

Could polyp pulsation be the key to species boundaries in the genus *Ovabunda* (Octocorallia: Alcyonacea: Xeniidae)?

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Abstract *Ovabunda* is a common genus of the family Xeniidae in the Red Sea. In the current study, 70 *Ovabunda* colonies were collected in Eilat (Gulf of Aqaba) and evaluated for inter- and intra-species variation, both morphologically and genetically, using three mitochondrial genes (mtMutS, COI, ND2) and the nuclear 28S rDNA. The purpose of the study was to elucidate species boundaries within the genus and determine which morphological characters are congruent with genetic clades. We found a large intra-specific variation in morphological characters and

therefore faced difficulties when trying to assign colonies to species based on classical taxonomy. Genetic analyses revealed that the morphospecies did not cluster according to their classification but, rather, in two groups: one comprising colonies with pulsating polyps and the other with non-pulsating polyps. Comparisons of SNP sites in 28S among parents and their respective offspring suggest that the pulsating and non-pulsating groups are reproductively isolated, as we did not find any of the heterozygotes that would be expected if cross-fertilization existed between the two clades. Based on these findings, we recommend further documentation of polyp pulsation in the study of other genera of the family Xeniidae.

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Introduction

The genus *Ovabunda* of the family Xeniidae (Octocorallia, Alcyonacea) is widespread and prevalent on coral reefs in both the Red Sea and Eilat (northern Gulf of Aqaba) (Reinicke, 1997; Benayahu & Loya, 1981; Benayahu et al., 2002; Halász et al., 2014). In the Eilat Nature Reserve, *Ovabunda* can constitute up to 90% of all soft corals on the reef (Shlesinger, 2014). The genus was established in 2001, when Alderslade separated it from *Xenia* on the basis of sclerite

microstructure. In a recent taxonomic revision of the genus (Halász et al., 2014), a total of 11 valid species were re-described and summarized based on examination of type material. In that study, three *Xenia* species were assigned to *Ovabunda*, three synonyms were established, and a neotype was designated for *O. macrospiculata*. Halász et al. (2014) applied the classical morphological features that were used in past studies (e.g., Gohar, 1940; Reinicke, 1997), including colony dimensions and color, stalk branching, numbers of pinnule rows and numbers of pinnules in the outermost row, their dimensions, and the gap-size between adjacent pinnules. Additionally, sclerite dimensions, shape, and microstructure were examined by SEM (Halász et al., 2014). Pulsation of polyps, namely the ability of the polyp tentacles to synchronously open and contract in a continuous measured rhythm, was noted following information derived from the literature (e.g., Gohar, 1940; Verseveldt & Cohen, 1971; Reinicke, 1997). In contrast to most other octocoral genera, the genus *Ovabunda* presents uniform sclerite shape, size, and surface microstructure, and thus sclerites are inadequate for species discrimination (Halász et al., 2014). Regarding the diagnostic value of polyp pulsation, Halász et al. (2014) considered it to be as important as the number of pinnule rows and the numbers of pinnules in the outermost row, and included it in the key to the species.

Pulsation imparts physiological benefits to the colony, such as increasing photosynthesis of the symbiotic algae (Kremien et al., 2013) and preventing re-filtration of surrounding water by neighboring polyps (Wild & Naumann, 2013). Gohar (1940) was the first to refer to polyp pulsation or non-pulsation among xeniid octocorals as a species-specific character. Since then it has been indicated for ten out of the 11 *Ovabunda* species (Halász et al., 2014). Among them, *O. macrospiculata* is pulsating, nine species are non-pulsating, and the trait is not mentioned in the literature for *O. verseveldti*.

The most studied species of the genus is *O. macrospiculata* Gohar, 1940, as several aspects of its biology and ecology have been studied in the Red Sea, its type locality. These include its sexual reproduction (Benayahu & Loya, 1984a, b), larval settlement behavior (Benayahu & Loya, 1984b, c; Achituv et al., 1992), colonization capabilities (Benayahu & Loya, 1985), natural chemistry products (Kashman &

Groweiss, 1980), and its response to ocean acidification (Gabay et al., 2013). These studies demonstrate that *O. macrospiculata* is a successful colonizer of reef substrate due to several traits, including the translocation capabilities of its colonies, early onset of reproduction, high fecundity, prolonged planulation period (Benayahu & Loya, 1984a, b), and substratum specificity (Benayahu & Loya, 1984c).

Until recently, few molecular systematic studies had included representatives of xeniids. These studies, using mitochondrial protein coding genes (McFadden et al., 2006), different barcode combinations (McFadden et al., 2011) or the 18S rDNA (Berntson et al., 2001), and only a handful of xeniid taxa, could only support the monophyly and intra-ordinal relationships of Xeniidae. Other studies have demonstrated some inter-species relationships using molecular data and a larger number of xeniid representatives. For instance, Haverkort-Yeh et al. (2013) found two clades of *Xenia*, with one lacking sclerites. Nonetheless, the same study could not resolve relationships among the five Red Sea *Ovabunda* species included in the analyses. Moreover, in other studies, the use of the variable nuclear SRP54, which among xeniids showed up to 17% divergence (Concepcion et al., 2008), did not resolve any inter-specific or intra-specific relationships among Red Sea *O. macrospiculata* and *O. faraunensis* (Stemmer et al., 2013). A recent comprehensive phylogeny of Indonesian xeniids (McFadden et al., 2014b) enabled an insight into the relationships between different *Xenia* spp. and also revealed a close relationship between two Red Sea *Ovabunda* spp. and Indonesian and Red Sea *Xenia*. The study of Janes et al. (2014) also showed a close relationship between Red Sea *Xenia* and *Ovabunda* from both the Red Sea and the Andaman Sea, and could not resolve relationships among several *Ovabunda* spp. from the Red Sea. Both the McFadden et al. (2014a, b) and Janes et al. (2014) studies implemented the use of a barcode combination of mitochondrial genes as well as the 28S rDNA used in our study.

In the current study, a phylogenetic approach was adopted using three mitochondrial genes (mtMutS, COI, and ND2) and the nuclear 28S rDNA in order to understand species boundaries within Red Sea *Ovabunda* and ascertain where morphological characters are congruent with genetic clades. We also examined whether reproductive boundaries exist between clades by comparing SNP sites in the 28S gene among sexual

offspring of different colonies. We conclude that the ten recognized species and seven unidentified morphospecies found belong to two reproductively isolated genetic groups, separated by a single, obvious morphological trait—polyp pulsation.

Methods

Colony collection

Collection of colonies was carried out using SCUBA during 2010–2011 in Eilat, Gulf of Aqaba (northern Red Sea) at eight sites at a depth of 3–42 m (detailed collection data in the Online Resource, Table S1). Collections were made haphazardly, without attempting to quantify abundance, but different sites were surveyed in order to collect a variety of samples. Pulsation of polyps was recorded during the dives, and the colonies were photographed using an Olympus 5060 camera. The colonies were gently separated from the substrate and placed in marked plastic Ziploc bags. Later, tissue samples from each colony were stored in 99% ethanol for molecular analyses; the rest of the colony was stored in 70% ethanol for morphological study. Specimens were deposited at the Tel Aviv University Zoological Museum (ZMTAU) (collection and field numbers are given in the Online Resource, Table S1).

Morphological analysis and taxonomic identification

A total of 70 *Ovabunda* colonies were included in both the morphological and phylogenetic analyses. Each ethanol-preserved colony was measured for its total height, and degree of stalk branching was recorded (i.e., when the stalk was undivided the number given was 1; a single split in the stalk was numbered 2, etc.). Pinnule length and width were not measured due to the large variation observed within a colony, especially due to new or small polyps in the colony. Distances between pinnules were represented by zero or one (i.e., zero representing no gap or a gap smaller than a pinnule-wide space, 1 representing a pinnule-wide space or larger). Number of rows of pinnules and numbers of pinnules in the outermost row were counted from at least five different polyps from each colony, and all of them were taken into consideration

in the analysis as a range (see Table 1; detailed data are presented in the Online Resource, Table S2). In order to characterize the sclerites, a few polyps from each colony were treated with 10% sodium hypochlorite and then rinsed repeatedly in distilled water. Wet preparations of the clean sclerites were examined and photographed under an Optiphot Nikon light microscope at 200× magnification. At least 18 sclerites from each colony were measured using ImageJ 1.440 (National Institute of Health, USA); all measurements were taken into consideration in the analysis as a range (see Table 1; detailed data are presented in the Online Resource, Table S3).

All colonies were assigned to species according to the key to *Ovabunda* species in Halász et al. (2014), based on evaluation of polyp pulsation, number of rows of pinnules, and numbers of pinnules in the outermost row. Colonies that could not be assigned based on the key were classified as unidentified morphospecies according to the traits used in the key.

Planulae collection

Three days prior to the first and last quarter-moon phase live *Ovabunda* colonies were collected using SCUBA in Eilat, during June–September 2012 and 2013 (timing of reproduction derived from data in Benayahu & Loya, 1984a). The colonies were carefully separated from the substratum without injuring the colony, placed in 5 l PVC tanks, and brought to the Interuniversity Institute for Marine Sciences in Eilat (IUI). There the colonies were placed in an open water-flow system in separate 3-l PVC containers supplied with running seawater at a flow rate of $\sim 2 \text{ l min}^{-1}$. Pulsation of live colonies was recorded for the colonies prior to collection and also in the water system at the IUI. Released planulae were prevented from washing away by a mesh net (200 μm) attached to the tank outflow opening. Planulation was monitored after dusk, at $\sim 22:00$ and $06:00$ (Yacobovitch et al., 2003). Planulating colonies were kept in the water system for two additional nights and planulation was monitored. Planulae were collected by net (200 μm), washed with 0.45 μm Millipore filtered seawater, and shipped by air to Tel Aviv University for further analysis. Following planulation, maternal colonies were preserved in 70% ethanol for morphological analysis and species identification; tissue samples were preserved in 99% ethanol for molecular

Table 1 Morphological characters of species and morphospecies based on collected material

Species ID	# of colonies	Characters used for species ID			Additional characters					
		Pulsation of polyps in live colonies	# of rows of pinnules	Minimum # of pinnules in the outermost row	Maximum # of pinnules in the outermost row	Minimum length of sclerites (mm)	Maximum length of sclerites (mm)	Maximum length of the colony (cm)	Gap between the pinnules	# of branches
<i>O. ainex</i>	5	No	3	13	22	0.019	0.044	2.2–3	0–1	1–3
<i>O. arabica</i>	1	No	2	22	26	0.021	0.032	3	1	1
<i>O. biseriata</i>	17	No	2	10	18	0.018	0.048	1.5–2.9	0–1	1–6
<i>O. crenata</i>	4	No	3/2(3)*	13	17	0.018	0.049	1.2–2	0	1–4
<i>O. faraunensis</i>	8	No	2	15	24	0.016	0.066	1.4–4	0–1	1–3
<i>O. gohari</i>	1	No	1	18	21	0.022	0.030	2.2	0	1
<i>O. hamsina</i>	2	No	3	16	22	0.019	0.038	3	0–1	1–2
<i>O. impulsatilla</i>	5	No	2	8	13	0.02	0.059	1.4–2	0–1	2
<i>O. verseveldti</i>	1	No	1	15	19	0.020	0.040	1.4	1	1
sp. 1	3	No	1	7	15	0.016	0.044	1.7–2.6	0–1	3–5
sp. 2	1	No	1	22	24	0.021	0.040	3.5	0	2
<i>O. macrospiculata</i>	9	Yes	3	12	18	0.02	0.046	0.7–2.5	0–1	1–3
sp. 3	1	Yes	2	8	11	0.021	0.035	1.8	0	3
sp. 4	2	Yes	2	10	16	0.019	0.04	1.1–2	0	1–2
sp. 5	7	Yes	2/2(3)*	14	20	0.017	0.046	1.2–2.3	0–1	1–5
sp. 6	1	Yes	2	19	23	0.022	0.038	2.8	1	3
sp. 7	2	Yes	3	15	25	0.016	0.036	1.5–2	0	2

Numbers in brackets indicate rare number of rows of pinnules; e.g., 2(3)*—mainly two rows of pinnules but rarely also three rows of pinnules

analyses. Colonies that did not release planulae were returned to the reef.

DNA sequencing and analysis

All 70 colonies used for the morphological analysis were sequenced for four genes: the octocoral-specific mitochondrial mutS (mtMutS), NADH dehydrogenase subunit 2 (ND2), cytochrome oxidase I (COI), and the nuclear ribosomal 28S rDNA (See Table 2 for references and PCR procedures). Planulae and their respective parent colonies were sequenced for 28S rDNA only. DNA was extracted from planulae without preservation using the DNeasy kit (Qiagen) applying protocol for animal tissue (v.07/2006). The incubation with proteinase K was set to ~3 h or until the tissue was dissolved; at the last step of the protocol, DNA was eluted with 75 µl AE buffer. Each 20 µl PCR reaction contained: 0.16 µl MYTaqTM DNA polymerase (Bioline), 2 µl 10X NH₄ reaction buffer (Bioline), 0.8 µl of 50 mM MgCl₂, 0.1 µl of 10 mM dNTPs, 0.24 µl (10 pmol) of each primer, 15.46 µl ultra pure water (Biological Industries), and 1 µl DNA extract. The primer pairs and PCR amplification settings used are presented in Table 2. PCR products were visualized using 2% agarose gel, stained with GelRedTM (Biotium). The products were sequenced at the Sequencing Unit at the G.S. Wise Faculty of Life Sciences, Tel Aviv University using an ABI 3500xl Genetic analyzer. All sequences were visually inspected using Geneious Pro 4.7.6 (Drummond et al., 2009) and aligned using both ClustalW, and MUSCLE plugins in Geneious Pro 4.7.6. 28S sequences with double peaks were sequenced in both forward and reverse directions;

the double peaks observed in both F and R sequences were coded according to the IUPAC ambiguity code. All sequences were submitted to GenBank [accession numbers: (mtMutS—KM371350-KM371421); (COI—KM371278-KM371349); (28S—KM224883-KM224954); (ND2—KM502891-KM502962), see also Online Resource, Table S1].

Phylogenetic analysis and parent offspring genetic affiliations

Optimal evolutionary models were selected for each gene separately using jModeltest 2.1.1 (Darriba et al., 2012) based on the Akaike Information Criterion (AIC) (Yang, 2005). Phylogenetic reconstructions were estimated based on maximum likelihood (ML) and Bayesian inference using RaxMLGUI v.1.3 and MrBayes v.3.1.2 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003), respectively; for the 70 *Ovabunda* colonies, each gene was analyzed separately followed by the concatenated sequence for all four genes based on a partitioned model (Stamatakis, 2006; Silvestro & Michalak, 2012). Parent and offspring 28S sequences were analyzed along with the 28S sequences of the 70 field-collected *Ovabunda* colonies. For the ML analyses, 1,000 bootstraps were run; MrBayes was run for 10 million generations; data were sampled every 1,000 generations with a burnin of 25%. The outgroups selected for all analyses included two closely related representatives of Xeniidae, *Heteroxenia fuscescens* (HF-A12-ZMTAU CO 36508, accession number KM224954), and *Anthelia glauca* (A133-ZMTAU CO 36506,

Table 2 Primers and PCR protocols used for *Ovabunda*

Gene	Primer name	Primer sequence	PCR protocol	References
COI	COII8068F	5'-CCATAACAGGACTAGCAGCATC-3'	57.7°C, 90:90:60, 35X	McFadden et al. (2004)
	HCO2198R	5'-AAACTTCAGGGTGACCAAAAAATCA-3'		
MtMutS	ND42625F	5'-TACGTGGYACAATTGCTG-3'	50°C, 90:90:60, 35X	Lepard (2003)
	Mut3458R	5'-TSGAGCAAAAGCCACTCC-3'		
ND2	16S647F	5'-ACACAGCTCGGTTTCTATCTACCA-3'	51°C, 30:30:60, 30X	McFadden et al. (2004)
	ND21418R	5'-ACATCGGGAGCCACATA-3'		
28S	28S-F	5'-CACGAGACCGATAGCGAACAAGTA-3'	50°C, 30:30:60, 35X	McFadden & van Ofwegen (2013)
	28S-R	5'-TCATTTTCGACCCTAAGACCTC-3'		

PCR protocols as follows: annealing temperature, seconds at 94°C: seconds at annealing temperature: seconds at 72°C, number of cycles

accession number KM224902) collected in Eilat. Mega 5.1 (Tamura et al., 2011) was used to calculate pairwise genetic distances (Kimura 2-parameter; Kimura, 1980) within and between morphospecies as well as within and between the groups found in the phylogenetic analyses.

Results

Morphology

Of the 70 colonies collected, 53 were identified to species level and represented ten species (Table 1). The remaining 17 colonies belonged to seven unidentified morphospecies (Table 1, sp. 1–7). No relationship was found between collection sites, depth, or collection time and the genetic clades or species identification (see the Online Resource, Table S1). No relationship was found between the total lengths of the colonies, the branching of their stalk, or the gap between the pinnules and species identification (Table 1) or the genetic groups (Fig. 1). Furthermore, these characteristics were highly variable among members of the same species or genetic clade (e.g., *O. biseriata* colonies ranged 1.5–2.9 cm long and with 1–6 branches; see Table 1).

We encountered difficulties in assigning the colonies to species due to a high variation in the number of pinnules in the outermost row (for example, samples: A13, A18, A93, A154, Online Resource, Table S2), and some difficulties determining the number of rows of pinnules (for example, samples: A90, A98, A115, Online Resource, Table S1). It is important to note that among the 70 colonies examined in the current study, the number of pinnules in the outermost row displayed a continuum and even an overlap among species, mentioned previously in Hickson's revision (1931, p. 146). Polyp-sclerite sizes of all colonies overlapped, ranging between $0.016\text{--}0.029 \times 0.03\text{--}0.066$ mm, with no significant differences between morphospecies (between pulsating and non-pulsating colonies: *T* test, *P* value = 0.878; between morphospecies: one-way ANOVA, *P* value = 0.801). The larger sclerite ranges found in *O. faraunensis* and *O. impulsatilla* (Table 1) were due to a single large sclerite in one sample of each species that might have been caused by measuring a complex of adhered sclerites, as discussed in Halász et al. (2014).

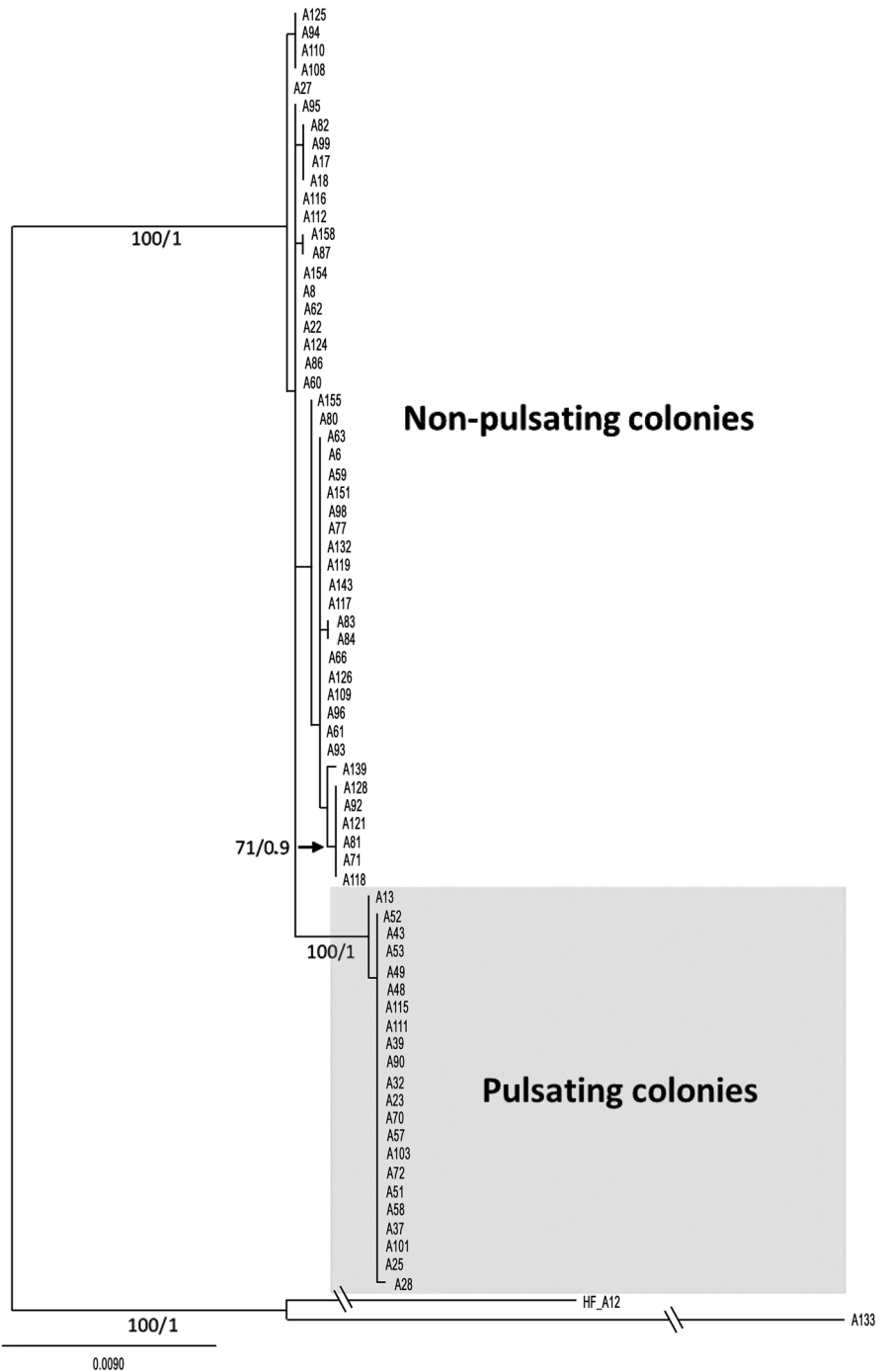
Phylogeny

All 70 colonies included in the morphological analysis were successfully sequenced for all four genes: three mitochondrial (mtMutS: 729 bp; COI: 774 bp; and ND2: 740 bp) and one nuclear (28S rDNA: 718 bp). The total length of the concatenated sequence was 2961 bp. The total number of nucleotides that varied within each gene was 6 for mtMutS (5 parsimony-informative); 5 for COI (all parsimony-informative); 1 parsimony-informative for ND2; and 9 for 28S (8 parsimony-informative) (only within *Ovabunda*, not including outgroups). The model search in jModelTest 2.1.1 resulted in the following models which were taken into consideration using Bayesian inference: MtMutS: TPM1uf; COI: TrN+G; ND2: HKY; 28S of *Ovabunda* colonies: SYM; 28S of parent colonies, planulae, and *Ovabunda* colonies: SYM+G.

ML and Bayesian phylogenetic tree topologies were highly congruent for each gene separately, differing only in some deeper nodes; therefore, sequences were concatenated and analyzed (Fig. 1). The concatenated analysis revealed that *Ovabunda* is a well-supported clade, distinct from the outgroup sequences selected. Within *Ovabunda*, there was one well-supported clade of colonies with pulsating polyps, representing *O. macrospiculata* and four unidentified pulsating morphospecies (spp. 3–7; Table 1; Fig. 1). The other colonies belonged to an unresolved group that includes colonies of the species *O. ainex*, *O. arabica*, *O. biseriata*, *O. crenata*, *O. faraunensis*, *O. gohari*, *O. hamsina*, *O. impulsatilla*, *O. verseveldti*, and two unidentified morphospecies (spp. 1–2; Table 1; Fig. 1), all with non-pulsating polyps.

The maximal variation between sequences was observed between the pulsating colonies and the non-pulsating colonies—0.55% in mtMutS, 0.41% in COI, 0.1% in ND2, and 0.55% in 28S rDNA. Pairwise distances between morphospecies, using mtMutS, COI and 28S, were always smaller than 0.3% when comparing two pulsating or two non-pulsating species, but when comparing one pulsating and one non-pulsating species, the distance was always larger than 0.3%. Divergence between species and between pulsating and non-pulsating groups using ND2 was lower, and reached only 0.14%. Furthermore, in each gene, a few nucleotide positions could discriminate the

Fig. 1 Maximum likelihood phylogram of 70 *Ovabunda* specimens using mtMutS, COI, ND2, and 28S rDNA. *Backslashes* indicates branch length trimmed by twofold. *Gray highlight* represents pulsating *Ovabunda* colonies. *Numbers on the branches* represent bootstrap support (larger than 70%) / pp from ML and Bayesian inference, respectively. *Scale bar* substitution/site



pulsating and the non-pulsating groups. These SNPs included three nucleotide positions in mtMutS (pos. 635, 690, 726); three in COI (pos. 472, 673, 709); one in ND2 (pos. 630); and three in 28S rDNA (positions 53, 156, and 500) (Table 3).

Reproductive boundaries

Seven colonies (3.2%) out of 218 monitored in the IUI running seawater system released planulae during the summer of 2012 and 2013. Six parent colonies and

Table 3 Nucleotide position differences between pulsating and non-pulsating colonies

Gene	Group	Position	Sequence	Position	Sequence	Position	Sequence
mtMutS	Pulsating	635	5'-ATA(G)AATATA-3'	690	5'-GTTAA(G)AATT-3'	726	5'-TTTCCC(G)TGT-3'
	Non-pulsating		5'-ATA(A)AATATA-3'		5'-GTTAA(A)ATTG-3'		5'-TTTCCC(A)TGT-3'
COI	Pulsating	472	5'-TGTT(C)ATAGA-3'	673	5'-ATT(T)GTATGG-3'	709	5'-TGTT(A)TTATC-3'
	Non-pulsating		5'-TGTT(T)ATAGA-3'		5'-ATT(C)GTATGG-3'		5'-TGTT(G)TTATC-3'
28S	Pulsating	53	5'-GCG(C)GCTCTT-3'	156	5'-TTG(T)AAGGAA-3'	500	5'-CTCAT(T)GAGA-3'
	Non-pulsating		5'-GCG(T)GCTCTT-3'		5'-TTG(C)AAGGAA-3'		5'-CTCAT(C)GAGA-3'
ND2	Pulsating	630	5'-GAGA(G)CTTGA-3'				
	Non-pulsating		5'-GAGA(A)CTTGA-3'				

Nucleotide position indicated in bold and in brackets in the sequence

Table 4 Details of parent *Ovabunda* colonies and their offspring

Parent colony	Pulsation in live colony	Species ID	Planulation date	Moon phase	# of offspring in the analysis
m1	Yes	sp. 4	12 July 2012	Day following third quarter	2
m2	Yes	sp. 4	11 August 2012	Day following third quarter	5
m5	No	<i>O. biseriata</i>	9–10 September 2012	Third quarter and consecutive night	16
m7	No	<i>O. biseriata</i>	29–31 July 2013	Day prior–day following third quarter	41
m8	Yes	sp. 4	29–31 July 2013	Day prior–day following third quarter	7
m11	Yes	sp. 4	30–31 July 2013	Third quarter and consecutive night	28

their respective offspring were included in the analysis (m1, m2, m5, m7, m8, m11). Timing of planulation, species identification of parent colonies, and number of offspring used for the molecular analysis are presented in Table 4.

ML and Bayesian inference revealed congruent topologies in an analysis that included parent colonies, their respective offspring, and the 70 *Ovabunda* colonies mentioned in the previous section. This analysis revealed that the *Ovabunda* colonies along with the parent colonies and their respective offspring formed a well-supported clade, separated from the outgroups selected. Moreover, it revealed a distinct clade that included the pulsating parent colonies, their offspring, and other pulsating colonies. The remaining un-resolved group represented the non-pulsating parent colonies, their respective offspring, and the other non-pulsating *Ovabunda* colonies (Fig. 2).

Divergence between the two groups was 0.58%, similar to the 0.55% divergence revealed between pulsating and non-pulsating *Ovabunda* colonies noted in the previous section.

Brooded larvae have the same mtDNA as the maternal colony, so our finding that offspring always had only alleles from the same clade as their parent indicates no gene flow between the pulsating and non-pulsating groups. This distinction among clades is noteworthy, because if there was sexual reproduction between the two clades, we would expect recombination to erase any linkage between the mitochondrial haplotype and a particular nuclear genotype. Contrary to this expectation, all offspring belonged to the same clade as their parent colony, and none were heterozygotes as would be expected if cross-fertilization existed between the two clades (Table 3). These results imply that the two groups are reproductively

isolated from one another. No other morphological trait could be compared between parent and offspring, such as polyp pulsation or number of pinnules, because only a few offspring survived to primary polyp stage or beyond, and also because most of the planulae were sacrificed for the molecular study.

Discussion

In the current study, ten nominal *Ovabunda* species and seven unidentified *Ovabunda* morphospecies were found in Eilat, in comparison to the recent revision of the genus that re-described and validated the existence

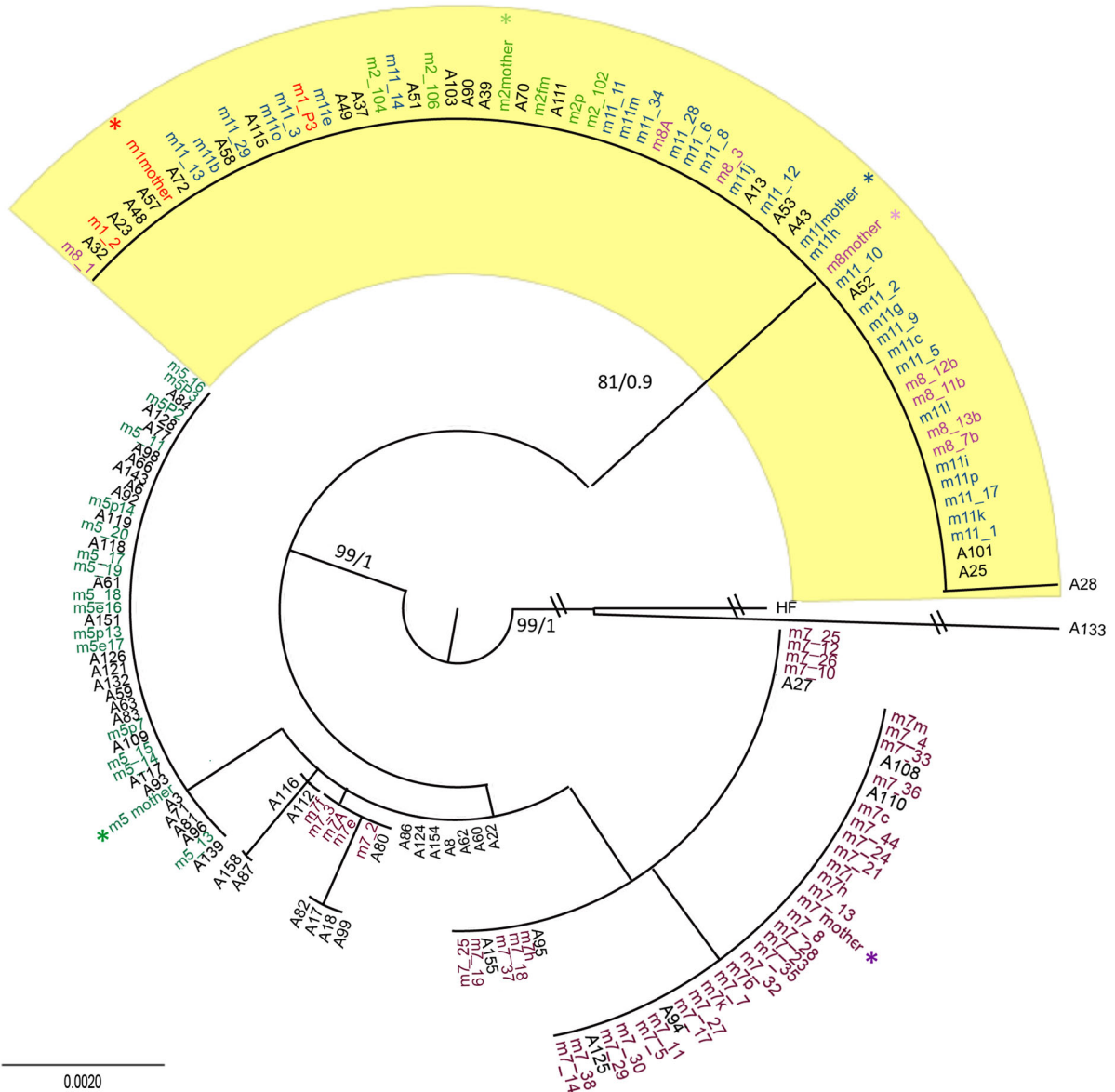


Fig. 2 Maximum likelihood phylogram using 28S rDNA of parents, offspring, and 70 *Ovabunda* colonies. Asterisk represents parent colonies, and their respective offspring is indicated with the same prefix (e.g., m5_20 is the offspring of m5mother). *Ovabunda* colonies not used in planulation experiments are indicated using their field numbers. Highlight represents

pulsating *Ovabunda* colonies, pulsating parent colonies, and their respective offspring. Backslashes indicates branches lengths trimmed by 25-fold. Numbers on the branches represent bootstrap support (larger than 70%)/pp from ML and Bayesian inference, respectively. Scale bar substitution/site

of 11 species for the entire Red Sea (Halász et al., 2014). The examination of 70 samples of the genus enabled us to recognize the range of variation of its morphological features as well as the inter- and intra-specific genetic variation that could not be examined using only the type material. Using a large number of colonies led to the conclusion that within a given morphospecies colony size, stalk branching and gap-size between pinnules are highly variable and essentially continuous among species. An overlap in polyp-sclerite dimensions was found among morphospecies, as noted for the type material of *Ovabunda* (Halász et al., 2014). Therefore, in contrast to other octocorals (Daly et al., 2007; McFadden et al., 2009; Benayahu, 2010), in the genus *Ovabunda* sclerite dimensions are not an informative trait for species delineation.

The number of pinnule rows and the number of pinnules in the outermost row have been commonly used for species identification in Xeniidae classical taxonomy (e.g., Gohar, 1940; Verseveldt & Cohen, 1971; Reinicke, 1997). These traits turned out to be quite confusing because in several colonies their arrangement in rows was not distinct (e.g., A90, A98, A115, Online Resource, Table S1). In most cases, even when a definite number of rows were present, some variation was observed, and the most common arrangement of pinnules was chosen. The observed number of pinnules in the outermost row was highly variable both among species and among polyps in the same colony (e.g., Online Resource, Table S2: A18, A93, A154; Gohar, 1940; Verseveldt, 1960; Reinicke, 1997). Moreover, examination of 70 colonies revealed a continuum in the number of pinnules in the outermost row among all colonies, as noted in Hickson's revision (1931) regarding examination of large numbers of *Xenia* colonies.

Polyp pulsation in live colonies was the only discrete trait that was congruent with the genetic groups, based on all genes tested, separately as well as with the concatenated sequence. Although polyp pulsation was mentioned in descriptions of some species of *Ovabunda* (Gohar, 1940: *X. macrospiculata*, Verseveldt & Cohen, 1971: *O. biseriata*, *O. faraunensis*, *O. impulsatilla*; Reinicke, 1997: *O. ainex*, *O. arabica*, *O. crenata*, *O. gohari*, *O. hamsina*, *O. verseveldti*) and *Heteroxenia* (Reinicke, 1997: *H. coheni* Verseveldt, 1974; *H. ghardaqensis* Gohar, 1940; *H. fuscescens* Ehrenberg, 1834), its diagnostic value for taxonomy has mostly been ignored.

Interestingly, polyp pulsation was already recorded by Lamarck in 1816 (in: Kremien et al., 2013) and then by Saville-Kent in 1893 (see: Hickson, 1931, p. 15). Gohar (1940, p. 82) claimed that "pulsation is a well-established physiological character. It is a perfectly constant specific character. A species is either pulsatile or it is not, and there is, as a rule, no difficulty in observing pulsation in living specimen. Such observation would be of much help to systematist as a diagnostic character." Gohar suggested that polyp pulsation can even be detected in preserved colonies, as they feature more feathery tentacles, with numerous and longer pinnules. However, our study could not confirm this assertion.

The separation of *Ovabunda* species into distinct pulsating and non-pulsating groups was supported by the phylogenetic analysis based on the four genes tested, mtMutS, COI, ND2, and 28S rDNA. Although the support is not strong, and only the pulsating clade is monophyletic, the two groups are clearly distinguished (Fig. 1). The genetic divergence found in this study between pulsating and non-pulsating morphospecies is similar to that among other octocoral and xeniid species studied (McFadden et al., 2014b). Furthermore, in all genes, one to three diagnostic SNPs (Table 3) enabled us to discriminate these two groups of *Ovabunda*, which could not be achieved using SRP54 (Stemmer et al., 2013).

The comparison of genetic affiliation of parent *Ovabunda* colonies and their respective offspring enabled us to gain for the first time an insight into the reproductive boundaries between the genetic groups we detected. Offspring always belonged to the same 28S clade as their mother, and not a single heterozygous offspring was found, thereby suggesting that the two groups are reproductively isolated (Fig. 2). Comparing SNP sites within 28S led to the same conclusion: all offspring shared the same nucleotides in these sites as their corresponding parent colony, indicating that no recombination (as expected with sexual reproduction) occurred among the pulsating and non-pulsating genetic groups.

It should be noted that *Ovabunda* species are brooders (Benayahu & Loya, 1984a; Benayahu, 1991), and therefore only the genetic affiliation of offspring and their respective maternal colonies could be tested. Also, it should be noted that a relatively low number of offspring were included in the analysis (Fig. 2) due to the low number of planulae obtained

and the difficulty in DNA extraction and amplification from larvae. It is noteworthy that the use of live planulae for the extraction, rather than preserved ones, yielded much better results.

The 28S rDNA was chosen for the comparison of the parent and offspring genetic affiliations despite its multi-copy nature (Gilbert et al., 2007). This gene was previously used in a study of xeniid phylogeny (McFadden et al., 2014b) and found to be as informative as other nuclear markers (i.e., SRP54, ITS, ATP5 α) used for that family (Haverkort-Yeh et al., 2013; Stemmer et al., 2013). The 28S rDNA gene is easy to amplify and analyze, and does not require cloning, i.e., any variation there might be among copies within an individual is not great enough to prevent direct sequencing of PCR products (McFadden et al., 2014a, b). Thus, if anything, the multi-copy nature of this marker would be expected to bias toward differences between the parent and offspring due to different copies being amplified in each, which makes our finding of identical types among parent and offspring robust.

In summary, the current study shows the limitations of the different morphological characters used for species delineation in *Ovabunda*. Our data indicate, using mtMutS, COI, ND2, and 28S rDNA, that there are two separate sympatric genetic groups of *Ovabunda* in Eilat, one pulsating and the other not, which can easily be distinguished by observation of live colonies. These data also show, for the first time in xeniids, based on a lack of recombination at 28S, that these two groups are reproductively isolated. We cannot yet determine whether there are additional reproductively isolated groups within the pulsating and non-pulsating clades. We are currently exploring intra-clade genetic variation further, using next-generation sequencing methods (Toonen et al., 2013; McFadden & Toonen, unpubl. data), but until those results are known we have two options: first, we can conclude that the morphological characters used to date discriminate species boundaries, in which case seven new species should be added to *Ovabunda*. Alternatively, we can conclude that the two genetic clades of *Ovabunda*, the pulsating and the non-pulsating, represent just two species, and that the morphological characters used to date are insufficient for taxonomic discrimination. Choosing the second option, supported by the evidence of reproductive isolation presented herein, might enable a better understanding of the ecology of the

genus. In any case, this study emphasizes the importance of polyp pulsation as a taxonomic trait, and further documentation of this phenomenon in live xeniids might be helpful in understanding their systematics.

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