Simnia patula (Penant, 1777)	RMNH.Mol.110064	North Sea, S side of Doggersbank	54°20'N 02°20'E	-
Simnialena uniplicata (Sowerby II, 1848)	RMNH.Mol.100770	Curaçao, Marie Pampoen/Carpile, CUR.05	12°05'42.1"N 68°54'43.0"W	18

Host organism	COI	16S	28S	H3	Reference
unknown	KT372453	KT372468	KT372489	KT372515	this publication
unknown	KT372454	KT372469	KT372490	-	this publication
Plexaurella dichotoma (Esper, 1791)	KT372441	KT372456	KT372478	KT372502	this publication
Gorgonia ventalina (Linnaeus, 1758)	KT372450	KT372465	KT372486	KT372512	this publication
-	KX360173	KX360214	KX360197	KX360184	this publication
Plexaurella nutans	-	KX360204	-	-	this publication
Plexaurella dichotoma	-	KX360210	KX360193	-	this publication
Plexaurella nutans	KX360169	KX360205	-	KX360178	this publication
Eunicea tourneforti Milne Edwards & Haime, 1857	KT372447	KT372462	KT372483	KT372509	this publication
Eunicea tourneforti Milne Edwards & Haime, 1858	KT372448	KT372463	KT372484	KT372510	this publication
Leptogorgia sarmentosa (Esper, 1789)	KT372442	KT372457	-	KT372504	this publication
Sarcophyton glaucum (Quoy & Gaimard, 1833)	KT372443	KT372458	KT372479	KT372505	this publication
Alcyonium digitatum Linnaeus, 1758	GU363450	GU363438	-	KT372503	Reijnen *et al*., 2010; this publication
Gorgonia flabellum Linnaeus, 1758	GU363445 *	GU363435	-	KT372492	Reijnen *et al*., 2010; this publication

Chapter 3

Bioactivity of Caribbean corals related to their associated fauna

Bastian T. Reijnen and Sancia E.T. van der Meij

Abstract

Natural products are commonly discovered in sessile coral reef organisms, after which their potential use in pharmacology is screened with the help of various bioactivity assays. In the present study a quick and easy assay is used to study the bioactivity of Caribbean corals, based on the luminescence of the marine bacterium *Aliivibrio fischeri*. EC_{50} values were determined for 77 anthozoan specimens collected from Curaçao (Octocorallia, n=52; Scleractinia, n=24; and one sponge species (Porifera)). In general sponge and octocoral specimens were more bioactive (= low EC_{50} values) than scleractinians, although some stony corals (e.g. *Madracis auretenra*) had EC_{50} values similar to some of the most bioactive soft corals. Scleractinians did not show specific bioactivity per family, genus or species, whereas some octocoral genera (*Antillogorgia*, *Gorgonia*, and *Pseudoplexaura*) had remarkable lower EC_{50} than other octocoral genera (*Pterogorgia*). There are discrepancies in bioactivity within a single coral species, which is in line with previous studies. Coral bioactivity was plotted on phylograms of Caribbean soft and stony corals, together with snail and crab associations (Ovulidae and Cryptochiridae, respectively). For the ovulid snails *Cyphoma gibbosum* and *Cymbovula acicularis*, both associated with Octocorallia, we found that host coral bioactivity is most probably unrelated to the snail's host specificity. The most bioactive host genera (*Antillogorgia* and *Gorgonia*) host two symbiotic species, and in contrast, less bioactive gorgonian genera are only predated by the generalist *C. gibbosum*. Cryptochirid crabs are more specific in their associations with certain scleractinian genera, but there is no clear association with the bioactivity of the corals. Nonetheless, the most bioactive coral (*Madracis auretenra*) does not have any cryptochirid symbionts.

Introduction

Many marine species are known to harbor secondary metabolites. These secondary organic compounds are not directly involved in the normal growth, development, or reproduction of an organism, contrary to primary metabolites. Sponges, ascidians, anemones, algae, soft corals and gorgonians, and stony corals all produce secondary metabolites (Chen *et al*., 2014; Liu *et al*., 2014; Daletos *et al*., 2015; Ebada *et al*., 2015). These compounds are subsequently screened by pharmacologists for useful properties in, for example, the biomedical sector and therefore referred to as 'marine drugs' (Marris, 2006; Benkendorff *et al*., 2015; De Jesus Raposo *et al*., 2015). The secondary metabolites or so-called marine natural products (MNPs) are divided into specific groups based on their chemical composition (e.g. terpenoids, lipids, and steroids), and many of these chemicals are cytotoxic, anti-inflammatory, antifouling or have anti-virulent effects (Rocha *et al*., 2011; Blunt *et al*., 2012). Discovery of new medicines or antibiotics from marine flora and fauna are, however, still uncommon. Two success stories are Prialt® (pain reliever) and Yondelis® (anti-tumor drug), which were produced from marine-derived precursors (Rocha *et al*., 2011).

Marine invertebrates, but also algae and bacteria, likely produce chemical compounds for defense against predators (fish, turtles, nudibranchs etc.) as well as for the competition for space with other sessile invertebrates (e.g. coral-coral interactions, coral-sponge interactions, overgrowing marine algae etc.) (McCook *et al*., 2001; Pawlik, 2012). These defensive metabolites are known to be toxic in pharmacological assays, but there is little evidence that specific unpalatable metabolites are toxic. Certain highly toxic metabolites tested in pharmaceutical assays are still quite palatable to fish and mollusks. No relationship was found between palatability and toxicity, but the number of available studies is still limited (Pawlik, 2012; and references therein).

The associated fauna of coral reefs makes up an important part of the diversity on reefs, and ranges from facultative to obligate associations (e.g. Zlatarski and Martínez-Estalella, 1982; Reijnen *et al*., 2010; Stella *et al*., 2011; Hoeksema *et al*., 2012; van der Meij, 2014). Species that live in close association with organisms such as sponges, corals and tunicates have to handle the chemical compounds of their host during their adult life (e.g. feeding), but also during larval settlement (Burke, 1986; Scheltema, 1986). Many obligate associations consist of species that are found on only one or a few species of closely related hosts, hence species-specific settlement cues are likely involved (Pawlik, 1992). The need to adapt to these compounds might be an important force in adaptive radiation and a driver in evolution for host-dependent marine organisms (Pawlik, 2012).

Here we used a quick assay based on light emitting bacteria to quantify the bioactivity of Caribbean anthozoans. Bioactivity is expressed by an EC_{50} value (half maximal effective concentration; expressed in mg/ml); a quantitative measure that indicates how much of a particular crude extract from biological origin is needed to inhibit half of the biological processes of the bacteria. With these EC_{50} values we identified differences in bioactivity between two different orders in the class Anthozoa (Scleractinia and Octo-

corallia), and between the genera and species in these orders. The results of the bioactivity assay were then used to study the link between the relative bioactivity of soft and stony corals and the host selection of associated fauna (false cowries – Ovulidae; gall crabs – Cryptochiridae). Cryptochirids are diminutive crabs that live in dwellings on their hosts, whereas Ovulidae snails roam over the branches of octocorals on which they prey, leaving large feeding scars behind. The snails and crabs are highly dependent on their host corals for survival, and therefore good candidates for a study on the effect of host bioactivity on associated fauna.

Material and methods

Collection and identification of specimens

Stony corals, soft corals and gorgonians, and a sponge sample were collected by SCUBA diving at the leeward site of Curaçao in November 2013. All samples were collected at 0-40 m depth and transported in large plastic bags with seawater to the Carmabi laboratory where the samples were drip-dried and stored in an -18°C freezer for further use. Stony coral identifications were based on Zlatarski and Martínez-Estalella (1982), Humann and DeLoach (2002), Coralpedia (http://coralpedia.bio.warwick.ac.uk) and the reference collections of Naturalis Biodiversity Center. Coral nomenclature was updated following Budd *et al*. (2012). Subsamples of the octocoral specimens were transported to Naturalis Biodiversity Center, where they were identified using Bayer (1961). Tissue of the octocorals was diluted in household bleach and the residual sclerites were washed with tap water and distilled water respectively. Sclerites were mounted on slides with Euparal for examination with an Olympus BX-53F microscope. The taxonomy of Caribbean octocorals is not yet completely resolved (Sánchez *et al*., 2003); hence not all specimens were identified to species level (nine specimens were identified to genus level). Additionally, octocoral samples were also subsampled for molecular analyses for the phylogeny reconstruction presented herein.

Background bioactivity assay

The luminometer method that was used in this study is developed to determine the toxicity of water-soluble samples in wastewater, fresh water and sediments and have been used as such since the 1980s (Chang *et al*., 1981; Dezwart and Slooff, 1983). The marine bacteria used in this assay (*Aliivibrio fischeri* (Beijerinck, 1889)) emit light by respiration. When the *A. fischeri* bacteria are exposed to toxic compounds, their respiration rate lowers or stops and the amount of emitted light decreases. In this study we use crude extracts from Anthozoa to measure the effect on the *A. fischeri* bacteria. By adding crude extracts obtained from stony corals and octocorals the bioactivity can be determined by measuring the decrease in light emission, which consequently is used to calculate the EC_{50} value. The lower the EC_{50} value, the more effect a sample has on the bacteria. Generally, only compounds displaying an $EC_{50} \leq 10.0$ µg/ml are considered, because these values are normally used in literature to ascertain relevant bioactivity (Rocha *et al*., 2011 and citations therein). Here we chose to show all bioactivity data related to the respective coral species.

Bioactivity assay materials and methods

Bioactivity assays on the anthozoan specimens were carried out using a Lumitester PD-20 (Kikkoman) in combination with an Aboatox BioTox™ Kit (1243-500) containing lyophilized *Aliivibrio fischeri* light emitting bacteria. The Lumitester PD-20 is a hand-held portable device, measuring light emission in Relative Light Units (RLU). Before bioactivity analyses can be started a 2% sodium chloride solution has to be prepared, which is used as buffer solution for the bacteria. The provided sodium chloride tablet was dissolved in distilled water (450 ml) to obtain the desired solution. The pH needs to be adjusted to 7 (± 0.2) with either HCl or NaOH. The *A. fischeri* bacteria need to be reconstituted before they can be used in the analysis. This involves adding the cold (4-7°C) reconstitution fluid (provided in the Aboatox BioTox™ Kit) to the bacteria and gently shake/swirl the vial until the solution is homogenous (no vigorous shaking). This reagent needs to stabilize at 15°C for at least 30 minutes. The reagent cannot be kept for long time intervals and should be used within 12 hours if kept at 4-7°C. To check if the bacteria are viable and emitting light at a stable rate, 500 μl of the bacteria solution was placed in a cuvette and measured with the luminometer. The bacteria should preferably emit >700 RLU and no less than 200 RLU. The same sample should be checked after 5 minutes to see if light emission has stabilized. If the RLU values are stable, they can be used for the bioactivity assay.

Samples (crude extracts of MNPs from anthozoans) were prepared in the field by scraping approximately 0.5 gram of soft tissue with a surgical blade from the samples. The amount of tissue was weighed using a digital analytical balance. A volumetric approach would be preferred above the gravimetric method (Pawlik, 2012), but this proved to be too problematic to collect tissue samples for the stony corals that could be used in the volumetric method. Soft tissue samples were ground up in 2 ml ethanol 96% for the stony corals and 3 ml for the soft corals and gorgonians using a mortar and pestle. Consequently this mixture was filtered (using a coffee filter) to obtain a clear solution without calcareous particles. The solution was then concentrated to 500 μl by evaporation and stored in micro vials before analyses (Tables 1-2). For every extracted coral specimen a dilution series was made. Several test runs showed that bioactivity differed greatly between Octocorallia and Scleractinia, hence two different dilution series were made with the 2% sodium chloride solution: 1:10, 1:20, 1:40, 1:80 for the Octocorallia and for the Scleractinia: 1:5, 1:10, 1:20, 1:40 and 1:80. Each cuvette was filled with 500 μl of the stabilized bacteria solution and their starting RLU values were measured and recorded. Consequently, the dilution series of the anthozoan extracts were added (also 500 μl) to their respective cuvettes and incubated for 30 minutes at approximately 15°C. For the negative control samples, 500 μl sodium chloride solution (2%) was added instead of anthozoan extract. All measurements were performed in duplicate. After the incubation period, samples were remeasured to record the light emission after incubation with the extracts. In some cases, the extracts proved to be hardly affecting the bacteria or were so toxic that the dilution series had to be adapted to measure their EC_{50} values.

Starting RLU values and values after incubation were entered into a spreadsheet (provided by Aboatox) to calculate the decrease of RLU and test whether the duplicate analyses were statistically sound (SD < 3% inhibition). Inhibition and concentration

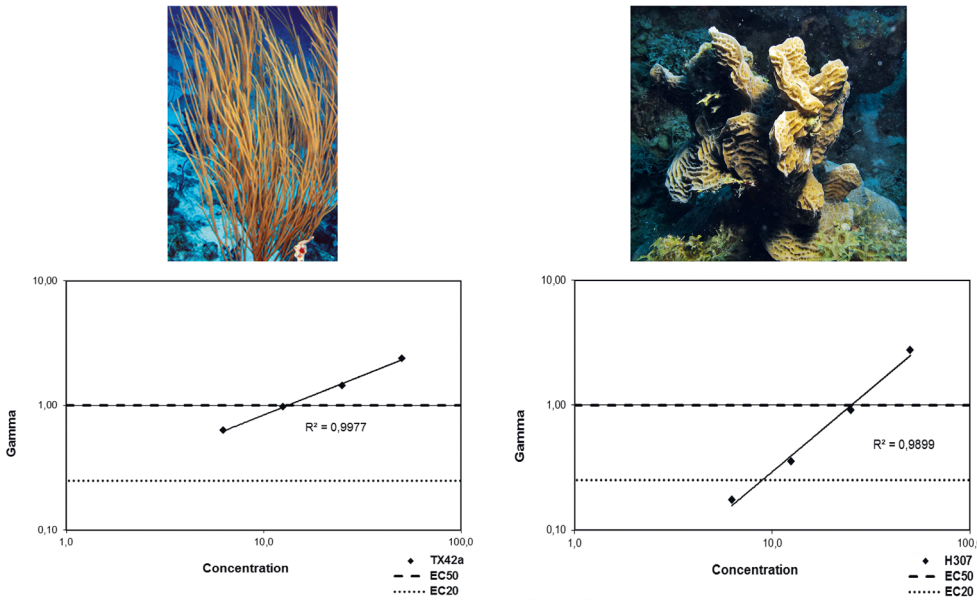


Fig. 1. Graphs showing anthozoan bioactivity data used for EC_{50} calculations: (left) the gorgonian *Ellisella barbadensis* (sample TX42a) and (right) the scleractinian coral *Agaricia agaricites* (sample H307). Gamma indicates log-transformed inhibition; concentration refers to the dilution series.

values were log transformed and consequently a fitted line of the linear regression was used to identify the EC_{50} values (Fig. 1).

Phylogeny reconstructions and host specificity

To study if there is a link between the bioactivity of the host corals and the phylogenetic relationships between the host species, phylogeny reconstructions were made of the host species. The octocoral phylogeny reconstruction was based on four markers (28S, COI, mtMutS and ND6) and the scleractinians on three markers (12S, CytB and COI). Octocorallia were sequenced at the Naturalis Barcoding lab, whereas the Scleractinia sequences were downloaded from GenBank (see supplementary material for the accession numbers).

A Maximum Likelihood (ML) analysis based on the GTR+I+G (Octocorallia) and HKY+G (Scleractinia) models were conducted in MrBayes (Ronquist and Huelsenbeck, 2003) (Octocorallia) and MEGA6 (Scleractinia) to infer phylogeny reconstructions for the host species. Both phylogeny reconstructions do not fully reflect the overall known relationships between corals (e.g. Fukami *et al*., 2004; Huang 2012; McFadden *et al*., 2006), for example due to paraphyletic relationships within the Caribbean (octo)corals and the relationships with Indo-Pacific corals. The phylogeny reconstructions are only used for the purpose of comparing the bioactivity of the Caribbean corals with symbiotic snails and crabs. Host data for the Ovulidae was based on Lorenz and Fehse (2009) and Reijnen *et al*., (2010), the host relations of the gall crabs were taken from a study by

van der Meij (2014). These associations were plotted on the phylogram to check if there is a relation with the bioactivity of the coral hosts.

Results

In total 77 specimens were analyzed, consisting of 52 Octocorallia, 24 Scleractinia and one Porifera sample. An overview of the measured ranges of toxicity values per genus shows that in general the Octocorallia have higher levels of bioactivity than the Scleractinia (Fig. 2). This is supported by the average EC_{50} calculated per anthozoan group. For the Scleractinia the average EC_{50} is 40.93 mg/ml (SD +/- 27.17), which is notably higher (= less bioactive) than the 10.17 mg/ml (SD +/- 8.13) average for the Octocorallia. Interestingly, some scleractinian corals have EC_{50} values close to those of some octocoral species. The sponge sample was among the most toxic samples in this study (EC_{50} = 1.62 mg/ml).

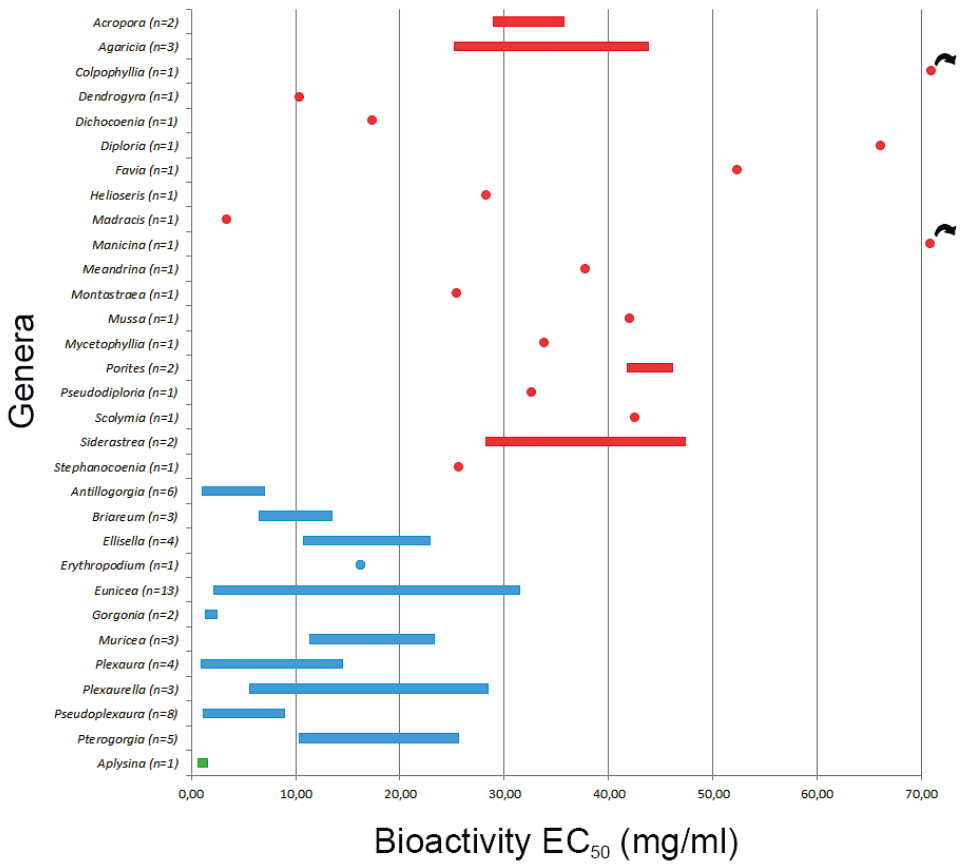


Fig. 2. Floating bar chart depicting the measured minimum and maximum EC_{50} values per genus (red = Scleractinia; blue = Octocorallia; green = Porifera). A circle shows a single data point. The EC_{50} value of *Colpophyllia* is 118.08 mg/ml and *Manicina areolata* is 117.18 mg/ml (off the chart).

Soft corals and gorgonians (Octocorallia)

Eleven genera of octocorals, comprising at least 22 species, were subjected to the bioactivity assay with the luminometer. Per species one to three specimens were analyzed (Table 1). It was observed that values between octocoral specimens of a single species had quite variable EC_{50} values, especially in the genera *Eunicea*, *Muricea* and *Plexaura* (Table 1, Fig. 2). It is therefore difficult to pinpoint the most bioactive octocoral species. The lowest EC_{50} value (most bioactive) for all octocoral specimens measured was 0.85 mg/ml for one of the *Plexaura homomalla* specimens. When the average bioactivity value was calculated over all *P. homomalla* specimens, the 'common sea fan' *Gorgonia ventalina* (n=2) turned out to have the lowest average EC_{50} value (1.91 mg/ml). The highest EC_{50} value (least bioactive) measured was 31.54 mg/ml for a specimen of *Eunicea mammosa*, but when EC_{50} values were averaged per species *Pterogorgia citrina* (n=3) had the highest EC_{50} value (23.81 mg/ml). However, the coefficient of determination (R^2) was low for *Plexaura homomalla* and might therefore be influencing the final EC_{50} value (Table 1). When samples with low R^2-values are not taken into account, *Pseudoplexaura* sp. and *G. ventalina* are the most bioactive species.

Differences in the average levels of bioactivity between coral genera were observed. The average EC_{50} values of unrelated *Antillogorgia* and *Pseudoplexaura* are low (ca. 4 mg/ml) when compared with *Pterogorgia* spp. (ca. 20 mg/ml). The average of all Octocorallia specimens in this study is 10.17 (SD +/- 8.13) mg/ml. Some species for which multiple specimens were analyzed showed a wide range of bioactivity. For example in *Eunicea flexuosa* EC_{50} values ranged from 2.5 to 17 mg/ml (Tables 1-2; Fig. 2).

Table 1. Overview of EC_{50} values and other metadata for Octocorallia samples analyzed in this study.

Family	Species (author)	EC_{50}	Wet tissue weight (gram)	R^2
Anthothelidae	*Erythropodium caribaeorum* (Duchassaing & Michelotti, 1860)	16.71	0.500	0.922
Briareidae	*Briareum asbestinum* (Pallas, 1766)	13.56	0.500	0.950
	Briareum asbestinum (Pallas, 1766)	9.31	0.500	0.827
	Briareum asbestinum (Pallas, 1766)	6.43	0.504	0.887
Ellisellidae	*Ellisella barbadensis* (Duchassaing & Michelotti, 1864)	11.97	0.513	0.962
	Ellisella barbadensis (Duchassaing & Michelotti, 1864)	13.19	0.492	0.998
	Ellisella barbadensis (Duchassaing & Michelotti, 1864)	10.72	0.505	0.837
	Ellisella elongata (Pallas, 1766)	22.89	0.500	0.892
Gorgoniidae	*Antillogorgia acerosa* (Pallas, 1766)	7.10	0.505	0.918
	Antillogorgia americana (Gmelin, 1791)	0.96	0.515	0.659
	Antillogorgia americana (Gmelin, 1791)	6.86	0.499	0.964
	Antillogorgia americana (Gmelin, 1791)	1.66	0.500	0.943
	Antillogorgia bipinnata (Verril, 1864)	5.36	0.509	0.844
	Antillogorgia bipinnata (Verril, 1864)	2.87	0.507	0.791

Table 1. Cont.

Family	Species (author)	EC_{50}	Wet tissue weight (gram)	R^2
	Pterogorgia citrina (Esper, 1792)	25.70	0.497	1.000
	Pterogorgia citrina (Esper, 1792)	23.46	0.502	0.883
	Pterogorgia citrina (Esper, 1792)	22.27	0.503	0.993
	Pterogorgia guadelupensis (Duchassaing & Michelin, 1846)	10.32	0.500	0.970
	Pterogorgia guadelupensis (Duchassaing & Michelin, 1846)	16.18	0.503	0.898
Plexauridae	*Eunicea calyculata* (Ellis & Solander, 1786)	8.14	0.502	0.936
	Eunicea calyculata (Ellis & Solander, 1786)	7.63	0.509	0.888
	Eunicea clavigera Bayer, 1961	12.39	0.508	0.911
	Eunicea clavigera Bayer, 1961	6.33	0.506	0.916
	Eunicea flexuosa (Lamouroux, 1821)	13.66	0.503	0.971
	Eunicea flexuosa (Lamouroux, 1821)	2.49	0.505	0.990
	Eunicea flexuosa (Lamouroux, 1821)	16.99	0.511	0.992
	Eunicea fusca Duchssaing & Michelotti, 1860	11.86	0.507	0.942
	Eunicea mammosa Lamouroux, 1816	31.54	0.504	0.906
	Eunicea pinta Bayer & Deichmann, 1958	0.00	0.497	0.588
	Eunicea succinea (Pallas, 1766)	2.15	0.504	0.932
	Eunicea succinea (Pallas, 1766)	2.15	0.518	0.958
	Eunicea succinea (Pallas, 1766)	5.06	0.508	0.963
	Gorgonia ventalina Linnaeus, 1758	2.48	0.513	0.964
	Gorgonia ventalina Linnaeus, 1758	1.34	0.496	0.986
	Muricea muricata (Pallas, 1766)	11.29	0.503	0.915
	Muricea muricata (Pallas, 1766)	23.35	0.504	0.828
	Muricea pinnata Bayer, 1961	12.05	0.511	0.986
	Plexaura homomalla (Esper, 1792)	7.15	0.505	0.944
	Plexaura homomalla (Esper, 1792)	0.85	0.505	0.600
	Plexaura homomalla (Esper, 1792)	4.64	0.513	0.980
	Plexaura nina Bayer & Deichmann, 1958	14.69	0.498	0.995
	Plexaurella dichotoma (Esper, 1791)	28.50	0.502	0.898
	Plexaurella dichotoma (Esper, 1791)	5.50	0.504	0.898
	Plexaurella dichotoma (Esper, 1791)	26.80	0.497	0.771
	Pseudoplexaura flagellosa (Houttuyn, 1772)	2.02	0.499	0.937
	Pseudoplexaura porosa (Houttuyn, 1772)	8.99	0.498	0.910
	Pseudoplexaura sp.	4.84	0.497	0.925
	Pseudoplexaura sp.	3.24	0.501	0.970
	Pseudoplexaura sp.	4.22	0.504	0.944
	Pseudoplexaura sp.	5.26	-	0.524
	Pseudoplexaura sp.	1.07	0.518	0.717
	Pseudoplexaura sp.	3.28	0.503	0.958

Table 2. Overview of EC_{50} values and other metadata for scleractinian corals analyzed in this study.

Family	Species (author)	EC_{50}	Wet tissue weight (gram)	R^2
Acroporiidae	*Acropora cervicornis* (Lamarck, 1816)	35.74	0.506	0.754
	Acropora palmata (Lamarck, 1816)	28.87	0.506	0.914
Agariciidae	*Agaricia agaricites* (Linnaeus, 1758)	25.15	0.506	0.990
	Agaricia lamarcki Milne Edwards & Haime, 1851	25.89	0.506	0.980
	Agaricia lamarcki Milne Edwards & Haime, 1851	43.90	0.515	0.932
	Helioseris cucullata (Ellis & Solander, 1786)	28.74	0.511	0.937
Astrocoeniidae	*Madracis auretenra* Locke, Weil & Coates, 2007	3.95	0.490	0.935
	*Stephanocoenia intersepta (*Lamarck, 1836*)*	26.09	0.495	0.987
Montastraeidae	*Montastraea cavernosa* (Linnaeus, 1767)	25.98	0.504	0.990
Meandrinidae	*Dendrogyra cylindrus* Ehrenberg, 1834	10.82	0.503	0.989
	Dichocoenia stokesi Milne Edwards & Haime, 1848	17.86	0.508	0.970
	Meandrina meandrites (Linnaeus, 1758)	38.19	0.499	0.997
Mussidae	*Colpophyllia natans* (Houttuyn, 1772)	118.08	0.495	0.953
	Diploria labyrinthiformis (Linnaeus, 1758)	66.64	0.497	0.989
	Favia fragum (Esper, 1795)	52.88	0.506	1.000
	Manicina areolata (Linnaeus, 1758)	117.18	0.506	0.400
	Mussa angulosa (Pallas, 1766)	42.40	0.498	0.989
	Mycetophyllia sp.	34.36	0.500	0.971
	Pseudodiploria clivosa (Ellis & Solander, 1786)	33.10	0.510	1.000
	Scolymia sp.	43.02	0.510	0.985
Poritidae	*Porites* cf. *divaricata* Le Sueur, 1820	41.71	0.511	0.980
	Porites porites (Pallas, 1766)	46.23	0.507	1.000
Siderastreidae	*Siderastrea siderea* (Ellis & Solander, 1786)	28.18	0.493	0.990
	Siderastrea siderea (Ellis & Solander, 1786)	47.45	0.501	1.000

Stony corals (Scleractinia)

Seventeen genera comprising 21 species (two specimens were not identified to species level) were analyzed. EC_{50} values varied greatly between species and ranged from 3.95 to 118.08 mg/ml (*Madracis auretenra* and *Colpophyllia natans*, respectively). Most stony coral species had EC_{50} values between ca. 25 and 45 mg/ml (average is 40.93 +/- 27.17 mg/ml). The least bioactive family appears to be the Mussidae, which is one of the largest Caribbean coral families. *Madracis auretenra* has the lowest EC_{50} value (3.95 mg/ml), but *Stephanocoenia intersepta* from the same family (Astrocoeniidae) does not appear to be very bioactive (26.09 mg/ml). In contrast to the Octocorallia, there are no clear similarities in EC_{50} values between families or genera (Table 2; Fig. 2).

As with the Octocorallia, we observed a range of bioactivity when multiple specimens of a single species were analyses. For *Agaricia lamarcki* and *Siderastrea siderea* the EC_{50} values ranged from 25.89 to 43.90 and 28.28 to 47.45, respectively (Table 2; Fig. 2).

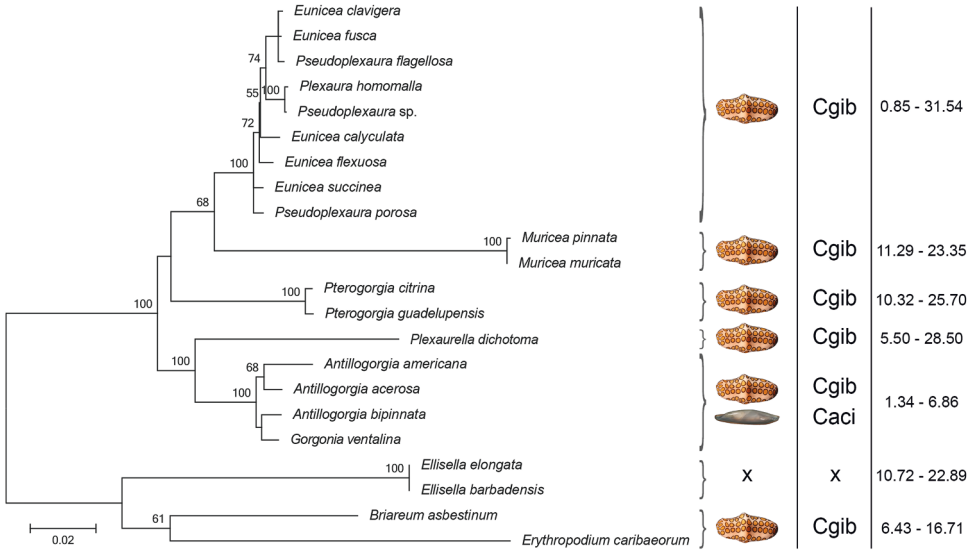


Fig. 3. Phylogeny reconstruction of Caribbean Octocorallia used in this study, including ovulid symbionts (Cgib = *Cyphoma gibbosum*; Caci = *Cymbovula acicularis*) and the octocorallian EC_{50} ranges as recorded in this study. Phylogeny reconstruction based on Bayesian inference analysis, bootstrap values > 50 are shown.

Sponge (Porifera)

For comparison with the cnidarian samples we also collected a specimen of the tube sponge *Aplysina archeri* (Higgin, 1875) as an outgroup. *Aplysina archeri* is highly bioactive with an EC_{50} value of 1.62 mg/ml ($R^2 = 0.992$).

Host specificity – Ovulidae

Cymbovula acicularis is a specialist predator associated with the bioactive octocoral genera *Antillogorgia* and *Gorgonia*, whereas the generalist species *C. gibbosum* is associated with a wide range of octocoral hosts, including the latter two octocoral genera (Fig. 3; Reijnen *et al*., 2010; Chapter 2).

By plotting the bioactivity values and host associations for the two symbiotic ovulid snails on a phylogram it becomes clear that there is no clear connection between these variables (Fig. 3). In fact, the two most toxic octocoral genera (*Antillogorgia*, *Gorgonia*) - which are closely related - have two instead of one ovulid symbiont (Fig. 3). The EC_{50} values as determined for *Ellisella* species are almost equal to the values of the genus *Muricea*, and in comparison with the other Octocorallia *Muricea* and *Ellisella* do not show high levels of bioactivity. *Ellisella* is the only genus included in this study that does not serve as a host for ovulid snails in the Caribbean, whereas *Muricea* is encountered with symbiotic *C. gibbosum* snails.

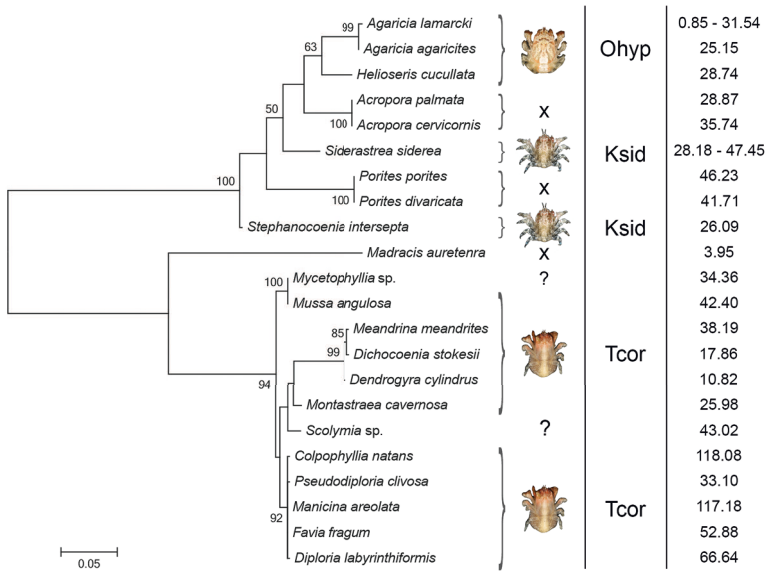


Fig. 4. Phylogeny reconstruction of the Caribbean stony corals used in this study, incl. their recorded cryptochirid symbionts (Ohyp = *Opecarcinus hypostegus*; Ksid = *Kroppcarcinus siderastreicola*; Tcor = *Troglocarcinus corallicola*; based on van der Meij, 2014) and the scleractinian EC_{50} values as recorded in this study. Question marks represent coral species for which gall crab specimens are recorded in literature (not found off Curaçao). Phylogeny reconstruction based on ML analysis, bootstrap values ≥ 50 are shown.

Host specificity – Cryptochiridae

Three shallow-water gall crabs species are associated with Caribbean stony coral hosts. *Opecarcinus hypostegus* inhabits corals of the family Agariciidae; *Kroppcarcinus siderastreicola* inhabits hosts of the genera *Siderastrea* and *Stephanocoenia*, and *Troglocarcinus corallicola* inhabits corals of a wide range of species - but does not occur in the former hosts. These associations were plotted on the phylogeny reconstruction of the Caribbean stony corals (Fig. 4). The most bioactive stony coral from this study (*Madracis auretenra*) is not a known host species for gall crabs.

Discussion

Bioactivity assay method

The luminescence bioassay has been used for some decades and provides scientist with empirical data on the bioactivity of compounds and products in a fast and easy way (Becerro *et al*., 1995; Marti *et al*., 2005). When using this bioactivity assay to determine EC_{50} values for eco-evolutionary research, three matters have to be considered.

Firstly, most marine invertebrates that act as hosts (e.g. corals, sponges, sea anemones) for associated fauna have zooxanthellae and bacteria in their tissue (Shnit-Orland and Kushmaro, 2013; Correa *et al*., 2013). The crude extract method therefore may

contain any possible secondary metabolites produced by these organisms. The exact degree of bioactivity of cnidarian species can therefore not be determined, as long as the source of the MNPs is unknown. It is likely that the coral tissue samples contained a mixture of compounds from different species groups, even beyond the animal kingdom. This cannot easily be overcome by any bioactivity assay.

Secondly, the density of calcareous skeletal parts from the stony corals and octocorals have effect on the amount of tissue containing MNPs subjected to the extraction. Pawlik (2012) mentions that calcite densities can differ per individual or even between locations in an individual. By using a volumetric approach instead of a gravimetric method the difference in skeletal tissue vs. actual tissue containing MNPs can be eliminated. This would be relatively easy for the octocorals, but proves to be almost impossible for the stony corals as most species have thin tissue layers. We therefore decided, despite the calcite density differences, to use the gravimetric method.

Thirdly, the bioactivity is measured on bacteria and not on the predators and associated fauna of the anthozoans, which are primarily vertebrates (fishes) and invertebrates (i.e. mollusks, crustaceans, worms). Since testing is performed on bacteria, some care has to be taken when extrapolating the results to the actual model organism. On the other hand, testing with bacteria also has the advantage of not becoming involved in any regulations for animal testing.

Bioactivity assay

As expected, soft corals are more bioactive that stony corals (Rocha *et al*., 2011), however, some stony coral species were more bioactive than certain soft coral species. When more specimens per species were analyzed for both the Scleractinia and the Octocorallia we observed that there is high intraspecific variety within species based on their respective EC_{50} values (Tables 1-2; Fig 2).

Octocorallia – It was not unexpected that *Gorgonia* and *Antillogorgia* are amongst the most bioactive octocoral genera. Pharmaceutical/chemical studies already focused on compounds obtained from these genera in their search for potential medicines because of high antimicrobial activity (Fenical, 1987). Both genera did not show a wide range of bioactivity whilst specimens from the genus *Eunicea* showed high variability in EC_{50} values and were amongst the most and least bioactive samples analyzed in this study. This fluctuating bioactivity can be the result of the differences in bioactive compounds from different tissue samples. Harvell and Fenical (1989) measured the bioactivity values in a single specimen between the polyps and the coenenchym of some Caribbean octocoral species (*Pseudopterogorgia = Antillogorgia*) and between the proximal and distal end of colony branches. They found that specific compounds observed in *Antillogorgia* could not be found in the coenenchym but were abundant in the polyp tissue or were found in significant higher doses at the distal end of a branch than at the colony base (which also harbors fewer polyps). Pawlik (2012), however, questions this result because a gravimetric method was used to weigh the animal tissue instead of a volumetric method, which would level out the variation in bioactivity values due to the effect of sclerite density vs. MNP containing tissue in the octocoral samples. As a result, it remains inconclusive what caused the

large variation observed between different specimens of the same species in some of the samples studied herein.

The variation in bioactivity among octocoral genera does not seem to reflect a phylogenetic relationship. Genera with similar EC_{50} values, for example *Antillogorgia* and *Gorgonia*, are phylogenetically closely related. In contrast, *Pseudoplexaura* clusters between *Plexaura* and *Eunicea*, which are not directly related to the genera *Antillogorgia* and *Gorgonia* (Fig. 3; Sánchez *et al*., 2003). Yet, the support values for the sister clades with *Muricea* and *Pterogorgia* species are not or moderately supported, which could indicate that species are actually more closely related than previously thought. The phylogenetic relationship between *Eunicea*, *Plexaura* and *Pseudoplexaura* is also unresolved. Recent molecular work by Lau *et al*., (unpubl. data) show that these genera cannot be separated easily based on four molecular markers and morphologically these genera are challenging to separate. We therefore analyzed the data generated for these genera as a single monophyletic group (Fig. 3). By grouping the data more genus-specific data might get lost, but future phylogenetic studies may reveal that only a single genus is involved.

Scleractinia – Scientific studies dealing with the bioactivity of scleractinians are a lot sparser than those available for Octocorallia and Porifera. The configuration of compounds obtained from *Tubastraea aurea*, *Cladocora cespitula* and *Tubastraea* sp. have been analyzed (Fusetani *et al*., 1986; Fontana *et al*., 1998; Marti *et al*., 2005; Meyer *et al*., 2009). Gunthorpe (1991) performed the first elaborate bioactivity assessment on scleractinian corals from different coral families at Heron Island, Australia. Additionally, Gunthorpe and Cameron (1990a, b) also investigated the intraspecific and intracolonial differences in bioactivity of scleractinian corals. Their findings of fluctuating EC_{50} values, within a nominal species, complement our findings.

Porifera – We only analyzed only one sample, which is among the most bioactive samples measured in this study.

Host specificity

Host species specialization is considered strong among insects, but not so much for marine invertebrates (Pawlik, 2012). This is primarily caused by the different life histories of insects and marine invertebrates. Insects such as butterflies have multiple generations in a season and have non-dispersive larvae, which are usually deposited by the adult on their host plant. In contrast, marine invertebrates have generation times measured in years and have larvae that are often pelagic and do disperse, sometimes over large distances. As a result, the evolutionary processes in insects are probably accelerated and more intense, when compared to most marine invertebrates (Pawlik, 2012). In the marine realm host specificity research between marine invertebrates is still scarce and only a few examples of tight associations are currently known (Lanterbecq *et al*., 2010; van der Meij, 2015). However, it is likely that many (marine) species associations still need to be discovered and that similar associations as described for insects will be revealed for (some) marine invertebrates.

Ovulidae – By plotting the host-symbiont data of the ovulids and the bioactivity data of the Octocorallia on the octocoral phylogeny it is shown that species symbiosis is not

based on the bioactivity of compounds in the host corals (Fig. 3). The octocoral genera with the lowest EC_{50} values are host to both ovulid species whereas one of the least bioactive genera, *Ellisella*, has no known ovulid symbionts in the Caribbean. *Ellisella* has similar-sized sclerites (approx. 0.10 mm) as *Gorgonia* which shows that structural defense is not the source for their lack of symbionts. Nonetheless, in the Indo-Pacific the family Ellisellidae is the most common host family for ovulid snails of the subfamily Aclyvolvinae (Lorenz and Fehse, 2009; Chapter 5).

One of the most iconic octocoral predators in the Caribbean is *Cyphoma gibbosum*. This brightly conspicuously coloured Caribbean snail has been found predating on a large number of different octocoral species (Lorenz and Fehse, 2009; Reijnen *et al*., 2010) and is considered a common generalist predator of Octocorallia. Another Caribbean ovulid is *Cymbovula acicularis*. This species is inconspicuous and is so far only found on the genera *Gorgonia* and *Antillogorgia* (Humann and Deloach, 2002; Lorenz and Fehse, 2009; Reijnen *et al*., 2010). The most bioactive octocoral genera in our assay (*Gorgonia* and *Antillogorgia*) are known to contain deterrent compounds against fishes and *C. gibbosum* (Van Alstyne and Paul, 1992). They used *C. gibbosum* in a feeding assay to test the chemical properties and structural defense of *Gorgonia ventalina*. Besides the repellent compounds in the crude extract of *G. ventalina* they also investigated the effect of *G. ventalina* sclerites (structural defense) in this assay. Their results showed that feeding was reduced by half when sclerites or crude extracts were introduced in the feeding pellets to *C. gibbosum* and the effect was even greater in fishes. Nevertheless, the deterrent effect by the crude extracts or the structural defense by sclerites does not seem hinder predation by *C. gibbosum* on *G. ventalina* in nature (Reijnen *et al*., 2010: Fig. 3). This is most probably because *C. gibbosum* can adapt to the chemical compounds due to biotransformation enzymes such as cytochrome P450 (Wahlen *et al*., 2010).

Cryptochiridae – As with the Octocorallia there is no clear connection between the coral phylogeny, bioactivity of the host and the symbiont fauna. Three shallow-water gall crabs species are associated with Caribbean stony coral hosts. Specialist species *Opecarcinus hypostegus* inhabits Agariciidae corals, whereas *Kroppcarcinus siderastreicola* inhabits the genera *Siderastrea* and *Stephanocoenia*. *Troglocarcinus corallicola* is a generalist species and inhabits a wide range of hosts (Fig. 4). The most bioactive stony coral from this study (*Madracis auretenra*) is not a known host of gall crabs (Kropp and Manning, 1987; van der Meij, 2014). It is unclear whether this is due to the presumed toxicity, the thin-branched, dendritic structure of the stony coral or the evolutionary history between the corals and crabs. Other genera that are not inhabited by gall crabs are *Acropora* and *Porites*. Caribbean *Acropora* is host to the xanthid crab *Domecia acanthophora* (Desbonne, in Desbonne and Schramm, 1867*)* that inhabit various types of structural deformations, somewhat similar to those of gall crabs (Patton, 1967). The genera *Madracis* and *Acropora* where, however, listed by Scott (1987) to be amongst the most hospitable corals, together with *Siderastrea* and *Agaricia lamarcki*. These species are ranked high on Lang's (1973) aggression hierarchy, used by Scott (1987), which is based on extracoelenteric digestion (Lang, 1973; Kropp, 1988). The absence of cryptochirid crabs in *Madracis* and *Acropora* is, therefore, likely linked to

other factors than the aggression or bioactivity of the host. In Scleractinia, presence of associates is significantly inversely correlated with polyp size according to Scott (1987). In his study, he included a wide range of infaunal associates of living coral, and many polychaetes, sipunculids and certain barnacles appear to be more common in (small-polyped) *Porites*.

Host specificity of associated fauna is perceived to be more specific in the Indo-Pacific (Stella *et al*., 2011), and hence the next step is to apply the described bioactivity assay on specimens from the Indo-Pacific to test whether the bioactivity of the host organism plays a role in the diversity of associated fauna.

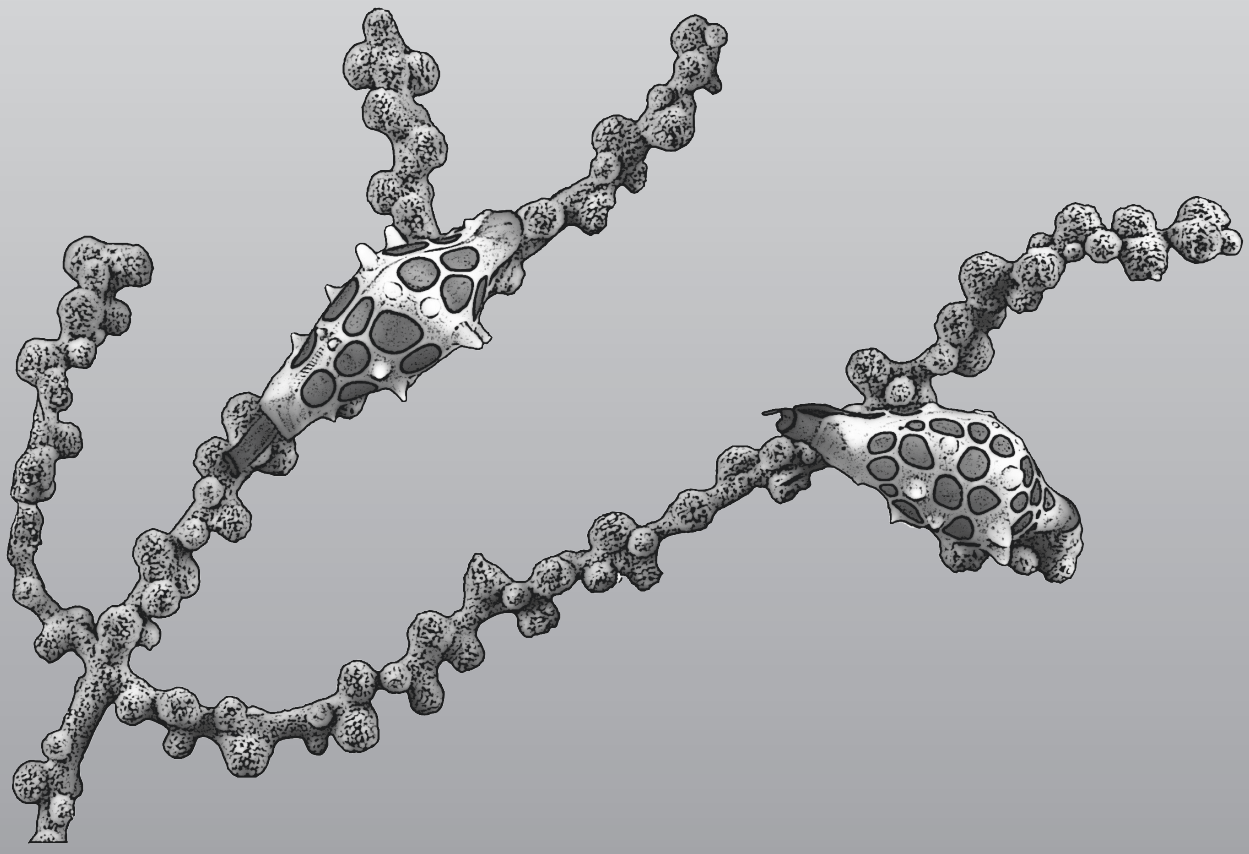
Acknowledgements

We are grateful to Juha Lappalainen from Aboatox for his help in for setting up the bioactivity assay for anthozoans. The CARMABI Research Station and DiveVersity Curaçao supported this research. For financial support we would like to thank the TREUB-maatschappij (Society for the Advancement of Research in the Tropics), and Academy Ecology Fund (formerly KNAW Fonds Ecologie). For reading and commenting on the manuscript we thank Bert Hoeksema and Edi Gittenberger (Naturalis). Leen van Ofwegen (Naturalis) assisted with the Octocorallia identifications and commented on the manuscript. Rob van Soest (Naturalis) is acknowledged for confirming the identity of the sponge sample.

Suppl. mat. GenBank accession numbers for the sequences used in the phylogeny reconstruction of the Scleractinia (Fig. 4), n/a = not available.

	COI	CytB	12S
Acropora cervicornis	AY451340	AF099654	EF597094
Acropora palmata	AY451341	AB441331	EF597092
Agaricia agaricites	AY451366	n/a	EF597080
Agaricia lamarcki	AY451369	n/a	EF597076
Colpophyllia natans	AB117228	AB117306	EF596998
Dendrogyra cylindrus	AB117299	AB117384	EF597024
Dichocoenia stokesii	AY451360	AB117383	EF597020
Diploria labyrinthiformis	AB117224	AB117302	EF597002
Favia fragum	AB117223	AB117301	EF597005
Helioseris cucullata	AB441220	AB441305	n/a
Madracis mirabilis	AB441227	NC011160	NC011160
Manicina areolata	AB117227	AB117305	EF597012
Meandrina meandrites	AB117296	AB117381	EF597032
Montastraea cavernosa	AF108712	AB117374	EF597007
Mussa angulosa	AB117239	AB117316	EF597011
Mycetophyllia aliciae	AB117235	AB117312	EF597039
Porites divaricata	AY451381	n/a	EF597058
Porites porites	AY451384	DQ643837	EF597056
Pseudodiploria clivosa	AB117226	AB117304	EF597001
Scolymia sp.	AB117248	AB117325	n/a
Siderastrea siderea	AB441211	AB441296	EF597067
Stephanocoenia intersepta	AB441229	AB441313	EF597073

The Indo-Pacific



Chapter 4

Molecular data for *Crenavolva* species (Gastropoda, Ovulidae) reveals the synonymy of *C. chiapponii*

Bastian T. Reijnen

Zookeys (2015) 501: 15-26.

Abstract

During fieldwork in Indonesia and Malaysia, eight lots containing 33 specimens belonging to the genus *Crenavolva* (Ovulidae) were collected. Species were initially identified as *C. aureola*, *C. chiapponii*, *C. striatula* and *C. trailli*, respectively. For *C. chiapponii* this is the second record. In contrast to the ecological data available from the original description of this species, it was found in shallow water on a gorgonian host coral, i.e. *Acanthogorgia* sp. A molecular analysis based on COI and 16S mtDNA markers, including sequence data obtained from GenBank, showed that *C. chiapponii* should be considered a junior synonym of *C. aureola* and that previously identified ovulid specimens are probably misidentified.

Introduction

The nominal taxon *Crenavolva* was introduced as a subgenus by Cate (1973), together with the subgenera *Crenavolva*, *Cuspivolva* and *Serratovolva*. In the most recent overview regarding Ovulidae these three taxa are considered genera (Lorenz and Fehse, 2009). At present 18 nominal species are recognized within *Crenavolva* (Rosenberg, 2014), most of which are considered rare (Lorenz and Fehse, 2009). These species are considered rare because few specimens have been collected, probably because they occur at depths greater than standard recreational diving depth of c. 30 m and/or are only known from a limited geographical area, usually just the type locality. This also accounts for *C. chiapponii* Lorenz and Fehse, 2009, which is only known from Balicasag Isl., Bohol, Philippines, where specimens were trawled from 70-120 m depth and, therefore, were considered rare and confined to deeper water (Lorenz and Fehse, 2009).

Table 1. Specimens used in the analyses, including locality, host, and GenBank accession data.

Collection number	Species (author)	Locality (Locality code)
RMNH.Mol.164072	*Crenavolva aureola* (Fehse, 2002)	Malaysia, Semporna, Si Amil Island (SEM.16)
RMNH.Mol.164085	*Crenavolva aureola* (Fehse, 2002)	Indonesia, Halmahera, Tidore, N of Desa Rum (TER.18)
RMNH.Mol.164209	*Crenavolva aureola* (Fehse, 2002)	Indonesia, Halmahera, Tanjung Ratemu (S of river) (TER.21)
RMNH.Mol.164211	*Crenavolva chiapponni* Lorenz and Fehse, 2009	Indonesia, Halmahera, Tanjung Ratemu (S of river) (TER.27)
RMNH.Mol.164217	*Crenavolva chiapponii* Lorenz and Fehse, 2009	Indonesia, Lembeh, Tanjung Kusukusu (LEM.31)
RMNH.Mol.164062	*Primovula rosewateri* (Cate, 1973)	Malaysia, Semporna, Kulapuan Island 2, N side (SEM.31)
RMNH.Mol.164186	*Crenavolva striatula* (Sowerby 1st, 1828)	Malaysia, Sabah, S Pulau Banggi, E Molleangan Besar Island, (TMP.37)
RMNH.Mol.164144	*Crenavolva trailli* (A. Adams, 1855)	Malaysia, Sabah, Kalang, (TMP.41)
RMNH.Mol.164189	*Crenavolva trailli* (A. Adams, 1855)	Malaysia, Sabah, Kalang, (TMP.41)
-	*Crenavolva* cf. *rosewateri* (Cate, 1973)	Philippines, Bohol, Balicasag Island
-	*Crenavolva tokuoi* Azuma, 1989	Philippines, Bohol, Balicasag Island
-	*Primovula beckeri* (Sowerby 3rd, 1900)	Indonesia, Sulawesi
-	*Ovula ovum* (Linnaeus, 1758)	Indonesia, Sulawesi, Spermonde Archipelago

Like almost all other ovulids, species of *Crenavolva* are associated with octocoral hosts (Schiaparelli *et al*., 2005; Reijnen, 2010) belonging to several families (e.g. Melithaeidae, Ellisellidae, Subergorgiidae and Plexauridae). However, the host species are usually not collected or are disregarded and therefore unknown, which is also the case for *C. chiapponii*.

Molecular data (16S and COI) obtained from *Crenavolva* was used by Meyer (2003) to root the phylogeny of the Cypraeidae. Later, the 16S sequence data were used by Schiaparelli *et al*., (2005) to produce the first molecular phylogenetic reconstruction of the Ovulidae, which included two *Crenavolva* species: *C.* cf. *rosewateri* (Cate, 1973) and *C. tokuoi* Azuma, 1989. In the present study, material of four additional nominal *Crenavolva* species, amongst other ovulids, have been used to reconstruct a phylogeny. The newly acquired molecular data are for *C. aureola* (Fehse, 2002), *C. chiapponii* Lorenz and Fehse, 2009, *C. striatula* (Sowerby I, 1828) (type species), and *C. trailli*

Coordinates	Date collected	Host species	GenBank Accession number (16S ; COI)	Reference
4°19’02.1”N; 118°52’30.7”E	4-12-2010	*Acanthogorgia* sp.	KP033143 ; KP033151	This publication
0°44’35.8”N; 127°23’06.3”E	4-11-2009	*Acanthogorgia* sp.	KP033144 ; KP033152	This publication
0°54’24.7”N; 127°29’17.7”E	5-11-2009	*Acanthogorgia* sp.	KP033148 ; KP033156	This publication
0°54’44.5”N; 127°29’09.9”E	8-11-2009	*Acanthogorgia* sp.	- ; KP033157	This publication
1°27’13.8”N; 125°14’13.0”E	16-2-2012	*Acanthogorgia* sp.	KP033149 ; KP033158	This publication
4°32’07.4”N; 118°50’18.2”E	9-12-2010	*Paratelesto* sp.	KP033142 ; KP033150	This publication
7°05’07.2”N; 117°03’33.8”E	19-9-2012	*Echinogorgia* sp.	KP033146 ; KP033154	This publication
6°59’48.1”N; 117°03’13.4”E	18-9-2012	*Subergorgia* sp.	KP033145 ; KP033153	This publication
6°59’48.1”N; 117°03’13.4”E	18-9-2012	*Paraplexaura* sp.	KP033147 ; KP033155	This publication
-	-	-	AY161394 ; AY161627	Meyer, 2003
-	-	-	AY161390 ; AY161623	Meyer, 2003
-	-	-	AJ868555 ; -	Schiaparelli *et al*., 2005
-	-	-	AY161399 ; AY161632	Meyer, 2003

(Adams, 1855). In addition to this phylogenetic reconstruction, data on host species and distributional records are given for this group of rarely recorded ovulid snails.

Material and methods

Collection and identification

During fieldwork in Indonesia (Halmahera, Ternate; Sulawesi, Lembeh Strait) and Malaysia (Borneo, Semporna and Kudat) specimens of *Crenavolva* species were collected by SCUBA diving (Table 1).

The snails and their octocoral hosts were photographed in situ (Fig. 1) whenever possible and subsequently fixed in 80% ethanol. The holotype of *C. chiapponii* was studied at the Muséum national d'Histoire naturelle (MNHN) in Paris. For the identification of the other ovulid species, Cate (1973), Fehse (2002b) and Lorenz and Fehse (2009) were used. For the identification of the host species, microscopy slides of their calcareous skeletal parts (sclerites) were made by dissolving the samples in a 4% solution of household bleach. The residual sclerites were rinsed with tap water followed by demineralised water before mounting on a slide or on a stub for Scanning Electron Microscopy (SEM). Stubs with sclerites were coated with Au/Pd before SEM images were made with a JEOL 6480 LV. Identification of the octocorals to genus level was based on Stiasny (1947) and Fabricius and Alderslade (2001).

Barcoding Ovulidae

Specimens were barcoded for the COI barcoding region and for additional phylogenetic research also for the 16S marker. Tissue samples obtained from the foot and/or mantle were extracted with the Machery-Nagel DNA extraction kit on a KingFisher Flex. The standard COI barcoding primers by Folmer *et al*. (1994) and the Palumbi (1996) 16S primers were used. PCR amplification was performed on a C1000 Touch Thermal Cycler (Bio-RAD). Sequencing of the PCR products was performed at Macrogen Europe on an ABI 3730xl Automated Sequencer. Sequences were edited in Sequencher 4.10.1

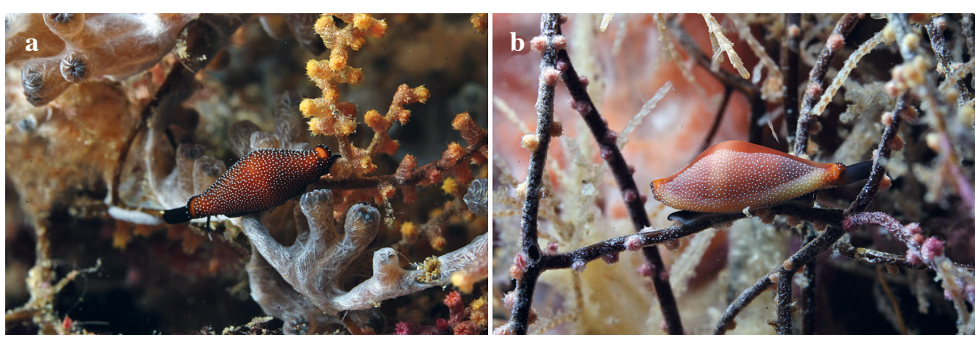


Fig. 1. a) In situ image of a) *Crenavolva aureola* (Fehse, 2002) (RMNH.Mol.164209) and b) *C. chiapponii* Lorenz and Fehse, 2009 (RMNH.Mol.164211) on *Acanthogorgia* sp. at Halmahera, Indonesia at 21 m and 17 m depth respectively.

and aligned with GUIDANCE (Penn *et al*., 2010) using the MAFFT algorithm (Katoh *et al*., 2005). Selecting an evolutionary model was done with jModeltest based on the Akaike Information Criterion score. MEGA 6.0.6 (Tamura *et al*., 2013) was used to perform Maximum Likelihood (ML) and Maximum Parsimony (MP) analyses and to calculate p-distances. Bayesian analyses were performed in MrBayes 3.2.0 (Ronquist and Huelsenbeck, 2003). MrBayes was run for 4,000,000 generations with six chains. Data were sampled every 100 generations. Sequence data for *Ovula ovum* (Linnaeus, 1758) from GenBank was used as an outgroup. GenBank data for *Crenavolva* cf. *rosewateri* (Cate, 1973), *C. tokuoi* Azuma, 1989 and *Primovula beckeri* (Sowerby III, 1900) was also included in the phylogenetic analyses.

Results

Collecting and morphology

Eight lots, containing 33 specimens representing four nominal *Crenavolva* species (*C. aureola*, *C. chiapponii*, *C. striatula* and *C. trailli*) were collected in Indonesia and Malaysia (Table 1; Fig. 2). For *C. chiapponii* this is the first record from shallow water. The specimens were assigned to these nominal species based on shell shape (rhomboid, inflated or slender) and the colour bands on the dorsum, which in case of *C. striatula* were also present on the labrum. For *C. aureola* and *C. chiapponii* the absence or presence of a white dorsal band on the shell is allegedly the most obvious character to distinguish the species.

After examination of the illustrations presented by Lorenz and Fehse (2009) and the newly collected material, minor morphological differences (strongly or weakly pronounced dentation, keeling angle, strongly or weakly produced funiculum, position of the widest part of the shell) do not clearly separate between *C. aureola* and *C. chiapponii* and can be considered morphological variation in a single species. The soft tissue colouration of both *C. aureola* and *C. chiapponii* is very similar (e.g. Fig. 1; Lorenz and Fehse 2009: A106, A107, p. 527). Both have a semi-transparent mantle which is entirely covered with small, irregularly placed, white dots, and both have a completely black or white foot, black tentacles with white tips, and a black siphon.

Molecular data

Nine specimens representing five species were sequenced for COI and 16S. For one sample of *C. chiapponii* (RMNH.Mol.164211) the 16S marker could not be amplified. Sequences were concatenated and aligned (GUIDANCE alignment score: 0.965034) which resulted in an alignment length of 1,080 base pairs per specimen including indels. Sequences obtained from GenBank are slightly shorter (~40 base pairs), these missing base pairs were coded as 'missing data'. The program jModeltest yielded in HKY+G as most optimal evolutionary model. This evolutionary model was implemented in the Bayesian and ML analysis. The results from the different phylogenetic reconstructions were congruent, therefore only the ML tree is shown (Fig. 3).

In the phylogenetic reconstructions, specimens of *Crenavolva striatula* and *C. tokuoi* form an unresolved trichotomy with the other *Crenavolva* specimens. The two

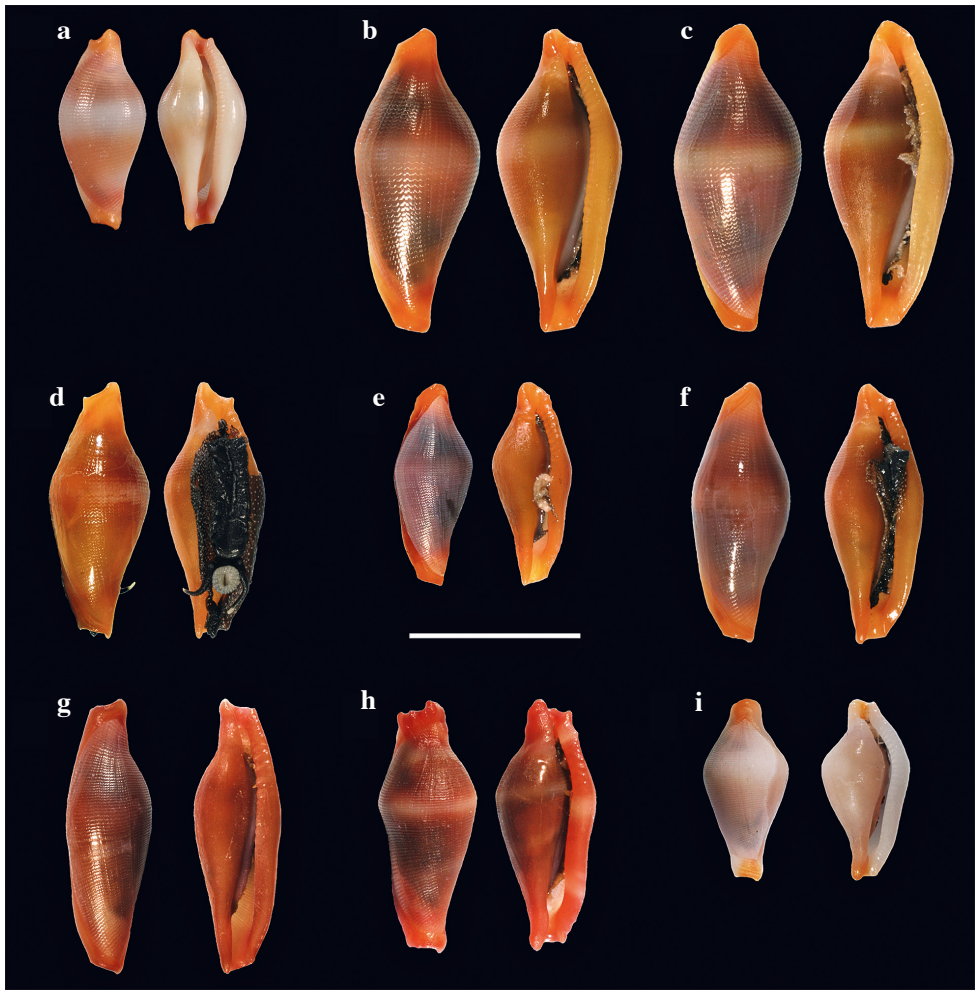


Fig. 2. Dorsal and ventral views of shells. a) Holotype of *Crenavolva chiapponii* Lorenz and Fehse, 2009 (MNHN 21244) b) *C. chiapponii* Lorenz and Fehse, 2009 (RMNH.Mol.164211) c) *C. chiapponii* Lorenz and Fehse, 2009 (RMNH.Mol.164217) d) *C. aureola* (Fehse, 2002) (RMNH.Mol.164085) e) *C. aureola* (Fehse, 2002) (RMNH.Mol.164072) f) *C. aureola* (Fehse, 2002) (RMNH.Mol.164209) g) *C. trailli* (Adams, 1855) (RMNH.Mol.164144) h) *C. striatula* (Sowerby I, 1828) (RMNH.Mol.164186) i) *Primovula rosewateri* (Cate, 1973) (RMNH.Mol.164062). Scale bars: 5 mm.

Primovula species cluster together and are well-supported sister species to all the *Crenavolva* species (with *C. striatula* as type species for the genus). This implies that the *Crenavolva* species used herein form a monophyletic group. The clustering of two *C. trailli* specimens is highly supported. Another well-supported clade holds three nominal species: *Crenavolva aureola*, *C. chiapponii* and *C.* cf. *rosewateri*. The pairwise p-distances between these three species are very low (16S: 0.2%; COI: 0.7%; concate-

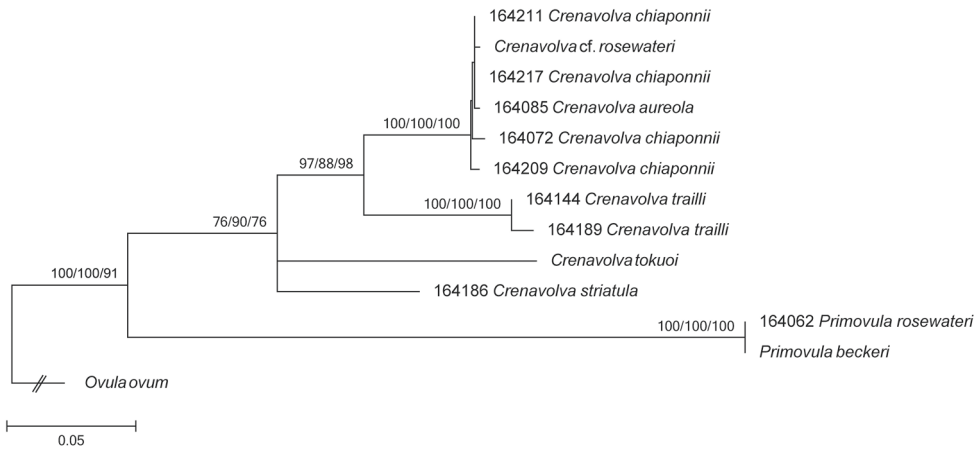


Fig. 3. Maximum Likelihood cladogram with support values for the ML/MP/BP analyses. Numbers preceding the species names represent RMNH.Mol. collection numbers of Naturalis Biodiversity Center; species names without numbers are obtained from GenBank for which additional data can be found in Table 1.

nated: 0.9%). In contrast, the sequence divergence between *C. trailli* and the *C. chiapponii* / *C. aureola* clade is almost ten times larger (16S: 5.2%; COI: 8.7%; concatenated: 8.2%). The sequence divergence between the two *C. trailli* specimens (16S: 0.6%; COI: 0.8%; concatenated: 0.8%) is almost equal to that between *C. aureola* and *C. chiapponii*. With the help of the Automatic Barcode Gap Discovery tool (ABGD) (Puillandre *et al*., 2011), the data were analysed to identify the MOTU's within the dataset. The results of this analysis showed that the barcode gap to identify the different species is 5-6% sequence divergence. This resulted in five groups containing the following species: 1, *C. aureola*, *C. chiapponii*, *C.* cf. *rosewateri*; 2, *C. trailli*; 3, *C. tokuoi*; 4, *C. striatula*; 5, *P. rosewateri*. One of the samples obtained from GenBank, viz. *Crenavolva* cf. *rosewateri* (= *Primovula* cf. *rosewateri*), clusters surprisingly within the clade containing *C. aureola* and *C. chiapponii* and not with the other *Primovula rosewateri* specimen. Instead, *Primovula beckeri* proves to be identical to the newly sequenced specimen of *Primovula rosewateri* from Malaysia.

Octocoral hosts

Almost all Ovulidae species are associated with Octocorallia hosts. By examining the sclerites and the habitus of the host corals, several new host species for ovulids of the genus *Crenavolva* could be identified. An overview of previously identified host species and new records is provided in Table 2. Some of the former host identifications were published with obsolete generic names, and therefore their names in the current literature are also provided.

Before *C. chiapponii* was synonymised, *Acanthogorgia* would have been a new host record. Yet, Reijnen (2010) already recorded *Acanthogorgia* sp. as a host for *C. aureola* and therefore it is not a new host record. Morphologically at least two different species

Table 2. Literature overview of the octocoral hosts of selected *Crenavolva* species including new records. Updated names of the octocoral hosts are provided between parentheses.

Ovulid species	Host genera	Reference
Crenavolva aureola	*Euplexaura*; *Astromuricea* (= *Echinogorgia*); *Acanthogorgia*	Lorenz and Fehse, 2009; Reijnen, 2010
Crenavolva chiapponii (= *C. aureola)*	*Acanthogorgia*	this publication; Reijnen, 2010
Crenavolva striatula	*Ellisella*; *Euplexaura*; *Echinogorgia*	Lorenz and Fehse, 2009; Yamamoto, 1973; Cumming, 1997; Mase 1989;
Crenavolva trailli	*Echinogorgia*; *Anthoplexaura* (= *Astrogorgia*); *Plexauroides* (= *Echinogorgia*); *Euplexaura*; *Subergorgia*	Goh *et al*., 1999; Mase, 1989
Primovula rosewateri	*Subergorgia*; *Dendronephthya*; *Stereonephthya; Paratelesto*	Goh *et al*., 1999; Lorenz and Fehse, 2009; this publication
Primovula beckeri	*Acanthogorgia*; *Acabaria* (= *Melithaea*);*Unicella* [sic] (= *Eunicella*); *Lophogorgia* (= *Leptogorgia*)	Schiaparelli *et al*., 2005; Lorenz and Fehse, 2009

of *Acanthogorgia* could be distinguished but these could not be identified since a revision of the family Acanthogorgiidae is lacking.

Furthermore, examination of the ovulid species and their octocoral hosts revealed that in two instances individuals formerly identified as *C. chiapponii* and *C. aureola* would have co-occurred on the same host coral, in both cases *Acanthogorgia* sp.

Discussion

Based on the molecular data and morphological observations listed above, *C. chiapponii* is considered a junior synonym of *C. aureola*. The systematic account is therefore as follows:

Systematic part

Family Ovulidae Fleming, 1822
Genus *Crenavolva* Cate, 1973

Crenavolva aureola (Fehse, 2002)

Primovula aureola Fehse 2002: 37, pl. 1, fig. 1.
Delonovolva formosa. — Gosliner *et al*. 1996: 136, fig. 469. Not *Delonovolva formosa* (Sowerby II in Adams and Reeve 1848). [= *Cuspivolva formosa* (Sowerby II in Adams and Reeve 1848)]
Primovula sp. — Coleman 2003: 51, fig. (Ovul: 121).
Crenavolva chiapponii Lorenz and Fehse 2009: 69, pl. 74, fig. 7-11.

The occurrence of *C. chiapponii* (= *C. aureola*) on Indonesian shallow water coral reefs would have represented new distribution records, both geographically and bathymetrically, before it was synonymised. However *C. chiapponii* proved to be a junior synonym of *C. aureola* and the new distribution records fall within the distribution range already known for *C. aureola*. Apparently, the dorsal white band and the minor morphological differences in shell shape are not indicative of species-level differences between *C. aureola* and *C. chiapponii*.

Molecular data

The species *Primovula rosewateri* was previously placed in the genus *Crenavolva* by Cate (1973) but Fehse (2002a) moved it to *Primovula*, primarily based on the triangular shape of the funiculum. The results of the molecular analyses (Fig. 3) support this decision. There is great genetic similarity between *C.* cf. *rosewateri* (= *Primovula* cf. *rosewateri*) obtained from GenBank, and *C. aureola*. However, the specimen from GenBank was collected from Balicasag Island, near Bohol, Philippines, which is the type locality of *C. chiapponii*. This location is approximately 85 km from Mactan Island of Cebu, Philippines which is the type locality of *C. aureola*. It is not unlikely that the so-called *C.* cf. *rosewateri* from GenBank (AY161394 (16S), AY161627 (COI)) was misidentified and actually represents *C. aureola*. Moreover, the newly sequenced specimen of *P. rosewateri* from Malaysia convincingly clusters with *Primovula beckeri*. According to Lorenz and Fehse 2009, *P. beckeri* has an E African distribution and was originally described from South Africa. The specimen obtained from GenBank is from Sulawesi, Indonesia (Schiaparelli *et al*., 2005). It is therefore unlikely that this sequence represents *P. beckeri* but instead is the quite similar species from the central Indo-Pacific, *P. rosewateri*.

Host species and distribution records

The ranges of the presently discussed species all fit within the Coral Triangle (see Hoeksema, 2007) and depend on the ranges of their host species. Species of the genus *Acanthogorgia* are not unique hosts for just *Crenavolva* spp. Reijnen (2010) already mentioned *Acanthogorgia* spp. as a host for *Dentiovula eizoi* Cate and Azuma, 1973 (in Cate, 1973) and *D. colobica* (Azuma and Cate, 1971). *Acanthogorgia* species and their ovulid associates are both known to occur from shallow to deep water in the Coral Triangle. In an overview of the Acanthogorgiidae by Stiasny (1947) the deepest record for an *Acanthogorgia* species is 4,239 m, collected SE of Seram, Indonesia (*Acalycigorgia densiflora* = *Acanthogorgia densiflora* (Kükenthal and Gorzawsky, 1908). Nevertheless, Stiasny (1947) doubts the identification and compared it to congeneric species which are found in waters not exceeding 400 m depth. As a result Stiasny (1947) doubts the entire record. Therefore the deepest reliable record for an *Acanthogorgia* species in the Malayan Archipelago is 1,254 m for *Acanthogorgia multispina* (Kükenthal and Gorzawsky, 1908). The deepest record for *Crenavolva* species is from approximately 1,000 m, which is the deepest record for any ovulid species found to date (Lorenz and Fehse, 2009).

Acknowledgements

Many thanks to Dr Bert W. Hoeksema who organized the Ternate and Lembeh Strait expedition together with Ir M.I. Yosephine Tuti Hermanlimianto under the umbrella of E-Win (Ekspedisi Widya Nusantara). LIPI Ternate and LIPI Bitung are acknowledged for accommodating the research at their field stations. LIPI and RISTEK granted research permits. The Semporna Marine Ecological Expedition (SMEE2010) was jointly organized by WWF-Malaysia, Universiti Malaysia Sabah's Borneo Marine Research Institute, Naturalis Biodiversity Center and Universiti Malaya's Institute of Biological Sciences, while research permission was granted by the Economic Planning Unit, Prime Minister's Department, Economic Planning Unit Sabah, Sabah Parks and Department of Fisheries Sabah. The MV Celebes Explorer accommodated the research in Semporna. The 2012 Tun Mustapha Park expedition (TMP) was jointly organised by WWF-Malaysia, Universiti Malaysia Sabah (UMS), Sabah Parks and Naturalis Biodiversity Center, the Netherlands. The research permits were granted by the Economic Planning Unit, Prime Minister's Department Malaysia and Sabah Biodiversity Centre. Both expeditions to Malaysia were co-organised by Ms Zarinah Waheed and WWF Malaysia, which was greatly appreciated. Dr Philippe Bouchet and Ms Virginie Heros were so kind to accommodate a visit to Muséum national d'Histoire naturelle (MNHN) in Paris to investigate the Ovulidae (type) collections. Dr Leendert P. van Ofwegen kindly provided help by identifying the octocoral hosts and Sancia van der Meij was a perfect dive buddy and ovulid hunter. Sequencing of the barcoding region of the ovulids was part of one of the barcoding initiatives within the Naturalis Biodiversity Center. Funding for fieldwork was provided by the Jan-Joost ter Pelkwijkfonds and A.M. Buitendijkfonds (Naturalis Biodiversity Center), Malacological Society of Australasia and the Percy Sladen Fund. I also would like to thank Prof Dr Catherine S. McFadden, an anonymous reviewer and Dr Thierry Backeljau for their constructive comments and remarks which improved the manuscript greatly.

Chapter 5

A new perspective on Ovulidae phylogenetics and systematics with special reference to the subfamily Aclyvolvinae

Bastian T. Reijnen

Abstract

Molecular phylogenetic research on species of the octocoral-associated family Ovulidae is still very limited. Phylogenetic relationships between subfamilies and genera are unclear and morphological characters can be confusing when dealing with species delimitations. Here four molecular markers (COI, 16S, 28S, and H3) and morphometrics are used to reconstruct the phylogeny and asses the systematics of the Aclyvolvinae, one of four subfamilies within the Ovulidae. These data are also analysed with 16S and COI sequences for other Ovulidae species from the remaining three subfamilies to identify the phylogenetic relationship of the subfamily Aclyvolvinae among the other ovulid subfamilies. The results show that two out of four subfamilies (viz. Aclyvolvinae and Simniinae) are polyphyletic. Within the subfamily Aclyvolvinae, the type species of *Hiatavolva*, *H. depressa*, does not cluster with the other *Hiatavolva* spp. Instead, the other species, *H. rugosa* and *H. coarctata*, cluster with the type and other species of the genus *Aclyvolva* and are therefore moved to that genus. Molecular and morphometric results show that *A. lamyi* and *A. nicolamassierae* are synonyms of *A. lanceolata* and that *A. rugosa* (n. comb.) is a synonym of *A. coarctata* (n. comb.). The genus *Kuroshiovolva* could not be retrieved in a fixed phylogenetic position within the Aclyvolvinae, but did not cluster with *Hiatavolva depressa* or *Aclyvolva* spp. Its taxonomic position remains therefore uncertain. In addition, photographs of type species are provided, as well as new information on the geographical distribution and host species of Aclyvolvinae.

Introduction

Species of the family Ovulidae occur in tropical, subtropical and temperate waters, but their diversity is highest in tropical waters of the Indo-Pacific (Lorenz and Fehse, 2009). Most of them are obligate symbionts of octocoral species. To provide camouflage against visual predation, their mantle colour is usually similar to that of their host octo-

Fig. 1. *In situ* images of Aclyvolvinae snails and their corals hosts. a) *Aclyvolva lanceolata* (RMNH. Mol.164192) on *Viminella* sp. at Kudat, Malaysia. b) *A. lamyi* (RMNH.Mol.164181) on *Junceella* sp. at Kudat, Malaysia. c) *Hiatavolva coarctata* (RMNH.Mol.164234) on *Ellisella* sp. at Lembeh Strait, Indonesia. d) *H. rugosa* (RMNH.Mol.164197) on *Ctenocella* sp. at Pulau Banggi, Malaysia. e) *H. depressa* (RMNH.Mol.164147) on *Alertigorgia orientalis* (Ridley, 1884) at Pulau Banggi, Malaysia. f) *Kuroshiovolva shingoi* at Bohol, Philippines. Photographs a-e by the author. f by E. Guillot de Suduiraut.

corals (Schiaparelli *et al*., 2005). Moreover, some ovulid species even mimic typical morphological octocoral host structures, such as their polyps (Fig. 1).

The family Ovulidae Fleming, 1882, has recently been revised (Fehse, 2007), which resulted in the recognition of four subfamilies, namely Ovulinae Fleming, 1822, Simniinae Schilder, 1925, Aclyvolvinae Fehse, 2007 and Prionovolvinae Fehse, 2007. The division into four separate subfamilies was partly based on a paper by Schiaparelli *et al*. (2005), in which the first molecular phylogeny reconstruction of the family Ovulidae was presented based on the mitochondrial 16S rRNA sequences. This phylogenetic reconstruction showed five groups in a polytomy (clades A-E after Schiaparelli *et al*., 2005). These clades were moderately to well-supported and could each represent a subfamily. Fehse (2007) combined the molecular 16S rRNA results of Schiaparelli *et al*. (2005) together with morphological characters as distinguished by Simone (2004) to erect the subfamilies Prionovolvinae and Aclyvolvinae. Of all four subfamilies, the Aclyvolvinae are morphologically well defined, easily recognisable as a subfamily, and holds the least number of species. Currently the Aclyvolvinae comprises eight recognised species (Lorenz and Fehse, 2009), divided over three genera (*Aclyvolva*, *Hiatavolva* and *Kuroshiovolva*). All are restricted to the central Indo-Pacific, except for *Aclyvolva nicolamassierae* Fehse, 1999, which occurs in the western Indian Ocean and the Red Sea (Fehse, 1999; Lorenz and Fehse, 2009). Species of *Aclyvolva* and *Hiatavolva* are hosted by gorgonians of the family Ellisellidae (Schiaparelli *et al*., 2005; Lorenz and Fehse, 2009; Reijnen, 2010), whereas members of *Kuroshiovolva* are found associated with primnoid corals of the genus *Plumarella* (Lorenz, 2009). Unfortunately, most ovulid material deposited in museum collections is not accompanied by data on the host species. The shells of Aclyvolvinae can easily be distinguished from those of other ovulids by their lanceolate form and the absence of a well-developed funiculum. Species-specific differences in the Aclyvolvinae are based on conchological characters such as the density and coarseness of the striae, or the presence or absence of longitudinal growth lines, or colour. Although these characters seem to be clear, large shell collections show much interspecific overlap in morphology, obscuring species differences. In juvenile shells the conchological characters are lacking or are expressed differently. As a consequence, many names have become available for similar lanceolate shells and there is disagreement among taxonomists. For example, Cate (1973) described two new genera and synonymized some species, while also describing or resurrecting others. Later, Lorenz and Fehse (2009) synonymized most of Cate's new species and many other available names, leaving only eight recognized species in the Aclyvolvinae. In the first molecular phylogenetic analysis of ovulids, Schiaparelli *et al*. (2005) included two species of Aclyvolvinae: *Aclyvolva lanceolata* (Sowerby II, 1848) and *A*. cf. *lamyi* (Schilder, 1932). These species clustered together in a monophyletic clade. The relationships between this clade of Aclyvolvinae and the other three subfamilies remained unresolved. These taxonomic uncertainties indicate the need for an integrated molecular and morphological approach to clarify the interspecific relationships in the Aclyvolvinae, which is the aim of the present study. To reconstruct the phylogenetic relationships between the Aclyvolvinae and the other ovulid subfamilies, to test generic assignments and to clarify the taxonomic status of the available species, material of seven nominal

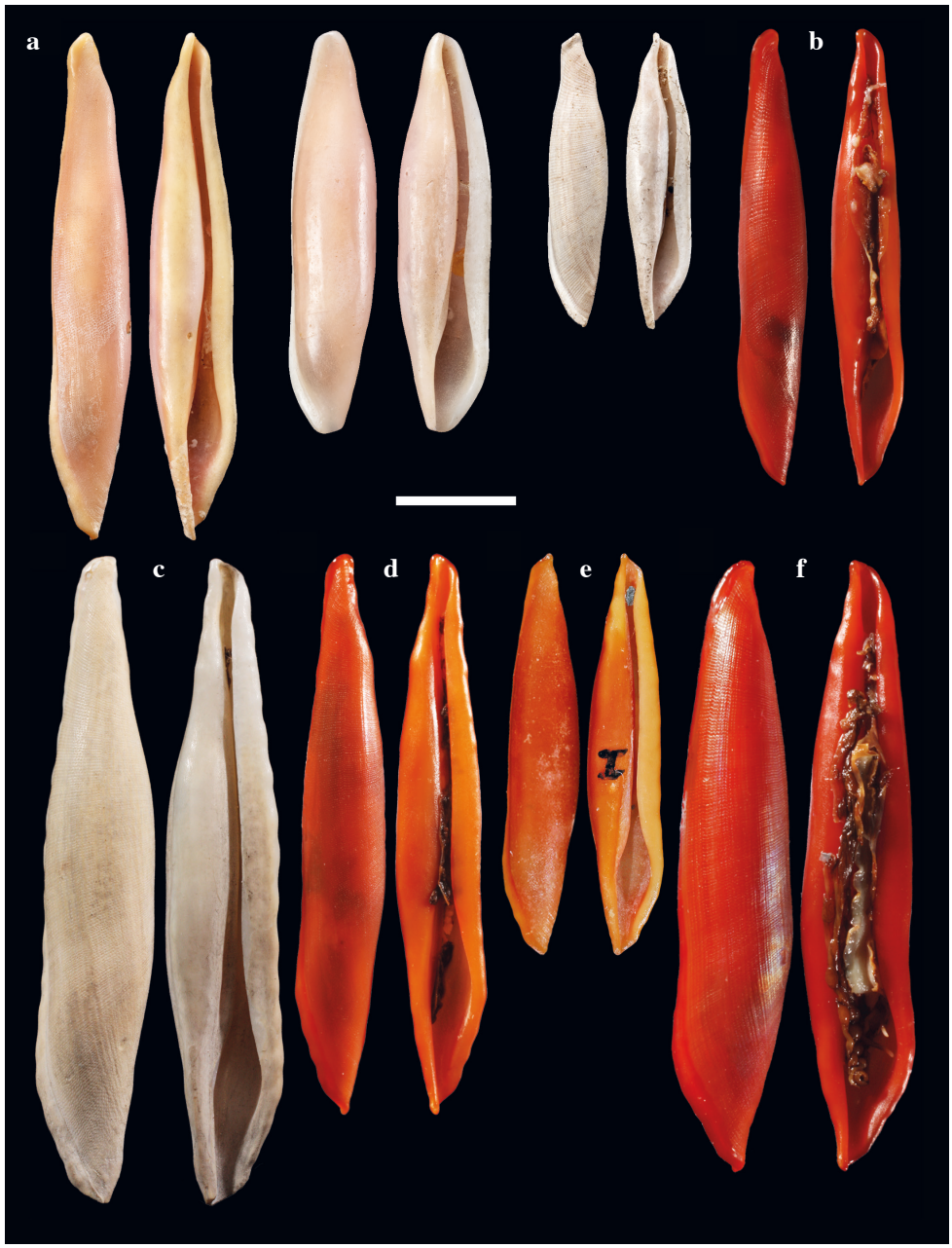


Fig. 2. Dorsal and ventral views of *Aclyvolva* (type)specimens. a) lectotype (left) and paralectotypes of *Ovulum lanceolatum* (=*Aclyvolva lanceolata*). b) *Aclyvolva lanceolata* (RMNH.Mol.164179). c) Holotype of *Neosimnia lamyi* (= *Aclyvolva lamyi*) (MNHN IM 2000-27664). d) *Aclyvolva lamyi* (RMNH.Mol.164165). e) Holotype of *Aclyvolva nicolamassierae*. f) *Aclyvolva nicolamassierae* (RMNH.Mol.337794). Photographs by author except for a) Andreia Salvador (BMNH) e) Dr. Vollrath Wiesse (Haus der Natur – Cismar). The scale bar represents 5 mm.

Aclyvolvinae species was sequenced. Morphological characters were evaluated to clarify species delimitations.

Material and methods

Abbreviations institutions

ANSP	The Academy of Natural Sciences of Drexel University, Philadelphia, USA
BMNH	Natural History Museum, London, UK
MNHN	Muséum national d'Histoire naturelle, Paris, France
Naturalis	Naturalis Biodiversity Center, Leiden, The Netherlands
SI	Smithsonian Institution, Washington D.C., USA

Sampling and identification

A total of 83 snail specimens and their cnidarian hosts were collected representing Ovulidae (n=79), Pediculariidae (n=3) and Cypraeidae (n=1). The latter two were used as outgroups. Snails belonging to the subfamily Aclyvolvinae represented seven nominal species: *Aclyvolva lamyi* (n=3), *A. lanceolata* (n=9), *A. nicolamassierae* (n=1), *Hiatavolva coarctata* (Sowerby II in Adams & Reeve, 1848) (n=13), *H. depressa* (Sowerby III, 1875) (n=2), *H. rugosa* (Cate & Azuma in Cate, 1973) (n=17) and *Kuroshiovolva shingoi* (Azuma & Cate, 1971) (n=1) (Fig. 2, 3). Two specimens included in the dataset represent type species of the genus, which are the type genera of their respective subfamilies; *Aclyvolva lanceolata* (Aclyvolvinae) and *Ovula ovum* (Linnaeus, 1758) (Ovulinae). For two other subfamilies, the Prionovolvinae and Simniinae, the type species are *Prionovolva brevis* (Sowerby I, 1828) and *Simnia nicaeensis* Risso, 1826, respectively, but unfortunately these are not represented due to the unavailability of suitable material.

The ovulid specimens were collected in Indonesia, Malaysia, Saudi Arabia and Thailand (see supplementary material S1 for more information). Voucher specimens were fixed in 70% ethanol and deposited in the mollusc collection of Naturalis (coded as RMNH.Mol) except for *Kuroshiovolva shingoi* Azuma and Cate, 1971. The voucher specimen for *K. shingoi* is curated by the SI and the sequences were provided by Dr. C.P. Meyer (SI). To identify the collected specimens a stereomicroscope (Leica MZ16) was used and specimens were compared with photographs of the Aclyvolvinae type specimens of *Aclyvolva nicolamassierae*, *Hiata rugosa*, *Neosimnia lamyi*, *Ovulum lanceolatum* and *O. coarctatum*) (Fig. 2, 3) and the ovulid monographs by Cate (1973) and Lorenz and Fehse (2009) amongst other Ovulidae literature.

DNA extraction and sequencing

Tissue for DNA extraction was obtained from the foot and/or mantle of the snails. The DNeasy Kit (QIAGEN) was used according to the corresponding protocol for animal tissue (v. 07/2006). Digestions were performed overnight for approximately 16 h and DNA elution was performed with 100 μl of buffer AE. DNA extracts were diluted (1:100 or 1:300) before PCR amplification. The PCR mixture contained: 2.5 μl PCR CoralLoad Buffer (containing 15 mM MgCl2) (QIAGEN), 0.5 μl dNTP's (2.5 mM), 1.0 μl per

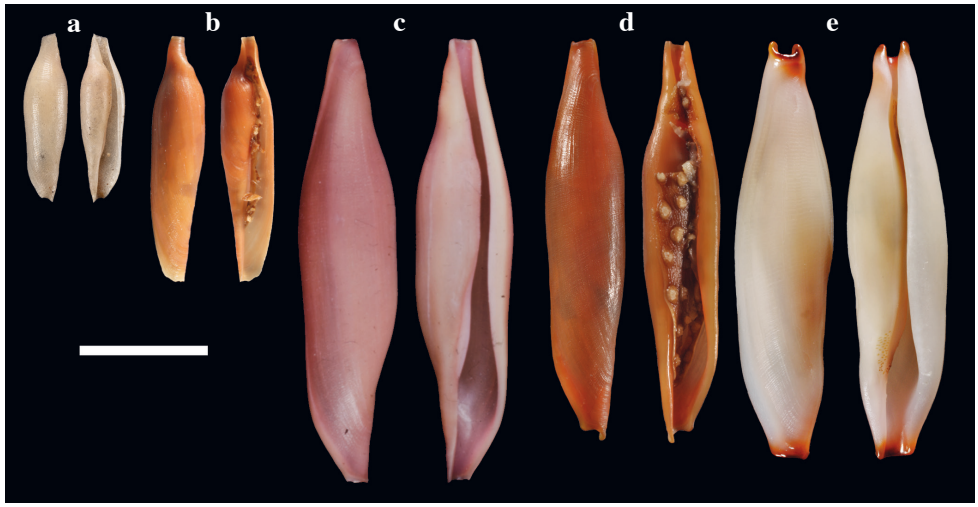


Fig. 3. Dorsal and ventral views of *Hiatavolva* (type)specimens. a) Holotype of *Ovulum coarctatum* (=*Hiatavolva coarctata*). b) *Hiatavolva coarctata* (RMNH.Mol.164185). c) Holotype of *Hiata rugosa* (= *Hiatavolva rugosa*). d) *Hiatavolva rugosa* (RMNH.Mol.164234). e) *Hiatavolva depressa* (RMNH.Mol. 164182). Photographs by author except for a) Andreia Salvador (BMNH). c) Prof. Gary Rosenberg (ANSP). The scale bar represents 5 mm.

primer (10 μM), 0.3 μl Taq polymerase (15 units/μl) (QIAGEN) and 18.7 μl of extra pure water and 1.0 μl (diluted) DNA extract. For amplification of the 28S marker 5.0 μl of water was replaced by 5.0 μl QSolution (QIAGEN). All PCR cycles consisted of an initial denaturing step of 95 °C for 1 min. followed by 39 cycles of 95°C for 10 s., preferred annealing temperature (see Table 1) for 1 min and an extension step of 72°C for 1 min. The final PCR cycle was followed by an elongated extension step of 72°C for 5 min. Successfully amplified samples were sent to Macrogen Europe for PCR cleaning and sequencing on an ABI Automated Sequencer 3730xl. Besides the Aclyvolvinae specimens, 41 specimens of 15 nominal ovulid species were sequenced for 16S and cytochrome c oxidase subunit I (COI). Not all markers were successfully amplified for all specimens, an overview of the sequence and provenance data is provided in Table S1. Sequence data for seven ovulid species (viz. *Crenavolva aureola* (4), *C. striatula* (1), *C. trailli* (2), *Cymbovula acicularis* (3), *Cyphoma gibbosum* (6), *Primovula rosewateri* (1) and *Simnia patula* (1)) was obtained from GenBank (see also Supplementary Material Table S1). All novel sequences are uploaded to GenBank (accession numbers: KP259314-KP259547 and KP271159-KP271161)

Molecular analyses

Sequences were edited using either Geneious Pro 5.6.4 or Sequencher 4.10.1 and aligned with ClustalW implemented in Bioedit (Hall, 1999) or the MAFFT algorithm used on the GUIDANCE server (Penn *et al*., 2010). All newly acquired sequences were checked against GenBank to check for resemblance with sequence data previously submitted by

Table 1. Information on primers, specific conditions and their references.

Primer names	Primer sequences	Region	Annealing T	Fragment size	Reference(s)
H3F & H3R	ATGGCTCGTACCAAGCAG ACVGC & ATATCCTTRGGC ATRATRGTGAC	Histone 3 (nuclear)	50	~ 380	Colgan *et al*., 2000
LSU5 & LSU800rc	TAGGTCGACCCGCTGAAY TTAAGCA & GACTCCTTGG TCCGTGTTTC	28S (nuclear)	50	~ 800	Littlewood *et al*., 2000; this publication
16Sar & 16Sbr	CGCCTGTTTATCAAAAA CAT & CCGGTCTGAACTCA GATCACGT	16S (mitochondrial)	52	~ 540	Palumbi *et al*., 1996
LCO-1490 & HCO-2198	GGTCAACAAATCATAAA GATATTGG & TAAACTTCA GGGTGACCAAAAATCA	COI (mitochondrial)	40-44	~ 660	Folmer *et al*., 1994

Meyer (2003) and Schiaparelli *et al*. (2005). Eventually sequences were concatenated with the help of SequenceMatrix (Vaidya *et al*., 2011) to create two datasets, one containing ovulid species representing all subfamilies (based on the 16S and COI markers) and a second dataset containing data of solely Aclyvolvinae (based on 16S, COI, histone H3 and 28S rRNA). The dataset containing all Ovulidae is 1,191 base pairs in length, including insertions and deletions (indels), and the Aclyvolvinae dataset is 2,296 base pairs long including indels. Each dataset was subjected to two model-testing algorithms, one implemented in MEGA 6.0.6 (Tamura *et al*., 2013) and to jModeltest2 (Darriba *et al*., 2012) (all AIC calculations). Subsequently the most optimal evolutionary model was selected for the various phylogeny reconstructions that were performed: Maximum Likelihood (ML) analyses (500 bootstrap iterations) in MEGA 6.06 and Bayesian inferences (BI) were calculated in MrBayes 3.2.2 (Ronquist and Huelsenbeck, 2003). Bayesian inferences were calculated over 10 million replicates using the dirichlet method. A tree was sampled every 100 iterations. The burnin was set to 50.000. The standard deviation of split frequencies was < 0.01. Support values for a MP analysis were determined over 500 bootstrap iterations using nearest neighbor interchange and the Tree-Bisection-Reconnection (TBR) branch swapping algorithm was used with ten initial trees. To check for non-arbitrary species delimitation, the molecular concatenated dataset for the four molecular markers was submitted to the online program ABGD (Automatic Barcode Gap Discovery) (Puillandre *et al*., 2012). Default settings with the Kimura (K80) TS/TV algorithm were used.

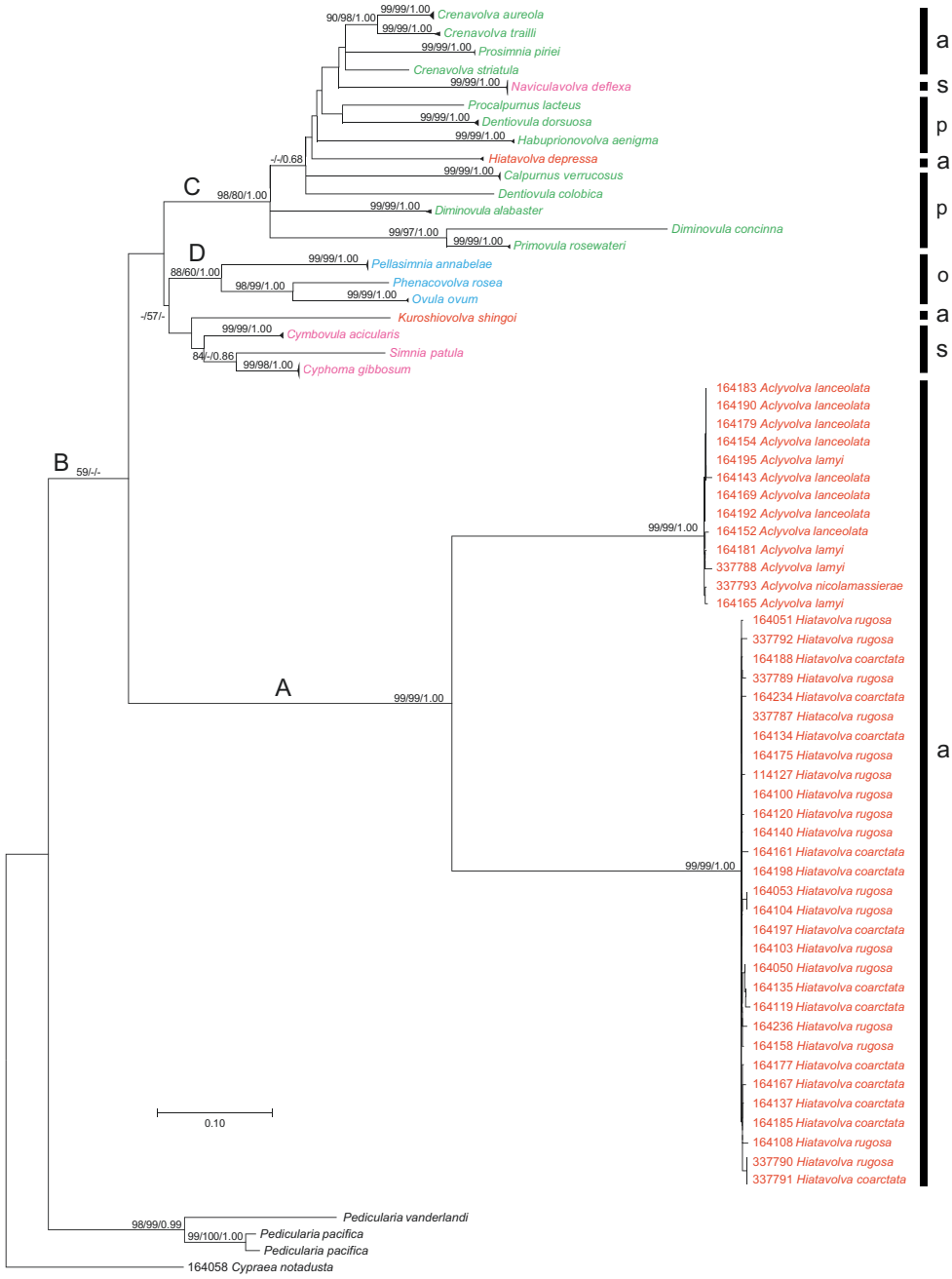


Fig. 4. Phylogeny reconstruction consisting of species representing each of the four subfamilies (Aclyvolvinae (= a), Ovulinae (= o), Prionovolvinae (= p) and Simniinae (= s)) within the Ovulidae. The phylogeny is based on 16S and COI. Support values are respectively bootstrap support values for MP and ML analyses, and posterior probabilities for BI. A-D refer to specific clades.

Morphological measurements and analyses

Shell morphological features were analysed by plotting 151 landmarks on photographs of the dorsal side of the sequenced specimens (in standard orientation as in Figs 2, 3), describing the entire shell outline. The Tps software package (tpsUtil, tpsDig2 and tpsRelw) (Rohlf, 2006) was used to create the morphological dataset and to calculate relative warps. The resulting relative warp data was exported to PAST (Palaeontological Statistics; Hammer *et al*., 2001) and was subjected to a principal component analysis (PCA). The length of all Aclyvolvinae specimens was measured with a calibrated digital calliper (Mitutoyo 500) as in Rosenberg (2010).

Results

Molecular analyses

In total 237 novel sequences for four molecular markers were generated including those from outgroup taxa. Sequences were combined into two datasets 1) All Ovulidae and 2) All Aclyvolvinae. The results from the different modeltest approaches were as follows: GTR+I+G by MEGA and TVM+I+G by jModeltest as most optimal evolutionary model. For the Aclyvolvinae dataset the GTR+I+G model was selected by both MEGA and jModeltest. Since the second best option in jModeltest Ovulidae dataset was the GTR+I+G model with only a small decimal difference in the likelihood calculations between the TVM+I+G and GTR+I+G model, the GTR+I+G model was selected for all analyses. The phylogeny reconstructions based on the Ovulidae dataset with representatives of all subfamilies showed that the relationships between the subfamilies, Simniinae, Prionovolvinae and Aclyvolvinae are unresolved (Fig. 4) only the Ovulinae are retrieved as a monophyletic group. Species of the subfamily Aclyvolvinae were retrieved at three different positions in the phylogeny reconstruction, which indicates that this subfamily is polyphyletic according to the definition of the subfamily Aclycolvinae of Fehse (2007). The Aclyvolvinae group that consists of *Aclyvolva lamyi*, *A. lanceolata*, *A. nicolamassierae*, *Hiatavolva coarctata* and *H. rugosa* (clade A in Fig. 4) is retrieved as a highly supported clade (99/99/100) to the clade containing members from all ovulid subfamilies. Phylogenetic relationships between these two clades are not supported (B in Fig. 4; 59/-/-). The type species of the genus *Hiatavolva*, *H. depressa*, is found among the Prionovolvinae. Phylogenetic relationships between the species in the group that contains *H. depressa* are unresolved, but all together this clade (clade C in Fig. 4), is well-supported (98/80/100). The other representatives of the genus *Hiatavolva* (*H. coarctata* and *H. rugosa*) are not retrieved as a sister species to the type species *H. depressa*, but cluster strongly as a sister group of *Aclyvolva* (clade A in Fig. 4). The genus *Kuroshiovolva*, here represented by *K. shingoi*, does not have a fixed position in the phylogeny reconstructions based on the ML, MP, and BI analyses. This species is either found unsupported as a sister species to all subfamilies or is retrieved in the group containing the Atlantic Ovulidae representatives *Cyphoma gibbosum*, *Cymbovula acicularis* and *Simnia patula*.

The cladogram based on four markers, representing only Aclyvolvinae species (Fig. 5), shows that there is almost no genetic distance between the nominal species *H. coarctata*/*H. rugosa* and *A. lanceolata*/*A. nicolamassierae*/*A. lamyi*. The non-arbitrary approach

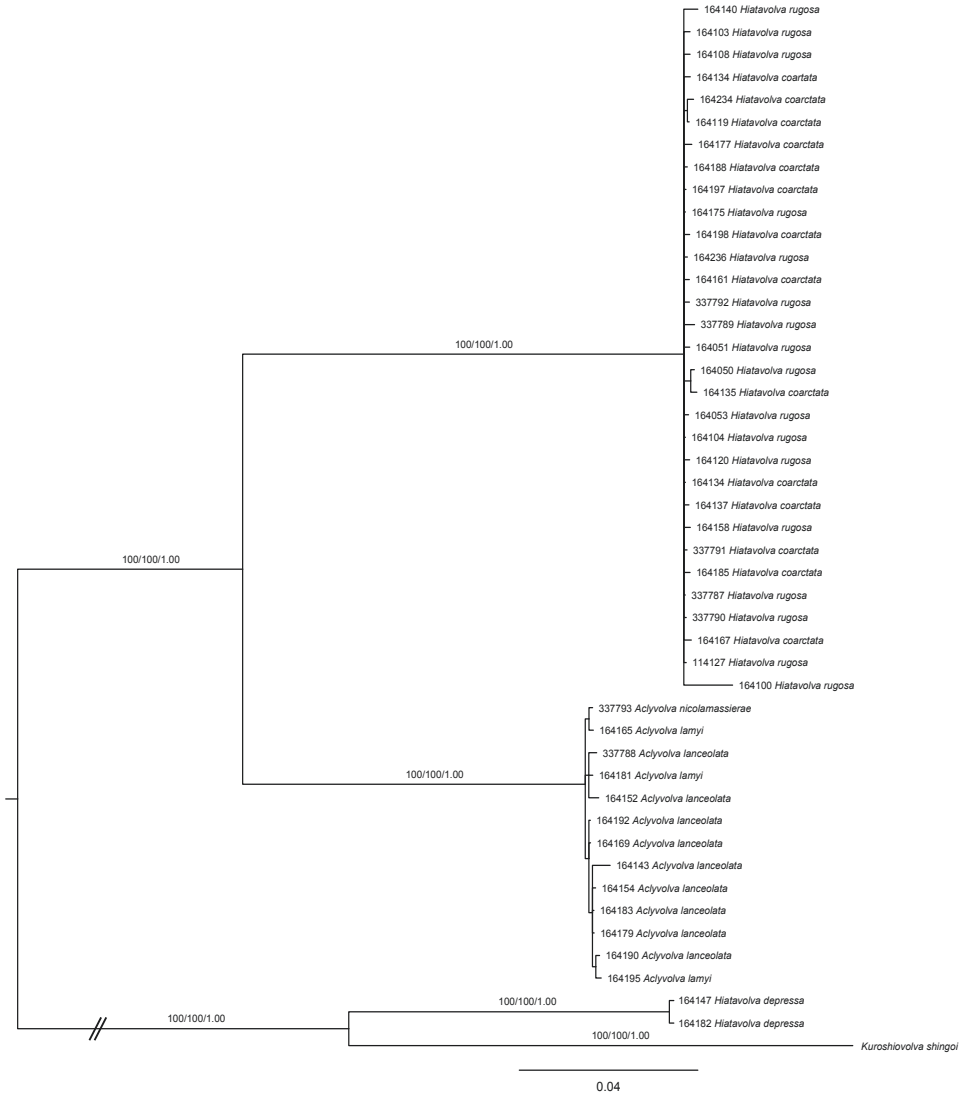


Fig. 5. Phylogeny reconstruction of the Aclyvolvinae based on 16S, COI, H3 and 28S. Support values are respectively bootstrap support values for MP and ML analyses, and posterior probabilities for BI.

for species delimitation in the ABGD analysis, supported these findings. Based on the differences in intra vs. interspecific sequence variation, the ABGD analysis resulted in four groups of species according to the clades in Figure 5, containing: 1) *A. lanceolata*/*A. nicolamassierae*/*A. lamyi*, 2) *H. coarctata*/*H. rugosa*, 3) *H. depressa* and 4) *K. shingoi*.

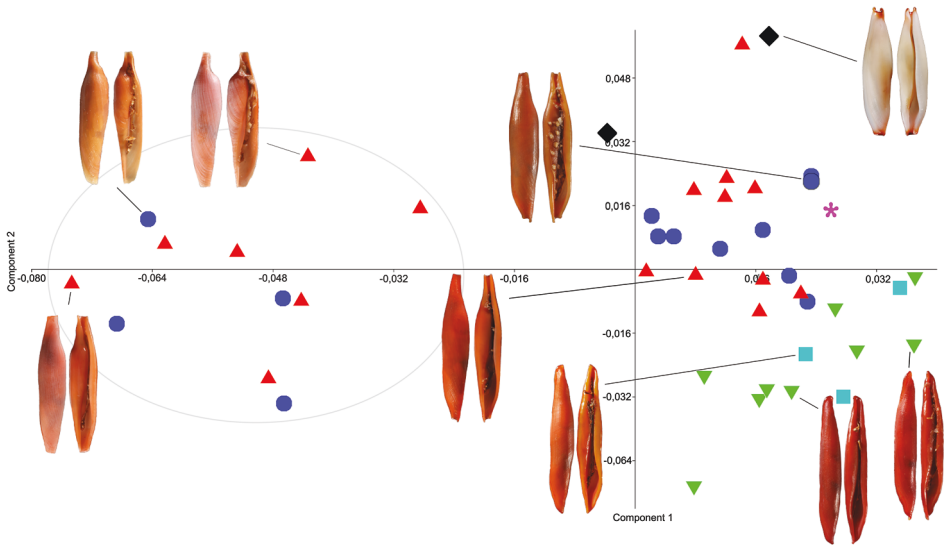


Fig. 6. Plot of the results of the principal component analysis (PC1 and PC2) on 44 relative warps and 151 landmarks. Square: *A. lamyi*; inverted triangle: *A. lanceolata*; asterix: *A. nicolamassierae*; circle: *H. coarctata*; diamond: *H. depressa*; triangle: *H. rugosa*. Images of the shells are not to scale.

Morphological analyses on Aclyvolvinae

The PCA was based on 44 relative warp coordinates of 151 landmarks. Principal component 1, 2, and 3 accounted for 88% of the variation amongst samples. *H. coarctata* and *H. rugosa* were scattered throughout the plot in two recognisable groups composed of each nominal species (Fig. 6).

Aclyvolva spp. also formed a group without further noticeable separation between species except for *A. nicolamassierae* which falls just outside the cluster near to the *Hiatavolva* spp. The two specimens of *H. depressa* did not cluster with the other *Hiatavolva* or *Aclyvolva* species. As a result of the separation into two groups, each containing specimens of *H. coarctata* and *H. rugosa*, shells were investigated more closely. The encircled specimens in the plot are all smaller in size (mean length = 12.14 mm, ± 2.56 mm, n=11) whereas the non-encircled specimens are longer (mean length = 15.81 mm ± 2.70 mm, n=19). Moreover, shells on the left have a less developed and less calloused shell, which is typical for juveniles or subadults, whilst specimens on the right side generally have a well-developed labrum and adapical and abapical canals.

Discussion

Molecular phylogeny and subfamily classification of Ovulidae

The deep phylogeny of the Ovulidae, as shown in the present phylogeny reconstruction (Fig. 4) is far from resolved. Many nodes are not or poorly supported, which hampers the higher taxonomic classification within the Ovulidae. Furthermore, the proposed

higher systematic classification as proposed by Fehse (2007), is inconsistent with the present molecular results. This study only deals with a limited number of representatives from the subfamilies, but already shows that the Simniinae, Prionovolvinae and Aclyvolvinae are not monophyletic subfamilies. The only monophyletic subfamily is the Ovulinae. Both Schiaparelli *et al*. (2005) and Fehse (2007) also concluded that the Ovulinae are monophyletic. In order to guarantee monophyly for the other three subfamilies, taxonomic rearrangements have to be made. Whether such rearrangements can be supported with morphological data is uncertain and requires additional studies. What can be observed from the phylogeny reconstruction is that ovulid shell shapes (e.g. rhomboid, lanceolate, globose or pyriform) are not restricted to specific clades in the phylogeny reconstructions. For example species having the lanceolate shells (e.g. Aclyvolvinae *sensu lato*) are retrieved at three positions in the cladogram, and likely reflects convergent evolution in ovulid shell shape rather than common ancestry. Studies on homoplasy and convergent evolution in marine gastropods (e.g. Marko and Vermeij, 1999; Johannesson, 2003) show that ecological factors can influence shell morphological features. Especially since ovulids live in close association with certain octocoral families or genera, homoplasy in ovulid shell shapes could indicate there is a functional requirement to live and survive on specific host species. Traditional taxonomic arrangements based on shell shapes are therefore biased by possible convergent evolution in Ovulidae, possibly triggered by host species symbiosis which finally troubles the higher systematics in the family Ovulidae.

Classification of Aclyvolvinae sensu stricto*: molecular and morphological evidence*
For the Aclyvolvinae, seven of the eight nominal species are retrieved at three different positions in the cladogram. Two species (*H. depressa* and *K. shingoi*) do not cluster near the clade containing the type species of the Aclyvolvinae. It is therefore clear that *H. depressa* does not belong in the subfamily Aclyvolvinae. *H. depressa* is morphologically also very distinct from all other Aclyvolvinae because of indented terminals creating two teeth-like projections at either terminal end. The phylogenetic position of *K. shingoi* is harder to justify due to unresolved grouping with other species from different subfamilies e.g. Simniinae. From a morphological perspective the placement of *K. shingoi* within the Simniinae is not in contrast with the shell-based diagnosis for this family, except for lacking a prominent transverse cord-like funiculum, which is lacking in all *Kuroshiovolva* species (Lorenz & Fehse, 2009). Besides the Simniinae, *K. shingoi* also clustered with members of the subfamily Ovulinae, although support values are low. The diagnosis for the subfamily Ovulinae states that shells can be ovate to spindle-shaped and that the funiculum is usually absent or indistinct and that the anal canal of their shells is slightly twisted. When *Kuroshiovolva* species are compared with this diagnosis, the shell shape and twisted anal canal cannot be matched. The position of this genus within the Ovulidae is therefore still unclear and requires additional research. The type specimen of *Hiatavolva coarctata* is a subadult shell and is therefore lacking most of the characters used in adult shells to distinguish species. Actually, the last sentence of the species description of *H. coarctata* by Sowerby II (1848) states: “It may, however, very possibly be a young shell”. Liltved (1989) agrees that the type of

H. coarctata is most probably a subadult shell, which does not fully resemble the characteristics in the adult shell morphology. Additionally, Liltved (1989: p. 132) also questioned the difference between *Phenacovolva coarctata* and *P. rugosa* (= *H. coarctata* and *H. rugosa*) based on their shell morphology. Nevertheless, Fehse (1999) disagreed with Liltved based on three shell characters: (1) small size, (2) shorter terminals, and (3) colour. Two of these characters are related to the subadult stage of the shell, e.g. the small size and shorter terminals. Colour was later disregarded by Lorenz and Fehse (2009) since they only identified two morphological differences to identify these species: (1) terminal length and (2) longitudinal sculpturing. Newly collected specimens in this study, from subadult and adult stages were morphologically assigned to either *H. coarctata* or *H. rugosa* based on these two characteristics. The morphometric analysis of these specimens shows that the juveniles are indeed morphologically distinct from adults (apart from just their size) whilst the molecular results do not show any support to separate these morphospecies into different species groups (Fig. 4, 6). This supports the assumption that juvenile shells are morphologically significantly different in comparison to their adult conspecifics. As can been observed in the successive growth stages of *Cyphoma gibossum*, juveniles in the family Ovulidae can differ morphologically very much from conspecific adults (Reijnen *et al*., 2010). This variable shape has probably caused problems in the identification of Aclyvolvinae specimens in earlier studies (e.g. Schiaparelli *et al*., 2005). Molecular data is one of the tools that could help to overcome difficulties in morphological species identifications. Molecular data for 16S, presented here, was checked against molecular data of Schiaparelli *et al*. (2005) deposited on GenBank. Specimens identified by the author as *H. coarctata/rugosa* match convincingly with *A. lanceolata* of Schiaparelli *et al*., 2005. Consequently, material herein identified as *A. lanceolata*, matched with *A. cf. lamyi* sequences from GenBank (Schiaparelli *et al*., 2005). Comparison of the photographs of the living animals and their respective shells (Suppl. Mat. Fig 3H, I, L, M and 4F-I, L) provided by Schiaparelli *et al*. (2005) with specimens figured in Cate (1973), Lorenz and Fehse (2009) and the images of the holotypes shown here, indicate that the GenBank specimens most probably have been misidentified (see also: Fehse, 2006, p. 19). However, in case of the Aclyvolvinae, shell morphological features are often indistinct but mantle patterns and structures provide an additional tool for identifying these species. *In situ* images show that *H. coartata* specimens have retractile mimetic gorgonian polyps whilst specimens representing *A. lanceolata* lack these mimetic polyps but have papillae on their mantle that might be of a different colour when compared to the rest of the mantle colour (Reijnen, 2011: Fig. 3 A,B; Lorenz and Fehse 2009: Fig A350-A365).

Remarks on distribution and host species of Aclyvolvinae

The distribution of ovulid species is highly dependent on the abundance of their host species. For example the coral hosts of the ovulid group containing *A. lanceolata* and its direct sister group containing some *Hiatavolva* spp. (clade A in Fig. 4), are different than those of *H. depressa* and *Kuroshiovolva* spp. *Hiatavolva depressa* is only known from *Alertigorgia orientalis* (Ridley, 1884) and *A. hoeksemai* van Ofwegen & Alderslade, 2007 from the octocoral family Anthothelidae. Species from the genus *Kuroshiovolva*

are only found in association with *Plumarella* spp. (family Primnoidae) and possibly *Astrogorgia* sp. (family Plexauridae). In comparison, all other Aclyvolvinae are found on octocorals of the family Ellisellidae (primarily *Ctenocella*, *Dichotella, Ellisella* and *Junceella*). As a consequence of these intricate associations, the absence of *H. depressa* in the Indian Ocean and Red Sea can be directly related to the absence of host corals of its host genus *Alertigorgia*. In contrast, *Aclyvolva* species are associated with Ellisellidae. Members of this family are found at the Indo-Pacific in shallow and deep water thereby fostering the distribution of *Aclyvolva* spp. In the collections of Naturalis there is also a specimen of *A. lanceolata* from the Persian Gulf (RMNH.Mol.187230). Like *A. nicolamassierae*, specimens from almost enclosed water bodies, such as the Red Sea and the Persian Gulf, are often considered endemic and/or new to science. Nevertheless, newly acquired molecular data for *A. nicolamassierae* from the Red Sea showed no

Table 2. Aclyvolvinae species, host species and ovulid distribution including their reference for *Aclyvolva*, *Hiatavolva* and *Kuroshiovolva* species.

Ovulid species (this study)	Original identification	Known octocoral host species	Geographical location	Reference
Aclyvolva coarctata	*Aclyvolva lanceolata*	*Ellisella* sp.; ?*Muricella* sp.	Sulawesi, Indonesia	Schiaparelli *et al*., 2005
Aclyvolva coarctata	*Hiata coarctata*	?*Echinogorgia rigida*	Kanagawa Prefecture, Japan	Mase, 1989
Aclyvolva coarctata	*Hiatavolva coarctata*	?*Echinogorgia* sp.; ?*Melithaea* sp.; ?*Muricella* sp.	Reunion to western Pacific	Lorenz and Fehse, 2009 (see also caption A356, A357 in Lorenz and Fehse, 2009)
Aclyvolva coarctata	*Hiatavolva coarctata*	*Dichotella* sp.; *Ellisella* sp.	Halmahera, Indonesia	Reijnen, 2010
Aclyvolva coarctata	*Hiatavolva coarctata*	*Ctenocella* sp.; *Ellisella* sp.; *Verrucella* sp.; *Viminella* sp.	Indonesia; Malaysia	This publication
Aclyvolva coarctata	*Hiatavolva rugosa*	?*Echinogorgia* sp.; *Ellisella* sp.; *Verrucella* sp.; *Viminella* sp.	Japan; Philippines; Indonesia; Queensland, Australia; E South Africa	Lorenz and Fehse, 2009
Aclyvolva lanceolata	*Aclyvolva* cf. *lamyi*	*Dichotella* sp.	Sulawesi, Indonesia	Schiaparelli *et al*., 2005
Aclyvolva lanceolata	*Aclyvolva lamyi*	*Ellisella* sp.; *Junceella* sp.	Philippines; Australia; Indonesia	Lorenz and Fehse, 2009
Aclyvolva lanceolata	*Aclyvolva lamyi*	*Ctenocella* sp.; *Junceella* sp.	Malaysia	This publication
Aclyvolva lanceolata	*Aclyvolva lanceolata*	*Dichotella* sp.; *Ellisella* sp.; *Junceella* sp.	Central Indo-Pacific	Lorenz and Fehse, 2009

genetic difference with *A. lanceolata* specimens from Indonesia and Malaysia and this species is therefore hereafter synonymised with *A. lanceolata*. As a result it can be concluded that *A. lanceolata* is distributed throughout the entire Indo-Pacific. *Kuroshiovolva* specimens are scarcely available in natural history collections and as a result not much is known about its host species. Three publications provide host data for species belonging to this genus (see Table 2). All publications mention *Plumarella* as the primary host genus, except for Lorenz (2009) who also mentions *Astrogorgia*. According to Fabricius and Alderslade (2001) there is only one *Plumarella* species known from shallow water (*Plumarella penna*), which occurs in Australia, all other species are from deeper and colder water. *Plumarella* spp. are considered to have a very limited distribution and it is therefore unclear what the effect is on the distribution of *Kuroshiovolva* spp.

Table 2. Cont.

Aclyvolva lanceolata	*Aclyvolva lanceolata*	*Ellisella* sp.	Halmahera, Indonesia	Reijnen, 2010
Aclyvolva lanceolata	*Aclyvolva lanceolata*	*Verrucella* sp.; *Junceella* sp.; *Ctenocella* sp.; *Viminella* sp.; *Dichotella* sp.	Malaysia; Thailand	This publication
Aclyvolva lanceolata	*Aclyvolva nicolamassierae*	*Ellisella* sp.	Red Sea, Tanzania to S Mozambique, Reunion	Lorenz and Fehse, 2009
Hiatavolva depressa	*Hiatavolva depressa*	*Alertigorgia orientalis*; *A. hoeksemai*	Central Indo-Pacific (Australia, Indonesia, Malaysia, New Caledonia)	Lorenz and Fehse, 2009
Hiatavolva depressa	*Hiatavolva depressa*	*Alertigorgia orientalis*	Malaysia	This publication
Kuroshiovolva lacanientae	*Kuroshiovolva lacanientae*	*Plumarella* sp.; *Astrogorgia* sp.	Philippines	Lorenz, 2009
Kuroshiovolva shingoi	*Kuroshiovolva shingoi*	*Plumarella* sp.	Japan; Philippines; New Caledonia; Fiji; New South Wales, Australia	Lorenz and Fehse, 2009
Kuroshiovolva shingoi	*Kuroshiovolva shingoi*	*Plumarella cristata* (= *Acanthoprimnoa cristata*)	Wakayama Prefecture, Japan	Yamamoto, 1972
Prosimnia draconis	*Prosimnia (Prosimnia) coarctata*	?*Melithaea flabellifera* (= *M. japonica*)	Wakayama Prefecture, Japan	Yamamoto, 1972

Doubtful host records
For *A. coarctata* all host genera in Table 2, except for *Echinogorgia*, *Melithaea* and *Muricella*, are representatives of the family Ellisellidae. The other three genera belong to the families Plexauridae, Melithaeidae and Acanthogorgiidae respectively. The host record for *Melithaea flabellifera* (= *M. japonica*, Matsumoto and Ofwegen, 2015) comes from Yamamoto (1973). Fortunately there are photographs of the living specimen included which clearly show that the ovulid species is actually a *Prosimnia* cf. *draconis* Cate, 1973 on a melithaeid, which is the common host genus for this ovulid species (Reijnen, 2010). For *A. coarctata* this host species record should therefore be neglected. Also the identification of *Muricella* and *Echinogorgia* as host genera (see caption A356, A357 in Lorenz and Fehse 2009) seems questionable. *Muricella* species are notoriously hard to identify (Reijnen *et al*., 2011) but based on the photographs it can be concluded the host species most likely represents a *Verrucella* sp. from the family Ellisellidae. *Verucella* and *Muricella* both have a planar and reticulated growth form. The other doubtful host record is that of *Echinogorgia* (Mase, 1989; Lorenz and Fehse, 2009). *Echinogorgia* is easily confused with other gorgonian genera (e.g. *Paraplexaura*) and cannot be identified *in situ* based on its habitus. Moreover, this genus is very uncommon in the Indo-Pacific which makes it more questionable as a possible host species. This specific host record remains doubtful unless tissue samples of the host can be examined.

Systematics
Based on the aforementioned phylogenetic and morphological analyses, three species names should be synonymised (*A. lamyi*, *A. nicolamassierae* and *H. rugosa* and/or placed into a different genus (*H. coarctata*). Due to the position of *A. lanceolata* as type of the name-bearing genus of the respective subfamily, the species represented in this clade (Clade A in Fig. 4) can be referred to as 'true' Aclyvolvinae. As a result of their morphological resemblance and phylogenetic affinity, the species *Hiatavolva rugosa* and *H. coarctata* should for that reason be transferred to the genus *Aclyvolva*.
The systematic account of the ovulid species is therefore as follows:

Ovulidae Fleming, 1822
Prionovolvinae Fehse, 2007
Hiatavolva Cate, 1973

Hiatavolva depressa (Sowerby III, 1875)

Ovulum depressum Sowerby III, 1875: 128, pl. 24, fig. 1
Phenacovolva depressa.— Iredale, 1935: 105
Neosimnia (*Pellasimnia*) *depressa*.— Allan, 1956: 130
Hiata depressa.— Cate, 1973: 87, fig. 194
Hiatavolva depressa.— Lorenz and Fehse, 2009: 135, pl. 192, fig. 1-6

Aclyvolvinae Fehse, 2007
Aclyvolva Cate, 1973

Aclyvolva lanceolata (Sowerby II, 1848)

Ovulum lanceolatum Sowerby II, 1848: 135
Ovula lanceolata.— Weinkauff, 1881: 207, pl. 52, fig. 10-11
Neosimnia lamyi Schilder, 1932: 54, pl. 4, fig. 44
Neosimnia lanceolata.— Allan, 1956: 127
Aclyvolva nicolamassierae Fehse, 1999: 51, pl. 2, fig. 1-2
Aclyvolva lanceolata.— Lorenz and Fehse, 2009:
Hiatavolva cf. *lamyi*.— Lorenz and Fehse, 2009: 615, A352
Aclyvolva (cf.) *lamyi*.— Lorenz and Fehse, 2009: 134, pl. 190, fig. 1-10, A353-A355

Aclyvolva coarctata (Sowerby II, 1848 in Adams and Reeve, 1848) comb. nov.

Ovulum coarctatum Sowerby II, 1848 (in Adams and Reeve, 1848): 21, pl. 6, fig 2a,b
Ovula coarctata.— Weinkauff, 1881: 188 pl. 48, fig 9, 12
Prosimnia (*Prosimnia*) *coarctata*.— Kuroda, 1958: 169
Hiata rugosa Cate and Azuma, 1973 (in Cate, 1973): 87, fig. 197
Phenacovolva coarctata.— Liltved, 1989: 132
Hiatavolva coarctata.— Lorenz and Fehse, 2009: 135, pl. 191, figs. 1-10, 18, A356-A359, not A360-A361 (= *Aclyvolva lanceolata*)
Hiatavolva rugosa.— Lorenz and Fehse, 2009: 135, pl. 191, figs. 11-17, A362-A365

Due to the above taxonomic and systematic changes, the diagnosis for the genus *Aclyvolva* should be extended with characters used to distinguish *Aclyvolva* from *Hiatavolva*. The modified diagnosis of *Aclyvolva* is as follows: "Shells elongate, narrow, rather cylindrical. Posterior terminal narrow, anterior broader. Canals open. Tips of terminals usually pointed but can also be blunt or have indented terminal tips. Aperture narrow and wideness in the fossular section, abruptly constricting to form the siphonal canal. Funiculum absent." (adapted from Lorenz and Fehse, 2009).

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Suppl. Mat. S1. Provenance data for Ovulidae used in this study.

Collection Code (RMNH.Mol.)	Genus	Species	Author	COI	16S	H3	28S
164165	*Aclyvolva*	*lamyi*	(Schilder, 1927)	KP259478	KP259362	KP259527	KP259408
164181	*Aclyvolva*	*lamyi*	(Schilder, 1927)	KP259486	KP259370	-	KP259414
164195	*Aclyvolva*	*lamyi*	(Schilder, 1927)	KP259494	KP259378	KP259537	KP259421
164143	*Aclyvolva*	*lanceolata*	(Sowerby II, 1848)	KP259466	KP259349	KP259522	-
164152	*Aclyvolva*	*lanceolata*	(Sowerby II, 1848)	KP259470	KP259354	KP259523	KP259404
164154	*Aclyvolva*	*lanceolata*	(Sowerby II, 1848)	KP259472	KP259356	KP259524	KP259405
164169	*Aclyvolva*	*lanceolata*	(Sowerby II, 1848)	KP259480	KP259364	-	KP259410
164179	*Aclyvolva*	*lanceolata*	(Sowerby II, 1848)	KP259485	KP259369	-	KP259413
164183	*Aclyvolva*	*lanceolata*	(Sowerby II, 1848)	KP259488	KP259372	KP259532	KP259416
164190	*Aclyvolva*	*lanceolata*	(Sowerby II, 1848)	KP259492	KP259376	KP259535	KP259419
164192	*Aclyvolva*	*lanceolata*	(Sowerby II, 1848)	KP259493	KP259377	KP259536	KP259420
337788	*Aclyvolva*	*lanceolata*	(Sowerby II, 1848)	KP259505	-	KP259543	KP259427
337793	*Aclyvolva*	*nicola-massierae*	Fehse, 1999	KP259510	KP259393	KP259547	KP259432
164077	*Calpurnus*	*verrucosus*	(Linnaeus, 1758)	KP259446	KP259327	-	-
164078	*Calpurnus*	*verrucosus*	(Linnaeus, 1758)	KP259447	KP259328	-	-
164079	*Calpurnus*	*verrucosus*	(Linnaeus, 1758)	KP259448	KP259329	-	-
164117	*Calpurnus*	*verrucosus*	(Linnaeus, 1758)	KP259455	KP259338	-	-
164072	*Crenavolva*	*aureola*	(Fehse, 2002)	KP033151	KP033143	-	-
164085	*Crenavolva*	*aureola*	(Fehse, 2002)	KP033152	KP033144	-	-
164209	*Crenavolva*	*aureola*	(Fehse, 2002)	KP033156	KP033148	-	-
164217	*Crenavolva*	*chiapponii*	Lorenz & Fehse, 2009	KP033158	KP033149	-	-
164186	*Crenavolva*	*striatula*	(Sowerby I, 1828)	KP033154	KP033146	-	-
164144	*Crenavolva*	*trailli*	(Adams, 1855)	KP033153	KP033145	-	-
164189	*Crenavolva*	*trailli*	(Adams, 1855)	KP033155	KP033147	-	-
100779	*Cymbovula*	*acicularis*	(Lamarck, 1810)	GU363447	GU363434	-	-
100792	*Cymbovula*	*acicularis*	(Lamarck, 1810)	GU363448	GU363436	-	-
100815	*Cymbovula*	*acicularis*	(Lamarck, 1810)	GU363449	GU363437	-	-
100744	*Cyphoma*	*gibbosum*	(Linnaeus, 1758)	GU363439	GU363427	-	-
100780	*Cyphoma*	*gibbosum*	(Linnaeus, 1758)	GU363440	GU363428	-	-
100781	*Cyphoma*	*gibbosum*	(Linnaeus, 1758)	GU363444	GU363432	-	-
100804	*Cyphoma*	*gibbosum*	(Linnaeus, 1758)	GU363443	GU363431	-	-
100809	*Cyphoma*	*gibbosum*	(Linnaeus, 1758)	GU363446	GU363433	-	-
100811	*Cyphoma*	*gibbosum*	(Linnaeus, 1758)	GU363441	GU363429	-	-
164058	*Notadusta*	*punctata*	(Linnaeus, 1771)	KP259441	KP259322	-	-
164099	*Dentiovula*	*colobica*	(Azuma & Cate, 1971)	KP259451	KP259333	-	-
164061	*Dentiovula*	cf. *dorsuosa*	(Hinds, 1844)	KP259442	KP259323	-	-
164095	*Dentiovula*	*dorsuosa*	(Hinds, 1844)	KP271160	KP259332	-	-
164150	*Dentiovula*	*dorsuosa*	(Hinds, 1844)	KP259469	KP259353	-	-

Locality	Date	Latitude (degrees)	Longitude (degrees)	Host species
Malaysia, Sabah, NE Pulau Banggi, NE Balundangan Besar Island, TMP.21	09/14/12	7°20'50.5" N	117°21'24.3" E	*Junceella* sp.
Malaysia, Sabah, Lubani Rock, TMP.03	09/07/12	6°53'45.0" N	117°23'15.8" E	*Junceella* sp.
Malaysia, Sabah, Lundayang, TMP.04	09/08/12	6°48'39.4" N	117°21'59.1" E	*Ctenocella* sp.
Malaysia, Sabah, Lundayang, TMP.04	09/08/12	6°48'39.4" N	117°21'59.1" E	*Verrucella* sp.
Malaysia, Sabah, Lundayang, TMP.04	09/08/12	6°48'39.4" N	117°21'59.1" E	*Junceella* sp.
Malaysia, Sabah, Lundayang, TMP.04	09/08/12	6°48'39.4" N	117°21'59.1" E	*Ctenocella* sp.
Malaysia, Sabah, Lundayang, TMP.04	09/08/12	6°48'39.4" N	117°21'59.1" E	*Ctenocella* sp.
Malaysia, Sabah, Lundayang, TMP.04	09/08/12	6°48'39.4" N	117°21'59.1" E	*Ctenocella* sp.
Malaysia, Sabah, Lundayang, TMP.04	09/08/12	6°48'39.4" N	117°21'59.1" E	*Junceella* sp.
Malaysia, Sabah, Lundayang, TMP.04	09/08/12	6°48'39.4" N	117°21'59.1" E	*Verrucella* sp.
Malaysia, Sabah, Lundayang, TMP.04	09/08/12	6°48'39.4" N	117°21'59.1" E	*Viminella* sp.
Thailand, Pattaya, Koh Thai Ta Mun	Feb-11	13°06'30.5" N	100°48'11.0" E	*Dichotella gemmacea*
Saudi Arabia (Red Sea), offshore of Farasan Banks, Shib Radib	08/03/13	16°46' N	41°56' E	?
Malaysia, Sabah, Ligitan Island 1 SW, SEM.13	12/03/10	4°11'13.4" N	118°47'29.6" E	*Sarcophyton* sp.
Indonesia, off Halmahera mainland, Teluk Dodinga; W Karang Ngeli, TER.40	11/15/09	0°46'25.3" N	127°32'22.0" E	*Sarcophyton trocheliophorum*
Malaysia, Sabah, Pasalat Reef, SEM.23	12/07/10	4°30'50.0" N	118°44'30.9" E	*Lobophytum pauciflorum*
Malaysia, Sabah, Selakan Island, SEM.42	12/12/10	4°34'23.6" N	118°43'02.5" E	*Sarcophyton* sp.
Malaysia, Sabah, Si Amil Island, SEM.16	12/04/10	4°19'02.1" N	118°52'30.7" E	*Acanthogorgia* sp.
Indonesia, Halmahera, Tidore, N of Desa Rum,TER.18	11/04/09	0°44'35.8" N	127°23'06.3" E	*Acanthogorgia* sp.
Indonesia, Halmahera mainland, Tanjung Ratemu (S of river), TER.21	11/05/09	0°54'24.7" N	127°29'17.7" E	*Acanthogorgia* sp.
Indonesia, N Sulawesi, Lembeh Strait, Tanjung Kusukusu, LEM.31	02/16/12	1°27'13.8" N	125°14'12.9" E	*Acanthogorgia* sp.
Malaysia, Sabah, S Pulau Banggi, E Molleangan Besar Island, TMP.37	09/19/12	7°05'07.2" N	117°03'33.8" E	*Echinogorgia* sp.
Malaysia, Sabah, Kalang, TMP.41	09/18/12	6°59'48.1" N	117°03'13.4" E	*Subergorgia* sp.
Malaysia, Sabah, Kalang, TMP.41	09/18/12	6°59'48.1" N	117°03'13.4" E	*Paraplexaura* sp.
Curaçao, Barank'i Karanito, CUR.15	05/19/05	12°02'13.5" N	068°48'14.2" E	*Antillogorgia acerosa*
Curaçao, Santa Martha, CUR.18	05/24/05	12°16'04.9" N	069°07'43.6" E	*Gorgonia ventalina*
Curaçao, Caracasbaai, CUR.17	06/03/05	12°03'01.6" N	068°52'01.2" E	*Antillogorgia bipinnata*
Curaçao, Marie Pampoen / Carpile, CUR.05	05/01/05	12°05'42.1" N	068°54'43.0" E	*Gorgonia flabellum*
Curaçao, Barank'i Karanito, CUR.15	05/19/05	12°02'13.5" N	068°48'14.2" E	*Gorgonia ventalina*
Curaçao, Barank'i Karanito, CUR.15	05/19/05	12°02'13.5" N	068°48'14.2" E	*Plexaurella nutans*
Curaçao, St. Michielsbaai, CUR.21	05/31/05	12°08'50.9" N	068°59'56.6" E	*Pseudoplexaura porosa*
Curaçao, Superior Producer (wreck), CUR.22	06/02/05	12°05'21.5" N	068°56'35.5" E	*Antillogorgia americana*
Curaçao, Superior Producer (wreck), CUR.22	06/02/05	12°05'21.5" N	068°56'35.5" E	*Muricea muricata*
Malaysia, Sabah, Kapikan Reef, SEM.33	12/09/10	4°39'04.9" N	118°49'18.2" E	-
Malaysia, Sabah, Ligitan Island 1 SW, SEM.13	12/03/10	4°11'13.4" N	118°47'29.6" E	*Acanthogorgia sp.*
Malaysia, Sabah, Timba Timba Island, SEM.27	12/08/10	4°33'39.2" N	118°55'29.3" E	*Chironephthya* sp.
Malaysia, Sabah, Darby Bank, SEM.04	11/30/10	4°08'23.0" N	118°10'14.6" E	*Siphonogorgia* sp.
Malaysia, Sabah, NE Pulau Banggi, NE Balundangan Besar Is., TMP.21	09/14/12	7°20'50.5" N	117°21'24.3" E	*Siphonogorgia* sp.

Suppl. Mat. S1. Cont.

Collection Code (RMNH.Mol.)	Genus	Species	Author	COI	16S	H3	28S
164174	*Diminovula*	*alabaster*	(Reeve, 1865)	KP259482	KP259366	-	-
164200	*Diminovula*	*alabaster*	(Reeve, 1865)	KP259497	KP259381	-	-
164163	*Diminovula*	*concinna*	(Sowerby II, *in* Adams & Reeve, 1848)	KP259477	KP259361	-	-
164215	*Habuprio-novolva*	*aenigma*	(Azuma & Cate, 1971)	KP259498	KP259382	-	-
164216	*Habuprio-novolva*	*aenigma*	(Azuma & Cate, 1971)	KP259499	KP259383	-	-
164119	*Hiatavolva*	*coarctata*	(Sowerby II, *in* Adams & Reeve, 1848)	KP259456	KP259339	KP259516	KP259399
164134	*Hiatavolva*	*coarctata*	(Sowerby II, *in* Adams & Reeve, 1848)	KP259462	KP259345	KP259518	KP259401
164135	*Hiatavolva*	*coarctata*	(Sowerby II, *in* Adams & Reeve, 1848)	KP259463	KP259346	KP259519	KP259402
164137	*Hiatavolva*	*coarctata*	(Sowerby II, *in* Adams & Reeve, 1848)	KP259465	KP259348	KP259520	KP259403
164161	*Hiatavolva*	*coarctata*	(Sowerby II, *in* Adams & Reeve, 1848)	KP259475	KP259359	KP259526	KP259407
164167	*Hiatavolva*	*coarctata*	(Sowerby II, *in* Adams & Reeve, 1848)	KP259479	KP259363	KP259528	KP259409
164177	*Hiatavolva*	*coarctata*	(Sowerby II, *in* Adams & Reeve, 1848)	KP259484	KP259368	KP259530	KP259412
164185	*Hiatavolva*	*coarctata*	(Sowerby II, *in* Adams & Reeve, 1848)	KP259489	KP259373	KP259533	KP259417
164188	*Hiatavolva*	*coarctata*	(Sowerby II, *in* Adams & Reeve, 1848)	KP259491	KP259375	KP259534	KP259418
164197	*Hiatavolva*	*coarctata*	(Sowerby II, *in* Adams & Reeve, 1848)	KP259495	KP259379	KP259538	KP259422
164198	*Hiatavolva*	*coarctata*	(Sowerby II, *in* Adams & Reeve, 1848)	KP259496	KP259380	KP259539	KP259423
164234	*Hiatavolva*	*coarctata*	(Sowerby II, *in* Adams & Reeve, 1848)	KP259501	KP259385	KP259540	KP259424
337791	*Hiatavolva*	*coarctata*	(Sowerby II, *in* Adams & Reeve, 1848)	KP259508	KP259391	KP259545	KP259430
164147	*Hiatavolva*	*depressa*	(G.B. Sowerby III, 1875)	-	KP259351	-	-
164182	*Hiatavolva*	*depressa*	(G.B. Sowerby III, 1875)	KP259487	KP259371	KP259531	KP259415
114127	*Hiatavolva*	*rugosa*	(Cate & Azuma *in* Cate, 1973)	KP259435	KP259316	-	-
164050	*Hiatavolva*	*rugosa*	(Cate & Azuma *in* Cate, 1973)	KP259438	KP259319	KP259511	KP259394
164051	*Hiatavolva*	*rugosa*	(Cate & Azuma *in* Cate, 1973)	KP259439	KP259320	KP259512	KP259395
164053	*Hiatavolva*	*rugosa*	(Cate & Azuma *in* Cate, 1973)	KP259440	KP259321	KP259513	KP259396
164100	*Hiatavolva*	*rugosa*	(Cate & Azuma *in* Cate, 1973)	KP259452	KP259334	KP259514	KP259397

Locality	Date	Latitude (degrees)	Longitude (degrees)	Host species
Malaysia, Sabah, S Pulau Banggi, Pancang Pukul, TMP.40	09/18/12	7°02'01.6" N	117°04'25.1" E	*Nephthea* sp.
Malaysia, Sabah, ENE Pulau Banggi, Latoan Patch, TMP.19	09/14/12	7°17'43.3" N	117°24'06.1" E	*Nephthea* sp.
Malaysia, Sabah, NE Pulau Banggi, NE Banggi Outer Reef, TMP.20	09/14/12	7°22'53.7" N	117°22'24.6" E	*Coelogorgia plumosa*
Indonesia, Halmahera mainland, Tanjung Ratemu (S of river), TER.27	11/08/09	0°54'44.5" N	127°29'09.9" E	*Dendronephthya* sp.
Indonesia, Raja Ampat Islands, W. Papua, Yeffam Isl., NW Pulau Keruo, RAJ.65	12/13/07	0°35'15.4" S	130°17'42.7" E	*Dendronephthya* sp.
Malaysia, Sabah, Bumbun Island W (channel), SEM.22	12/05/10	4°27'27.5" N	118°34'14.9" E	*Ctenocella* sp.
Indonesia, N Sulawesi, Bunaken Island, Raymond, MEN.11	12/02/08	1°37'44.6" N	124°44'07.0" E	*Ellisella ceratophyta*
Indonesia, N Sulawesi, Bunaken Island, Mandolin, MEN.16	12/04/08	1°36'43.8" N	124°43'56.7" E	*Ellisella ceratophyta*
Indonesia, N Sulawesi, Bunaken Island, Lekuan III, MEN.14	12/12/08	1°36'20,3" N	124°46'08,0" E	*Ellisella ceratophyta*
Malaysia, Sabah, NE Pulau Banggi, NE Balundangan Besar Is., TMP.21	09/14/12	7°20'50.5" N	117°21'24.3" E	*Ctenocella* sp.
Malaysia, Sabah, E Pulau Banggi, Purukan Sibaliu, TMP.15	09/11/12	7°12'41.5" N	117°28'13.7" E	*Verrucella* sp.
Malaysia, Sabah, NE Pulau Banggi, NE Balundangan Besar Is., TMP.21	09/14/12	7°20'50.5" N	117°21'24.3" E	*Ctenocella* sp.
Malaysia, Sabah, NE Pulau Banggi, NE Balundangan Besar Is., TMP.21	09/14/12	7°20'50.5" N	117°21'24.3" E	*Ctenocella* sp.
Malaysia, Sabah, Lubani Rock, TMP.03	09/07/12	6°53'45.0" N	117°23'15.8" E	*Viminella* sp.
Malaysia, Sabah, SE Pulau Banggi, SW Carrington Reef, TMP.38	09/20/12	7°07'49.4" N	117°13'41.9" E	*Ctenocella* sp.
Malaysia, Sabah, Kalang, TMP.41	09/18/12	6°59'48.1" N	117°03'13.4" E	*Verrucella* sp.
Indonesia, N Sulawesi, Lembeh Strait, Tanjung Nanas I, LEM.01	01/30/12	1°27'40.4" N	125°13'36.4" E	*Ellisella* sp.
Indonesia, N Sulawesi, Lembeh Strait, Tanjung Kusukusu, LEM.31	02/16/12	1°27'13.8" N	125°14'13.0" E	*Ellisella* sp.
Malaysia, Sabah, SE Pulau Banggi, SW Carrington Reef, TMP.38	09/20/12	7°07'49.4" N	117°13'41.9" E	*Alertigorgia orientalis*
Malaysia, Sabah, E Pulau Balambangan, SE Tanjung Siagut, TMP.30	09/23/12	7°20'10.5" N	117°01'24.3" E	*Alertigorgia orientalis*
Indonesia, W. Papua, Raja Ampat Islands, Jelly Point, RAJ.03	11/21/07	0°32'15.0" S	130°57'06.7" E	*Ellisella* sp.
Malaysia, Sabah, Bohayen Island, SEM.26	12/08/10	4°28'05.6" N	118°56'50.8" E	*Viminella* sp.
Malaysia, Sabah, Sipadan Island, Mid Reef, SEM.59	12/18/10	4°06'47.8" N	118°38'10.1" E	*Viminella* sp.
Malaysia, Sabah, Bohayen Island, SEM.26	12/08/10	4°28'05.6" N	118°56'50.8" E	*Ellisella* cf. *ceratophyta*
Malaysia, Sabah, Si Amil Island, SEM.16	12/04/10	4°19'02.1" N	118°52'30.7" E	*Viminella* sp.

Suppl. Mat. S1. Cont.

Collection Code (RMNH.Mol.)	Genus	Species	Author	COI	16S	H3	28S
164103	*Hiatavolva*	*rugosa*	(Cate & Azuma *in* Cate, 1973)	-	KP259335	-	-
164104	*Hiatavolva*	*rugosa*	(Cate & Azuma *in* Cate, 1973)	KP259453	KP259336	KP259515	KP259398
164108	*Hiatavolva*	*rugosa*	(Cate & Azuma *in* Cate, 1973)	-	KP271159	-	-
164120	*Hiatavolva*	*rugosa*	(Cate & Azuma *in* Cate, 1973)	KP259457	KP259340	KP259517	KP259400
164140	*Hiatavolva*	*rugosa*	(Cate & Azuma *in* Cate, 1973)	KP271161	-	KP259521	-
164158	*Hiatavolva*	*rugosa*	(Cate & Azuma *in* Cate, 1973)	KP259474	KP259358	KP259525	KP259406
164175	*Hiatavolva*	*rugosa*	(Cate & Azuma *in* Cate, 1973)	KP259483	KP259367	KP259529	KP259411
164236	*Hiatavolva*	*rugosa*	(Cate & Azuma *in* Cate, 1973)	KP259502	KP259386	KP259541	KP259425
337787	*Hiatavolva*	*rugosa*	(Cate & Azuma *in* Cate, 1973)	KP259504	KP259388	KP259542	KP259426
337789	*Hiatavolva*	*rugosa*	(Cate & Azuma *in* Cate, 1973)	KP259506	KP259389	KP259544	KP259428
337790	*Hiatavolva*	*rugosa*	(Cate & Azuma *in* Cate, 1973)	KP259507	KP259390	-	KP259429
337792	*Hiatavolva*	*rugosa*	(Cate & Azuma *in* Cate, 1973)	KP259509	KP259392	KP259546	KP259431
-	*Kuroshiovolva*	*shingoi*	Azuma & Cate, 1971	-	-	-	-
164066	*Naviculavolva*	*deflexa*	(Sowerby II, 1848)	KP259443	KP259324	-	-
164094	*Naviculavolva*	*deflexa*	(Sowerby II, 1848)	KP259450	KP259331	-	-
164130	*Naviculavolva*	*deflexa*	(Sowerby II, 1848)	KP259459	KP259342	-	-
164145	*Naviculavolva*	*deflexa*	(Sowerby II, 1848)	KP259467	KP259350	-	-
164149	*Naviculavolva*	*deflexa*	(Sowerby II, 1848)	KP259468	KP259352	-	-
164153	*Naviculavolva*	*deflexa*	(Sowerby II, 1848)	KP259471	KP259355	-	-
164155	*Naviculavolva*	*deflexa*	(Sowerby II, 1848)	KP259473	KP259357	-	-
164173	*Naviculavolva*	*deflexa*	(Sowerby II, 1848)	KP259481	KP259365	-	-
164069	*Ovula*	*ovum*	(Linnaeus, 1758)	KP259444	KP259325	-	-
164080	*Ovula*	*ovum*	(Linnaeus, 1758)	KP259449	KP259330	-	-
337786	*Pedicularia*	*pacifica*	Pease, 1865	KP259503	KP259387	-	-
099021B	*Pedicularia*	*pacifica*	Pease, 1865	KP259434	KP259315	-	-
099021A	*Pedicularia*	*vanderlandi*	Goud & Hoeksema, 2001	KP259433	KP259314	-	-
164047	*Pellasimnia*	*annabelae*	Lorenz & Fehse, 2009	KP259436	KP259317	-	-
164076	*Pellasimnia*	*annabelae*	Lorenz & Fehse, 2009	KP259445	KP259326	-	-

Locality	Date	Latitude (degrees)	Longitude (degrees)	Host species
Indonesia, off Halmahera mainland, Teluk Dodinga, Karang Ngeli W, TER.40	11/15/09	0°46’25,3” N	127°32’22,0” E	*Ellisella* sp.
Indonesia, Halmahera, off Tidore, Pulau Pilongga S, TER.35	11/12/09	0°42’44,1” N	127°28’47,3” E	*Dichotella gemmacea*
Indonesia, Halmahera, Maitara, Maitara W, TER.09	10/29/09	0°43’47,6” N	127°21’44,7” E	*Ellisella* sp.
Malaysia, Sabah, Ligitan Island 1 SW, SEM.13	12/03/10	4°11’13.4” N	118°47’29.6” E	*Viminella* sp.
Indonesia, Halmahera, Tidore, Tanjung Ebamadu, TER.08	10/28/09	0°45’23,4” N	127°24’26,5” E	*Ellisella* sp.
Malaysia, Sabah, Lubani Rock, TMP.03	09/07/12	6°53’45.0” N	117°23’15.8” E	*Ctenocella* sp.
Malaysia, Sabah, S Pulau Banggi, S Patanunan Island, TMP.36	09/19/12	7°05’59.7” N	117°05’21.1” E	*Ctenocella* sp.
Indonesia, N Sulawesi, Lembeh Strait, Tanjung Nanas I, LEM.33	02/17/12	1°27’39.5” N	125°13’35.8” E	*Ellisella* sp.
Indonesia, Pulau Pulau Gura Ici, Pulau Lelai S, TER.29	11/09/09	0°01’58.3” S	127°14’56.8” E	*Dichotella gemmacea*
Indonesia, N Sulawesi, Lembeh Strait, Teluk Makawide, LEM.19	02/09/12	1°29’05.1” N	125°14’26.1” E	*Ellisella* sp.
Indonesia, N Sulawesi, Lembeh Strait, N Pulau Dua, LEM.25	02/13/12	1°23’28.6” N	125°12’58.7” E	*Ellisella* sp.
Indonesia, N Sulawesi, Lembeh Strait, N Sarena Kecil, LEM.32	02/16/12	1°27’26.9” N	125°13’37.6” E	*Viminella* sp.
-	-	-	-	-
Malaysia, Sabah, S Boheydulang Island 1, SEM.37	12/11/10	4°35’00.4” N	118°46’40.5” E	*Hicksonella* sp.
Malaysia, Sabah, S Ligitan Reef 1, SEM.09	12/01/10	4°14’07.7” N	118°33’22.7” E	*Hicksonella* sp.
Malaysia, Sabah, Alert Patches 2, SEM.05	11/30/10	4°09’37.7” N	118°15’37.3” E	*Hicksonella* sp.
Malaysia, Sabah, NE Pulau Banggi, NE Banggi Outer Reef, TMP.20	09/14/12	7°22’53.7” N	117°22’24.6” E	*Rumphella*sp.
Malaysia, Sabah, NE Pulau Malawali, S Sibaliu, TMP.14	09/11/12	7°06’50.0” N	117°22’36.8” E	*Rumphella*sp.
Malaysia, Sabah, ENE Pulau Banggi, Latoan Patch, TMP.19	09/14/12	7°17’43.3” N	117°24’06.1” E	*Rumphella*sp.
Malaysia, Sabah, ENE Pulau Banggi, Latoan Patch, TMP.19	09/14/12	7°17’43.3” N	117°24’06.1” E	*Rumphella*sp.
Malaysia, Sabah, SWLigitan Island 1, SEM.13	12/03/10	4°11’13.4” N	118°47’29.6” E	*Hicksonella* sp.
Malaysia, Sabah, Kapalai Island, SEM.10	12/02/10	4°13’04.8” N	118°40’20.1” E	*Sarcophyton glaucum*
Indonesia, Halmahera, Ternate, Tanjung Pasir Putih, TER.16	11/02/09	0°51’50.4” N	127°20’36.7” E	*Sarcophyton trocheliophorum*
Indonesia, Halmahera, Ternate, Restaurant Floridas, TER.03	10/25/09	0°45’35.8” N	127°21’25.4” E	*Stylaster* sp.
Indonesia, Bali, N side of Nusa Lembongan, Tanjung Taal	04/22/01	8°39’33” S	115°26’37” E	*Distichopora vervoorti*
Indonesia, Bali, N side of Nusa Lembongan, Tanjung Taal	04/22/01	8°39’33” S	115°26’37” E	*Distichopora vervoorti*
Malaysia, Sabah, S Larapan Island 2, SEM.57	12/17/10	4°32’51.1” N	118°36’32.3” E	*Annella* sp.
Indonesia, Halmahera, Hiri, Tanjung Ngafauda, TER.14	10/31/09	0°54’38.3” N	127°19’02.7” E	*Annella* sp.

Suppl. Mat. S1. Cont.

Collection Code (RMNH.Mol.)	Genus	Species	Author	COI	16S	H3	28S
164114	*Pellasimnia*	*annabelae*	Lorenz & Fehse, 2009	KP259454	KP259337	-	-
164162	*Pellasimnia*	*annabelae*	Lorenz & Fehse, 2009	KP259476	KP259360	-	-
164187	*Pellasimnia*	*annabelae*	Lorenz & Fehse, 2009	KP259490	KP259374	-	-
164128	*Phenacovolva*	*rosea*	(Adams, 1854)	KP259458	KP259341	-	-
164049	*Primovula*	*rosewateri*	(Cate, 1973)	KP259437	KP259318	-	-
164062	*Primovula*	*rosewateri*	(Cate, 1973)	KP033150	KP033142	-	-
164221	*Procalpurnus*	*lacteus*	(Lamarck, 1810)	KP259500	KP259384	-	-
164132	*Prosimnia*	*piriei*	(Petuch, 1973)	KP259460	KP259343	-	-
164133	*Prosimnia*	*piriei*	(Petuch, 1973)	KP259461	KP259344	-	-
164136	*Prosimnia*	*piriei*	(Petuch, 1973)	KP259464	KP259347	-	-
110064	*Simnia*	*patula*	(Pennant, 1777)	GU363450	GU363438	-	-

Locality	Date	Latitude (degrees)	Longitude (degrees)	Host species
Malaysia, Sabah, Darby Bank, SEM.04	11/30/10	4°08’23.0” N	118°10’14.6” E	*Annella* sp.
Malaysia, Sabah, NE Pulau Banggi, NE Balundangan Besar Is., TMP.21	09/14/12	7°20’50.5” N	117°21’24.3” E	*Annella* sp.
Malaysia, Sabah, S Pulau Banggi, S Balak-Balak Island, TMP.39	09/19/12	7°20’22.6” N	117°08’36.0” E	*Annella* sp.
Malaysia, Sabah, Horn Reef, SEM.08	12/01/10	4°14’32.0” N	118°26’24.1” E	*Acanthogorgia* sp.
Malaysia, Sabah, Sipadan Island, Hanging Gardens, SEM.60	12/18/10	4°06’40.3” N	118°37’29.3” E	*Villogorgia* sp.
Malaysia, Sabah, N Kulapuan Island 2, SEM.31	12/09/10	4°32’07.4” N	118°50’18.2” E	*Paratelesto* sp.
Indonesia, N Sulawesi, Lembeh Strait, Batu Kapal, LEM.35	02/18/12	1°32’56.8” N	125°17’31.8” E	*Sinularia pavida*
Indonesia, N Sulawesi, Bunaken Island, Lekuan II, MEN.04	11/27/08	1°36’00.4” N	124°45’58.4” E	*Euplexaura* sp.
Indonesia, N Sulawesi, Bunaken Island, Sachiko’s Point, MEN.10	12/01/08	1°37’52.5” N	124°46’20.4” E	*Euplexaura* sp.
Indonesia, N Sulawesi, Manado Tua, Muka Gereja, MEN.17	12/05/08	1°36’57.4” N	124°41’40.0” E	*Euplexaura* sp.
North Sea, South side of Doggersbank	09/19/03	54°20’ N	02°20’ E	*Alcyonium digitatum*

Chapter 6

A molecular and morphological exploration of the generic boundaries in the family Melithaeidae (Coelenterata: Octocorallia) and its taxonomic consequences

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Abstract

The validity of the currently recognized melithaeid genera (*Acabaria*, *Clathraria*, *Melithaea*, *Mopsella*, *Wrightella*) with the exception of the recently added genus *Asperaxis*, has puzzled scientists for almost a century. Diagnostic morphological characters are often missing or are obscured by the variation in sclerite forms. Consequently, species are difficult to assign to genera. In this study the current genera and their taxonomic positions are reviewed and reassessed based on material collected from the Indo-Pacific, Red Sea and Indian Ocean as far south as South Africa. Molecular data were obtained for four different loci, both mitochondrial (COI, mtMutS, ND6) and nuclear (28S rDNA). Combining the molecular and morphological data revealed that all former genera, except for the monotypic genus *Asperaxis* and the genus *Wrightella* are paraphyletic. Molecular data for the two subfamilies (Asperaxinae and Melithaeinae) within the Melithaeidae, in comparison with the outgroup, indicated that the family is also paraphyletic. Furthermore we observed that species did not cluster according to their present morphological classification but instead clustered according to a biogeographical pattern. Species from the Red Sea, Indian Ocean and Central Pacific, respectively, grouped into well-supported clades. Consequently, we did not find morphological- or phylogenetic support to maintain the generic names *Acabaria*, *Clathraria*, *Mopsella* and *Wrightella*. Therefore these names are synonymised with the oldest available generic name, *Melithaea*. As a result, five secondary homonyms originated; these junior homonyms are herein renamed, viz. *Melithaea hendersoni* nom. nov, *Melithaea mcqueeni* nom. nov., *Melithaea shanni* nom. nov., *Melithaea thorpeae* nom. nov., and *Melithaea wrighti* nom. nov. Additionally, neotypes are selected for *Melithaea ochracea* to stabilize the genus *Melithaea*, and for *Acabaria rubra*.

Introduction

The Melithaeidae (Cnidaria: Anthozoa) are gorgonians (also commonly known as sea fans), distributed from the Red Sea (Grasshoff, 2000), Indian Ocean (Thomson, 1916, Ofwegen, 1987, Ofwegen, 1989 and Williams, 1992) and Indo-West Pacific (Ofwegen, 1987, Grasshoff, 1999 and Ofwegen *et al*., 2000) to Hawai'i (Bayer, 1956). Based on their internal skeletal elements called sclerites, which are used for genus and species identifications, five genera have traditionally been distinguished. These are *Acabaria* Gray, 1859, *Clathraria* Gray, 1859, *Melithaea* Linnaeus, 1758, *Mopsella* Gray, 1857 and *Wrightella* Gray, 1870. Recently, *Asperaxis* Alderslade (2006) was added. Unfortunately, the sclerites do not always demonstrate clear diagnostic characteristics to assign species to a specific genus. In many cases, species exhibit characters that are consistent with their placement in multiple genera. Therefore the taxonomic position and validity of the genera within the family Melithaeidae have puzzled taxonomists for over a century. Confusion at the generic level is also caused by the many intermediate sclerite forms observed when large numbers of specimens are studied. Often these extensive investigations revealed that specimens may show much variation in morphological characters (Hickson, 1937), obscuring the pre-determined generic borders and keeping taxonomists debating the validity and status of most of the described genera (Hickson, 1937, Broch, 1939 and Fabricius and Alderslade, 2001). Although his overview seemed straightforward, Hickson (1937, p. 89) himself found his proposed classification problematic: "The division of them [Melithaeidae] into definite generic and even specific forms is quite artificial and represents nothing in Nature". Despite these taxonomic uncertainties, species belonging to the Melithaeidae have frequently been used in ecological and chemical studies (Goh *et al*., 1999, Goh and Chou, 1994, Matsumoto, 2004, Oppen *et al*., 2005, Shin and Seo, 1995 and Kobayashi and Kanda, 1991) and in studies of associated fauna such as crustaceans, molluscs, echinoderms and fish (Goh *et al*., 1999 and Kumagai and Aoki, 2003).

As recently as 1999, Grasshoff proposed alteration of Hickson's classification by suggesting synonymising the genera *Melitella*, *Mopsella* and *Wrightella* with the genus *Melithaea*. Consequently, only three genera would have been maintained: *Acabaria*, *Clathraria* and *Melithaea*. Subsequently, Grasshoff (2000) revised his proposed classification and resurrected the genus *Mopsella*. However, Fabricius and Alderslade (2001) maintained the classification as proposed earlier by Hickson (1937) with an additional comment, saying that based on the considerable overlap in sclerite morphology between the alleged genera, they probably represent a single genus.

The latest addition to the family Melithaeidae is the genus *Asperaxis*. This genus was considered to be morphologically so markedly different compared to the other genera, that it was even placed in a new subfamily, Asperaxinae Alderslade, 2006. Only recently molecular data were used to investigate the phylogenetic relationships among the genera and species within the Melithaeidae. Aguilar-Hurtado *et al*. (2012) included the genera *Acabaria*, *Melithaea* and *Mopsella* in their phylogenetic reconstruction based on two genetic markers, COI and 28S rDNA. Their results suggest that the genetic boundaries of these three genera are in concordance with the morphological classification as suggested by Hickson (1937). However, their study includes only specimens

collected from subtropical Japanese waters, thereby excluding *Clathraria* Gray, 1859 and *Wrightella* Gray, 1870, genera that are predominately found in the Red Sea and the Indian Ocean. To gain more comprehensive insights into the phylogenetic history of the genera and species within the Melithaeidae, in addition to museum specimens already available, samples for this study were collected from most areas within the known geographic distribution of Melithaeidae, and subsequently used for phylogenetic studies.

Material and methods

Specimen collection

Melithaeidae were collected in Australia, Chagos Archipelago, Eritrea, Indonesia, Israel, Japan, Malaysia, Maldives, New Caledonia, Palau, Seychelles, South Africa, Tanzania and Vietnam (Fig. 1). In total, specimens are from 18 different eco-regions (Marine Ecoregions of the World (MEOW)) (Spalding *et al*., 2007).

All voucher specimens and respective subsamples were stored in either 70% or 96% ethanol except for the Malaysian and some of the Indonesian samples, which were stored in a 20% salt-saturated DMSO-buffer. All specimens are stored in the collections of the Naturalis Biodiversity Center, the Netherlands. An overview of the specimens and their locality and collection data are presented in Table 1.

In addition, 44 type specimens were studied, in an attempt to identify the specimens used in the molecular phylogeny (Table 2; App. 2, Pl. 1-44).

For each (type) specimen microscope slides were made. A small piece (<1 cm) of the distal part of the octocoral was dissolved in a 4% household bleach solution to isolate sclerites. The sclerites were washed with tap water (five times), followed by the same number of wash steps with demineralised water. Sclerites were dried on a hot plate and subsequently embedded in Euparal for visualisation with a Leica DM LB2 light microscope. In addition, sclerites of specimens that represent specific clades or needed further morphological investigations were mounted on SEM stubs and coated with Pd/Au for imaging on a JEOL JSM6490LV scanning electron microscope operated at high vacuum

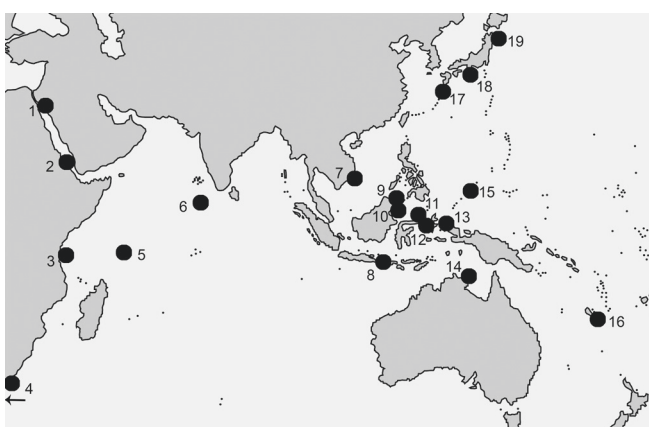

Fig. 1. Overview of the 19 localities where Melithaeidae were collected for this study. See Table 1 for more specific locality data.

Table 1. Overview of all samples including species information, locality data and additional collecting data.

Catalogue number	Species (author)	New species name (author)	Locality	Latitude (decimal)
AKM0615	*Acabaria* sp.	*Melithaea* sp.	Japan, Iwate Prefecture, Otsuchi Bay, entrance of Otsuchi Bay	39.365283 N
AKM0664	*Acabaria tenuis* Kükenthal, 1908	*Melithaea tenuis* (Kükenthal, 1908) comb. nov.	Japan, Wakayama Prefecture, off Tanabe Bay	33.650833 - 33.649333 N
AKM0724	*Acabaria* sp.	*Melithaea* sp.	Japan, Kagoshima prefecture, off Tanegashima Island	30.410333 - 30.415833 N
AKM0743	*Mopsella* sp.	*Melithaea* sp.	Japan, Kagoshima Prefecture, Sata Misaki Cape	30.93333 - 30.938333 N
AKM0980	*Melithaea* sp.	*Melithaea* sp.	Japan, Kagoshima Prefecture, Tsukara-se	31.315833 - 31.308333 N
AKM1148	*Acabaria* sp.	*Melithaea* sp.	Japan, Okinawa Prefecture, off Kerama Island	26.1575 - 26.160833 N
AKM1175	*Acabaria* sp.	*Melithaea* sp.	Japan, Okinawa Prefecture, off Kerama Island	26.0765 - 26.076 N
AKM1200	*Acabaria* sp.	*Melithaea* sp.	Japan, Iwate Prefecture, Otsuchi Bay, off Ohako-zaki	39.35N
AKM1252	*Acabaria* sp.	*Melithaea* sp.	Japan, Iwate Prefecture, Otsuchi Bay, off Ohako-zaki	39.35N
RMNH.Coel.19852	*Melithaea ochracea* (Linnaeus, 1758)	*Melithaea ochracea* (Linnaeus, 1758) comb. nov.	Indonesia, Moluccas, Ambon, Hitu, N coast Mamala	3.537997 S
RMNH.Coel.24373	*Wrightella coccinea* (Ellis & Solander, 1786)	*Melithaea coccinea* (Ellis & Solander, 1786) comb. nov.	Chagos Archipelago, Salomon atoll, off Ile Fouquet	5.339111 S
RMNH.Coel.39452	*Mopsella* sp.	*Melithaea* sp.	Australia, Wigram Island, The English Companys Islands, eastern Arnhem Land, NT	11.7637 S
RMNH.Coel.39455	*Melithaea* sp.	*Melithaea* sp.	Australia, Cotton Island at mouth of the northern bay, The english Companys Islands, eastern Arnhem Land, NT	11.798125 S
RMNH.Coel.39466	*Melithaea* sp.	*Melithaea* sp.	Australia, off Rimbija Island, 3.2 km W of Cape Wessel, Wessel Islands, eastern Arnhem Land, NT	11.006967 S
RMNH.Coel.40034	*Mopsella* sp.	*Melithaea* sp.	Indonesia, Papua, Raja Ampat, S Gam, near mangrove border	0.51895 S
RMNH.Coel.40041	*Wrightella coccinea* (Ellis & Solander, 1786)	*Melithaea coccinea* (Ellis & Solander, 1786) comb. nov.	Seychelles, St. François atoll, W rim	7.25 S
RMNH.Coel.41099	*Acabaria* sp.	*Melithaea* sp.	Indonesia, NE Kalimantan, Berau Islands, Maratua Island, NE side	2.291444 N
RMNH.Coel.41100	*Acabaria* sp.	*Melithaea* sp.	Malaysia, Borneo, Semporna, Mid Rock Roach Reef	4.1775 N

					accession numbers			
Longitude (decimal)	Date	Depth (m)	Collector	Clade	ND6	COI	mtMutS	28S
142.000517 E	9/12/2005	78	A.K. Matsumoto	A	KC845768	KC802190	KC845667	KC845880
135.164833 - 135.169333 E	11/26/2005	180	A.K. Matsumoto	A	KC845778	KC802203	KC845668	KC845885
131.141 - 131.138667 E	2/23/2007	468-502	A.K. Matsumoto	A	KC845797	-	KC845684	KC845912
130.737167 - 130.723333 E	2/23/2007	116-120	A.K. Matsumoto	A	KC845782	KC802205	KC845669	KC845891
129.769167 - 129.766 E	3/6/2008	154-155	A.K. Matsumoto	A	KC845781	KC802204	KC845670	KC845890
127.448333 - 127.480167 E	12/15/2008	71-85	A.K. Matsumoto	B	KC845780	KC802201	KC845671	KC845889
127.461667 - 127.465833 E	12/16/2008	160-153	A.K. Matsumoto	A	KC845779	KC802202	KC845672	KC845888
142.00E	3/21/2008	ca. 90	K. Morita / A.K. Matsumoto	A	KC845769	KC802188	KC845666	KC845882
142.00E	5/9/2008	ca. 75	K. Morita / A.K. Matsumoto	A	KC845767	KC802189	KC845665	KC845881
128.206414 E	11/21/1990	10-15	J.C. den Hartog	B	KC845766	KC802206	KC845653	KC845879
72.276447 E	3/8/1996	-	G.B. Reinicke	D	KC845761	KC802114	KC845582	KC845868
136.531667 E	3/29/2004	13-16	H. Nguyen	B	KC845698	KC802185	KC845585	KC845878
136.48415 E	3/30/2004	13-16	D. DeMaria	A	KC845699	KC802116	KC845586	KC845824
136.7276 E	4/1/2004	30-40	P. Colin	B	KC845700	KC802117	KC845587	KC845856
130.641117 E	11/30/2007	22	F.R. Stokvis	A	KC845764	KC802145	KC845652	KC845867
52.733333 E	5/6-01-1993	<27	L.P. van Ofwegen	D	KC845759	KC802156	KC845580	KC845869
118.591389 E	10/10/2003	28	L.P. van Ofwegen & M. Slierings	A	KC845799	-	KC845693	KC845923
118.303361 E	11/29/2010	12	B.T. Reijnen	A	KC845701	KC802118	KC845596	KC845827

Table 1. Cont.

Catalogue number	Species (author)	New species name (author)	Locality	Latitude (decimal)
RMNH.Coel.41101	*Acabaria* sp.	*Melithaea* sp.	Malaysia, Borneo, Semporna, Alert Patches 3	4.162972 N
RMNH.Coel.41102	*Acabaria* sp.	*Melithaea* sp.	Malaysia, Borneo, Semporna, Bohayen Island	4.466917 N
RMNH.Coel.41103	*Acabaria* sp.	*Melithaea* sp.	Malaysia, Borneo, Semporna, Bohayen Island	4.466917 N
RMNH.Coel.41104	*Acabaria* sp.	*Melithaea* sp.	Malaysia, Borneo, Semporna, Timba timba Island	4.560472 N
RMNH.Coel.41105	*Acabaria* sp.	*Melithaea* sp.	Malaysia, Borneo, Semporna, Pandanan Island	4.576667 N
RMNH.Coel.41106	*Acabaria* sp.	*Melithaea* sp.	Malaysia, Borneo, Semporna, Pandanan Island	4.576667 N
RMNH.Coel.41107	*Acabaria* sp.	*Melithaea* sp.	Malaysia, Borneo, Semporna, Pandanan Island	4.576667 N
RMNH.Coel.41108	*Acabaria* sp.	*Melithaea* sp.	Malaysia, Borneo, Semporna, Mataking Island	4.582667 N
RMNH.Coel.41109	*Acabaria* sp.	*Melithaea* sp.	Malaysia, Borneo, Semporna, Kulapuan Island 2 N	4.536 N
RMNH.Coel.41110	*Acabaria* sp.	*Melithaea* sp.	Malaysia, Borneo, Semporna, Kulapuan Island 2 N	4.536 N
RMNH.Coel.41111	*Acabaria* sp.	*Melithaea* sp.	Malaysia, Borneo, Semporna, Mantabuan Island	4.632222 N
RMNH.Coel.41112	*Acabaria* sp.	*Melithaea* sp.	Malaysia, Borneo, Semporna, Mantabuan Island	4.632222 N
RMNH.Coel.41113	*Acabaria* sp.	*Melithaea* sp.	Malaysia, Borneo, Semporna, Gaya Island 1 SE	4.624722 N
RMNH.Coel.41114	*Acabaria* sp.	*Melithaea* sp.	Indonesia, Sulawesi, Lembeh, Pulau Abadi	1.43354 N
RMNH.Coel.41115	*Melithaea* sp.	*Melithaea* sp.	Indonesia, NE Kalimantan, Berau Islands, Berau delta, Lighthouse 2 reef	2.159417 N
RMNH.Coel.41116	*Melithaea* sp.	*Melithaea* sp.	Malaysia, Borneo, Semporna, Bumbun Island W (channel)	4.461306 N
RMNH.Coel.41117	*Melithaea* sp.	*Melithaea* sp.	Seychelles, St. Joseph atoll, NW rim	5.4 S
RMNH.Coel.41118	*Melithaea* sp.	*Melithaea* sp.	Indonesia, Sulawesi, Lembeh, Tanjung Labuhankompeni	1.43218 N
RMNH.Coel.41119	*Melithaea* sp.	*Melithaea* sp.	Indonesia, Lombok	8.408333 S
RMNH.Coel.41120	*Acabaria* sp.	*Melithaea* sp.	Republic of Palau, Short Drop-Off, outer slope	7.258883 N
RMNH.Coel.41121	*Acabaria* sp.	*Melithaea* sp.	Malaysia, Borneo, Semporna, Church Reef 2	4.68625 N
RMNH.Coel.41122	*Acabaria* sp.	*Melithaea* sp.	Indonesia, Sulawesi, Lembeh, Pulau Abadi	1.43354 N
RMNH.Coel.41123	*Mopsella* sp.	*Melithaea* sp.	Malaysia, Borneo, Semporna, Kulapuan Island 1 S	4.511472 N
RMNH.Coel.41124	*Melithaea* sp.	*Melithaea* sp.	Malaysia, Borneo, Semporna, Selakan Island	4.572806 N

					accession numbers			
Longitude (decimal)	Date	Depth (m)	Collector	clade	ND6	COI	mtMutS	28S
118.276611 E	11/30/2010	12	B.T. Reijnen	A	KC845702	KC802119	KC845597	KC845828
118.947667 E	12/8/2010	14	B.T. Reijnen	A	KC845709	KC802120	KC845599	KC845840
118.947667 E	12/8/2010	15	B.T. Reijnen	A	KC845710	KC802121	KC845600	KC845829
118.925111 E	12/8/2010	22	B.T. Reijnen	A	KC845711	KC802122	KC845601	KC845836
118.920583 E	12/8/2010	4	B.T. Reijnen	A	KC845712	KC802123	KC845602	KC845837
118.920583 E	12/8/2010	12	B.T. Reijnen	A	KC845713	KC802124	KC845603	KC845838
118.920583 E	12/8/2010	14	B.T. Reijnen	A	KC845714	KC802125	KC845604	KC845830
118.94625 E	12/8/2010	13	B.T. Reijnen	A	KC845715	KC802126	KC845592	KC845839
118.8385 E	12/9/2010	14	B.T. Reijnen	A	KC845703	KC802127	KC845607	KC845831
118.8385 E	12/9/2010	11	B.T. Reijnen	A	KC845704	KC802128	KC845608	KC845832
118.796833 E	12/10/2010	20	B.T. Reijnen	A	KC845705	KC802129	KC845609	KC845833
118.796833 E	12/10/2010	12	B.T. Reijnen	A	KC845706	KC802130	KC845664	KC845834
118.777472 E	12/10/2010	23	B.T. Reijnen	A	KC845707	KC802131	KC845610	KC845835
125.20628 E	2/3/2012	12	B.T. Reijnen	A	KC845783	KC802199	KC845663	KC845903
118.169833 E	10/5/2003	12	L.P. van Ofwegen & M. Slierings	A	KC845765	KC802158	KC845651	KC845842
118.635861 E	12/5/2010	14	B.T. Reijnen	A	KC845722	KC802187	KC845598	KC845841
53.316667 E	12/26/1992	-	L.P. van Ofwegen	A	KC845720	KC802186	KC845588	KC845825
125.18629 E	2/4/2012	6	B.T. Reijnen	A	KC845770	KC802198	KC845660	KC845904
116.07 E	8/4/2011	5	B.W. Hoeksema	A	KC845721	KC802138	KC845619	KC845826
134.52555 E	5/26/2011	24.4	C.S. McFadden	A	KC845741	KC802179	KC845628	KC845865
118.649028 E	12/13/2010	30	B.T. Reijnen	A	KC845708	KC802132	KC845614	KC845866
125.20628 E	2/3/2012	15	B.T. Reijnen	A	KC845784	KC802200	KC845659	KC845902
118.866222 E	12/9/2010	9	B.T. Reijnen	B	KC845733	KC802146	KC845606	KC845852
118.717861 E	12/12/2010	8	B.T. Reijnen	B	KC845730	KC802152	KC845613	KC845853

Table 1. Cont.

Catalogue number	Species (author)	New species name (author)	Locality	Latitude (decimal)
RMNH.Coel.41125	*Mopsella* sp.	*Melithaea* sp.	Malaysia, Borneo, Semporna, Mata Pahi Is.	4.580806 N
RMNH.Coel.41126	*Melithaea* sp.	*Melithaea* sp.	Malaysia, Borneo, Semporna, Mata Pahi Is.	4.580806 N
RMNH.Coel.41127	*Melithaea* sp.	*Melithaea* sp.	Indonesia, Sulawesi, Lembeh, N Sarena Kecil	1.45746 N
RMNH.Coel.41128	*Melithaea* sp.	*Melithaea* sp.	Indonesia, Halmahera, Tidore, Pulau Pilongga N	0.713833 N
RMNH.Coel.41129	*Melithaea* sp.	*Melithaea* sp.	Republic of Palau, Short Drop-Off, inside wall	7.525383 N
RMNH.Coel.41130	*Melithaea* sp.	*Melithaea* sp.	Republic of Palau, Siaes Tunnel	7.311433 N
RMNH.Coel.41131	*Melithaea* sp.	*Melithaea* sp.	Republic of Palau, Wonder Channel	7.18115 N
RMNH.Coel.41132	*Melithaea* sp.	*Melithaea* sp.	Republic of Palau, Turtle Cove	7.084633 N
RMNH.Coel.41133	*Melithaea* sp.	*Melithaea* sp.	Republic of Palau, KB Channel	7.309867 N
RMNH.Coel.41134	*Melithaea* sp.	*Melithaea* sp.	Indonesia, Papua, Raja Ampat, SE Gam, Desa Besir	0.463367 S
RMNH.Coel.41135	*Acabaria* sp.	*Melithaea* sp.	Indonesia, Papua, Raja Ampat, Mayalibit Bay, E Manil Island	0.304133 S
RMNH.Coel.41136	*Melithaea* sp.	*Melithaea* sp.	Republic of Palau, Wonder Channel	7.18115 N
RMNH.Coel.41137	*Melithaea* sp.	*Melithaea* sp.	Republic of Palau, Ngerikuul Gap	7.3209 N
RMNH.Coel.41138	*Melithaea* sp.	*Melithaea* sp.	Republic of Palau, Turtle Cove	7.084633 N
RMNH.Coel.41139	*Melithaea* sp.	*Melithaea* sp.	Republic of Palau, KB Channel	7.309867 N
RMNH.Coel.41140	*Acabaria* sp.	*Melithaea* sp.	Malaysia, Borneo, Semporna, Gaya Island 1 SE	4.624722 N
RMNH.Coel.41141	*Acabaria* sp.	*Melithaea* sp.	Malaysia, Borneo, Semporna, Selakan Island	4.572806 N
RMNH.Coel.41142	*Acabaria* sp.	*Melithaea* sp.	Malaysia, Borneo, Semporna, Timbun Mata Island	4.633222 N
RMNH.Coel.41143	*Acabaria* sp.	*Melithaea* sp.	Malaysia, Borneo, Semporna, Balusuan Island	4.685528 N
RMNH.Coel.41144	*Melithaea* sp.	*Melithaea* sp.	Vietnam, Con dao, Bong Lan Island, S side of Bong Lan	8.650667 N
RMNH.Coel.41145	*Melithaea* sp.	*Melithaea* sp.	Indonesia, Halmahera, Ternate, Sulamadaha Beach	0.863222 N
RMNH.Coel.41146	*Melithaea* sp.	*Melithaea* sp.	Indonesia, Sulawesi, Lembeh, Lobangbatu	1.43407 N
RMNH.Coel.41147	*Mopsella* sp.	*Melithaea* sp.	Indonesia, Bali, Tanjung Benoa, Loloan Benoa	8.762778 N
RMNH.Coel.41148	*Mopsella* sp.	*Melithaea* sp.	Republic of Palau, Neco Channel	7.205267 N
RMNH.Coel.41149	*Melithaea* sp.	*Melithaea* sp.	South Africa, Port Elizabeth, Algoa Bay, Table Top	33.982 S
RMNH.Coel.41150	*Melithaea* sp.	*Melithaea* sp.	South Africa, Port Elizabeth, Algoa Bay, Riy Banks 1	33.984483 S
RMNH.Coel.41151	*Melithaea* sp.	*Melithaea* sp.	South Africa, Cape Peninsula, Vulcan Rock	34.066167 S

					accession numbers			
Longitude (decimal)	Date	Depth (m)	Collector	clade	ND6	COI	mtMutS	28S
118.547056 E	12/17/2010	10	B.T. Reijnen	B	KC845731	KC802153	KC845617	KC845854
118.547056 E	12/17/2010	10	B.T. Reijnen	B	KC845732	KC802154	KC845618	KC845855
125.22711 E	2/16/2012	28	B.T. Reijnen	B	KC845774	KC802194	KC845661	KC845908
127.479278 E	11/12/2009	16	B.T. Reijnen	B	KC845727	KC802140	KC845595	KC845862
134.525383 E	5/19/2011	-	C.S. McFadden	B	KC845734	KC802172	KC845620	KC845863
134.2266 E	5/21/2011	24.4	C.S. McFadden	B	KC845744	KC802173	KC845621	KC845859
134.3602 E	5/21/2011	16.2	M. Janes	B	KC845735	KC802174	KC845622	KC845860
134.262167 E	5/24/2011	15.2	M. Janes	B	KC845739	KC802177	KC845626	KC845861
134.52475 E	5/26/2011	6.0	C.S. McFadden	B	KC845742	KC802180	KC845593	KC845864
130.687383 E	12/2/2007	13	J. van Egmond	B	KC845728	KC802136	KC845605	KC845857
130.904333 E	12/12/2007	20	B.T. Reijnen	B	KC845729	KC802137	KC845632	KC845858
134.3602 E	5/21/2011	13.6	C.S. McFadden	B	KC845736	KC802175	KC845623	KC845844
134.487567 E	5/22/2011	14.0	M. Janes	B	KC845737	KC802182	KC845624	KC845850
134.262167 E	5/24/2011	4.3	C.S. McFadden	B	KC845738	KC802176	KC845625	KC845849
134.52475 E	5/26/2011	6.5	C.S. McFadden	B	KC845743	KC802181	KC845633	KC845883
118.777472 E	12/10/2010	2	B.T. Reijnen	B	KC845719	KC802141	KC845611	KC845845
118.717861 E	12/12/2010	5	B.T. Reijnen	B	KC845723	KC802142	KC845612	KC845846
118.589333 E	12/15/2010	17	B.T. Reijnen	B	KC845724	KC802143	KC845615	KC845847
118.541556 E	12/15/2010	12	B.T. Reijnen	B	KC845725	KC802144	KC845616	KC845848
106.676333 E	7/25/2008	18.3	CRRF	B	KC845694	KC802113	KC845579	KC845843
127.334472 E	10/26/2009	7	B.T. Reijnen	B	KC845726	KC802139	KC845594	KC845851
125.2027 E	2/6/2012	28	B.T. Reijnen	B	KC845771	KC802197	KC845662	KC845905
115.233611 E	4/7/2001	<20	L.P. van Ofwegen & M. Slierings	B	KC845798	-	KC845689	KC845921
134.377433 E	5/21/2011	11.9	C.S. McFadden	B	KC845740	KC802178	KC845627	KC845884
25.693167 E	3/13/2008	10-12	C.S. McFadden	E	KC845752	KC802162	KC845649	KC845813
25.864017 E	3/11/2008	15-20	C.S. McFadden	E	KC845754	KC802164	KC845640	KC845809
18.31045 E	3/22/2004	15-27	C.S. McFadden	E	KC845808	-	KC845687	KC845922

Table 1. Cont.

Catalogue number	Species (author)	New species name (author)	Locality	Latitude (decimal)
RMNH.Coel.41152	*Melithaea* sp.	*Melithaea* sp.	South Africa, Port Elizabeth, Algoa Bay, Riy Banks 2	33.984483 S
RMNH.Coel.41153	*Melithaea* sp.	*Melithaea* sp.	South Africa, Port Elizabeth, Algoa Bay, Riy Banks 1	33.984483 S
RMNH.Coel.41154	*Melithaea* sp.	*Melithaea* sp.	South Africa, Port Elizabeth, Algoa Bay, Riy Banks 1	33.984483 S
RMNH.Coel.41155	*Melithaea* sp.	*Melithaea* sp.	South Africa, Port Elizabeth, Algoa Bay, Riy Banks 1	33.984483 S
RMNH.Coel.41156	*Melithaea* sp.	*Melithaea* sp.	South Africa, Port Elizabeth, Algoa Bay, Riy Banks 1	33.984483 S
RMNH.Coel.41157	*Melithaea* sp.	*Melithaea* sp.	South Africa, Port Elizabeth, Algoa Bay, Riy Banks 1	33.984483 S
RMNH.Coel.41158	*Melithaea* sp.	*Melithaea* sp.	South Africa, Port Elizabeth, Algoa Bay, Bell Buoy 2	33.980717 S
RMNH.Coel.41159	*Melithaea* sp.	*Melithaea* sp.	South Africa, Port Elizabeth, Algoa Bay, Bell Buoy 2	33.980717 S
RMNH.Coel.41160	*Melithaea* sp.	*Melithaea* sp.	South Africa, Port Elizabeth, Algoa Bay, Bell Buoy 2	33.980717 S
RMNH.Coel.41161	*Melithaea* sp.	*Melithaea* sp.	South Africa, Port Elizabeth, Algoa Bay, Bell Buoy 3	33.980267 S
RMNH.Coel.41162	*Melithaea* sp.	*Melithaea* sp.	South Africa, Kwazulu-Natal, Park Rynie, Lander's Reef	30.332883 S
RMNH.Coel.41163	*Melithaea* sp.	*Melithaea* sp.	Seychelles, NW of Alphonse Atoll	7.000 S
RMNH.Coel.41164	*Melithaea* sp.	*Melithaea* sp.	Seychelles, NW of Praslin Island	4.266667 S
RMNH.Coel.41165	*Acabaria* sp.	*Melithaea* sp.	Indonesia, Papua, Raja Ampat, S Gam, shoal near mangrove border	0.51895 S
RMNH.Coel.41166	*Acabaria* sp.	*Melithaea* sp.	Indonesia, Papua, Raja Ampat, S Friwin Island	0.481817 S
RMNH.Coel.41167	*Melithaea caledonica* Grasshoff, 1999	*Melithaea caledonica* Grasshoff, 1999	New Caledonia, Baie de Nakety	21.509467 S
RMNH.Coel.41168	*Melithaea caledonica* Grasshoff, 1999	*Melithaea caledonica* Grasshoff, 1999	New Caledonia, Passe de Goyeta	21.249117 S
RMNH.Coel.41169	*Wrightella coccinea* (Ellis & Solander, 1786)	*Melithaea coccinea* (Ellis & Solander, 1786) comb. nov.	Seychelles, E of Mahé, N of Moyenne Island	4.616667 S
RMNH.Coel.41170	*Acabaria formosa* Nutting, 1911	*Melithaea formosa* (Nutting, 1911) comb. nov.	Indonesia, Sulawesi, Lembeh, SW Sarena Kecil	1.45551 N
RMNH.Coel.41171	*Acabaria formosa* Nutting, 1911	*Melithaea formosa* (Nutting, 1911) comb. nov.	Indonesia, Sulawesi, Lembeh, Tanjung Kungkungan	1.46622 N
RMNH.Coel.41172	*Acabaria formosa* Nutting, 1911	*Melithaea formosa* (Nutting, 1911) comb. nov.	Indonesia, Sulawesi, Lembeh, Tanjung Nanas I	1.46097 N
RMNH.Coel.41173	*Clathraria maldivensis* van Ofwegen, 1987	*Melithaea maldivensis* (van Ofwegen, 1987) comb. nov.	Maldives, Lankanfinolhu	4.285753 N

					accession numbers			
Longitude (decimal)	Date	Depth (m)	Collector	clade	ND6	COI	mtMutS	28S
25.864017 E	3/11/2008	14-17	S. Parker-Nance	E	KC845745	KC802163	KC845639	KC845823
25.864017 E	3/11/2008	15-20	C.S. McFadden	E	KC845746	KC802167	KC845642	KC845816
25.864017 E	3/11/2008	15-20	C.S. McFadden	E	KC845757	KC802168	KC845643	KC845817
25.864017 E	3/11/2008	15-20	C.S. McFadden	E	KC845750	KC802169	KC845644	KC845818
25.864017 E	3/11/2008	15-20	C.S. McFadden	E	KC845749	KC802165	KC845645	KC845819
25.864017 E	3/11/2008	15-20	C.S. McFadden	E	KC845751	KC802170	KC845646	KC845820
25.6601 E	3/13/2008	18-22	C.S. McFadden	E	KC845753	KC802166	KC845648	KC845821
25.6601 E	3/12/2004	18-22	C. McFadden	E	KC845804	-	KC845685	KC845920
25.6601 E	3/12/2004	18-22	S. Parker-Nance	E	KC845805	-	KC845690	KC845919
25.69295 E	3/14/2008	12-15	C.S. McFadden	E	KC845756	KC802171	KC845650	KC845822
30.79205 E	3/17/2004	22-28	C.S. McFadden	E	KC845806	-	KC845686	KC845918
52.716667 E	1/3/1993	-	L.P. van Ofwegen	A	KC845802	-	KC845682	KC845913
55.666667 E	12/17/1992	25	L.P. van Ofwegen	A	KC845800	-	KC845683	KC845914
130.641117 E	11/30/2007	23	F.R. Stokvis	A	KC845803	-	KC845691	KC845917
130.69835 E	12/7/2007	21	F.R. Stokvis	A	KC845801	-	KC845692	KC845916
166.097083 E	4/22/2012	<20	B.W. Hoeksema	B	KC845776	KC802192	KC845655	KC845900
164.775917 E	4/7/2012	20	B.W. Hoeksema	B	KC845777	KC802191	KC845654	KC845899
55.516667 E	12/25/1992	7	L.P. van Ofwegen	D	KC845760	KC802157	KC845581	KC845870
125.22362 E	12/7/2012	10	B.T. Reijnen	A	KC845772	KC802196	KC845657	KC845906
125.23396 E	2/10/2012	6	B.T. Reijnen	A	KC845773	KC802195	KC845656	KC845907
125.22661 E	2/17/2012	10	B.T. Reijnen	A	KC845775	KC802193	KC845658	KC845901
73.546742 E	5/3/2011	<20	B.T. Reijnen	D	KC845716	KC802133	KC845589	KC845875

Table 1. Cont.

Catalogue number	Species (author)	New species name (author)	Locality	Latitude (decimal)
RMNH.Coel.41174	*Clathraria maldivensis* van Ofwegen, 1987	*Melithaea maldivensis* (van Ofwegen, 1987) comb. nov.	Maldives, Lankanfinolhu	4.285753 N
RMNH.Coel.41175	*Clathraria maldivensis* van Ofwegen, 1987	*Melithaea maldivensis* (van Ofwegen, 1987) comb. nov.	Maldives, Lankanfinolhu	4.285753 N
RMNH.Coel.41176	*Acabaria rubra* Esper, 1798	*Melithaea rubra* (Esper, 1798) comb. nov.	South Africa, Port Elizabeth, Algoa Bay, Riy Banks 1	33.98495 S
RMNH.Coel.41177	*Acabaria rubra* Esper, 1798	*Melithaea rubra* (Esper, 1798) comb. nov.	South Africa, Port Elizabeth, Algoa Bay, White Sands 15	33.998333 S
RMNH.Coel.41178	*Acabaria rubra* Esper, 1798	*Melithaea rubra* (Esper, 1798) comb. nov.	South Africa, Port Elizabeth, Algoa Bay, Bell Buoy 2	33.980717 S
RMNH.Coel.41179	*Acabaria rubra* Esper, 1798	*Melithaea rubra* (Esper, 1798) comb. nov.	South Africa, Cape Peninsula, Oudekraal, Justin's Caves	33.98165 S
RMNH.Coel.41180	*Acabaria rubra* Esper, 1798	*Melithaea rubra* (Esper, 1798) comb. nov.	South Africa, Cape Peninsula, Oudekraal, Justin's Cave	33.98165 S
RMNH.Coel.41181	*Chironephthya* sp.	n/a	Malaysia, Borneo, Semporna, Sebangkat Island	4.55525 N
RMNH.Coel.41182	*Siphonogorgia* sp.	n/a	Malaysia, Borneo, Semporna, Sebangkat Island	4.55525 N
RMNH.Coel.41183	*Annella* sp.	n/a	Indonesia, Halmahera, Hiri, Tanjung Ngafauda	0.910639 N
RMNH.Coel.41184	*Chironephthya* sp.	n/a	Malaysia, Borneo, Semporna, Timba timba Island	4.560883 N
RMNH.Coel.41185	*Solenocaulon* sp.	n/a	Malaysia, Borneo, Semporna, Pandanan Island	4.577967 N
RMNH.Coel.41186	*Chironephthya* sp.	n/a	Malaysia, Borneo, Semporna, 2 N Kulapuan Island	4.5354 N
RMNH.Coel.41187	*Siphonogorgia* sp.	n/a	Malaysia, Borneo, Semporna, Church Reef 1	4.681683 N
RMNH.Coel.41188	*Chironephthya* sp.	n/a	Malaysia, Borneo, Sipadan Island, Barracuda Point	4.12035 N
RMNH.Coel.41189	*Euplexaura* sp.	n/a	Indonesia, Sulawesi, Lembeh, N Sarena Kecil	1.45746 N
ZMTAU.CO.32749	*Acabaria* sp.	*Melithaea* sp.	Tanzania, Pemba Island, Nijao Gap, mega wall	4.961111 S
ZMTAU.CO.32939	*Clathraria rubrinodes* Gray, 1859	*Melithaea rubrinodes* (Gray, 1859) comb. nov.	Eritraea, Dahlak Archipelago, between Nocra Island and Dhlak Island, southern entrance channel	15.689333 N
ZMTAU.CO.34054	*Clathraria rubrinodes* Gray, 1859	*Melithaea rubrinodes* (Gray, 1859) comb. nov.	Israel, Gulf of Aqaba, Eilat, Interuniversity Institute for Marine Sciences	29.502333 N

					accession numbers			
Longitude (decimal)	Date	Depth (m)	Collector	clade	ND6	COI	mtMutS	28S
73.546742 E	5/3/2011	<20	B.T. Reijnen	D	KC845717	KC802134	KC845590	KC845876
73.546742 E	5/3/2011	<20	B.T. Reijnen	D	KC845718	KC802135	KC845591	KC845877
25.8629 E	3/11/2008	15-20	C.S. McFadden	E	KC845755	KC802159	KC845641	KC845810
25.7087 E	3/19/2008	14-16	C.S. McFadden	E	KC845748	KC802160	KC845634	KC845812
25.6601 E	3/13/2008	18-22	C.S. McFadden	E	KC845758	KC802161	KC845647	KC845814
18.359833 E	3/23/2004	7-11	C.S. McFadden	E	KC845807	-	KC845688	KC845915
18.359833 E	3/24/2008	7-11	C.S. McFadden	E	KC845747	KC802155	KC845635	KC845811
118.655217 E	12/12/2010	15	B.T. Reijnen	Out-group	KC845790	KC802212	KC845676	KC845896
118.655217 E	12/12/2010	15	B.T. Reijnen	Out-group	KC845791	KC802213	KC845677	KC845894
127.317417 E	10/31/2009	16	B.T. Reijnen	Out-group	KC845788	KC802214	KC845678	KC845892
118.924817 E	12/8/2010	17	B.T. Reijnen	Out-group	KC845794	KC802215	KC845679	KC845893
118.9204 E	12/8/2010	22	B.T. Reijnen	Out-group	KC845796	KC802207	KC845680	KC845887
118.838383 E	12/9/2010	20	B.T. Reijnen	Out-group	KC845795	KC802208	KC845681	KC845895
118.658017 E	12/13/2010	23	B.T. Reijnen	Out-group	KC845792	KC802209	KC845673	KC845898
118.629433 E	12/18/2010	24	B.T. Reijnen	Out-group	KC845793	KC802210	KC845674	KC845897
125.22711 E	2/16/2012	28	B.T. Reijnen	Out-group	KC845789	KC802211	KC845675	KC845886
39.664167 E	29-112004	10-20	Y. Benayahu	C	KC845762	KC802183	KC845583	KC845815
39.934667 E	2/14/2005	0-5	Y. Benayahu	C	KC845763	KC802184	KC845584	KC845871
34.917917 E	7/23/2007	23.2	Y. Benayahu	C	KC845695	KC802115	KC845636	KC845872

Table 1. Cont.

Catalogue number	Species (author)	New species name (author)	Locality	Latitude (decimal)
ZMTAU.CO.34200	*Acabaria sinaica* Grasshoff, 2000	*Melithaea sinaica* (Grasshoff, 2000) comb. nov.	Israel, Gulf of Aqaba, Eilat, North Oil jetty	29.5235 N
ZMTAU.CO.34216	*Acabaria erythraea* (Ehrenberg, 1834)	*Melithaea erythraea* (Ehrenberg, 1834) comb. nov.	Israel, Gulf of Aqaba, Eilat, Speed boat jetty	29.548 N
ZMTAU.CO.35497	*Acabaria sinaica* Grasshoff, 2000	*Melithaea sinaica* (Grasshoff, 2000) comb. nov.	Israel, Gulf of Aqaba, Eilat, North Oil jetty	29.5235 N
ZMTAU.CO.35499	*Acabaria sinaica* Grasshoff, 2000	*Melithaea sinaica* (Grasshoff, 2000) comb. nov.	Israel, Gulf of Aqaba, Eilat, North Oil jetty	29.5235 N
ZMTAU.CO.35500	*Acabaria sinaica* Grasshoff, 2000	*Melithaea sinaica* (Grasshoff, 2000) comb. nov.	Israel, Gulf of Aqaba, Eilat, North Oil jetty	29.5235 N

at 10 kV. Consequently, microscope slides and SEM images were used to assign specimens to the nominal genera according to the following key based on van Ofwegen (1987).

(1) Sclerites at coenenchymal surface predominantly spindles and occasionally a few thorn-clubs in some species. (*Acabaria*/*Asperaxis*).
(2) Sclerites at coenenchymal surface consisting of predominantly double discs. Leaf-clubs and thorn-clubs might be present but in few number. (*Melithaea*).
(3) Predominantly leaf-clubs and thorn-clubs in the coenenchymal surface. (*Mopsella*).
(4) Coenenchymal surface is formed by large foliate spheroids forming a complete pavement-like protection. (*Wrightella*).
(5) Coenenchymal surface without any predominant sclerite type, and includes spindles, clubs and small leafy spheroids. (*Clathraria*).

Species were identified, where possible, based on the traditional characters used for Octocorallia identification such as: overall shape and size of sclerites, absence and presence of projections and/or tuberculation and the occurrence of certain sclerite types.

Molecular analysis
DNA was extracted using the DNEasy Kit (QIAGEN) with the corresponding protocol for animal tissue (v. 07/2006). Approximately 1 cm of the gorgonian was cut into small pieces before the tissue was added to the extraction buffers. The digestion was performed overnight (c. 16 h). In some cases the extract had to be diluted before DNA amplification. The PCR mixture contained: 2.5 µl PCR CoralLoad Buffer (containing 15 mM $MgCl_2$) (QIAGEN), 0.5 µl dNTP's (2.5 mM), 1.0 µl per primer (10 pmol), 0.3 µl

					accession numbers			
Longitude (decimal)	Date	Depth (m)	Collector	clade	ND6	COI	mtMutS	28S
34.935667 E	7/26/2007	15.2	Y. Benayahu	C	KC845696	KC802148	KC845638	KC845874
34.953667 E	7/27/2007	0.3-0.6	Y. Benayahu	C	KC845697	KC802147	KC845637	KC845873
34.935667 E	2/15/2012	16	Y. Benayahu	C	KC845785	KC802151	KC845630	KC845909
34.935667 E	2/15/2012	16	Y. Benayahu	C	KC845787	KC802150	KC845631	KC845910
34.935667 E	2/15/2012	16	Y. Benayahu	C	KC845786	KC802149	KC845629	KC845911

Taq polymerase (15 units/µl) (QIAGEN) and 18.7 µl of extra pure PCR water and 1.0 µl (diluted) DNA extract. The primer pairs and PCR amplification settings used are presented in Table 3. Apart from the different annealing temperatures, all PCR cycles consisted of an initial denaturing step of 95°C for 1 min. followed by 39 cycles of 95°C for 10 s, preferred annealing temperature (see Table 3) for 1 min. and an extension step of 72°C for 1 min. The final PCR cycle was followed by an elongated extension step of 72°C for 5 min.

PCR products were analysed on a 1% agarose gel and stained with ethidium bromide, and visualized on a Cell Biosciences Red. Amplified samples were sent to Macrogen Europe for PCR cleaning and sequencing on an ABI Automated Sequencer 3730xl or were purified by PEG-precipitation (Sánchez *et al*., 2003) and sent to htSeq (University of Washington, Seattle) for sequencing. Sequences were assembled with Sequencher 4.10.1. The resulting consensus sequences were aligned in BioEdit (Hall, 1999), except for the 28S data. The 28S data contained insertions and/or deletions, therefore nucleotides were aligned with the help of the GUIDANCE server (Penn *et al*., 2010) using the MAFFT algorithm. All consensus sequences were also blasted against GenBank to check for nonspecific amplification or contamination. All novel sequences have been submitted to GenBank (accession numbers: KC802113 – KC802215 (COI); KC845579 – KC845693 (mtMutS); KC845694 – KC845808 (ND6); KC845809 – KC845923 (28S)). The outgroup species for our analyses were selected based on the phylogenetic tree of McFadden *et al*. (2006, 521, Fig. 3) from which direct sister species (*Siphonogorgia* spp. and *Chironephthya* spp.) and other less related species (*Annella* sp., *Solenocaulon* sp., and *Euplexaura* sp.) were selected for inclusion in our phylogenetic analyses.

Table 2. List of type specimens of Melithaeidae studied. BMNH = British Museum of Natural History, London, Un
de Strasbourg, France; NTM = Museum and Art Gallery of the Northern Territory, Australia; ZMA = Naturalis Biod
für Naturkunde, Berlin, Germany.

Original species (author)	Collection code	Type	Depth
Acabaria amboinensis Hentschel, 1903	MZS.Cni.105	Holotype	n/a
Acabaria baladea Grasshoff, 1999	MNHN.HG.198	Paratype	33m
Acabaria biserialis Kükenthal, 1908	ZMB.5810	Syntype	litoral
Acabaria cinquemiglia Grasshoff, 1999	MNHN.HG.172	Paratype	30m
Acabaria divaricata Gray, 1859	BMNH 1846.7.30.59	Holotype	n/a
Acabaria formosa Nutting, 1911	ZMA.Coel.2100	Holotype	9-45m
Acabaria haddoni Hickson, 1937	BMNH 1937.7.14.8	Paratype	18-31m
Acabaria hicksoni Nutting, 1911	ZMA.Coel.2102	Holotype	23m
Acabaria kuea Grasshoff, 1999	MNHN.HG.164	Paratype	10-15m
Acabaria laevis Wright & Studer, 1889	BMNH.1947-03-22-007	Holotype	27-37m
Acabaria nuttingi Hickson, 1937	ZMA.Coel.2103	Holotype	937m
Acabaria ouvea Grasshoff, 1999	MNHN.HG.165	Paratype	n/a
Acabaria planoregularis Kükenthal, 1910	ZMB.5818	Syntype	8-10m
Acabaria ramulosa Kükenthal, 1910	ZMB.5804	Holotype	18m
Acabaria serrata Ridley, 1884	BMNH 1882.2.23.129-132	Paratype	12-22m
Acabaria squarrosa Kükenthal, 1910	ZMB.5806	Syntype	15m
Acabaria triangulata Nutting, 1911	ZMB.5802	Syntype	57m
Acabaria valdiviae Kükenthal, 1908	ZMB.5802	Syntype	318m
Asperaxis karenae Alderslade, 2006	NTM.C.13575	Paratype	4-6m
Birotulata splendens (Nutting, 1911)	ZMA.Coel.1684	Holotype	n/a
Clathraria robusta Kükenthal, 1919	ZMB.5106	Holotype	litoral
Clathraria roemeri Kükenthal, 1908	ZMB.5800	Holotype	n/a
Melitella elongata Gray, 1859	BMNH 1983.3.2.11	Holotype	n/a
Melithaea caledonica Grasshoff, 1999	MNHN.HG.163	Paratype	33m
Melitodes africana (Kükenthal, 1908)	ZMB.5819	Syntype	70m
Melitodes albitincta Ridley, 1884	BMNH 1881.10.21.189	Holotype	22-37m
Melitodes contorta Dean, 1932	BMNH 1937.7.14.2	Holotype	n/a
Melitodes esperi (Wright & Studer, 1889)	BMNH.1889-05-27-148	Holotype	n/a
Melitodes flabellum Thomson & Mackinnon, 1910	BMNH 1912.2.24.59	Holotype	11m
Melitodes mertoni (Kükenthal, 1910)	ZMB.5808	Syntype	18m
Melitodes modesta (Nutting, 1911)	ZMA.Coel.2860	Holotype	13m
Melitodes philippinensis Wright & Studer, 1889	BMNH 1889.5.27.145	Syntype	n/a
Melitodes rugosa Wright & Studer, 1889	BMNH 1889.5.27.114	Holotype	70-73m
Melitodes sinuata Wright & Studer, 1889	BMNH 1889.5.27.144	Holotype	n/a
Melitodes squamata (Nutting, 1911)	ZMA.Coel.2867	Holotype	34m
Melitodes stormii (Studer, 1895)	ZMB.3762	Syntype	4-10m
Melitodes sulphurea (Studer, 1895)	ZMB.3766	Holotype	4-10m
Mopsella clavigera Ridley, 1884	BMNH 1881.10.21.116-125	Syntype	7-26m
Mopsella spinosa Kükenthal, 1910	ZMB.5811	Syntype	10m
Mopsella spongiosa Nutting, 1911	ZMA.Coel.2902	Holotype	13m
Mopsella studeri Nutting, 1911	ZMA.Coel.2904	Syntype	13m
Mopsella zimmeri Kükenthal, 1908	ZMB.5812	Syntype	n/a
Wrightella robusta Shann, 1912	BMNH 1937.7.14.12	Holotype	shallow water
Wrightella tongaensis Kükenthal, 1908	ZMB.5813	Holotype	n/a

nited Kingdom; MNHN = Muséum national d'Histoire naturelle, Paris, France; MZS = Musée zoologique de la ville liversity Center, Leiden, The Netherlands (former collection of Zoological Museum of Amsterdam); ZMB = Museum

	Locality	Remarks
	Ambon	is synonym of *Acabaria laevis*
	New Caledonia, Banc Gail stn. 114	
	Red Sea, sta.95, 34°47.7 E; 29°12.7 N (nowadays Gulf of Aqaba)	
	New Caledonia, S lagon, Canal Woodin stn. 252	
	n/a	
	Indonesia, Sta. 240 Banda anchorage	
	N Australia, Saibai Channel	
	Indonesia, Samau Island near Timor, sta. 60 Haingsisi	
	New Caledonia, S Grecife/rext Recife Kue stn. 445	
	Indonesia, Ambon	
	Indonesia, near Ternate, Siboga expedition sta. 139	
	New Caledonia, no precise locality data	
	Indonesia, Aru Islands, SW of Lola	
	Indonesia, Aru Islands, Sungi Barkai	
	N coast of Australia, Port Darwin	
	Indonesia, Aru Islands, Sungi Barkai	
	Indonesia, Siboga expedition Sta. 274, 5°28.2 S 134°53.9 E	
	South Africa, Cape of Good Hope	
	Australia, Tasmania , Port Davey, Mundy Island, Bathurst Channel	
	Indonesia, Siboga expedition Sta. 213, anchorage in Saleyer (South Celebes)	is *Melithaea mcqueeni* nom. nov.
	Neu Pommern (=New Britain), Tallasia	is *Melithaea shanni* nom.nov.
	Indonesia, Ambon	is synonym of *Melithaea ochracea*
	n/a	
	New Caledonia, S lagon, Chenal des cinq milles, stn 242	
	South Africa, Simons Bay	
	Australia, Queensland, Port Molle	
	Indonesia, Papua, Sorong	
	Australia, Torres Strait	
	Seychelles, Providence	
	Indonesia, Aru Islands, Sungi Barkai	
	Indonesia, East Coast of the Aru Islands, Siboga expedition sta. 273, anchorage off Pulau Jedan	is synonym of *Acabaria planoregularis*
	N Philippines, Saboangan, Reefs,	
	Australia, Tasmania, Bass strait, East moncouer Isl., Sta. 162	
	N Philippines, Saboangan, Reefs,	
	Indonesia, Siboga expedition Sta. 299, 10°52.4 S 123°01.1 E	
	Singapore, Bintang Isls.	
	Singapore, Bintang Isls.	is synonym of *Melithaea stormii*
	Australia, Queensland, Port Curtis/Port Molle/Thursday Isl	
	Indonesia, Aru Islands	
	Indonesia, East Coast of the Aru Islands (Pearl Banks), Siboga expedition Sta. 273, anchorage off Pulau Jedan	
	Indonesia, East Coast of the Aru Islands (Pearl Banks), Siboga expedition Sta. 273, anchorage off Pulau Jedan	
	Australia, Sydney	
	Near Singapore	
	Tonga Isl.	

Table 3. Overview of primer pairs, targeted area, fragment size, annealing temperatures and references.

Name	Targeted gene area	Fragment size	Forward primer (length)
Alc_715_Fw & Alc_1303_RV	ND6	~600	GGG CCA ATC CAG TAG AGG (18)
COII8068xF & COIOCTr	COI	~900	CCA TAA CAG GAC TAG CAG CAT C (22)
ND42599F & MUT3458R	mtMutS	~800	GCC ATT ATG GTT AAC TAT TAC (21)
msh-2761F & msh-3270R	mtMutS	~530	TAT GAA CTT TGG CAT GAG CC (20)
MSH_mod_F & MSH_mod_R	mtMutS	~800	TTA CCG TTT ACG TGG CAC AA (20)
28S-F252 & 28S-R1283	28S	~900	CAC GAG ACC GAT AGC GAA CAA GTA (24)

Phylogenetic analyses

Molecular datasets were analysed in MEGA 5.0.5 (Tamura *et al*., 2011) and jModeltest 2.1.1 (Darriba *et al*., 2012) to test for the most optimal evolutionary model based on the Akaike Information Criterion (AIC) (Yang, 2005). Phylogeny reconstructions were estimated based on the maximum parsimony (MP) method and Maximum Likelihood (ML) algorithm implemented in MEGA 5.0.5. For the ML and MP analyses 1,000 bootstrap replicates for which the heuristic search method Nearest-Neighbor-Interchange and Close-Neighbor-Interchange were used respectively. Gaps and missing data were treated as complete deletion. Additionally, datasets were also subjected to MrBayes 3.2.0 to check for congruency with the MP and ML analyses. MrBayes was run for 5,000,000 generations, with six chains (four cold and two heated ones). Data were sampled every 100 generations and the burnin was set to 12,500. For *Asperaxis karenae* Alderslade, 2006, mtMutS data was already available in GenBank (accession number DQ302847.1) (McFadden *et al*., 2006). To investigate the position of this subfamily compared to our Melithaeinae specimens, we have included the sequence in our mtMutS dataset. As outgroup we have used the same specimens as in the previous analyses.

Results

Sampling and molecular datasets

In total 103 specimens, including the outgroup selection, were sequenced for four molecular markers, three mitochondrial (ND6, COI, mtMutS) and one nuclear (28S rDNA). Among these specimens ten species could be identified with the help of type specimens and original species descriptions. The total length of the concatenated sequences ranged from 2,294-2,579 bp due to insertions, deletions or missing data. In particular the 28S sequences proved to be variable in length. Unfortunately not all samples amplified well with the standard mtMutS primers, therefore new nested primers were developed (Table 3). As a result approx. 270 bp less were amplified for four specimens included in this study. Some double peaks were observed while editing the 28S data, which were coded according to the IUPAC ambiguity codes. The most difficult marker to amplify proved to be COI. Therefore a second dataset was prepared from which the COI marker was removed. Consequently it was possible to include sequence data for 12 additional spec-

Reverse primer (length)	Annealing temp.	Reference
AGG TGA ATT TGG YTG CTT RG (20)	50°C	This publication
ATC ATA GCA TAG ACC ATA CC (20)	58°C	McFadden *et al*. (2011)
TSG AGC AAA AGC CAC TCC (18)	48°C	France and Hoover (2002); Sánchez *et al*. (2003)
TGC CCA AAT TAC TAT TTC TCT AAT ACG (27)	48°C	This publication
ATT GGG CGA TGT TTC CAT AA (20)	48°C	This publication
TCA TTT CGA CCC TAA GAC CTC (21)	50°C	McFadden and Ofwegen (2013)

imens. This resulted in a dataset containing 115 sequences. The dataset containing four molecular markers had 1,861 constant characters, 206 parsimony uninformative, variable characters and 533 parsimony informative characters while the dataset based on three molecular markers had 1,226 constant characters, 162 parsimony uninformative, variable characters and 397 parsimony informative characters. The alignment scores for both datasets calculated by GUIDANCE under the MAFFT algorithm were respectively 0.998603 and 0.999201. Alignments can be requested from the corresponding author.

The model searching analysis in both MEGA 5.0.5 and jModelTest 2.1.1 resulted in GTR + I + Γ being the most general model for the concatenated datasets as well as some of the single-gene datasets. On two occasions, jModeltest 2.1.1 selected different models for the COI and ND6 datasets respectively viz. TVM + I + Γ and TrN + I + Γ. Neither of these evolutionary models are implemented in MrBayes therefore the best approximation of the model in MrBayes was selected (GTR + I + Γ) (Hofman *et al*., 2007). Based on the results of both model testing programs, and the congruent topology for the single-gene trees we did not partition the dataset for the phylogenetic analyses. Molecular datasets including the parameters for the best evolutionary model were subjected to (ML)- (dataset with four markers, best log likelihood was –9051.4462, three markers –7200.6663), Maximum Parsimony (MP)- (dataset with four markers, 93 most parsimonious trees; length 1,055, three markers 103 most parsimonious trees; length 872) and Bayesian analyses. For the Bayesian analyses the final average split frequency after 5.000.000 runs for the datasets was, respectively, 0.0038 and 0.0049. Unidentified specimens or species groups that formed well-supported clades are illustrated with SEM images (App. 1, Pl. 1-39). At least one representative per species group is illustrated, except for the deep water Melithaeidae which are published separately (Matsumoto & van Ofwegen, 2015).

Phylogenetic analyses

The results from the ML, MP and Bayesian analyses revealed highly congruent phylograms. In Fig. 2 and Fig. 3 the ML trees of the datasets with and without the COI marker are shown. Both phylograms have a very similar tree topology representing five basal clades based on the biogeographic origin of the specimens viz. clade A-E which will be discussed below.

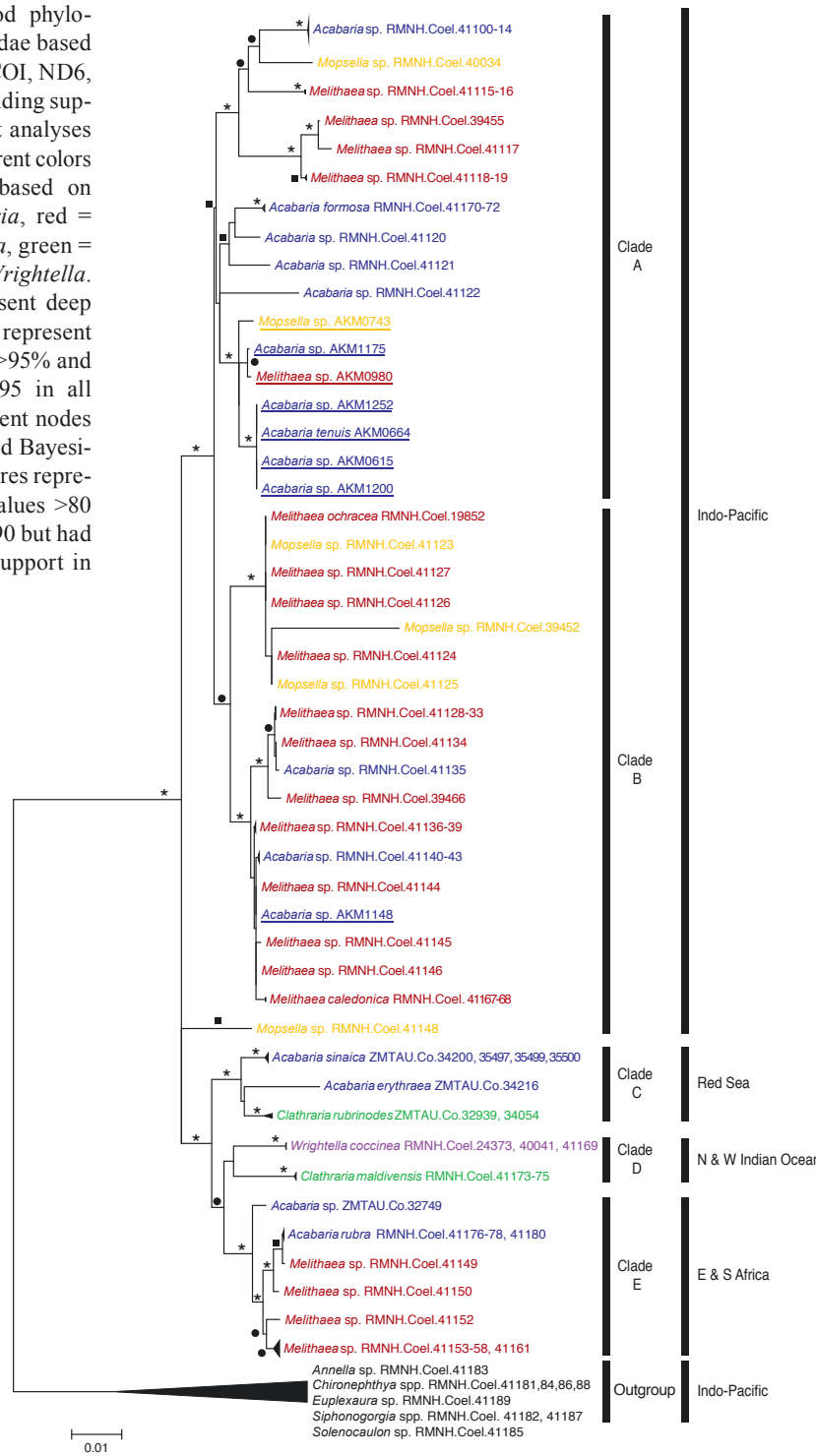

Fig. 2. Maximum likelihood phylogram of the family Melithaeidae based on four molecular markers (COI, ND6, mtMutS and 28S rDNA) including support values of three different analyses (ML/MP/MrBayes). The different colors represent different genera based on morphology: blue = *Acabaria*, red = *Melithaea*, yellow = *Mopsella*, green = *Clathraria* and purple = *Wrightella*. Underlined specimens represent deep water specimens. Asterisks represent nodes with bootstrap values >95% and Bayesian probabilities of >95 in all three analyses, circles represent nodes with bootstrap values >80 and Bayesian probabilities >90 and squares represent nodes with bootstrap values >80 and Bayesian probabilities >90 but had a considerable lower or no support in the MP analysis.

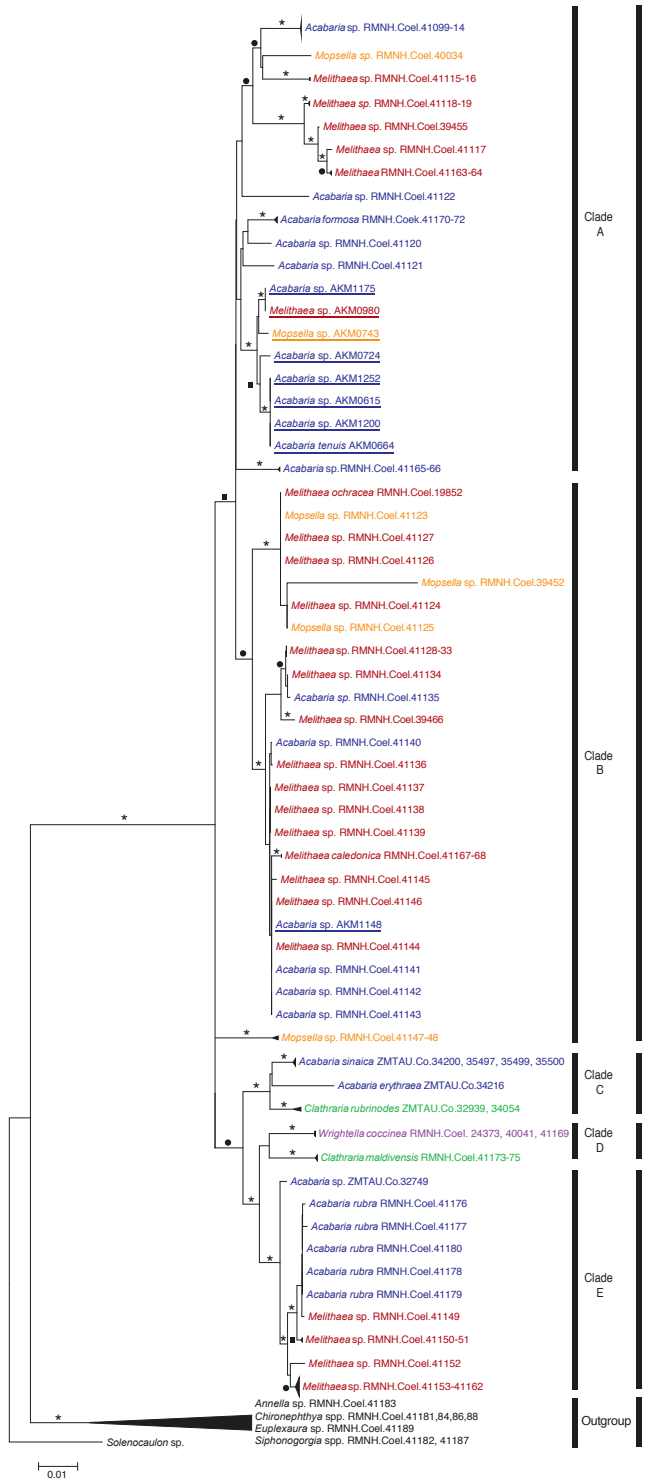

Fig. 3. Maximum likelihood phylogram of the family Melithaeidae based on three molecular markers (ND6, mt-MutS and 28S rDNA) including support values of three different analyses (ML/MP/MrBayes). The different colors represent different genera based on morphology: blue = *Acabaria*, red = *Melithaea*, yellow = *Mopsella*, green = *Clathraria* and purple = *Wrightella*. Underlined specimens represent deep water specimens. Asterisks represent nodes with bootstrap values >95% and Bayesian probabilities of >95 in all three analyses, circles represent nodes with bootstrap values >80 and Bayesian probabilities >90 and squares represent nodes with bootstrap values >80 and Bayesian probabilities >90 but had a considerable lower or no support in the MP analysis.

Clade A

In both trees, clade A consists of central Pacific specimens except for one specimen from the Seychelles (*Melithaea* sp. RMNH.Coel.41117). In the phylogenetic tree based on three markers two additional specimens cluster with this specimen *Melithaea* sp. (RMNH.Coel.41163–64), also from the Seychelles. These three specimens are the only ones that do not cluster according to the biogeographic pattern found for all other specimens in Fig. 2 and Fig. 3. Other species or genera represented in Clade A (based on the phylogeny consisting of four genetic markers) are *Acabaria formosa* Nutting, 1911, nine unidentified species of *Acabaria*, five of *Melithaea* and two *Mopsella* specimens. Both shallow as well as deep water species are represented in this clade. The deep water samples were collected at 71-502 m and are nested within the shallow water clade. One of the deep water specimens does not cluster with the other deep water specimens and is not retrieved in this clade (see section Clade B). The specific localities represented in clade A are: Northern Territory (Australia); Lombok, East Kalimantan, North Sulawesi, Papua (Indonesia); North Honshu, Ryukyu Archipelago (Japan); Semporna (Malaysia); Seychelles and Palau.

Clade B

Clade B, like clade A, also consists of central Pacific specimens but has a different species and generic composition than clade A. Clade A predominantly consists of species belonging to the genus *Acabaria* whereas this genus is only represented by three species in clade B. More specifically, clade B consists of: *Melithaea caledonica* Grasshoff, 1999, *M. ochracea* (Linnaeus, 1758), and an additional ten unidentified species of *Melithaea*, three of *Mopsella* and three of *Acabaria*. The only deep water specimen that was not retrieved in clade A (*Acabaria* sp. AKM1148) falls among all other representatives in this clade. Some species e.g. *Melithaea* sp. (RMNH.Coel.41128-34, 41136-39, 41144-45) appear morphologically very similar to the type specimen of *Melithaea squamata* (Nutting, 1911) but are genetically different. Additional morphological investigation of these specimens did not provide any strong characters to assign one or more of these specimens as representatives of *M. squamata*. Noticeable in our phylogenies is the relatively long branch length for *Melithaea* sp. (RMNH.Coel.39452). By investigating the concatenated alignment it clearly shows that only within the 28S marker this individual sequence has a consecutive region of approximately 100 bp that is unique in comparison to all other sequences. Since the 28S marker is known for having pseudogenes, the elongated branch is most probably an effect of these genes and does not represent actual species differences. The specific localities represented in clade B are: Northern Territory (Australia); West Halmahera, Moluccas, North Sulawesi, Papua (Indonesia); Okinawa Prefecture (Japan); Semporna (Malaysia); New Caledonia; Palau and South Vietnam.

Clade C

Clade C is represented by three species (in total seven specimens) all from the Red Sea viz. *Acabaria erythraea* (Ehrenberg, 1834), *Acabaria sinaica* Grasshoff, 2000 and *Clathraria rubrinodes* Gray, 1859. Together they form a well-supported clade (all sup-

port values >95%) and are considered a highly supported sister group (all support values >95%) to the representatives from the NW Indian Ocean and S and E Africa (clades D and E). Support for the phylogenetic position of *A. erythraea* is low (bootstrap and parsimony support <80%; Bayesian support <90%) and this species alternates between being a sister species of *C. rubrinodes* and *A. sinaica* in the two phylograms. Localities represented are: Dahlak Archipelago (Eritrea) and Eilat (Israel).

Clade D

Clade D is the smallest clade and is not well supported (bootstrap and parsimony support below 80%; Bayesian support below 90%). This clade consists of two species: *Clathraria maldivensis* van Ofwegen, 1987 and *Wrightella coccinea* (Ellis & Solander, 1786) both found in the North West Indian Ocean. *Clathraria maldivensis* is represented by three specimens from the Maldives. *Wrightella coccinea* is also represented by three specimens, one from the Chagos Archipelago and two from the Seychelles. The individual specimens cluster together with high support values (all support values >95%), but the relationship between both species is not very well supported (bootstrap and parsimony support <80%; Bayesian support <90%). As a result the support for this clade is low, but the split between clades D and E is rather well supported (bootstrap and parsimony support >80%; Bayesian support >90%). Therefore we decided to maintain the specimens as representatives of a separate clade and did not include the specimens in clade E. Localities represented are: Lankanfinolhu (Maldives); Salomon Atoll (Chagos Archipelago), and the Seychelles.

Clade E

The final clade, clade E, consists of an East African species and many South African species. One of the South African specimen groups could be identified as *Acabaria rubra* (Esper, 1798) (see also the neotype designation). The other species are considered to be an unidentified *Acabaria* and four *Melithaea* species. The East African species (*Acabaria* sp. (ZMTAU.Co.32749)) is sister to the South African specimens, a relationship that is very well supported (bootstrap and parsimony support >80%; Bayesian support >90%) in both phylogenies. Within the South African specimens, excluding *A. rubra*, four additional clades are identified possibly representing different species. Unfortunately there are more names available for African melithaeid species (Williams, 1992) than there are species in our phylogeny. Without a revision of the African melithaeids and investigations of the type species we are unable to identify the other species represented in this clade. Remarkably, all specimens in the South African clade have sclerites that often exceed 0.2 mm in length, which is large in comparison to melithaeid species from Indonesia and Malaysia. Localities represented in clade E are: Tanzania (East Africa); Cape of Good Hope, Natal (South Africa).

Relationships among clades

In contrast to the expectations that species representing the different nominal genera would cluster together, all genera except for *Wrightella* were found to be paraphyletic. For example, the genus *Acabaria* is represented in all clades except for clade D and

species which morphologically belong to the genus *Melithaea* are represented in clades A, B and E. To further investigate the relationships among clades we added the COI data from Aguilar-Hurtado *et al*. (2012), to our dataset. The intrageneric genetic variability in COI is relatively low, and therefore does not provide enough resolution to satisfactorily resolve clades. But although large polytomies are present, all sequences from Aguilar-Hurtado *et al*. (2012) cluster within our Indo-Pacific group viz. clades A and B. We were unable to include their 28S rDNA data in our phylogeny because they sequenced a different region of that gene.

Status of the subfamilies Melithaeinae and Asperaxinae

The results of the phylogenetic analyses revealed that the sequence of *Asperaxis karenae* does not cluster within the subfamily Melithaeinae, but although the coenenchymal sclerites are morphologically similar to the other members of the Melithaeidae, it is positioned in between the outgroup specimens, *Solenocaulon* sp. and the sister group containing *Annella* sp., *Euplexaura* sp., *Chironephthya* spp. and *Siphonogorgia* spp. (Fig. 4). These results indicate that based on the position of the mtMutS sequence of *Asperaxis karenae*, the family Melithaeidae is paraphyletic. Since this result was unexpected we obtained additional material of the type specimen sequenced by McFadden *et al*. (2006), to check the validity of their sequence. Although several attempts were made, and different methods used, we were not able to re-amplify DNA from the type material.

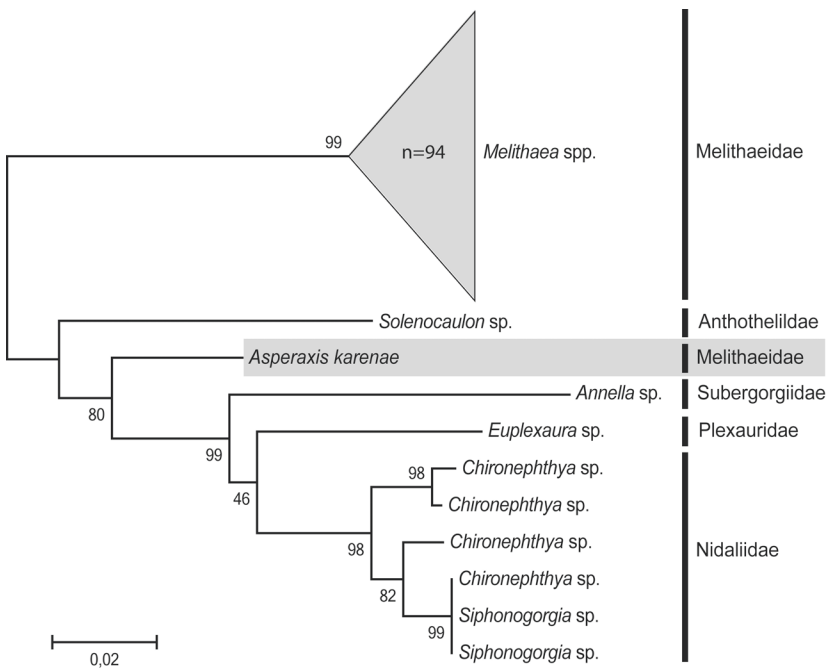

Fig. 4. ML analysis with bootstrap values showing the paraphyly of the Melithaeidae based on the mtMutS marker.

Systematic consequences within the family Melithaeidae

In this phylogenetic study we did not find molecular support to maintain the traditional morphologically defined genera. Therefore we synonymise the genera *Acabaria*, *Clathraria*, *Mopsella* and *Wrightella* with the earliest established genus in the Melithaeidae, *Melithaea*. As a result of synonymising the former genera only the genera *Asperaxis* and *Melithaea* will remain in their respective subfamilies Asperaxinae and Melithaeinae within the family Melithaeidae. According to the World Register of Marine Species (WoRMS) database (accessed on 22-01-2013) there are currently 114 names accepted for Melithaeidae. Yet, for five of these species identical species names are already in use and have become secondary homonyms, for which substitute names are given (Table 4).

Neotype designations for some Melithaeidae species

Besides renaming species due to secondary homonyms, neotype specimens are designated for the type species of *Melithaea*, namely *Melithaea ochracea* (Linnaeus, 1758), and for the South African species *Melithaea rubra* (Esper, 1798), neotypes are designated hereafter. Numerous type specimens in the family Melithaeidae are in very poor condition or lost.

Neotype designation of Melithaea ochracea (Linnaeus, 1758)

The original description of *Melithaea ochracea* is by Linnaeus *as Isis oc*[*h*]*racea* and is as follows: '*Stirpe coralline, articulis decorticates, geniculus nodosis*'. The type locality mentioned is: M.[Mare] indico. Linnaeus used the habitus drawing of *Accarbarium rubrum* from Rumphius (1750) for his description, of which the actual specimen is now considered lost. Additionally, in their descriptions Rumphius and Linnaeus only described

Table 4. Secondary homonyms and new substitute names for six melithaeid species after synonymising the genera *Acabaria*, *Clathraria*, *Mopsella* and *Wrightella* with *Melithaea*.

Secondary homonyms	New senior homonym combinations and new substitute names
Acabaria fragilis Wright & Studer, 1889	*Melithaea fragilis* (Wright & Studer, 1889) comb. nov.
Wrightella fragilis Thomson, 1917	*Melithaea wrighti* nom. nov.
Acabaria hicksoni Nutting, 1911	*Melithaea hicksoni* (Nutting, 1911) comb. nov.
Mopsella hicksoni Thorpe, 1928	*Melithaea thorpeae* nom. nov.
Acabaria modesta Kükenthal, 1908	*Melithaea modesta* (Kükenthal, 1908) comb. nov.
Melitodes modesta Nutting, 1911	*Melithaea kukenthali* nom. nov.
Wrightella robusta Shann, 1912	*Melithaea robusta* (Shann, 1912) comb. nov.
Clathraria robusta Kükenthal, 1919	*Melithaea shanni* nom. nov.
Melitodes splendens Thomson & McQueen, 1908	*Melithaea splendens* (Thomson & McQueen, 1908) comb. nov.
Birotulata splendens Nutting, 1911	*Melithaea mcqueeni* nom. nov.
Melitodes variabilis Hickson, 1905	*Melithaea variabilis* (Hickson, 1905) comb. nov.
Wrightella variabilis Thomson & Henderson, 1906	*Melithaea hendersoni* nom. nov.

the colony form. Therefore we designate RMNH.Coel.19852 as neotype for *M. ochracea* which was collected at the type locality viz. Ambon, Moluccas, Indonesia.

Melithaea ochracea (Linnaeus, 1758) (Neotype)
(Fig. 5 (habitus); Fig. 6 (sclerites))

Isis ocracea Linnaeus, 1758: 799
Isis ochracea Linnaeus, 1766: 1287
Clathraria roemeri Kükenthal, 1908: 201; 1919: 195 pl. 37 fig. 39; 1924: 87 fig. 60.; Hickson, 1937: 188.
Melithaea ochracea van Ofwegen, 1987: 7 Fig. 1 and Fig. 2

Locality: Sta.21 of the Rumphius Biohistorical Expedition 1990, Indonesia, Moluccas, Ambon, Hitu, N coast, Mamala, 3.537997° S; 128.206414° E, depth: 10-15 m, date: 21-09-1990. coll. L.P. van Ofwegen. RMNH.Coel.19852; Clade B, Fig. 2 and Fig. 3; GenBank accession numbers: KC845766 (ND6), KC802206 (COI), KC845653 (mtMutS), KC845879 (28S).

Description: The colony is approximately 25 cm long and dichotomously branched. Both the nodes and internodes are red, the calyces are yellow and the polyps are white.

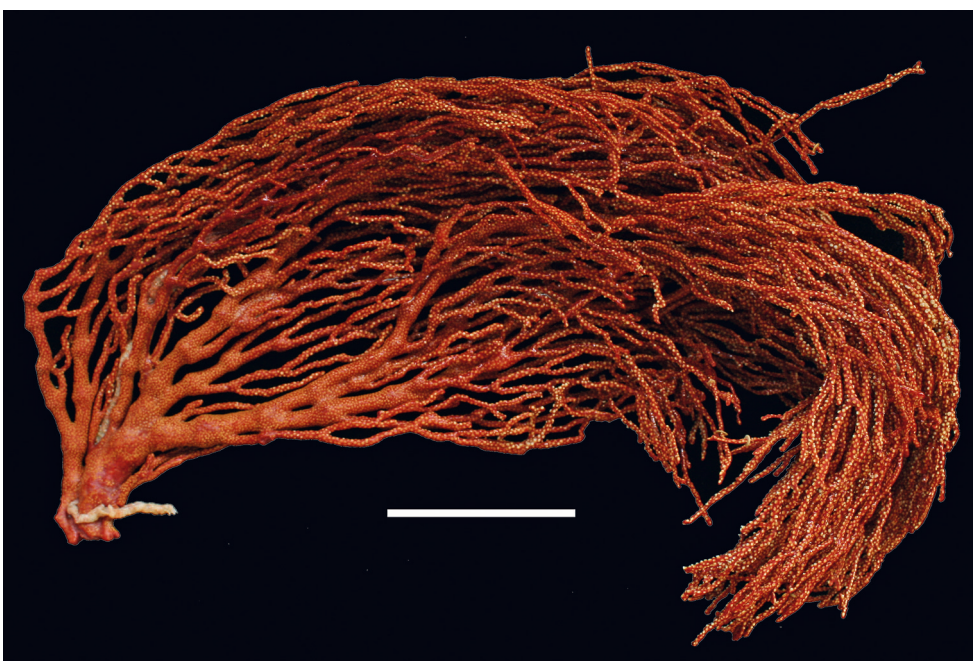

Fig. 5. Habitus overview of the neotype specimen for *Melithaea ochracea* (Linnaeus, 1758). Scale bar 5.0 cm.

The polyps are irregularly situated around the branches, contracted, and relatively small (<1.0 mm). Calyces project slightly above the coenenchyme. Sclerites of the coenenchyme include capstans, double discs (both 0.05-0.10 mm long) (Fig. 6g), leaf clubs

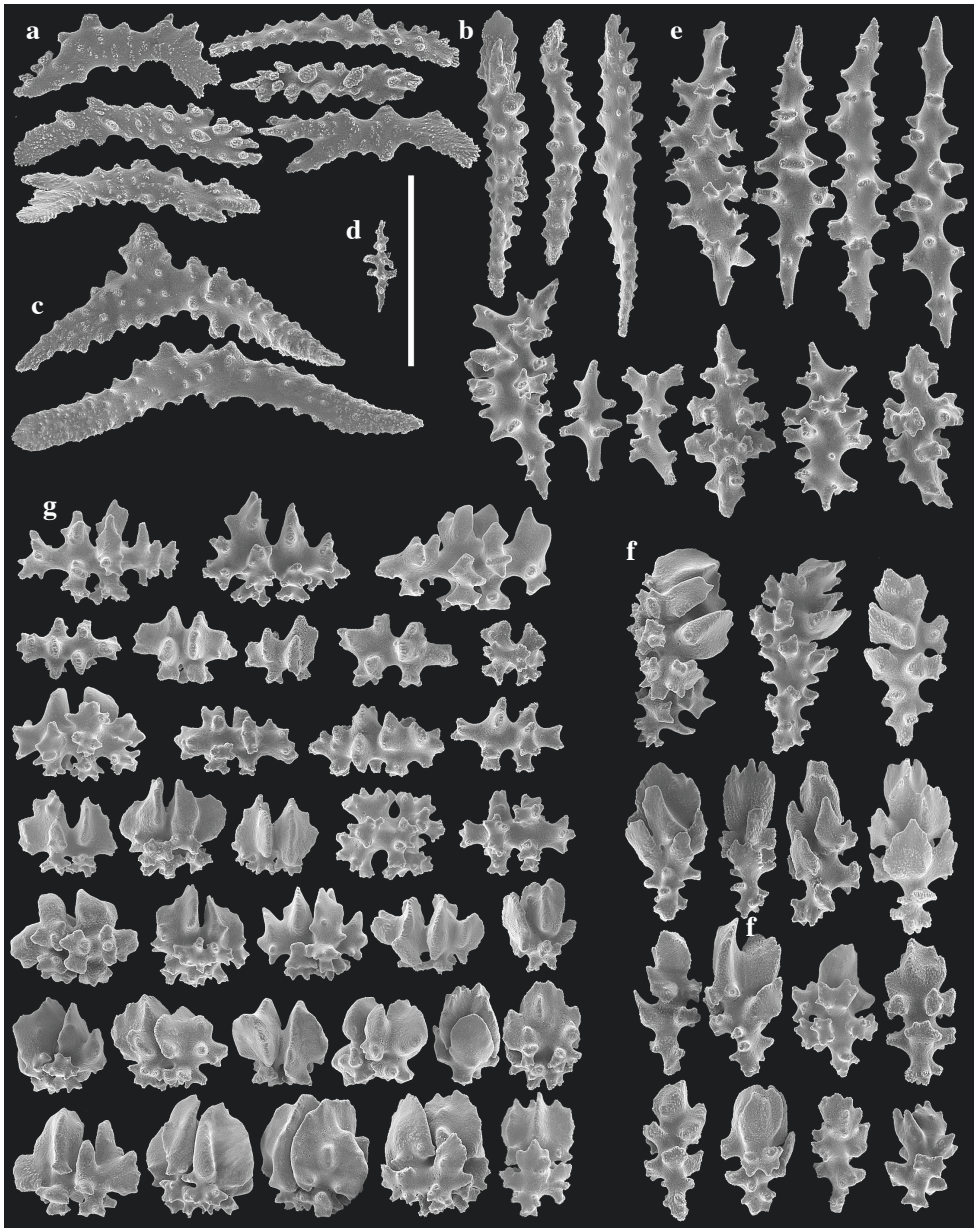

Fig. 6. Sclerite diversity in the neotype specimen of *Melithaea ochracea* (Linnaeus, 1758) (RMNH. Coel.19852); a) tentacle sclerites; b) point sclerites; c) collaret sclerites; d) pharynx sclerite; e) spindles; f) club sclerites; g) double discs and capstans. Scale bar 0.1 mm.

(0.06-0.12 mm long, longer ones are from the calyces) (Fig. 6f) and spindles (0.06-0.20 mm long) (Fig. 6e). The point sclerites are tuberculate, slightly curved spindles, and have spines or leaf-like projections appearing at one of the tips. These sclerites are 0.11-0.19 mm long (Fig. 6b). Collaret has flattened spindles with an ornamentation that is tuberculate in the middle and becomes less tuberculate and more granular at the distal end. Some collaret sclerites have an additional projection at the central bend, approaching a triradiate shape (Fig. 6c). The tentacles have flat, tuberculate, slightly crescent shaped platelets which are 0.07-0.12 mm long (Fig. 6a). The surface of the tentacle platelets appears somewhat granular. The pharynx sclerites are straight rods with spines on the middle area. They are on average 0.06 mm long (Fig. 6d).

Remarks: We examined and compared many melithaeid type specimens. While comparing them with the specimens included in the phylogeny we also compared the sclerites of *Clathraria roemeri* (App. 2, Pl. 18) with those of the neotype specimen of *Melithaea ochracea*. Based on this comparison, *C. roemeri* proved to be a synonym of *M. ochracea*. Both species were collected from Ambon (Moluccas, Indonesia) and are very similar in sclerite morphology. Morphologically the species *Melithaea* sp. (RMNH. Coel.39452, 41124-27; App. 1, Pl. 13-17) and *M. ochracea* have the same sclerite composition (double wheels and small clubs) but the shapes of the sclerites vary, ranging from small and pointed to large and very rounded (App. 1; Pl. 12-17). The taxonomic value of this type of variation in sclerites has to be studied before this variety can be positively identified as *M. ochracea*.

We were also able to check identifications of *M. ochracea* specimens from Singapore (van Ofwegen *et al*., 2000) which proved to be *M. stormii* (Studer, 1895), and of *M. ochracea* from New Caledonia (Grasshoff, 1999) which proved to be *M. caledonica*. So far the only other specimens we consider to truly belong to the species *M. ochracea* are those from Seram (van Ofwegen, 1987). Therefore the distribution of *M. ochracea* is limited to the Moluccas, Indonesia.

Neotype designation of Melithaea rubra Esper, 1798

This species was originally described by Esper (1798) as *Isis dichotoma cortice rubro*. Therein a piece of the octocoral is figured and the type locality ("das Vorgebürg der Guten Hoffnung" [Cape of Good Hope]) is provided. Details on the sclerite morphology are lacking. Grasshoff and Scheer (1990) provided an extensive overview of Esper's work and noted that the type material is lost and that *M. africana* (Kükenthal, 1908) is a possible synonym of *M. rubra*. The current status of the taxonomy and systematics of South African Melithaeidae is not considered satisfactory (Williams, 1992). Fortunately Williams (1992), who reassigned the species to the genus *Acabaria* mentions this is the commonest species around Cape of Good Hope. Therefore we designate a neotype for *M. rubra*, of which the habitus matches the description of Esper (1798) and was collected from the type locality.

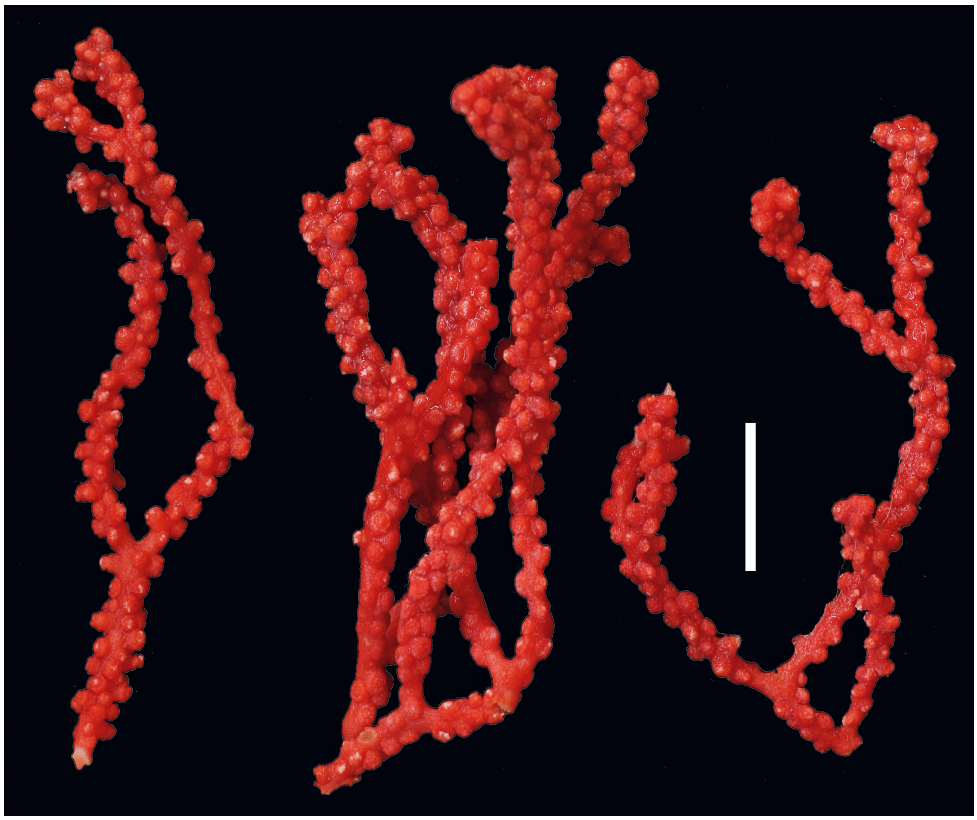

Fig. 7. Habitus overview of the neotype fragments for *Melithaea rubra* (Esper, 1798). Scale bar: 1.0 cm.

Melithaea rubra comb. nov. (Esper, 1798) (Neotype) (Fig. 7 (habitus); Fig. 8 and Fig. 9 (sclerites))

Isis dichotoma cortice rubro Esper, 1798: 6 pl. 1 Fig. 4 and Fig. 5.
Acabaria rubra Williams (1992): 197 Figs. 1A-B, 10–13 (in part).

Locality: South Africa, Cape Peninsula, Oudekraal, Justin's Cave, 33.98165° S; 18.359833° E, depth: 7-11 m, date: 24-3-2008. coll. C.S. McFadden. RMNH.Coel.41180; Clade E, Fig. 2 and Fig. 3; GenBank accession numbers: KC845747 (ND6), KC802155 (COI), KC845635 (mtMutS), KC845811 (28S).

Description: The colony consists of 3 fragments, which are all dichotomously branched, 4.0–5.5 cm long and a light red to pinkish colour. The calyces are of the same colour but the polyps are white. Nodes are not visible. The polyps are large (1.0-1.6 mm in diameter), irregularly situated around the branches giving them a thick and rugged appearance. Calyces project prominently above the coenenchyme. Sclerites of the

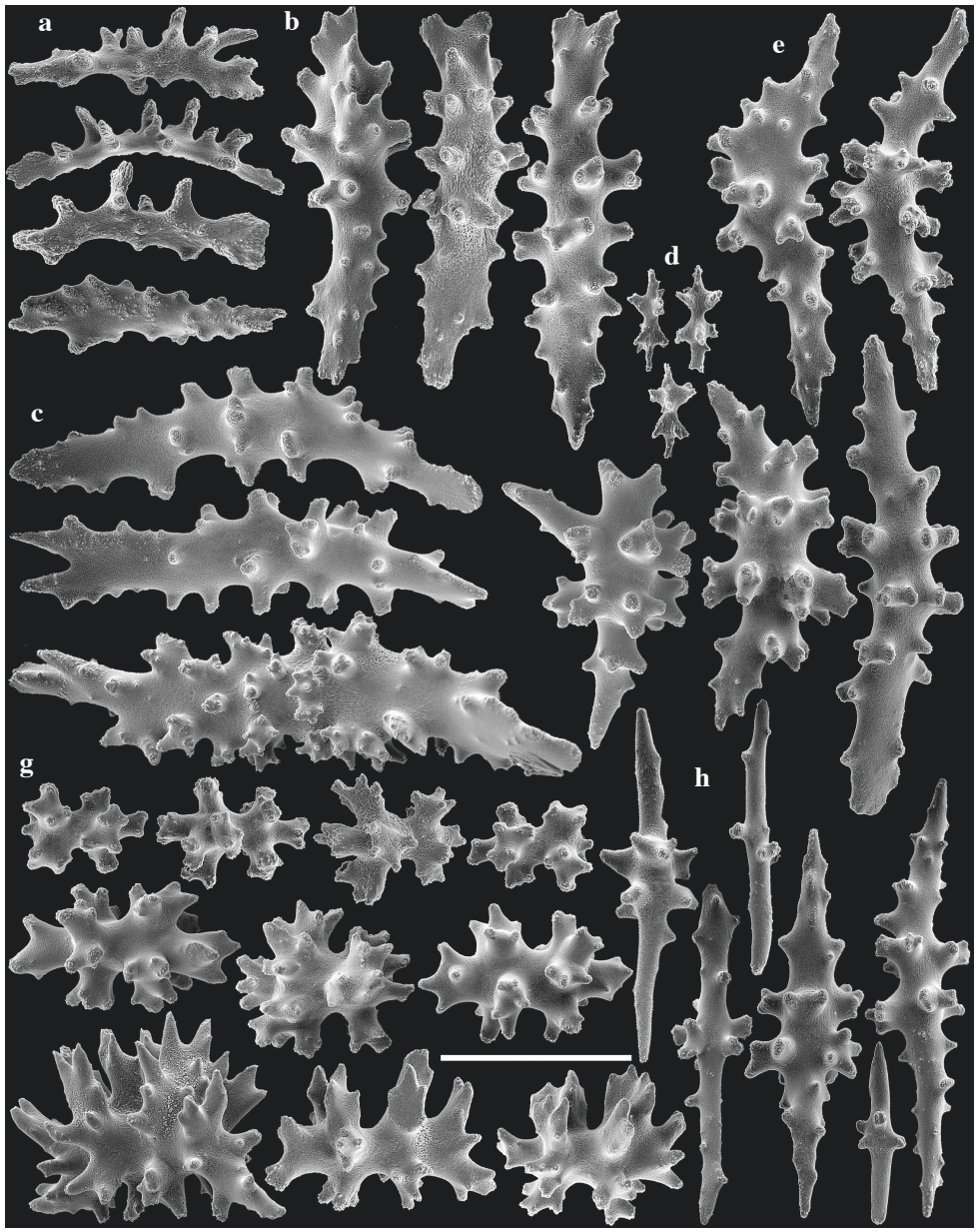

Fig. 8. Overview of the sclerite diversity in the neotype specimen for *Melithaea rubra* (Esper, 1798) (RMNH.Coel.41180); a) tentacle sclerites; b) point sclerites; c) collaret sclerites; d) pharynx sclerites; e) spindles; g) unilaterally spinose spindles and capstans; h) sclerites from nodes and internodes. Scale bar: 0.1 mm.

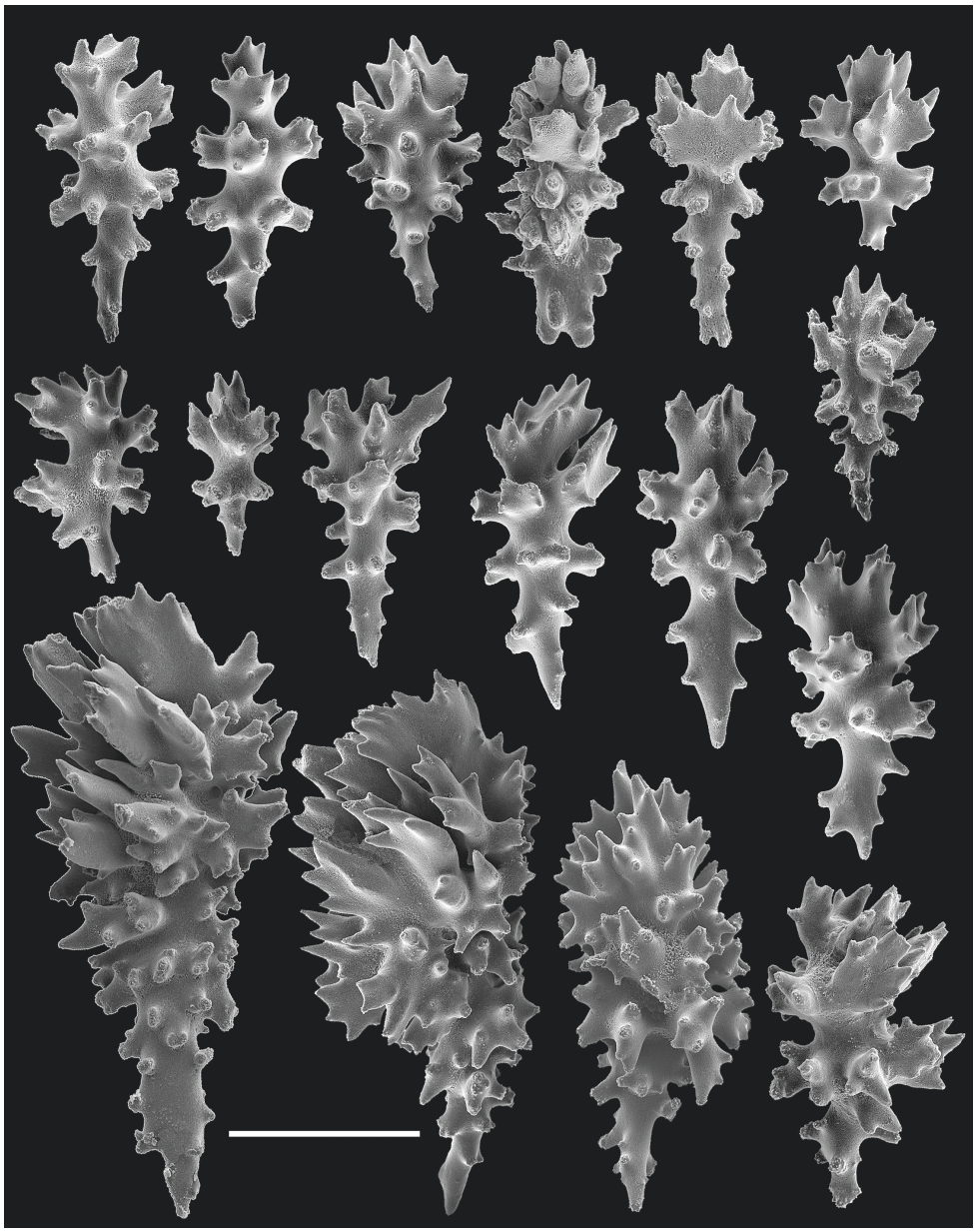

Fig. 9. Overview of the club sclerites in the neotype specimen for *Melithaea rubra* (Esper, 1798) (RMNH.Coel.41180). Scale bar: 0.1 mm.

coenenchyme include capstans (0.08-0.15 mm long) (Fig. 8g), leaf clubs (0.10-0.32 mm long) (Fig. 9) and spindles (0.09-0.28 mm long) (Fig. 8e). Capstans can also have leafy or spinose projections almost giving them a double disk appearance. The leaves on the clubs are very spinose and may take up ⅔ of the total length of the club (Fig. 9). Most spindles in the coenenchyme are slightly crescent shaped and are tuberculate to spiny in the middle area. The point sclerites (Fig. 8b) are relatively thick and blunt on one side and have large projecting tubercles. Point spindles are 0.24-0.27 mm long. The collaret and point spindles can be very similar in appearance, but in general the collaret spindles have more tapered endings, projecting tubercles in the middle and become less tuberculate at the distal end (Fig. 8c). The tentacles contain flat, branched platelets (Fig. 8a), 0.09-0.17 mm long. Spindles or rods from the nodes and internodes (Fig. 8h) often have large median projections resembling some of those of *Asperaxis karenae*. The pharynx sclerites (Fig. 8d) are 0.05-0.06 mm long, straight, and have a waist situated between two girdles of spines and large tubercles.

Discussion

Phylogenetic results

The phylogenetic results obtained in this study differ from those presented earlier by Aguilar-Hurtado *et al*. (2012). Based on their molecular and morphological data at least three melithaeid genera could be validated: *Acabaria*, *Melithaea* and *Mopsella*. Based on our molecular phylogenies these results are not supported. In our case, the phylogenetic results indicate that four genera are paraphyletic, and have been reorganized into the single genus *Melithaea*. The difference in results between our study and Aguilar-Hurtado *et al*. (2012) are most probably the result of a sampling bias. Although the sampling was limited to Japan, comparison between the phylogenetic tree by Aguilar-Hurtado *et al*. (2012) and the phylogenies presented herein show similar, basal topologies. For example, clade A which predominantly consists of *Acabaria* spp. resembles the clade containing *Acabaria* sp. A-D in Aguilar-Hurtado *et al*. (2012). Consequently, Clade B is comparable to the clades containing *Mopsella* and *Melithaea* spp. in Aguilar-Hurtado *et al*. (2012). Clade B in our phylogenies is also predominantly composed of *Mopsella* and *Melithaea* spp. Aguilar-Hurtado *et al*. (2012) sampled solely from tropical Japanese reefs, which automatically excludes species and genera primarily occurring in the Indian Ocean and Red Sea. It therefore appears that intensive sampling of Melithaeidae in a relatively small biogeographic area biases the subsequent molecular phylogenies and as a result different conclusions are reached. The four different markers that were used in this phylogenetic study provided enough information to support decisions on the generic level and in most cases also on species level for the species that could be identified. Unfortunately in some species groups, genetic resolution at the species level was lacking. In these specific cases morphological features and molecular data also contradict each other, which provokes the discussion on species variation and sequence diversity related to species identifications. To fully resolve these taxonomic issues within the Melithaeidae, new approaches such as next generation sequencing are needed because species-specific molecular markers are still lacking. However, barcoding efforts can still help in the iden-

tification of species. Case studies on the genus *Alcyonium* and species collected during a biodiversity assay in Eilat, Israel (McFadden *et al*., 2011) revealed that approximately 70% of the morphospecies can be recognized by means of DNA barcoding with multiple markers. These rapid advancements in sequencing techniques and genomic research on Octocorallia might therefore help to identify gene regions useful for species level identifications in the near future which will provide more insight into the evolution and species numbers in the family Melithaeidae.

Distributional patterns within the Melithaeidae

Both phylogenies (Fig. 2 and Fig. 3) presented in this paper reveal that species do not cluster according to their original morphological classification. Instead a pattern based on their larger scale biogeographic distribution was observed. Specimens from the Indo-Pacific and Red Sea that were formerly classified as *Acabaria* do not form a well-defined group, but are divided among several different clades. Additionally the well-supported sister clade relationship between the Indo-Pacific (clade A and B) and the other three clades (C-E) suggests an ancient divergence with independent diversification in each region. Within the Indian Ocean, the monophyly of these three clades suggests that those most probably originated from ancient one-time events.

To our knowledge this is the first time that such a distributional pattern has been observed within a family of octocorals. Historically the distribution of most melithaeid species such as *M. ochracea* was considered widespread. For example Hickson (1937) stated that *M. ochracea* occurs from Singapore to Fiji. Our findings contradict these historical opinions and indicate that species seem to be distributed according to regional endemism based on oceanic basins. Investigation of type specimens has also shown that species formerly identified as *Wrightella tongaensis* Kükenthal, 1908 collected at Tonga Island stretched the distribution of this species from its sister species in the Indian Ocean towards the East Pacific. Recent investigation of the type specimen showed that this species does not concur with the description of the genus *Wrightella* but represents the original concept of the genus *Melithaea*. In many cases these incorrect identifications have obscured the distribution patterns of species and genera.

Studies on other marine organisms that involve both molecular phylogenetic research and distributional patterns are limited. Cowman and Bellwood (2013) studied three marine fish families occurring circum-globally and found that Atlantic and East-Pacific lineages have been largely independent and isolated from the Indo-Pacific since the Oligocene. Therefore, there was no influx on the Indo-Pacific biota, which in our case can explain why the different clades retrieved from our phylogenetic analyses each represent the different biogeographic areas. For Scleractinia, Fukami *et al*. (2004) found that Atlantic representatives of a specific genus were according to the phylogenetic analyses more closely related to other Atlantic genera than to their Indo-Pacific congeners (Fukami *et al*., 2004). In that specific case the morphological convergence has probably obscured the evolutionary distinctiveness of these corals. Consequently the scleractinian taxonomy is currently being revised based on these results. Close examination of Melithaeidae specimens has not revealed such morphological convergence, but species of e.g. *Acabaria* from the Indo-Pacific versus the Red Sea resemble one

another more closely than they resemble other former genera from the same biogeographic region. Instead the phylogenetic history shows resemblance to the biogeographical patterns found by Cowman and Bellwood (2013).

Deep phylogenetic divergence between western Atlantic and Indo-Pacific fauna is most often explained by lack of genetic connectivity following the formation of the Panama isthmus (Knowlton *et al*., 1993, Williams *et al*., 2001 and Reimer *et al*., 2012). In contrast, the distribution of melithaeids is primarily limited to sub-tropical and tropical waters and ranges from the Red Sea, Indian Ocean and Central Pacific to New Caledonia, east to Hawai'i. Additionally species also occur in deeper or colder waters e.g. South Africa and northern Japan. One species (*Acabaria erythraea* (Ehrenberg, 1834)) has also invaded the Mediterranean Sea (Fine *et al*., 2005). Accordingly species distributions cannot be explained by the formation of physical barriers. Therefore, the regulatory factors in melithaeid distribution are most probably oceanic currents. Within the Central Pacific, the North Equatorial Current feeds e.g. the Mindanao- and the Indonesian Through-flow current which via various ways connects the water bodies around southern Japan as far south as North East Australia. These currents primarily explain the distribution of species within the Central Pacific but do not seem to directly influence the distribution outside this area. However, the average sea level is higher in the Central Pacific than in the East Indian Ocean, and Pacific water can therefore permeate through the Indo-Malayan region into the Indian Ocean (Hoeksema, 2007). This one directional route enables some exchange between the Central Pacific and Indian Ocean. In our case this might be expressed by the occurrence of three specimens with their origin in the Seychelles clustering within clade A, for which all other specimens are from the Central Pacific. If this is a recent dispersal event the larvae have probably come from the central Indo-Pacific into the Indian Ocean. In favourable conditions the larvae of other Octocorallia (e.g. *Dendronephthya hemprichi* Klunzinger, 1877) can survive up to 59 days (Dahan and Benayahu, 1998), which could be long enough to reach coral reefs in the Indian Ocean via these oceanic currents.

Taxonomic implications

Most of the genera (*Acabaria*, *Clathraria*, *Melithaea* and *Mopsella*) as defined in the identification key of Hickson, 1937 and Ofwegen, 1987 were found to be paraphyletic in our phylogeny. The findings were supported by each of the single locus analyses as well as in the analyses of the concatenated sequence datasets with and without the COI marker. Several solutions can reconcile the taxonomy with the phylogeny e.g.: (1) The paraphyly of several genera can be maintained as it is. In addition the assumption has to be made that identical morphological characters have evolved in different regions over time by convergent evolution; (2) All former genera (except for *Asperaxis*) can be synonymized within the genus *Melithaea*; or (3) The existing genera can be maintained but split based on biogeographic affinity. If we were to adopt either 1 or 3, the taxonomy of the Melithaeidae would become more confused by unclear characters that will not help to differentiate between genera or species. In particular, morphological features to clearly describe these (new) genera are lacking.

By adopting the second solution the genus *Melithaea* will contain almost all species (n = 114; WoRMS database, accessed 22-01-2013) described in the Melithaeinae. *Asperaxis karenae* is the only exception and will remain in its separate subfamily (Asperaxinae). Although this solution creates a genus representing over one hundred species, future studies of the Melithaeidae will likely show that there are more species names than valid species. The type specimens we examined suggest that several species morphologically resemble each other and should be synonymized such as suggested for *M. roemeri* with *M. ochracea*. *Melithaea amboinensis* (Hentschel, 1903) (App. 2; Pl. 1) (formerly *Acabaria amboinensis*) can be synonymised with *Melithaea laevis* (Wright and Studer, 1889) (App. 2; Pl. 10) (formerly *Acabaria laevis*); *Melithaea sulphurea* (Studer, 1895) (App. 2; Pl. 37) (formerly *Melitodes sulphurea*) which is a synonym of *Melithaea stormii* (Studer, 1895) (App. 2; Pl. 36) (formerly *Melitodes stormii*) [Hickson (1935) already mentioned that *M. amboinensis*, *M. fragilis* and *M. laevis* are very similar but he never formerly synonymised these species, and we did not investigate the type specimen of *M. fragilis* so the status of this species remains tentative]; and *Melithaea modesta* (Nutting, 1911) (App. 2; Pl. 31) (formerly *Melitodes modesta*) is a synonym of *Melithaea planoregularis* (App. 2; Pl. 13) (formerly *Acabaria planoregularis* Kükenthal, 1910). Another species that closely resembles the former two species is *Melithaea esperi* (Wright and Studer, 1889) (App. 2; Pl. 28) (formerly *Melitodes esperi*), but based on the differences in tuberculation of the sclerites we refrain from synonymising this species with *M. planoregularis* until more is known about the sclerital variety within species.

Likely there are more species that should be synonymised, but studying type specimens is a time-consuming and meticulous process. With the figures of the sclerites of type specimens added as Appendix 2 we provide a baseline for future taxonomic and phylogenetic research on the family Melithaeidae.

Validity of Asperaxinae

The mtMutS phylogeny (Fig. 4), which includes the single representative of the subfamily Asperaxinae (*Asperaxis karenae*), shows a well-supported distinction between the Melithaeinae and Asperaxinae. As a result the molecular data suggest that the family Melithaeidae should be considered paraphyletic. However, when the morphological features of *A. karenae* are taken into account a different conclusion is reached. Based on the morphological features described for *Asperaxis karenae* by Alderslade (2006) (App. 2, Pl. 19A-B), this species would formerly be referred to as a "true" Melithaeidae species which resembles the characteristics of the genus formerly recognised as *Acabaria*. According to Hickson's classification (1937) the main feature of the genus *Acabaria* is the dominance of spindles in the coenenchyme and the absence of clubs and capstans. Likewise, *A. karenae* is also dominated by spindles and lacks double discs, clubs or foliate capstans. The characters Alderslade (2006) used to separate the Asperaxinae from the Melithaeinae are axial sclerites in the form of rods and sticks that are often sinuous and branched and possess simple, sparse, tubercles. Morphological examinations of our material revealed that these characteristics are also found in other species that are placed in the Melithaeinae. For example in specimens of *Melithaea rubra* similar (branched) rods

with tubercles can be found and therefore these characters do not clearly differentiate between the two subfamilies. The phylogenetic research on the position of Asperaxinae, as performed herein, is only based on a single mtMutS sequence from GenBank. Since additional data could not be obtained its position remains inconclusive, but with the current morphological and phylogenetic data it is doubtful whether *A. karenae* deserves its own subfamily and should most probably be included in the Melithaeinae pending new specimens for molecular studies.

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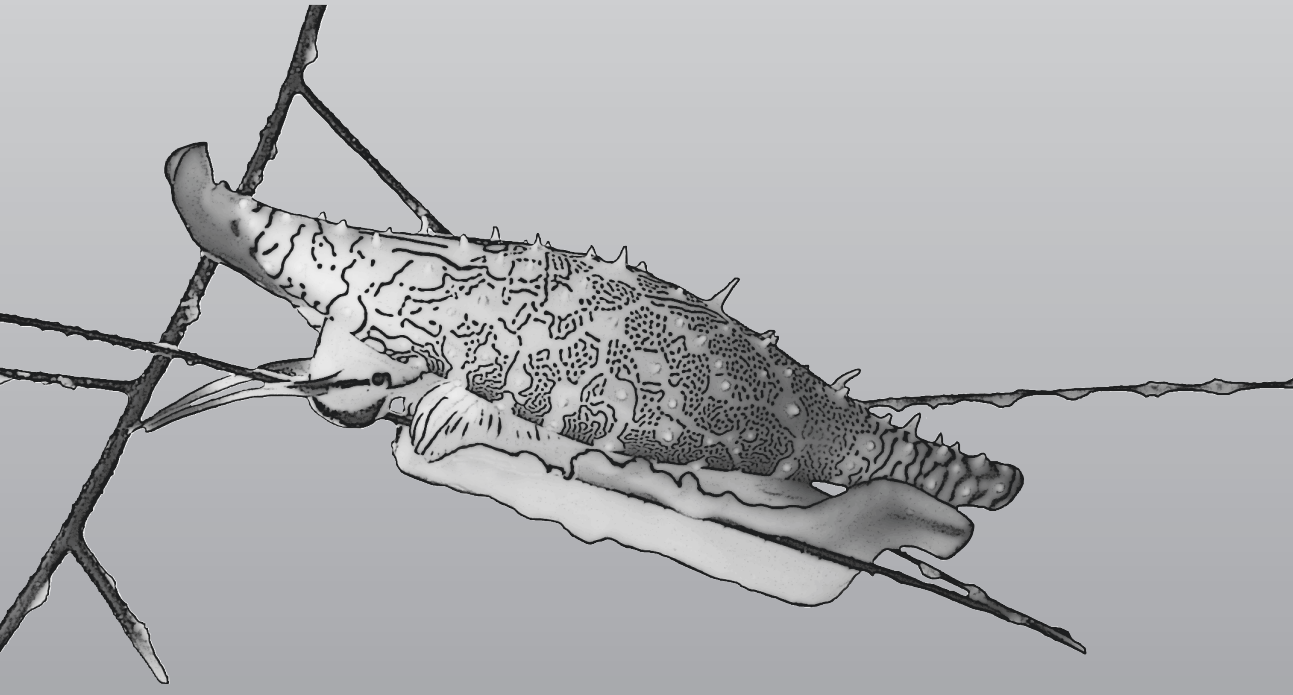
Appendices are available in the online version of Molecular Phylogenetics and Evolution.

Global



Chapter 7

Following the octocorals on a snails' pace: the evolutionary history of Octocorallia and Ovulidae

Bastian T. Reijnen

Abstract

Host-symbiont associations can on an evolutionary time scale affect the evolution of both animal groups. Associations between species that appear intrinsic or obligate are usually thought to be the result of coevolution or cospeciation. This also accounts for Octocorallia hosts and their Ovulidae snail associates. Most Ovulidae species are perfectly well-adapted to the appearance of their host species of Octocorallia by their specialised morphology and colour patterns resulting in camouflage as a defence mechanism against potential predators like fish. Such associations may be thought to result from coevolution or cospeciation. Specialisation related to a specific host assumes obligate relationships formed over long periods of time between the snails and the corals. To investigate the evolutionary histories of both the Octocorallia and the Ovulidae, a tanglegram was made to identify all possible associations based on phylogeny reconstructions of Octocorallia and Ovulidae. Phylogeny reconstructions were based on mitochondrial as well as nuclear markers: 28S, igr-COI, mtMutS, ND6 for the Octocorallia and 16S, 28S, COI, H3 for the Ovulidae. The tanglegram data was subjected to event-based analyses in Jane 4 and CoRe-PA to study the presence of coevolution between Octocorallia and Ovulidae. Both programs identified cospeciation events and statistical tests showed that these results can be considered significant. To calibrate the speciation events, molecular clock analyses were performed on the datasets for both Octocorallia and Ovulidae in BEAST2. Occurrences of fossils for Octocorallia and Ovulidae were used to calibrate nodes in the phylogenies. The analyses showed that the diversification in the Octocorallia started earlier (100-50 mya) than in the Ovulidae (40-15 mya). This excludes strict coevolution or cospeciation and suggests that the association between Octocorallia and Ovulidae is most probably based on sequential evolution, by which the host affects the symbiont but not vice versa. The host-symbiont defence strategies have therefore not an evolutionary background but prove to be the results of adaptation within the symbionts' generation.

Introduction

Coevolution is a widely used umbrella term referring to separate lineages of hosts and symbionts that are mutually influenced by each other's evolution (de Vienne *et al*., 2013). In heterospecific associations, the partner species may change together (coadaptation) and eventually speciate simultaneously (cospeciation) (Lanterbecq *et al*., 2010). Co-evolutionary relationships between host species and symbionts develop by intertwined physiological and ecological interactions over millions of years. These relationships between species can be based on symbiosis, commensalism, mutualism or parasitism.

Coevolution generally encompasses two modes of species interactions, e.g. sequential evolution and strict evolution (also known as 'Fahrenholz rule') (Ridley, 1996; Lanterbecq *et al*., 2010). Alternative to strict evolution, which has been proven to be rare (probably less than 7% convincing cases: De Vienne *et al*., 2012), sequential evolution is a non-reciprocal mode of evolution. In the latter mode of evolution morphological or physiological changes and the phylogeny of the symbionts are influenced by the host evolution but not vice versa. De Vienne *et al*. (2012) concluded that in many instances symbionts/parasites have diverged more recently than their hosts, mostly by host-shift speciation. This result points to sequential evolution as the primary driver of evolutionary diversification in close associations between species.

Cophylogenetic studies on marine species interactions are far less common than on terrestrial systems. Studies dealing with marine species often focus on fish and their parasites, because of the commercial value of the fish (Kirk, 2003). The only published investigations involving marine invertebrate species' interactions are those for myzostomid flatworms, which are obligate associates on crinoids (Lanterbecq *et al*., 2010) and gall crabs (Cryptochiridae) that live in close association with stony corals (van der Meij *et al*., 2015).

The present paper deals with the evolutionary ecology of Octocorallia (Cnidaria) and Ovulidae (Gastropoda) associations. Octocorallia, mainly soft corals and sea fans, are often a dominant and abundant species group in deep and shallow coral reef environments. Worldwide there are approximately 3,200 species, of which almost 3,000 are gorgonians and soft corals (Appeltans *et al*., 2012; van Ofwegen, 2015). This species group plays an important role in the ecosystem because many species depend on octocorals as a host, e.g. crustaceans (Humes, 1990; Buhl-Mortensen and Mortensen, 2004), echinoderms (Neves *et al*., 2007), and gastropods (Goh *et al*., 1999; Mase, 1989; Reijnen *et al*., 2010; chapter 2).

Ovulidae belong to the superfamily Cypraeoidea and are the sister group of the Cypraeidae and Pediculariidae (Meyer, 2003; chapter 5). All Ovulidae (~200 spp.) and Cypraeidae have a retractable mantle that can cover the entire shell. Therefore, in active snails, the shell is hidden underneath the mantle, which is shown to the outside world when the animals are not disturbed. The Ovulidae differ from the Cypraeidae in the fact that almost all species are obligate symbionts of Octocorallia (Lorenz and Fehse, 2009). For survival on their coral hosts, many ovulids have an adapted morphology. In some species the mantle may be provided with mimicked, retractable polyps. Other species have a bright, vividly coloured mantle, advertising their noxious properties

(aposematism), which they may have obtained from their host by feeding (Schiaparelli *et al*., 2005).

Gastropods of some families and genera are found in particular associations with various taxa of marine invertebrates e.g. Phyllidiidae nudibranchs - Porifera (Brunckhorst, 1993), Eulimidae - echinoderms (Warén, 1983; Takano and Kano, 2014), Epitoniidae - Scleractinia (Gittenberger and Gittenberger, 2005; Gittenberger and Hoeksema, 2013), *Leptoconchus* - Scleractinia (Gittenberger and Gittenberger, 2011), but cospeciation has not been demonstrated with coevolutionary analyses in any of these cases so far. Because of the striking similarities between the appearance of the mantle in Ovulidae and that of the octocoral hosts, coevolution between these snails and their hosts is supposed to have occurred. To investigate this hypothesis, phylogeny reconstructions have been made: 1) to assess the interspecific relationships of the Octocorallia and the Ovulidae species; 2) to elucidate cospeciation and host-symbiont switches, and 3) to use molecular clock dating for the speciation events in both species groups.

Material and methods

Collecting and identification

Octocoral specimens and their ovulid symbionts were collected during fieldwork by the author and colleagues in Curaçao, Florida (USA), North Sea, Spain, Saudi Arabia, Oman, Maldives, Malaysia, Indonesia and New Caledonia (Suppl. mat. 1). Octocoral samples are coded RMNH.Coel and Ovulidae samples are coded RMNH.Mol. Unless stated otherwise, voucher specimens are stored in the collections of Naturalis Biodiversity Center (Naturalis).

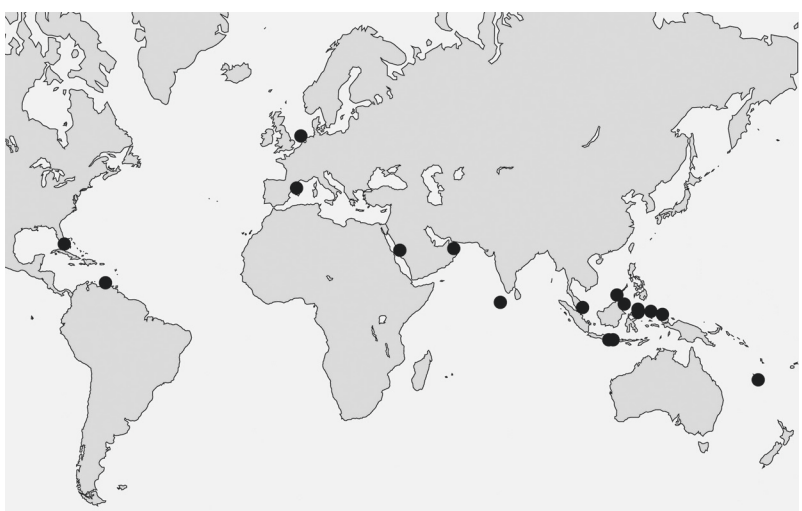


Fig 1. All localities (n=17) where Ovulidae snails were collected. Provenance data can be found in Suppl. mat. 1.

Octocoral species were identified with microscope slides of the sclerites. To make these slides, a small fragment of the tip of a gorgonian was dissolved in a common household bleach solution (approximately 4% active hypochlorite). In case of soft corals, tissue from the base of the colony and of the polyps was used to make such slides. After dissolving the tissue in hypochlorite solution, the residual sclerites were washed with tap water and double distilled water, and eventually embedded in Euparal for visualisation under a microscope (Olympus BX53F). Sclerites were compared with those illustrated by Fabricius and Alderslade (2001) and were identified to genus level and where possible to species level.

Ovulidae specimens were studied under a Leica MZ16 stereomicroscope. Specimens were compared with photographs and species descriptions in the literature (Cate, 1973; Kaicher, 1991; Lorenz and Fehse, 2009).

DNA extraction, sequencing and sequence processing

DNA extractions were performed with the Machery Nagel kit or the Qiagen Tissue kit on a Kingfisher Flex. Tissue samples from the foot or mantle of ovulids, or terminal tips from octocoral colonies, were subjected to tissue lysis overnight (approximately 16 hours). All other DNA extraction steps were performed according to the manufacturers' protocol except for the final DNA elution step. For Ovulidae samples DNA was eluted with 100 μl and for Octocorallia with 150 μl. For both Octocorallia and Ovulidae several dilutions (1:100-1:300) were made for amplification of the targeted gene regions by PCR. For the Octocorallia we targeted the following four genes: COI, mtMutS, 28S, ND6 and for the Ovulidae COI, 16S, 28S and H3. The amplified PCR products were

Table 1. Molecular markers used and additional amplification data for PCR.

	Forward primer	Reverse primer	Targeted area	Annealing temperature	Reference
Ovulidae	LCO-1490	HCO-2198	Cytochrome oxidase I	50	Folmer *et al*., 1994
	16Sar	16Sbr	16S	52	Palumbi, 1996
	H3F	H3R	Histone 3	50	Colgan *et al*., 2000
	LSU5	LSU800rc	28S	50	Chapter 2, 5
Octocorallia	Alc_715_Fw	Alc_1303_Rv	ND6	50	Reijnen *et al*., 2014
	Alc_715_Car	Alc_1303_Car	ND6	50	This chapter
	ND42599F	MUT3458R	mtMutS	48	France and Hoover, 2002; Sánchez *et al*., 2003
	28S-Far	28S-Rar	28S	50	McFadden and van Ofwegen, 2013
	COII8068xF	COIOCTr	Cytochome oxidase I (incl. igr)	58	McFadden *et al*., 2011

sequenced (bidirectional) at Macrogen Europe or BaseClear (both on an ABI 3730XL). Raw sequence data was edited with Sequencher 4.10.1 and Geneious 5.6.4.

For three octocoral host genera sequence data was missing (*Alcyonium digitatum*, *Eunicella singularis* and *Studeriotes* sp.). To include data for these hosts, sequence information was obtained from GenBank (*Alcyonium digitatum*: JX203641 (28S), GQ342381 (COI), AF530498 (ND6); *Eunicella singularis*: AY827538 (COI); *Studeriotes* sp.: GQ342443 (COI), GQ342515 (mtMutS)).

Preliminary alignments were made in BioEdit and some minor adjustments were made manually. Final single gene alignments were constructed with GUIDANCE2 (online server) (Sela *et al*., 2015) using the MAFFT algorithm. For the Octocorallia the alignments contained many indels. To partly overcome the problems of analysing a dataset that contains many indels, columns with a probability lower than 0.9 were deleted from the dataset.

The alignments for each marker were separately tested for the best fitting evolutionary model in jModeltest2.0 by using the AIC (Akaike Information Criterion). Eventually the single marker alignments were concatenated into two datasets: one containing all data for the ovulids and a dataset containing all sequence data for the octocorals.

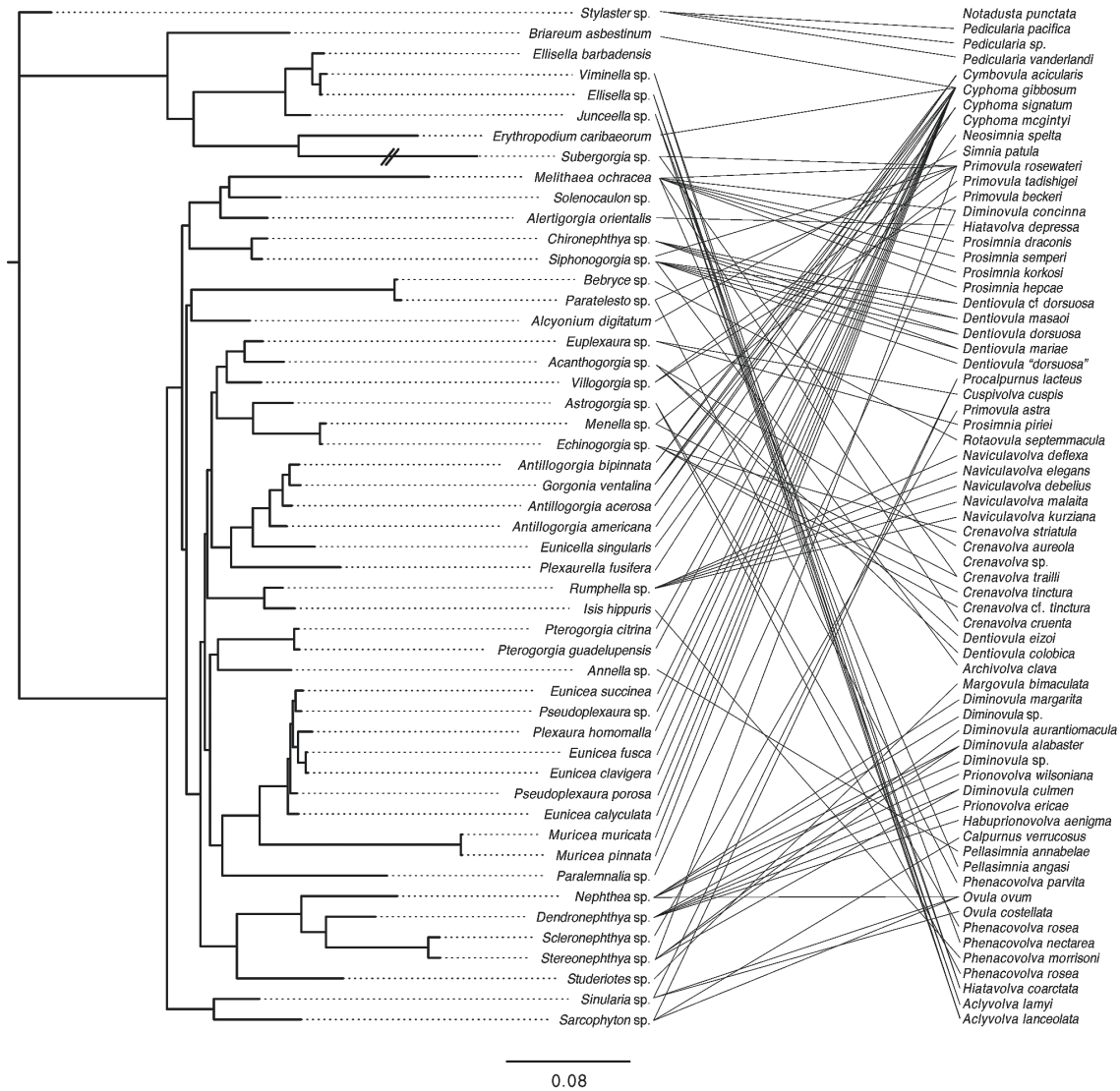
Host and symbiont phylogenetic analyses

Phylogeny reconstructions were performed in MrBayes 3.2.5 (Bayesian inference) and Garli 2.0 (Maximum Likelihood approach). The cypraeid species *Notadusta punctata* was used as an outgroup in the Ovulidae analyses and the lace coral *Stylaster* was selected as outgroup for the Octocorallia. The actual Stylasteridae sequences were not included in the DNA dataset. Firstly, because only non-homologous sequence data was available from GenBank (e.g. mtMuts is only known from bacteria and Octocorallia (McFadden *et al*., 2006)). Secondly because lace corals are from a different class, which would make aligning the Octocorallia dataset even more challenging than it already was. A recently published paper by Zapata *et al*. (2015) shows that Filifera (which includes the Stylasteridae) are eligible as outgroup for Octocorallia. Moreover, by including *Stylaster* as an outgroup for the Octocorallia, the host associations between Pediculariidae and Stylasteridae could also be assessed in the cophylogeny analyses.

MrBayes was run for 4 million generations with evolutionary models set for each

Table 2. Best evolutionary models estimated with the jModeltest analyses for Ovulidae and Octocorallia including their respective AIC scores.

Ovulidae	Evolutionary model	Likelihood score (AIC)	Octocorallia	Evolutionary model	Likelihood score (AIC)
16S	TPM3uf+I+G	13757.7416	28S	GTR+I+G	9785.7953
28S	GTR+I+G	8466.4251	igr-COI	GTR+G	9927.8210
COI	TIM2+I+G	19353.7919	mtMutS	TPM3uf+G	10755.5549
H3	TIM3+I+G	2941.0842	ND6	TIM1+G	6198.1305



of the different gene partitions on both the concatenated dataset of the Octocorallia and Ovulidae. Not all evolutionary models are represented in MrBayes. For some gene regions, instead of the jModeltest result for the particular gene region, the GTR+I+G model was the most appropriate model and was consequently used. The sample frequency was set at 100 and the burnin fraction was 0.25. Average standard deviations of split frequencies were 0.004 for the Ovulidae and 0.007 for the Octocorallia.

Garli is more versatile with implementing evolutionary models. Therefore, the selected models by jModeltest were used in the partitioned garli config file. Garli was run with 500 bootstrap iterations with each two replicates.

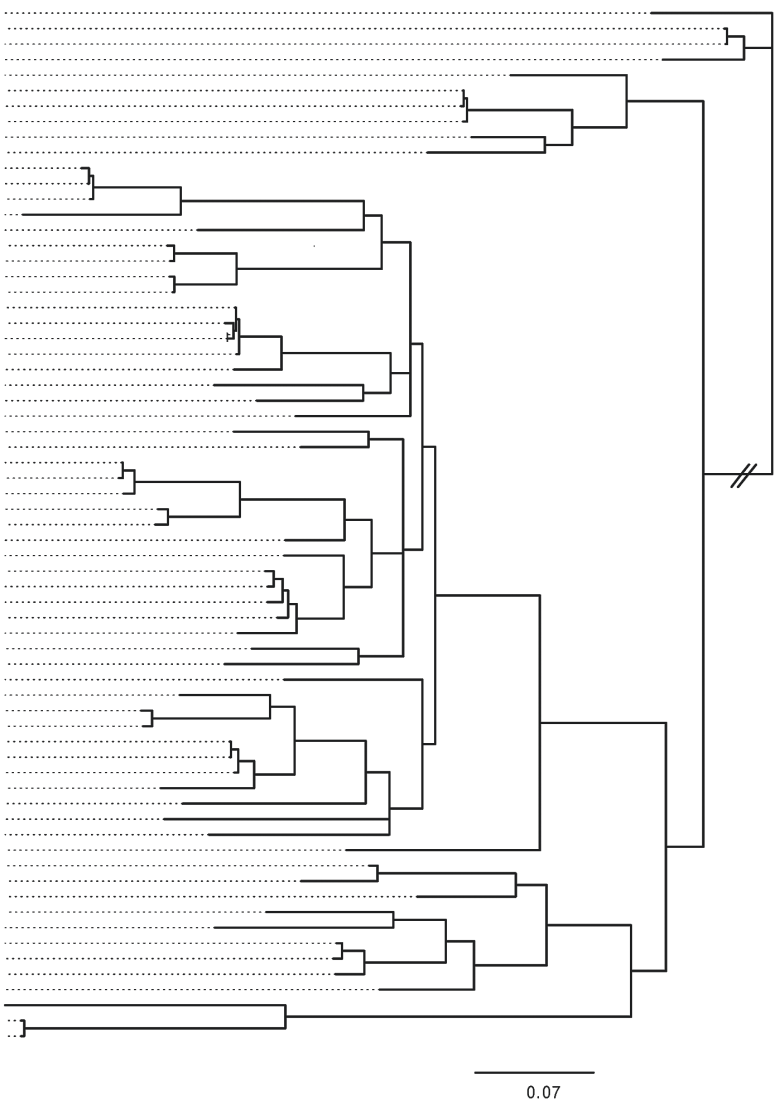


Fig. 2. Tanglegram showing the species associations between Octocorallia (left) and Ovulidae (right).

Cospeciation analyses

The phylogeny reconstructions based on the Bayesian and Maximum Likelihood analyses were used to create a tanglegram providing an overview of the species' associations between the Ovulidae and Octocorallia (Fig. 2).

The tanglegram was redrawn after the host and parasite trees in Jane 4.0 (Conow *et al*., 2010) for an event-based cospeciation analysis (50 host tips and 62 parasite tips). This method uses a parsimonious approach where costs for evolutionary events such as host switching and duplication events of the associated species are superimposed on the host phylogeny. Jane (solve mode) was run for 1,000 generations with 250 populations.

All other settings were default and Jane (stats mode) was run 10 generations with 100 populations and all other settings at default. Likewise the phylogeny reconstructions were used to create tanglegrams in CORe-PA (Merkle *et al*., 2010). Both Jane and CORe-PA are event-based concepts for reconciliation analyses, but in contrast to Jane, CoRe-PA has a different algorithm that uses another parameter-adaptive approach, i.e., no costs have to be assigned to the coevolutionary events in advance (Merkle *et al*., 2010). CORe-PA was run for 10,000 random cycles to investigate coevolutionary patterns between the cladograms of the Octocorallia and Ovulidae.

Molecular clock analysis

The BEAST2 package (Bouckaert *et al*., 2014) was used to calibrate the phylogenies of the Ovulidae and the Octocorallia. BEAUti datasets were set up with the help of fossil data and sequence divergence estimates (only for the Ovulidae) obtained from literature (Table 3 and 4).

For dating the Ovulidae phylogeny the following fossil calibrations were used: *Cyphoma* (20.44 – 15.97 mya); *Pedicularia* (37.8 – 33.9 mya); *Pellasimnia* (28.1 – 23.03 mya) and *Prosimnia* (23.03 – 20.44 mya). For the Octocorallia three calibration points (of 3 genera) were used, viz. *Isis* (83.6 – 72.1 mya), *Melithaea* (66.0 – 61.6 mya) and *Verrucella* (66.0 – 61.6 mya). All calibration points were regarded as 'stem fossils' because of the uncertainty of the exact taxonomic position of the fossils in the phylogeny reconstructions. This is considered the most conservative option for calibrating phylogeny reconstructions with fossil data (Forest, 2009).

Divergence estimates can also be determined, in addition to fossil data, by using the rates of sequence evolution. Such data was not available for the Octocorallia but for molluscs the molecular marker COI is the best-researched gene region for sequence rate estimates. Previously estimated values range between 0.7% / my (Marko *et al*., 2002) to 2.6% / my (Williams and Reid, 2004) for various gastropod groups. For the Ovulidae in particular, such data is not yet available, but can be retrieved from other relatively

Table 3. Records obtained from the literature for Indo-Pacific and Caribbean Octocorallia fossils. Records in **bold** were used for dating the phylogeny of the Octocorallia.

Family/Genus	Epoch	Mya	Reference	Remarks
Acabaria	Danian	66.0 - 61.6	Kusmicheva, 1980.	is *Melithaea* (See Reijnen *et al*., 2014)
Isis	**Campanian**	**83.6 - 72.1**	Kusmicheva, 1980.	
Melithaea	**Danian**	**66.0 - 61.6**	Kusmicheva, 1980.	
Verrucella	**Danian**	**66.0 - 61.6**	Sepkoski, 2002	
Nicella	Maastrichtian	72.1 - 66.0	Kusmicheva, 1980.	
Gorgoniidae	Rupelian	33.9	Kocurko and Kocurko, 1992	Caribbean origin
Plexauridae	Rupelian	33.9	Kocurko and Kocurko, 1992	Caribbean origin
Anthothelidae	Rupelian	33.9	Kocurko and Kocurko, 1992	Caribbean origin

closely affiliated marine gastropod species. One of the most closely related taxa for which a value for the COI region has been estimated is *Nucella* (1.3%; Marko *et al*., 2014). Therefore, this value was used as the substitution rate under the site model for COI in BEAUti. For both species groups the relaxed clock log normal was selected. The Yule model was selected as the speciation model prior with a uniform birth rate and the upper limit set at 10,000. Gene regions were treated as gamma site models and the gamma shape was considered exponential. Substitution rates were also set as gamma distributed.

The .xml files can be obtained from the author. The BEAST2 analyses for the Ovulidae and Octocorallia had problems to converge; therefore a starting tree was added manually to the xml file. The starting tree was obtained by performing an initial analysis with the models and priors set as described above. The initial analysis was run for 15 million generations. The consensus tree (newick) was implemented manually in the xml file created by BEAUti. Final analyses were run for 30 million iterations (Ovulidae) or 50 million iterations (Octocorallia) with samples saved for every 1,000 iterations. The effective sampling size (ESS) values (>100) were investigated in Tracer 1.6 (Rambaut *et al*., 2014).

Table 4. Records of non-Eocypraeinae fossil Ovulidae (and one pediculariid). Records in **bold** were used for dating the Ovulidae phylogeny.

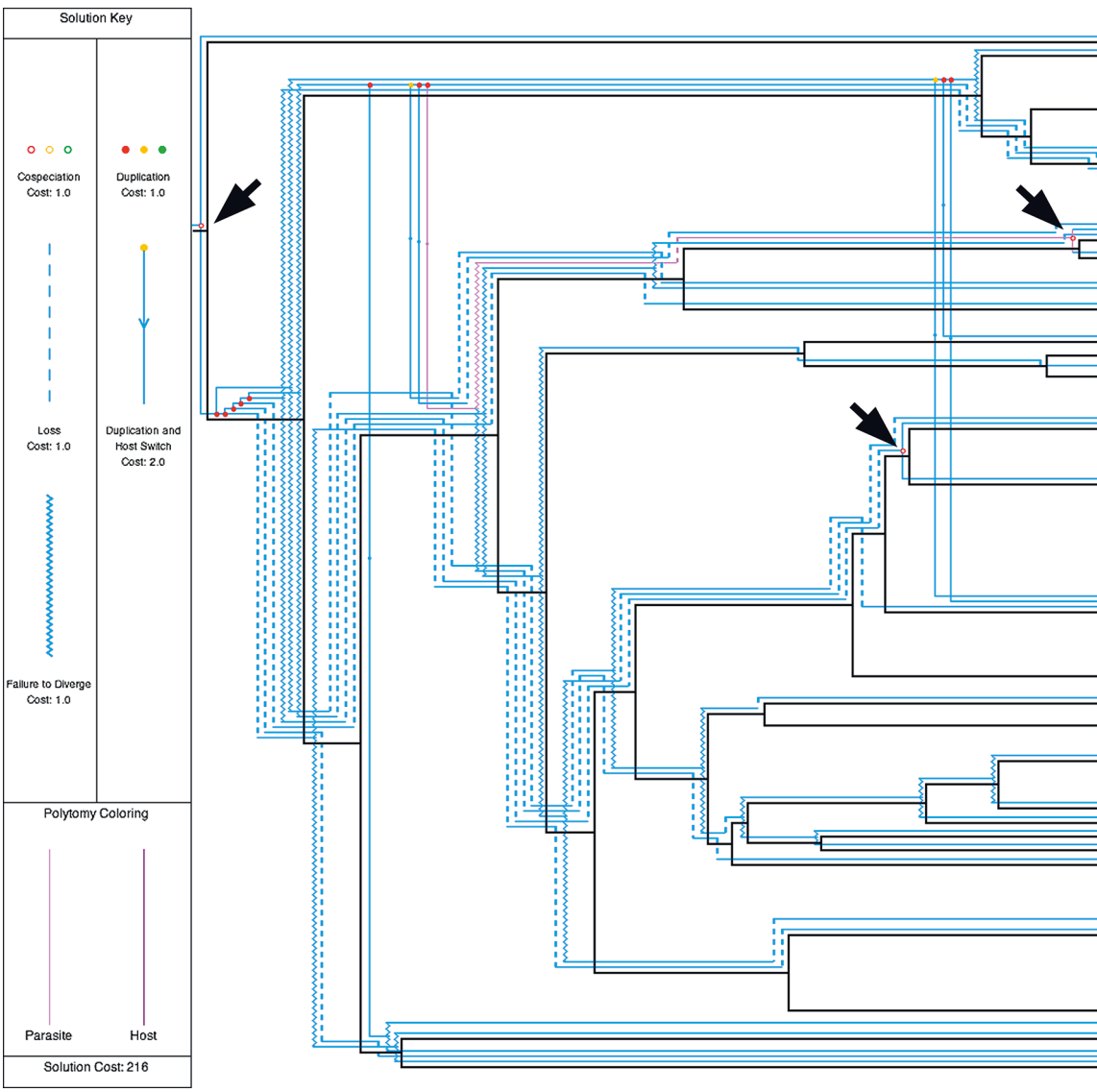
	Genus	Epoch	Mya	Reference
Ovulidae	*Amphiperas*	Langhian and Serravallian	15.97 - 11.63	Darragh, 1985
	Cyphoma	**Burdigalian**	**20.44 - 15.97**	Woodring, 1973
	Globovula	Middle Miocene	15.97 - 11.63	Dharma, 2005
	Jenneria	Bartonian	41.2 - 37.8	Signor, 1990
	Margovula sp. 1	Middle Miocene	15.97 - 11.63	Dharma, 2005
	Margovula sp. 2	Upper Pliocene	3.6 - 2.58	Dharma, 2005
	Neosimnia	Ypresian	56.0 - 47.8	Schilder, 1932
	Pellasimnia	**Chattian;**	**28.1 - 23.03;**	Signor,1990;
		Late Eocene	**37.6 - 33.9**	Beu and Marshall, 2011
	Phenacovolva	Pliocene	5.3 - 2.58	Ladd, 1977
	Primovula	Pliocene	5.3 - 2.58	Ladd, 1977
	Prionovolva	Pleistocene	2.58 - 0.0117	Sepkoski, 2002
	Prionovolva brevis	Upper Pliocene	3.6 - 2.58	Dharma, 2005
	Procalpurnus	Pleistocene	2.58 - 0.0117	Schilder, 1932
	Prosimnia	**Upper Pliocene;**	**3.6 - 2.58;**	Schilder, 1932;
		Lower Miocene	**23.03 - 20.44**	Beu and Marshall, 2011
	Simnia	Langhian and Serravallian	15.97 - 11.63	Schilder, 1932
	Volva	Upper Pliocene	3.6 - 2.58	Schilder, 1932; Dharma, 2005
Pediculariidae	***Pedicularia***	**Priabonian**	**37.8 - 33.9**	Ladd, 1977

Results

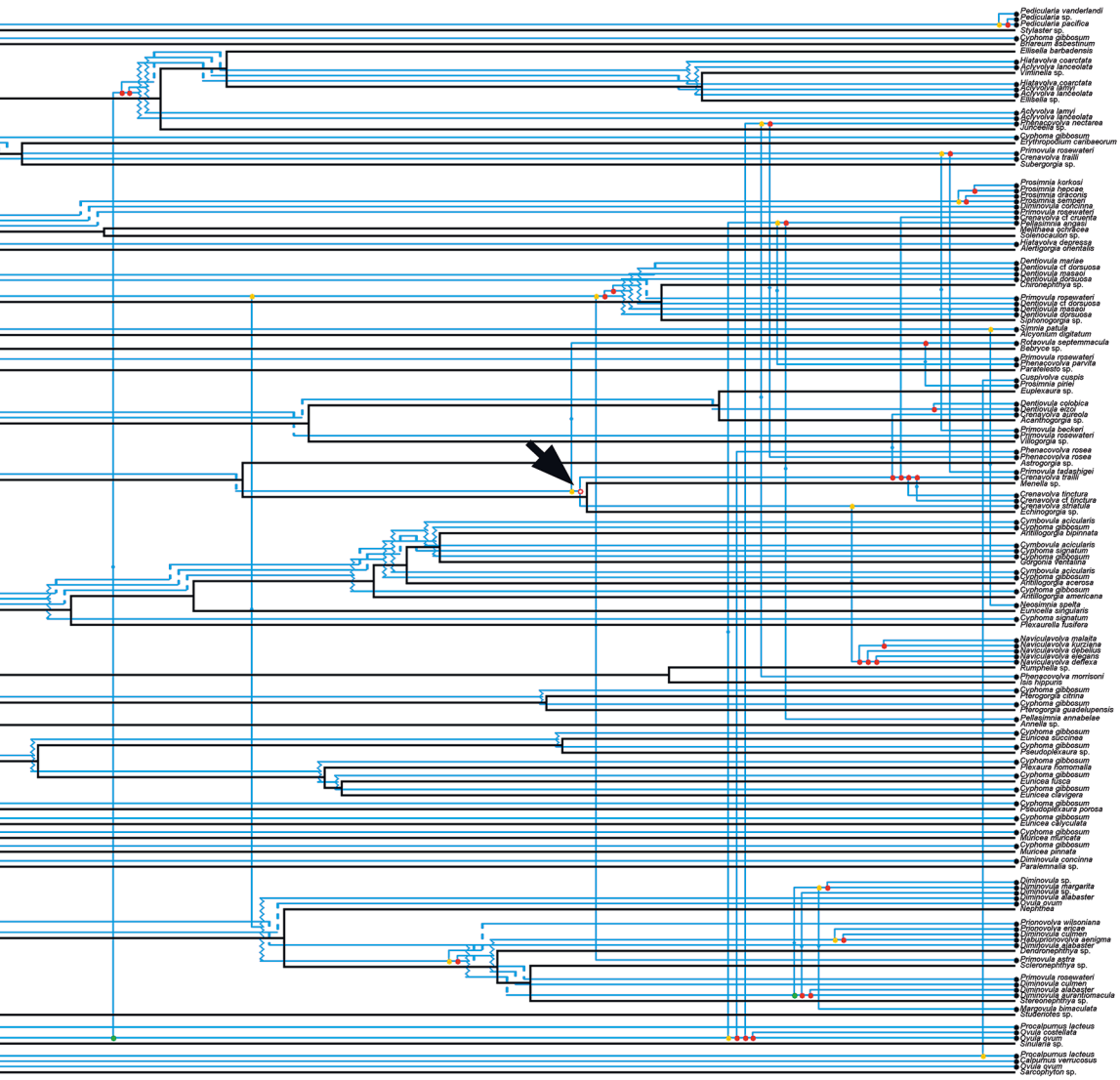
Octocorallia and Ovulidae – host and symbiont phylogenies

The phylogenetic relationships within the Octocorallia have proven to be difficult to assess. Phylogeny reconstructions by McFadden *et al*. (2006) and McFadden and Ofwegen, 2013 based on multiple markers (mtMutS, COI and ND2 or 28S), show that the basic

Fig. 3. Output of isomorphic tree by Jane 4.0 showing all events reflected on an overlay of the parasite-host trees. The black arrows indicate the cospeciation events.



relationships between octocoral genera or even families (sensu Fabricius and Alderslade, 2001) are generally not supported. Adding another molecular marker (ND6) as was performed to identify the evolutionary associations between Octocorallia and the Ovulidae did not improve these results. Yet, some families (Alcyoniidae, Ellisellidae, Nephtheidae [except for *Paralemnalia*] and Nidaliidae) were retrieved as monophyletic groups, in contrast with the results by McFadden and Ofwegen (2013) for the Alcyoniidae and Nephteidae (see also Suppl. mat. 2). The Atlantic genera and species do not form a monophyletic group but cluster together with Indo-West Pacific (IWP) genera in a single clade. These groups are poorly supported, however. To be able to use a phylogeny reconstruction for



the cospeciation analyses the tree should be fully resolved. Therefore the cladogram is represented according to the majority-rule consensus method showing al groupings (even below 50% majority).

For the Ovulidae the increase of molecular data has provided new insights in the relationships between species and some higher taxa (Suppl. mat. 2) as compared to the large-scale phylogeny reconstruction by Schiaparelli *et al*. (2005). The current opinion regarding the subfamily level is largely consistent with that of previous studies except for some species or genera. In contrast to the Octocorallia the Caribbean / Atlantic Ovulidae species form a clade together, which is the sistergroup to all IWP Ovulidae whereas the family Pediculariidae is the sistergroup of all Ovulidae species. According to the molecular data, some Ovulidae species should be classified in other subfamilies than hitherto accepted. The genus *Naviculavolva* allegedly belonging to the Simniinae clustered in the subfamily Prionovolvinae as well as *Hiatavolva depressa* supposedly belonging to the subfamily Aclyvolvinae, was also clustering in the subfamily Prionovolvinae (see chapter 5). The phylogenetic relationships between the species of the Prionovolvinae are still unclear. While aiming at monophyletic groups as genera, extensive revisions are inevitable.

Cospeciation

Almost all ovulids are more or less obligate symbionts with one or more octocoral genera. The tanglegram of the Octocorallia and Ovulidae (Fig. 2) shows that the Caribbean species *Cyphoma gibbosum* and the Indo-Pacific *Primovula rosewateri* are facultative symbionts of Octocorallia. These ovulid species are found associated with eight and six different octocoral genera, respectively. It is striking that the latter two species show aposematic colour patterns and do not rely on camouflage like most other ovulids. Both *P. rosewateri* and *C. gibbossum* are not closely related and occur in different oceans (Indo-West Pacific vs. Atlantic) what indicates that aposematic characters in the Ovulidae have originated at least twice.

In contrast, other species are found to be symbiotic with host species from only a single genus, for example the ovulid species *Dentiovula eizoi* and *D. colobica*. These species were found only on *Acanthogorgia* spp. whilst congeners *D. dorsuosa*, *D. mariae* and *D. masaoi* are obligate symbionts of the octocoral genera *Chironephthya* and *Siphonogorgia*. This would exemplify a typical host-switch event regarding *Dentiovula* spp. but the phylogeny reconstruction shows that these *Dentiovula* species are not closely related to one another. In this case convergent evolution of shell morphology has probably troubled the systematics of this genus. An ovulid genus that is dependent on a single octocoral genus is for example *Naviculavolva*, in which all species are associated with *Rumphella* sp. Furthermore, *Phenacovolva annabelae* was only found on *Annella* and the ovulids of the genus *Prosimnia* are solely associated with *Melithaea* species, except for *Prosimnia piriei* which is an obligate symbiont on *Euplexaura*. *Prosimnia piriei* and the allegedly congeneric species are not closely related and do not share a common ancestor, so this is also not a convincing host-switch event. Anyway, the tanglegram shows that a large number of octocoral genera play an important role as host species for Ovulidae. Specifically the genera from the families Nephtheidae (*Dendronephthya*, *Nephthea*), Nidaliidae (*Siphonogorgia*, *Chironephthya*) and Melithaeidae (*Melithaea*) are important as hosts for

more than one ovulid species. Some of these ovulid species, like *Habuprionovolva aenigma*, solely depend on this single host genus (*Dendronephthya* sp.). Less strict is the association between the octocoral genera in the family Ellisellidae (*Ellisella*, *Junceella*, *Viminella* a.o.), which are important as host genera for *Aclyvolva* species.

All species association data was used for a cospeciation analysis in Jane 4.0. This analysis resulted in nine isomorphic solutions (having the same topological structure when the associated species are superimposed on the host phylogeny) of which one solution contained 115 trees. The costs for all nine isomorphic solutions were 216, and were based on 26 duplications and 39 failures to diverge. Depending on which of the the nine different isomorphic solutions was investigated, there were three or four cospeciation events, 32 or 31 duplications / host switches, and 84 or 85 losses. The selected tree (with the most isomorphic trees) (Fig. 3) had four cospeciation events, 31 duplications / host switches and 85 losses.

Cospeciation events are retrieved for 1) *Stylaster – Pedicularia*, 2) *Melithaea – Prosimnia* spp. and 3) the clade containing *Euplexaura – Cuspivolva cuspis* / *Prosimnia piriei*, *Acanthogorgia – Dentiovula colobica* / *D. eizoi* / *Crenavolva aureola*, *Villogorgia – Primovula beckeri* / *P. rosewateri*, *Phenacovolva rosea – Astrogorgia*, *Primovula tadashigei* / *Crenavolva trailli – Menella*, *Crenavolva* spp. – *Echinogorgia*. The cospeciation event of *Primovula tadashigei* / *Crenavolva trailli – Menella*, *Crenavolva* spp. – *Echinogorgia*) is considered the fourth cospeciation event.

The output data resulting from Jane was tested against randomly generated trees in the 'stats mode'. This test compares the obtained coevolutionary signal from the 'solve mode' versus randomly generated data of the coevolution data. Based on the costs for the obtained optimal coevolutionary analysis versus the randomised data, a cost histogram was generated. This histogram (Fig. 4) shows that randomly generated data has much higher costs (average = 443; median = 436) than the lowest costs observed (216) in the coevolution analysis, showing that there is a coevolutionary signal.

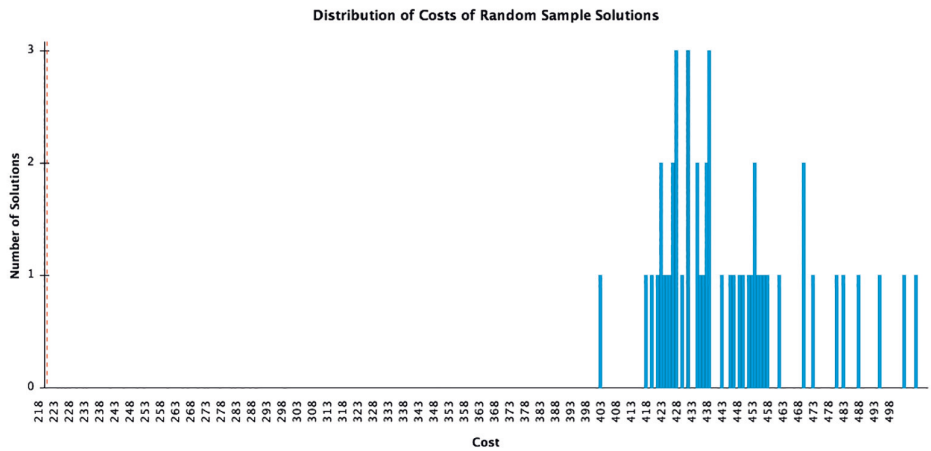


Fig. 4. Histogram produced by Jane 4 with randomly generated cospeciation tree costs versus the costs of the isomorphic solutions (red dotted line).

An identical tanglegram was created in CoRe-PA and tested for coevolutionary signals. CoRe-PA found 102 isomorphic trees. In contrast to the analysis in Jane, CoRe-PA found 19 cospeciation events, 75 sortings, 32 duplications and 11 host switches. CoRe-PA found more cospeciation events compared to Jane (19 vs 3 or 4), which could be the effect of different algorithms and definitions used by the programs.

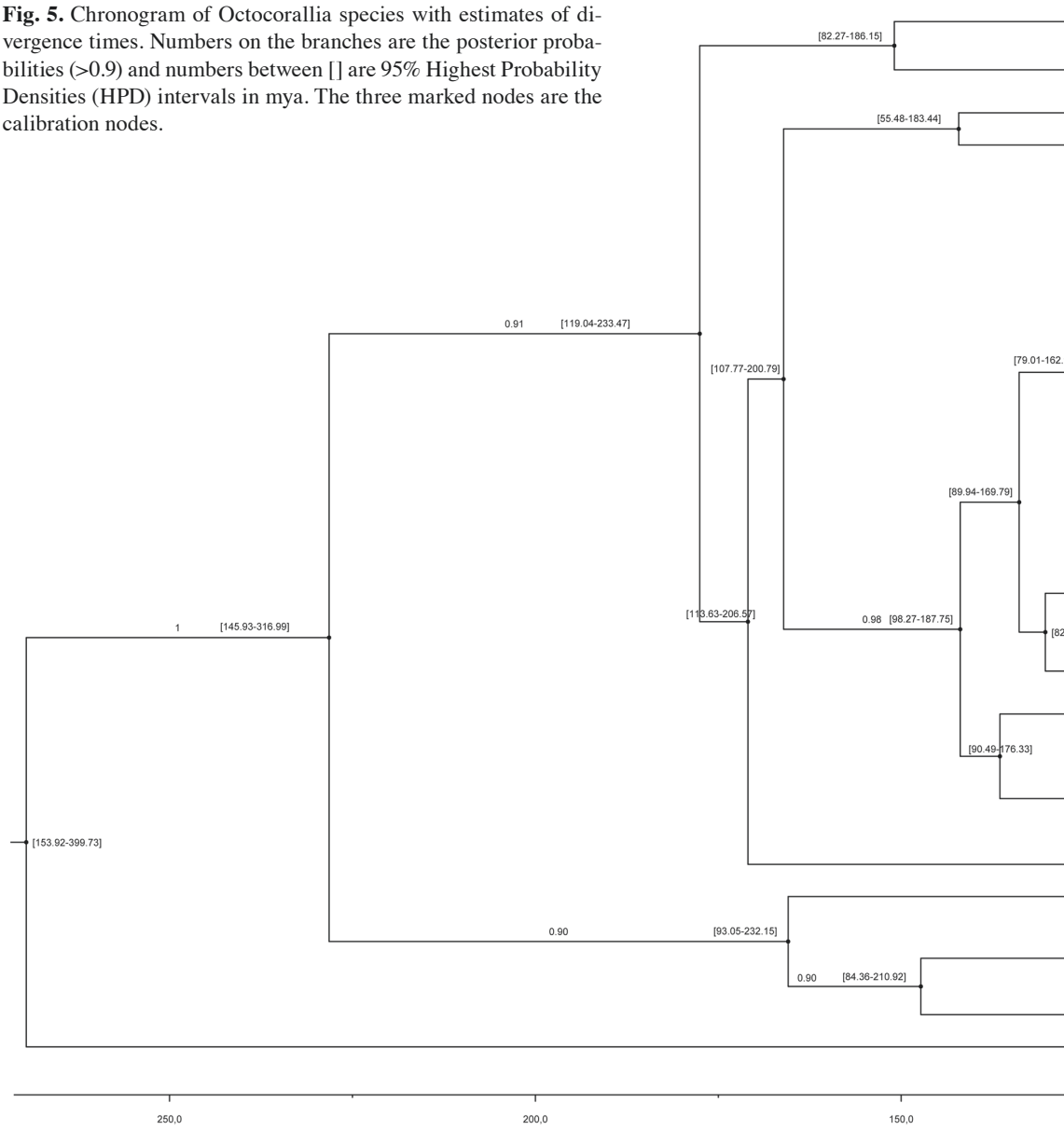
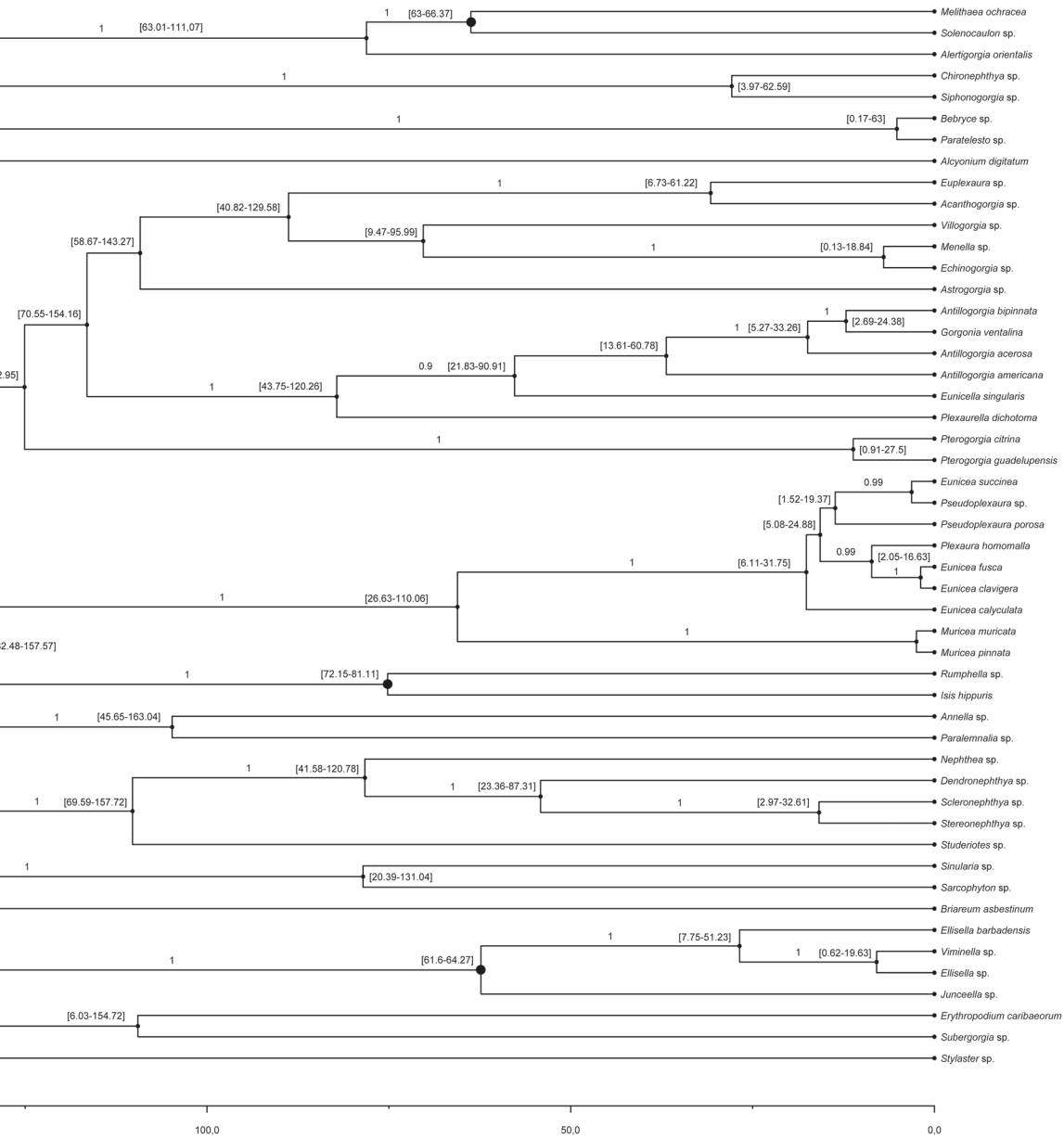


Fig. 5. Chronogram of Octocorallia species with estimates of divergence times. Numbers on the branches are the posterior probabilities (>0.9) and numbers between [ ] are 95% Highest Probability Densities (HPD) intervals in mya. The three marked nodes are the calibration nodes.

Molecular clock analysis

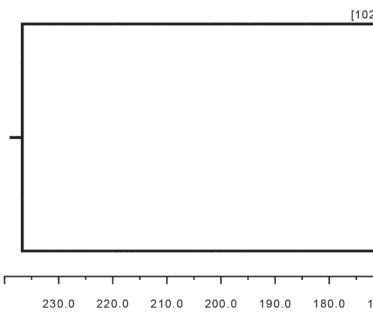
One of the requirements for divergence time estimates are calibration points that are based on the fossil record and can be assigned to specific nodes in the phylogenies. Therefore, two data sets were composed for Octocorallia (Table 3) and for Ovulidae (Table 4).



Octocorallia – Data on fossil Octocorallia are scarce and mostly based on axial remains and holdfasts, which lack most diagnostic characters to identify the fossil remains to genus level, let alone species level. However, Kocurko and Kocurko (1992) reported on actual sclerites found in the Red Bluff formation. These sclerites were well preserved and could be assigned to families or genera. In Table 3 eight records of octocoral fossils are mentioned, three of which were used to estimate divergence times in the octocoral phylogeny. The other records were neglected because of the lack of representatives in the phylogeny reconstruction or because of difficulties in assigning these dates to specific nodes in the cladogram.

The calibrated phylogeny reconstruction of the Octocorallia in BEAST2 shows that 95% Highest Probability Densities (HPD) are covering large time spans and most branches are not or only poorly supported (Fig. 5). Nevertheless the divergence estimates show that most octocoral genera originated probably between 100 mya and 50 mya. Most generic splits occur within this time period. One of the oldest families is probably the Ellisellidae, which was found together with *Subergorgia* and *Erythropodium* as the sistergroup of all other octocoral genera. According to the divergence time estimates by BEAST2 the family Ellisellidae (here represented by *Ellisella barbadensis*, *Ellisella* sp., *Viminella* sp. and *Junceella* sp.) probably originated 210 to 84 mya (Upper Triassic - Upper Cretaceous). Other families, e.g. Nephtheidae, Nidaliidae and Alcyoniidae originated later, i.e. 121-42 mya (Lower Cretaceous - Eocene), 62-4 mya (Palaeocene - Pliocene) and 131-20 mya (Lower Cretaceous - Miocene), respectively.

Ovulidae – Most ovulid fossils belong to the Eocypraeinae (Groves, 1994; Dolin and Lozouet, 2001), a subfamily that morphologically most closely resembles the Cypraeidae. In the extant ovulid fauna the Eocypraeinae is represented by only a single species, *Sphaerocypraea incomparabilis* (Briano, 1993), which is not included in the molecular dataset. Consequently, the data of the fossil Eocypraeinae can only provide little holdfast in dating the phylogeny of the Ovulidae. These records had to be ignored. A non-extensive overview of fossil, non-Eocypraeinae Ovulidae species (and one pediculariid) is provided in Table 4. Four records for fossil taxa were used to estimate the divergence times in the Ovulidae phylogeny. These four fossil records were selected because they are represented by extant conspecifics in the cladogram, are relatively easy identified compared to other ovulids and also represent three subfamilies (Ovulinae, Prionovolvinae and Simniinae) in the Ovulidae.



The divergence time estimation in BEAST2 resulted in a chronogram (Fig. 6) indicating that the Pediculariidae (clade E) diverged from the Ovulidae between 200 and 100

Fig. 6. Chronogram of Ovulidae species with estimates of divergence times. Numbers on the branches are the posterior probabilities (>0.9) and numbers between [ ] are 95% HPD intervals in mya. The four marked nodes are the calibration nodes. A-E refer to specific clades.

mya according to the 95% highest probability density (HPD). This divergence is very well supported (1.00). Other highly supported groups are the Atlantic ovulids (clade A), which diverged from the Indo-West Pacific (IWP) Ovulidae (clade B-D) 120-68 mya, i.e. during the Cretaceous. In the IWP Ovulidae (clade B-D), between 40 mya and 15 mya (middle Eocene- middle Miocene), branch lengths are shorter and most species divergences occur, these events could be indications of a large species radiation in that era.

The oldest split-off in the family Ovulidae are the Aclyvolvinae (clade D), which are endemic to the IWP, and diverged from the other IWP Ovulidae between the Lower Cretaceous and Upper Cretaceous (95% HPD = 137-76 mya). Therefore, the Aclyvolvinae might contain the oldest Ovulidae species.

Discussion

Octocorallia and Ovulidae phylogenies

The traditional morphological character states that are used to identify octocoral species and genera are mostly obtained from their sclerites. However, the different types of sclerites, the variation within a specific type, and the obvious interspecific overlap made it difficult to identify the specimens. Here, molecular analyses were used to investigate the value of these morphological features. This did not result in unambiguous results. Most gene regions turned out to be conserved and the 28S RNA gene region (which was found to contain pseudogenes) proved to result in phylogeny reconstructions that are incongruent with those of the mitochondrial data (McFadden *et al*., 2010, 2011). Nevertheless, a combination of genes was used to overcome the problems with incongruence and lack of phylogenetic signal (McFadden *et al*., 2006). For example in the Melithaeidae (Aguilar-Hurtado *et al*., 2012; Reijnen *et al*., 2014), a multiple-marker approach partially provided new insight in the relationships between genera and species, but some clades remained unresolved. The lack of phylogenetic resolution for the molecular markers is also represented in the tanglegram by the short and unsupported branches for Octocorallia genera and families. Due to the difficulties in identifying octocoral species and lack of resolution of the molecular markers at genus or family level the taxa in the tanglegram represent genera, unless accurate species identifications were possible (mostly for Caribbean species). The so-called species interdependencies that are indicated are therefore mostly not strict species-to-species associations but representatives of a host octocoral genus and their ovulid symbionts.

The phylogeny reconstruction of the Ovulidae (Fig. 6) is congruent with the topology of the only other large-scale molecular phylogeny reconstruction of Ovulidae, which was published by Schiaparelli *et al*. (2005). Their model is based on the mitochondrial marker 16S, and shows a polytomy for a large number of species currently placed in the subfamily Prionovolvinae. In the present article this clade was reconfirmed with even better support when compared with Schiaparelli *et al*. (2005). The subfamily classification published by Fehse (2007) is largely consistent with the one presented in the present study with the exception of some aberrant species and genera (*Hiatavolva depressa*, *Naviculavolva*, *Prosimnia*). Obviously, these subfamilies have to be redescribed, at least regarding shell morphology. The position of the *Pedicularia*

species has been under dispute. This genus has been placed in the Ovulidae (Schilder, 1931; Von Salvini-Plawen, 1972; Goud and Hoeksema, 2001), but has also been considered a separate family (Yamamoto, 1973; Fehse, 2007; Braga-Henriques *et al*., 2011). The molecular data support the interpretation of the *Pedicularia* species as a separate family, the Pediculariidae. This finding is supported by a difference in host species between the Pediculariidae and the Ovulidae. All *Pedicularia* species are affiliated with Stylasteridae from the class Hydrozoa, whilst all ovulid species are associated with species from the class Anthozoa. In their introduction to the Pediculariidae, Lorenz and Fehse (2009) indicate that there are differences in radula morphology between Ovulidae and the Pediculariidae, which could be an adaptation to the different hosts.

Host species associations can be relevant in identifying the status of symbiotic species. Gittenberger and Gittenberger (2011) for example found that for *Leptoconchus* spp. (Gastropoda) the rudimentary shell characters and the anatomical details do not allow identification. Molecular data, however, enabled the identification of a radiation of several morphologically cryptic species. When the phylogeny reconstruction for the snail species was linked to that of the host species, associations between fungiid coral species and *Leptoconchus* species were discovered.

Similarly, van der Meij *et al*. (2015) found that in the coral-associated crab family Cryptochiridae, species that morphologically looked almost identical belonged to an array of different coral genera. Later on, molecular analyses showed that these morphologically similar gall crabs actually represent a complex of cryptic species, each of which occurring in association with only one coral genus. Similar results were found when the associations between Ovulidae and Octocorallia were studied, as shown in the tanglegram (Fig. 2). Some genera are paraphyletic according to the molecular phylogeny, such as *Dentiovula* and *Prosimnia*. Two species in the genus *Dentiovula* (*D. eizoi* and *D. colobica*) are morphologically quite similar to their supposed conspecifics according to their shell morphology, but the two groups of *Dentiovula* species do not share a recent common ancestor. Moreover, species of these paraphyletic groups are found with different host genera, e.g. *D. eizoi* and *D. colobica* are associated with the octocoral genus *Acanthogorgia* (family Acanthogorgiidae) and the other *Dentiovula* species occur with the octocoral genera *Chironephthya* and *Siphonogorgia* from the family Nidaliidae. In this case, convergent morphological evolution has probably caused confusion, because both the molecular data and the host genera suggest that two different groups are involved. This also accounts for the *Prosimnia* species. All species in this genus except for one, i.e. *Prosimnia piriei*, are found on octocorals of the family Melithaeidae, whilst *P. piriei* is associated with the genus *Euplexaura* of the family Plexauridae. *Prosimnia piriei* is not closely related to the other so-called congeneric species, and its shell shape is unique among the Ovulidae.

Fossils and molecular clock analyses

For both the Ovulidae and the Octocorallia only few fossil records are available. Dating molecular phylogenies of either one of these groups therefore heavily relies on the small number of young (mostly Miocene) fossil remains or disputed old fossils. The lack of

fossils is probably the result of poor taphonomic conditions, such as the absence of strong endo- or exoskeletons and an environment that is unsuitable for fossilisation. Most ovulid species lack strong, big shells and even larger thick-shelled species such as Caribbean *Cyphoma* species, are hardly found as fossils. This could be because *Cyphoma* species are relatively young and are therefore not found in older deposits. Despite their recent common occurrence in the Caribbean, there are only eight records of fossil occurrences (Paleobiology Database; accessed October 2015), the oldest record being from the Aquitanien (23.0-20.4 mya; Woodring, 1973). Fossil remains of Octocorallia are also scarce. Most octocorals have fragmentary calcareous sclerites, which usually do not exceed 0.5 mm in length. Because of their small size, fossil sclerites are hardly found and if so, they are difficult to study while embedded in the concretion. Nevertheless, some fossilised sclerites have been found in the Caribbean and IWP (Kocurko and Kocurko, 1992; Jeng *et al*., 2011). Where Kocurko and Kocurko found a small number of sclerites, Jeng *et al*. (2011) found thick layers of them (also referred to as spiculite layers) in Taiwan, belonging to the genus *Sinularia*. These spiculite layers were found as young fossils as well as extant spiculite layers. Yet, the layers of fossilised sclerites were not correlated to a specific era, system or epoch and could therefore not be used in the molecular divergence estimates.

Instead of sclerites, more commonly the axis or holdfasts of gorgonians have been found as fossil remains (Kocurko, 1988), but these remains are often poorly conserved and can hardly be identified to family level, let alone to a lower taxonomical level. There is more fossil data reported, but the nature of these records is disputed (Taylor and Rogers, 2015) and they could not be related to the shallow-water species that are currently represented in the phylogeny reconstructions.

There are few scientific publications that deal with divergence time estimates for Octocorallia. Ardila *et al*. (2012) focussed on precious corals (Corallidae) and their species delimitations, Park *et al*. (2012) dealt with shallow-water Octocorallia mitogenomes amongst other cnidarian mitogenomes, Taylor and Rogers (2015) used a classical approach by combining molecular data for several gene regions of the family Primnoidae (deep-water, Antarctic), whilst Herrera and Shank (2015) use RAD sequencing on species from the deep-water octocoral genus *Paragorgia* to estimate the divergence splits. The divergence estimates calculated in these articles largely resemble the outcome presented here for the shallow-water, mostly tropical Octocorallia. According to the chronogram published by Park *et al*. (2012), modern Octocorallia have originated from tMRCA during the late Paleozoic and early Mesozoic (421-173 mya) and must therefore have survived the Permian-Triassic mass extinction 251 mya. The 95% HPD intervals from the chronogram (Fig. 5) fall within that range (400-153 mya). The generic diversification in the shallow water Octocorallia must have taken place between 100 and 50 million years ago, which is more or less in correspondence with the dates indicated by Park *et al*. (2012), but the 95% HPD confidence intervals can also exceed these times, up to more than 200 mya for *Sarcophyton glaucum*.

Herrera and Shank (2015) found that the split between the family Corallidae and Paragorgiidae must have taken place approximately 150-80 mya while Park *et al*. (2012) found that the MRCA of Corallidae is 50 my old, with a 95% confidence interval of

100-25 mya. Even though most divergences show overlap, there are also contradictions, which could result from differences in the data that were used (mitogenomes vs. RAD sequencing).

One of the oldest families in the Octocorallia chronogram (Fig. 5) is most probably the Ellisellidae (Bilewitch *et al*., 2014). This family has split-off from the group consisting of *Erythropodium caribaeorum* and *Subergorgia* sp. between 211 and 84 mya (95% HPD). According to a phylogenetic study by McFadden *et al*. (2006) and axial, morphological features by Bayer *et al*. (in Moore, 1956) Elliselllidae are the sister group of the pennatulaceans (Sea Pens), which are among the oldest known Octocorallia fossils, dating back to the Silurian (approximately 444-419 mya). These (trace) fossils are disputed however, and more reliable fossils belonging to the Pennatulacea are from the Cretaceous (Williams, 1999).

The Ovulidae species that are associated with Ellisellidae (Aclyvolvinae) are among the oldest ones. These could be hypothesised to be the first associations between ovulids and Octocorallia, although the fossil record for Ovulidae is poor and inconclusive and for the Aclyvolvinae there are no known fossil species. Conventionally, species of the genus *Archivolva* are considered to be the oldest species in the Ovulidae (hence the name *Archivolva*) because in contrast to all other Ovulidae species, the protoconch is not internalised and still visible (Lorenz and Fehse, 2009). This character was believed to represent an ancestral state, but instead of a basal position, *Archivolva* clusters within the Prionovolvinae and not as the sister species to all other ovulids (a position taken by the Aclyvolvinae).

The large radiation of octocoral genera in the IWP around 100-50 mya is not shared by Ovulidae genera. According to the chronogram (Fig. 6), most ovulid genera came into being 40-15 mya, which is much later than the octocorals. These divergence times are quite similar to the dates estimated by Williams and Duda (2008) for three gastropod species from the IWP. They reconstructed calibrated phylogenies for *Conus*, *Echinolittorina* and *Turbo* spp. and found that for each of these species groups an increased diversification in the IWP occurred between 23.7 and 21.0 mya. Williams and Duda (2008) matched the diversification of those species with the collision of the Australian and New Guinean plate with the southeast boundary of the Eurasian plate, approximately 25 mya. The collision of the tectonic plates is supposed to have resulted in an increase in shallow-water areas and, thereby, an increased number of different habitats triggering the diversification in IWP gastropods and zooxanthelate (octo)coral species. Maybe, most Ovulidae species and genera have originated around that same time in the IWP.

A small number of Atlantic ovulid representatives (*Cyphoma* spp., *Cymbovula acicularis*, *Neosimnia spelta* and *Simnia patula*) separated earlier than their IWP congeners according to the chronogram (Fig. 6). This Atlantic group diverged approximately 121-68 mya. There are fossil remains of *Neosimnia* spp. from the lower Eocene of Europe (Schilder, 1932), indicating that these species occurred in what is nowadays considered Europe 56-41 mya. Temperatures in Europe during the Eocene were higher and the climate could be considered tropical and might be comparable to the current climate in the Caribbean. Species in the Atlantic clade might therefore be relicts from the Eocene North Atlantic ovulid fauna.

Coevolution, cospeciation, or sequential evolution?

The chronograms for the Octocorallia (Fig. 5) and the Ovulidae (Fig. 6) indicate that the Octocorallia originated long before Ovulidae did. This, and given that both topologies of the Octocorallia and Ovulidae in the tanglegram (Fig. 2) are not mirror images, proves that coevolution and cospeciation did not govern the evolutionary history of the combined Octocorallia and Ovulidae. The evolutionary relationships between Octocorallia and Ovulidae are best described as sequential evolution. The changes in the host corals have influenced the symbionts (Ovulidae) without reciprocity. Van der Meij *et al*. (2015) found the same result for Cryptochiridae and scleractinian corals. The interactions between Octocorallia and Ovulidae that are reported in the present study are not based on strict coevolution and cospeciation. Sequential evolution is the most likely evolutionary model for the Octocorallia and Ovulidae.

Defence strategies in Ovulidae

As a result of the specific colouration, colour patters, and sometimes even the mimicry of ornaments resembling polyps, the relationships between Octocorallia and Ovulidae was thought to exemplify coevolution or cospeciation. The results show, however, that sequential evolution is most probably the evolutionary model here. This raises the question how ovulid species got morphologically adapted (mantle ornamentation and colour patterns) to their specific host corals for their own defence. Gastropods use wide-ranging defence strategies against potential predators such as shell thickness, spines, operculum, chemical defence, deep withdrawal and colouration. Shell colour as camouflage is already known for marine gastropods from the Mesozoic era (Vermeij, 2015). Especially in one of the sister groups of the Ovulidae, the Cypraeidae, shells can be vividly coloured whilst the pattern on the mantle that covers the shell can be highly contrasting, possibly confusing potential predators (Vermeij, 2015). Colour is also often used as a defensive strategy in Ovulidae but is more related to the mantle covering the shell rather than the shell itself. Most shells of Ovulidae are monochromous with only a limited number of species that exhibit patterns such as spots on the shells. In contrast, the mantles of Ovulidae can be vibrantly coloured with very abundant colour patterning. These patterns provide camouflage or communicate aposematic behaviour and in some rare occasions, can even mimic other species. The physiological origin of the colour pigments is under dispute but the colouration is believed to be obtained via alimentary homochromy (Salvini-Plawen, 1972; Rosenberg, 1994; Schiaperelli *et al*., 2005). For example, ovulid snails, which have been placed on a differently coloured host coral, changed their shell colour accordingly over time (Salvini-Plawen, 1972). Sacoglossans (*Elysia* spp.) do also change colour when they feed on differently coloured algae. These species are believed to take up the chlorophyll in their digestive tract for photosynthesis and are therefore also referred to as "solar-powered seaslugs" (Middlebrooks *et al*., 2012).

The majority of the Ovulidae species use camouflage as a defence strategy and these species occur in all clades in the phylogeny reconstruction. Some ovulid genera (*Aclyvolva*, *Dentiovula*) even take camouflage a step further and mimic host structures such as the retractable polyps on the mantle (Schiaparelli *et al*., 2005; Reijnen, 2010).

According to the chronogram (Fig. 6), Aclyvolvinae are among the oldest ovulids, so perhaps its MRCA were also camouflaged. The genera *Aclyvolva* and *Dentiovula* are not closely related; the mimicry of octocoral polyps on the mantle of these snails must therefore have arisen more than once in the Ovulidae. Aposematic species are more rare than camouflaged ones in the Ovulidae. Caribbean *Cyphoma* spp. are considered aposematic because they are very brightly coloured and easily observed. The unpalatability of *Cyphoma* is probably a consequence of their gregarious feeding behaviour on a large array of Caribbean octocorals (Reijnen *et al*., 2010; chapter 2) from which they acquire (precursors of) these unpalatable compounds. Its IWP equivalent is *Cuspivolva tigris* which has a conspicuous mantle pattern resembling tiger stripes. *Primovula rosewateri* / *P. beckeri*, *Calpurnus verrucosus* and *Ovula ovum* can be considered aposematic, but only for *O. ovum* there is data on toxicity or unpalatability (Coll *et al*., 1983). Aposematism has therefore arisen at least twice in ovulid evolution, once in the Atlantic (*C. gibbossum*) and once in the IWP (*O. ovum*). It is expected that more cases of aposematism will be discovered in the Ovulidae when the unpalatability of other candidate species will be investigated.

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Suppl. mat. 1. Provenance data for the Octocorallia.

Species (author)	Locality	Latitude	Longitude	Date	Depth (m)
Acanthogorgia sp.	Indonesia, Sulawesi, Lembeh, Tanjung Kuning, LEM.23	1.38633 N	125.17312 E	02/11/12	16
Alertigorgia orientalis (Ridley, 1884)	Malaysia, Borneo, Kudat, Pulau Banggi, TMP.38	7.13040 N	117.22832 E	09/20/12	23
Annella sp.	Indonesia, Halmahera, Hiri, Tanjung Ngafauda, TER.14	0.910639 N	127.317417 E	10/31/09	16
Antillogorgia acerosa (Pallas, 1766)	Curaçao, Marie Pampoen, CAO.21	12.090761 N	68.904956 W	11/05/13	10
Antillogorgia americana (Gmelin, 1791)	Curaçao, Waterfabriek I, CAO.02	12.108611 N	68.950333 W	10/19/13	-
Antillogorgia bipinnata (Verrill, 1864)	Curaçao, Grote Knip, CAO.22	12.351139 N	69.151917 W	11/06/13	37
Astrogorgia sp.	Indonesia, Halmahera, Ternate, Sulamadaha Beach, TER.04	0.863222 N	127.334472 E	10/26/09	8
Bebryce sp.	Malaysia, Semporna, Church Reef 2, SEM.46	4.686117 N	118.64855 E	12/13/10	10
Briareum asbestinum (Pallas, 1766)	Curaçao, Playa Jeremy, CAO.07	12.329028 N	69.079194 W	10/22/13	6
Chironephthya sp.	Malaysia, Sipadan Isl., Mid Reef, SEM.59	4.113333 N	118.636111 E	12/18/10	24
Dendronephthya sp.	Malaysia, Semporna, Pom Pom Isl., SEM.32	4.591944 N	118.861389 E	12/09/10	23
Echinogorgia sp.	Indonesia, Sulawesi, Bitung, Tanjung Nanas II, LEM.05	1.46213 N	125.22823 E	02/01/12	14
Ellisella barbadensis (Duchassaing & Michelotti, 1864)	Curaçao, Blauwbaai, CAO.04	12.134917 N	68.984306 W	11/02/13	37
Ellisella sp.	Malaysia, Semporna, Bohayen Isl., SEM.26	4.468333 N	118.9475 E	12/08/10	24
Erythropodium caribaeorum (Duchassaing & Michelotti, 1860)	Curaçao, Playa Forti, CAO.18	12.366139 N	69.15375 W	11/01/13	15
Eunicea calyculata (Ellis & Sollander, 1786)	Curaçao, Waterfabriek II, CAO.10	12.110278 N	68.954528 W	10/31/13	15
Eunicea clavigera Bayer, 1961	Curaçao, Slangenbaai, CAO.09	12.139722 N	68.997194 W	10/24/13	5
Eunicea fusca Duchassaing & Michelotti, 1860	Curaçao, Hilton Reef, CAO.01	12.121789 N	68.969508 W	10/31/13	25
Eunicea succinea (Pallas, 1766)	Curaçao, W Piscadera Baai, CAO.03	12.12275 N	68.970428 W	11/02/13	6
Euplexaura sp.	Indonesia, Sulawesi, Lembeh, N Sarena Kecil, LEM.32	1.45746 N	125.22711 E	02/16/12	28
Gorgonia ventalina Linnaeus, 1758	Curaçao, Marie Pampoen, CAO.21	12.090761 N	68.904956 W	11/05/13	8
Isis hippuris Linnaeus, 1758	Malaysia, Borneo, Kudat, Pulau Malawali, TMP.08	6.98313 N	117.50312 E	09/09/12	12
Junceella sp.	Republic of the Maldives, Maghoodoo Isl., Nilandhoo. MAD.17	3.049972 N	72.878778 E	02/24/15	25

Suppl. mat. 1. Cont.

Species (author)	Locality	Latitude	Longitude	Date	Depth (m)
Melithaea ochracea (Linnaeus, 1758)	Indonesia, Mollucas, Ambon, Hitu, N coast Mamala	3.537997 S	128.206414 E	11/21/90	10-15
Menella sp.	Malaysia, Johor, Pulau Sibu Tengah, Malang Acha	2.185033 N	104.105117 E	05/26/12	11
Muricea muricata (Pallas, 1766)	Curaçao, Waterfabriek II, CAO.10	12.110278 N	68.954528 W	10/31/13	8
Muricea pinnata Bayer, 1961	Curaçao, Slangenbaai, CAO.09	12.139722 N	68.997194 W	10/25/13	37
Nephthea sp.	Saudi Arabia, Thuwal, Al Dgiyg, THU.11	22.213861 N	38.983111 E	11/12/14	17
Paralemnalia sp.	Saudi Arabia, Thuwal, Um Albalam, THU.10	22.193556 N	38.9475 E	11/12/14	10
Paratelesto sp.	Malaysia, Semporna, Larapan Isl., SEM.47	4.574283 N	118.658017 E	12/13/10	23
Plexaura homomalla (Esper, 1792)	Curaçao, Playa Manzaliña, CAO.13	12.245611 N	69.105222 W	10/26/13	8
Plexaurella dichotoma (Esper, 1791)	Curaçao, Playa Lagun, CAO.16	12.318472 N	69.150167 W	10/29/13	8
Pseudoplexaura porosa (Houttuyn, 1772)	Curaçao, Playa Lagun, CAO.16	12.318472 N	69.150167 W	10/29/13	16
Pseudoplexaura sp.	Curaçao, Playa Manzaliña, CAO.13	12.245611 N	69.105222 W	10/26/13	14
Pterogorgia citrina (Esper, 1792)	Curaçao, Playa Kanoa, CAO.20	12.174722 N	68.865028 W	11/04/13	4
Pterogorgia guadelupensis Duchassaing & Michelin, 1846	Curaçao, Playa Lagun, CAO.16	12.318472 N	69.150167 W	10/29/13	5
Rumphella sp.	Saudi Arabia, Thuwal, Al Dgiyg, THU.11	22.213861 N	38.983111 E	11/12/14	29
Sarcophyton sp.	Saudi Arabia, Thuwal,Fsar+, THU.16	22.247639 N	39.003639 E	11/18/14	17
Scleronephthya sp.	Indonesia, Sulawesi, Bitung, Batu Kapal, LEM.35	1.54912 N	125.29218 E	02/18/12	23
Sinularia sp.	Saudi Arabia, Thuwal, Fsar+, THU.16	22.247639 N	39.003639 E	11/18/14	16
Siphonogorgia sp.	Malaysia, Semporna, Church Reef I, SEM.45	4.681667 N	118.658056 E	12/13/10	23
Solenocaulon sp.	Malaysia, Semporna, Boheydulang Isl. 2 outer reef, SEM.38	4.56755 N	118.757533 E	12/11/10	21
Stereonephthya sp.	Saudi Arabia, Thuwal, Al Bilut (Rose Reef), THU.20	22.31 N	38.886333 E	11/20/14	12
Subergorgia sp.	Republic of the Maldives, Maghoodoo Isl., Beyrufushi, MAD.07	3.108056 N	73.01875 E	02/26/15	31
Villogorgia sp.	Republic of the Maldives, Maghoodoo Isl., Free Climbing, MAD.18	3.065611 N	72.920972 E	02/24/15	23
Viminella sp.	Malaysia, Sipadan Isl., Mid Reef, SEM.59	4.113333 N	118.636111 E	12/18/10	32

Suppl. mat. 2. Provenance data for the Ovulidae.

Species (author)	Locality	Latitude	Longitude	Date	Depth (m)
Notadusta punctata (Linnaeus, 1771)	Malaysia, Borneo, Kapikan Reef, SEM.33	4.651367 N	118.821733 E	12/09/10	-
Pedicularia sp.	Indonesia, Sulawesi, Bitung, Tanjung Pandea, LEM.24	1.39797 N	125.16637 E	02/11/12	18
Pedicularia sp.	Malaysia, Semporna, Tabawan Island, SEM.51	4.78725 N	118.417033 E	12/16/10	12
Pedicularia vanderlandi Goud & Hoeksema, 2001	Indonesia, Bali, N side of Nusa Lembongan, Tanjung Taal	8.659167 S	115.443611 E	04/22/01	<35
Cymbovula acicularis (Lamarck, 1810)	Curacao, Barankí Karanito, CUR.15	12.037083 N	68.803944 W	05/19/05	25
Cyphoma gibbosum (Linnaeus, 1758)	Curacao, Barankí Karanito, CUR.15	12.037083 N	68.803944 W	05/19/05	9
Cyphoma signatum Pilsbry & McGinty, 1939	Curacao, Marie Pampoen, CUR.05	12.095028 N	68.911944 W	06/10/05	5
Cyphoma mcgintyi Pilsbry, 1939	USA, Florida, N of St. Petersburg	28.537806 N	84.272694 W	03/13/11	26
Neosimnia spelta (Linnaeus, 1758)	Spain, Begur, Aigua Blava	41.935542 N	3.218031 W	2008	<15
Simnia patula (Pennant, 1777)	North Sea, South side of Doggersbank	54.333333 N	2.333333 E	09/19/03	-
Primovula rosewateri (Cate, 1973)	Malaysia, Semporna, Sipadan Isl., Hanging Gardens, SEM.60	4.1112 N	118.624817 E	12/18/10	22
Primovula tadashigei (Cate, 1973)	Indonesia, W. Papua, Raja Ampat Isls., Yeffam Isl., NW Pulau Keruo, RAJ.65	0.5876 S	130.295183 E	12/13/07	18
Primovula beckeri (Sowerby III, 1900)	Republic of the Maldives, Maghoodoo Isl., Beyrufushi, MAD.07	3.108056 N	73.01875 E	02/26/15	31
Diminovula concinna (G.B. Sowerby II in Adams & Reeve, 1848)	Malaysia, Borneo, Kudat, NE Pulau Banggi, TMP.20	7.38158 N	117.37349 E	09/14/12	8
Hiatavolva depressa (G.B. Sowerby III, 1875)	Malaysia, Borneo, Kudat, NE Pulau Balambangan, TMP.30	7.33625 N	117.02342 E	09/23/12	12
Prosimnia draconis Cate, 1973	Malaysia, Borneo, Kudat, W of Pulau Tigaba, TMP.03	6.89584 N	117.35427 E	09/07/12	8
Prosimnia semperi (Weinkauff, 1881)	Indonesia, NW Lombok (mainland), Teluk Narat, underneath jetty	8.40833 S	116.07 E	08/04/11	5
Prosimnia korkosi Fehse, 2005	Saudi Arabia, Tiger Head Isl., SAU.23	16.47458 N	42.11919 E	03/10/13	10
Prosimnia hepcae Lorenz & Fehse, 2011	Saudi Arabia, Thuwal, Al Fahal S, THU.05	22.246528 N	38.959194 E	11/09/14	6
Dentiovula cf. *dorsuosa*	Malaysia, Semporna, Timba Timba Isl., SEM.27	4.560883 N	118.924817 E	12/08/10	17
Dentiovula masaoi Cate, 1973	Malaysia, Semporna, Kulapuan Isl. 2 N, SEM.31	4.5354 N	118.838383 E	12/09/10	20
Dentiovula dorsuosa (Hinds, 1844)	Malaysia, Semporna, Darby Bank, SEM.04	4.139722 N	118.170722 E	11/30/10	15
Dentiovula mariae (Schilder, 1941)	Malaysia, Borneo, Kudat, S Pulau Banggi, TMP.36	7.09993 N	117.0892 E	09/19/12	20

Suppl. mat. 2. Cont.

Species (author)	Locality	Latitude	Longitude	Date	Depth (m)
Dentiovula "*dorsuosa*"	Republic of the Maldives, Maghoodoo Isl., Free Climbing, MAD.18	3.065611 N	72.920972 E	02/24/15	23
Procalpurnus lacteus (Lamarck, 1810)	Indonesia, Sulawesi, Bitung, Batu Kapal, LEM.35	1.54912 N	125.29218 E	02/18/12	10
Cuspivolva cuspis (Cate, 1973)	Malaysia, Borneo, Kudat, S Pulau Banggi, TMP.39	7.12294 N	117.14334 E	09/19/12	14
Primovula astra Omi & Iino, 2005	Indonesia, N Sulawesi, Siladen Isl., Siladen Timur, MEN.08	1.635467 N	124.806717 E	11/30/08	30
Prosimnia piriei (Petuch, 1973)	Indonesia, N Sulawesi, Bunaken Isl., Lekuan II, MEN.04	1.6001 N	124.766217 E	11/27/08	10
Rotaovula septemmacula (Azuma, 1974)	Malaysia, Semporna, Larapan Isl. 2 S, SEM.57	4.547517 N	118.6087 E	12/17/10	12
Naviculavolva deflexa (G.B. Sowerby II, 1848)	Malaysia, Semporna, Boheydulang Isl. 1 S, SEM.37	4.58345 N	118.777917 E	12/11/10	21
Naviculavolva elegans Fehse, 2009	Indonesia, N Sulawesi, Manado, Tiwoho, MEN.19	1.596333 N	124.837633 E	12/06/08	10
Naviculavolva debelius Lorenz & Fehse, 2011	Saudi Arabia, Thuwal, Al Dgiyg, THU.11	22.213861 N	38.983111 E	11/12/14	23
Naviculavolva malaita (Cate, 1976)	Malaysia, Semporna, Horn Reef, SEM.08	4.242233 N	118.440033 E	12/01/10	6
Naviculavolva kurziana (Cate, 1976)	Indonesia, Halmahera, Maitara Isl., Maitara NW, TER.10	0.742222 N	127.364139 E	10/29/09	14
Crenavolva striatula (G.B. Sowerby I, 1828)	Malaysia, Borneo, Kudat, S Pulau Banggi, TMP.37	7.08533 N	117.05938 E	09/19/12	20
Crenavolva aureola (Fehse, 2002)	Malaysia, Semporna, Si Amil Isl., SEM.16	4.31725 N	118.875183 E	12/04/10	23
Crenavolva sp.	Oman, SE of Muscat, Bandar Al-Jissah	23.524983 N	58.739183 E	06/01/09	-
Crenavolva trailli (A. Adams, 1855)	Malaysia, Borneo, Kudat, NE of Kudat, TMP.41	6.9967 N	117.05372 E	09/18/12	10
Crenavolva tinctura (Garrard, 1963)	Indonesia, Halmahera, Tidore Isl., N of Desa Rum, TER.18	0.743278 N	127.385083 E	11/04/09	38
Crenavolva tinctura (Garrard, 1963)	Indonesia, Halmahera, Tidore Isl., N of Desa Rum, TER.18	0.743278 N	127.385083 E	11/04/09	38
Crenavolva cruenta Gowlett-Holmes & Holmes, 1989	Indonesia, Sulawesi, Bitung, Tanjung Nanas II, LEM.05	1.46213 N	125.22823 E	02/01/12	28
Dentiovula eizoi Cate & Azuma in Cate, 1973	Malaysia, Semporna, Pom Pom Isl., SEM.32	4.591944 N	118.861389 E	12/09/10	18
Dentiovula colobica (Azuma & Cate, 1971)	Indonesia, Halmahera, Pasir Lamo (W side), TER.26	0.889028 N	127.4595 E	11/08/09	15
Archivolva clava (Habe, 1991)	Indonesia, Halmahera, Tidore Isl., Desa Tahua, TER.07	0.75275 N	127.392028 E	10/28/09	11
Margovula bimaculata (Adams, 1854)	Malaysia, Semporna, Creach Reef, SEM.20	4.315933 N	118.605117 E	12/05/10	16
Diminovula margarita (G.B. Sowerby I, 1828)	Indonesia, Halmahera, Teluk Dodinga, Karang Galiasa Besar E, TER.38	0.846 N	127.585389 E	11/14/09	7

Suppl. mat. 2. Provenance data for the Ovulidae.

Species (author)	Locality	Latitude	Longitude	Date	Depth (m)
Diminovula sp.	Saudi Arabia, Thuwal, Al Asoul, THU.07	22.265361 N	39.002139 E	11/10/14	13
Diminovula aurantiomaculata (Cate & Azuma, 1973)	Malaysia, Borneo, Kudat, NE of Kudat, TMP.40	7.03377 N	117.07363 E	09/18/12	8
Diminovula alabaster (Reeve, 1865)	Malaysia, Borneo, Kudat, NE of Kudat, TMP.40	7.03377 N	117.07363 E	09/18/12	17
Diminovula sp.	Malaysia, Borneo, Kudat, NE of Pulau Bilang Bilangan, TMP.19	7.29537 N	117.40169 E	09/14/12	11
Prionovolva wilsoniana Cate, 1973	Indonesia, Sulawesi, Bitung, Tanjung Kubur, LEM.06	1.47908 N	125.24976 E	02/01/12	18
Diminovula culmen (Cate, 1973)	Malaysia, Semporna, Second Reef, SEM.02	4.175694 N	118.298472 E	11/29/10	3
Prionovolva ericae (Cossignani & Calo, 2002)	Indonesia, W. Papua, Raja Ampat Isls., S Friwin Isl., RAJ.55	0.481817 S	130.69835 E	12/07/07	20
Habuprionovolva aenigma (Azuma & Cate, 1971)	Indonesia, Halmahera, Tanjung Ratemu (S of river), TER.27	0.912361 N	127.486083 E	11/08/09	10
Calpurnus verrucosus (Linnaeus, 1758)	Malaysia, Semporna, Ligitan Isl. 1 SW, SEM.13	4.187056 N	118.79155 E	12/03/10	6
Pellasimnia annabelae Lorenz & Fehse, 2009	Malaysia, Semporna, Larapan Isl. 2 S, SEM.57	4.547517 N	118.6087 E	12/17/10	12
Pellasimnia angasi (Reeve, 1865)	Malaysia, Johor, Pulau (Babi) Besar, Teluk Bakau	2.451433 N	103.971017 E	05/30/12	3
Phenacovolva parvita Cate & Azuma in Cate, 1973	Malaysia, Semporna, Horn Reef, SEM.08	4.242233 N	118.440033 E	12/01/10	24
Ovula ovum (Linnaeus, 1758)	Malaysia, Semporna, Kapalai Isl., SEM.10	4.218 N	118.67225 E	12/02/10	3
Ovula costellata Lamarck, 1810	New Caledonia, Baie de Nakety	21.509467 S	166.097083 E	04/21/12	<30
Phenacovolva rosea (Adams, 1854)	Malaysia, Semporna, Horn Reef, SEM.08	4.242233 N	118.440033 E	12/01/10	20
Phenacovolva nectarea Iredale, 1930	Malaysia, Borneo, Kudat, S of Pulau Malawali, TMP.01	6.95247 N	117.28374 E	09/07/12	11
Phenacovolva morrisoni Lorenz & Fehse, 2009	Malaysia, Borneo, Kudat, E of Pulau Bilang Bilangan, TMP.17	7.24367 N	117.40119 E	09/12/12	6
Phenacovolva cf. *rosea*	Oman, SE of Muscat, Bandar Al-Jissah	23.524983 N	58.739183 E	06/01/09	-
Hiatavolva coarctata (G.B. Sowerby II in Adams & Reeve, 1848)	Malaysia, Semporna, Bohayen Isl., SEM.26	4.468333 N	118.9475 E	12/08/10	37
Aclyvolva lamyi (Schilder, 1932)	Malaysia, Borneo, Kudat, NE of Pulau Banggi, TMP.21	7.34737 N	117.35674 E	09/14/12	14
Aclyvolva lanceolata (G.B. Sowerby II, 1848)	Malaysia, Borneo, Kudat, SW of Pulau Tigaba, TMP.04	6.81093 N	117.36642 E	09/08/12	6

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Summary

Octocorals, most commonly represented by soft corals and gorgonians, live in all oceanic basins and continental shelves, in shallow and deep water. The majority of the species can be found on tropical and temperate coral reefs, where they may act as a host to associated taxa such as shrimps, echinoderms, fish, worms, and snails. The focus of this PhD thesis is on the association between symbiotic snails of the family Ovulidae and their octocoral hosts. These snails are mostly found on the branches of gorgonians and on the body of massive soft corals. They are either remarkably well-camouflaged or flamboyantly coloured. Such interspecific relations raise the question how these associations have evolved and how species have adapted their appearance as trait to enable survival on their host.

In **chapter 1**, the associations between Caribbean octocorals and Ovulidae were studied. The results showed that *Cyphoma gibbosum* is a generalist symbiont found on 21 octocorals species, belonging to nine genera, whilst *Cymbovula acicularis* is a specialist species. The latter was only found on closely related species of the genera *Gorgonia* and *Pseudopterogorgia* (= nowadays *Antillogorgia*). In addition, the phylogeny of Atlantic Ovulidae was reconstructed based on 16S and COI sequences. The sequence data of 13 specimens belonging to four nominal Caribbean ovulid species was combined with sequence data of Indo-Pacific ovulids to get insight into the interspecific relationships. A specimen of *Simnialena uniplicata* clustered within the clade of *C. gibbosum*. Shell morphological features of different growth stages of *C. gibbosum* were compared with the shell of *S. uniplicata* to seek for morphological evidence for this seemingly aberrant clustering. Based on this comparison it was hypothesised that *S. uniplicata* exemplifies a case of paedomorphosis. In **chapter 2,** the results of chapter 1 are revisited with molecular data from four loci (16S, COI, H3 and 28S), and an additional 23 specimens belonging to five nominal species and an undescribed morphotype. The newly acquired data showed that the mantle patterns in Ovulidae, which were thought to be diagnostic at species level, are not indicative in case of the *Cyphoma* species. Morphological results from earlier studies were in agreement with these results, and consequently the species' names *C. signatum* and *C. mcgintyi* are synonymised with *C. gibbosum*. The black morph (previously identified as *S. uniplicata*) should also be referred to as *C. gibbosum*. Three hypotheses were opted (rapid divergence, supergenes, and recent speciation) to explain the differences in the mantle colour pattern and the lack of molecular differentiation. In **chapter 3** an experimental approach was used to identify the bioactivity of the host corals of Ovulidae and Cryptochiridae (coral-dwelling crabs) to detect if bioactivity of host species is a possible trigger in symbiont speciation. A luminometer with light emitting *Aliivibrio fisheri* bacteria was used to investigate the bioactivity, by means of EC_{50}-values, for the host corals (stony corals [=Scleractinia] and octocorals). It showed that investigated octocorals were more bioactive than the examined scleractinians. Among the Octocorallia, studied species of the genera *Antillogorgia* and *Gorgonia* were the most bioactive and *Madracis auretenra* for the Scleractinia. When bioactivity values and the associated symbionts were plotted on the host cladograms, the most bioactive octocoral genera appeared to have most (two) symbionts. Host coral bioactivity is therefore most probably unrelated to the snail's host preference. In contrast, cryptochirid

crabs were not found on the most bioactive scleractinian, but were found in association with less bioactive coral hosts, implying that bioactivity might be an evolutionary driver. Speciation linked to host shifts, however, cannot be ruled out as the evolutionary mechanism.

In **Chapter 4** the phylogenetic relationships among five *Crenavolva* species are discussed. This chapter discussed the second record for the species *C. chiapponii* and concerned a sample from shallow water, whereas this species previously was considered to be restricted to deep water. This was also the first time that *Acanthogorgia* sp. was identified as its host species. The phylogenetic relationships of *C. chiapponii* with its congeners *C. aureola*, *C. striatula, C. tokuoi* and *C. trailli* were assessed based on two molecular markers (16S and COI) in combination with GenBank data available for two of the *Crenavolva* species. The phylogram showed that *C. chiapponii* did not genetically differ from *C. aureola*. This was supported by a lack of diagnostic morphological features separating these nominal species and therefore *C. chiapponii* was synonymised with *C. aureola*.

In the next chapter, **chapter 5**, a large-scale molecular data set was presented to investigate the traditional subfamily division within the family Ovulidae. It appeared that the subfamilies Aclyvolvinae and Simniinae are paraphyletic. Especially the species within the Aclyvolvinae (n = 8) have been troublesome in their identification; with a four-marker dataset (16S, COI, H3 and 28S) the phylogenetic relationships between these species was re-assessed. The type species of two of the three genera (*Hiatavolva depressa* and *Aclyvolva lanceolata*) did not cluster together with other species in these genera. *Hiatavolva depressa* was retrieved in the subfamily Prionovolvinae and did not cluster with its congeners. Instead, the congeners *H. rugosa* and *H. coarctata,* cluster with the type and other species of the genus *Aclyvolva* and were therefore moved to that genus. Molecular and morphometric results show that *A. lamyi* and *A. nicolamassierae* are synonyms of *A. lanceolata* and that *A. rugosa* (n. comb.) is a synonym of *A. coarctata* (n. comb.). The genus *Kuroshiovolva* could not be retrieved in a fixed phylogenetic position within the Aclyvolvinae, but did not cluster with *Hiatavolva depressa* or *Aclyvolva* spp. Its taxonomic position therefore remains uncertain. Photographs of type species are provided in this chapter, as well as new information on the geographical distribution and host species of Aclyvolvinae. In **chapter 6** an approach as in chapter 5 was used, but instead of ovulids the speciose octocoral family Melithaeidae - host to many ovulid species - was investigated. The taxonomic position of six genera (*Asperaxis*, *Acabaria*, *Clathraria*, *Melithaea*, *Mopsella*, *Wrightella*) divided over two subfamilies were reviewed and reassessed. Material collected from the Central Pacific, Red Sea and Indian Ocean (as far south as South Africa) was used for sequencing of four different loci, both mitochondrial (COI, mtMutS, ND6) and nuclear (28S rDNA). A combination of molecular and morphological data revealed that all former so-called genera, except for the monotypic genus *Asperaxis* and the genus *Wrightella,* are paraphyletic. Moreover, molecular data on the two subfamilies in the Melithaeidae (Asperaxinae and Melithaeinae) indicated that the family is also paraphyletic. Furthermore, it was observed that species did not cluster according to their present morphology-based classification, but instead clustered according to a biogeographical pattern. Melithaeid species from the Red Sea, Indian Ocean and Central Pacific, grouped each into well-supported clades related to their respective distribution

ranges. Consequently, we did not find morphological or molecular phylogenetic support to maintain the generic names *Acabaria*, *Clathraria*, *Mopsella* and *Wrightella*. These names are synonymised with the oldest available generic name: *Melithaea*. As a result, six secondary homonyms originated and were renamed, viz. *Melithaea hendersoni* nom. nov., *Melithaea kukenthali* nom. nov., *Melithaea mcqueeni* nom. nov., *Melithaea shanni* nom. nov., *Melithaea thorpeae* nom. nov., and *Melithaea wrighti* nom. nov. Additionally, neotype specimens were selected for *Melithaea ochracea* to stabilize the genus *Melithaea*, and for *Acabaria rubra*. **Chapter 7** is the all-enfolding chapter in which all available data on the associations between Octocorallia and Ovulidae was combined to investigate coevolutionary scenarios on large molecular and host species association datasets. A tanglegram, identifying all known associations between Octocorallia and Ovulidae, was constructed and subsequently subjected to coevolutionary analyses in Jane 4 and CoRe-PA. To create the tanglegram, phylogeny reconstructions of the Ovulidae and Octocorallia were made, each based on four loci (16S, 28S, COI, H3 and 28S, igr-COI, mtMutS, ND6, respectively). Statistical tests show that the number of coevolutionary events was higher than expected by chance alone, thereby indicating a coevolutionary scenario between Octocorallia and Ovulidae. For a coevolutionary or cospeciation scenario the phylogenies are expected to be mirror images with identical branch lengths. To estimate these speciation times, molecular dating was carried out on the octocoral and ovulid datasets in BEAST2. Fossil data for the Octocorallia and Ovulidae was used to calibrate the nodes in their respective phylogeny reconstructions. The molecular clock analyses showed that diversification in the Octocorallia started before diversification in the Ovulidae (100-50 mya vs. 40-15 mya). Diversification in the Ovulidae corresponds with diversification of other gastropod groups from the Indo-Pacific, and is linked to the collision of the Australian plate with the southeast boundary of the Eurasian plate approximately 25 million years ago. This resulted in an increase in shallow-water areas, which possibly fostered the diversification of gastropods and zooxanthelate (octo)corals. The discrepancy between the diversification times excludes coevolution or cospeciation as evolutionary scenario. Most probably sequential evolution, where the host affects the symbiont but not vice versa, is the evolutionary scenario explaining the intrinsic association between Octocorallia and Ovulidae.

Samenvatting

Octocorallia, vooral vertegenwoordigd door zachte koralen en gorgonen, komen voor in alle oceanen, zowel in ondiep als diep water. De grootste verscheidenheid aan soorten is te vinden op tropische koraalriffen en in gematigde zeeën waar ze fungeren als gastheer voor veel geassocieerde taxa, zoals garnalen, stekelhuidigen, vissen, wormen en slakken. In dit proefschrift gaat het met name om de associatie tussen symbiotische slakken van de familie Ovulidae en Octocorallia. Deze slakken zijn meestal te vinden op of tussen de takken van zachte koralen en gorgonen en zijn ofwel opmerkelijk goed gecamoufleerd of juist opvallend gekleurd. Dergelijke specifieke interacties roepen vragen op over het ontstaan van associaties tussen verschillende diergroepen en hoe soorten hun uiterlijk hebben aangepast aan de specifieke voorwaarden om te kunnen samenleven met hun gastheer.

In **hoofdstuk 1** werden de associaties tussen Caraïbische Octocorallia en Ovulidae bestudeerd. De resultaten toonden aan dat *Cyphoma gibbosum* een generalistische symbiont is. De soort werd gevonden op 21 verschillende gastheersoorten, behorende tot negen geslachten, terwijl *Cymbovula acicularis* daarentegen meer gespecialiseerd is. Die soort werd alleen op nauw verwante soorten uit de geslachten *Gorgonia* en *Pseudopterogorgia* (= tegenwoordig *Antillogorgia*) waargenomen. De fylogenie van de Atlantische Ovulidae werd gereconstrueerd op basis van 16S en COI sequenties van 13 exemplaren die behoren tot vier nominale soorten. Deze data werden geanalyseerd in combinatie met moleculaire gegevens aangaande Indo-Pacifische Ovulidae, om zo inzicht te krijgen in de fylogenetische relaties tussen de soorten. Een exemplaar geïndentificeerd als *Simnialena uniplicata* clusterde binnen de clade van *C. gibbosum*. De schelpmorfologische kenmerken van de verschillende groeistadia van *C. gibbosum* werden daarom vergeleken met de schelp van *S. uniplicata* om te zoeken naar morfologische aanwijzingen voor deze afwijkende clustering. Op basis van deze vergelijking werd verondersteld dat de schelpmorfologie van *S. uniplicata* een voorbeeld is van paedomorphosis. Echter, in **hoofdstuk 2**, werden de resultaten van hoofdstuk 1 herzien met behulp van moleculaire gegevens van vier verschillende loci (16S, COI, H3 en 28S), en 23 extra exemplaren behorende tot vijf nominale soorten en een onbeschreven morfotype. Uit de nieuw verworven gegevens bleek dat mantelpatronen in Ovulidae, die geacht werden diagnostisch te zijn op soortniveau, die rol niet vervullen in het genus *Cyphoma*. Morfologische resultaten uit eerdere studies waren in overeenstemming met deze resultaten en daarom zijn de soortnamen *C. signatum* en *C. mcgintyi* gesynonimiseerd met *C. gibbosum*. Het zwarte morfotype (voorheen aangeduid als *S. uniplicata*) moet ook worden aangeduid als *C. gibbosum*. Drie hypothesen werden voorgesteld (snelle divergentie, supergenen en recente speciatie) om de verschillen in de mantelpatroonmorfologie en het gebrek aan moleculaire differentiatie te verklaren. In **hoofdstuk 3** werd een experimentele aanpak gebruikt om de bioactiviteit van de gastheerkoralen van Ovulidae en Cryptochiridae (galkrabben: kleine in koraal levende krabben) te identificeren om vervolgens te bepalen of de bioactiviteit van een gastheersoort een mogelijke *driver* is in de soortvorming van de geassocieerde fauna. Een luminometer in combinatie met lichtgevende *Aliivibrio fisheri* bacteriën werd gebruikt om de biologische activiteit, uitgedrukt in EC_{50}-waarden, te bepalen voor de gastheerkoralen (steenkoralen [= Scleractinia] en Octocorallia). Uit deze

analyses bleek dat de Octocorallia meer bioactief waren dan Scleractinia. Voor de betreffende Octocorallia behoorden de nauwverwante geslachten *Antillogorgia* en *Gorgonia* tot de meest bioactieve geslachten, terwijl *Madracis auretenra* de meeste bioactieve steenkoraalsoort is. Wanneer de bioactiviteitswaarden en de bijbehorende symbionten op fylogeniereconstructies van de gastheercladogrammen werden geplot, bleek dat de meest bioactieve genera in de Octocorallia het hoogste aantal symbionten hebben. De bioactiviteit van het gastheerkoraal is dus waarschijnlijk niet gerelateerd aan gastheervoorkeur van de slak. Galkrabben daarentegen werden niet in associatie gevonden met het meest bioactieve koraal, maar bewoonden wel minder bioactieve koraalgastheren. Bioactiviteit zou in dit geval een mogelijke evolutionaire stimulans kunnen zijn. Sequentiële evolutie kan echter niet worden uitgesloten als een alternatief mechanisme voor soortvorming in galkrabben.

In **hoofdstuk 4** werd de fylogenetische relatie tussen vijf *Crenavolva* soorten onderzocht. Dit hoofdstuk bespreekt ook de tweede waarneming voor de soort *C. chiapponii*. Deze soort werd geacht alleen voor te komen in diep water, maar werd tijdens expedities in Indonesië verzameld van ondiepe koraalriffen. Dit was eveneens de eerste keer dat de gastheersoort *Acanthogorgia* sp. geïdentificeerd kon worden. De fylogenetische relaties tussen *C. aureola*, *C. striatula*, *C. tokuoi* en *C. trailli* werden bepaald op basis van twee genregio's (16S en COI), in combinatie met GenBankdata voor twee van de *Crenavolva* soorten. De fylogeniereconstructie toonde aan dat *C. chiapponii* genetisch niet te onderscheiden is van *C. aureola*. Dit werd ondersteund door een gebrek aan morfologische kenmerken om beide zogenaamde soorten te onderscheiden. *Crenavolva chiapponii* werd daarom gesynonimiseerd met *C. aureola*.

In het volgende hoofdstuk, **hoofdstuk 5**, werd een grootschalige moleculaire dataset gebruikt om de traditionele onderscheiding in subfamilies binnen de familie Ovulidae te onderzoeken. Het bleek dat de subfamilies Aclyvolvinae en Simniinae parafyletisch waren. Vooral de soorten binnen de Aclyvolvinae (n = 8) zijn lastig te identificeren en daarom zijn met een vier-marker-dataset (16S, COI, H3 en 28S) de fylogenetische relaties tussen deze soorten bepaald. De typesoorten van twee van de drie genera (*Hiatavolva depressa* en *Aclyvolva lanceolata*) clusterden niet samen met andere soorten uit deze geslachten, *Hiatavolva depressa* werd zelfs geplaatst in de subfamilie Prionovolvinae en clusterde helemaal niet met de beoogde soortgenoten. De soorten *H. rugosa* en *H. coarctata*, clusterden met de typesoort en andere soorten in het geslacht *Aclyvolva*. Als gevolg hiervan zijn de twee *Hiatavolva* soorten verplaatst naar het geslacht *Aclyvolva*. Moleculaire en morfometrische resultaten laten zien dat andere 'soorten' in het geslacht *Aclyvolva*, *A. lamyi* en *A. nicolamassierae*, synoniemen zijn van *A. lanceolata*. *Aclyvolva rugosa* (comb. nov.) is een synoniem van *A. coarctata* (comb. nov.). Het geslacht *Kuroshiovolva* had geen vaste fylogenetische positie binnen de Aclyvolvinae, en clusterde niet met *Hiatavolva depressa* of *Aclyvolva* soorten. De taxonomische positie van dit geslacht blijft dus onzeker. Als aanvulling zijn foto's van de diverse typesoorten toegevoegd aan dit hoofdstuk en nieuwe informatie over de geografische verspreiding en de gastheersoorten van de Aclyvolvinae. In **hoofdstuk 6** werd een vergelijkbare aanpak als in hoofdstuk 5 gebruikt, maar in plaats van Ovulidae is de soortenrijke gorgonenfamilie Melithaeidae onderzocht, die als gastheer voor vele Ovulidae soorten dient. De taxonomische positie van zes genera (*Asperaxis*, *Acabaria*, *Clathraria*, *Melithaea*, *Mopsella* en *Wrightella*), verdeeld over twee subfamilies, werden herzien en opnieuw geëvalueerd.

Materiaal verzameld in de Stille Oceaan, de Rode Zee en de Indische Oceaan (zuidelijk tot Zuid-Afrika) werd gebruikt voor het sequencen van vier verschillende loci; zowel de mitochondriale genen (COI, mtMutS, ND6) als een nucleair gen (28S rDNA) werden gebruikt. Uit de combinatie van de moleculaire en morfologische gegevens bleek dat alle hiervoor genoemde genera, met uitzondering van het monotypische genus *Asperaxis* en *Wrightella* parafyletisch zijn. Bovendien wezen de moleculaire gegevens uit dat de twee subfamilies binnen de Melithaeidae (Asperaxinae en Melithaeinae) ook parafyletisch zijn. Verder bleek dat soorten niet clusteren op basis van hun huidige morfologische indeling, maar in plaats daarvan clusteren ze op basis van een biogeografisch patroon. Soorten uit de Rode Zee, de Indische Oceaan en de Stille Oceaan zijn gegroepeerd in goed onderbouwde clades per regio. Hieruit volgt dat er geen morfologische of fylogenetische ondersteuning is om de genusnamen *Acabaria*, *Clathraria*, *Mopsella* en *Wrightella* te behouden. Deze namen werden daarom gesynonimiseerd met de oudste beschikbare generieke naam: *Melithaea*. Door ontstonden er vijf secondaire homoniemen, welke hernoemd werden als *Melithaea hendersoni* nom. nov., *Melithaea kukenthali* nom. nov., *Melithaea mcqueeni* nom. nov., *Melithaea shanni* nom. nov., *Melithaea thorpeae* nom. nov., en *Melithaea wrighti* nom. nov. Daarnaast zijn neotypes geselecteerd voor *Melithaea ochracea,* om de naam van het geslacht *Melithaea* te stabiliseren, en voor *Acabaria rubra*. **Hoofdstuk 7** is het allesomvattende hoofdstuk waarin alle beschikbare gegevens van de Octocorallia en Ovulidae werden gecombineerd om mogelijke coevolutionaire scenario's te onderzoeken aan de hand van grote moleculaire datasets en gastheerspecificiteit. Een *tanglegram* werd gemaakt om alle associaties tussen Octocorallia en Ovulidae weer te geven en vervolgens te onderwerpen aan coevolutionaire analyses in Jane 4 en CoRe-PA. Om de *tanglegram* te maken, werden fylogeniereconstructies van de Ovulidae en Octocorallia gemaakt, elk gebaseerd op vier loci (respectievelijk 16S, 28S, COI, H3 en 28S, igr-COI, mtMutS, ND6). Statistische testen tonen aan dat het aantal coevolutionaire gebeurtenissen hoger was dan verwacht door toeval alleen, wat impliceert dat er een coevolutionaire relatie bestaat tussen de Octocorallia en Ovulidae.

Voor een dergelijk coevolutionair scenario wordt aangenomen dat fylogenieën van de host en symbiont elkaars topologische spiegelbeeld zijn met identieke taklengtes. Moleculaire dateringen werden uitgevoerd op de Octocorallia en Ovulidae datasets in BEAST2 om de tijd van speciatie tussen soorten te benaderen. Gegevens van fossiele soorten werden gebruikt om de fylogeniereconstructies te kalibreren. Uit deze moleculaire-klok-analyses bleek dat de diversificatie in de Octocorallia begon vóór de diversificatie in de Ovulidae (100-50 ten opzichte van. 40-15 miljoen jaar geleden). De diversificatie in de Ovulidae komt overeen met de diversificatieperiode van andere gastropoden uit de Stille Oceaan, en is mogelijk gerelateerd aan de tektonische botsing van de Australische plaat met de zuidoostelijke grens van de Euraziatische plaat (ca. 25 miljoen jaar geleden). Dit resulteerde in een toename van ondiepe gebieden, die mogelijk de diversificatie van gastropoden en zoöxanthellate koralen heeft bevorderd. De discrepantie tussen de diversificatieperioden van Octocorallia en Ovulidae sluit coevolutie of cospeciatie uit als mogelijk evolutionair scenario. Waarschijnlijk is sequentiële evolutie, waarbij de gastheer invloed heeft op de symbiont maar deze niet op zijn gastheer, het meest waarschijnlijke scenario om de intrinsieke associatie tussen Octocorallia en Ovulidae te verklaren.

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Curriculum vitae

Bastian Theodoor Reijnen was born in The Hague, The Netherlands on June 24th, 1982. He enjoyed his childhood in Zoetermeer where he started higher general secondary education (HAVO) in 1994 at the Erasmus College and graduated in 1999 majoring in natural sciences. In the summer holidays of 1994, Bastian got his first introduction to SCUBA diving in a swimming pool at a French campsite. Within a single year he was licensed as an "Open Water Diver" and in the summer of 1995 he got his "Advanced Open Water Diver" license.

After passing high school he continued his education in Leiden at the University of Applied Sciences to study organic chemistry, where he successfully graduated in 2003. For his internship at the Synthetic and Bio-organic Chemistry lab (VU University Amsterdam), he investigated a synthetic route to prepare a precursor for a compound, which could become a new anti-biotic. In this way he got introduced to the academic research world and he acquired a taste for doing research, but not in the field of organic chemistry. Therefore he joined a three-month program at Leiden University to become eligible for an MSc study in Biology. In 2004 he started his MSc track Biology, Evolutionary and Ecological Sciences. At a trip to Artis Royal Zoo in this MSc track, biodiversity lecturer Dr. Rinny Kooi noticed his interest in marine life and systematic zoology. She advised him to contact Prof. dr. Edmund Gittenberger for an internship at the National History Museum, Naturalis (nowadays Naturalis Biodiversity Center at Leiden). In 2004, Bastian started his first internship under supervision of PhD candidate Arjan Gittenberger on the molecular systematics of Fungiidae corals. By hearing all the good stories about fieldwork, he wanted to join a scientific diving expedition himself. Fortunately, for his second and final MSc internship at Naturalis, Dr. Bert Hoeksema offered him the opportunity to organise fieldwork in Curaçao in 2005 with three fellow students. This was his first introduction to Octocorallia and Ovulidae, since the project concerned: "The Ovulidae from Curaçao, The Netherlands Antilles: a phylogenetic and ecological approach". After almost three months in the field, and many hours in the molecular laboratory at the Van der Klauw-building, he finished his MSc at Leiden University early 2007. To financially support his study and life in Leiden he worked during the weekends and holidays as front of house at the exhibition part of the NBC. In 2007 he continued his research career at Naturalis, besides being front of house in the weekends, as a research assistant for various (molecular) projects. In 2008 he ventured out for a little while and worked as a project assistant at the Royal Netherlands Institute for Sea Research (NIOZ), to help in finishing molecular work for a European research project (MarPace) for Dr. Katja Philippart. Meanwhile he worked at Naturalis on various projects dealing with collections and research until 2011. From 2007 to 2011 he also continued his research on Octocorallia and Ovulidae in his spare time. In 2011 he applied for a PhD research project at Naturalis, sponsored by the FES programme, on the relationship between Octocorallia and Ovulidae and started with this PhD project on January 1st, 2012. Approximately eleven years after getting introduced to Naturalis, after 400 research dives, visits to many tropical countries such as Indonesia, Malaysia and the Maldives, countless hours spent in the molecular laboratory and behind the microscope, this PhD thesis has come to completion in 2016.

Publications

Publications resulting from thesis

Reijnen BT, van der Meij SET, submitted. Coat of many colours – DNA reveals polymorphism of mantle patterns and colouration in Caribbean Cyphoma Röding, 1798.

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