Correlating Molecular Phylogeny with Venom Apparatus Occurrence in Panamic Auger Snails (Terebridae)

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Abstract

Central to the discovery of neuroactive compounds produced by predatory marine snails of the superfamily Conoidea (cone snails, terebrids, and turrids) is identifying those species with a venom apparatus. Previous analyses of western Pacific terebrid specimens has shown that some Terebridae groups have secondarily lost their venom apparatus. In order to efficiently characterize terebrid toxins, it is essential to devise a key for identifying which species have a venom apparatus. The findings presented here integrate molecular phylogeny and the evolution of character traits to infer the presence or absence of the venom apparatus in the Terebridae. Using a combined dataset of 156 western and 33 eastern Pacific terebrid samples, a phylogenetic tree was constructed based on analyses of 16S, COI and 12S mitochondrial genes. The 33 eastern Pacific specimens analyzed represent four different species: *Acus strigatus*, *Terebra argyosia*, *T. ornata*, and *T.* cf. *formosa*. Anatomical analysis was congruent with molecular characters, confirming that species included in the clade *Acus* do not have a venom apparatus, while those in the clade *Terebra* do. Discovery of the association between terebrid molecular phylogeny and the occurrence of a venom apparatus provides a useful tool for effectively identifying the terebrid lineages that may be investigated for novel pharmacological active neurotoxins, enhancing conservation of this important resource, while providing supplementary information towards understanding terebrid evolutionary diversification.

Introduction

The auger snails (family Terebridae) are a distinctive group of carnivorous, sand-dwelling gastropods included in the superfamily Conoidea, along with cone snails and turrids [1]. Species in this large gastropod superfamily (>10,000 species) generally use venom to capture their prey [2,3]. Conoidean venoms are of considerable interest as they are a rich source of neuroactive peptides, widely used to investigate cellular communication in the nervous system [4–6]. Some Conoidean venom components have been used directly for a variety of biomedical applications [7,8]. Several peptides from cone snail venoms have reached human clinical trials, and one (Prialt) has been approved as a drug for intractable pain [9,10].

In contrast to cone snail toxins (conotoxins), terebrid toxins are largely uncharacterized and no physiological target for any terebrid venom peptide has been defined. However, the very preliminary characterization carried out to date suggests that the venoms of the Terebridae have novel components, distinct from other conoidean venoms [11,12]. Thus, terebrid venoms are potentially a rich, unexplored pharmacological resource.

A significant fraction of the ~300–400 species in the Terebridae do not have the characteristic anatomical structures that comprise the venom delivery apparatus of conoidean snails, namely a venom bulb, venom duct, and radula sac [13–16]. Analysis of shell morphology alone cannot generally determine whether or not a species in the Terebridae has a venom apparatus. The non-monophyly of most of the terebrid genera makes the attribution of a specimen to a particular genus challenging. Therefore, identifying *a priori* which species to collect for the analysis of venom components is problematic.

The first molecular phylogeny of the Terebridae based on a three-gene matrix of molecular markers 12S, 16S, and cytochrome oxidase I (COI), was recently published[16]. The data suggest that the family Terebridae could be divided into at least 5 distinctive generic clades: *Acus*, *Terebra*, *Hastula*, *Myurella*, and a sister clade of the four others that includes *Terebra jungi* (recently revised to *Pellifronia jungi* [17]). Furthermore, based on species clusters, it was suggested that molecular data may be a useful tool to identify which terebrid species have a venom apparatus and which do not. For these molecular criteria to reliably indicate which species of terebrids are venomous, the criteria should be applicable to all Terebridae.

The original correlation between venom apparatus and molecular phylogeny was established using only western Pacific species [16]. This paper examines the validity of correlating

molecular phylogeny and venom apparatus by increasing the diversity of taxa sampled and the geographic coverage to include terebrid samples from the eastern Pacific. There are currently 55 described species of terebrids found in the Panamic fauna as defined by Keen [18]. In terms of geographic distribution, the Panamic tropical molluscan marine fauna is arguably highly divergent from that of the western Pacific. Thus, whether the molecular phylogeny/venom apparatus correlation established for western Pacific terebrid samples can be used to assess eastern Pacific terebrid snails is a central issue addressed by this study. Presented here is the first molecular analysis of Panamic Terebridae, which is used to highlight both phylogenetic and taxonomic issues for this group.

Materials and Methods

Material

Panamic specimens used were dredged from the Las Perlas Archipelago in 2008, using The Smithsonian Tropical Research Institute research vessel RV-Urraca. The collected material was specifically fixed for molecular and anatomical analysis. Living specimens were anesthetized in $MgCl_2$ isotonic with seawater for 1 or 2 hours. Samples were dissected and a piece of tissue (usually foot) was fixed in 95% ethanol. Table 1 lists all terebrid specimens used in this study, including the specific geographical coordinates of where they were collected (for map, see Figure 1). Taxonomic assignments were made based on shell morphology. Vouchers of the Panamic specimens are deposited in the Muséum National d'Histoire Naturelle (MNHN) of Paris. Included with the 33 Panamic taxa are sequences from specimens collected in the western Pacific and analyzed in Holford *et al.* 2009 [16]. Outgroups are identical to those used in Holford et al. 2009 [16] and identified in Table 1.

Sequencing

DNA was extracted from foot or other tissue using Qiagen QIAamp Dneasy Tissue kit. Fragments of mitochondrial genes 12S, 16S and COI were amplified using universal primers 12S1/12S3 [19], 16Sar/16Sbr [20], and LCO1490/HCO2198 [21] respectively. PCR reactions were performed in 25 µl, containing 3 ng of DNA, 10X reaction buffer, 2.5 mM $MgCl_2$, 0.26 mM dNTP, 0.3 mM each primer, 5% DMSO, and 1.5 units of Qbiogene Q-Bio Taq or Advantage® 2 PCR Kit from Clontech. Amplification was performed as previously described [16]. PCR products were purified using USB ExoSAP-IT® or Quiagen PCR purification kit and sequenced. All genes were sequenced in both directions. Sequences were deposited in GenBank (Genbank accession numbers: FJ707376-FJ707472). Specimens data and COI sequences were also deposited in BOLD (Barcode of Life Data Systems, project CONO - Conoidea barcodes and taxonomy).

Molecular and Phylogenetic analyses

COI sequences were manually aligned and 12S and 16S were automatically aligned using ClustalW multiple alignment implemented in BioEdit version 7.0.5.3 [22]. The accuracy of automatic alignments was confirmed by visual inspection. Hyper-variable regions of 12S and 16S genes were excluded from further analyses due to ambiguities in the alignments. All the western Pacific terebrid sequences obtained by Holford *et al.* 2009 [16] were included in this new dataset.

Phylogenetic analyses were based on reconstructions using two approaches: (i) Maximum Likelihood (ML) using PhyML 2.4.4 [23], where support of nodes were estimated with 100 bootstrap replicates, and (ii) Bayesian Analyses (BA) consisting of six Markov chains, 10,000,000 generations each, with a sampling frequency of one tree each thousand generations, run in four parallel analyses using MrBayes [24]. The number of swaps that are tried each time the chain stops for swapping was 4, and the chain temperature was set at 0.05. Twenty-five percent of the first generations were discarded as burnin, which correspond to the time the chain took to reach stationarity. For both ML and BA, the best-fitting model of evolution was applied, as determined by Modelgenerator V.85 following the Hierarchical Likelihood Ratio Test (with four discrete gamma categories). Variation was partitioned among genes and gene-specific model parameters were used. Each gene was first analysed separately and then the combined dataset was analysed. For the combined dataset one model of evolution for the concatenation of the three genes was used for the ML analysis. For the BA, a different model was applied for each gene as determined by Modelgenerator.

Results

Distribution of the Panamic Terebridae

The 33 Panamic specimens analyzed were assigned to four different terebrid species: *Acus strigatus, Terebra argyosia, T. ornata,* and *T.* cf. *formosa.* All taxonomic assignments made are based on shell morphology and later confirmed by molecular results. The *T. argyosia* specimens (collection sites 1, 2, 3, and 4) appear to be present both in the northern and southern ends of the archipelago (Figure 1A). *A. strigatus* was found between Punta Coco on Isla Del Rey and San Jose (sites 4 and 6). *T. ornata* was collected along the eastern coast of San Jose (site 5) and *Terebra* cf. *formosa* at site 7. Examples of the actual specimens analyzed are shown in Figure 1B.

Phylogenetic analyses

After alignment, DNA fragments of 658, 534, and 455 bp were obtained for COI, 12S, and 16S genes, respectively. No contradictions were observed when independently constructed gene trees for COI, 12S, and 16S genes were analyzed (results not shown). These Panamic sequences were combined with sequences from western Pacific terebrid specimens to reconstruct the phylogeny illustrated in Figure 2. The best model of evolution for the COI, 12S and 16S and for the combined dataset is GTR+I+G (General Time Reversible model, with invariant sites and a gamma law parameter) for all genes, with $I = 0.51$ and $\alpha = 0.68$ for COI, $I = 0.6$ and $\alpha = 0.62$ for 12S, $I = 0.34$ and $\alpha = 0.32$ for 16S and $I = 0.41$ and $\alpha = 0.4$ for the combined dataset. Results obtained with Maximum Likelihood (ML) and Bayesian analyses (BA) are highly similar, however, the support values for ML were generally weaker.

Of the 5 distinct terebrid clades previously identified, Clade A (*P. jungi*), Clade B (*Acus*), Clade C (*Terebra*), Clade D (*Hastula*), and Clade E (*Myurella*), the Panamic sequences reported here fall into the *Acus* and *Terebra* clades. As a result, in order to reduce the size of the tree and to focus on the Panamic clades, only the *Acus* and *Terebra* clades are detailed in Figure 2. The other clades, represented by a single branch, are identical to those in Holford *et al.* 2009 [16].

The phylogenetic analysis strongly indicates that the Panamic *Acus strigatus* specimens in our sampling are within the *Acus* clade (Posterior Probablity (PP) = 1; Bootstraps (B) = 98). The *Acus* clade comprises a prevalence of western Pacific species (*A. felinus, A. chloratus, A. maculatus, A. areolatus, A. crenulatus,* and *A. dimidatus*). The monophyly of the Panamic specimens identified as belonging to the *Terebra* clade is well-supported (PP = 1; B = 96) within this group. As

Table 1. List of terebrid samples used in this study. VA = venom apparatus.

Genus	Species	COI	12S	16S	VA	Station number - Coordinates/Depth	MNHNnumber
Panamic Specimens							
Acus	strigatus (Sowerby, 1825)	x	x	x	No	3–08°11.8′N, 078°57.1′W/21.4 m	42093
Acus	strigatus (Sowerby, 1825)	x	x	x	No	4–08°11.8′N, 078°57.5′W/22.4 m	42105
Acus	strigatus (Sowerby, 1825)	x	x	x	No	5–08°14.7′N, 079°05.55′W/17.5 m	42136
Acus	strigatus (Sowerby, 1825)	x	x	x	No	5–08°14.7′N, 079°05.55′W/17.5 m	42137
Acus	strigatus (Sowerby, 1825)		x	x	No	9–08°30.1′N, 079°06.0′W/21 m	42159
Terebra	argosyia (Olsson, 1971)	x	x	x	Yes	1–08°37.18′N, 079°01.12′W/25 m	42068
Terebra	argosyia (Olsson, 1971)	x	x	x	Yes	1–08°37.18′N, 079°01.12′W/25 m	42069
Terebra	argosyia (Olsson, 1971)			x	Yes	2–08°15.61′N, 078°51.57′W/24.1 m	42071
Terebra	argosyia (Olsson, 1971)	x	x	x	Yes	2–08°15.61′N, 078°51.57′W/24.1 m	42072
Terebra	argosyia (Olsson, 1971)	x	x	x	Yes	2–08°15.61′N, 078°51.57′W/24.1 m	42073
Terebra	ornata (Gray, 1834)			x	Yes	1–08°37.18′N, 079°01.12′W/20 m	42074
Terebra	argosyia (Olsson, 1971)	x	x	x	Yes	3–08°11.8′N, 078°57.1′W/21.4 m	42084
Terebra	argosyia (Olsson, 1971)	x	x	x	Yes	3–08°11.8′N, 078°57.1′W/21.4 m	42085
Terebra	argosyia (Olsson, 1971)	x	x	x	Yes	3–08°11.8′N, 078°57.1′W/21.4 m	42086
Terebra	argosyia (Olsson, 1971)	x	x	x	Yes	3–08°11.8′N, 078°57.1′W/21.4 m	42087
Terebra	argosyia (Olsson, 1971)	x	x	x	Yes	3–08°11.8′N, 078°57.1′W/21.4 m	42089
Terebra	argosyia (Olsson, 1971)	x	x	x	Yes	3–08°11.8′N, 078°57.1′W/21.4 m	42090
Terebra	argosyia (Olsson, 1971)	x	x	x	Yes	3–08°11.8′N, 078°57.1′W/21.4 m	42091
Terebra	argosyia (Olsson, 1971)	x	x	x	Yes	3–08°11.8′N, 078°57.1′W/21.4 m	42092
Terebra	argosyia (Olsson, 1971)	x	x	x	Yes	4–08°11.8′N, 078°57.5′W/24 m	42099
Terebra	argosyia (Olsson, 1971)	x	x	x	Yes	4–08°11.8′N, 078°57.5′W/24 m	42100
Terebra	argosyia (Olsson, 1971)			x	Yes	4–08°11.8′N, 078°57.5′W/24 m	42102
Terebra	argosyia (Olsson, 1971)	x	x	x	Yes	4–08°11.8′N, 078°57.5′W/22.4 m	42103
Terebra	argosyia (Olsson, 1971)	x	x	x	Yes	4–08°11.8′N, 078°57.5′W/22.4 m	42104
Terebra	argosyia (Olsson, 1971)	x	x	x	Yes	4–08°11.8′N, 078°57.5′W/22.4 m	42118
Terebra	argosyia (Olsson, 1971)	x	x	x	Yes	4–08°11.8′N, 078°57.5′W/22.4 m	42119
Terebra	argosyia (Olsson, 1971)	x	x	x	Yes	4–08°11.8′N, 078°57.5′W/22.4 m	42120
Terebra	argosyia (Olsson, 1971)	x	x	x	Yes	4–08°11.8′N, 078°57.5′W/22.4 m	42121
Terebra	argosyia (Olsson, 1971)	x	x	x	Yes	4–08°11.8′N, 078°57.5′W/22.4 m	42122
Terebra	argosyia (Olsson, 1971)	x	x	x	Yes	4–08°11.8′N, 078°57.5′W/22.4 m	42123
Terebra	argosyia (Olsson, 1971)	x	x	x	Yes	4–08°11.8′N, 078°57.5′W/22.4 m	42124
Terebra	argosyia (Olsson, 1971)	x	x	x	Yes	4–08°11.8′N, 078°57.5′W/22.4 m	42125
Terebra	ornata (Gray, 1834)	x	x	x	Yes	6–08°14.94′N, 079°05.7′W/14.3 m	42131
Terebra	cf. formosa	x	x	x	Yes	7–08°16.86′N, 079°02.67′W/39.2 m	42152
Terebra	argosyia (Olsson, 1971)		x	x	Yes	8–08°24.50′N, 079°04.66′W/18.4 m	42153
IndoPacific Specimens							
Acus	maculatus (Linnaeus, 1758)	x	x	x	No	9°37.4′N, 123°46.9′E, 3–20 m	30370
Acus	dimidiatus (Linnaeus, 1758)	x	x	x	No	15°32.5′S, 167°10.5′E, 5–10 m	30372
Acus	dimidiatus (Linnaeus, 1758)	x	x	x	No	15°36.9′S, 167°10.5′E, 6–33 m	30373
Acus	crenulatus (Linnaeus, 1758)	x	x	x	No	15°34.4′S, 167°13.1′E, 9 m	30377
Acus	dimidiatus (Linnaeus, 1758)	x	x	x	No	15°32.5′S, 167°10.5′E, 5–10 m	30379
Acus	dimidiatus (Linnaeus, 1758)	x	x	x	No	15°35.4′S, 166°59.7′E, 3–37 m	30381
Acus	maculatus (Linnaeus, 1758)	x	x	x	No	15°28.7′S, 167°15.2′E, 19 m	30389
Acus	dimidiatus (Linnaeus, 1758)	x	x	x	No	15°38.1′S, 167°05.9′E, intertidal	30428
Acus	felinus (Dillwyn, 1817)	x	x	x	No	9°37.4′N, 123°54.5E, 6–8 m	30443
Acus	felinus (Dillwyn, 1817)	x	x	x	No	9°37.4′N, 123°54.5E, 6–8 m	30445
Acus	chloratus (Lamarck. 1822)	x	x	x	No	15°22.6′S, 167°11.6′E, intertidal	30490
Acus	crenulatus (Linnaeus, 1758)	x	x	x	No	15°34.4′S, 167°13.1′E, 9 m	30494
Acus	areolatus (Link, 1807)	x	x	x	No	9°37.4′N, 123°46.9′E, 3–20 m	30587

Table 1. Cont.

| Genus	Species	COI	12S	16S	VA	Station number - Coordinates/Depth	MNHNnumber
Cinguloterebra	cf. fujitai (Kuroda & Habe, 1952)	x	x	x	Yes	9°27.4'N, 123°49.4'E, 273–356 m	15724
Cinguloterebra	cf. fenestrata (Hinds, 1844)	x	x	x	Yes	9°36.2'N, 123°43.8'E, 382–434 m	16735
Cinguloterebra	cf. fenestrata (Hinds, 1844)	x	x	x	Yes	9°29.4'N, 123°44.4'E, 271–318 m	30390
Cinguloterebra	triseriata (JE Gray, 1824)	x	x	x	Yes	9°35.3'N, 123°52.2'E, 84–87 m	30404
Cinguloterebra	fenestrata type I	x	x	x	Yes	9°39.2'N, 123°47.5'E, 255–268 m	30410
Cinguloterebra	fenestrata type II	x	x	x	Yes	9°39.2'N, 123°47.5'E, 255–268 m	30418
Cinguloterebra	lima (Deshayes, 1857)	x	x	x	Yes	15°32.5'S, 167°10.5'E, 5–10 m	30485
Cinguloterebra	lima (Deshayes, 1857)	x	x	x	Yes	8°39.5' S, 157°23.0' E, 214–243 m	30487
Cinguloterebra	jenningsi (RD Burch. 1965)	x	x	x	Yes	15°28.6'S, 167°15.1'E, 3–31 m	30544
Cinguloterebra	anilis (Röding, 1798)	x	x	x	Yes	15°35.2'S, 167°59.4'E, intertidal	30552
Hastula	strigilata (Linnaeus, 1758)	x	x	x	Yes	15°35.2'S, 167°59.4'E, intertidal	30420
Myurella	affinis (JE Gray 1834)	x	x	x	No	9°37.4'N, 123°54.5'E, 6–8 m	30430
Terebra	guttata (Röding, 1798)	x	x	x	Yes	15°33.1'S, 167°12.2'E, 3–40 m	30376
Terebra	babylonia (Lamarck. 1822)	x	x	x	Yes	15°31.1'S, 167°10.5'E, 7 m	30380
Terebra	subulata (Linnaeus, 1767)	x	x	x	Yes	15°36.6'S, 167°10.1'E, 8–20 m	30386
Terebra	guttata (Röding, 1798)	x	x	x	Yes	15°33.1'S, 167°12.2'E, 3–40 m	30387
Terebra	laevigata (JE Gray, 1834)	x	x	x	Yes	15°36.9'S, 167°10.5'E, 6–33 m	30394
Terebra	tricolor(GB Sowerby I, 1825)	x	x	x	Yes	15°33.1'S, 167°17.8'E, 15–25 m	30409
Terebra	laevigata (JE Gray, 1834)	x	x	x	Yes	9°36.8'N, 123°52.2'E, intertidal	30431
Terebra	subulata (Linnaeus, 1767)	x	x	x	Yes	9°37.4'N, 123°54.5E, 6–8 m	30444
Terebra	subulata (Linnaeus, 1767)	x	x	x	Yes	9°32.8'N, 123°42.1'E, 3–35 m	30483
Terebra	tricolor(GB Sowerby I, 1825)	x	x	x	Yes	15°38.5'S, 167°15.1'E, 13 m	30493
Terebra	laevigata (JE Gray, 1834)	x	x	x	Yes	15°26.6'S, 167°15.2'E, intertidal	30597
Terebra	laevigata (JE Gray, 1834)	x	x	x	Yes	15°43.4'S, 167°15.0'E, 6	30603
Terebra	laevigata (JE Gray, 1834)	x	x	x	Yes	15°31'S, 167°09'E, intertidal	30613
Terebra	laevigata (JE Gray, 1834)	x	x	x	Yes	15°31'S, 167°09'E, intertidal	30632
Pellifronia	jungi (Lai, 2001)	x	x	x	Yes	9°37.5'N, 123°40.2'E, 606–631 m	30395
Outgroups							
Cochlespira sp. (Turridae)		x	x	x		21°10'S, 158°39'E, 650–723 m	40568
Conus nereis (Conidae)		x	x	x	Yes	9°32.5'N, 123°41.8'E, 111–115 m	17922
Harpa sp. (Harpidae)		x	x	x		9°32.5'N, 123°41.8'E, 111–115 m	40569
Iotyrris cingulifera (Turridae)		x	x	x		15°33.6'S, 167°16.6'E, 8–9 m	17685

illustrated in the tree there are three distinct Panamic species present, *Terebra argyosia*, *Terebra ornata*, and *Terebra* cf. *formosa*.

Character evolution

All Panamic specimens collected were dissected and the presence or absence of a venom apparatus was noted (Table 1). The presence/absence of a venom apparatus is a character trait that can be correlated with the molecular phylogeny of these specimens. The character evolution of the venom apparatus in the Terebridae was mapped previously for western Pacific specimens [16], indicating this group has lost the venom apparatus at least twice during its evolution. As indicated in Figure 2, the Panamic species placed in the *Acus* clade, *A. strigatus*, did not have a venom apparatus (highlighted with a white box). However, *T. ornata*, *T. argyosia*, and *T.* cf. *formosa*, all have a venom apparatus and fall within the genus *Terebra*, which contains other terebrid species identified as having a venom apparatus [13,25] (highlighted by a black box).

Discussion

Predatory marine snails of the superfamily Conoidea produce several neurotoxins in their venom that are used to capture and subdue prey [26–28]. The characteristic venom apparatus of conoideans is not present in a significant fraction of species in the family Terebridae. For this work, four Panamic species, *Acus strigatus*, *Terebra argyosia*, *Terebra ornata*, and *Terebra* cf. *formosa*, were analyzed using a combination of molecular phylogeny and character trait evolution based on the presence or absence of a venom apparatus (Figure 2). The molecular characters are completely congruent with anatomical data: all specimens without a venom apparatus are in the *Acus* clade, and all specimens with a venom apparatus are in the *Terebra* clade. Thus, DNA sequences can be used to infer if a terebrid species has a venom apparatus or not. This study confirms the correlation between phylogeny and the presence or absence of the venom apparatus previously established [16]. The present findings can be used to broaden the current knowledge of the Terebridae as it pertains to their
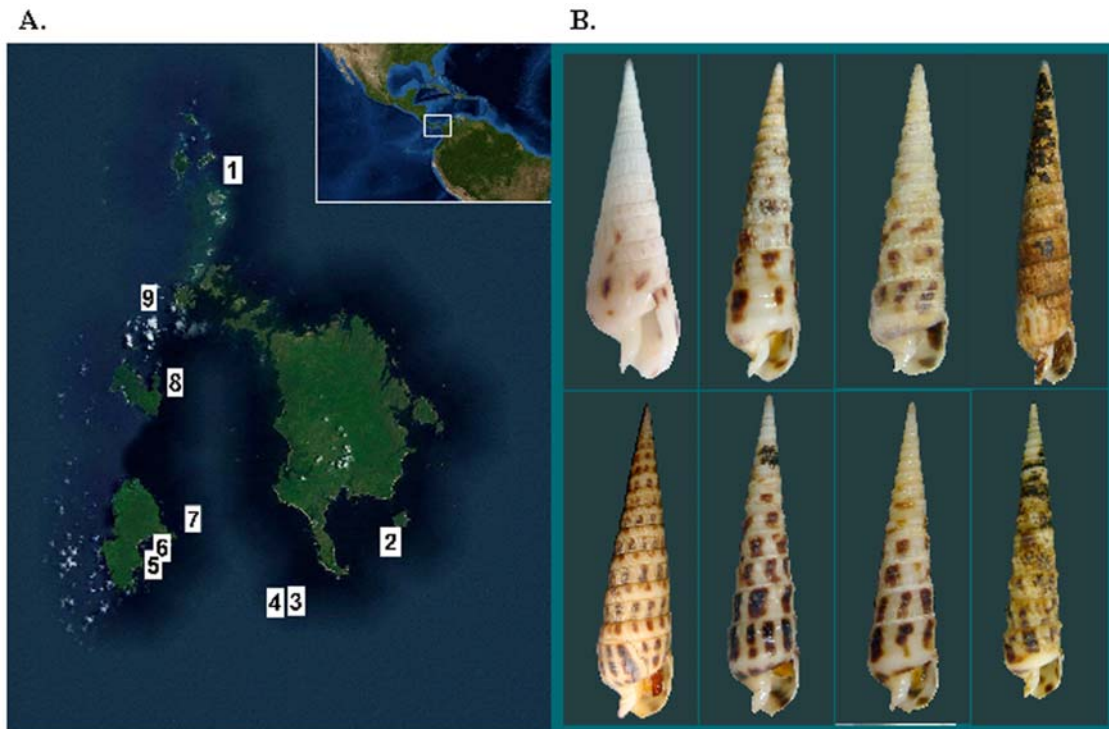
Figure 1. Panamic terebrid collection site and specimens. A. The Las Perlas Archipelago, located off the west coast of Panama (see Inset), is the collection site for the terebrids analyzed. The numbers shown on the map refer to the stations for the Panamic specimens listed in Table 1. B. Las Perlas specimens of *Acus* and *Terebra* analyzed in this study. Top left, *Acus strigatus*. Bottom left, *Terebra ornata*. Top right-most specimen, *Terebra* cf. *formosa*. All other specimens are *Terebra argyosia*.
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taxonomy and the potential use of their toxins to characterize ion channels and receptors in the nervous system.

Terebrid taxonomic considerations

The three Panamic species *T. argyiosa*, *T. cf formosa* and *T. ornata* form a well supported monophyletic branch (PP = 1; B = 96) within the clade that includes the type species of the genus *Terebra*, *T. subulata*. Therefore we provisionally treat all species in this clade as belonging to the genus *Terebra*. Subgeneric divisions may be feasible, but it seems best to defer the comprehensive taxonomic treatment of the genus *Terebra* until greater taxon sampling has been achieved.

The species-level taxonomy of *Terebra* species from the Panamic region is generally problematic. The results obtained so far provide a guide for suggesting which Panamic forms are likely to belong to *Terebra*, and thus have a venomous apparatus. However, considerable care should be taken before assigning definitive species designations for forms in this group. This problem is highlighted by the specimens of a variety of eastern Pacific terebrids shown in Figure 3. Note that the specimens assigned to *T. argyosia* and *T. ornata* from Mexico are quite different in shell pattern from the specimens from Panama. Two non-Panamic species are also included in the figure, a western Pacific species, *T. subulata*, and an Atlantic species that we expect will also belong to the same *Terebra* clade, *T. taurina*.

In this instance the molecular characters used in the phylogenetic analyses confirmed the shell-based morphological characters used to identify different terebrid species. The specimens of *Terebra argyosia* comprise the largest group of Las Perlas specimens collected that have a venom apparatus. Molecular analysis implies that *T. argyosia*, *T. ornata* and *T.* cf.

formosa are indeed three different species. However, the relatively small number of specimens included for *T. ornata* and *T.* cf. *formosa* does not allow an estimation of the intra and interspecific variability, and species delimitation hypotheses would be more accurately tested by adding replicates. The type locality for *T. formosa* is Panama [29]. The shell of the *T.* cf. *formosa* specimen used in this study (Figure 1B) is very worn and therefore not readily identified, but appears to have the three characteristic squarish brown spots on the body whorl, a short columella that is recurved and heavily plicated, and a smooth subsutural band as described in Bratcher & Cernohorsky [29]. Therefore, as a test of the shell-based ID, the resulting relationships for *T. argyosia*, *T.* cf. *formosa*, and *T. ornata* are in agreement with what is expected.

Terebrid toxin characterization

The Panamic *Terebra argyosia/ornata/formosa* complex used in this study have the traits necessary for probing the biochemical characterization of their venom, namely they are found in large quantities and can be easily collected. A combined phylogenetic and toxinological approach will accelerate the investigation of the Terebridae significantly. Genes that encode venom peptides belong to a special category termed ''exogenes,'' as their gene products act outside the organism [5,6,30]. Such genes are expected to diverge from each other extremely rapidly. If the various Panamic forms in the *Terebra* clade are separate species, then their exogenes should have diverged and an entirely different spectrum of venom components would be found in each species. If, however, these are morphological variants of the same species, the same gene sequences (with minor allelic variation) should be observed. Correlating molecular phylogeny with the presence of venom apparatus is a significant advance that will aid in the
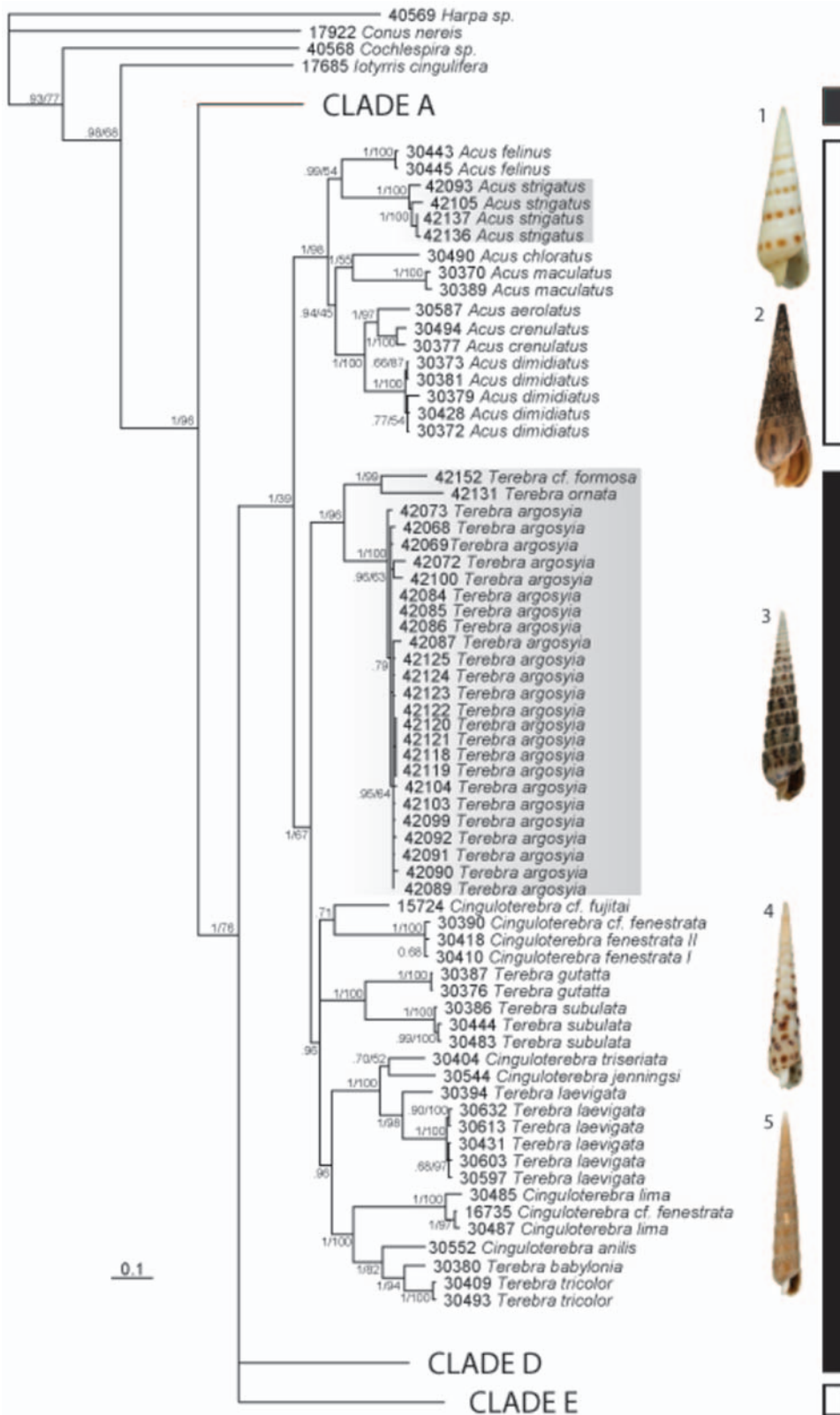
Figure 2. Combined Phylogenetic analysis of Panamic and western Pacific Terebridae. Shown is a consensus tree (BA) using COI, 16S, and 12S data sets. Posterior probabilities and bootstrap values are specified for each node. Shaded clades were collected in Panama. The bar on the right shows which taxa have venom glands (black bars) and which do not (white bars). Clade A refers to the sister group that includes *Pellifronia jungi*, Clades D and E refer to the *Hastula* and *Myurella* clades respectively; these clades were identified previously. Representative shells are shown as follows: 1. *Acus felinus*. 2. *Acus strigatus*. 3. *Terebra argosyia*. 4. *Terebra subulata*. 5. *Cinguloterebra anilis*.
doi:10.1371/journal.pone.0007667.g002
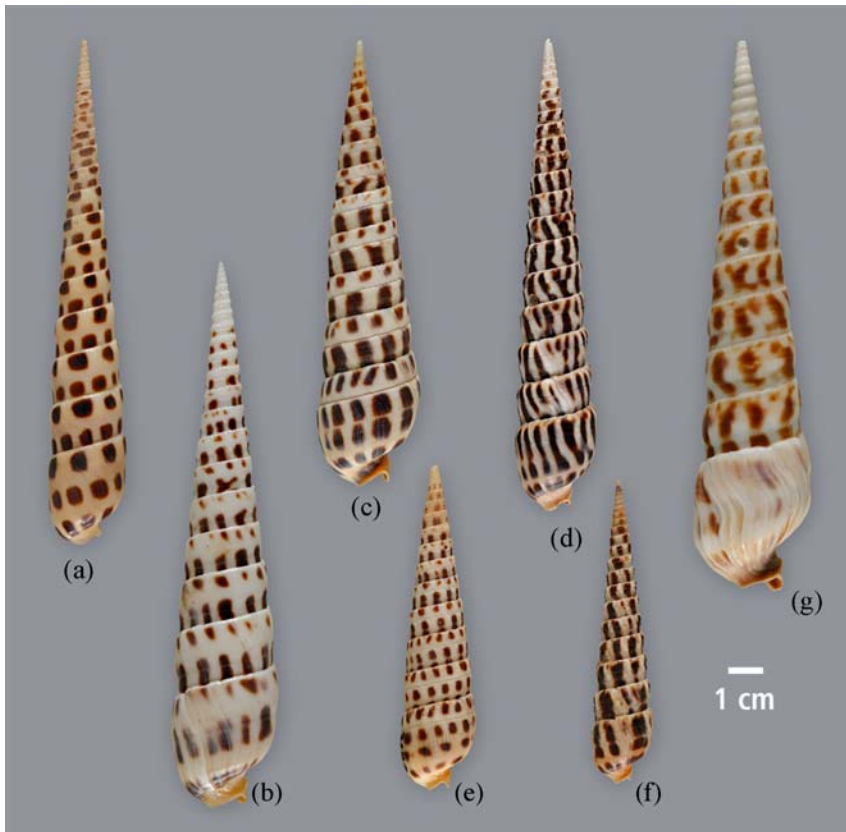
Figure 3. Diversity of Eastern Pacific *Terebra*. The figure shows the diversity of the venomous eastern Pacific forms tentatively assigned to Clade C, *Terebra*. The samples from Mexico, labeled (b–d), appear different to the samples from Panama, which are labeled (e–f). These are compared to the left-most specimen (a), *Terebra subulata* from the western Pacific and the right-most specimen (f), *Terebra taurina* from the western Atlantic.
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efficient discovery of new pharmacologically-active compounds from the Terebridae, and also inform the taxonomy and phylogeny of this group.

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Author Contributions

References

1. Puillandre N, Samadi S, Boisselier MC, Sysoev AV, Kantor YI, Cruaud C, Couloux A, Bouchet P (2008) Starting to unravel the toxoglossan knot: molecular phylogeny of the "turrids" (Neogastropoda Conoidea). Mol Phy & Evol 43(3): 1122–1134.

2. Kohn AJ (1959) The Ecology of Conus in Hawaii. Ecology Monograph 29: 47–90.

3. Taylor JD, Kantor Y, Sysoev AV (1993) Foregut anatomy, feeding mechanisms, relationships and classifications of the Conoidea (= Toxoglossa) (Gastropoda). Bull Nat Hist Mus Lond (Zool) 59: 125–170.

4. Terlau H, Olivera BM (2004) Conus venoms: a rich source of novel ion channel-targeted peptides. Physiol Rev 84: 41–68.

5. Olivera BM (2006) Conus peptides: biodiversity-based discovery and exogenomics. Journal of Biological Chemistry 281: 31173–31177.

6. Olivera BM, Teichert RW (2007) Diversity of the Neurotoxic Conus peptides: A Model for Concerted Pharmacological Discovery. Molecular Interventions 7(5): 253–262.

7. Han T, Teichert RW, Olivera BM, Bulaj G (2008) Conus venoms- A Rich Source of Peptide-Based Therapeutics. Current Pharmacuetical Design 14: 2461–2479.

8. Bulaj G (2008) Integrating the discovery pipeline for novel compounds targeting ion channels. Current Opinion in Chemical Biology 12: 1–7.

9. McIntosh JM, Cruz LJ, Hunkapiller MW, Gray WR, Olivera BM (1982) Isolation and structure of a peptide toxin from the marine snail Conus magus. Arch Biochem Biophys 218: 329–334.

10. Miljanich G (2004) Ziconotide: neuronal calcium channel blocker for treating severe chronic pain. Curr Med Chem 11: 3029–3040.

11. Imperial JS, Watkins M, Chen P, Hillyard DR, Cruz LJ, Olivera BM (2003) The augertoxins: biochemical characterization of venom components from the toxoglossate gastropod Terebra subulata. Toxicon 41: 391–398.

12. Imperial J, Kantor Y, Watkins M, Heralde III FM, Stevenson B, Chen P, Hansson K, Stenfo J, Ownby JP, Bouchet P, Olivera BM (2007) Venomous Auger Snail Hastula (Impages) hectica (Linnaeus, 1758): Molecular Phylogeny, Foregut Anatomy, and Comparative Toxinology. J Experimental Zoology 308B: 1–13.

13. Miller BA (1970) Studies on the biology of Indo-Pacific Terebra (Ph.D. dissertation) Durham, NH: University of New Hampshire.

14. Miller BA (1970) Feeding mechanisms of the family Terebridae. Ann Rep Am Mal Union 1971: 72–74.

15. Taylor JD (1990) The anatomy of the foregut and relationships in the TEREBRIDAE. Malacologia 32: 19–34.

16. Holford M, Puillandre N, Terryn Y, Cruaud C, Olivera BM, Bouchet P (2009) Evolution of the Toxoglossa Venom Apparatus as Inferred by Molecular Phylogeny of the Terebridae. Molecular Biology and Evolution 26(1): 15–25.

17. Terryn Y, Holford M (2008) The Terebridae of the Vanuatu Archipelago with a Revision of the Genus Granuliterebra Oyama 1961. Visaya December Supplement 3. ISSN 1656-4.650. ISBN 978-3-929767-20-6.

18. Keen AM (1972) Sea Shells of Tropical West America. Marine Mollusks from Baja California to Peru. Second Edition. Stanford, CA: Stanford University Press.

19. Simon C, Franke A, Martin A (1991) The Polymerase Chain Reaction: DNA Extraction and Amplification, in Molecular Techniques in Taxonomy, G. Hewitt, Johnson AWB, Young JPW, eds. New York: Springer-Verlag. pp 329–355.

20. Palumbi S (1996) Nucleic Acids II: The Polymerase Chain Reaction, in Molecular Systematics, D. Hillis, Moritz C, Mable BK, eds. Sunderland, Massachusetts: Sinauer Associates. pp 205–247.

21. Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R (1994) DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Mol Mar Biol Biotechnol 3: 294–299.

22. Hall T (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl Acids Symp Ser 41: 95–98.

23. Guindon S, Gascuel O (2003) A simple, fast and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst Biol 52: 696–704.

24. Huelsenbeck JP, Ronquist F, Hall B (2001) MRBAYES: Bayesian inference of phylogeny. Bioinformatics 17: 753–755.

25. Miller BA (1975) The Biology of Terebra gouldi Deshayes, 1859, and a Discussion of Life History Similarities among Other Terebrids of Similar Proboscis Type. Pacific Science 29(3): 227–241.

26. Remigio E, Duda Jr, TF (2008) Evolution of ecological specialization and venom of predatory marine gastropod. Molecular Ecology 17: 1156–1162.

27. Holford M, Zhang MM, Gowd KH, Azam L, Green BR, Watkins M, Ownby JP, Bulaj G, Olivera BM (2009) Pruning Nature: Biodiversity-Derived Discovery of Novel Sodium Channel Blocking Conotoxins from Conus bullatus. Toxicon 53: 90–98.

28. McIntosh JM, Corpuz GP, Layer RT, Garrett JE, Wagstaff JD, Bulaj G, Vyazovkina A, Yoshikami D, Cruz LJ, Olivera BM (2000) Isolation and characerization of a novel Conus peptide with apparent antinociceptive activity. J Biol Chem 275: 32391–32397.

29. Bratcher T, Cernohorsky WO (1987) Living Terebras of the World. New York, NY: American Malacologists, Inc.

30. Imperial J, Silverton N, Olivera BM, Bandyopadhyay PK, Sporning A, Ferber M, Terlau H (2007) Using Chemistry to Reconstruct Evolution: On the Origins of Fish-hunting in Venomous Cone Snails. Pro Amer Phil Soc 151(2): 185–200.