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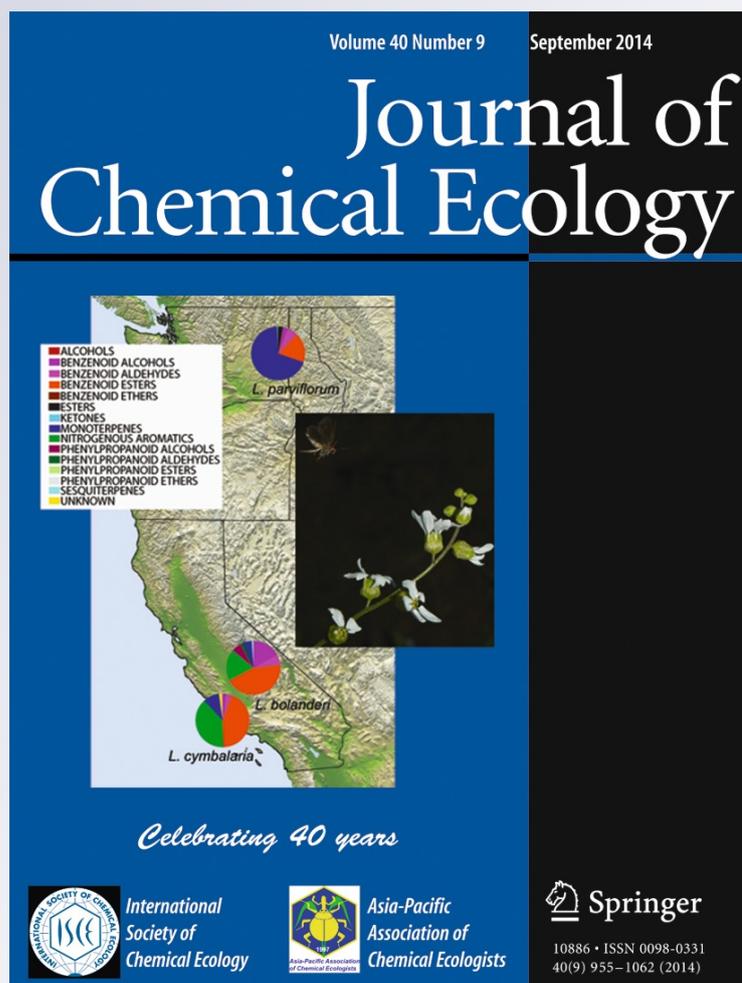
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# Defense in the Aeolidoidean Genus *Phyllodesmium* (Gastropoda)

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**Abstract** The genus *Phyllodesmium* (Aeolidoidea, Gastropoda) comprises shell-less marine snails, whose defense strategies are not well investigated yet. Here we report results of the first chemical investigation of *P. briareum*, as well as a re-investigation of *P. longicirrum* and *P. magnum*. Briarane diterpenes were isolated from *P. briareum*, and their origin could be traced to its prey organism *Briareum* sp. (Octocorallia). Considerable enrichment of the soft coral secondary metabolites in the slug was shown. Re-investigation of *P. magnum* led to isolation of cembrane diterpenes, 2-phenylethylamide, and furano sesquiterpenes. Sequestration of chemicals seems to have influenced speciation and evolution of *Phyllodesmium* species. Structural similarity or dissimilarity of particular slug metabolites suggests a closer, or more distant relationship of the respective *Phyllodesmium* taxa.

**Keywords** *Phyllodesmium* · Evolution · Chemoecology · Heterobranchia · Nudibranchia · Natural compounds

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## Introduction

Marine gastropods with probably 150.000 extant species usually are protected by their heavy calcified shell. However, within the various opisthobranch groups, shell reduction is a common feature and has occurred in nearly all lineages independently (Wägele and Klussmann-Kolb 2005), including the species richest group of the Nudibranchia with about 3000 species described. Shell reduction is often compensated by evolution of alternative defense strategies, including mimicking the surrounding environment, producing calcareous needles, production of acidic sulfates, and/or sequestration of toxic compounds from the respective food (Cattaneo-Vietti et al. 1993; Edmunds 1968, Edmunds 1987; Wägele et al. 2006).

One of the most enigmatic defense strategies is performed by the Aeolidoidea, which incorporate stinging cells (cnidocysts, then called kleptocnides) from their favored food (usually Hydrozoa) in a special morphological structure, the cnidosac (Greenwood 2009). These cnidosacs are highly exposed at the end of dorsal appendages (cerata) and thus in the preferred part for potential predators like fish and crabs. The evolution of this special morphological structure is certainly a key character that has led to a high radiation of the Aeolidoidea (Wägele 2004).

There is, however, one aeolidoidean genus, *Phyllodesmium*, which has switched to Octocorallia as a food source (Table 1). The appearance of *Phyllodesmium* is typical aeolid, with long cerata (Fig. 1a, b), but its cnidosacs have become useless, since they do not obtain kleptocnides from their food (Fig. 1c). Thus, other defense strategies have had to be developed. One intriguing feature contributing to protection within this group is certainly the stunning mimicry of most species with their home coral (Burghardt and Gosliner 2006; Rudman 1991). Probably the highest deceptive appearance can be observed in *P. rudmani*, where the cerata perfectly mimic the polyps of the home coral

**Table 1** *Phyllodesmium* spp. and their food sources. Not yet described *Phyllodesmium* species with the numbers 5, 8 and 12 refer to the designation as it is used in the seaslug forum species list: <http://www.seaslugforum.net/specieslist.htm>. Information on food is also taken from this website, when not observed by ourselves

<i>Phyllodesmium</i> species	Food source	Remarks
<i>P. briareum</i>	<i>Briareum</i> spp.	
<i>P. colemani</i>	<i>Tubipora musica</i>	
<i>P. crypticum</i> , <i>P. hyalinum</i> , <i>P. jakobsenae</i> , <i>P. kabiranum</i> , <i>P. lembehensis</i> , <i>P. lizardensis</i> , <i>P. parangatum</i> , <i>P. pecten</i> , <i>P. rudmani</i> , <i>P. tuberculatum</i> , <i>Phyllodesmium</i> sp. 12	Xeniid corals	
<i>P. guamensis</i> , <i>P. magnum</i>	<i>Sinularia</i> spp.	<i>P. magnum</i> was also observed feeding <i>Capnella</i>
<i>P. horridum</i>	<i>Acabaria</i> sp.	
<i>P. koehleri</i>	<i>Lemnalia</i> sp.	
<i>P. longicirrum</i>	<i>Sarcophyton trocheliophoru</i>	Also observed on <i>Lobophyton</i> sp.
<i>P. macphersonae</i>	<i>Erythropodium</i> sp.	
<i>P. poindimiei</i>	<i>Carijoa riisei</i>	
<i>P. serratum</i>	<i>Carijoa</i> sp., <i>Clavularia</i> sp., <i>Junceella</i> sp., <i>Steronephya</i> sp. and unidentified gorgonian	
<i>P. iriomotense</i> , <i>P. karenae</i> , <i>P. opalescens</i> , <i>P. undulatum</i> , <i>Phyllodesmium</i> sp. 8	Unknown (non zooxanthellate coral)	<i>Phyllodesmium</i> sp. 8 feeds on xanthellate coral
<i>Phyllodesmium</i> sp. 5	<i>Melithaea</i> (non zooxanthellate)	

(Fig. 1d). Currently, about 30 species of *Phyllodesmium* are documented, with 26 of them enlisted in the WoRMS database (<http://www.marinespecies.org>). Approximately half of the species form a monophyletic clade (Fig. 2) (Moore and Gosliner 2009, 2011; Wägele et al. 2010), and interestingly are specialized on the octocoral family Xenidiidae (Table 1).

Only recently the number of recognized species has increased due to several projects dealing with the photosynthetic ability of these slugs (Burghardt and Gosliner 2006; Burghardt et al. 2008a; Burghardt and Wägele 2004; Moore and Gosliner 2009; Wägele et al. 2010). Most of the octocoral food organisms of *Phyllodesmium* spp. use the single celled dinophyte *Symbiodinium* (usually referred to as zooxanthellae) to obtain energy via photosynthesis. Depending on the presence of zooxanthellae in the food and the ability to incorporate these, the color of the slugs varies from more transparent with tinges

to bluish and reddish (Fig. 1a; non zooxanthellate species), to brownish (Fig. 1b, d; zooxanthellate species). In earlier studies (Wägele 2004; Wägele et al. 2010), we assumed that the mutualistic symbiosis with *Symbiodinium* has driven speciation in this enigmatic genus, since photosynthates from the zooxanthellae may sustain the slugs for weeks to months (Burghardt et al. 2005, 2008b) and thus provide an advantage. However, the specific radiation of *Phyllodesmium* on Xenidiidae cannot be explained by this symbiotic relationship, and thus chemical defense strategies are discussed as additional factors that probably influenced evolution. Many octocorals, and especially some known as food sources of *Phyllodesmium*, like *Lemnalia*, *Sinularia*, *Sarcophyton*, *Briareum*, *Erythropodium*, and *Tubipora*, exhibit a high level of secondary metabolites (Berrue and Kerr 2009; Coll 1992; Iguchi et al. 1986; Kamel and Slattery 2005). This also applies to a xeniid member investigated earlier by us, *Heteroxenia* sp. (Affeld et al. 2009), which was recently reassigned to the genus *Bayerxenia* (Stemmer et al. 2013).

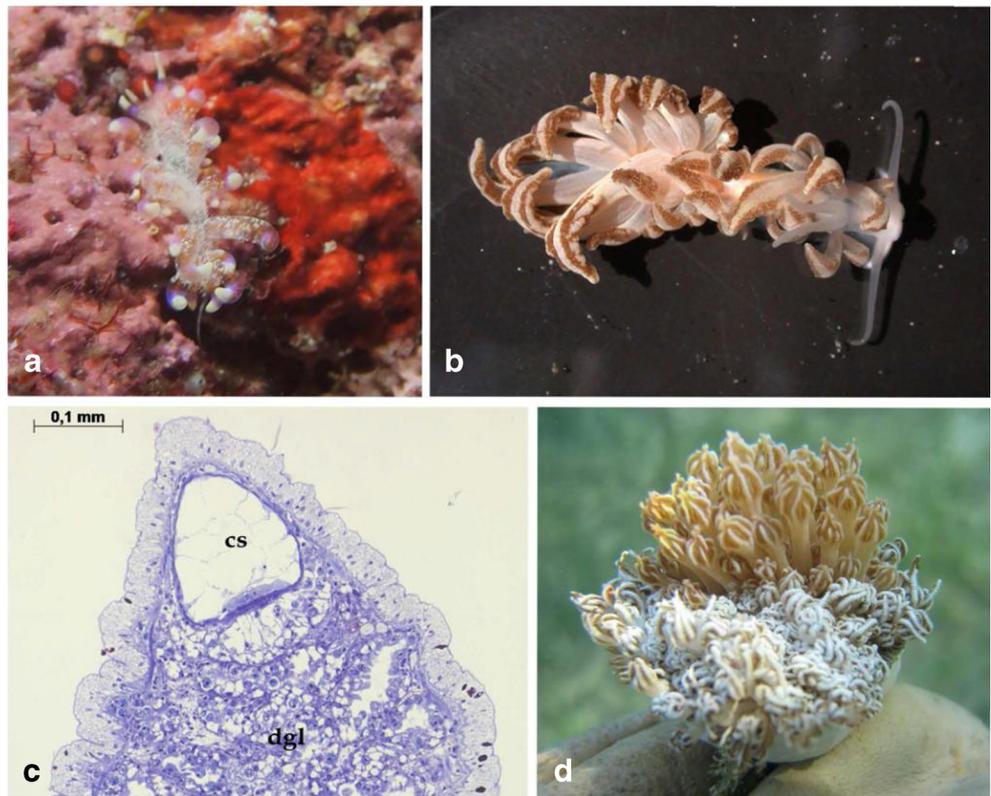
Here, we include new results on the sequestration of chemicals in a *Phyllodesmium* species not investigated before, and correlate this with the change of its food, as well as data on already investigated species from localities not yet targeted, in order to shed light on the ecology and evolution of the defensive system in these slugs. Most intriguing is the correlation of *Phyllodesmium* species relatedness with species relatedness of its octocoral prey. Table 2.

## Methods and Material

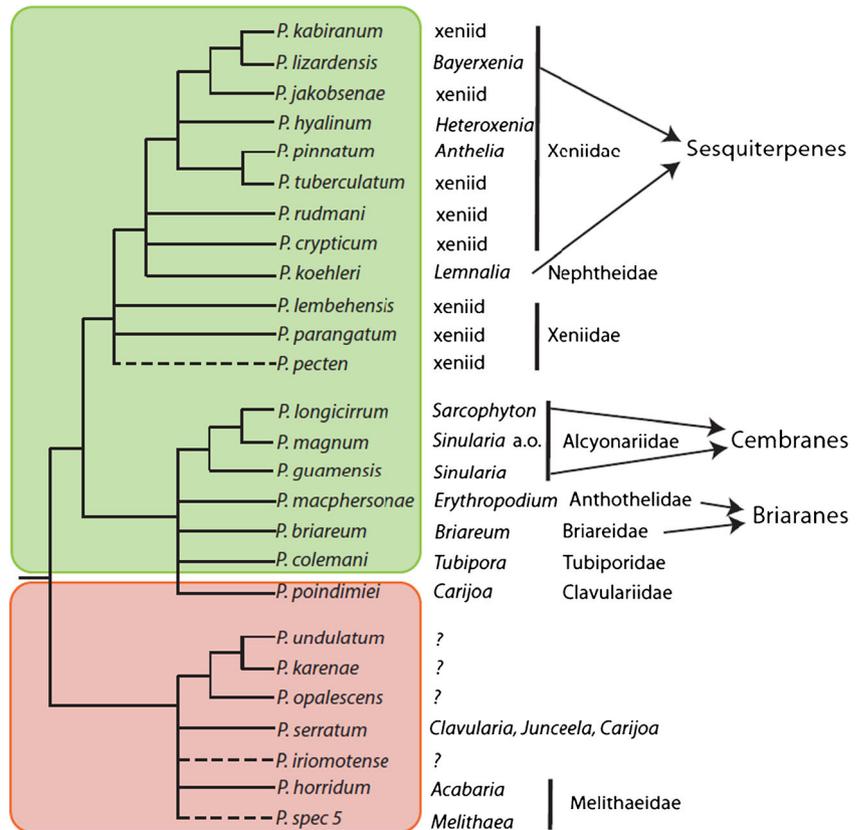
**Samples** The sample of *P. magnum* was commercially obtained and identified by H. Wägele. *Phyllodesmium briareum* and *Briareum* sp. also were commercially obtained and subsequently cultured in Wuppertal by S. Bleidissel. Juveniles and adults were preserved separately in order to obtain information on ontogenetic variances within the species. The sample of *P. longicirrum* was collected during a field trip to Lizard Island (Great Barrier Reef, Australia) by H. Wägele. All specimens were stored in ethanol (96 %) until further extraction and processing in the laboratories at the University of Bonn.

**General Procedures** All NMR spectra were recorded in MeOH-*d*<sub>4</sub> using BrukerAvance 300 DPX or BrukerAvance 500 DRX spectrometers, respectively. Spectra were referenced to residual solvent signals with resonances at  $\delta_{H/C}$  3.35/49.0. The fractions were purified on a Merck Hitachi HPLC system equipped with a L-6200A pump, L-4500A photodiode array detector, a D-6000A interface with D-7000A HSM software, a Rheodyne 7725i injection system, and a Waters Atlantis T3 RP<sub>18</sub> column (250 mm x 4.6 mm; 5  $\mu$ m). LC-ESIMS was performed using an Agilent 1100

**Fig. 1** **a** *Phylloidesmium poindimiei*, an azooxanthellate species here hiding between calcareous red algae and sponges. Note the transparency. **b** *P. jakobsenae*, a zooxanthellate species, usually associated with xeniid octocorals. **c** Histological slide (*P. lizardensis*) showing tip of a dorsal appendage with cnidosac devoid of cnidocysts. Cs cnidosac, dgl digestive gland. **d** *P. rudmani* (darker brown) sitting on top of its home coral, a xeniid octocoral (with lighter coloured polyps). Note the dorsal appendages of the slug which mimic the polyps perfectly



**Fig. 2** Cladogram of *Phylloidesmium* species combined from published phylogenetic analyses based on morphology (Moore and Gosliner 2009) and molecular data (Wägele et al. 2010; Moore and Gosliner 2011). Green (above): zooxanthellate, red (below): non zooxanthellate species. The food source is given in the right hand column. Dotted lines indicate putative position of respective species according to preliminary and unpublished results. Please note that *P. poindimiei* is a non zooxanthellate species grouping with zooxanthellate species. Arrows indicate relevant secondary metabolites from soft coral genera



**Table 2** *Phyllodesmium* species, their distribution and in the case of our own studies, the origin of the material, food source and secondary metabolites thereof (References are cited in text)

Species	Distribution/Origin	Food source	Secondary metabolites in slugs	Secondary metabolites in food organisms	Remarks/activity
<i>Phyllodesmium</i> spp. investigated previously					
<i>P. guamensis</i>	Guam, Micronesia	<i>Sinularia maxima</i> , <i>Sinularia polydactyla</i> , <i>Sinularia</i> sp.	Cembrane diterpene: 11 $\beta$ -acetoxypukalide ( <b>16</b> )	Terpenes	<b>1</b> showed feeding deterrence against pufferfish
<i>P. lizardensis</i>	Only known from Lizard Island, Australia	<i>Bayerxenia</i> sp.	Sesquiterpenes: (+)-3 $\beta$ -hydroxy- $\alpha$ -muurolene ( <b>17</b> ), (+)-3 $\beta$ -acetoxo- $\alpha$ -muurolene ( <b>18</b> ), (+)- $\alpha$ -muurolene ( <b>19</b> )	( <b>2</b> ) and ( <b>3</b> )	Metabolites did not show any antibacterial or antifungal activities
<i>Phyllodesmium</i> spp. investigated in this study					
<i>P. longicirrum</i>	Indonesian Archipelago, Great Barrier Reef / Lizard Island	<i>Sarcophyton trocheliophorum</i>	Cembrane diterpenes: (+)-thunbergol ( <b>13</b> ), (+)-epoxythunbergol ( <b>14</b> ), diterpene alcohol <b>15</b>	<b>13–15</b> , further terpenes e. g. <b>9–12</b>	Cembrane diterpenes show feeding deterrence
<i>P. magnum</i>	Widespread, Indian and western Pacific oceans/ This study: Aquaristik Bonn, with Philippinean origin	<i>Sinularia</i> spp., <i>Capnella</i> sp. our observation	Sesquiterpenes: asteriscane ( <b>20</b> ), africanane ( <b>21–22</b> ), elemene ( <b>23</b> ), selinane ( <b>24–25</b> ) and furano ( <b>26–27</b> ) types. This investigation: cembrane diterpenes: 11 $\beta$ ,12 $\beta$ -epoxypukalide ( <b>4</b> ), 11- <i>epi</i> -sinulariolide acetate ( <b>5</b> ), 2-phenylethylamide ( <b>6</b> ), furanosesquiterpenes <b>7–8</b>	Terpenes	Isolated metabolites are known from <i>Sinularia</i> spp. and <i>Leptogorgia</i> spp. <b>4</b> showed feeding deterrence and antifouling, <b>5</b> is cytotoxic against P-388, HT-29, HL-60 with ED <sub>50</sub> 1.2, 1.9 and 0.8 $\mu$ g/ml respectively, <b>6</b> displayed a trial stimulance.
<i>P. briareum</i>	Tropical western Pacific, Indonesian Archipelago / This study: Aquaristik Wuppertal with Indonesian origin	<i>Briareum</i> spp.	No lit. data Briarane diterpenes: brianthein W ( <b>1</b> ), excavatolides B and C ( <b>2–3</b> ), trace amounts of more terpenoids	Briarane diterpenes Our results: <b>1–3</b>	<b>1</b> is cytotoxic against P-388 - ED <sub>50</sub> 0.76 $\mu$ g/ml, <b>3</b> cytotoxic activity against P-388 - ED <sub>50</sub> 0.3 $\mu$ g/ml and KB, A-549, HT-29 - ED <sub>50</sub> 1.9 $\mu$ g/ml cell lines

\* data in original study is confusing concerning assignment to *P. magnum* or *P. guamensis*

system with an API 2000 Triple Quadrupole LC/MS/MS with ESI source (Applied Biosystems/MDS Sciex) and a photodiode array detector. HRESIMS were recorded on a LTQ Orbitrap mass spectrometer. UPLC-HRMS analysis was performed on Thermo Scientific Qexactive with HESI source, Phenomenex Kinetex RP<sub>18</sub> column (150 mm $\times$ 4.6 mm, 2.6  $\mu$ m, 100 Å).

**Extraction and Isolation** The ethanolic storage solutions were combined with the methanol extract of the slug biomass and the solvents were evaporated. After liquid-liquid separation of the methanolic extracts between water and ethyl acetate (EtOAc), the organic phases were further separated using RP<sub>18</sub> VLC or SPE with methanol/water mixtures as eluent. In the case of *P. briareum* (very low amount of extract, i.e., 40 mg), the obtained extract after liquid-liquid separation was directly submitted to a RP<sub>18</sub> HPLC column in order to minimize losses. The identification of pure metabolites was

performed by comparing their spectroscopic data with those in literature reports.

The extraction of *P. briareum* (approx. 15 animals) gave 84 mg crude extract, which was separated between water and 3  $\times$  30 ml EtOAc. The latter phase (40 mg) was submitted to RP-HPLC (methanol:water, 65:35; column: Waters Atlantis T3, 1 ml/min) to yield 1 mg (2.5 % of EtOAc solubles) brianthein W (**1**), 3 mg (7.5 %) excavatolide B (**2**), and 2 mg excavatolide C (5.0 %) (**3**).

The extraction of *P. magnum* (1 animal) gave 117 mg crude extract. After separation between water and 3  $\times$  30 ml EtOAc, the lipophilic phase (41 mg) was further fractionated on Bakerbond RP<sub>18</sub> SPE 2000 (J.T.Baker), using a methanol-water step gradient (1. 50:50, 2. 75:25, 3. 90:10, 4. 100:0, 30 ml for each fraction). Fraction 2 (10.4 mg) was submitted to RP-HPLC (methanol:water, 70:30; column: Waters Atlantis T3, 1 ml/min) and yielded 3 mg (7.3 % of EtOAc solubles) 11 $\beta$ ,12 $\beta$ -epoxypukalide (**4**) and 2 mg (4.9 %) 11-

episinulariolide acetate (**5**). HPLC separation (methanol:water, 75:25; column: Waters Atlantis T3, 1 ml/min) of fraction 3 (8.7 mg) led to isolation of 1 mg (2.4 %) 2-phenylethylamide **6** and 1 mg of a mixture of furano sesquiterpenes **7** and **8**.

The crude extract of *P. longicirrum* (4.52 g) was separated between water and 3 × 100 ml EtOAc. The lipophilic part (2.16 g) was fractionated by vacuum liquid chromatography (VLC) over Polygoprep 60–50 RP<sub>18</sub> stationary phase (Macherey-Nagel) using gradient elution from 20:80 (methanol:water) to 100 % methanol to yield 11 fractions. Fractions 3–8 (6 mg, 16 mg, 207 mg, 763 mg, 696 mg, 160 mg, respectively) were analyzed with UPLC HRMS using the following solvent gradient program: A. water+0.1 % formic acid and B. acetonitrile+0.1 % formic acid; 5 % B 0–2 min, 5–95 % B 2–14 min, 95 % B 14–17 min, 95–5 % B 17–22 min. The column oven was adjusted to 30 °C.

The crude extract (40 mg) of the specific food specimen of *P. briareum*, the octocoral *Briareum* sp. was obtained by extracting a small piece (approx. 1 cm<sup>3</sup>) of soft coral exhaustively with methanol. Separation between 20 ml water and 3 × 20 ml EtOAc resulted in 1 mg of EtOAc solubles, which were submitted to UPLC-HRMS using the same conditions as already applied for analysis of *P. longicirrum* fractions (see above).

**Phylogenetic Analyses** The here proposed phylogeny derives from those reported by Moore and Gosliner 2009, 2011, and Wägele et al. 2010. Information was combined and nodes were depicted as resolved only when this was without conflict among the various analyses. Positioning of some species is based on unpublished data of *S. Bleidissel* (*P. pecten*, *P. iriomotense*). In cases where no analysis is available, the position was assumed according to available morphological data (*Phyllodesmium* spec 5; see Rudman, 2002). The position of these latter species is indicated with a dotted line in Fig. 2.

## Results

***Phyllodesmium briareum* and *Briareum* spec** The ethyl acetate solubles of the *P. briareum* extract yielded upon separation (using RP-HPLC) brianthein W (**1**) (1 mg, 2.5 % of the ethyl acetate solubles), and in the more polar fractions excavatolide B (**2**) (3 mg, 7.5 %) and excavatolide C (**3**) (2 mg, 5 %, Fig. 3). Comparison of our spectroscopic data with those described in the literature confirmed the structures of these compounds (Cardellina et al. 1984; Sheu et al. 1998). Spectroscopic data of other metabolites obtained from the *P. briareum* extract indicated the presence of further related terpenoids, but due to low yields unambiguous identification of the latter was not possible. Juveniles from our cultivation efforts and adult species of *P. briareum* were extracted

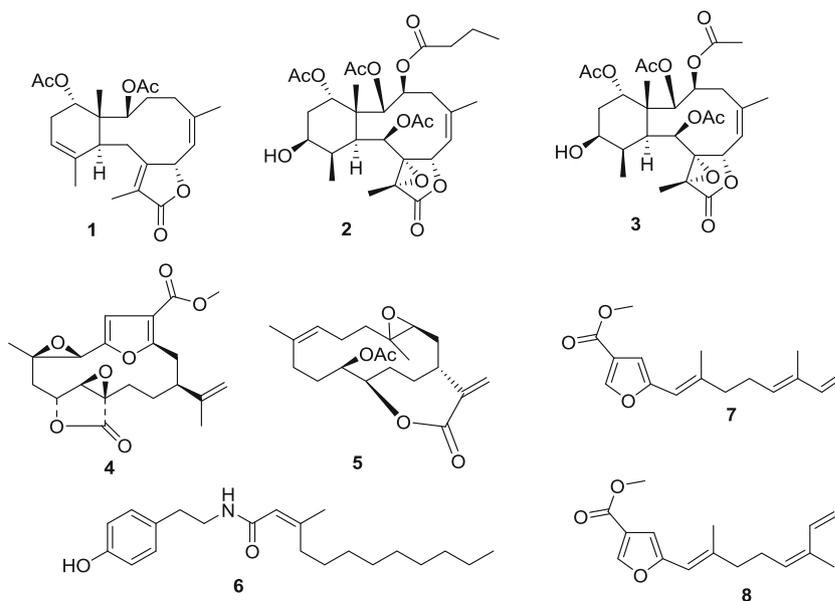
separately, and extracts compared using NMR spectroscopy. However, no chemical differences among specimens of different ontogenetic phases were discernible.

The food source of *P. briareum* also was investigated. Despite the small amount of the available *Briareum* spec. extract, HR LCMS analysis clearly showed the presence of the compounds brianthein W, excavatolide B, and excavatolide C (**1–3**) in this soft coral, and thus confirmed the origin of these metabolites in the slugs as derived from their specific food item (Fig. 4). Even though mass spectrometric analysis did not provide data for a reliable quantification of secondary metabolites in this particular soft coral extract, we compared the amount of identified *P. briareum* isolates with that of published data. Thus, Sheu et al. (1998) obtained 1.85 % excavatolide B (**2**) and 0.44 % excavatolide C (**3**) from the crude ethyl acetate extract of *Briareum*. The yield of brianthein W (**1**) was 0.47 % of a crude dichloromethane extract of *B. polyanthes* (Cardellina et al. 1984; Grode et al. 1983). This indicates a significant enrichment of octocoral metabolites in our *P. briareum* samples, where concentration ranges from 2.5–7.5 % (Table 3).

***Re-investigation of *Phyllodesmium magnum* and *P. longicirrum* Specimens*** Several cembrane diterpenes were present in the ethyl acetate part of the methanolic extract of *P. magnum*. RP-HPLC of SPE fraction 2 yielded 11 $\beta$ ,12 $\beta$ -epoxypukalide (**4**) (3 mg, 7.3 % of the ethyl acetate solubles) and 11-episinulariolide acetate (**5**) (2 mg, 4.9 %). Surprisingly, a more lipophilic SPE fraction contained an unusual 2-phenylethylamide, i.e., **6** (ca. 1 mg, 2.4 %). The structures of these compounds were established by comparing their spectrometric data with those of literature reports (Hsieh et al. 2003; Kazlauskas et al. 1980; Ksebati et al. 1984). Besides these compounds, a not further separated mixture of furano sesquiterpenes **7** and **8** was identified, as NMR resonances observed for this mixture clearly were comparable to those of furano sesquiterpenes described previously for *Simularia* corals (Bowden et al. 1983). Again, the concentrations of the here isolated compounds **5** and **6** in *P. magnum* were much higher than those reported for the soft corals (for **5**: 4.9 % vs. 0.19 %, for **6**: 2.4 % vs. 0.018 %, see also Table 3). A significant enrichment of these metabolites in *Phyllodesmium* is thus likely.

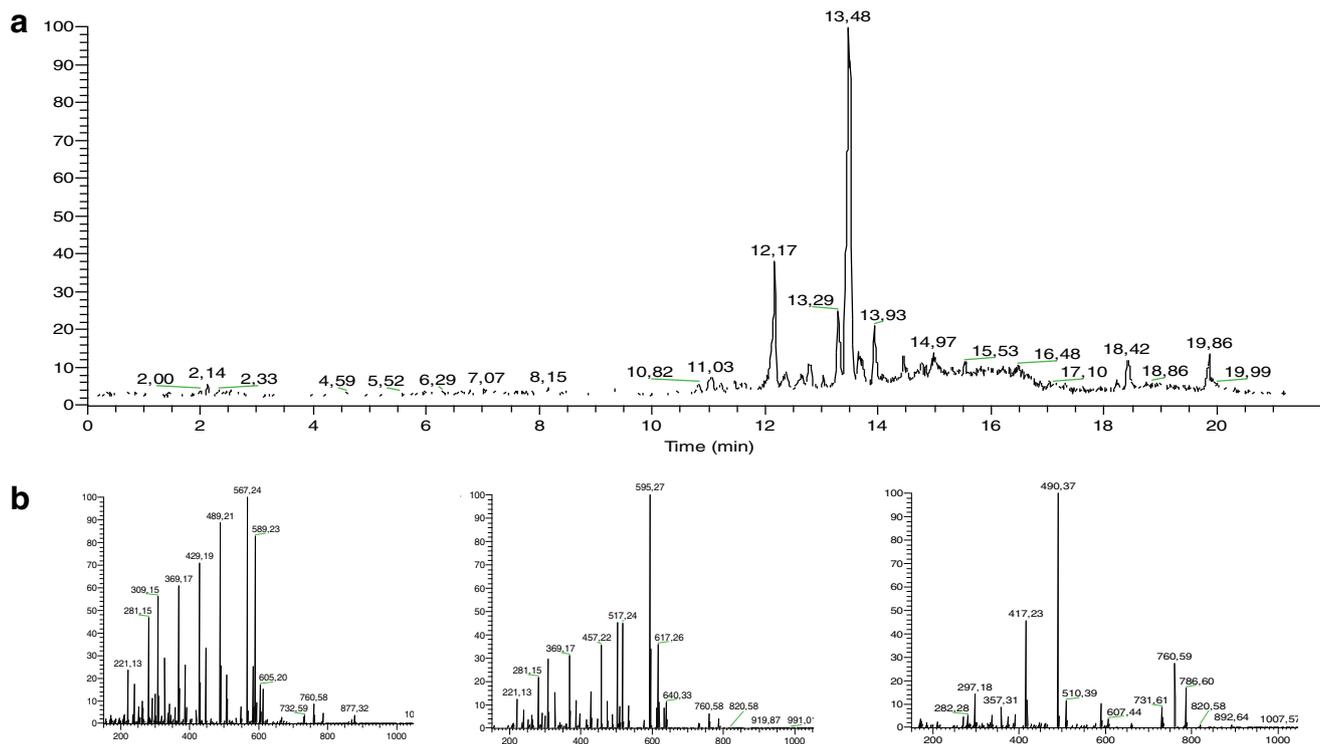
After extraction and liquid-liquid partitioning, the lipophilic residue of *P. longicirrum* was separated by RP-VLC and 10 fractions obtained. Using NMR experiments, VLC fractions 3–8 were found to contain terpenoid metabolites. In order to verify the presence of those cembranes reported in the previous study of *P. longicirrum* by Coll et al. (1985), VLC fractions 3–8 were analyzed using UPLC-HRMS. Complex chromatograms were obtained indicating the presence of far more secondary metabolites than reported previously. The molecular masses point towards known metabolites from

**Fig. 3** Secondary metabolites isolated in this study from *Phylloidesmium briareum* (1–3) and *P. magnum* (4–8)



*Sarcophyton* spp.: e.g., molecular masses of 287.2367 Da (M+H) and 301.2160 Da (M+H) may relate to sarcophytonin A (9) and B (10), respectively (Kobayashi et al. 1979; Kobayashi and Hirase 1990). A molecular mass of 317.2110 Da (M+H) may indicate the presence of sarcophine (11) (Bernstein et al. 1974) or sarcophytolide (12) (Uchio et al.

1983) (see Fig. 5). The exact structures could not be retrieved from the high resolution mass data alone, since the presence of stereo- and positional isomers with an identical molecular mass has to be considered. However, and most importantly, the previously reported cembranes 13–15 (Coll et al. 1985) could not be found in the complex metabolite mixture (Fig. 6).



**Fig. 4** **a** LCMS chromatogram of the ethyl acetate solubles of the *Phylloidesmium briareum* food coral *Briareum* sp. **b** MS spectra of LC-peaks at 12.17 (left), 13.48 (middle) and 13.93 min (right) proving the

presence of excavatolide C (3) M+H 567.24, excavatolide B (2) M+H 595.27, and brianthin W (1) M+H 417.23, respectively

**Table 3** Quantity of secondary metabolites in *Phyllodesmium* species as compared to the respective food source

<i>Phyllodesmium</i> species	Isolated secondary metabolites	% Occurrence in prey organisms	% Occurrence in slugs	Enrichment factor
<i>P. guamensis</i>	11 $\beta$ -acetyypukalide ( <b>16</b> )	1.1 ( <i>S. maxima</i> ) <sup>*</sup>	2.6–7.2 <sup>*</sup>	2.4–6.5
<i>P. lizardensis</i>	(+)-3 $\beta$ -hydroxy- $\alpha$ -muurolene ( <b>17</b> ),	**	9.9	
	(+)-3 $\beta$ -acetoxy- $\alpha$ -muurolene ( <b>18</b> ),		2.1	
	(+)- $\alpha$ -muurolene ( <b>19</b> )		34.1	
<i>P. magnum</i>	11-episinulariolide acetate ( <b>5</b> )	0.19 ( <i>S. flexibilis</i> )	4.9	25
	2-phenylethylamide ( <b>6</b> )	0.018 ( <i>S. flexibilis</i> )	2.4	133
<i>P. briareum</i>	brianthein W ( <b>1</b> )	0.47 ( <i>B. polyanthes</i> )	2.5	5.3
	excavatulide B ( <b>2</b> )	1.85 ( <i>B. excavatum</i> )	7.5	4.1
	excavatulide C ( <b>3</b> )	0.44 ( <i>B. excavatum</i> )	5.0	11.3

<sup>\*</sup> Dry weight in *P. guamensis* and percentage of lipophilic extract for *P. lizardensis*, *P. magnum*, *P. briareum*; <sup>\*\*</sup> no data available, terpene content should be comparable to other soft corals mentioned in this table

**Phylogeny of *Phyllodesmium*** To date, only three phylogenetic analyses of *Phyllodesmium* are available, with one based purely on morphological data and including 26 species (Moore and Gosliner 2009) and two based on molecular data, including 19 and 14 species, respectively (Moore and Gosliner 2011; Wägele et al. 2010). Obviously, the three analyses only partly overlapped with regard to included species and branching pattern. Additionally, resolution of clades differed within the various analyses. For this study, we combined the information on resolved nodes and only depicted a node as resolved, when this was without conflict among the various analyses. Our combined tree (Fig. 2) comprises 26 species.

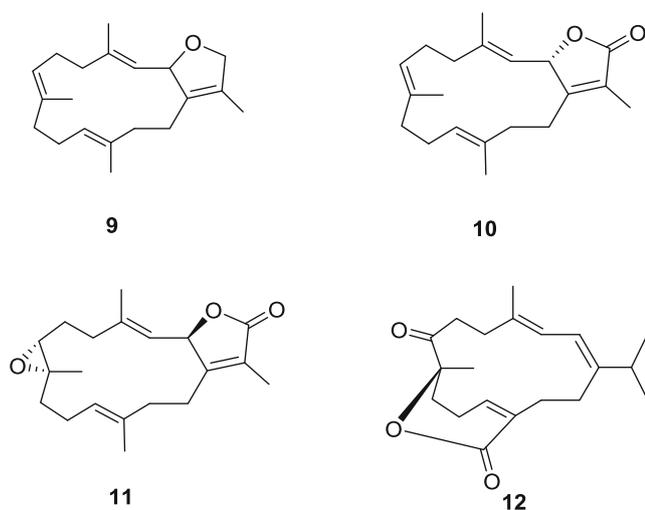
## Discussion

**Secondary Metabolites of *Phyllodesmium* Species and their Food Sources** *Phyllodesmium briareum* was originally named after its preferred food, i.e., Scleraxonian octocorals of the

genus *Briareum*. To date, no data have been reported concerning the presence and/or type of secondary metabolites in *P. briareum* that may function in defensive chemistry. Soft corals of the genus *Briareum* are, however well investigated revealing a range of highly substituted diterpenoid compounds (Berrue and Kerr 2009). Chemical investigation of *P. briareum* indeed has revealed that it contains briarane diterpenes **1–3**, which are typical for its food source. Brianthein W (**1**) was described as a metabolite from *B. polyanthes* (Cardellina et al. 1984), excavatulide B (**2**) and excavatulide C (**3**) are both known from *B. excavatum* (Sheu et al. 1998).

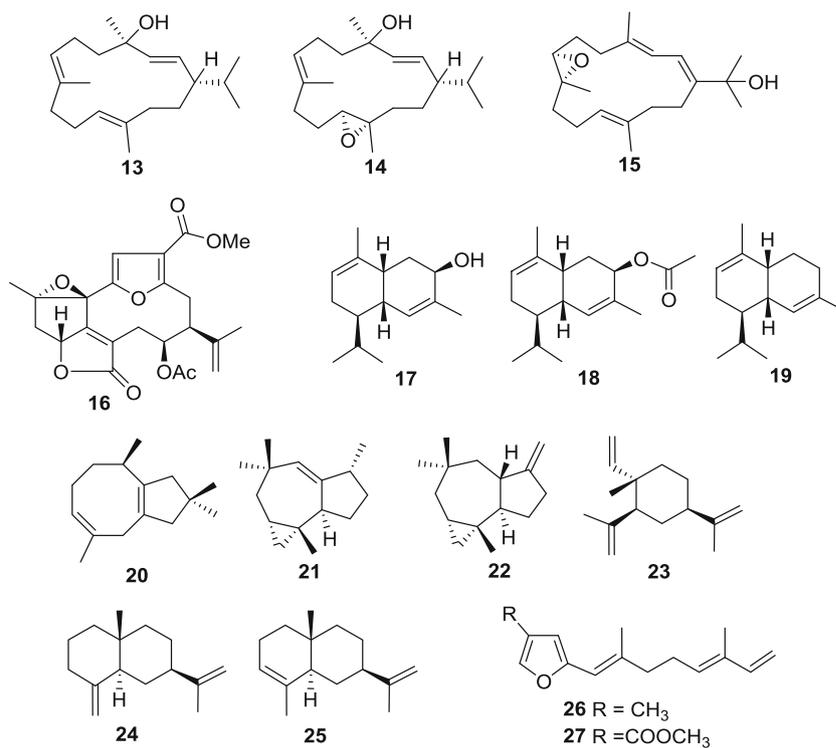
*Phyllodesmium magnum* with a size of up to 14 cm, is besides *P. longicirrum*, one of the biggest *Phyllodesmium* species. A recent chemical investigation of a specimen collected in the South China Sea led to the isolation of compounds **20–27** (Fig. 6; Mao et al. 2011). These reported metabolites are all sesquiterpenes, belonging to the asteriscane-, africanene-, elemene-, selinane- and furano-type of terpenoid compounds, respectively (Fig. 7). In our sample of *P. magnum*, we identified furano sesquiterpenes as reported earlier by Mao et al. (2011). Additionally, 11 $\beta$ ,12 $\beta$ -epoxyypukalide (**4**), first described from the Holaxonian *Leptogorgia setacea* by Ksebati et al. (1984), and 11-episinulariolide acetate (**5**), a *Simularia flexibilis* metabolite, were obtained (Hsieh et al. 2003). We report here the first cembranes from *P. magnum*. Most astonishing, however, is the presence of 2-phenylethylamide, i.e., **6** in a concentration as high as 2.4 %, which was reported earlier in a study by Kazlauskas et al. (1980) as a minor metabolite in *S. flexibilis* (0.018 % of the dichloromethane extract) (Table 3). Taking literature reports and our results together, *P. magnum* shows the broadest array of terpenoids so far, including sesqui- and diterpenes, and, found here for the first time, the amide **6**.

An earlier investigation of *P. longicirrum* collected at Orpheus Island, Great Barrier Reef, (Coll et al. 1985) led to isolation of the three cembranoid compounds **13–15**, all

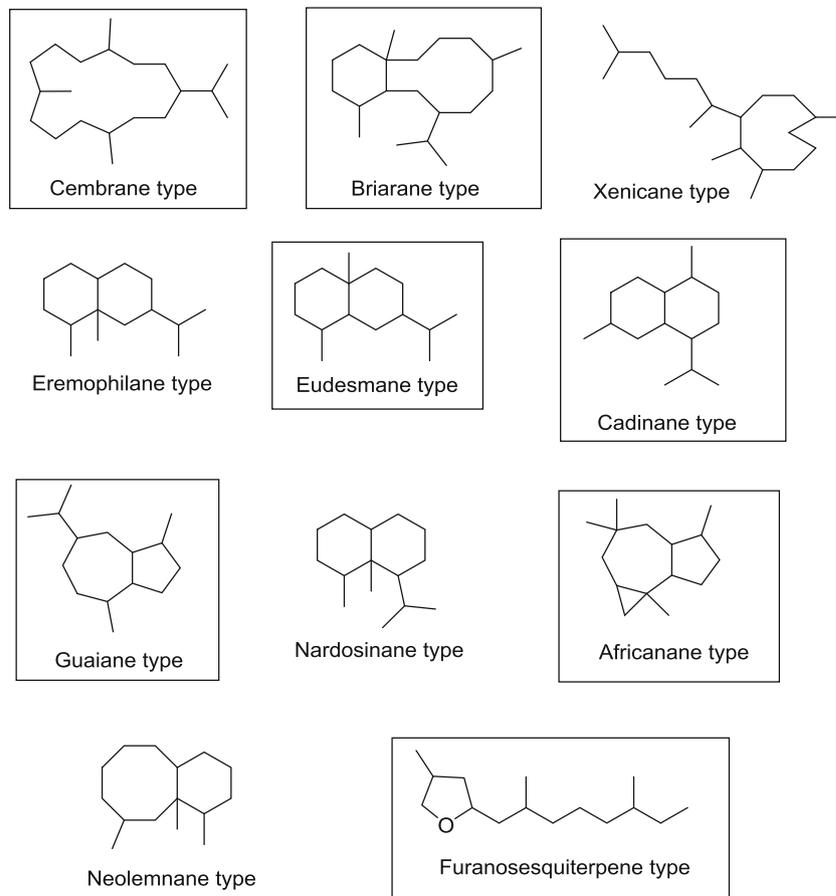


**Fig. 5** Cembrane diterpenes from *Sarcophyton* spp

**Fig. 6** Structures of reported secondary metabolites from *Phyllodesmium longicirrum* (13–15), *P. guamensis* (16), *P. lizardensis* (17–19), and *P. magnum* (20–27)



**Fig. 7** Most relevant terpene structural classes found in food sources of slugs of the genus *Phyllodesmium*. Structural types in boxes have to date also been detected in *Phyllodesmium* species



known from the slug's food soft coral *Sarcophyton trocheliophorum*. In our study these cembranes could not be detected at all, even though our sample also originated from the Great Barrier Reef (however, from Lizard Island, about 500 km North to Orpheus Island). Instead, UPLC-MS data indicate the occurrence of other cembrane derivatives.

To date, only five representatives of the genus *Phyllodesmium* have been chemically investigated, i.e., *P. briareum*, *P. guamensis*, *P. lizardensis*, *P. longicirrum* and *P. magnum* (Affeld et al. 2009; Coll et al. 1985; Mao et al. 2011; Slattery et al. 1998). From these animals a large variety of secondary metabolites (13–27) were isolated (Fig. 6), often in higher quantities than from the respective food source from which they originated. With the exception of the rare amide 6, all thus far isolated secondary metabolites from the genus *Phyllodesmium* are terpenes. The predominance of terpenes is in agreement with former results on aeolids and other related cladobranchs (Putz et al. 2009). Terpenes encountered can be grouped into the diterpenes such as 13–15 in *P. longicirrum*, 16 in *P. guamensis*, 4–5 in *P. magnum*, and 1–3 in *P. briareum*, and sesquiterpenes such as 17–19 in *P. lizardensis*, and 20–27, as well as 7–8 in *P. magnum*. The structural classes of the detected sesquiterpenes are in general not very unusual, e.g., compounds 17–19 belong to cadinane sesquiterpenes, which are also widely distributed in terrestrial plants. A variety of sesquiterpenes have been isolated (Mao et al. 2011), having asteriscane (20), africanane (21–22), elemene (23), selinane (24–25) skeletons or are furano sesquiterpenes (26–27) (Fig. 6).

Most of the diterpenes can be attributed to the cembranoids, a long known class of metabolites with a prominent 14-membered ring skeleton. Concerning marine habitats, cembranoids are to date described from different soft corals, i.e., Alcyonacea, Holaxonia, and Pennatulacea (Berrue and Kerr 2009; Guerriero et al. 1990; Kamel and Slattery 2005) but they also occur in terrestrial higher plants, e.g., in the resin of pines and in tobacco plants (Hamm et al. 2005; Kimland and Norin 1968; Weinheimer et al. 1979). Here, we report for the first time on briarane-type diterpenes from *Phyllodesmium*. Briaranes are biosynthetically related to cembranes in that they may arise from the latter by further cyclization to produce a fused 6- and 10-membered cyclic skeleton (Li et al. 2012). Briaranes have to date been found in marine organisms only, especially in Scleraxonian and Calcaxonian soft corals (Berrue and Kerr 2009).

**Locality and Population Variations** in secondary metabolite profiles depending on the collection site have been reported for various slugs such as *Aplysia dactylomela* (Wessels et al. 2000). To our knowledge, only one study exists that addresses chemical variability in *Phyllodesmium* (Slattery et al. 1998). Such studies are important in order to understand intraspecific variability in one and the same slug population or between

different populations. Slattery et al. (1998) investigated *P. guamensis* collected in various locations on Guam and although the animals were found feeding on different *Simularia* corals, the chemical constituents of the slugs were consistently the same, i.e., they all contained the sequestered 11 $\beta$ -acetoxypukalide (16). Even though the here presented data on chemical variability are still preliminary, they indicate for *P. magnum* and *P. longicirrum* differences in secondary metabolite profiles possibly due to differing localities, seasonality or food sources. It has to be said though, that often the meager biomass available limits the possibilities to determine the exact structure of all metabolites. Variability of chemical components in the food, or even the lack of compounds in some octocoral populations usually consumed by *Phyllodesmium* species, might have driven the evolution of the genus by forcing the slugs to switch to other octocoral species providing similar compounds.

**Accumulation of Toxins** According to literature data, sea slugs seem to accumulate the compounds, as judged from the much higher quantity in the slugs compared to the food (Table 3). For example, the 11 $\beta$ -acetoxypukalide (16) concentration in *Simularia polydactyla* and *S. maxima* was reported to be 1.1 % of the colony dry mass (Slattery et al. 2001), whereas *P. guamensis* accumulated this compound in ranges of 2.6–7.2 % of dry body mass (Slattery et al. 1998). Dietary derived secondary metabolites (17–19) isolated by Affeld et al. (2009) made up to 34 % of the crude extract of *P. lizardensis*, that is orders of magnitude higher than the usual metabolite content in soft corals. Our results also confirm the postulated enrichment of soft coral secondary metabolites in the slugs (Table 3).

**Bioactivity and Defensive Role** A direct proof of the ecological function of food-extracted secondary metabolites in *Phyllodesmium* is still missing. Some of the compounds identified in *Phyllodesmium* species were, however formerly investigated for their bioactivity. A significant cytotoxic effect was demonstrated for briarane 1 (P-388 - ED<sub>50</sub> 0.76  $\mu$ g/ml) and 3 (P-388 - ED<sub>50</sub> 0.3  $\mu$ g/ml; KB, A-549, HT-29 - ED<sub>50</sub> 1.9  $\mu$ g/ml), as well as for cembrane 5 (P-388 ED<sub>50</sub> - 1.2  $\mu$ g/ml, HT-29–1.9  $\mu$ g/ml, HL-60–0.8  $\mu$ g/ml) (Hsieh et al. 2003; Sheu et al. 1996, 1998). Qi et al. (2009) reported antifouling and antifeedant properties of briaranes from the octocoral *Junceella juncea*. Various pharmacological activities of briaranes such as anti-inflammatory, cytotoxic, and antibacterial effects also have been described (Sung et al. 2002). Cembranoid secondary metabolites have to some extent been studied concerning their ecological roles. Bernstein et al. (1974) reported the isolation of the cembranoid sarcophine from *Sarcophyton glaucum* and postulated it to be one of the substances responsible for protecting the soft coral from being devoured by numerous predators in the coral reef environment. Slattery et al. (1998) described the sequestered 11 $\beta$ -

acetoxypukalide (**16**) in *P. guamensis* as a feeding deterrent against pufferfish at a concentration (0.5 % of dry mass), which is even lower than the abundance of **16** in the slug tissue. Of the here isolated cembranes, 11 $\beta$ ,12 $\beta$ -epoxypukalide (**4**) was reported to be deterrent against fish predators, as well as antifouling at concentrations found in living coral (Epifanio et al. 1999, Epifanio et al. 2006). Kamel and Slattery (2005) summarized in their review the known terpenoids from *Sinularia* and the ecological and pharmacological properties thereof, such as antimicrobial, anti-inflammatory, and cytotoxic activities. Furanoic acid is structurally closely related to the *Phyllodesmium*-derived furano sesquiterpenes **7** and **8**, and showed anti-inflammatory effects (Grace et al. 1994). Moreover, furano sesquiterpenes such as dendrolasin are known to function as alarm pheromones and defensive agents in some ant species (Breitmaier 2005). A recent publication describes the sesquiterpene  $\alpha$ -muurolene (**19**) emitted by the plant *Ficus racemosa* as a volatile organic compound responsible for attracting wasps for pollinating the plant (Borges et al. 2013). Thus, in general it can be stated that many terpenoid constituents play a role in chemical communication within complex environments. Finally, the 2-phenylethylamide **6** has been reported to be an arial stimulant (Kazlauskas et al. 1980). Moreover, the unusual acid-moiety of **6** has been found as part of the methylester (Z)-3-methylododec-2-enoate in the terrestrial endophyte *Epichloë*. The compound attracts *Botanophila* flies, which are important for the sexual reproduction of the fungus (Steinebrunner et al. 2008). In light of these data, it seems likely that the compounds identified by us and others in *Phyllodesmium* spp. are relevant in the biological system, including defense and antifouling, as well as blending in to the substrate on which these animals are mainly living. Ongoing studies may demonstrate this.

**Ecology of *Phyllodesmium*** The number of extant *Phyllodesmium* species known to date is 30, and does not seem high when compared with other successful marine taxa. The snail genus *Conus*, e.g., is described as having more than 500 extant species and represents the largest marine gastropod group (Duda and Kohn 2005). However, when comparing the genus *Phyllodesmium* with related slug genera, like *Austraeolis* or *Godiva*, the species number is up to 10 times higher. *Phyllodesmium* species successfully incorporate and accumulate chemicals provided by the various octocoral groups, possibly for their defense. This might have been one of the successful traits that allowed higher radiation in this group, compared to the related forms that rely on incorporation of cnidocysts from their hydrozoan prey.

Figure 6 displays the main terpene classes found in those Octocorallia that have been reported as food sources for the genus *Phyllodesmium*. It seems likely that slugs feeding on closely related octocorals, thus extracting similar compounds,

also share a close relationship. *Vice versa* we can hypothesize that particular metabolites are chemical markers that indicate a phylogenetic relationship (Fig. 2). All members that have been identified to enrich cembranes (*P. longicirrum*, *P. magnum*, and *P. guamensis*) form a monophyletic clade. *Phyllodesmium longicirrum* feeds on soft corals of the genus *Sarcophyton*, and *P. magnum* and *P. guamensis* on *Sinularia* spp. According to published octocoral phylogenies (McFadden et al. 2006), *Sarcophyton* and *Sinularia* are closely related. A closer relationship between *P. briareum* and *P. macphersonae* cannot be shown yet, due to the lack of a phylogenetic signal in the applied genes, but both species feed on scleraxonian octocorals with briarane compounds, i.e., *Briareum* and *Erythropodium* (Berrue and Kerr 2009), respectively. We, thus, assume that future investigations will reveal that *P. macphersonae* contains briaranes and is a sister group - or at least in close - relationship of these two *Phyllodesmium* taxa. It also is noteworthy, that the biosynthetic relationship between cembranes and briaranes is mirrored in the proposed close phylogenetic relation between the *Phyllodesmium* taxa storing these chemicals.

Within the *Phyllodesmium* radiation, one big clade of at least 12 species has speciated on XenIIDae as their prey (Fig. 2). *Phyllodesmium lizardensis* and its food source *Bayerxenia* is the only system described chemically in detail within this clade (Affeld et al. 2009). This species was shown to extract sesquiterpenes from *Bayerxenia*, compounds, which we found missing in another sympatric xeniid octocoral genus, *Xenia*. We postulate that all *Phyllodesmium* species belonging to this xeniid feeding group will prefer members of the XenIIDae that can provide structurally similar terpenoid constituents. However, there is one exception here, *P. koehleri*. This species, located within the xeniid feeders, preys on the nephthaeid *Lemnalia*. Bishara et al. (2008) mentioned the closer phylogenetic relationship of *Lemnalia* nephthaeids to XenIIDae than to other members of the nephthaeid group based on molecular analyses, which is in line with our own unpublished analyses. Kitagawa et al. (1986) isolated guaiane sesquiterpenes from *Xenia* species identical to those reported from *Nephthea chabrolii* and *Lemnalia africana*. Furthermore, a range of sesquiterpenes, e.g., of the africanol (Tursch et al. 1974), nardosinane, neolemnane, and eremophilane type (Bishara et al. 2008; Izac et al. 1981; Jurek and Scheuer 1993) have been isolated. The similarity of the chemical structure of secondary metabolites in XenIIDae and *Lemnalia* would certainly facilitate a food switch.

## Conclusion

In summary, appropriate defense mechanisms that include sequestration of chemicals and unusual feeding strategies

(including energy supply *via* incorporation of photosynthetically active micro-algae of the genus *Symbiodinium*) appear to have influenced speciation and evolution of *Phyllodesmium* species. The chemical structures encountered in *Phyllodesmium* share many structural features. This may facilitate food switches to other closely related food items and in this way enhance speciation. The structural similarity or dissimilarity of particular slug metabolites also seems to suggest closer, or more distant relationship of the respective *Phyllodesmium* taxa.

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