The Indo-Pacific *Gemmula* species in the subfamily Turrinae: Aspects of field distribution, molecular phylogeny, radular anatomy and feeding ecology

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The biology, feeding ecology and phylogenetic relationships of marine snails in the family Turridae remain poorly understood. Here we report our study on four deep-water species in the genus *Gemmula*, a major group in this family. The four species *G. speciosa* (Reeve 1843), *G. sogodensis* (Olivera 2005), *G. kieneri* (Doumet 1940) and *G. diomedea* (Powell 1964) were collected at five different sites in the Philippines, and their pattern of distribution in the sites, their feeding behavior as well as their phylogenetic relationships with each other and with other members of the subfamily Turrinae were investigated. The radular morphology (of two *Gemmula* species) and potential prey (for one *Gemmula* species) were also examined. Actual feeding observations were also conducted for *Gemmula speciosa* and compared with two turrids from other genera.

All four *Gemmula* species showed strikingly different patterns of distribution; each species was found to be relatively much more abundant at one site but not at the other sites. Molecular phylogenetic analysis based on 16S sequences correlated with previously reported 12S sequences and revealed that the four species all belong to a well-supported *Gemmula* clade within the subfamily Turrinae; and that this clade appeared more closely related to the clades *Xenuroturris*, *Turris* and *Lophiotoma* than to the other clades in the subfamily (i.e.,*Turridrupa, Unedogemmula* and *Polystira)*. Morphological analysis of the radula of both *G. speciosa* and *G. sogodensis* revealed that the radulae of the two species were similar but differed from the other turrids, *Lophiotoma acuta* and *Unedogemmula bisaya*, by the presence of central teeth, consistent with the separation of the *Gemmula* clade from the *Lophiotoma* and *Unedogemmula* clade.

To identify the polychaete group that is targeted as prey by species of *Gemmula*, analysis of regurgitated food fragments was made; phylogenetic analysis of an mtCOI gene fragment that was PCR-amplified from the regurgitated tissue of one specimen (*G. diomedea)* indicated close affinity of the prey to the terebellid polychaete *Amphitritides*. Specimens of *Gemmula speciosa,* when challenged with the terebellid polychaete *Loimia* sp., were observed to attack the worm suggesting that *Gemmula* species feed on terebellid polychaetes. *Lophiotoma acuta* were also observed to feed on the same species of terebellid but were usually group-feeding in contrast to the solitary feeding of *G. speciosa*. *Unedogemmula bisaya* did not feed on the terebellid which also supports the separation of the *Gemmula* and *Unedogemmula* clade. The 16S-based clustering

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prediction of a likely terebellid foodtype for the *Gemmula/Lophiotoma* clade was validated by the feeding challenge experiments in contrast with the central teeth pattern of the radula.

Two lines of proof (i.e. the molecular phylogenetic analysis and the feeding challenge) supporting the toxin homology findings previously reported, provide consistent evidence that *Gemmula* is a distinct clade of worm-hunting Turrinae that feeds on Terebellidae.

KEYWORDS

Gemmula ecology, Turrinae taxonomy, molecular phylogeny, 16S clustering, central teeth, terebellid foodtype, feeding challenge, regurgitate analysis

INTRODUCTION

Marine snails belonging to the family Turridae (the "turrids") comprise a large and highly diverse subgroup within the superfamily Conoidea, a group consisting of venomous marine gastropods. In fact, turrids comprise the largest family of deep-sea gastropods (Rex et al. 2000) and account for the vast majority of conoidean biodiversity (Bouchet et al. 2002). However, the biology, ecology, and species-level phylogenetic relationships of these organisms are still poorly known. A number of factors contribute to the difficulty of studying these organisms, such as high morphological similarity among species, poor accessibility of their habitats (50 -500 m depth), their small size, and their nocturnal and burrowing behavior. Recent studies have started to shed light on the biochemistry, toxinology and toxin gene expression of this group, revealing its potential as a source of bioactive peptides (López-Vera et al. 2004; Watkins et al. 2006; Heralde et al. 2008). Understanding the ecology, i.e., feeding behavior, and genetic diversity of these organisms will therefore be of interest to marine biologists as well as marine biotechnologists.

As part of an initial effort to characterize this group, this study focuses on the genus *Gemmula* (the "gem turrids"). The members of this genus largely occur in deeper tropical waters (at depths of 50-500 meters) and comprise a major group in the subfamily Turrinae (Powell 1964). Other genera conventionally included in this subfamily are *Turris, Lophiotoma, Unedogemmula, Turridrupa* and *Polystira* (Heralde et al. 2007; Powell 1964; Taylor et al. 1993). Several species of *Gemmula* have been collected in relatively large numbers in Philippine waters. In this study, we particularly focused on four deep-water *Gemmula* species, namely, *G. speciosa* (Reeve 1843) (the "splendid gem turrid"), *G. sogodensis* (Olivera 2004), *G. diomedea* (Powell 1964), and *G. kieneri (*Doumet 1840) (Figure 1). We investigated their distribution and phylogeny, as information on the pattern of their distribution is scant and the phylogeny of these species has not yet been elucidated. In the superfamily Conoidea, most previous investigations of molecular phylogeny have been carried out on *Conus* (Duda and Palumbi 1999; Duda et al. 2001; Espiritu et al. 2001; Monje et al. 1999); phylogenetic relationships for the vast biodiversity of turrids (Powell 1964; Taylor et al. 1993; Bouchet et al. 2002; and Puillandre et al. 2008) are still poorly understood. So far, only one study has been carried out (Heralde et al. 2007) which defined clades in the subfamily Turrinae.

To further discriminate the species, we examined and compared the foregut anatomy, i.e., radula, of three species. Because their habitats are inaccessible, little is also known of their feeding biology. Although turrids in general are known to feed on polychaetes, there are no available data on which species of polychaetes are preyed on by *Gemmula* (or any turrid) species. We therefore collected new data on feeding behavior and potential prey preferences.

MATERIALS AND METHODS

Sample Collection

Snails were purchased from local fishermen as by-catch in trawl nets along the mouth of Manila Bay (from Bataan to Cavite and Batangas) and tangle nets in the seas of Cebu and Bohol. Live snails were kept in seawater until they were processed for anatomical or molecular work. The relative distribution and abundance of *Gemmula speciosa* along the periphery of Manila Bay was initially assessed by monitoring the collections per trawl of selected boats in August 2005 and from October 2005 to January 2006. The abundance in all the sampling sites was monitored from the snails collected by the fishermen from February to May 2006.

Specimen Preparation and DNA Extraction

The snails were segregated by putative species and preserved for various uses. The snails were cracked and tissue samples (hepatopancreas and foot) were obtained and preserved in approximately 10 volumes of RNALater. Voucher specimens were kept in 70% ethanol. DNA extraction was performed in fifty mg tissue samples (hepatopancreas or foot) using the Puregene DNA kit (Invitrogen) or the DNeasy tissue kit (Qiagen) and aliquots were prepared..

Gene amplification

The 16S mitochondrial rRNA gene was amplified using the primers 16SL (5'-GTTTACCAAAAACATGGCTTC-3') and 16SH (5'-CCGGTCTGAACTCAGATCACGT-3') with uracil adaptor sequences. A PCR mix containing 20-40 ng genomic DNA, 2μM of each primer, 2μM of dNTP and 2μM of Taq Polymerase was prepared and cycled with the following profile: 95ºC 1 min initial denaturation; 40 cycles of 95ºC 20 sec denaturation, 55ºC 20 sec annealing and 72ºC 30 sec extension; and 72ºC 5 min final extension. The PCR product was ligated to pNEB206A (USER Friendly Cloning, New England Biolabs) and introduced into *E. coli* (DH5a) through chemical transformation. Plasmids from transformants with inserts were sequenced through the ABI 377 DNA Sequencer or submitted to

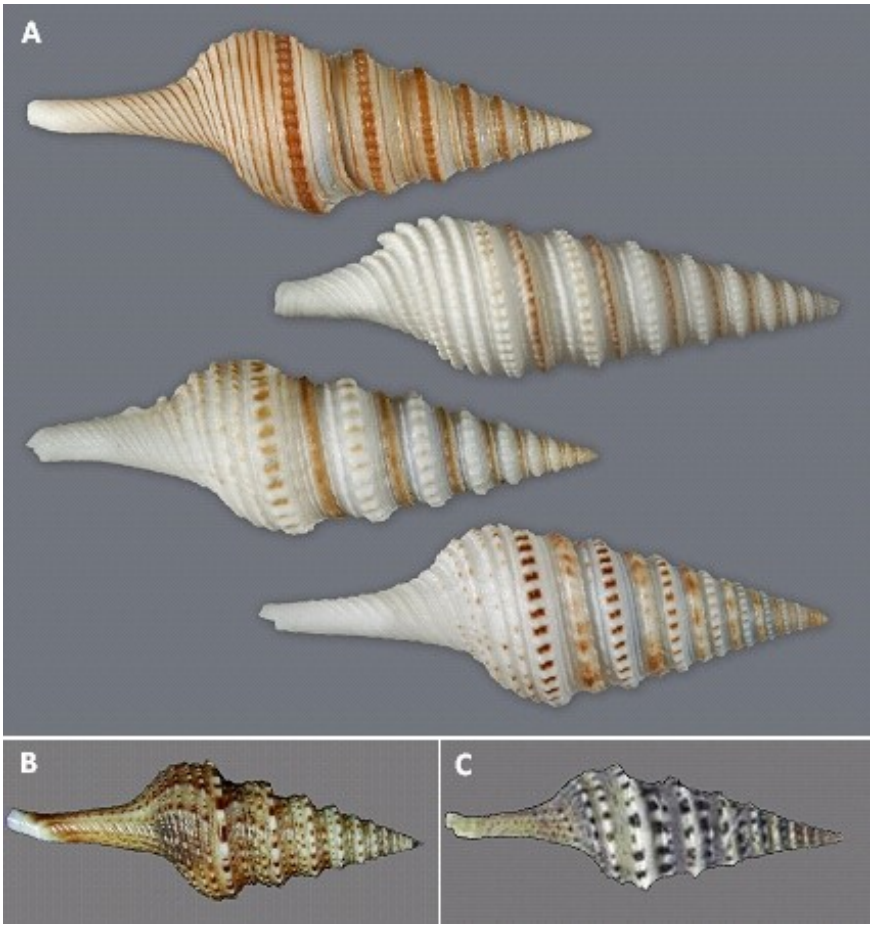

Figure 1. Shells of *Gemmula* species and other turrids analyzed in this study. A, from top to bottom, *Gemmula speciosa*, *Gemmula diomedea*, *Gemmula sogodensis*, *Gemmula kieneri*; B, *Unedogemmula bisaya* and C, *Lophiotoma acuta*.

the University of Utah Sequencing Facility.

Phylogenetic Analysis

The 16S rDNA sequences of 22 conoidean samples analyzed in the study were aligned with Genbank-derived sequences for coniids with *Rhinoclavis aspera*, a mesogastropod as root (Table 2). A minimum evolution-based phylogenetic reconstruction was made using MEGA 3.1 (Kumar et al. 2001). Bootstrap values were calculated and putative clades were marked accordingly. The genetic distances were computed using the Kimura-2-parameter model to determine the range of distances of the specimens that belong to a food type cluster.

A second phylogenetic analysis was made using the combined 12S rDNA and 16S rDNA (12S previously reported in Heralde et al. 2007) sequences. The concatenated sequences were aligned using Clustal X. The alignments were refined manually using MacClade 4.08. The process was repeated for some highly variable regions as long as further refinement by eye seemed possible.

Trees were optimized using the individual rRNA gene sequence alignments and the concatenated alignments (presented herein). Final analyses were restricted to model-based maximum likelihood (PAUP4b10) and Bayesian inference (MrBayes 3.1.2) to account for the complexity of sequence evolution. Sequence evolution parameters were optimized by a GTR+I+G model that includes six possible substitution types (GTR), allows some sites to be invariant (I), allows across-site rate heterogeneity (G) and allows unequal base frequencies.

The maximum likelihood optimization used TBR branch swapping with 10 searches, each using a random addition of taxa. The analysis ended when the PAUP default criteria for convergence of the log-likelihood were met.

The Bayesian analysis was run for two million generations with the first 500,000 generations discarded as burn-in trees. Two MCMCMC runs (Metropolis-Coupled Markov-Chain Monte-Carlo), using four chains each, were used to thoroughly

explore tree space. Convergence of the likelihoods was judged adequate by monitoring the ASED (Average Standard Error of the Difference) in split frequencies between the two runs and by comparing plots of the tree log-likelihood trees from generation 500,000 to 2 million. By the last generation, average standard error was 0.0039; the plot of likelihoods versus generation had stabilized. Furthermore, the PSRF (Potential Scale Reduction Factor) reached 1.00 for the total tree length and for each model parameter.

regurgitated tissues were recovered. Genomic DNA was extracted from the tissues using the DNeasy tissue kit (Qiagen). An aliquot was prepared as template for mtCOI amplification using modified universal primers with USER adaptor sequences (Simison 2000). Subsequent cloning into pNEB206A, transformant screening and plasmid sequencing were as described above. The mtCOI sequence obtained was searched in the Genbank database using BLAST (Basic Local Alignment Search Tool).

Table 1. *Gemmula* species collected at various sites in the period February-May, 2006.

Species	Sampling Sites 1-3: Bataan, Corregidor Is., Batangas, Luzon		Sampling Site 4: Sogod, Cebu		Sampling Site 5: Panglao Is., Bohol	
	Total Number	(%)	Total Number	(%)	Total Number	(%)
G. speciosa	868	87.7%	1	0.2%	0	0.0%
G. sogodensis	0	0.0%	333	76.6%	1	0.8%
G. diomedea	0	0.0%	4	0.9%	108	81.2%
G. kieneri	0	0.0%	0	0.0%	24	18.0%
U. bisaya	122	12.3%	97	22.3%	0	0.0%

Since maximum likelihood and Bayesian analyses converged to the same tree, only the Bayesian results are presented below (ML results are available from PSC).

Species in the phylogenetic analysis included the following: Turrinae: *Gemmula speciosa, Gemmula diomedea, Gemmula kieneri, Gemmula sogodensis, Lophiotoma albina, Lophiotoma acuta, Lophiotoma cerithiformis, Lophiotoma olangoensis, Lophiotoma cingulifera, Lophiotoma kingae, Lophiotoma jickelli, Lophiotoma polytropa, Turris garnonsii, Turris babylonia, Turris spectabilis, Turris normandavidsoni, Turris grandis, Turridrupa elongata, Turridrupa bijubata, Unedogemmula bisaya, Unedogemmula leucotropis, Unedogemmula tayabasensis, Unedogemmula indica, Unedogemmula panglaoensis, Polystira oxytropis, Polystira picta; and* Terebridae: *Hastula hectica and Terebra guttata,*.

Scanning Electron Microscopy (SEM) of the Radula

Live snails were relaxed in 1% cold magnesium chloride ($MgCl_2$) for 2-3 hours and preserved in 95% ethanol; the SEM of their radulae was carried out as described previously (Imperial et al. 2007).

Molecular regurgitate analysis

Six samples of *Gemmula diomedea* caught by trawling at depths of 231-271 meters in the Panglao 2005 Expedition were relaxed in cold 1% magnesium chloride for at least 2 hours and

Tank feeding preference and competition experiments

The snails used for the feeding experiments were maintained indoor using a 56-liter aquarium containing seawater with salinity maintained at a range between 35-37 ppt. A filtration system and an aerator were in place while the feeding behavior of *G. speciosa* and other turrids was observed. The snails used in the experiment had been in the tanks for a period of 2-4 weeks with artificial lighting following a 12 hour light-dark cycle. The introduction of live terebellid worms into the tank was done at night.

RESULTS

Species distribution

The four species of *Gemmula* were obtained from two different biogeographic regions. The first three sites came from Manila Bay in the South China Sea region: site 1 is close to the Bataan peninsula, site 2 is off Corregidor Island and site 3 is off the Batangas coast. At these three sites, *Gemmula* specimens were obtained as by-catch of commercial fish trawlers operating in these areas. The only larger *Gemmula* species collected was *G. speciosa* (Olivera 2005; Powell 1964); no specimens of *G. sogodensis* or *G. diomedea* were found at sites 1-3 (Table 1). Specimens were mostly trawled at depths of 50 to 100 meters.

G. speciosa was only rarely collected at the southern sites within the Visayan Seas biogeographic region (off Sogod, Cebu and off the island of Panglao in Bohol). The primary method for collection at the latter sites was tangle nets mostly laid at greater depths. At the Sogod, Cebu site, the major *Gemmula* species collected was *G. sogodensis* (Olivera 2005). Off Panglao, Bohol, *G. diomedea* was the dominant *Gemmula* species; specimens of *G. kieneri* were also collected. A summary of the number of specimens collected at these sites is shown in Table 1.

be noted that the fishing gear used effectively captured only those organisms that were either on or close to the surface of the substrate (unlike dredges that go deeper).

Two attempts were made to collect *G. speciosa* during the day. A dredging trip to the Batangas area was carried out; this was unproductive (only three specimens of *G. speciosa* were collected). However, between November 2003 and April 2004, there were commercial divers operating in the Batangas area using the hookah method (air compressor/ tanks onboard boats are used to supply air to divers). Apparently, it was necessary to

Table 2. List of gastropod species utilized for analysis in this study.

Species	Source	12S Sequence	16S Sequence
Gemmula sogodensis	Sogod, Cebu, Philippines	EF467337*	GU434132
Gemmula speciosa	Batangas, Philippines	EF467338*	GU434131
Gemmula diomedea	Panglao Is. Bohol, Philippines	EF467334*	GU434133
Gemmula lisajoni	Sogod, Cebu, Philippines	EF467335*	GU434134
Gemmula rosario	Sogod, Cebu, Philippines	EF467336*	GU434135
Gemmula ambara	Marinduque, Philippines	-	GU471196
Lophiotoma acuta	Marinduque, Philippines	EF467339*	GU471195
Lophiotoma albina	Marinduque, Philippines	-	GU471186
Lophiotoma kingae	Cawoy, Olango Island, Philippines	GU585767	GU434137
Lophiotoma olangoensis	Cawoy, Olango Island, Philippines	EF467345*	GU434136
Lophiotoma polytropa	Bohol, Philippines	EF467347*	GU471190
Turris garnonsii	Cawoy, Olango Island, Philippines	EF467352*	GU434139
Turris grandis	Sogod, Cebu, Philippines	EF467353*	GU434138
Turris babylonia	Cawoy, Olango Island, Philippines	EF467351*	GU434140
Turris spectabilis	Cawoy, Olango Island, Philippines	EF467355*	GU471188
Turris normandavidsoni	Sogod, Cebu, Philippines	EF467354*	GU471189
Unedogemmula bisaya	Batangas, Philippines	EF467340*	GU471187
Unedogemmula tayabasensis	Sogod, Cebu, Philippines	EF467348*	GU471185
Clavus unizonalis	Cawoy, Mactan Is, Cebu, Philippines	-	GU585764
Drillia regius	Panglao Is. Bohol, Philippines	EF467333*	GU471193
Turridrupa bijubata	Sogod, Cebu, Philippines	GU585769	GU471192
Terebra subulata	Buenavista, Marinduque, Philippines	-	AF174213*
Terebra areolata	Buenavista, Marinduque, Philippines	-	GU471184
Terebra crenulata	Buenavista, Marinduque, Philippines	-	GU471194
Conus rolani	Sogod, Cebu, Philippines	-	GU471191
Conus emaciatus	-	-	AF126018*
Conus virgo	-	-	AF086616*
Conus flavidus	-	-	AF160704*
Conus terebra	-	-	AF103815*
Conus pulicarius	-	-	AF143992*
Conus vexillum	-	-	AF108822*
Conus capitaneus	-	-	AF126014*
Conus miles	-	-	AF108821*
Rhinoclavis aspera	-	-	AF174212*
Lophiotoma cingulifera	Cawoy, Olango Island, Philippines	EF467342*	GU585765
Lophiotoma cerithiformes	Oahu, Hawaii	EU682298*	EU682307*
Unedogemmula panglaoensis	Panglao Is., Bohol, Philippines	EF467346*	GU827613
Hastula hectica	Panglao Is. Bohol, Philippines	GU585763	GU585766
Terebra guttatta	Cawoy, Olango Island, Philippines	GU827606	GU827607
Polystira oxytropis	-	GU827610	GU827611
Polystira picta	-	GU827608	GU827609
Unedogemmula indica	-	EF467343*	GU827612
Unedogemmula leucotropis	-	GU300025	GU345773
Turridrupa elongata	Cawoy, Olango Island, Philippines	GU300032	GU345780
Lophiotoma jickeli	Cawoy, Olango Island, Philippines	EF467344*	GU585770
Gemmula kieneri	Panglao Is. Bohol, Philippines	GU585768	-

* Genbank-derived sequences

Note: The GU series of accession numbers are the sequences obtained in this paper.

Of the three species, the largest number of specimens collected was *G. speciosa* from sites 1-3. Between August 2005 and January 2006, the by-catch was systematically analyzed from site 3 (Table 1). The mean number of *G. speciosa* collected per trawl was 20±9 or a mean catch rate of 1.3±0.6 per trawl-hour. The specimens ranged in length from 2.0 to 6.7 centimeters (mean: 4.0±1.1 cm.) and collected at night. It must go down to a depth of around 50 meters, and then to dig in the sandy-muddy bottom with shovels, followed by sieving the substrate to recover the *G. speciosa* and other mollusks. The conoidean taxa found to co-occur with *G. speciosa* using the shoveling/sieving technique included *Conus longurionus, Conus mucronatus, U bisaya*, *T. crispa* and sporadically, four to five other small turrid species. In aquarium set-ups and based on

the fishers' knowledge, *G. speciosa* appear to be nocturnal species, burrowing into the substrate during the day and active at night.

At Sites 1-3 and 4, the major species of Turridae collected in addition to *Gemmula* was *U. bisaya* (= *L. bisaya* (Olivera 2004)). At both localities, fewer specimens of *Unedogemmula* were collected compared to the dominant *Gemmula* species (see Table 1). It is notable that although a considerable number of specimens of *U. bisaya* were collected at the two sites, there was little overlap in the species of *Gemmula* collected.

1/Lophiotoma 1 clade. This clade is where the food type/preference analysis was focused. The occurrence of *Unedogemmula* as a separate clade in the Turrinae (Heralde et al. 2007) is also well-supported as indicated by a high bootstrap value (82%).

The genetic distances calculated for the members of the putative turrid clades were compared to the *Conus* clades which have similar food type/ preference (i.e., among worm-hunting cone species) (Table 3). The working hypothesis applied was that closely related species would assume similar food type/ preference; this observation was positively demonstrated in

Table 3. Comparison of genetic distance between 16S rRNA gene sequences of *Gemmula* species and selected vermivorous cone clades. Clade S1, sedentary polychaete feeders, mainly *Terebellidae*; Clade S2, sedentary polychaete feeders, mainly *Capitellidae*; and Clade E6, errant polychaete feeders, mainly *Eunicidae*.

						S1				S2		E6		
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1. *Gemmula speciosa*	0.00													
2. *Gemmula sogodensis*	**0.01**	0.00												
3. *Gemmula diomedea*	**0.03**	**0.03**	0.00											
4. *Gemmula lisajoni*	**0.04**	**0.05**	**0.04**	0.00										
5. *Gemmula rosario*	**0.04**	**0.03**	**0.04**	**0.04**	0.00									
6. *Conus emaciatus*	0.16	0.16	0.17	0.17	0.17	0.00								
7. *Conus terebra*	0.17	0.17	0.17	0.16	0.18	**0.02**	0.00							
8. *Conus virgo*	0.16	0.17	0.17	0.17	0.18	**0.04**	**0.03**	0.00						
9. *Conus flavidus*	0.19	0.19	0.18	0.19	0.19	**0.10**	**0.09**	**0.08**	0.00					
10. *Conus rolani*	0.16	0.16	0.16	0.16	0.17	0.09	0.09	0.09	0.10	0.00				
11. *Conus pulicarius*	0.17	0.17	0.17	0.17	0.18	0.08	0.08	0.08	0.09	**0.05**	0.00			
12. *Conus capitaneus*	0.18	0.19	0.19	0.19	0.19	0.11	0.11	0.11	0.12	0.09	0.09	0.00		
13. *Conus miles*	0.17	0.19	0.18	0.18	0.19	0.10	0.10	0.10	0.12	0.08	0.09	**0.04**	0.00	
14. *Conus vexillum*	0.17	0.18	0.18	0.18	0.19	0.10	0.10	0.10	0.11	0.07	0.07	**0.05**	**0.03**	0.00

Molecular phylogeny

The molecular analysis on selected members of Turrinae (Table 2) for 16S rRNA gene marker has shown congruency in the phylogenetic tree earlier reported for 12S rRNA (Heralde et al. 2007) (Figure 2). Here, the three major genera under the Subfamily Turrinae, namely *Gemmula*, *Turris* and *Lophiotoma* were observed to form distinct clusters, except for the *Gemmula*

coniids (Duda et al. 2001; Espiritu et al. 2001). We selected three clades of worm-hunting coniids (i.e. S1– sedentary polychaete feeders, mainly *Terebellidae*, S2– sedentary polychaete feeders, mainly *Capitellidae* and E6 –errant polychaete feeders, mainly *Eunicidae*) based on the clade grouping of Duda et al. (2001) for the genetic distance calculation. We noted the distances in *Conus* (S1:0.02-0.10,

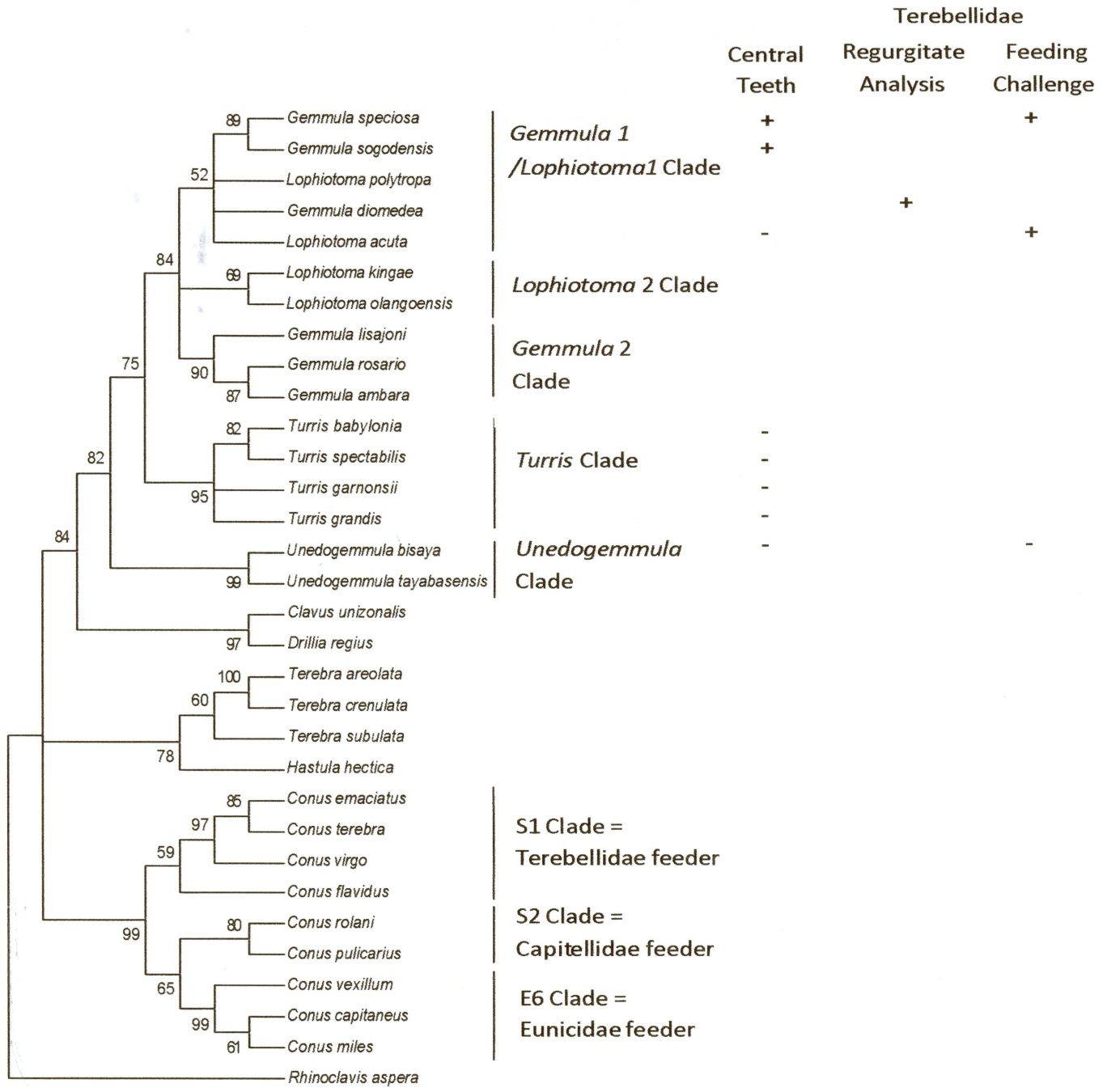

Figure 2. Phylogeny of 31 conoideans based on mitochondrial 16S rRNA gene. *Rhinoclavis aspera*, a mesogastropod was utilized as outgroup. The bootstrap values indicate separation of turrid and coniid clades. The clustering of worm-hunting coniids are shown: S1– sedentary polychaete feeders, mainly *Terebellidae*, S2– sedentary polychaete feeders, mainly *Capitellidae* and E6 –errant polychaete feeders, mainly *Eunicidae*) based on the clade grouping of Duda et al. (2001). On the right side columns are indicated, the presence (+) or absence (-) of central teeth (Note: the *Turris* clade data are published elsewhere); the molecular regurgitate analysis result indicating *G. diomedea* as a *Terebellidae* feeder (shown by a "+" sign), and the validation of 16S clustering prediction through a *Terebellidae* feeding challenge where *L. acuta* and *G. speciosa* showed feeding responses to *Loimia* (indicated by "+" sign) and no feeding response from *U. bisaya* (-).

S2:0.05 and E6:0.03-0.05) to be larger than, if not similar to, those calculated for *Gemmula* (0.01-0.05) (Table 3). The largest distance range occurs among Terebellidae feeders (i.e., the S1 clade), thus in *Conus*, there are more distantly related species feeding on terebellid worms than in *Gemmula*.

The phylogenetic relationship of the four species of *Gemmula* (*G. speciosa, G. sogodensis, G. kieneri* and *G. diomedea;* images of their shells are shown in Figure 1) with each other and with other forms in the subfamily Turrinae was further inferred from the 12S and 16S mitochondrial rRNA gene sequences. Both Bayesian and Maximum Likelihood methods, as described under Experimental Procedures, were used. The phylogenetic tree, shown in Figure 3, groups the species into seven well-supported clades (labelled I to VII) and reveals that the four *Gemmula* species comprise a distinct well-supported group (the *Gemmula* clade), separate from other groups in the subfamily Turrinae that were included in the analysis. Furthermore, the analysis suggests that the sister group of the

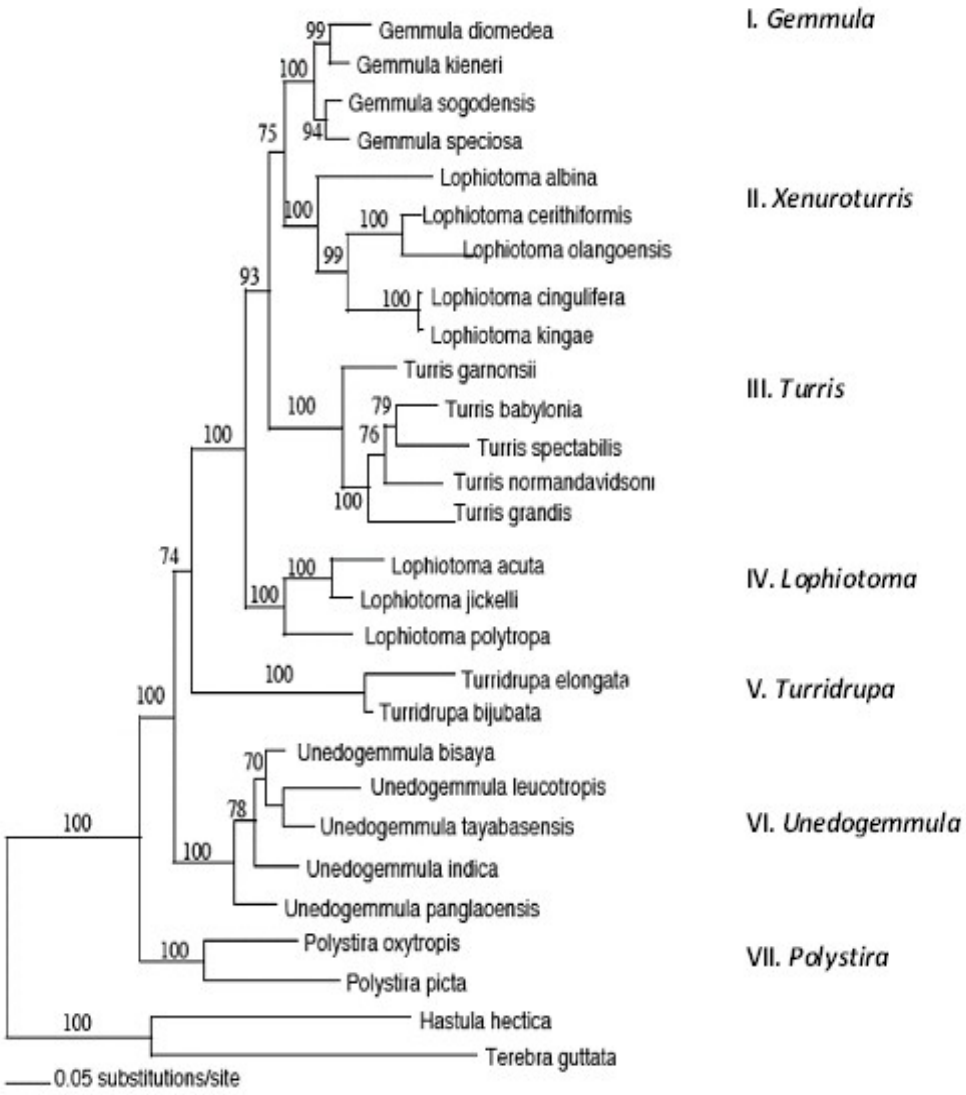

Figure 3. Phylogenetic tree. Optimal tree for the combined 12S rRNA and 16S rRNA gene sequences for *Gemmula* and their relatives based on Bayesian inference. (An identical tree was returned by a full maximum likelihood analysis of the sequence data.) Branches are labeled with Bayesian confidence values expressed as percentages. For clarity, some of the outgroup species used in the analysis have been pruned from this figure (see Methods for the full list). Shown are various forms in the subfamily Turrinae, including the four species that are the subject of this article (shells of these species are shown in Figure 1). The seven clades identified by Roman numerals all have 100% support based on both Bayesian and Maximum Likelihood analysis and have the following generic/subgeneric assignments within the subfamily Turrinae: I. *Gemmula;* II. *Xenuroturris* (presently a subgenus of *Lophiotoma*); III. *Turris*; IV. *Lophiotoma* s.s.; V. *Turridrupa*; VI. *Unedogemmula*; VII. *Polystira.*

Gemmula species is clade II, i.e., the *Xenuroturris* clade (Olivera 2002; Powell 1964), and that the four groups *Gemmula, Xenuroturris, Turris* and *Lophiotoma* (clades I to IV, respectively, in Figure 3) form a major monophyletic group within the Turrinae, which is strongly supported by the analysis.

Anatomy and morphology

There were significant morphological variations in the shells of *G. speciosa* specimens collected (i.e., gemmule shape, inter-gemmule distance, length and diameter ratio, etc). However, when molecular analysis was done to evaluate the specimens with different shell morphotypes, no significant differences could be detected in the rRNA gene sequences of the morphological variants. Thus, the shell morphological variation does not appear to be correlated with any significant genetic (12S and 16S rRNA gene) divergence.

A morphological analysis of the foregut anatomy of *G. speciosa* revealed a strong similarity to that previously reported for *Gemmula deshayesi* (Taylor et al. 1993) The radula had type 2 wishbone teeth that were robust, short and curved, sometimes with a knifelike cutting edge on the main limb and a large accessory limb with a formula of 1+0+1+0+1 (following Powell's system). An analysis of the radular structure of *G. sogodensis* revealed similar radular morphology. However, both *G. speciosa* and *G. sogodensis* differed from the radula of *L. acuta* and *U. bisaya* by the presence of central teeth. The relevant radular preparations are shown in Figure 4. The data support the clustering of *Gemmula* species into one group of the phylogenetic tree (Figure 3) and their separation from the *Lophiotoma* and *Unedogemmula* clades.

Feeding ecology

Little is known regarding the prey preference of any species of *Gemmula.* A freshly-collected specimen of *G. diomedea* was observed to regurgitate its gut contents when placed in cold 1% $MgCl_2$ solution (Figure 5); a fragment of mtCOI gene was PCR-amplified from the regurgitate, sequenced, and compared with sequences in the GenBank.

A high sequence similarity between the regurgitate COI and the COI sequence of the tube-dwelling polychaete *Amphitritides harpa* (Hutchings and Glasby 1988) was found (Figure 6). *A. harpa* belongs to the family Terebellidae, a sedentary clade of polychaetes. Given the significant homology in the toxin sequences from the three *Gemmula* species previously reported (Heralde et al. 2007), it comes as a reasonable working hypothesis that the prey of *Gemmula* species are sedentary polychaetes belonging to the family Terebellidae.

This hypothesis was experimentally evaluated by challenging the species that could be maintained successfully in an aquarium, *G. speciosa*, with a terebellid polychaete. The species of Terebellidae most accessible was a *Loimia* species. The addition of a terebellid to the tank containing turrids elicited activities such as movement of siphon and the active hunt for the prey for many of the turrids including *G. speciosa*.

In the first *Gemmula-Loimia* interaction observed, the snail detected and located the worm ~5 minutes after the latter was dropped into the aquarium. The snail moved toward one end of the worm and pinned it down using its muscular and flexible foot. It rolled its foot into a barrel-like form that can be easily mistaken as a mouth. The snail tried to fully engulf the worm with its foot but was unsuccessful. A smaller *Loimia* sp. was dropped into the aquarium. The same "foot-folding" behavior was exhibited by the snail. The rostrum was observed to have expanded as it extended towards it captured prey. The muscular foot was observed to not only pin down the worm but also helped to bring it near the snail's mouth. The snail remained motionless after ingesting ~50% of the worm's body length. The entire worm was consumed after 2 hours.

L. acuta attacked the terebellid in groups. However, in some feeding events, it was also capable of feeding alone. In both cases, the *L. acuta* extended its proboscis and quickly stabbed the worm. It then attached itself and remained motionless for an extended period of time. A closer look at the snail's mouth shows that it takes in a small portion of the worm tissue and is not limited to just sucking. It is most likely that digestion is already taking place even in the mouth. After leaving a single worm being fed on by a *L. acuta* overnight, the latter was able to eat up the anterior portion of the worm including the tentacles. When *L. acuta* are group feeding, the prey is completely consumed.

U. bisaya was not observed to react to the addition of *Loimia* sp. into the tank. In most feeding experiments, it remained submerged in the substrate. The closest interaction between *U. bisaya* and *Loimia* sp. documented was when the snail crawled on top of the worm being fed on by an *L. acuta* (http://msiconusproject.multiply.com/video).

To test the specificity of the snails' prey, other worms (< 2 cm.), e.g., blood worms (*Glycera* sp.) and fireworms, were also introduced in the tank. Several trials yielded no result as the turrids showed no activity after introduction of the worms both during day and night time. The snails remained partially burrowed under the substrate and the worms remained untouched.

DISCUSSION

The four species of *Gemmula* investigated in this study occur in relatively deep waters (>50 meters). What is interesting is the striking difference in the pattern of their abundance across the collection sites. Each species appeared to exhibit a similar distribution pattern (Table 1), being much more abundant at one site and scarce at the other sites, but the site where a species was most abundant was different for each species. The other turrid species, *U. bisaya,* showed another distribution pattern, being abundant at two sites (Sites 1-3 and 4) but not at the third site (Site 5, which is geographically close to Site 4). The field distribution of gastropods is usually associated with their larval life cycles (i.e. planktonic or non-planktonic) (Jablonski and Lutz 1983). These larval life cycles are determined from the type of protoconch that each species possess. The *Gemmula*

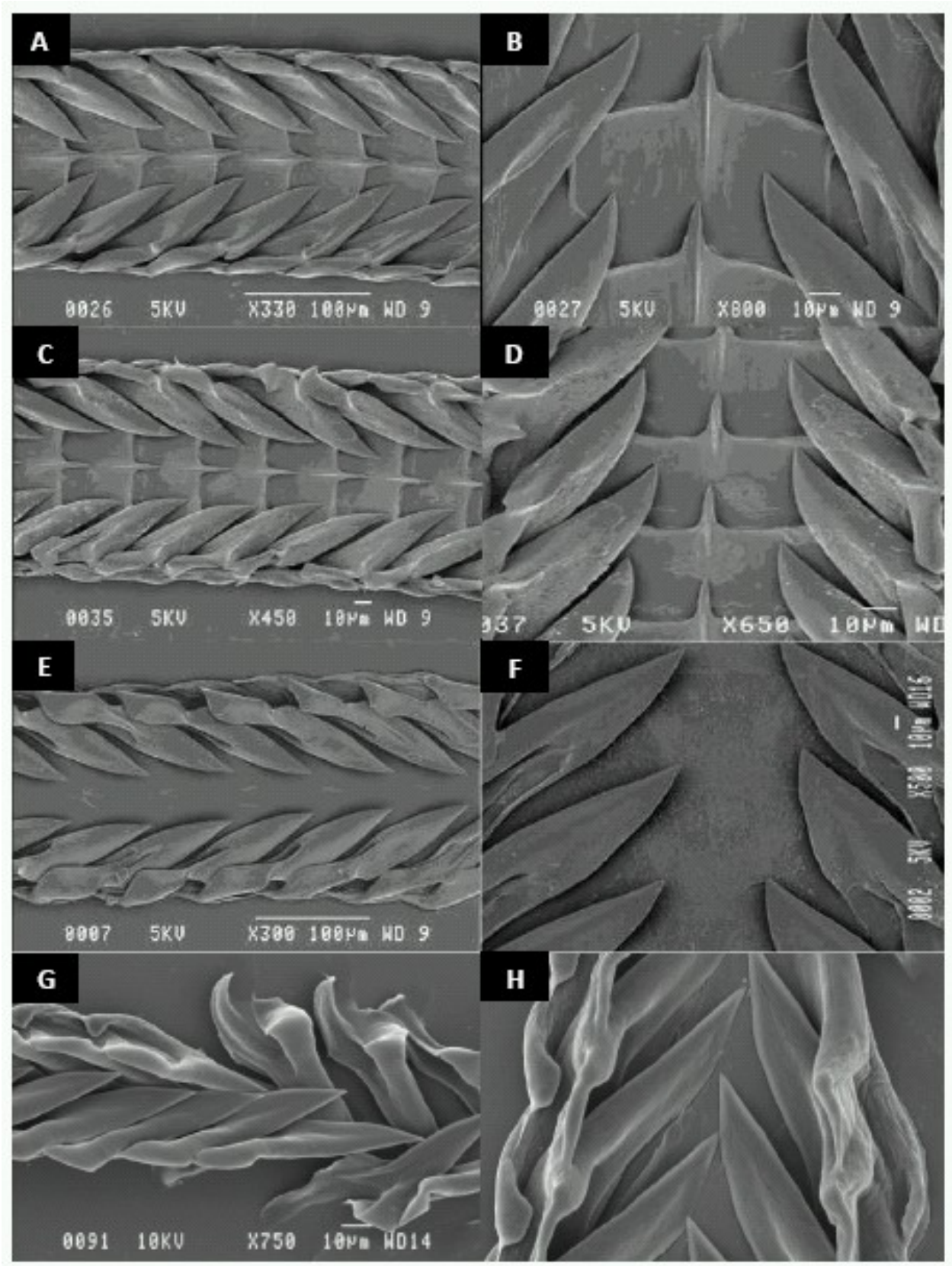

Figure 4. Radular morphology. Scanning electron microscopy images of the radula of *Gemmula speciosa* (A-B). *Gemmula sogodensis* (C-D), *Unedogemmula bisaya* (E-F) and *Lophiotoma acuta* (G-H). The central tooth is prominent in both *Gemmula* species and absent in *U. bisaya* and *L. acuta.*

species have polygyrate protoconch (Olivera 2005) and thus have planktonic larval life cycle (Jablonski and Lutz 1983), while *Unedogemmula* species have paucispiral protoconch and have short planktonic life (Olivera 2004). Surprisingly, the observed field distribution of *Gemmula* and *Unedogemmula* runs in contrast with the expected pattern of larval distribution. This result warrants further exploration to explain the ecological factors that govern this distribution pattern.

The differences in distribution pattern among the four species could also be seen when comparing their abundance. From the collection by commercial fishing vessels near Manila Bay, the number of specimens of *G. speciosa* collected was greater than the other three (*G. sogodensis, G. diomedea* and *G. kieneri*); the latter two species were entirely absent from site 1-3 (see Table I). In sites 4 and 5 respectively, *G. sogodensis* and *G. diomedea* were the predominant species found, with only a minor amount of overlap. The fourth species, *G. kieneri*, was only collected at site 5. Again, the ecological factors that could explain these interspecies differences in the spatial pattern of abundance remain to be investigated.

The phylogeny of Turrinae was reconstructed based on the 16S rRNA gene sequence and congruency was observed with 12S rDNA-based tree reported by Heralde et al. (2007). The agreement of results from two independent gene markers provides strong support for the phylogenetic relationship of members of Turrinae under study. The *Gemmula species* in particular have shown a consistent clustering where three major groups emerge: the *Gemmula speciosa* group, the *Gemmula diomedea* group and the *Gemmula lisajoni* group. The close association reflected between *G. diomedea* group and *G. speciosa* group more than the *G. lisajoni* group, indicates a sharing of biological characteristics (like food type/preference). We have shown, by molecular regurgitate analysis, the diet of *Gemmula diomedea* to be a tube-dwelling polychaete belonging to the Terebellid group. Similar attempts in *G. speciosa* have been unsuccessful. We utilized the molecular approach of inferring food type from the phylogenetic relatedness based on the 16S rRNA gene sequence (Duda et al. 2001; Espiritu et al. 2001) and validated this prediction with a Terebellidae feeding challenge.

In the combined 12S and 16S sequence analysis, the four *Gemmula* species (*G. speciosa, G. sogodensis, G. kieneri* and *G. diomedea*) are clearly closely related phylogenetically (Figure 3). The morphological similarity of their shells is shown in Figure 1. These species form a distinct well-supported *Gemmula* clade in the tree relative to the other groups in the subfamily Turrinae.

Figure 5. Prey determination. A freshly collected *G. diomedea* with regurgitated prey tissue.

This result further warrants the *Gemmula* clade as likely Terebellid worm feeders analogous to the worm-hunting coniids, the S1– sedentary polychaete feeders, feeding mainly on Terebellidae (Duda et al. 2001).

The phylogenetic results also have implications on the current understanding of the phylogeny of the group. If the phylogenetic scheme revealed by our analysis is confirmed by a more extensive analysis, it would seem justified to separate *Xenuroturris* (i.e., clade II, consisting of what is currently recognized as *Lophiotoma* species) from *Lophiotoma* (clade IV) at the generic level. It must be noted that in this scheme, the form *Lophiotoma albina*, which is generally not included in *Xenuroturris* (but in *Lophiotoma* s.s.) based on shell morphology, is grouped with the *Xenuroturris* (clade II). This is consistent with the previous observation by Olivera (2002) that *L. albina* was very closely related to species assigned previously to *Xenuroturris* (Powell 1964). The outgroup taxa used were in the family Terebridae (*Hastula hectica* and *Terebra guttata*). Our results further indicate that the subfamily Turrinae is a

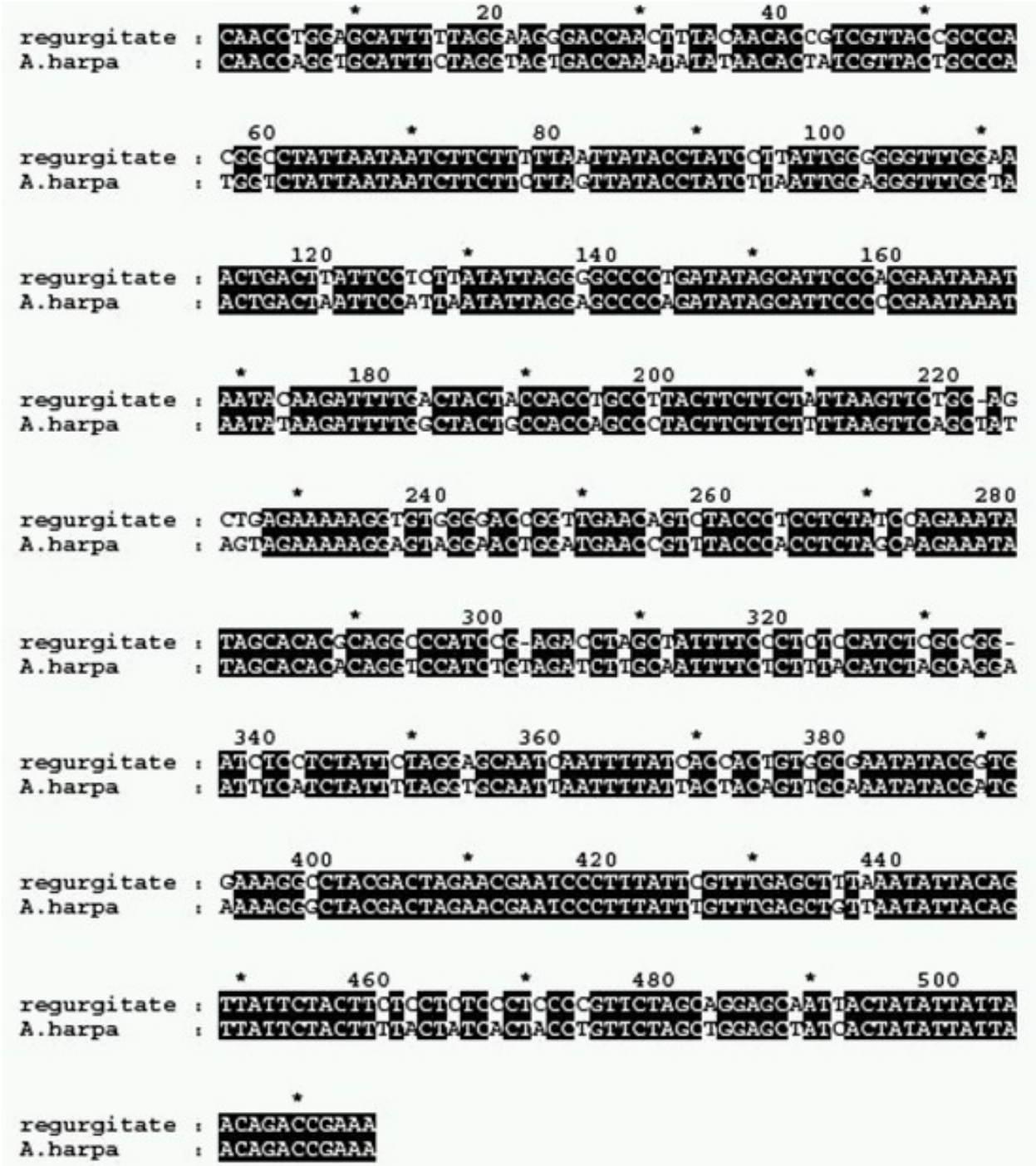

Figure 6. Molecular regurgitate analysis. BLAST hit for COI gene from regurgitated tissue (Query sequence) indicating the generic identity of the polychaete to be *Amphitritides* sp. (Subject sequence).

monophyletic assemblage; the closer kinship of *Gemmula, Xenuroturris, Turris,* and *Lophiotoma* to each other than to the other turrine groups is strongly supported by this analysis. All of the taxa shown are in the Pacific, except for the two *Polystira* species which appear to be outliers with respect to the other branches shown within the subfamily. This result also points out the value of combined 12S and 16S rDNA gene markers in generating a reasonably sound phylogenetic inference for the members of Turrinae.

The analysis of the radular structure shows a clear distinction between *G. speciosa* and *G. diomedea*, in one hand, versus *L. acuta* and *U. bisaya*, on the other hand. The radula is

an important structure for prey hunting of venomous gastropods. In coniids, the hypodermic type of radula is found in fish-hunting cones (Olivera 1997) while a typical hypodermic type radula, enrolled, with a wide opening at the base is found in the terebrid, *Hastula hectica* (Imperial et al. 2007); thus radular structure may define the likely prey to a particular snail. The emergence of the central tooth to the *Gemmula* radulae suggests a prey-specificity that could be validated by the polychaete feeding challenge.

The recent molecular analysis of Heralde et al. (2007) demonstrated that contrary to the phylogenetic scheme proposed by Powell (1964), *Unedogemmula* is not a subgenus of *Gemmula* and in fact, within the subfamily Turrinae, it is one of the more distant groups from *Gemmula*. The radular differences documented here provide additional support for the generic separation of *Gemmula* from *Unedogemmula*. This conclusion is further strengthened by the phylogenetic analysis carried out in this study (Figure 3). The data shown provide the basis for a comparison of molecular phylogeny with the sequences of toxins (described in studies presented elsewhere (Heralde et al. 2008). Both the present data and the toxinological data are consistent with the four species of *Gemmula* defining a distinct clade within the subfamily Turrinae. It should be noted, however, that the taxonomic status of the genus *Gemmula* needs reevaluation. Recent unpublished data (C. Meyer, personal communication) suggest that Atlantic and Eastern Pacific forms of *Gemmula* do not belong in the same clade as the Indo-Pacific species in Figures 1 and 3. This would create a problematic taxonomic situation because the type species of *Gemmula* is *Pleurotoma gemmata* (= *G. hindsiana*) from the Eastern Pacific.

Finally, we have provided the first data suggesting what species (or group) of polychaetes the *Gemmula* species might target as prey. A regurgitate from *G._diomedea* collected in the field was analyzed by the barcode (COI gene) sequence; the match with the polychaete*, A. harpa,* provided the first evidence for *Gemmula* being predators of sedentary polychaetes in the family Terebellidae. We have tested this hypothesis more directly: a *Gemmula* species that could be maintained in an aquarium for extended periods was challenged with the Terebellid species most readily collected alive, a species in the genus *Loimia.* When *Loimia* was presented to *Gemmula*, this triggered an attack; the worm was engulfed and ingested by the *Gemmula*. The molecular analysis combined with the aquarium challenge experiment is consistent with members of Terebellidae being major prey of this clade of *Gemmula* species. The feeding experiment on *L. acuta* expanded the group of turrids that preys on terebellids. This response of *L. acuta* was consistent with the prediction of the 16S-based clustering of turrids with similar prey type/preference. The non-responsive behavior of *U. bisaya* towards *Loimia* sp. indicated that it may not be its prey preference. This also supports the generic separation of *Gemmula* from *Unedogemmula*. Correlating this result with radular data, the presence/absence of central teeth does not appear to support prey type/preference as observed in *L. acuta.*

Hence, two lines of proof were provided to demonstrate *Gemmula* as a distinct clade of worm-hunting Turrinae feeding on Terebellidae. The molecular phylogenetic analyses (i.e., the 16S-based clustering of snails with similar food type and combined 12S and 16S rRNA gene sequences that define the *Gemmula* clade) and the polychaete feeding challenge provide consistent evidence of this distinction. The radular anatomy did not support prey preference in contrast with the 16S clustering data. The radular anatomy's non-correlation with prey preference could be validated in the genus *Turris*, where the central teeth presence/absence is diverse within a specific 16S cluster. Furthermore, the 16S clustering further validated the expanded spectrum of Terebellidae feeding turrids which includes *L. acuta*. Meanwhile, the field distribution study demonstrates an unusual pattern that requires further investigation.

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AUTHORS CONTRIBUTIONS

FMH contributed majority of the 16S and 12S rDNA sequences while MW provided the others; FMH performed the 16S clustering and the molecular regurgitate analysis under the guidance of GPC and ADS; RG provided the field collection data under the guidance of PA; YIK provided the radula images; MQA performed the polychaete feeding challenge and provided the video documentation under the guidance of AOL; PSC provided the combined 16S and 12S rDNA sequence analysis; BMO provided the shell images and was mainly responsible for the preparation of the manuscript.

REFERENCES

Bouchet P, Lozouet P, Maestrati P, Heros V. Assessing the magnitude of species richness in tropical marine environments: exceptionally high numbers of molluscs at a New Caledonia site. Biol J Linnaean Soc 2002; 75: 421-436.

Duda TFJr, Palumbi SR. Molecular genetics of ecological diversification: duplication and rapid evolution of toxin genes of the venomous gastropod *Conus*. Proc Nat Acad Sci USA 1999; 96: 6820-3.

Duda TFJr, Kohn AJ, Palumbi SR. Origins of diverse feeding ecologies within *Conus*, a genus of venomous marine gastropods. Biol J Linnaean Soc 2001; 73: 391-409.

Espiritu JD, Watkins M, Monje VD, Cartier GE, Cruz LJ, Olivera BM. Venomous cone snails: molecular phylogeny and the generation of toxin diversity. Toxicon 2001; 39: 1899-1916.

Heralde FMIII, Imperial J, Bandyopadhyay PK, Olivera BM, Concepcion GP, Santos AD. A rapidly diverging superfamily of peptide toxins in venomous *Gemmula* species. Toxicon.2008; 51: 890-897.

Heralde FMIII, Watkins M, Ownby J-P, Bandyopadhyay PK, Santos AD, Concepcion GP, Olivera BM. Molecular phylogeny of some Indo-Pacific genera in the family Turrinae H. Adams and A. Adams. Nautilus 2007; 121: 131-138.

Hutchings PA, Glasby, CJ. The Amphitritinae (Polychaeta: Terebellidae) from Australia. Records of the Australian Museum 1988; 40: 1-60.

Imperial J, Kantor Y, Watkins M, Heralde FMIII, Stevenson BJ, Chen P, Ownby P-J, Bouchet P, Olivera BM. The venomous auger snail *Hastula (Impages) hectica* (Linnaeus, 1758): molecular phylogeny, foregut anatomy and comparative toxinology. J Exp Zoo 2007; Part B: 6,744-756.

Jablonski D, Lutz R. Larval ecology of marine benthic invertebrates: paleobiological implications. Biol Rev 1983; 58:21-89.

Kumar S, Tamura K, Jakobsen IB, Nei M. MEGA2: molecular evolutionary genetics analysis software. Bioinformatics 2001; 17:1244–1245.

López-Vera E, Heimer de la Cotera EP, Maillo M, Riesgo-Escovar JR, Olivera BM, Aguilar MB. A novel structural class of toxins: the methionine-rich peptides from the venoms of turrid marine snails (Mollusca, Conoidea). Toxicon 2004; 43: 365–374

Monje VD, Ward R, Olivera BM, Cruz LJ. 16S mitochondrial ribosomal RNA gene sequences: a comparison of seven *Conus* species. Phil J Sci 1999; 128: 225-237.

Olivera B M. The gastropod genus *Xenuroturris* (Iredale, 1929) evaluated and a new turrid *Lophiotoma olangoensis*, described from the central Philippines. Sci Diliman 2002; 14: 39-49.

Olivera BM. Larger forms of *Lophiotoma*: four new species described in the Philippines and three from elsewhere in the Indo-Pacific. Sci Diliman 2004; 16: 1-28.

Olivera BM. Evaluation of Philippine *Gemmula:* I. Forms related to *G. speciosa* and *G. kieneri.* Sci Diliman 2005; 17: 1-14.

Olivera BM. Conus venom peptides, receptor and ion channel targets, and drug design: 50 million years of neuropharmacology. Essay E.E. Just Lecture, 1996. Molec Bio Cell 1997; 8:2101–2109.

Powell AWB. The family Turridae in the Indo-Pacific: Part 1 - The subfamily Turrinae. Indo-Pacific Mollusca 1964; 1: 227-340.

Puillandre N, Samadi S, Boisselier M-C, Sysoev AV, Kantor YI, Cruaud C, Couloux A, Bouchet P. Starting to unravel the toxoglossan knot: molecular phylogeny of the "turrids" (Neogastropoda: Conoidea). Mol Phylogen Evol 2008; 47:1122-1134.

Rex M.A, Stuart CT, Coyne G. Latitudinal gradients of species richness in the deep-sea benthos of the North Atlantic. Proc Nat Acad Sci USA 2000; 97: 4082–4085

Simison WB. Evolution and phylogeography of new world gastropod faunas. PhD Dissertation, UC-Berkeley 2000; 202 pp.

Taylor JD, Kantor YI, Sysoev AV. Foregut anatomy, feeding mechanisms, relationships and classification of the Conoidea (=Toxoglossa) (Gastropoda). Bull Nat His Mus London (Zool) 1993; 59: 125-170.

Watkins M, Hillyard DR, Olivera BM. Genes expressed in a turrid venom duct: divergence and similarity to conotoxins. J Mol Evol 2006; 62: 247-56.