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Morphological and genetic analyses of xeniid soft coral diversity (Octocorallia; Alcyonacea)

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Abstract Studies on the biodiversity and evolution of octocorals are hindered by the incomplete knowledge of their taxonomy, which is due to few reliable morphological characters. Therefore, assessment of true species diversity within abundant and ecologically important families such as Xeniidae is difficult. Mitochondrial genes provide a reliable solution to this problem for a wide range of taxa. However, low mutation rates of the mitochondrial DNA in octocorals result in insufficient variability for species discrimination. We compared the variation of a fragment of the Signal Recognition Particle 54 gene (SRP54, proposed for octocorals) and the mitochondrial ND6/ND3 marker among members of the xeniid genera *Ovabunda*, *Xenia*, *Heteroxenia* and *Bayerxenia*. The mean uncorrected pairwise sequence divergence was 39 % for SRP54 compared to 2 % for ND6/ND3. Morphological assignments were not always supported by genetics: Species diversity was underestimated (one case) or overestimated, probably reflecting intraspecific polymorphisms or hinting at recent speciations. ND6/ND3 is informative for some species-level assignments, whereas SRP54 shows the variability needed for species delimitations within this understudied taxon. Our results on both genes show their potential for evolutionary and biodiversity studies in Xeniidae.

Keywords Xeniidae · SRP54 · ND6/ND3 · Molecular marker · Systematics · Phylogeny

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Introduction

Species within the alcyonacean soft coral family Xeniidae, in particular the genera *Ovabunda,* Alderslade (2001), *Xenia,* Lamarck (1816), *Bayerxenia,* Alderslade (2001) and *Heteroxenia,* Kölliker (1874), are essential members of tropical reef communities throughout the Indo-West-Pacific and the Red Sea. They play an important role in recolonising destroyed reef areas even before algae can grow (Reinicke 1995). Furthermore, their natural products are of interest to biochemists (Affeld et al. 2009; Anta et al. 2002). Since xeniid soft corals have a mutualistic symbiotic relationship with the dinoflagellate *Symbiodinium,* ecologists are concerned that they may be affected severely by climate change (Strychar et al. 2005). Xeniids are also an important food source for stenophagous nudibranchs, especially the genus *Phyllodesmium* (Burghardt and Waegele 2004; Burghardt et al. 2008a), and a radiation of the genus on this enigmatic soft coral family has been discussed recently (Waegele et al. 2010).

The main problem in soft coral research is the identification of most corals to species level due to few reliable

morphological characters in this understudied taxon (Reinicke 1995; Alderslade 2001; Berntson et al. 2001; McFadden et al. 2006). Hence, species identification prior to any phylogenetic or ecological studies is challenging, and using genetic markers additional to morphological characters becomes very important (Hebert et al. 2003a, b). In most eukaryotic organisms, mitochondrial DNA (mtDNA), in particular a fragment of the cytochrome c oxidase subunit I (COI), has been established as a barcode marker because of its relatively high mutation rate (Hebert et al. 2003b, 2004; Ward et al. 2005) and has been used successfully in phylogenetic analyses on the genus and family level (e.g. Hülsken et al. 2011). Unfortunately, COI is less variable in Cnidaria (Anthozoa and Medusozoa) than in most other taxa (Huang et al. 2008). Particularly, the substitution rate in the mitochondrial genome of the anthozoans has been reported to be about 100 times slower than in other metazoan taxa (France and Hoover 2001, 2002; Shearer et al. 2002). Therefore, the discrimination power of mitochondrial markers is limited within anthozoans (Hellberg 2006; McFadden et al. 2010a, b; Park et al. 2012; Shearer and Coffroth 2008).

In recent studies, even the fastest evolving mitochondrial regions lacked the resolution necessary to distinguish soft coral species within genera (McFadden and Hutchinson 2004; McFadden et al. 2006). For example, McFadden et al. (2006) used different mitochondrial markers (e.g. ND2, msh1) for phylogenetic analysis of octocorals, which unfortunately showed insufficient intrageneric resolution within the Xeniidae. COI and the extended mitochondrial DNA barcode COI+igr1+msh1, recently analysed by McFadden et al. (2010b), could not distinguish *Xenia* and *Heteroxenia*. In contrast, the nuclear DNA of anthozoans appears to accumulate mutations at the same rate or even faster as compared to other animal groups (Hellberg 2006; Chen et al. 2008). Consequently, most molecular coral research currently focuses on nuclear DNA markers for species-level studies. Several nuclear intron markers have been investigated for this purpose in scleractinian corals (Hatta et al. 1999; van Oppen et al. 2000, 2001, 2004), with limited success in only few taxa. The multi-copy marker ITS-1 (Internal Transcribed Spacer) has been used to reconstruct species-level relationships in some octocoral and scleractinian genera (Fukami et al. 2004; McFadden et al. 2001; McFadden and Hutchinson 2004; van Oppen et al. 2000, 2002; Forsmann et al. 2010; Flot et al. 2011) but the marker is not always reliable for species-level phylogeny (Vollmer and Palumbi 2004; Wei et al. 2006). Recently, Concepcion et al. (2008) introduced a hypervariable, single-copy nuclear marker that can be used for phylogenetic investigation of closely related soft coral taxa: the 54-kDa subunit of the Signal Recognition Particle (SRP54). Their results revealed a great number of differences between sequences even between closely related taxa. Pairwise sequence divergences within octocorals were 8–13 times greater for SRP54 than for mtDNA. Among scleractinian corals, within the same genus, even up to 2.8 % pairwise sequence divergence was found for the SRP54 fragment, whereas no variation was found for the mtDNA markers at all. Concepcion et al. (2008) sequenced eight individuals of xeniids and reported up to 17 % pairwise sequence divergence among specimens based on a 129-bp SRP54 alignment.

Due to the reported high variability, we applied this promising and highly variable nuclear marker to analyse species-level assignments and phylogenetic relationships between species of the xeniid genera *Ovabunda, Xenia*, *Heteroxenia* and *Bayerxenia*. We also analysed the slow evolving ND6/ND3 gene fragment for assessing and comparing its suitability for biodiversity studies on xeniid soft corals. Furthermore, we tested and discussed species assignments based upon morphological characters with the SRP54 and ND6/ND3 markers. Finally, we discussed the potential use of these genes as possible barcode markers.

Methods

Sampling

Specimens were collected from selected sites in the Indo-Pacific and the Red Sea by SCUBA diving or snorkelling (Table 1; Supplementary material). Samples were initially preserved in either absolute ethanol for further DNA analysis or in 7 % formalin in seawater for morphological investigation. All samples were transferred again into absolute ethanol and stored at 4 °C. Alternatively, when no suitable ethanol for preservation was available, a high percentage spirit such as gin was used for specimen preservation.

Species determination and morphological analyses

Taxonomic identification to the genus and, when possible, species level was performed by applying character analysis according to Reinicke (1995, 1997) and the systematic revisions from Alderslade (2001).

Morphology was investigated under a stereomicroscope. For investigation of sclerites, whole tissue material was dissolved in 10 % NaClO. The sclerites were then washed in distilled water, centrifuged, mounted and finally spattered with gold. Electron microscope images were taken with a scanning electron microscope (ZEISS DSM 950, Fig. 2).

DNA analysis

The DNeasy® Mini Kit (Qiagen, Valencia, CA) was used to extract octocoral genomic DNA according to the animal

Table 1 Species, GenBank accession numbers (Gbn #) from SRP54 and ND6/ND3, collection site and date for the xeniid specimens analyzed. The column "clade" refers to the genetically defined phylogenetic lineage based on the SRP54 alignment

Species	SRP Gbn #	ND6/ND3 Gbn #	Clade	Collection date	Collection site
Ovabunda faraunensis_04	KC341803	KC341874	1	2008.04.25	Dahab: Housereef
Ovabunda faraunensis_06	KC341805	KC341875	1	2008.04.25	Dahab: Housereef
Ovabunda faraunensis_09	KC341813	KC341876	1	2008.04.26	Dahab: Three pools
Ovabunda faraunensis_12	KC341804	KC341877	1	2008.04.28	Dahab: Moray eel garden
Ovabunda faraunensis_13	KC341808	KC341878	1	2008.04.30	Dahab: Three pools
Ovabunda faraunensis_17	KC341806	KC341879	1	2008.04.30	Dahab: Three pools
Ovabunda faraunensis_22	KC341815	KC341880	1	2008.05.05	Dahab: Lagoon
Ovabunda macrospiculata_02	KC341807	KC341881	1	2008.04.27	Dahab: Moray eel garden
Ovabunda macrospiculata_03	KC341814	KC341882	1	2008.05.05	Dahab: Lagoon
Ovabunda macrospiculata_06	KC341818	KC341883	1	2008.05.05	Dahab: Lagoon
Ovabunda macrospiculata_07	KC341819	KC341884	1	2008.05.05	Dahab: Lagoon
Ovabunda macrospiculata_08	KC341812	KC341885	1	2008.05.05	Dahab: Lagoon
Ovabunda macrospiculata_09	KC341816	KC341886	1	2008.05.13	Dahab: Lagoon
Ovabunda macrospiculata_10	KC341809	KC341887	1	2008.05.13	Dahab: Lagoon
Ovabunda macrospiculata_11	KC341810	KC341888	1	2008.05.13	Dahab: Lagoon
Ovabunda macrospiculata_12	KC341811	KC341889	1	2008.05.13	Dahab: Lagoon
Ovabunda macrospiculata_13	KC341817	KC341890	1	2008.05.13	Dahab: Lagoon
Xenia sp.*1_01*	KC341820	KC341891	2	2007.07.13	Palawan: Dimakya
Xenia sp.*2_01*	KC341821	KC341892	2	2007.07.15	Palawan: Dimakya
Xenia sp.*3_*03		KC341893			
Xenia sp.*3_04*	KC341822	KC341894	3	2007.06.28	Lizard Island: on pipe
Xenia sp.*3_05*	KC341823	KC341895	3	2007.06.29	Lizard Island: on pipe
Xenia sp.*3_06*	KC341824	KC341896	3	2007.06.29	Lizard Island: on pipe
Xenia sp.*3_08*	KC341825	KC341897	3	2007.07.01	Lizard Island: Loomis beach
Xenia sp.*3_09*	KC341831	KC341898	3	2007.07.01	Lizard Island: Loomis beach
Xenia sp.*3_10*	KC341832	KC341899	3	2007.07.01	Lizard Island: Loomis beach
Xenia sp.*3_11*	KC341826	KC341900	3	2007.07.01	Lizard Island: Loomis beach
Xenia sp.*3_12*	KC341827	KC341901	3	2007.07.01	Lizard Island: Loomis beach
Xenia sp.*3_13*	KC341828	KC341902	3	2007.07.01	Lizard Island: Loomis beach
Xenia sp.*4_02*	KC341829	KC341903	4	2007.06.29	Lizard Island: on pipe
Xenia sp.*4_03*	KC341830	KC341904	4	2007.07.02	Lizard Island: on pipe
Xenia sp.*5_01*	KC341773	KC341905	5	2007.07.01	Lizard Island: Loomis beach
Xenia sp.*6_01(a/b)*	KC341774/75	KC341906	7	2007.07.13	Palawan: Dimakya
Heteroxenia ghardaqensis_02	KC341794	KC341865	4	2008.04.28	Dahab: Moray eel garden
Heteroxenia ghardaqensis_03	KC341795	KC341866	4	2008.04.28	Dahab: Moray eel garden
Heteroxenia ghardaqensis_04	KC341796	KC341867	4	2008.04.28	Dahab: Moray eel garden
Heteroxenia ghardaqensis_05	KC341797	KC341868	4	2008.04.28	Dahab: Moray eel garden
Heteroxenia ghardaqensis_06	KC341798	KC341869	4	2008.04.28	Dahab: Moray eel garden
Heteroxenia ghardaqensis_07	KC341799	KC341870	4	2008.05.05	Dahab: Lagoon
Heteroxenia ghardaqensis_08	KC341800	KC341871	4	2008.05.05	Dahab: Lagoon
Heteroxenia ghardaqensis_09	KC341801	KC341872	4	2008.05.05	Dahab: Lagoon
Heteroxenia guardaqensis_10	KC341802	KC341873	4	2008.05.05	Dahab: Lagoon
Bayerxenia sp.*1_01*	KC341757	KC341833	5	2007.08	Bali: Uthamuda
Bayerxenia sp.*1_02*	KC341758	KC341834	5	2007.08	Bali: Uthamuda
Bayerxenia sp.*1_03*	KC341759	KC341835	5	2007.08	Bali: Uthamuda
Bayerxenia sp.*1_04*	KC341760	KC341836	5	2007.08	Bali: Uthamuda
Bayerxenia sp.*1_05*	KC341761	KC341837	5	2007.08	Bali: Uthamuda
Bayerxenia sp.*1_06*	KC341762	KC341838	5	2007.08	Bali: Tempokchantik

Table 1 (continued)

Species	SRP Gbn #	ND6/ND3 Gbn #	Clade	Collection date	Collection site
Bayerxenia sp.*1_07*	KC341763		5	2007.08	Bali: Tempokchantik
Bayerxenia sp.*1_09*	KC341764	KC341839	5	2007.08	Bali: Tempokchantik
Bayerxenia sp.*1_10*	KC341765		5	2007.08	Bali: Tempokchantik
Bayerxenia sp.*1_11*	KC341766	KC341840	5	2007.08	Bali: Tempokchantik
Bayerxenia sp.*2_01*	KC341767	KC341841	5	2007.06.22	Lizard Island: Casuarina beach
Bayerxenia sp.*2_03*	KC341768	KC341842	5	2007.06.28	Lizard Island: Loomis beach
Bayerxenia sp.*2_05*	KC341769	KC341843	5	2007.06.28	Lizard Island: Loomis beach
Bayerxenia sp.*2_06*	KC341770	KC341844	5	2007.06.29	Lizard Island: on pipe
Bayerxenia sp.*2_09*	KC341771	KC341846	5	2007.06.29	Lizard Island: on pipe
Bayerxenia sp.*2_04*	KC341789	KC341850	6	2007.06.29	Lizard Island: on pipe
Bayerxenia sp.*2_08*	KC341790	KC341845	6	2007.07.01	Lizard Island: Loomis beach
Bayerxenia sp.*2_10*	KC341791	KC341847	6	2007.07.01	Lizard Island: Loomis beach
Bayerxenia sp.*2_11*	KC341792	KC341851	6	2007.07.02	Lizard Island: on pipe
Bayerxenia sp.*2_12*	KC341793	KC341848	6	2007.06.25	Lizard Island: Loomis beach
Bayerxenia sp.*3_01*	KC341772	KC341849	5	2007.06.22	Lizard Island: Vicky's Reef
Heteroxenia fuscescens_01	KC341777	KC341853	8	2008.04.28	Dahab: Moray eel garden
Heteroxenia fuscescens_02	KC341778	KC341854	8	2008.04.28	Dahab: Moray eel garden
Heteroxenia fuscescens_03	KC341779	KC341859	8	2008.04.28	Dahab: Moray eel garden
Heteroxenia fuscescens_04	KC341780	KC341860	8	2008.04.29	Dahab: Front of Sinai Divers
Heteroxenia fuscescens_05	KC341781	KC341855	8	2008.04.29	Dahab: Front of Sinai Divers
Heteroxenia fuscescens_06	KC341782	KC341856	8	2008.04.29	Dahab: Front of Sinai Divers
Heteroxenia fuscescens_07	KC341783	KC341857	8	2008.05.05	Dahab: Lagoon
Heteroxenia fuscescens_08	KC341784	KC341861	8	2008.05.07	Dahab: Nabaq
Heteroxenia fuscescens_10	KC341785	KC341862	8	2008.05.07	Dahab: Nabaq
Heteroxenia fuscescens_11	KC341776	KC341863	8	2008.05.07	Dahab: Nabaq
Heteroxenia fuscescens_12	KC341786	KC341864	8	2008.05.07	Dahab: Nabaq
Heteroxenia fuscescens_13	KC341787	KC341852	8	2008.05.13	Dahab: Lagoon
Heteroxenia fuscescens_14	KC341788	KC341858	8	2008.05.13	Dahab: Lagoon

tissue protocol. Approximately 2 mg of tissue was cut from a polyp of each sample with sterilised scissors and dried on a sterile paper. Instead of 2×200 μl AE-buffer as outlined in the protocol, only 2×100 μl was added to the spin column and incubated for 5 min before elution. Protocols for amplifying the fragment of the mitochondrial NADH subunit 6 and NADH subunit 3 (ND6 and ND3) were adapted from McFadden et al. (2004). For the amplification of SRP54, three different primer pairs were tested: one primer pair published by Concepcion et al. (2008) and two newly designed pairs (Table 2). The new primers were designed using sequence alignment information of xeniid sequences from GenBank (Concepcion et al. 2008). Due to the great variability, several wobble bases were introduced to the primers (Table 2). The optimal annealing temperature was assessed using a gradient PCR. A concentration of 0.03 U/μl of EuroTaq polymerase (Biocat) and a final concentration of 1.5 mM $MgCl_2$ were used for the PCR. Thermal cycling conditions were: an initial denaturation at 94 °C for 2 min followed by 35 cycles, each with 94 °C for 20 s, annealing at the species-specific temperature for 30 s and an extension at 72 °C for 25 s, followed by a final 5-min extension step. Using an annealing temperature of 45 °C for samples identified as *Heteroxenia* and *Bayerxenia* produced the most distinct bands, whereas the best results were achieved at a higher temperature of 56 °C for samples identified as *Xenia* and *Ovabunda*. For the elimination of residual oligonucleotides and dNTPs from the PCR mixes, 3 μl of shrimp alkaline phosphatase (SAP, 1 U/μl) and 0.75 μl exonuclease I (Exo, 1 U/μl) were added to 16.25 μl of the PCR products, following an incubation at 37 °C for 15 min and an inactivation step at 80 °C for 15 min. The purified products were sent to the commercial sequencing companies Macrogen, Inc. (Seoul, Korea) and GATC-Biotech (Konstanz, Germany).

Phylogenetic analysis

DNA sequences were assembled, corrected and edited using the GENEIOUS 5.5 (Drummond et al. 2012).

Table 2 Primers developed for amplification of a SRP54 fragment in xeniids. Only primers SRP54-f2/r1 amplified successfully for xeniid specimens in this study

Primer name	Sequence
CrSRP54f	5′-CGAACTAAAATTAGAAGAAAACGAAG-3′
CrSRP54r	5′-TCATACATGTCTCTCAGCGTAAAC-3′
SRP54-f1	5′-GAAGGACTGATNGATAAAGTC-3′
SRP54-f2	5′-GAAGGACTGATNGATAAAGTCA-3′
SRP54-r1	5′-CAAWGTRAAYTGYCCTGAAGT-3′
SRP54-r2	5′-TGGAATTGNTCATACATGTC-3′

Multiple sequence alignment was performed using MAFFT v6.240 (Katho et al. 2002). *Asterospicularia* sp. (EU006867 for SRP54 and AF530513 for ND6/ND3) (Alderslade 2001), a member of the clearly distinct genus within the Xeniidae, was selected from GenBank as an additional member of the ingroup. Representatives of the genera *Muricea* (*M. purpurea*, GQ293342), *Alaskagorgia* sp. (GQ293337) and *Alcyonium* (*A. digitatum*, AF530498) were chosen as outgroup taxa for the ND6/ND3 analysis and *Sympodium caeruleum* (EU006855) for the SRP54 analysis. Maximum likelihood (ML) bootstrap trees with 10,000 replicates were computed with RAxML, version 7.3.0, using the GTRCAT model (Stamatakis 2006) with four rate categories. Bayesian tree reconstructions were performed using MrBayes v3.1.2 (Huelsenbeck 2001) by computing 10,000,000 generations in two runs, with four chains each. Trees were sampled every 100th generation. A burn-in was determined (1) by requiring that split frequencies were <0.01 and (2) by inspecting the time series of log posterior probabilities to ensure convergence. Appropriate DNA substitution models were determined using the Akaike information criterion (AIC) implemented in jModeltest (Posada 2008) (Table 3).

Results

Morphological analysis and taxonomy

In total, six different morphospecies of *Xenia*, two of the genus *Ovabunda*, two different morphospecies of *Heteroxenia* and three of the genus *Bayerxenia* could be identified. The four genera were distinguished on the basis of presence or absence of polyp dimorphism (Fig. 1) as well as the size and structure of sclerites (Fig. 2, Table 4) (see Alderslade 2001). Species assignment was based on colony morphology (general shape, size and organisation) and polyp morphology (especially pinnules and sclerite structure). All characters are listed in Table 4. Picture tables of all morphospecies are provided as an online resource and will be further referred to as "Figure (S1–6)". Voucher specimens have been deposited in the Museum of Stralsund (Germany) if not entirely used for DNA or sclerite analyses. Voucher numbers are also listed in Table 4.

Presence of distinct siphonozooids in analysed specimens led to an assignment to the genera *Heteroxenia* and *Bayerxenia* (Fig. 1a). Thirteen colonies studied from Dahab (Egypt, Red Sea) with unbranched single syndete growth forms but variable colours were referred to *Heteroxenia fuscescens* (Ehrenberg 1834) (Table 4). Analysis of the sclerite structure revealed a surface appearance similar to the sclerites of the genus *Xenia* (Fig. 2k, l). The corpusculars were more of rodlet shape rather than triangular (comparison with *Bayerxenia* in Fig. 2i and j). The colonies of *H. fuscescens* were the only ones associated with the nudibranch *Phyllodesmium hyalinum,* Ehrenberg (1831) (Figure S6 B). Nine colonies from two locations at Dahab were of brownish colour and distinctly branched with pulsating polyps (autozooids) arising from the terminal dome-shaped capitula, with siphonozooids present and sclerites lacking (Table 4, Figure S6 C, D). These characters allow assigning the specimens to *Heteroxenia ghardaqensis,* Gohar (1940), described from Hurghada and the Gulf of Aqaba (Red Sea) (Gohar 1940; Reinicke 1997). The colonies collected from Uthamuda (n=5) and Tempokchantik (n=5, all Bali, Indonesia, here named *Bayerxenia* sp. 1) as well as those from Lizard Island (Great Barrier Reef, Australia, *Bayerxenia* sp. 2) revealed very similar sclerite surface structures with prominently triangular-shaped corpusculars as described by Alderslade 2001 (Fig. 2i, j). The long and stretched anthocodiae of *Bayerxenia* sp. 1 were also very similar to those of the *H. fuscescens* colonies found in the Dahab Lagoon (Figure S4 A, Figure S5 C, respectively).

All other specimens without siphonozooids were assigned to the genera *Ovabunda* Alderslade 2001, and *Xenia* Lamarck 1816. One specimen exhibited distinct triangles in the sclerites (Fig. 2h) and was therefore also assigned to the genus *Bayerxenia* (sp. 3), despite the lack of siphonozooids. *Ovabunda* is characterised by sclerites with round corpuscular-shaped microsclerites (Aharonovich and Benayahu 2011). Seven colonies from different sites at Dahab (Egypt, Red Sea) were identified as *Ovabunda faraunensis* (Verseveldt and Cohen 1971) and ten colonies as *Ovabunda macrospiculata* (Gohar 1940) (Figure S1). Both have rather large sclerites, which in addition show species-specific spherical corpusculars (Fig. 2a–c).

About six species of *Xenia* were collected from various locations in the Philippines, Indonesia and Australia. Their taxonomic affiliation to the genus is based on the lack of siphonozooids and rodlet-form corpusculars of the sclerites (Fig. 2d–g), but an assignment to specific species was not possible because of the lack of appropriate descriptions and revisions for the Indo-Pacific region. *Xenia* sp. 1 lacked sclerites.

Table 3 Pairwise genetic distances (uncorrected) of the SRP54 (below diagonal) and the ND6/ND3 gene fragments (above diagonal) of selected taxa. For clades with several identical taxa one representative has been chosen. Shading indicates intraspecific comparisons. For *Xenia* sp. 6 we distinguish the two SRP54 alleles *Xenia* sp. *6_a* and *Xenia* sp. *6_b*. ND6/ND3 has just one allele denoted as *Xenia* sp. *6_a* and the missing values of Xenia sp. 6_b are indicated by "-" symbols

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
1	*Ovabunda faraunensis _13*	*	0.0000	0.0000	0.0020	0.0000	0.0000	0.0000	0.0020	0.0000	0.0000	0.0000	0.0000	0.0000	0.0130	0.0190	0.0150	0.0150	0.0220	0.0220	-	0.0240	0.0220	0.0240	0.0240	0.0230	0.0240
2	*Ovabunda faraunensis_22*	0.0496	*	0.0000	0.0020	0.0000	0.0000	0.0000	0.0020	0.0000	0.0000	0.0000	0.0000	0.0000	0.0130	0.0190	0.0150	0.0150	0.0220	0.0220	-	0.0240	0.0220	0.0240	0.0240	0.0230	0.0240
3	*Ovabunda faraunensis_17*	0.0455	0.0121	*	0.0020	0.0000	0.0000	0.0000	0.0020	0.0000	0.0000	0.0000	0.0000	0.0000	0.0130	0.0190	0.0150	0.0150	0.0220	0.0220	-	0.0240	0.0220	0.0240	0.0240	0.0230	0.0240
4	*Ovabunda faraunensis_12*	0.0413	0.0161	0.0124	*	0.0020	0.0020	0.0020	0.0000	0.0020	0.0020	0.0020	0.0020	0.0000	0.0130	0.0220	0.0170	0.0170	0.0240	0.0240	-	0.0260	0.0240	0.0260	0.0260	0.0250	0.0260
5	*Ovabunda faraunensis_04*	0.0372	0.0199	0.0163	0.0121	*	0.0000	0.0000	0.0020	0.0000	0.0000	0.0000	0.0000	0.0000	0.0130	0.0190	0.0150	0.0150	0.0220	0.0220	-	0.0240	0.0220	0.0240	0.0240	0.0230	0.0240
6	*Ovabunda faraunensis_09*	0.0496	0.0202	0.0081	0.0207	0.0204	*	0.0000	0.0020	0.0000	0.0000	0.0000	0.0000	0.0000	0.0130	0.0190	0.0150	0.0150	0.0220	0.0220	-	0.0240	0.0220	0.0240	0.0240	0.0230	0.0240
7	*Ovabunda faraunensis_06*	0.0537	0.0276	0.0242	0.0202	0.0159	0.0323	*	0.0020	0.0000	0.0000	0.0000	0.0000	0.0000	0.0130	0.0190	0.0150	0.0150	0.0220	0.0220	-	0.0240	0.0220	0.0240	0.0240	0.0230	0.0240
8	*Ovabunda macrospiculata_03*	0.0413	0.0315	0.0202	0.0161	0.0279	0.0282	0.0354	*	0.0020	0.0020	0.0020	0.0020	0.0000	0.0130	0.0220	0.0170	0.0170	0.0240	0.0240	-	0.0260	0.0240	0.0260	0.0260	0.0250	0.0260
9	*Ovabunda macrospiculata_09*	0.0537	0.0197	0.0081	0.0202	0.0239	0.0161	0.0315	0.0276	*	0.0000	0.0000	0.0000	0.0000	0.0130	0.0190	0.0150	0.0150	0.0220	0.0220	-	0.0240	0.0220	0.0240	0.0240	0.0230	0.0240
10	*Ovabunda macrospiculata_10*	0.0537	0.0202	0.0161	0.0207	0.0245	0.0242	0.0323	0.0363	0.0242	*	0.0000	0.0000	0.0000	0.0130	0.0190	0.0150	0.0150	0.0220	0.0220	-	0.0240	0.0220	0.0240	0.0240	0.0230	0.0240
11	*Ovabunda macrospiculata_13*	0.0455	0.0282	0.0161	0.0207	0.0245	0.0242	0.0323	0.0202	0.0242	0.0323	*	0.0000	0.0000	0.0130	0.0190	0.0150	0.0150	0.0220	0.0220	-	0.0240	0.0220	0.0240	0.0240	0.0230	0.0240
12	*Ovabunda macrospiculata_06*	0.0413	0.0161	0.0040	0.0083	0.0122	0.0121	0.0202	0.0161	0.0121	0.0202	0.0121	*	0.0000	0.0130	0.0190	0.0150	0.0150	0.0220	0.0220	-	0.0240	0.0220	0.0240	0.0240	0.0230	0.0240
13	*Ovabunda macrospiculata_08*	0.0455	0.0204	0.0082	0.0124	0.0082	0.0163	0.0163	0.0204	0.0163	0.0245	0.0163	0.0041	*	0.0140	0.0200	0.0150	0.0150	0.0210	0.0210	-	0.0230	0.0210	0.0230	0.0230	0.0230	0.0230
14	*Xenia* sp. *1_01*	0.3077	0.3038	0.2996	0.3034	0.2869	0.3038	0.2996	0.3080	0.3038	0.2911	0.3080	0.2996	0.2954	*	0.0000	0.0100	0.0100	0.0240	0.0240	-	0.0210	0.0240	0.0260	0.0260	0.0250	0.0260
15	*Xenia* sp. *2_01*	0.3034	0.2996	0.2954	0.2991	0.2827	0.2996	0.2954	0.3038	0.2996	0.2869	0.3038	0.2954	0.2911	0.0032	*	0.0150	0.0150	0.0280	0.0280	-	0.0280	0.0280	0.0300	0.0300	0.0290	0.0300
16	*Xenia* sp. *3_09*	0.2275	0.2322	0.2275	0.2275	0.2133	0.2322	0.2275	0.2417	0.2322	0.2227	0.2417	0.2275	0.2227	0.2169	0.2129	*	0.0000	0.0220	0.0220	-	0.0240	0.0220	0.0240	0.0240	0.0230	0.0240
17	*Xenia* sp. *4_04*	0.2275	0.2322	0.2275	0.2275	0.2133	0.2322	0.2275	0.2417	0.2322	0.2227	0.2417	0.2275	0.2227	0.2169	0.2129	0.0277	*	0.0220	0.0220	-	0.0240	0.0220	0.0240	0.0240	0.0230	0.0240
18	*Xenia* sp. *5_01*	0.2675	0.2771	0.2771	0.2719	0.2641	0.2814	0.2771	0.2814	0.2771	0.2771	0.2771	0.2727	0.2727	0.3333	0.3370	0.2686	0.2769	*		-	0.0090	0.0220	0.0020	0.0020	0.0020	0.0020
19	*Xenia* sp. *6_a*	0.2763	0.2857	0.2857	0.2807	0.2727	0.2900	0.2857	0.2900	0.2944	0.2857	0.2900	0.2814	0.2814	0.3370	0.3407	0.2727	0.2810	0.0534	*	-	0.0090	0.0000	0.0020	0.0020	0.0020	0.0020
20	*Xenia* sp. *6_b*	0.2675	0.2814	0.2814	0.2719	0.2684	0.2857	0.2814	0.2857	0.2900	0.2814	0.2857	0.2771	0.2771	0.3297	0.3333	0.2603	0.2686	0.0463	0.0214	*	-	-	-	-	-	-
21	*Heteroxenia ghardaqensis_10*	0.2981	0.3146	0.3146	0.3077	0.2986	0.3192	0.3099	0.3146	0.3239	0.3146	0.3192	0.3099	0.3081	0.3411	0.3372	0.2431	0.2523	0.2262	0.2024	0.2024	*	0.0090	0.0110	0.0110	0.0110	0.0110
22	*Heteroxenia fuscescens_01*	0.2711	0.2622	0.2622	0.2578	0.2578	0.2711	0.2622	0.2667	0.2711	0.2622	0.2667	0.2578	0.2578	0.3221	0.3258	0.2645	0.2727	0.1018	0.0982	0.0873	0.2439	*	0.0020	0.0020	0.0020	0.0020
23	*Bayerxenia sp. 1_01*	0.2675	0.2771	0.2771	0.2719	0.2641	0.2814	0.2771	0.2814	0.2771	0.2771	0.2771	0.2727	0.2727	0.3333	0.3370	0.2686	0.2769	0.0071	0.0534	0.0463	0.2262	0.1018	*	0.0000	0.0000	0.0000
24	*Bayerxenia sp. 2_01*	0.2675	0.2771	0.2771	0.2719	0.2641	0.2814	0.2771	0.2900	0.2944	0.2857	0.2900	0.2814	0.2814	0.3333	0.3370	0.2686	0.2769	0.0036	0.0498	0.0427	0.2222	0.1018	0.0036	*	0.0000	0.0000
25	*Bayerxenia sp. 2_04*	0.2585	0.2627	0.2627	0.2585	0.2500	0.2669	0.2627	0.2627	0.2627	0.2627	0.2669	0.2585	0.2585	0.3525	0.3561	0.2727	0.2810	0.0866	0.1011	0.0903	0.2205	0.1091	0.0866	0.0830	*	0.0000
26	*Asterospicularia* sp.	0.2573	0.2500	0.2459	0.2448	0.2336	0.2500	0.2459	0.2500	0.2541	0.2500	0.2459	0.2418	0.2418	0.3368	0.3333	0.2390	0.2470	0.2384	0.2491	0.2384	0.2711	0.2545	0.2349	0.2349	0.2500	*
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26

Genetics

SRP54

Amplification of the partial SRP54 gene with the *Carijoa*-specific primers CrSRP54f and CrSRP54r failed completely for the samples analysed (Table 2). Of the newly developed primer pairs (SRP54-f1/SRP54-r1/SRP54-f2/SRP54-r2) only the combination SRP54_f2 with SRP54_r1 resulted in a successful amplification (Table 2). Similar to the study of Concepcion et al. (2008), the PCR success rate for SRP54 varied depending on the taxon. A total of 75 xeniid specimens were sequenced, resulting in 25 distinct haplotypes identified from a 453-bp alignment. Fragment sizes ranged from 214 bp to 338 bp (GenBank accession numbers; Table 1). The alignment of the 79 sequences, which also included two NCBI sequences and the second allele of *Xenia* sp. 6, consisted of 37.3 % gap sites. Not considering the gaps, 213 sites were variable and 173 parsimony-informative. The best substitution model suggested by the AIC for this data set was the GTR+I model.

The Bayesian phylogenetic tree is shown in Fig. 3 (left side). The ML bootstrap tree (not shown) is less resolved

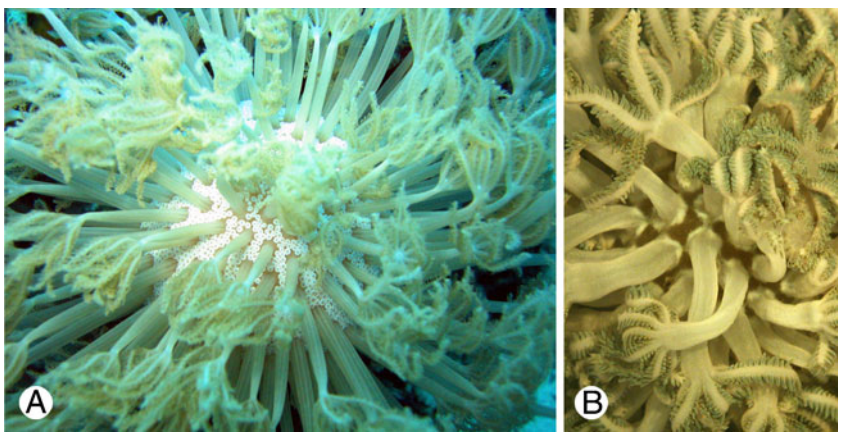


Fig. 1 Colony morphology of Xeniidae: **a** *Heteroxenia* and *Bayerxenia* (here *Bayerxenia* sp.1). Siphonozooids are seen as white rings between the polyps. **b** *Ovabunda* and *Xenia* (here *Xenia* sp. 5*)*. These genera lack siphonozooids

and has no conflicts with the Bayesian tree. The phylogenetic analyses reveal eight well-supported clades and the *Asterospicularia* sp. specimen in the ingroup. All clades differ from each other by more than 6 % pairwise distances with the highest divergence of up to 35.6 % (Fig. 3, Table 3). The uncorrected pairwise distances between the 25 different haplotypes are listed in Table 3 (lower panel). Divergences within clades are less than 6 %. In the well-supported clade 1, the two morphospecies of the genus *Ovabunda* (*O. macrospiculata* and *O. faraunensis*) cluster together in a comb-like structure. Thus, the two morphologically well-characterised species are not recovered by the SRP54 gene, although sequences within the *Ovabunda* clade show up to 5.4 % sequence divergence. Clade 2 and clade 3 encompass four morphospecies of the genus *Xenia* (here labelled as *Xenia* sp. 1, 2, 3 and 4*)*. The morphologically distinct *Xenia* sp. 1 and *Xenia* sp. 2 (clade 2) show no differences in their SRP54 gene. Similarly, in clade 3 two distinct morphospecies (*Xenia* sp. 3 and *Xenia* sp. 4 from Lizard Island) cluster together. Although there are two well-supported subclades within clade 3, they do not correspond to the morphologically described species boundaries (Fig. 3).

In this analysis, clade 1, 2 and 3, represented by *Xenia* and *Ovabunda* specimens, form a monophyletic group supported with a posterior probability of 0.99 and a ML bootstrap of 93 %. Clades 4 to 8 contain *Heteroxenia* and *Bayerxenia* specimens. Both dimorphic genera are supported with a posterior probability of 1 and a ML bootstrap support of 95 %. However, two specimens with monomorphic polyps, determined as *Xenia* sp., cluster within the dimorphic clade (Fig. 3: *Xenia* sp. 5_01 and the two alleles of the specimen *Xenia* sp. 6, 6a and 6b). Clade 4 hosts all individuals identified as *H. ghardaqensis* whereas all clade 8 animals are identified as *H. fuscescens*. Within clade 5, all specimens of *Bayerxenia* sp. 1, which form a weakly supported monophyletic taxon, group together with one *Heteroxenia* sequence from GenBank (accession no. EU006856), some sequences of *Bayerxenia* sp. 2, the one sequence of *Bayerxenia* sp. 3 and one morphologically determined *Xenia* species (sp. 5). The sister group to clade 5 is *Xenia* sp. 6 (clade 6). Individuals 04, 08, 10, 11 and 12 from Australia, morphologically assigned to *Bayerxenia* sp. 2, form a strongly supported separate clade (clade 7). According to SRP54, neither *Heteroxenia* nor *Bayerxenia* are resolved as monophyletic but specimens of both of these genera form one group. The single sequence of *Asterospicularia* sp. grouped as sister taxon to the dimorphic clade.

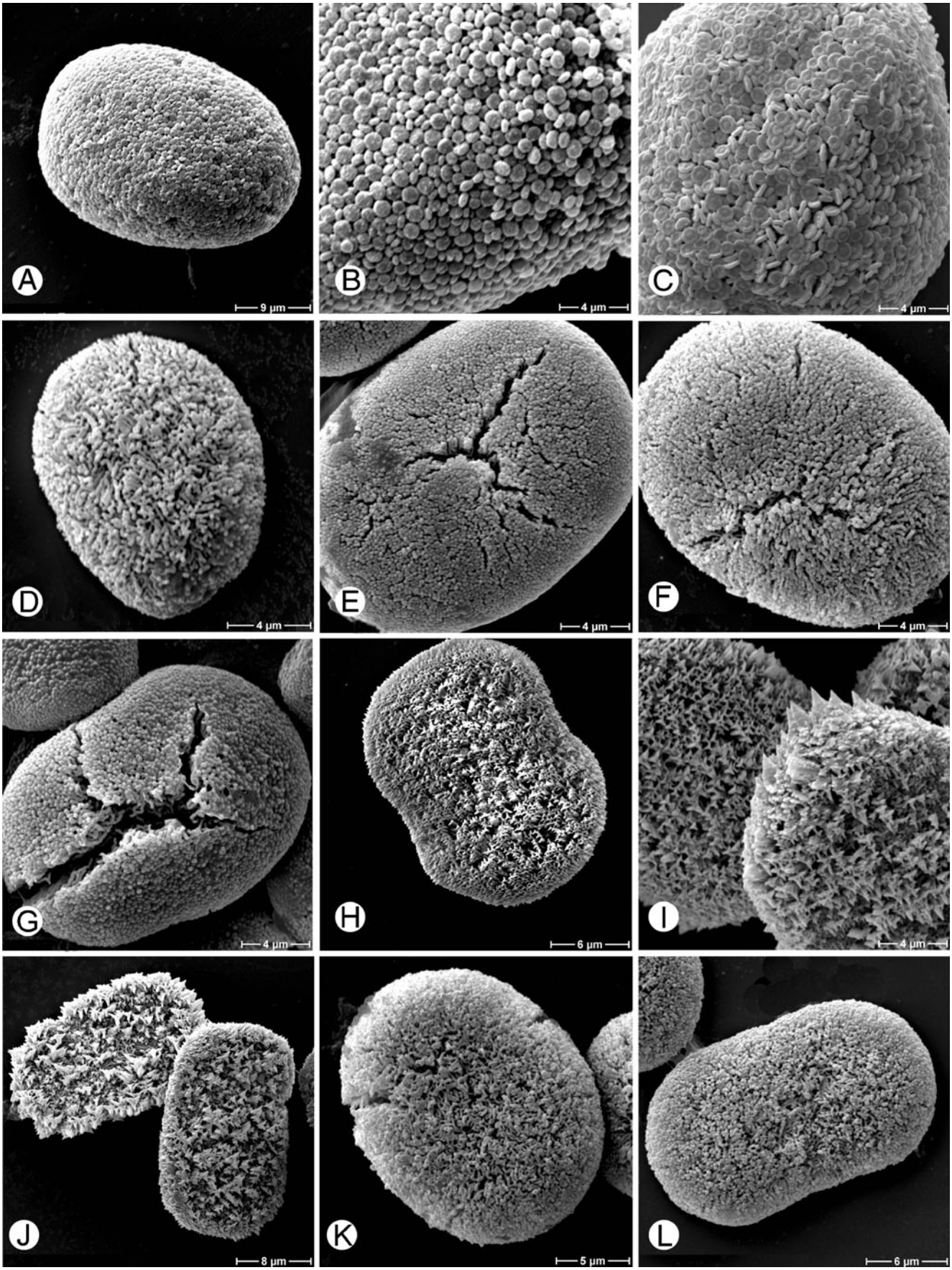
For the whole SRP54 data set, seven individuals were found to have heterozygous genotypes. Thus, for all specimens analysed, 9.3 % could not be sequenced directly because of heterozygous genotypes. For *Xenia* sp. 6, both alleles could be identified from the electropherograms because of phase differences in the fluorescence peaks of the second allele, which had only a limited number of differences (3 %).

ND6/ ND3

A partial fragment of the ND6 and ND3 gene could be obtained for 76 xeniid specimens using the primers of McFadden et al. (2004), resulting in 12 distinct haplotypes (and three outgroup taxa) identified from a 545-bp MAFFT alignment (GenBank accession numbers; sequences will be submitted to GenBank before publication). The alignment of the 80 sequences also included one species of the genus *Asterospicularia* (AF530513) and three outgroup species. Sequences of nine specimens contained "Ns". Sixty-three positions in the alignment were variable (11.56 %), 50 of which were parsimony-informative. While the AIC suggested the HKY+I model of sequence evolution as most adequate for this data set, we computed Bayesian phylogenetic trees for the HKY+I as well as the GTR+I model. The GTR+I model was used because of the general advice (see MrBayes manual) that posterior probabilities are more realistic if more model parameters are varied during the analysis. The two trees show no conflicts. The tree computed with the GTR model is slightly more resolved and has marginally higher posterior probabilities. Only this tree is shown in Fig. 3 (right side). Branch labels indicate posterior probabilities and ML bootstrap values. The phylogenetic analyses reveal five well-supported clades that differ from each other by a maximum of 3 % uncorrected pairwise distances, which is only about one tenth of the divergence found within the SRP54 gene fragment (see scale bar in Fig. 3). The uncorrected pairwise distances between the different haplotypes are listed in Table 3 (upper diagonal).

Similar to the results from the SRP54 analysis, the *Ovabunda* species, *O. macrospiculata* and *O. faraunensis,* clustered in a well-supported clade (posterior probability of 1, ML bootstrap of 100) and formed the sister taxon to the monophyletic genus *Xenia* (Fig. 3, posterior probability 1, ML bootstrap 100). *Xenia* sp. 1 and *Xenia* sp. 2, as well as *Xenia* sp. 3 and *Xenia* sp. 4, have identical ND6/ND3 gene fragments (see Table 3, Fig. 3).

All *Heteroxenia* and *Bayerxenia* sequences (and sequences of *Xenia* sp. 5 and sp. 6, as well as the GenBank sequence *Xenia* sp. AF530512 and *Asterospicularia* AF530513) cluster in one major clade, with the exception of *H. ghardaqensis*. Among the morphospecies, only *H. ghardaqensis* (clade 4) forms a distinct monophyletic group that is consistent with the morphological determination and the SRP54 results. All other morphologically distinct species (*H. fuscescens*, *Bayerxenia* sp. 1, sp. 2 and sp. 3) cluster in an internally unresolved clade. Similar to the SRP54


A
9 µm
B
4 µm
C
4 µm
D
4 µm
E
4 µm
F
4 µm
G
4 µm
H
6 µm
I
4 µm
J
8 µm
K
5 µm
L
6 µm

◀ **Fig. 2** Sclerites. **a** *Ovabunda faraunensis*_03; **b** *Ovabunda faraunensis*_03; **c** *Ovabunda macrospiculata*_04; **d** *Xenia* sp.1_01; **e** *Xenia* sp.3_05; **f** *Xenia* sp.4_02; **g** *Xenia* sp.5_01; **h** *Bayerxenia* sp.3_01; **i** *Bayerxenia* sp.1_10; **j** *Bayerxenia* sp.2_09; **k** *Heteroxenia fuscescens*_14; **l** *Heteroxenia fuscescens*_13

analyses the two specimens morphologically assigned to the genus *Xenia* (Xenia sp. 5, and sp. 6) as well as the GenBank sequence *Xenia* sp. AF530512 cluster within this last clade.

The GenBank sequence of *Asterospicularia* sp. AF530513 is again part of the Xeniidae, but in contrast to the SRP54 analysis, its position is not resolved.

Discussion

Species and/or genus assignment based on morphological characters is supported by the nuclear and mitochondrial gene fragments for some of the taxa investigated here. These include the genus *Ovabunda* and the species *Heteroxenia ghardaqensis*. Conflicting results are obtained especially for the dimorphic clade with regard to the species *Bayerxenia* sp. 1, sp. 2 and sp. 3 as well as two species morphologically determined as members of the genus *Xenia*, and *Heteroxenia fuscescens*.

The two genes investigated differ in the resolution within the dimorphic clade with *Heteroxenia* and *Bayerxenia*, whereas results are very similar with regard to the two *Ovabunda* species and the position of the *Xenia* species. *Asterospicularia* is part of the ingroup in both gene analyses. Whereas *Xenia* clusters with *Ovabunda* in the SRP54 analysis, it is a sister group to the clade *Heteroxenia*/*Bayerxenia* in the ND6/ND3 gene fragment analysis.

Comparing the nucleotide alignments, almost half of the alignment sites are variable for the SRP54 gene fragment and only about 10 % of the positions are variable in the 545-bp alignment of the mitochondrial ND6/3 gene fragment. Both genes, however, are sufficiently variable to distinguish the genera *Ovabunda, Asterospicularia* and *Xenia* as well as the group of *Bayerxenia* and *Heteroxenia* (under the premises that *Xenia* sp. 5 and sp. 6 are misidentified members of one of the genera *Heteroxenia* and *Bayerxenia*; see below). Furthermore, it is possible to distinguish several clades within these genera: With the SRP54 marker, nine genetically distinct clades with deep phylogenetic breaks between the clades, indicative of separate species, are obtained, whereas only six can be distinguished with the mitochondrial marker (two of which are weakly supported). The SRP54 gene also concurs with morphological results found in the main clades.

Ovabunda

The two identified morphospecies of the genus *Ovabunda, (O. faraunensis* and *O. macrospiculata*) form one well-supported monophyletic group (clade 1) in both genes analysed. However, the two morphospecies are not recovered. This is interesting, since both taxa could be well distinguished according to the morphological descriptions in Reinicke (1995) and Alderslade (2001): *O. faraunensis* appeared in big branching upright colonies connected by stolons. Each clavate colony was only up to 2 cm high and polyps showed a random arrangement on the upper surface of the coenenchyme. The monomorphic polyps were not pulsating and showed only one or two rows of pinnules (Figure S1 A, B). *O. macrospiculata* could be distinguished from *O. faraunensis* by the pulsating and feathery polyps. The specimens were also found in aggregations, often in the vicinity of *O. faraunensis* (Figure S1 E). Instead of the grey-white colour exhibited by *O. faraunensis*, the colonies of *O. macrospiculata* were characterised by a more yellowish appearance and a noticeably dense sclerite aggregation around the oral opening of the autozooids forming a white star around the mouth of the autozooid (Figure S1 C, D). The sclerites of both morphospecies had the same size of about 30×20 μm and showed "closely packed, round and flattened corpusculars" (Benayahu 1990) at the surface (Fig. 2a–c). The form of these corpusculars also distinguished the two species. *O. macrospiculata* exhibited deep pits in these corpusculars, whereas *O. faraunensis* showed spherically shaped corpusculars without these pits. The incongruence between morphology and both independent genetic markers may be due to a very recent divergence of *O. faraunensis* and *O. macrospiculata* that has not been manifested in the two markers yet (incomplete lineage sorting and the presence of ancestral polymorphisms). We consider the range of morphological characters that distinguish these two species as sufficiently distinctive and do not doubt their validity at the moment.

Xenia

The sister taxon relationship of *Xenia* sp. 1 and *Xenia* sp. 2 (clade 2 in Fig. 3) is characterised by an extremely low sequence divergence for both molecular markers (0.3 % and 0 % for SRP54 and ND6/ND3, respectively). This is in contrast to morphological data indicating two distinct species: *Xenia* sp. 1 lacked sclerites and the polyps exhibited a very low number of pinnules at the outer row of the tentacles (Figure S2 A, B). In *Xenia* sp. 2, sclerites were present. Since the same preservatives were used, it seems very unlikely that the absence of sclerites in *Xenia* sp. 1 is an artefact due to preservation. But possibly this colony was very young and sclerites have not yet been formed. As a consequence, the presence or absence of sclerites, as a species-specific character, has to be considered carefully (see below).

The morphospecies *Xenia* sp. 3 was distinguished from *Xenia* sp. 4 with the aid of size differences in the syndete

Table 4 List of different taxon characters sorted according to geographic and systematic criteria. (Loc. = location: Red Sea, Philippines, Indonesia, Great Barrier Reef; S = siphonozooids; P = pulsating polyps)

Species[a]	Voucher no.	Loc.	S	No. of rows of pinnules	No. of pinnules (outer) Row	Max. sclerites ø [μ]	Colony size ø [cm]	Colony morphology	P	Colour	Depth [m]	SRP54 clade/ monophyletic?	ND6/3 clade/ monophyletic?
Ovabunda faraunensis	DMM II-C/3701	RS		1 to 2	16 to 20	30×20	2	Aggregation, branched syndetes connected by stolons		Grey white	5 to 18	1/no	1/no
Ovabunda macrospiculata	DMM II-C/3702	RS		2 to 3	12 to 16	30×20	2	Aggregation, short branched syndetes	x	White, yellow	8 to 13	1/no	1/no
Heteroxenia ghardaqensis	DMM II-C/3703	RS	x	2 to 3	20	-	5	Branched, terminated with domed polyp bearing capitulum	x	Brown	4 to 9	4/yes	4/yes
Heteroxenia fuscescens		RS—moray eel garden	x	4 to 5	26 to 30	22×12	7	Short syndete with convex capitulum	x	White brown	5	8/yes	5/no
Heteroxenia fuscescens	DMM II-C/3704	RS—lagoon	x	4	30	22×12	7	Short syndete with convex capitulum	x	Light brownish	12	8/yes	5/no
Heteroxenia fuscescens	DMM II-C/3705	RS—Nabaq	x	4	20	22×12	4	Aggregation, short syndete	x	Orange brown	1	8/yes	5/no
Heteroxenia fuscescens		RS—Sinai front	x	4	20	22×12	4	Short syndete with convex capitulum	x	Orange brown	8	8/yes	5/no
Xenia sp. 1		Philip.		2 to 3	7	-	2	Short syndete with polyp bearing region		Light yellow	2	2/no	2/no
Xenia sp. 2		Philip.		3	20	15×13	3	Short syndete with polyp bearing region		Light brownish	6	2/no	2/no
Xenia sp. 6		Philip.		2 to 3	16 to18	22×16	7	Short syndete with convex capitulum	x	Light brownish	11	7/yes	5/no
Xenia sp. 5	DMM II-C/3706	Indon.		3 to 4	30	20×17	7	Short syndete with convex capitulum, fleshy tentacles		Creamy white	1 to 2	5/no	5/no
Bayerxenia sp.1		Indon.	x	2 to 3	30	22×15	6	Short syndete with convex capitulum	x	Light brownish	13 to 22	5/no	5/no
Xenia sp. 3	DMM II-C/3707	GBR		3	29	19×14	6	Aggregation, with fleshy short syndete		Light blue	1 to 2	3/no	3/no
Xenia sp. 4	DMM II-C/3708	GBR		2 to 3	18	15×13	2	Short syndete with polyp bearing region		Light yellow	1	3/no	3/no
Bayerxenia sp.3	DMM II-C/3709	GBR		3 to 4	20	24×16	4	Short syndete with convex capitulum	x	Light brownish	6	5/no	5/no
Bayerxenia sp.2	DMM II-C/3710	GBR	x	3 to 4	25	20×17	4	Short syndete with convex capitulum	x	Red brown	1 to 2	6/yes	5/no

[a] Picture tables of all morphotypes are provided in the "Supplementary material"

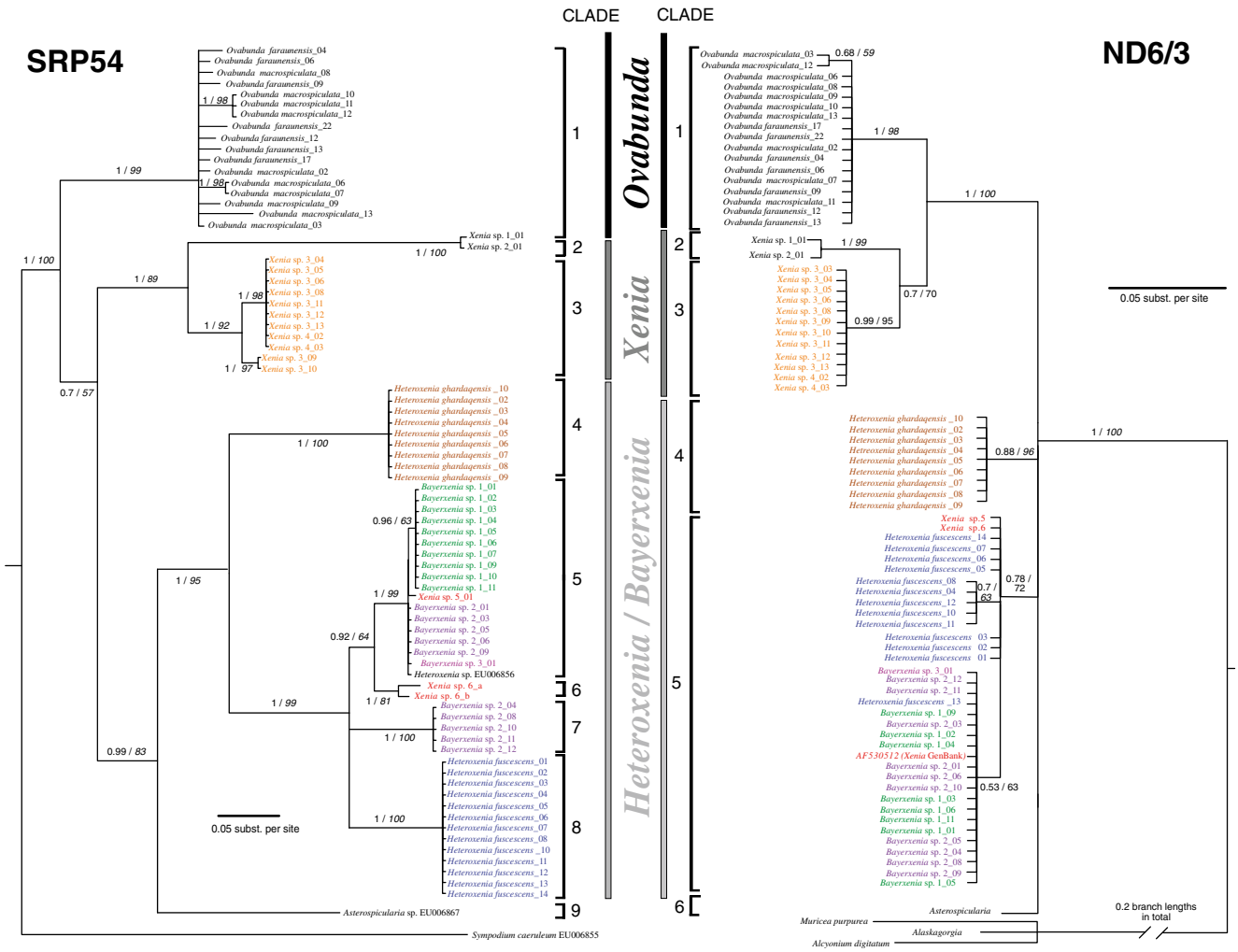


Fig. 3 Bayesian phylogenetic tree of *Ovabunda*, *Xenia, Bayerxenia* and *Heteroxenia*. *Node labels* indicate Bayesian posterior probabilities and maximum likelihood bootstrap support values (*in italics*) based on 10,000 replications. The non-vanishing terminal branch lengths in multifurcations of identical sequences are an artefact of the Bayesian inference as well as due to the occurrence of Ns in the alignment

(6 cm versus 2 cm, respectively). No differences between the two species were observed in the mitochondrial gene fragment, except that the sequence reads of *Xenia* sp. 3 were shorter than those from *Xenia* sp. 4 and terminal Ns had to be added. In the SRP54 gene two distinct subclades are found (Fig. 3): one subclade encompassing specimens of *Xenia* sp. 3 and *Xenia* sp. 4 and the other containing only two *Xenia* sp. 3 specimens. Distinction of these two species is mainly based on the size of the sclerites and the syndete. Both species (some specimens of *Xenia* sp. 3 and both specimens of *Xenia* sp. 4) were collected at exactly the same locality, i.e. the water pipeline of the Lizard Island Research station. Following the results of the genetic analyses, it seems unlikely that *Xenia* sp. 3 and 4 represent two distinct species. Morphological differences may be a consequence of daily exposure to air, wave action or other ecological factors. Nevertheless, a recent speciation process might have resulted in the two *Xenia* sp. 3 specimens (*Xenia* sp. 3_09 and *Xenia* sp. 3_10) with morphologically similar but genetically distinct features from all other specimens of clade 3.

All other specimens assigned to the genus *Xenia* based on the absence of siphonozooids (Xenia sp. 5, sp. 6a and 6b) cluster within the dimorphic genera *Heteroxenia* and *Bayerxenia*.

Heteroxenia

Heteroxenia ghardaqensis is supported as a monophyletic species by both genes. The species can be distinguished easily from all other samples by the dark brown colour of the clearly branched colonies and by the lack of sclerites (Reinicke 1995). Interestingly, not all colonies showed polyp dimorphism, reflecting Gohar's (1940) statement that siphonozooids appear during the reproductive season in spring. Thus, the use of this trait as a diagnostic character is limited.

All 13 specimens identified *a priori* as *Heteroxenia fuscescens* have the same haplotype of the SRP54 gene and form a monophyletic group supported by a posterior probability value of 1 and ML bootstrap value of 100 in the analysis. This result is not supported by the analysis of the mitochondrial gene fragment, where *H. fuscescens* is paraphyletic, clustering with other members of the genera *Heteroxenia* and *Bayerxenia*. All 13 investigated specimens had siphonozooids, the same number of rows of pinnules (4) and a large number of pinnules at the outer row. Additionally, the sclerites exhibited a uniform diameter in all specimens (22×12 μm). Noticeable is a variable growth form, which seems to be related to their locality (Figure S5 and S6): The specimens collected at a depth of 8 m ("moray eel garden") formed single cylindrical colonies (<7 cm) with a convex capitulum and dense, feathery and pulsating polyps (Figure S5 A, B). At the lagoon of Dahab, similar specimens were collected at a depth of 12 m with much longer and thinner anthocodiae (Figure S5 C, D), similar to specimens of the genus *Bayerxenia* within clade 5. Compared to the latter, specimens from "Sinai front" (3 m depth) (Figure S5 E, F) and "Nabaq" (1 m depth) (Figure S6 A, B) had a much smaller colony size of 4 cm, very short anthocodiae and did not appear as cylindrical as the first two morphs. These latter *Heteroxenia* specimens, preliminarily assigned to *H. fuscescens*, had a similar growth form as those described as *Heteroxenia elisabethae* by Reinicke (1995). But this species distinction is not supported by our genetic data. Specimens collected in "Nabaq" appeared in aggregations slightly underneath the surface. On two morphospecies the nudibranch *Phyllodesmium hyalinum,* Ehrenberg (1831) was found in a pouch between the siphonozooids (Figure S6 B). No evaluation of the morphological characters was possible for *Heteroxenia* sp. EU 006856 (clade 5) from GenBank. Since it groups together in clade 5 with *Bayerxenia* specimens, it may possible be a misidentified specimen.

Bayerxenia

Bayerxenia sp. 2, identified as a distinct and well-defined species on the basis of various morphological characters, is paraphyletic in the SRP54 analysis with two distinct and strongly supported groups, but remains unresolved in the ND6/ND3 analysis. Specimens of *Bayerxenia* sp. 2 occurred in dense aggregations of distinguishable and sometimes fused colonies and were collected from three different locations along the same beach on Lizard Island (Figure S6 E). The red-brownish colonies had a size of about 4 cm and the autozooids were pulsating. Siphonozooids were visible between the dense, feathery autozooids. At the same locality, also colonies of *Xenia* sp. 3 were found. Both species formed huge and dense intermingling colonies (Figure S6 F). Only *Bayerxenia* sp. 2 was colonised by the slug *Phyllodesmium lizardensis*, Burghardt et al. (2008b) (Figure S4 C). Affeld et al. (2009) showed that two new secondary metabolites (sesquiterpenes) were only present in the dimorphic xeniid and the associated slug, but not in the sympatric species *Xenia* sp. 3. In their study, the dimorphic xeniid was still assigned to the genus *Heteroxenia* because the sclerite structure of their material was not analysed until now in this study.

It is astonishing that in the SRP54 gene analysis some of the specimens identified as *Bayerxenia* sp. 2 from Lizard Island group together with a morphotype classified as *Bayerxenia* sp. 1 from Bali (SRP54 clade 5). Differences between these *Bayerxenia* sp. 2 specimens are 8.3 % uncorrected pairwise distances for SRP54. Specimens assigned to *Bayerxenia* sp. 1 can be distinguished from *Bayerxenia* sp. 2 by larger sclerites, longer anthocodiae and a smaller number of pinnule rows with similarities to *Heteroxenia pinnata*, described for the Philippine Sea by Roxas (1933). This discrepancy can potentially be explained by ancestral polymorphisms together with incomplete lineage sorting. Alternatively, members of *Bayerxenia* sp. 2 may belong to two different species (clade 5 and clade 7 in SRP54, Fig. 3), and the morphological characters used for the delimitation of species may be phenotypically plastic and thus of limited use. However, both morphospecies can be assigned unambiguously to the genus *Bayerxenia* because of the distinct sclerite surface structures with triangular corpusculars as described by Alderslade (2001). This also applies to *Bayerxenia* sp. 3, which we initially determined as a *Xenia* species based on the lack of siphonozooids. The analysis of the sclerites revealed the typical triangular corpusculars of *Bayerxenia* (Fig. 2h), and both genetic analyses confirmed its assignment to this genus. Achituv and Benayahu (1990) have shown that siphonozooids are not present throughout the whole life cycle in dimorphic xeniid species. This clearly shows that the absence of siphonozooids is an ambiguous character and ontogenetic variability has to be taken into consideration in the process of species identification. Similar results were obtained for two further specimens identified preliminarily as members of the genus *Xenia* because of the absence of siphonozooids (*Xenia* sp. 5 and sp. 6). They clearly group within the dimorphic clade (*Heteroxenia*/*Bayerxenia*, Fig. 3), but the two genetic analyses are not congruent in the assignment to a certain genus. Whereas SRP54 indicates a closer relationship of these two species to one of the *Bayerxenia* clades (clade 5), the ND3/ND6 analysis shows no resolution. We assume that the *Xenia* sequence taken from GenBank (AF530512) also represents a misidentification.

In this study, *Asterospicularia* is resolved as the sister group to the dimorphic clade in the SRP54 analysis but shows no particular affiliation to any xeniid genus in the less resolved tree of the ND6/ND3 analysis. Its grouping

within the Xeniidae analysed in this study confirms the rejection of the monotypic family Asterospiculariidae and the placement of the monogeneric *Asterospicularia* within the family Xeniidae (Alderslade 2001).

In summary, resolution was much higher for the fast-evolving nuclear marker SRP54 compared to the mitochondrial gene fragment ND6/ND3. Furthermore, several incongruencies have been found between morphological and genetic characters: On the one hand, the molecular genetic data cannot confirm the validity of some distinct morpho-species, whereas, on the other hand, the morphologically identical specimens of *Xenia* sp. 3 revealed high sequence divergence in the fast-evolving SRP54 gene, indicative of overlooked or cryptic species. A reasonable explanation for these incongruencies between genetic markers and morphological characters could lie in the properties of the very fast-evolving gene SRP54 and its heterozygote nature. But they could also be the result of the usage of polymorphic morphological characters as diagnostic features, which hence are of limited use for species classification. Similar observations were made by Concepcion et al. (2008) for the octocoral genus *Carijoa*. Furthermore, sampling of differing ontogenetic stages may lead to a misidentification. We need more information on the ontogeny, life cycle and environmentally induced changes in the morphology of Xeniidae to re-evaluate diagnostic characters used for discriminating species of the family Xeniidae and also other phenotypic plastic octocorals. So far, it cannot be determined whether the genetically distinct clades in this study represent cryptic species that have not been recognised previously or whether they represent other, already described species whose morphs are difficult to distinguish or have been synonymised (McFadden et al. 2006). At this point it is also interesting to note that the specimens within each of the eight clades always originated from the same sampling site (Table 1), indicating a clear genetic differentiation with respect to geographic location.

Our results give evidence that SRP54 is a suitable marker for phylogenetic analyses on the generic and species levels within Xeniidae, whereas ND6/ND3 probably will contribute more to the generic and higher taxa levels. It is also evident that SRP54 is a good marker for discriminating several xeniid species, whereas morphological characters showed limitations and therefore have to be re-evaluated. Even though ND6/ND3 is less variable than SRP54, it was sufficiently variable for genus and even species delimitation in some cases.

The mitochondrial gene msh1 (MutS homolog 1) has been in the focus with regard to octocoral phylogenies (McFadden et al. 2006). But according to McFadden et al. (2006), this marker shows a low genetic divergence among xeniid species. For the octocoral *Narella*, Baco and Cairns (2012) showed that 83 % of the species within this group could be resolved by a combination of COI and msh1 along with the ND2 marker. Future studies should focus on comparing existing and finding additional markers for studying biodiversity and evolution in the xeniid genera.

Amplification and sequencing problems

The main advantage of SRP54, i.e. its high variability, but it also has major drawback compared to the mitochondrial genes, which can be amplified without problems. The primers used by Concepcion et al. (2008) failed in the present analysis and new primers with several wobble bases had to be designed. Also for these primers, several DNA samples could not be amplified. So far, no primers have been found that work well for a wider range of alcyonarian species. Most likely, this is the effect of variability at the 3′ terminus of the priming sites, a problem already recognised by Concepcion et al. (2008). Baco and Cairns (2012) also mention difficulties in sequencing SRP54 across diverse octocoral taxa. Another drawback of the SRP54 gene is that several heterozygous specimens were found for the SRP54 region. These heterozygous specimens were excluded from the data set and will be analysed in subsequent studies by sequencing the clones of different allelic variants. It should be mentioned that the high variability of this marker and the high genetic distances found between sequence fragments (Table 3) are partly the result of regions of low complexity. In these, mutations can quickly introduce a long insertion or deletion in one single event. Flot and coworkers (2011) realised that despite the drawbacks of sequencing problems, di-allelic nuclear markers were superior to haploid mt-markers on the species-level.

Benefits and limitation of SRP54

Nuclear gene fragments can pose a problem when analysing di- or even polyploid species with heterozygous genotypes. They require more intensive analyses and are therefore regarded to be inappropriate as a barcode marker (Hebert et al. 2003a). However, since the haploid mtDNA evolves too slowly to resolve species relationships in some cases, sufficiently variable nuclear markers must be considered. In soft corals, but also several other taxa, multicopy markers such as the ITS-1 have been investigated (Pillay et al. 2006; Wei et al. 2006). For soft corals, however, the ITS analyses were of limited success (Aguilar and Sanchez 2007; Dorado and Sanchez 2009). The results of our study using the single-copy nuclear SRP54 marker introduced by Concepcion et al. (2008) showed that (at least) 9.3 % of the genotypes analysed were heterozygous and had to be analysed separately or excluded from the analysis. Within eight of nine individuals screened, differences between alleles were below 3 %. However, for one heterozygous

Ovabunda specimen we found two alleles that differed by 6.4 % uncorrected pairwise distance. Hence, using nuclear markers such as SRP54 has the downside that analyses cannot be performed as convenient as for haploid mitochondrial markers of other animal taxa (Hebert et al. 2004). But, in the case where useful haploid molecular markers such as ND6/ND3 do not have the necessary variability, SRP54 seems to be a good marker that is worth testing in other octocoral families. To avoid time-consuming and laboratory-intensive cloning methods, other techniques could be used that allow the discrimination of heterozygous genotypes by confirming the presence of one of a set of known alleles, such as single-strand conformation polymorphism (SSCP) or denaturing gradient gel electrophoresis (DGGE).

Conclusions

The short SRP54 gene fragment amplified with the primers established in this study is highly variable and provides sufficient resolution to distinguish the genera *Ovabunda*, *Xenia, Bayerxenia, Heteroxenia* and *Asterospicularia*, as well as several clades therein. High bootstrap support values also indicate good resolution concerning intergeneric relationships, thus promoting this gene as a valuable marker for broader phylogenetic analyses including more xeniid genera. The gene has an about 10× higher variation than the mitochondrial ND6/ND3 gene, although the variation of ND6/ND3 was sufficiently high for genus and even species delimitation in several cases. Therefore, ND6/ND3 should not be completely discarded for future phylogenetic analyses in combined data sets and when including further xeniid genera or other octocoral taxa. Comparing genetic and morphological analyses revealed one likely case of overlooked species diversity but several cases of polymorphic species: Only nine distinct clades were found in the SRP54 and six in the mitochondrial ND6/ND3 analysis, whereas 14 morphospecies have been identified (*Asterospicularia* included). Both of these problems, i.e. overlooking species and splitting of morphologically variable species, can systematically bias biodiversity estimates and should be avoided. Since molecular and morphological analyses provide different and partly contradicting pictures, the morphological characters used in the past for species and even genus discrimination have to be re-evaluated carefully by taking into account variation that may be due to differences in the life cycle as well as geographic variations. Additionally, life history traits influenced by environmental factors (symbiotic relationship with zooxanthellae including depth and exposure to irradiance, hydrodynamics and seasonality) may influence the growth form. These effects are hardly known at all. But also the different allelic variants of SRP54 need to be investigated further for a full picture of the discriminating power of this marker.

Our study shows that SRP54 constitutes a promising candidate marker for evolutionary studies within octocoral families and even suggests its potential use as barcode marker. We recommend that future studies with a similar broad sampling should also include the msh1 marker and the extended COI+igr1+msh1 barcode for comparison.

Our analyses on the Xeniidae represent an important step toward resolving and understanding the systematics and evolution of this difficult and poorly known group.

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Authors' contributions KS, FL, IB, HW and RT designed the study. KS and FL did the laboratory work. KS, FL and CM performed the computer analyses. IB and GR helped with the morphological investigations. KS, FL, CM and HW drafted the manuscript and interpreted the data. RT and GR made valuable contributions to the interpretation of the data. All authors have read and approved the final version of the manuscript.

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