Molecular phylogeny of some Indo-Pacific genera in the subfamily Turrinae H. Adams and A. Adams, 1853 (1838) (Gastropoda: Neogastropoda)

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ABSTRACT

We have carried out a phylogenetic analysis of a group of Indo-Pacific species in the subfamily Turrinae (Swainson, 1840) using 12S mitochondrial ribosomal RNA gene sequences. Most of the species analyzed are conventionally assigned to one of three genera, *Turris* Röding, 1798, *Gemmula* Weinkauff, 1875 or *Lophiotoma* Casey, 1904. The molecular analysis revealed that while the species of *Turris* and *Gemmula* analyzed in this study comprise monophyletic groups, the species presently assigned to *Lophiotoma* definitely do not constitute a monophyletic assemblage and can be separated into two very distinctive groups of species based on the molecular analysis. The species presently designated as *Lophiotoma tayabasensis* Olivera, 2004, *Lophiotoma panglaoensis* Olivera, 2004, *Lophiotoma indica* (Röding, 1798) and *Lophiotoma bisaya* Olivera, 2004, are related to *Lophiotoma* (*Unedogemmula*) *unedo* (Kiener, 1839 in 1834–80) by molecular criteria, and are clearly widely separated from *Turris*, *Gemmula* or *Lophiotoma* (as redefined). We propose that *Unedogemmula* (MacNeil, 1960) be recognized as a full genus; *Unedogemmula unedo* (Kiener, 1839 in 1834–80) is the type species, and the species above are transferred from *Lophiotoma* to *Unedogemmula*.

INTRODUCTION

Venomous gastropods comprise three groups: the cone snails, the auger snails or terebrids and the "turrids", classically included in a single family, Turridae (H. Adams and A. Adams, 1853); these families are generally assigned to the superfamily Conoidea (Ponder and Waren, 1988; Taylor, Kantor, and Sysoev, 1993). It has been apparent for some time that the "turrids" comprise the largest species group in the superfamily (see for example, Powell, 1966); however, the groundbreaking work of Bouchet and co-workers (Bouchet et al., 2002; 2004) in New Caledonia, has provided evidence that >90% of Conoidean biodiversity probably resides in the "turrids" (broadly defined).

"Turrids" are problematic at all levels: not only are they a megadiverse group (>10,000 species) with a large fraction of species that remain undescribed, but the phylogenetic relationships within the group are poorly understood. The number of different "turrid" genera that have been proposed is >600; although in traditional molluscan taxonomic work all turrids had been assigned to the family Turridae, in most of the more recent systematic treatments, the group has been split into 3–6 different families (Taylor et al., 1993; Bouchet and Rocroi, 2005). However, some standard taxonomic treatments retain the old nomenclature (see for example Kohn, 1998).

To complement our ongoing study of turrid venoms (see for example Watkins et al., 2006) we initiated a study of "turrid" molecular phylogeny; the first results at the generic level are reported below. The genus *Turris* Röding, 1798, is the nominate genus of the family Turridae, with *Turris babylonia* (Linnaeus, 1758) as the type species. This is an exclusively Indo-pacific genus. However, there has been inconsistency in the definition of those genera which are traditionally grouped together with *Turris* in the subfamily Turrinae. Two other groups largely from the Indo-Pacific, *Lophiotoma* Weinkauff, 1875, and *Gemmula* Casey, 1904, are included in the

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subfamily by most workers; in the western Atlantic and eastern Pacific, the genus *Polystira* Woodring, 1928, is also thought to have a close affinity to *Turris* (the type species for these genera are *Lophiotoma acuta* (Perry, 1811), *Gemmula hindsiana* (Berry, 1958) and *Polystira albida* (Perry, 1811)). Additionally, a number of other groups (such as *Turridrupa* (Hedley, 1922)) are regarded as turrine by most workers.

A major motivation for these studies is to investigate the toxin genes expressed in the venom ducts of conoidean gastropods. Among the different groups of turrids, we have initially concentrated on studying the gene products expressed in the venom ducts of species in the subfamily Turrinae, since they are larger and more easily collected than are most other turrid groups. We hope to correlate the gene families expressed in venom ducts with the molecular phylogeny of the species analyzed. Thus, the molecular analysis reported below has focused on larger Indo-Pacific species in the subfamily Turrinae, e.g. *Turris*, *Lophiotoma* and *Gemmula* spp. The data that we present below demonstrates that two groups of species presently assigned to the genus *Lophiotoma*, which appear to be closely related by shell morphology, prove to be unexpectedly divergent when evaluated by molecular criteria and need to be placed in different genera.

MATERIALS AND METHODS

Specimen Collection: Species analyzed in this study, shown in Table 1, were mostly collected by commercial shell collectors in the Philippines, except for *Polystira albida* (Perry, 1811), a generous gift of Drs. Estuardo Lopez Vera and Ed Heimer, and *Lophiotoma cerithiformis* (Powell, 1964), which was collected in Oahu, Hawaii. Specimens of each were preserved either in RNAlater® (Ambion Inc., Tx) or 95% ethanol, and DNA extracted as described below. In most cases, the digestive gland was used as the source of DNA; however, for alcohol preserved specimens where the shell had not been cracked, the digestive gland was often degraded, and DNA was extracted from foot tissue.

Identification and sequencing of clones encoding 12S mitochondrial rRNA gene segments: Genomic DNA was prepared from tissue (~20 mg) from each turrid species using the Gentra PUREGENE DNA isolation kit (Gentra Systems, Minneapolis, MN) according to the manufacturer's standard protocol. Genomic DNA from each species (~10ng) was used as a template for polymerase chain reaction (PCR) with oligonucleotides corresponding to 12S-I (5′ TGC CAG CAG YCG CGG TTA) and 12S-III (5′ AGA GYG RCG GGC GAT GTG T) mitochondrial rRNA segments (Oliverio and Mariottini, 2001). The 5′ and 3′ primers included adapters GGACA-CAU and GGGAAACGU respectively for annealing to the cloning vector pNEB206A. The PCR cycling profiles were as follows: initial denaturation (95°C, 60s); followed by 40 cycles of denaturation (95°C, 20s); annealing (55°C, 20s) and extension (72°C, 30s). The PCR products were purified using the PureLink PCR Product Purification Kit (Invitrogen Life Technologies, Carlsbad, California) following the manufacturer's suggested protocol. The eluted DNA fragments were digested with uracil specific excision reagent, annealed to pNEB206A vector (USER™ Friendly Cloning kit, New England BioLabs, Inc., Beverly, Massachusetts) and the resulting products transformed into competent DH5α cells (Sambrook and

Table 1. List of species analyzed in this study.

Species	Locality
Lophiotoma acuta (Perry, 1811)	Buenavista, Marinduque, Philippines
*Lophiotoma bisaya** Olivera, 2004	Batangas, Philippines
Lophiotoma cerithiformis (Powell, 1964)	Oahu, Hawaii
Lophiotoma cinguliferа (Lamarck, 1822)	Cawoy, Olango Island, Philippines
*Lophiotoma indica** (Röding, 1798)	Aligway Is. Dipolog, Philippines
Lophiotoma jickelii (Weinkauff, 1875)	Cawoy, Olango Island, Philippines
Lophiotoma olangoensis Olivera, 2002	Cawoy, Olango Island, Philippines
*Lophiotoma panglaoensis** Olivera, 2004	Panglao Is. Bohol, Philippines
Lophiotoma polytropa (Hellbling, 1779)	Batan, Luzon, Philippines
*Lophiotoma tayabasensis** Olivera, 2004	Sogod, Cebu, Philippines
*Lophiotoma unedo** (Kiener, 1839 in 1834–80)	Panglao Is. Bohol, Philippines
Gemmula speciosa (Reeve, 1843)	Batangas, Philippines
Gemmula diomedea Powell, 1964	Sogod, Cebu, Philippines
Gemmula rosario Shikama and Hayashi, 1977	Sogod, Cebu, Philippines
Gemmula tisajoni Olivera, 2000	Sogod, Cebu, Philippines
Gemmula sogodensis Olivera, 2005	Sogod, Cebu, Philippines
Turris garmonsii (Reeve, 1843)	Cawoy, Olango Island, Philippines
Turris grandis (Gray, 1833)	Sogod, Cebu, Philippines
Turris normandavidsoni Olivera, 2000	Sogod, Cebu, Philippines
Turris babylonia (Linnaeus, 1758)	Cawoy, Olango Island, Philippines
Turris spectabilis (Reeve, 1843)	Cawoy, Olango Island, Philippines
Turris totiphyllis Olivera, 2000	Cawoy, Olango Island, Philippines
Polystira albida (Perry, 1811)	Bay of Campeche, Mexico
Drillia regius (Habe and Murakami, 1970)	Panglao Is., Bohol, Philippines

* These species are proposed to be transferred from *Lophiotoma* to *Unadogemmula* (see text).

Russell, 2001). Plasmid DNAs were isolated from ampicillin resistant colonies and the nucleic acid sequences of the inserts determined using ABI DNA sequencer with ABI Big Dye chemistry (Foster City, CA). DNA sequences have been submitted to GenBank and the accession numbers are: EF467333, EF4673334, EF467335, EF467336, EF467337, EF467338, EF467339, EF467340, EF467341, EF467342, EF467343, EF467344, EF467345, EF467346, EF467347, EF467348, EF467349, EF467350, EF467351, EF467352, EF467353, EF467354, EF467355, and EF467356.

Sequence Analysis: Nucleic acid sequences (the longest of which had 593 nucleotides) were aligned manually using MEGA version 3.1 (Kumar, 2004). One tree was created from two independent runs using the software program MrBayes (Huelsenbeck, 2001; Ronquist, 2003). 5,000,000 trees were made in each run, 50,000 of which were saved. Two hundred and fifty of each of those 50,000 were also discarded as burn-in. Each run had four chains (one cold and three heated). The two independent runs were combined into a single tree where branches were preserved if they were found in 70% or more of those trees not discarded. The standard deviation after 5,000,000 generations was 2.401×10^{-3}. A general time reversible (GTR) model was used, with the rate variation of some sites kept invariable and the remaining rates drawn from a gamma distribution. The other tree was created using the software program PHYML (Guindon, 2003). A thousand trees were obtained using nonparametric bootstrap analysis and combined into a single tree where branches were preserved if they were found in 70% or more of the given trees. A GTR model was used, with the base frequency estimates found empirically and the proportion of invariable sites estimated. Four substitution rates were used, with the gamma distribution parameter estimated.

RESULTS AND DISCUSSION

PCR Amplification of 12S Sequencing: The sequences of 12S rDNA from 23 species in the subfamily Turrinae (see Table 1) were obtained as described above. The 12S sequence of a *Drillia* species, *Drillia regius* (Habe and Murakami, 1970), was used as the outgroup for the phylogenetic analysis. The sequences obtained are shown in Table 2; these were aligned for maximal overlap.

Phylogenetic Analysis: A phylogenetic tree, shown in Figure 1, was constructed as described under Methods. The species that are presently assigned to two of the major turrine genera, *Turris* and *Gemmula*, appear as monophyletic clades in the phylogenetic tree obtained through Bayesian methods. However, the *Lophiotoma* species analyzed clearly split into two distinct, well-separated groups.

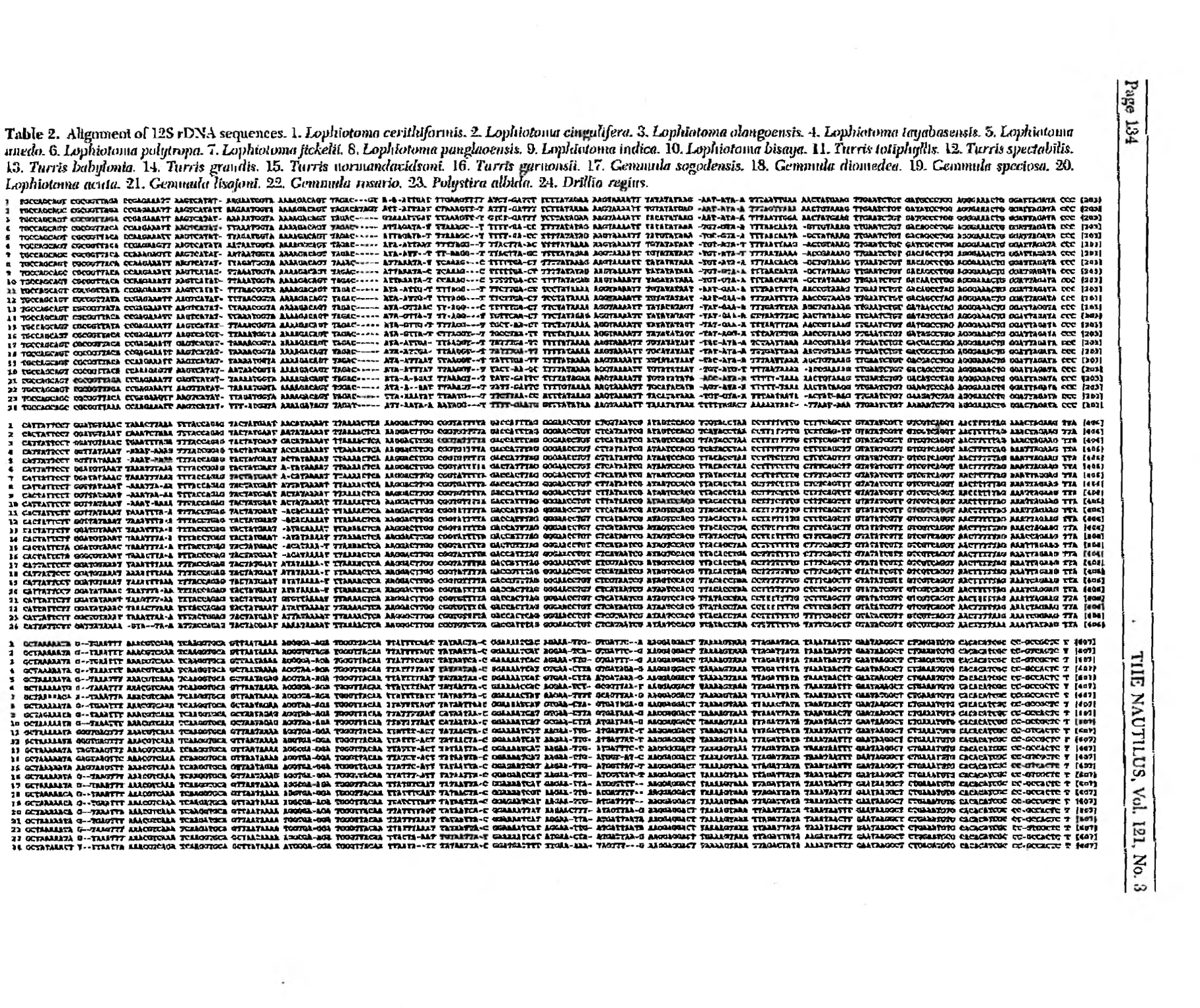
Thus, the species presently assigned to *Lophiotoma* analyzed in this study do not appear to constitute a monophyletic assemblage. A large separation is found between two groups of *Lophiotoma* species; one group includes *Lophiotoma unedo* (Kiener, 1839 in 1834–80), *Lophiotoma tayabasensis* Olivera, 2004, *Lophiotoma panglaoensis* Olivera, 2004, *Lophiotoma indica* (Röding, 1798), and *Lophiotoma bisaya* Olivera, 2004. These species appear to be much more divergent from the *Turris* and *Gemmula* branches than the other group of *Lophiotoma*, which includes the type species of *Lophiotoma*, *Lophiotoma acuta* (Perry, 1811); the latter comprises two sub-branches, one branch including species such as *Lophiotoma cingulifera* (Lamarck, 1822), assigned by many systematists to the subgenus *Xenuroturris* Iredale, 1929, which is regarded as a separate genus by some workers (Powell, 1966).

Generic Classification and Nomenclature: The unexpected phylogenetic separation between two groups of species conventionally assigned to the genus *Lophiotoma*, makes the present assignment of these species into the conventional Indo-Pacific turrine genera, *Turris*, *Lophiotoma*, and *Gemmula* inconsistent with the phylogenetic tree shown in Figure 2. One potential solution would be to lump these Indo-Pacific genera together under one genus, *Turris*, and use subgeneric designations for each large clade of species (this might be called the "*Conus* alternative"; the major group of Indo-Pacific Turrinae comprise a phylogenetic branch that does not appear to be more divergent by molecular criteria than is the divergence within the species presently assigned to the genus *Conus*; Espiritu, 2001). Although this alternative may have some merit, the substantial literature referring to species in the traditional genera *Turris*, *Gemmula*, and *Lophiotoma* (including a significant paleontological component of the research literature) would make this a radical (and probably impractical) alternative.

Because *Lophiotoma acuta* is the designated type species for the genus *Lophiotoma*, the species presently in *Lophiotoma* that are in the branch not including *L. acuta* require a new generic designation. There are two generic/subgeneric designations potentially available for the group. One is a name proposed originally by Powell, *Lophioturris* Powell, 1964; Powell envisioned *Lophioturris* as a genus allied to *Lophiotoma* with *Lophiotoma indica* (Röding, 1798) as type. *Lophioturris* was set up specifically for forms that have blunt paucispiral protoconchs; since most of the species in this clade have polygyrate (multispiral) protoconchs, *Lophioturris* does not seem to be an appropriate taxonomic designation (see Powell, 1964, for a discussion of differences in protoconch morphology).

The other available generic name for this group of species (which would have priority) is *Unedogemmula* MacNeil 1960: as originally proposed, *Unedogemmula* was a separate genus, with *Unedogemmula unedo* (Kiener, 1839 in 1834–80) as type. However, Powell relegated *Unedogemmula* to be a subgenus of *Gemmula*. Subse-

Table 2. Alignment of 12S rDNA sequences. 1. *Lophiotoma cerithiformis*. 2. *Lophiotoma cingulifera*. 3. *Lophiotoma olangoensis*. 4. *Lophiotoma tayabasensis*. 5. *Lophiotoma unedo*. 6. *Lophiotoma polytropa*. 7. *Lophiotoma fickelii*. 8. *Lophiotoma panglaoensis*. 9. *Lophiotoma indica*. 10. *Lophiotoma bisaya*. 11. *Turris totiphyllis*. 12. *Turris spectabilis*. 13. *Turris babylonia*. 14. *Turris grandis*. 15. *Turris normandavidsoni*. 16. *Turris garmonsii*. 17. *Gemmula sogodensis*. 18. *Gemmula diomedea*. 19. *Gemmula speciosa*. 20. *Lophiotoma acuta*. 21. *Gemmula bisajoni*. 22. *Gemmula rosario*. 23. *Polystira albida*. 24. *Drillia regius*.



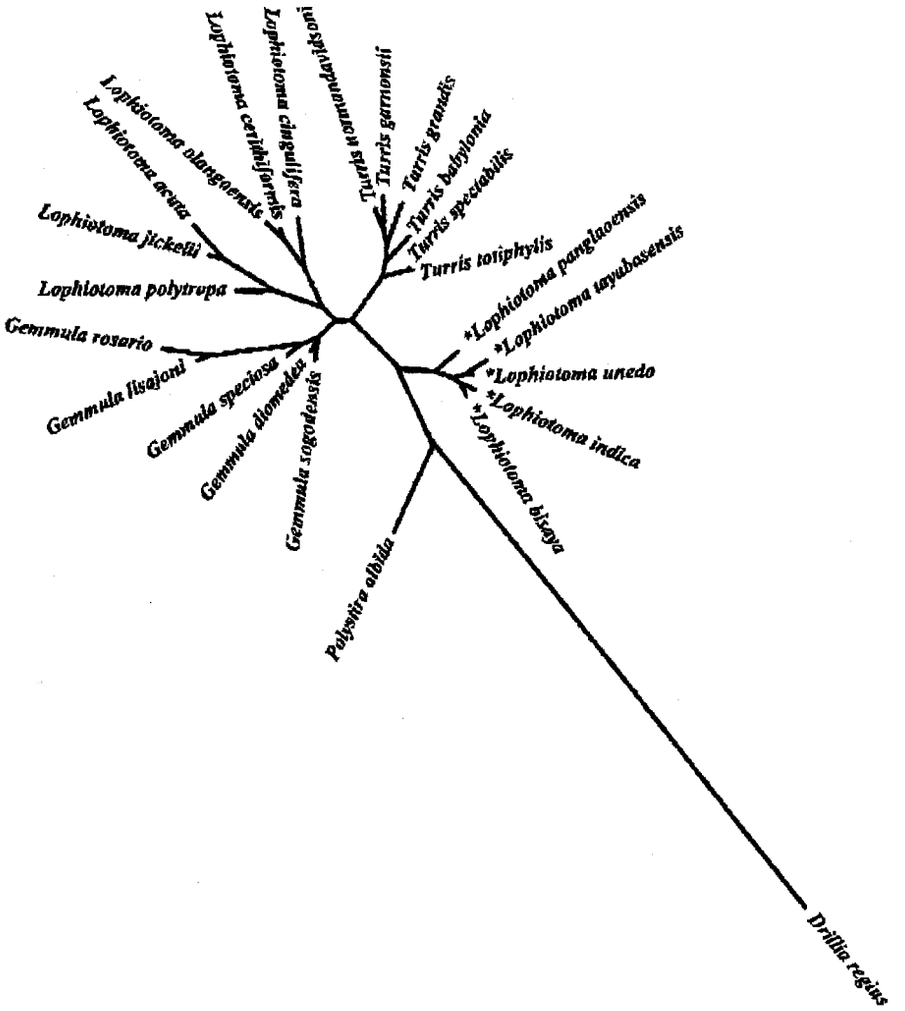


Figure 1. Phylogenetic tree of species listed in Table 1. A tree based on 12S rDNA sequences was constructed (see Methods). Species from three Indo-Pacific families in the subfamily Turrinae were analyzed (*Turris*, *Gemmula*, *Lophiotoma*). In addition, one Atlantic species, *Polystira albida* and a species in the subfamily Drillinae (or Clavinae), *Drillia regius* were included. Note that the species of *Lophiotoma* are split into two widely separated groups. As discussed in the text, species marked by an asterisk are proposed to be moved to another genus. *Lophiotoma jickelii* and *Turris grandis* are non-standard taxonomic assignments for the figured specimens: these are widely regarded as synonymous to *Lophiotoma acuta* and *Turris crispa*, respectively (see Powell, 1964). However, the molecular data clearly separates *Lophiotoma acuta* and *Lophiotoma jickelii*, and the type of *Turris crispa* is sufficiently divergent from the form shown that using *Turris grandis* seems a preferable name for this species.

quently, Kilburn (1983) suggested that based on general shell morphology, *Unedogemmula* seems much closer to species traditionally assigned to *Lophiotoma*, and that it should more appropriately be regarded as a subgenus of *Lophiotoma*; this suggestion has been adopted in most of the recent taxonomic treatments of turrine genera.

The molecular results reported above demonstrate that *Unedogemmula unedo* is indeed closely related to some of the species most taxonomists presently include in *Lophiotoma*, (such as *Lophiotoma indica*, *Lophiotoma bisaya*, and *Lophiotoma tayabasensis*). Furthermore, the molecular analysis clearly shows that there is no justification for designating *Unedogemmula* as a subgenus of either *Gemmula* or *Lophiotoma*, since *Unedogemmula unedo* is in a very divergent branch of the phylogenetic tree. Thus, our results support the original designation of *Unedogemmula* as a full genus, although the species comprising the genus need to be somewhat redefined. *Unedogemmula unedo* is the type species, and the larger, strongly maculated forms previously assigned to *Lophiotoma* (such as *Lophiotoma indica*, *Lophiotoma tayabasensis*, and *Lophiotoma bisaya*) are transferred to *Unedogemmula* from *Lophiotoma*. A recent analysis of Philippine forms related to these species, clarifying the relationships between these forms and the *Unedogemmula unedo* group, was recently published (Olivera,

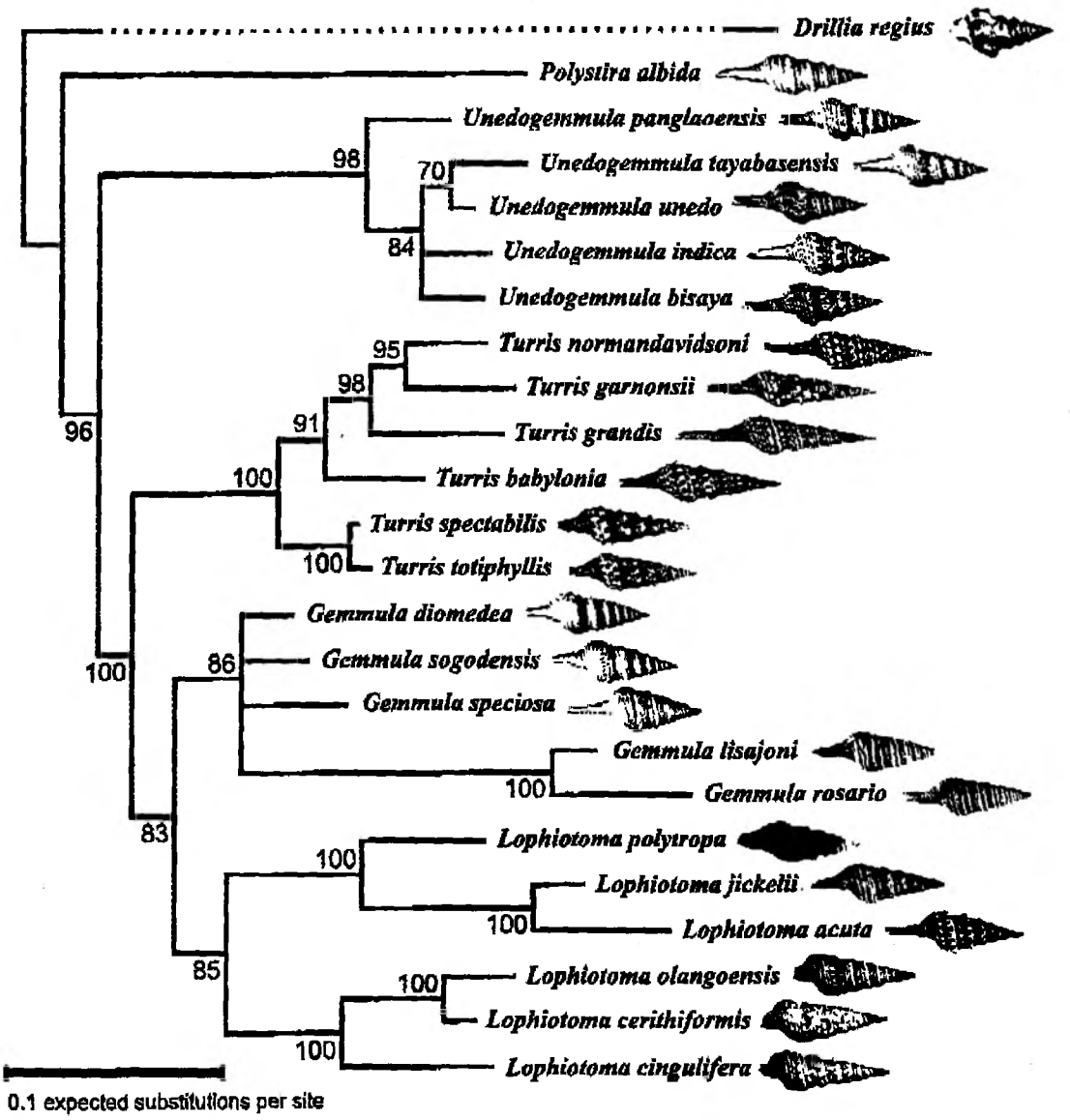


Figure 2. The phylogenetic tree in Figure 1 is re-plotted, except that the confidence limits are included (calculated after 10 million generations). Note that all the major groups are highly supported; as is explained in the discussion, one of the groups formerly assigned to *Lophiotoma* is now regarded as a full genus, *Unedogemmula*, with *Unedogemmula unedo* as type species; thus, in this figure, these species (still labeled *Lophiotoma* in Figure 1) are designated as *Unedogemmula* spp.

2004); it seems likely that all of the species level taxa treated in that work are properly placed in the genus *Unedogemmula*, as redefined below, although this should be verified by obtaining molecular data for those species.

Genus *Unedogemmula* MacNeil, 1960

Description: Shell large, 30–105mm, fusiform with tall spire, long, straight, anterior canal unnotched. Protoconch variable, from blunt paucispiral to multispiral, often with the transitional part of the larval shell decorated with brephic axials or axially costate whorls. Sinus is peripheral, deep, and narrow at the termination of the sinus rib.

Remarks: The genus has shell morphology with strong similarities to *Lophiotoma*: most species have a smooth peripheral keel but, in some species, there are distinct peripheral granulations. In other species, these granulations continue to the body whorl, but these tend toward obsolescence in most forms. In contrast to *Lophiotoma*, the peripheral keel does not consist of two raised cords at the edges with a depressed area in the center; rather, in

some forms, there is a single smooth rib exhibiting a variable level of peripheral granulation.

In order to test the veracity of the proposals based on the Bayesian analysis and outlined above, a second approach to the phylogenetic analysis of the sequences was also carried out. A Maximum Likelihood method was employed as described under Methods. The results of this analysis are shown in Figure 3. The separation of the species described above originally in *Lophiotoma* into two distinct groups is strongly supported using this analysis. Thus, both methods support raising *Unedogemmula* to a full genus, as described above. The analyses differ, however, in the results with *Gemmula*; the maximum likelihood method does not group all of the species analyzed into a single monophyletic clade, but into two groups of species. Thus, given this discrepancy between the two methods, the monophyly of *Gemmula* clearly requires further investigation.

A brief summary of a proposed revision of Indo-Pacific genera in the subfamily Turrinae is given in Table III. The cladogram in Figures 2 and 3 give support to *Turris* and *Unedogemmula*. However, *Lophiotoma* (redefined to exclude the species transferred to *Unedogemmula*) has two branches, both strongly supported: the conjoining of the branches has less than 90% support in the Bayesian analysis, and is not supported above the cutoff level

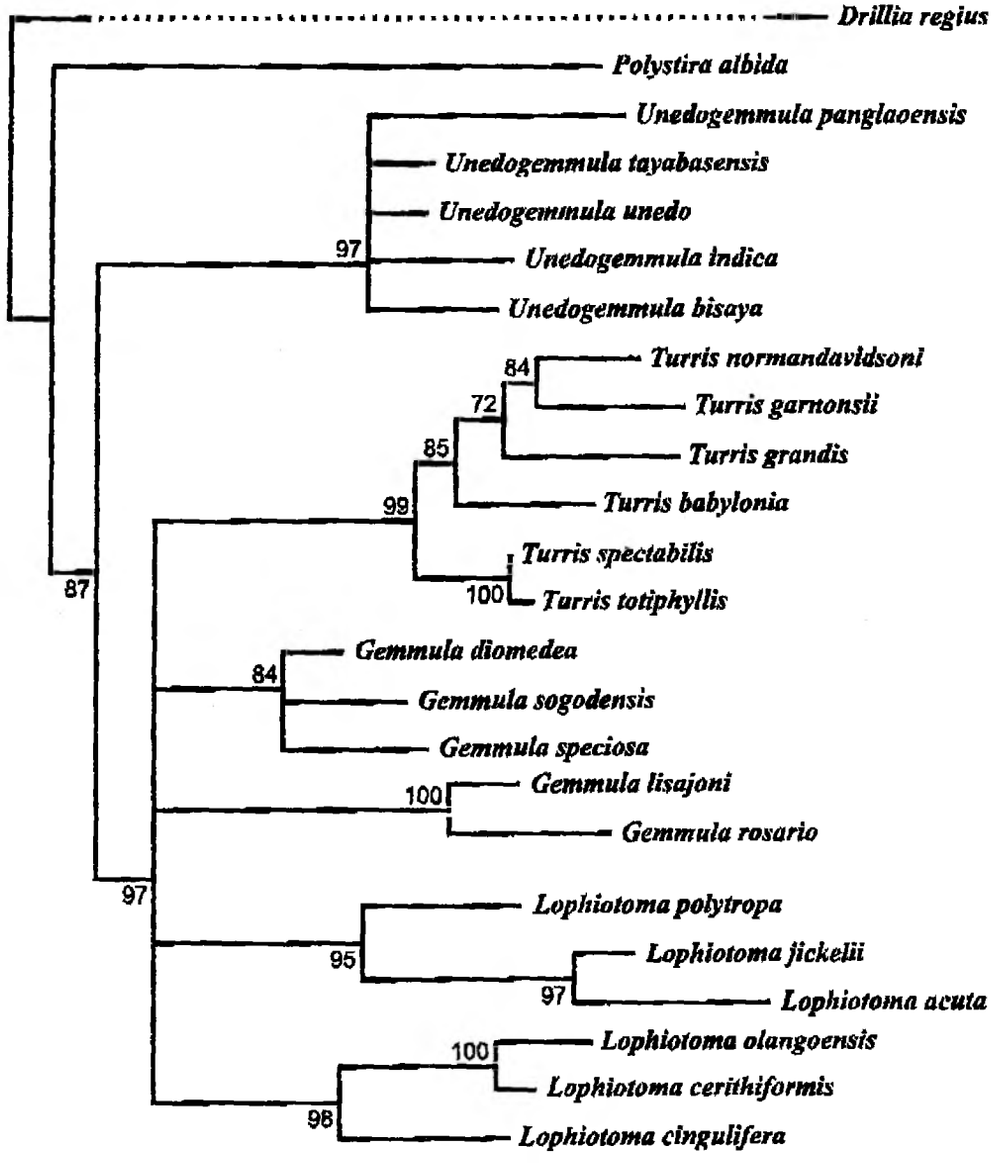


Figure 3. Phylogenetic tree of species listed in Table 1. This tree was made using the same sequence alignment as that used for the tree in Figure 1, but was constructed using the PHYML software program.

(70%) in the Maximum Likelihood analysis. Given these data, the solution would be to split *Lophiotoma* into two separate genera, *Lophiotoma* and *Xenuroturris*. We feel that at this time the more conservative approach of retaining the genus *Lophiotoma*, and dividing it into two subgenera, *Lophiotoma* (s.s.) and *Xenuroturris* (with *Lophiotoma* (*Lophiotoma*) *acuta* and *Lophiotoma* (*Xenuroturris*) *cingulifera* as types, respectively) is preferable until a wider range of species has been analyzed. There are a number of species (presently in *Lophiotoma*) that are problematic to assign (such as *Lophiotoma ruthveniana* (Melvill, 1923)), and we believe that a molecular analysis of these forms needs to be carried out before we fully understand the relationship between *Lophiotoma* (s.s.) and *Xenuroturris*. It may well turn out that, when the analysis is completed, the separation between the two branches (*Lophiotoma* and *Xenuroturris*) will be definitive; at that point, separating the two groups of species into different genera will be justified.

The major conclusion from this work is that *Unedogemmula* should be recognized as a full genus, and is a sister group to the major branch that includes *Turris*, *Gemmula*, and *Lophiotoma* (as redefined).

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