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## Abstract

Since 2000, eighteen epitoniid species that were found in association with corals, were described as new to science in addition to the four such species that were already known. Three genera of coral-associated epitoniids were also described as new. Most of these taxa could only be diagnosed by their ecology and by the morphology of the radulae, jaws, opercula and egg-capsules. Using an original molecular data set, it is demonstrated that these data support the existence of the recently described, coral-associated species as separate gene pools and the alleged genera as monophyletic groups. The nominal genus *Epitonium*, as it shows up in most of the recent literature, turns out to be polyphyletic. To some extent, co-evolution has played a role in the evolutionary history of the associations between wentletraps and their coelenterate hosts.

## Contents



## Introduction

This is the fourth contribution in a series of papers aiming at a better knowledge of epitoniid species (Gastropoda: Epitoniidae) associated with corals (Scleractinia). For an introduction about the ontogeny and ecology of these snails, and detailed descriptions of the morphology of their shells, radulae, jaws, opercula and egg-capsules, see also A. Gittenberger (2003), A. Gittenberger and E. Gittenberger (2005), A. Gittenberger and Hoeksema (chapter 10) and A. Gittenberger et al. (2000). The snails and shells that were examined in this study came from many localities (fig. 1).

Before 2000, only four epitoniid species were known to be associated with corals (Scleractinia: Fungiidae or Dendrophyllidae), i.e. *Epidendrium billeeanum* (Dushane and Bratcher, 1965), *Epidendrium dendrophylliae* (Bouchet and Warén, 1986), *Epifungium ulu* (Pilsbry, 1921) and *Surrepifungium costulatum* (Kiener, 1838). Since then, eighteen additional species were found in association with corals. All of these were described as new to science (Bonfitto and Sabelli, 2001; A. Gittenberger, 2003; A. Gittenberger and E. Gittenberger, 2005; A. Gittenberger et al., 2000). Three genera of coral-associated species were described as new to science, i.e. *Epidendrium*, *Epifungium* and *Surrepifungium* (A. Gittenberger and E. Gittenberger, 2005). Most of these species and genera cannot be identified on the basis of conchological characters alone, because of the apparent parallel or convergent evolution in shell shape, size and sculpture (A. Gittenberger and E. Gittenberger, 2005). Most of these taxa can be diagnosed by their ecology and by the morphology of the radulae, jaws, opercula and egg-capsules, however.

Using an original molecular data set, we discuss in this paper the following research questions: [1] do the molecular data support the existence of the recently described, coral-associated species as separate gene pools; [2] are the so-called genera of the Epitoniidae that are associated with corals monophyletic groups;

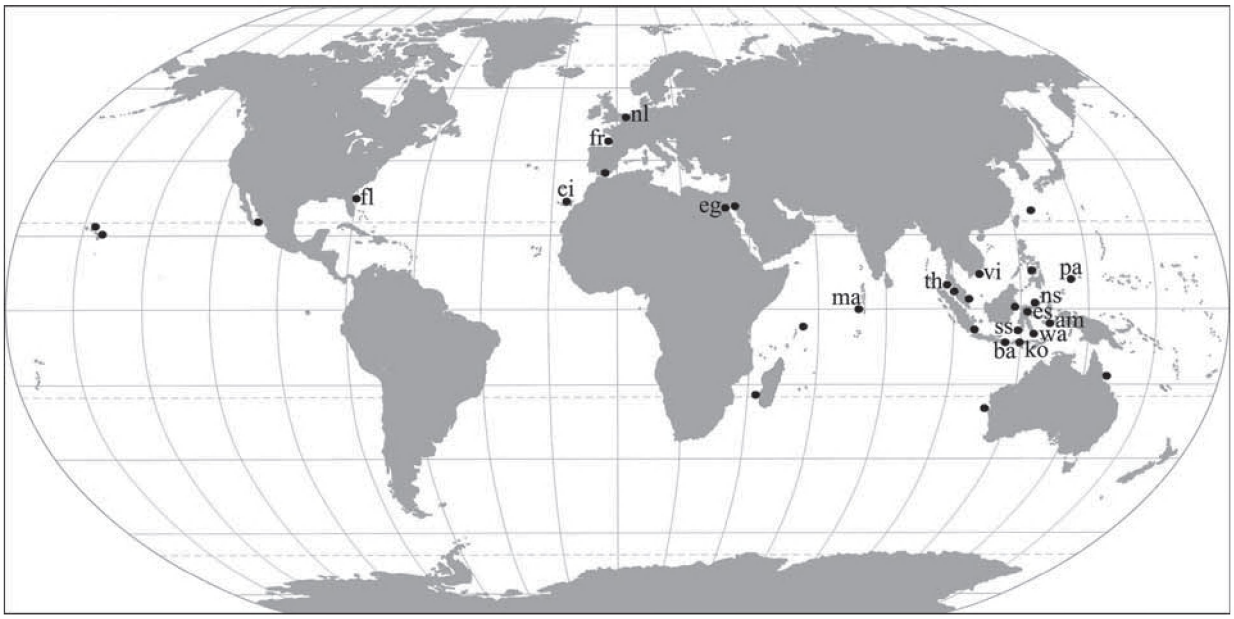



*Fig. 1.* World map. Black dots indicate localities of which snails and/or shells were examined by the authors. The dots accompanied by the two letter abbreviations, indicate localities from which epitoniid snails were successfully sequenced (see also fig. 2). Abbreviations: am, Ambon, Indonesia; ba, Bali, Indonesia; ci, Canary Islands; eg, Egypt (Red Sea); es, east Sulawesi, Indonesia; fl, Florida, USA; fr, France; nl, The Netherlands; ns, north Sulawesi, Indonesia; ko, Komodo, Indonesia; ma, Maldives; pa, Palau; ss, south Sulawesi, Indonesia; th, Thailand; vi, Vietnam; wa, Wakatobi, Indonesia.

[3] what can be concluded about the status of the nominal genus *Epitonium*; [4] what evolutionary mechanisms, like co-evolution, may have played a prominent role in the evolutionary history of the associations between wentletraps and their coelenterate hosts?

## Material and methods

### Fieldwork

All snails used in the molecular analyses were identified by the first author. The ones that are associated with corals are described in detail in A. Gittenberger and E. Gittenberger (2005). They were collected by searching approximately 60,000 stony corals of the families Fungiidae, Dendrophylliidae and Euphylliidae for gastropod parasites in the Indo-West Pacific off Egypt, Maldives, Thailand, Malaysia, Japan, Palau, Philippines, Indonesia and Australia. The fungiid hosts were usually identified twice, from photographs and/or specimens, independently by A. Gittenberger and B.W. Hoeksema. H. Ditlev identified the euphylliids from photographs. The dendrophylliids were not identified. Most of the specimens used in this study were collected in a three years period (2001-2003) while scuba-diving in Indonesia and Palau during several excursions organized by the National Museum of Natural History Naturalis. This material was preserved in ethanol 96% to enable DNA-analyses. For making comparisons, epitoniid species that are known to be associated with sea anemones, were also included in the molecular analyses. These snails are found in both the Atlantic and the Indo-Pacific Ocean. The localities from which material was used for the molecular analyses are indicated in figure 1.

### DNA extraction and sequencing

Until DNA-extraction, most snails were preserved in ethanol 96%, some in ethanol 70%, and the specimens from Thailand in a 1:1 mixture of rum (c. 40% alcohol) and 70% ethanol. In relatively small specimens, the complete snail without its shell was used for the

extraction. In larger specimens a piece of the foot tissue was cut off with a scalpel. A minute, curved needle, stuck into a wooden match, was used to pull the snails out of their shells without breaking them. The tissue sample was dissolved by incubation at 60° C, for c. 15 hours, in a mixture of 0.003 ml proteïnase K (20 mg/ml) and 0.5 ml CTAB buffer, i.e. 2% CTAB, 1.4M NaCl, 0.2% mercapto-ethanol, 20mM EDTA and 100mM TRIS-HCl pH8. After incubation the solution was mixed with 0.5 ml Chloroform/Isoamyl alcohol, and centrifuged for 10' at 8000 rpm. The supernatant was extracted, mixed with 0.35 ml isopropanol, put aside for c. 15 hours at 4° C and finally centrifuged for 10' at 8000 rpm to precipitate the DNA. The supernatant was discarded and the remaining DNA-pellet was washed at room temperature with 0.5 ml of an ethanol/ ammonium-acetate solution for 30'. After centrifugation for 10' at 8000 rpm, this solution was discarded. The pellet was dried in a vacuum centrifuge and than dissolved in 0.020 ml MilliQ. The DNA quality and quantity were tested by electrophoresis of the stock-solution through an agarose gel, and by analyzing a 1:10 dilution of the stock in a spectrophotometer.

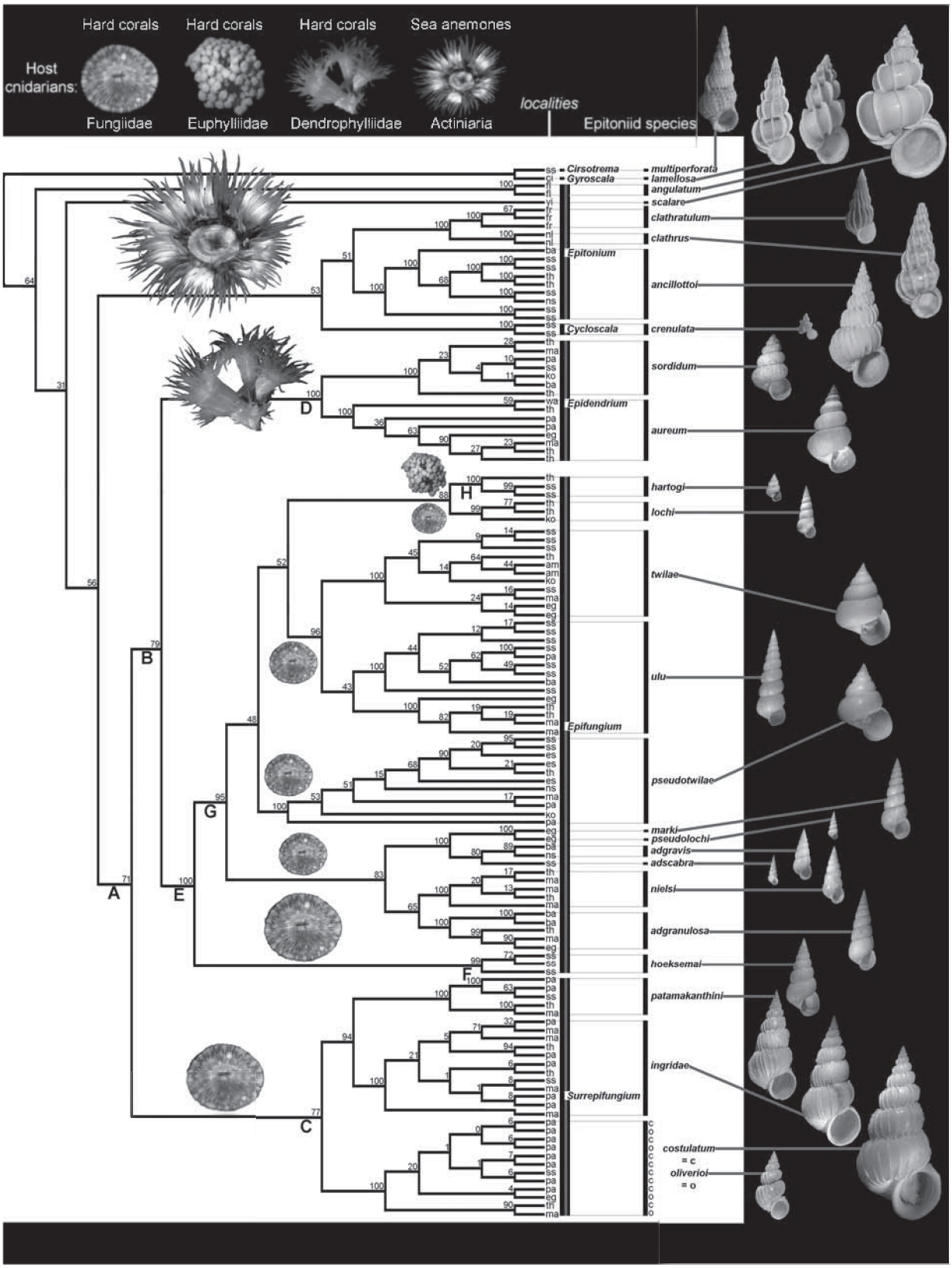
The COI region was amplified using the primers and annealing temperatures (AT) as specified in table 1 in a Peltier Thermal Cycler PTC-200. The epitoniid specific COI primers were developed on the basis of 15 wentletrap sequences retrieved using Folmer Universal COI primers (table 1). The sequences of these primers were made wentletrap-specific by comparing them with the Folmer COI-sequences (A. Gittenberger, Reijnen and Hoeksema, chapter 3) of their fungiid hosts, making sure that the primers would not fit on the COI-region of these corals. The optimized PCR-program consisted of 1 cycle of 94° C for 4' and 60 cycles of 94° C for 5"; AT for 1'; 0.5° C/s to AT + 5° C; 72° C for 1'. After the PCR, the samples were kept on 4° C until purification by gel extraction using the QIAquick Gel Extraction Kit from QIAGEN. The PCR reaction mix consisted of 0.0025 ml PCR buffer (10x), 0.0005 ml MgCl2 (50mM), 0.0010 ml forward primer (10 pM), 0.0010 ml reverse primer (10 pM), 0.0005 ml dNTP's (10 mM), 0.0003 ml Taq polymerase (5 units / 0.001 ml), 0.0132 ml MilliQ and 0.0010 ml 1:10 DNA stock-solution (= c. 100 ng DNA). The samples were kept at 4° C until cycle sequencing. Cycle sequencing was done in both directions of the amplified region, with a program consisting of 45 cycles of 96°C for 10", 50°C for 5" and 60°C for 4'. The reaction mix used was 0.0020 ml Ready Reaction Mix (Big DyeTM by PE Biosystems), 0.0020 ml Sequence Dilution-buffer, 0.0005 ml primer (5 pM forward or reverse primer solution) and 0.0055 ml amplified DNA (= half the PCR-product, evaporated to 0.0055 ml by vacuum centrifugation). The cycle sequence products were purified with Autoseq G50 columns (Amersham Pharmacia Biotech) and kept on 4°C until they were run on an ABI 377 automated sequencer (Gene Codes Corp.), using the water run-in protocol as described in the User Bulletin of the ABI Prism 377 DNA Sequencer (PE Biosystems, December 7, 1999). The consensus sequences that were used in further analyses, were retrieved by combining the forward and reverse sequences in Sequencher 4.05 (Genes Codes Corp.).

## Sequence alignment and phylogenetic analyses

The COI sequences were imported in BioEdit v7.0.5 (Hall, 1999) and subsequently aligned using the

*Table 1.* Primers used for amplifying COI in Epitoniidae

| Primers for COI region | AT | Primer seq. | Primer length | Reference |
|---|---|---|---|---|
| Folmer Universal primer Forward: LCO-1490 | 45 | 5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3' | 25-mer | Folmer et al., 1994 |
| Folmer Universal primer Reverse: HCO-2198 | 45 | 5'-TAA ACT TCA GGG TGA CCA AAA ATC A-3' | 25-mer | Folmer et al., 1994 |
| Wentletrap specific primer Forward: WenCOI-for | 51 | 5'-TAT AAT GTA ATT GTA ACT GCT CA-3' | 23-mer | Newly developed primer |
| Wentletrap specific primer Reverse: WenCOI-rev | 51 | 5'-GGG TCA AAA AAT GAA GTA TT-3' | 23-mer | Newly developed primer |


Host cnidarians:
Hard corals
Hard corals
Hard corals
Sea anemones
Fungiidae
Euphylliidae
Dendrophylliidae
Actiniaria
localities
Epitoniid species
Cirsotrema
Gyroscala
Epitonium
Cycloscala
Epidendrium
Epifungium
Surrepifungium
multiperforata
lamellosa
angulatum
scalare
clathratulum
clathrus
ancillottoi
crenulata
sordidum
aureum
hartogi
lochi
twilae
ulu
pseudotwilae
marki
pseudolochi
adgravis
adscabra
nielsi
adgranulosa
hoeksemai
patamakanthini
ingridae
costulatum = c
oliverioi = o
A
B
C
D
E
F
G
H

Clustal-W plugin in the default parameter settings. The alignment was than exported in nexus format and MacClade 4.0 (Maddison and Maddison, 2000) was used for manual editing of the alignment. The codon positions were identified by checking the amount of variation. The positions were than calculated. A translation to amino acids was made using the *Drosophila* genetic code and the protein sequence was checked for stop codons. The alignment is available from the authors. The only samples included in this data set that may be miss-identified, because the shells in question closely resemble each other (A. Gittenberger and E. Gittenberger, 2005), are those of *Surrepifungium costulatum* and *S. oliverioi*.

The homogeneity of base frequencies in the sequences was tested. Paup* 4.0b10 (Swoford, 2002) was used to perform a chi-square for the complete data set, and for the first, second and third codon positions separately. To test for the presence of phylogenetic signal we did the G1 skewness statistic based on 1000 random trees (Hillis and Huelsenbeck, 1992). MrModeltest 2.2 (Nylander, 2004) was used to calculate a best fitting model for the data. Likelihoods for 24 models of evolution were calculated using PAUP* and the command file provided with MrModeltest. MrBayes 3.1 (Ronquist and Huelsenbeck, 2003) was used for Bayesian inference analysis.

Bayesian inference was performed with five incrementally (T = 0.20) heated Markov chains and a cold one, which were run 4,000,000 generations and sampled once every 50 generations, using the best-fit model for nucleotide substitution as suggested by MrModeltest output. Standard deviations (SD) between posterior probabilities of both simultane runs were observed to identify the burnin of suboptimal trees. SD value below 1% was considered significant convergence. The remaining trees were than imported in PAUP* and a majority rule consensus tree with compatible groupings was calculated.

*Fig. 2.* Majority rule consensus tree with compatible groupings, resulting from a Bayesian inference analysis. The ancestral species A-H are indicated underneath the branches. Hosts are indicated as photos on those lineages that do not show a mayor host switch, i.e. a switch between coral families or corals and sea anemones, assuming maximum parsimony. See fig. 1 for locality abbreviations: fr, France; nl, The Netherlands; ns, north Sulawesi, Indonesia; ko, Komodo, Indonesia; ma, Maldives; pa, Palau; ss, south Sulawesi, Indonesia; th, Thailand; vi, Vietnam; wa, Wakatobi, Indonesia.

**Results and discussion**

The COI alignment of a stretch of 503 bases contains 211 variable positions 201 of which are potentially parsimony informative. The data set showed no stop codons. A single triplet gap was found in the sequence of *Epifungium twilae* from the Spermonde archipelago, Indonesia. The data set has a highly significant phylogenetic signal, as is indicated by the G1 skewness test, i.e. g1= -0.509. Base frequencies in the complete data set and in the first and second codon positions, are significantly homogeneous across taxa, i.e. P = 1 in all cases. The third codon position has a strong AT bias as is shown in base frequencies (A = 0.35, C = 0.06, G = 0.13, T = 0.46). The best fit model of nucleotide substitution proved to be the General Time Reversal model, including the proportion of invariant sites and gamma shape parameter (GTR + I + G). Bayesian analysis showed a convergence (SD < 0.01) of both simultaneous runs after approximately 3.5 billion generations.

The molecular analyses (fig. 2) support the three nominal, epitoniid genera *Epidendrium*, *Epifungium* and *Surrepifungium* as monophyletic groups. Furthermore, the identification of the individual snails on the basis of the criteria published by A. Gittenberger and E. Gittenberger (2005) was paralleled by the results of the analyses of the DNA sequences. Except for *Epifungium ulu*, all clades representing a species or a genus were supported by 100% or in rare cases by bootstrap values of at least 82%. The *E. ulu* sequences form a clade in the 50% consensus tree with compatible groupings (fig. 2), which is not significantly supported however, i.e. with a value of 43%, and should be considered therefore a "compatible grouping". The two sister clades that are combined here as *E. ulu* are supported by 100% each, however. These two clades represent exclusively specimens from Pacific Ocean localities, i.e. Indonesia and Palau, versus Indian Ocean localities, i.e. Maldives, Thailand and Egypt (Red Sea). Within these two clades a geographical pattern cannot be recognized. There seem to be two allopatric population groups of *E. ulu*, i.e. two panmictic gene pools that are separated by a geological barrier, with little or no gene-flow in between.

With very low support values (less than 60%) the epitoniid genera *Cycloscala*, *Epidendrium*, *Epifungium* and *Surrepifungium*, cluster within the *Epitonium*

clade, indicating that the latter taxon does not represent a monophyletic group in the actual interpretation in the literature. On the basis of such low support values in a Mr Bayes analysis, taking into account that many more alleged *Epitonium* species are known from shells only, additional conclusions on the status of this nominal genus would be premature. The most parsimonious, molecular phylogeny reconstruction (fig. 2) indicates that the ancestor of the Epitoniidae dealt with here was associated with sea-anemones, whereas only once in evolutionary history an epitoniid species switched to hard corals. That is surprising in view of the fact that it could be demonstrated experimentally, that at least under artificial circumstances in an aquarium the coral-associated species *Epifungium ulu* may switch its diet to sea-anemones when no corals are available (Bell, 1985). This induced change in host species was not accompanied by any clear disadvantages, the snails still completed an entire life cycle within 36 days (Bell, 1985). What mechanism[s] kept epitoniids from switching from sea-anemone to coral host species more often in evolutionary history remains unclear.

In conformity with A. Gittenberger and Hoeksema (chapter 10), the recent epitoniid species and their suggested ancestors are referred to as either specialists or generalists, dependent on being associated with either (1) only one or a monophyletic group of host species, or (2) some distantly related hosts. For a molecular phylogeny reconstruction of the coral host species, see A. Gittenberger, Reijnen and Hoeksema (chapter 10).The here molecular phylogeny reconstruction (fig. 1) indicates that ancestors [A], [B], [C], [E] and [F] have been generalists associated with Fungiidae. All species in the *Surrepifungium* lineage, descending from ancestor [C], have remained generalists associated with Fungiidae. The descendants of ancestor [F], i.e. the *Epifungium hoeksemai* lineage, also remained generalists associated with Fungiidae. The ancestor of the sister group of the *E. hoeksemai* clade, i.e. species [G], also remained associated with Fungiidae, but changed its life-history strategy in comparison to its ancestor [E] by becoming a specialist. All descendants of ancestor [G] remained specialists. Remarkably, ancestor [H] and its descendants, i.e. the *Epifungium hartogi* clade, changed from Fungiidae to Euphylliidae as coral hosts.

Like its ancestor [B], ancestor [D] was a generalist. It switched from an association with the Fungiidae to the Dendrophylliidae, however. All descendants of ancestor [D], i.e. the species in the *Epidendrium* clade, have remained generalists associated with Dendrophylliidae.

Here we refer to co-evolution as the evolutionary mechanism in which the evolution of one taxon, e. g. the family Epitoniidae, is influenced by the evolution of another, unrelated taxon, e.g. the phylum Cnidaria, and not necessarily vice versa. Co-evolution may have played a role in the evolutionary history of the clade including *Epifungium marki* and *E. adgravis* and the clade including *E. nielsi* and *E. adgranulosa.* The epitoniid sister species *E. marki* and *E. adgravis* are associated with *Fungia* spec. A and *Fungia gravis*, which are also sister species (A. Gittenberger, Reijnen and Hoeksema, chapter 3). Similarly, the sister species *E. nielsi* and *E. adgranulosa* are associated with two closely related fungiid clades, which may be sister clades (A. Gittenberger, Reijnen and Hoeksema, chapter 3), i.e. *Fungia* (*Pleuractis*) spp. and *Fungia* (*Wellsofungia*) *granulosa.* In both cases an application of the molecular clock model, combined with the phylogeny reconstructions of both the parasites and their hosts, would give more certainty. It could indicate to what extent the speciation events in both the corals and the snails are interdependent in time. However, at present no data are available to calibrate such a molecular clock for both phylogenies.

The conchological similarities between the coral- and sea-anemone-associated wentletraps indicate that parallel or convergent evolution has played a mayor role in the evolutionary history of this group (A. Gittenberger and E. Gittenberger, 2005). In some cases, as for example in *Epifungium twilae* and *E. pseudotwilae*, this convergent evolution is clearly adaptive. The shells of these two species are very similar in all aspects, and conspicuously broader than those of all other *Epifungium* species. With a support value of 98% molecular analyses indicate that *E. twilae* is more closely related to *E. ulu* than to *E. pseudotwilae*, however (fig. 2). The broad shells of *E. twilae* and *E. pseudotwilae* might have evolved in both species independently because of selection pressure by fish predators (A. Gittenberger and Hoeksema, chapter 10). Snails with broad shells may be more difficult to grasp, depending on the size of the mouths of the potential predator fishes. *Epifungium twilae* and *E. pseudotwilae* in general encounter more

of these predators than do the other *Epifungium* species because they are hosted by corals that have the potential of becoming relatively large, leaving space for fishes to get underneath them.

After the generically separate classification of the coral-associated, epitoniid taxa, the remaining so-called genus *Epitonium*, with *E. scalare* (L., 1758) as its type species, became more than ever an unsatisfactory clustering of species, next to somewhat better defined taxa, like *Cycloscala* Dall, 1889, *Cirsotrema* Mörch, 1852, and *Gyroscala* de Boury, 1887, all of which represented by at least one species in the molecular phylogeny reconstruction (fig. 2). This is also illustrated by the positions of the eastern Atlantic species *E. clathrus* (L., 1758) and *E. clathratulum* (Kanmacher, 1798), the type species of the nominal taxa *Clathrus* Oken, 1915, and *Hyaloscala* de Boury, 1890, respectively. These species look quite different in shell characters and are placed in separate subgenera by several authors (Fretter and Graham, 1982). They show up as sister species in the molecular phylogeny analysis (fig. 2), however. Obviously, far more species should be studied to achieve a more convincing, phylogenetically based classification of the Epitoniidae.

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