

The relationships of the enigmatic gastropod *Tritonoharpa* (Neogastropoda): New data on early neogastropod evolution?

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ABSTRACT

In this paper, the relationships of *Tritonoharpa* Dall, 1908, within Neogastropoda are discussed. *Tritonoharpa* is indeed similar to *Colubraria* in the morphology of its head-foot, pallial complex, reproductive and excretory systems, in the presence of an extremely long and coiled proboscis, and a very large stomach. However, it differs from *Colubraria* in the rest of its foregut anatomy, revealing a cancellariid affinity, and a typical nematoglossan radula. The molecular data confirms Beu and Maxwell's placement of *Tritonoharpa* in the Cancellariidae, close to *Plesiotriton*. It is also suggested that cancellariids may be the sister-group to the rest of neogastropods. *Tritonoharpa* has a rather large and well developed midgut gland, resembling the gland of Leiblein. As previously studied cancellarioideans have been shown to lack a well differentiated gland of Leiblein, the present study raises some interesting questions about the evolution of the foregut in Neogastropoda. In fact, if this glandular structure were confirmed as a true homologue of the gland of Leiblein, and the cancellarioideans proved to be the sister group to the remaining neogastropods, the possession of the gland should be considered a synapomorphy of the Neogastropoda.

Additional keywords: Anatomy, phylogeny, molecular systematics, Neogastropoda, Cancellariidae

INTRODUCTION

Tritonoharpa antiquata (Hinds in Reeve, 1844) belongs to a small group of 19 Recent species, most occurring in the tropical Indo-West Pacific (Beu and Maxwell, 1987). These species had previously been referred to a *Colubraria*-like group, together with members of at least four families (Beu and Maxwell, 1987). Elongate and varicose shells, typical of *Colubraria*, have evolved through convergence several times in the families Ranellidae, Muricidae, Buccinidae, and Cancellariidae. A number of genera with columellar plaits and a nematoglossan radula, morphologically similar to *Plesiotriton*, Fisher, 1884, were placed in the Cancellarioidea. Among those, the genus *Tritono-*

harpa Dall, 1908 (type species by original designation, *Tritonoharpa vexillata* Dall, 1908, Recent, from western America and the Galapagos Islands) was distinguished from *Plesiotriton* only by the absence of columellar plaits and the absence of radula (Beu and Maxwell, 1987).

Information on the anatomy of Cancellariidae is available (Harasewych and Petit, 1982; 1984; 1986), based on representatives of the subfamilies Cancellariinae and Admetinae. The anatomy and phylogenetic relationships of the Plesiotritoninae to the other cancellariids are still unknown.

Herein we describe the foregut anatomy of *Tritonoharpa antiquata* (Figure 18) and compare it with anatomical data already available for other cancellariids. A molecular dataset, based on two mitochondrial markers (12S and 16S rDNA) was used to construct a molecular phylogenetic framework for the systematics of the Plesiotritoninae.

MATERIALS AND METHODS

TAXON SAMPLING AND SPECIMEN COLLECTION: The material for the present study was collected during field work and expeditions to the West Pacific (PANGLAO 2004, Philippines, and SANTO 2006, Vanuatu, organized by the Muséum national d'Histoire naturelle, Paris), Panama (Neogastropod Workshop 2006 at the Smithsonian Tropical Research Institution, Panama), the Mediterranean Sea, and other localities, and supplemented by specimens provided by Museums and colleagues (see Table 1 for details). Vouchers are stored at BAU (Department of Animal and Human Biology, Rome), MNHN (Muséum national d'Histoire naturelle, Paris), NMSA (Natal Museum, Pietermaritzburg).

Representatives of 21 additional neogastropods, including representatives of 13 families were sequenced to provide a phylogenetic framework for the relationships of *Tritonoharpa* to other cancellariids and within the Neogastropoda. The cypraeid *Cypraea cervinetta* Kiener, 1843 has been chosen as an outgroup (see Table 2 for details).

Table 1. Species included in the molecular analysis, with collecting data, voucher numbers, length of the 12S and 16S sequences, and EMBL accession numbers. BAU, Department of Animal and Human Biology, Rome; MNHN, Muséum National d'Histoire Naturelle, Paris; NMSA, Natal Museum, Pietermaritzburg; and EMBL, The European Molecular Biology Laboratory, Heidelberg.

| Family | Species | Locality | Voucher Number | 12S | | 16S | | References |
|----------------|--|--|-------------------------------|----------|-----|----------|-----|----------------------------------|
| | | | | EMBL | bp | EMBL | bp | |
| Cypraeidae | <i>Cypraea cervinetta</i> Kiener, 1843 | Venado (Panama), 8.89° N, 79.59° W, intertidal | BAU00799 | FM999072 | 521 | FM999103 | 492 | Oliverio and Modica, in press |
| Cancellariidae | <i>Cancellaria cancellata</i> Linné, 1767 | Off Malaga (Spain), 40–50 m | BAU00224 | FM999074 | 541 | FM999105 | 652 | Oliverio and Modica, in press |
| Cancellariidae | <i>Cancellaria cooperi</i> Gabb, 1865 | Off La Jolla (California, USA), 40 m | MNHN IM-2009-4611 BAU00797 | FM999073 | 537 | FM999104 | 616 | Oliverio and Modica, in press |
| Cancellariidae | <i>Tritonoharpa antiquata</i> (Hinds in Reeve, 1844) | Mactan Is. (Philippines), 10.32° N, 124.03° E, 40–120 m, tangle nets, 15 May 2006 | BAU00270 | FN392228 | 521 | FN392229 | 489 | This work |
| Cancellariidae | <i>Plesiotriton vius</i> Habe and Okutani, 1981 | Bohol/Sulu sea sill (Philippines), PANGLAO 2005, st CP2359 | MNHN32123 | FM999075 | 523 | FM999106 | 656 | Oliverio and Modica, in press |
| Comidae | <i>Conus textile</i> Linnaeus, 1758 | 8.83° N 123.58° E, 437–476 m Philippines | – | DQ862058 | 535 | DQ862058 | 609 | Bandyopadhyay et al., 2007 |
| Turridae | <i>Lophiotoma cerithiformis</i> Powell, 1964 | Philippines | – | DQ284754 | 532 | DQ284754 | 625 | Bandyopadhyay et al., 2006 |
| Muricidae | <i>Nucella lapillus</i> Linnaeus, 1758 | Portobello (UK), 55.95° N, 3.10° W, intertidal | MNHN IM-2009-4617 BAU00187 | FM999088 | 527 | FM999119 | 679 | Oliverio and Modica, in press |
| Muricidae | <i>Cronia</i> sp. 1 | Tolo Channel, Hong Kong, 22.45° N, 114.26° E, 1 m depth | MNHN IM-2009-5118 BAU00619 | FN391982 | 521 | FM999120 | 669 | Oliverio and Modica, in press |
| Muricidae | <i>Stramonita haemastoma</i> (Linné, 1767) | S. Marinella (Italy), 42.0° 3' N, 11.90° E, intertidal | BAU00696 | FM999090 | 525 | FM999121 | 661 | Oliverio and Modica, in press |
| Muricidae | <i>Drupella cornus</i> Röding, 1798 | Panglao Is., Catarman (Philippines), PANGLAO 2004, st. R18, 9.60° N, 123.86° E, 2–46 m | MNHN IM-2009-4601 BAU00192 | FM999091 | 521 | FM999122 | 657 | Oliverio and Modica, in press |
| Buccinulidae | <i>Paraethria plumbea</i> (Philippi, 1841) | Ushuaia (Argentina), 54.78° S, 68.23° W, intertidal | MNHN IM-2009-4613 BAU00697 | FM999095 | 530 | FM999126 | 637 | Oliverio and Modica, in press |
| Buccinidae | <i>Neobuccinum eatoni</i> (Smith, 1875) | Terra Nova Bay (Antarctic), 74.69° S, 164.1° 2' E | MNHN IM-2009-4614 BAU00785 | FM999096 | 535 | FM999127 | 657 | Oliverio and Modica, in press |
| Nassaridae | <i>Ilyanassa obsolleta</i> (Say, 1822) | Not available | | DQ238598 | 535 | DQ238598 | 563 | Simison et al., 2006 |
| Nassaridae | <i>Nassaritis pagodus</i> (Reeve, 1844) | Las Perlas Is. (Panama), 8.74° N, 79.20° W, 50 m | MNHN IM-2009-4620 BAU00237 | FM999094 | 528 | FM999125 | 659 | Oliverio and Modica, in press |
| Melongenidae | <i>Melongena patula</i> (Broderip and Sowerby, 1829) | Venado (Panama), 8.89° N, 79.59° W, intertidal | MNHN IM-2009-4621 BAU00794 | FM999093 | 533 | FM999124 | 671 | Oliverio and Modica, in press |
| Melongenidae | <i>Volema myristica</i> (Röding, 1798) | Panglao Is., Sungcolan (Philippines) PANGLAO 2004, st. M11, 9.64° N, 123.83° E, 0–3 m | MNHN IM-2009-4602 BAU00225 | FM999091 | 534 | FM999123 | 662 | Oliverio and Modica, in press |

| | | | | | | | | |
|------------------|---|---|-------------------------------|----------|-----|----------|-----|----------------------------------|
| Oliviidae | <i>Oliva spicata</i> (Röding, 1798) | Las Perlas (Panama), 8.53° N, 79.09° W, 20–22 m | MNHN IM-2009-4616 BAU00278 | FM999083 | 524 | FM999114 | 672 | Oliverio and Modica, in press |
| | <i>Olivella volutella</i> (Lamarck, 1811) | Venado (Panama), 8.89° N, 79.59° W, intertidal | MNHN IM-2009-4615 BAU00241 | FM999082 | 534 | FM999113 | 665 | Oliverio and Modica, in press |
| Pseudolividae | <i>Sylvanocochlis ancilla</i> (Hanley, 1859) | SW of Mossel Bay, Agulhas Bank, Western Cape (South Africa), 81 m | NMSA-E-5279 | FM999084 | 532 | FM999115 | 489 | Oliverio and Modica, in press |
| Costellariidae | <i>Vexillum plicarium</i> (Linnaeus, 1758) | Panglao Is., Tangibilaran-Panglao Channel (Philippines), PANGLAO- 2004, st. R67, 9.64° N, 123.86° E, 3.0–3.5 m | MNHN IM-2009-4603 BAU00207 | FM999081 | 535 | FM999112 | 489 | Oliverio and Modica, in press |
| Volutomitridae | <i>Microvoluta</i> sp. | Bohol/Sulu Seas sill (Philippines), PANGLAO 2005 St. CP2358, 8.87° N, 123.62° E, 569–583 m | MNHN IM-2009-4609 BAU00699 | FM999080 | 525 | FM999111 | 651 | Oliverio and Modica, in press |
| Ptychatriactidae | <i>Latromittra</i> sp. | Bellona West (New Caledonia), Coral Sea, EBISCO, st. CP2556, 21.1° S, 158.53° E, 741–791 m | MNHN IM-2009-4610 BAU00612 | FM999085 | 525 | FM999116 | 653 | Oliverio and Modica, in press |

Table 2. The specimens of *Tritonoharpa antiquata* with their shell measurements (in mm) and their use in this study. Abbreviations: **H**, shell length; **h**, length of the last whorl; **al**, aperture length.

| Specimen/Voucher ID | Locality | H | w | h | al | Sex |
|---------------------|--|------|-----|------|-----|--------|
| BAU00268 | Aliguay Is. (Philippines), 8.75° N, 123.23° E, 30–150 m, tangle nets, May 2006 | 15.7 | 5.4 | 9.4 | 6.8 | male |
| BAU00269 | Aliguay Is. (Philippines), 8.75° N, 123.23° E, 30–150 m, tangle nets, May 2006 | 18.5 | 6.1 | 9.9 | 7.2 | female |
| BAU00270 | Mactan Is. (Philippines), 10.32° N, 124.03° E, 40–120 m, tangle nets, 15 May 2006 | 14.1 | 4.6 | 8.4 | 6 | female |
| BAU00301 | Santo Is. (Vanuatu), SANTO 2006, sta. DR74, SE Matewulu, 15.38° S, 167.19° E, 6 m (J. Pelorce leg.) | 15.3 | 5.1 | 9.1 | 6.6 | female |
| BAU00302 | Santo Is. (Vanuatu), SANTO 2006 sta. DR74, SE Matewulu, 15.38° S, 167.19° E, 6 m (M. Oliverio leg.) | 20 | 6.5 | 10.3 | 8.1 | female |
| BAU00303 | Santo, Vanuatu, SANTO 2006 sta. DR55, Palikulo Bay, 15.48° S, 167.25° E, 3–7 m (J. Pelorce leg.) | 19.1 | 6.3 | 10.1 | 7.3 | female |

In the Results and the Discussion sections, we have used collective taxonomic names within quotation marks (e.g.: 'volutoid', 'buccinoid') as descriptive terms in the traditional context of the names (e.g., Ponder, 1974), but without attributing a specific taxonomic rank to them.

ANATOMICAL METHODS: Four specimens of *Tritonoharpa antiquata* were manually dissected (two from the Philippines BAU00268-9 and two from Vanuatu BAU00301, BAU00303). One female (from Vanuatu, BAU00302) was embedded in paraffin and serially sectioned at a thickness of 7 μm . The sections were stained either with hematoxylin and alcoholic eosin, or with hematoxylin, eosin and Alcian Blue. Radulae were cleaned in liquid bleach [NaOCl], air-dried, coated with gold, and examined using a JEOL scanning electron microscope.

DNA EXTRACTION, PCR, CLONING, AND SEQUENCING: Total DNA was extracted following a standard Phenol/Chloroform/Ethanol protocol (Hillis et al., 1990) with slight modification as previously described by Oliverio and Mariottini (2001). The QIAGEN QiAmp Extraction Kit was used for extraction of DNA from difficult samples, according to manufacturer's instructions.

Partial sequences of two mitochondrial genes encoding ribosomal DNA were PCR amplified. A region of the gene encoding 16S rDNA encompassing the domains IV and V (Gutell and Fox, 1988) was amplified using primers 16SA (5'-CGCCTGTTTATCAAAAACAT-3') (Palumbi et al., 1991) and 16SH (5'-CCGGTCTGAACTCAGATCAC-3') (Espirito et al., 2001) or CGLeuR (5'-TATTTAGGGCTTAAACCTAATGCAC-3') (Hayashi, 2005). A portion of the gene encoding 12S rDNA corresponding to the domains II and III was amplified with primers 12SI (5'-TGCCAGCAGCCGCGGTTA-3') and 12SIII (5'-GAGCGACGGCGRRTTWGTAC-3') (Oliverio and Mariottini, 2001). Amplification conditions were as follows (30–35 cycles): 94°C for 30 seconds, 45–50°C for 30 seconds, 72°C for 60 seconds. When a single band was obtained, the PCR product was purified using the Exo-Sap enzymatic method. In cases of persistent aspecific amplification, the PCR product was ligated into the pGEM-T-Easy vector according to manufacturer's (Promega) instructions and then used to chemically transform *E. coli* JM109 cells. Transformed colonies were selected by blue-white selection and clones containing the correct insert size were PCR-screened. Then, they were purified using the SIGMA miniprep kit. Purified products (amplicons and clones) were then double-strand sequenced with BigDye v. 2.0 (Applied Biosystems, Foster City, CA, USA) using the PCR primers and sequences visualized on automatic sequencer. Sequencing was performed by Macrogen Inc. (Seoul, South Korea). Chromatograms were analysed using the Staden Package (Version-1.6.0, Staden et al., 1998, 2005). All sequences have been deposited at EMBL (The European Molecular Biology Laboratory, Heidelberg; see Table 1 for accession numbers).

SEQUENCE AND PHYLOGENETIC ANALYSIS: Sequences were aligned using Clustal X (Thompson et al., 1994; 1997)

using the default settings, then edited manually. The aligned dataset is available from the authors upon request. Analyses of nucleotide sequences were performed using Mega3.1 (Kumar et al., 2004). The uncorrected 'p' and the ML distances between the sequences were calculated. To test for the presence of mutational saturation, uncorrected 'p' pairwise distances, transition (Ts) and transversion (Tv) were plotted against the estimated ML distance (Nichols, 2005; Philippe et al., 1994) in DAMBE (Xia and Xie, 2001; Xia, 2000). The χ^2 test implemented in PAUP* v. 4b10 (Swofford, 2002) was used to test for base composition homogeneity of the aligned sequence data. The aligned sequences were analysed under the assumptions of Maximum Parsimony, Maximum Likelihood (ML, Felsenstein, 1981) and with a Bayesian approach (Rannala and Yang, 1996), using the packages PAUP* v. 4b10 (Swofford, 2002), Modeltest v. 3.7 (Posada and Crandall, 1998), MrModeltest v. 2.2 (Nylander, 2004), MrBayes v. 3.1.2 (Ronquist and Huelsenbeck, 2003), and Treefinder, June 2007 version (Jobb et al., 2004; Jobb, 2007). Each locus (12S and 16S) was first analysed separately. A partition homogeneity test (Mickeych and Farris, 1981; Farris et al., 1995a, 1995b; Cunningham, 1997), implemented as ILD test in PAUP*, was performed before combining the two loci (but see Darlu and Lecointre, 2002, and Yoder et al., 2001 for criticisms on ILD's efficiency in determining data compatibility). The combined dataset was analyzed by MP, and partitioned ML and Bayesian analyses. ML analyses were performed by Treefinder, using for each partition the substitution models chosen after evaluation by Modeltest using the Akaike information criterion. Base frequencies, relative rates of the six substitution types and model parameters were estimated separately for each partition by the software during phylogenetic reconstruction. Confidence for the nodes was estimated in Treefinder using 1000 bootstrap replicates and compared with the LR-ELW Edge Support (Expected Likelihood Weights on the Local Rearrangements: Strimmer and Rambaut, 2002; Jobb, 2007). A Bayesian analysis (BI) was performed to obtain posterior probabilities of branches using the software MrBayes, which adopts the Markov Chain Monte Carlo method to sample from posterior densities (Larget and Simon, 1999; Yang and Rannala, 1997). The substitution model used was estimated for each partition using the software MrModeltest. Base frequencies, the relative rates of the six substitution types and model parameters were estimated during the analysis, separately for each partition (using the command 'unlink' in MrBayes). A four chain metropolis-coupled Monte Carlo analysis was run twice in parallel for 10^6 generations, and trees were sampled every 1.000 generations, starting after a burn-in of 250,000 generations. Stationarity was considered to be reached when the average standard deviation of split frequencies shown in MrBayes was less than 0.01 (Ronquist and Huelsenbeck, 2003). Bayesian posterior probabilities (BPP) of a branch were estimated as the percentage of trees (after burn-in) which showed that specific node.

RESULTS

Anatomy of *Tritonoharpa antiquata*: EXTERNAL MORPHOLOGY: Animal uniform cream in base color, with bright orange spots most frequently situated on surface of kidney and digestive gland (Figures 1–3). Foot (Figures 1–3, **ft**) partly contracted, with a deep propodial groove separating narrow propodium. Operculum absent in all specimens. Head small (Figure 4), on well-defined neck, with short, narrow, apparently non-retractable snout (**sn**) and pair of long, thick tentacles (**t**), each with a large black eye (**e**) on outer side of a basal swelling. Penis (Figure 7, **p**) of male (spm. No. 2) rather large, flattened, slightly widening distally, with small rounded orifice (**so**) at right upper angle.

MANTLE: Mantle margin smooth (Figure 8). Siphon (**s**) short, muscular. Osphradium (**os**) occupying 1/3 of mantle length, approximately 1/10 of mantle width. Osphradium with broad axis, 2 equal rows of short lamellae. Ctenidium (**ct**) long, crescent-curved, slightly wider than osphradium, occupying almost entire mantle length. Females with broad capsular gland (**cg**) covering rectum. Female genital orifice (**fo**) small, slit-like, terminal. Area between ctenidium and capsular gland occupied by numerous high folds of hypobranchial gland (**hg**).

DIGESTIVE SYSTEM: Proboscis extremely long, narrow (Figure 6, **pr**), folded within body haemocoel into > 10 coils (Figure 13, **pr**). In histological sections, proboscis wall consisting of columnar epithelium with basal nuclei (Figure 12, **ep**), a layer of circular muscles (**cml**) and a thick inner layer of longitudinal fibers (**lm**). Mouth opening large, terminal (Figure 6, **m**). Oral tube short, lined with thick cuticle (Figure 16, **ctc**). Buccal mass short, thick (Figure 5, **bm**), occupying ~1/10 proboscis length, consisting of buccal musculature and folded cartilages (Figures 9, 11, 15, **crt**). Buccal mass surrounded by well-developed, cuticularized, funnel-like jaw plate (Figures 9, 15, 16 **jw**, **ctc**), tubular anteriorly, expanded posteriorly into two small wings surrounding odontophore. Radula slightly shorter than odontophore (Figure 5, **r**), nematoglossan, consisting of a thin membrane and one central longitudinal row of rachidian teeth (Figure 19). Each tooth long, narrow (length >10×width), with three short cusps on distal end. Median cusp bearing vertical row of short secondary cusps (Figures 20, 21). Teeth closely set, distance between them approximately equal to their width.

Accessory salivary glands paired, strongly-coiled, thick-walled, tubular (Figure 5, **asg**), running parallel to buccal mass, tapering toward buccal tube, opening by two ducts (**asd**) into medial region of buccal cavity. Glands consisting of very thin layer of circular fibers and layer of tall columnar glandular epithelium with basal nuclei (Figure 16, **asg**). Lumen of gland filled with mucous secretion (staining blue with Alcian: Figure 16, **asg**). Proximal ends of accessory salivary glands fused together and connected to ventral part of proboscis wall by a strip of connective tissue (Figure 5, **cnt**). Buccal mass attached to

bottom of buccal tube by multiple retractor muscles. Anterior esophagus thin-walled (Figure 5, **aoe**). Proboscis cavity containing thick proboscis nerves (Figure 5, **n**) and ducts of primary salivary glands.

Single proboscis retractor muscle running from base of proboscis to floor of body haemocoel (Figure 6, **prr**). Esophagus penetrating massive nerve ring (**nr**) then continuing ventrally. Spirally coiled valve of Leiblein (**vl**) situated within proboscis. Long midgut gland posterior to nerve ring, provisionally referred to as gland of Leiblein (Figure 6, **gl**), running along posterior part of esophagus. Gland well developed, easily recognized by its dark-brown color. Tissue of gland compact in histological sections, represented by globular cells with large nuclei and multiple granules, indicating strong apocrine secretion (Figure 16, 17, **gl**). Globular cells with large nuclei situated along septa internally dividing gland into distinct lobes. Gland filled with vesicles containing multiple secretion granules. Duct of this gland not found. Anterior aorta thick, running parallel to gland of Leiblein after passing through nerve ring. Primary salivary glands paired, whitish, tightly fused (Figure 6, **sg**), situated posterior to gland of Leiblein. In histological sections (Figure 17, **sg**), primary salivary glands appear clearly tubular, consisting of thin outer layer of connective tissue, and thick layer of high columnar epithelium, with cells having long necks and basal nuclei. Ducts of primary salivary glands (Figure 6, **sd**) thin, not passing through nerve ring, forming a loop, entering proboscis base parallel to esophagus. Ducts entering buccal mass posterior to ducts of accessory salivary gland.

Stomach long, narrow, situated beneath kidney and digestive gland, spanning one whorl. Stomach imperfectly preserved, transversal folds on its walls could not be clearly recognized.

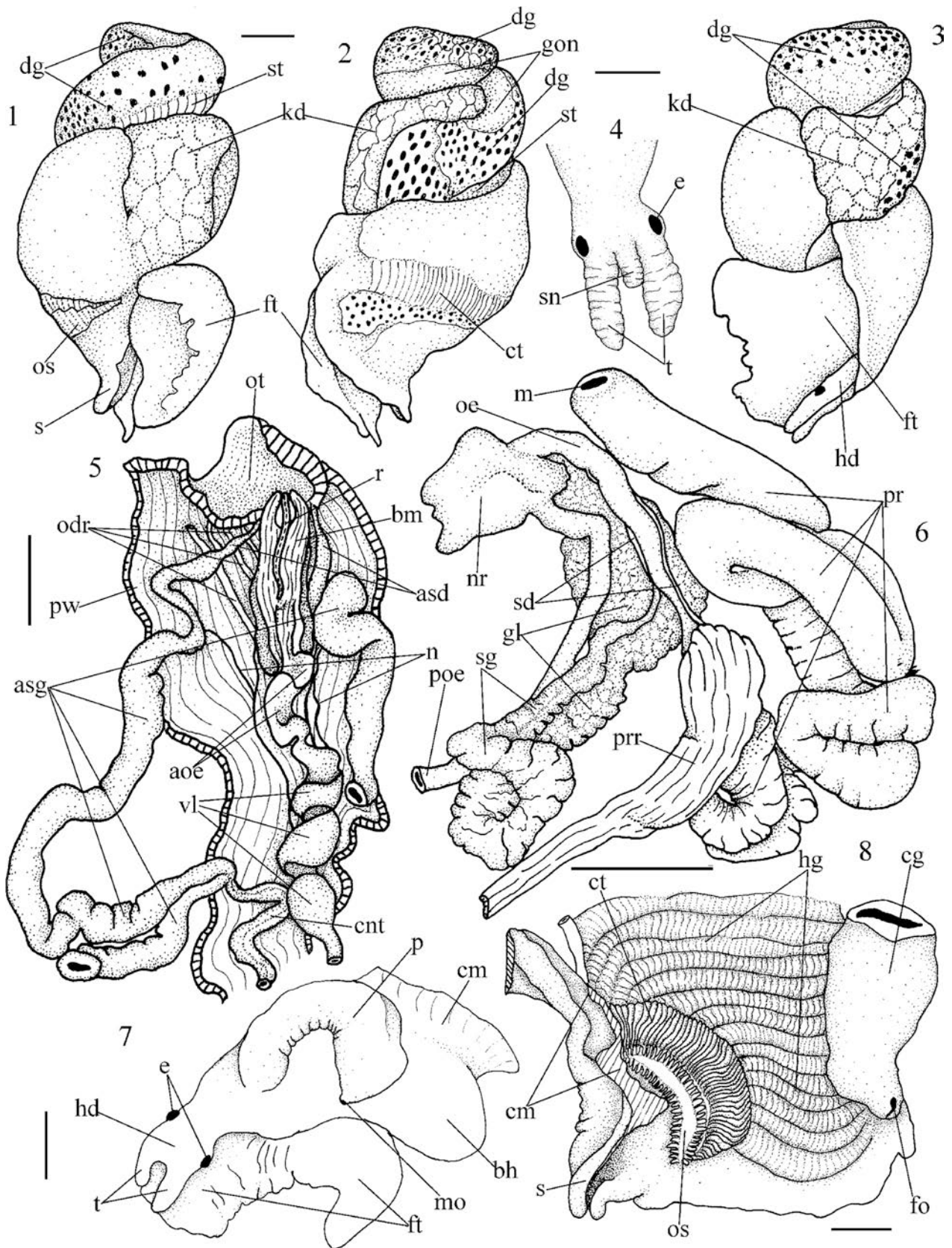
DNA Analysis: A total of 23 sequences were obtained for each of the two genes (including the outgroup *Cypraea cervinetta*). The sequences in the trimmed alignment were 521–541 bp for 12S and 489–679 bp for 16S. A χ^2 test of base homogeneity, uncorrected for phylogeny, indicated that base composition at each partition was not significantly different across all sites (16S: P=1.000; 12S: P=0.999).

Mutational saturation plots (results not shown) displayed evidence of saturation for both 12S and 16S sequences at the level of the ingroup-outgroup comparisons.

A partition homogeneity test performed in PAUP* (Swofford, 2000) did not reveal significant incongruence between the 16S and 12S datasets (P value=0.65).

The combined aligned dataset comprised 1300 nucleotide positions (12S: 581; 16S: 719), with the alignment of 301 positions considered uncertain, and thus excluded from subsequent analysis. Of the 999 included positions 536 were constant, 136 variable positions were parsimony-uninformative and 327 variable positions were parsimony-informative.

The MP analyses of each partition and of the combined dataset, produced topologies with very few nodes



supported by $bs > 50\%$ (Figure 22). In all MP trees, the Rachiglossa, the Toxoglossa, the Muricidae, and the Buccinidae emerged as polyphyletic. In the analysis of the combined dataset, *Tritonoharpa*+*Plesiotriton* and the *Cancellaria* spp. comprised a nematoglossan clade, sister to the Olividae. Only seven nodes received a bootstrap support $> 90\%$.

Model test 3.7 selected by AIC the following models of nucleotide evolution: the TrN+I+G for 12S rDNA only and the TVM+I+G (transversal model) for 16S rDNA only. These models were adopted for ML analysis. MrModelTest2.2 selected by AIC the GTR+I+G substitution model both for 16S rDNA and for 12S rDNA; this model was used in the Bayesian analysis.

In the ML topology obtained for the concatenated dataset (Figure 23), a sister-group relationship between *Tritonoharpa* and *Plesiotriton* was strongly supported ($bs=99$ and $BPP=1$). The Plesiotritoninae emerged as the sister group of the other Cancellariidae included in our analysis (*C. cooperi* and *C. cancellata*), albeit without strong support ($bs=50$ and $BPP=0.89$); the clade comprising all the nematoglossans (Cancellarioidea) was the sister-group of the remaining neogastropods (rachiglossans and toxoglossans). Toxoglossans (Conoidea) emerged as polyphyletic and basal to the stenoglossans. Within the rachiglossate group, a clade Olividae was basal ($bs=95$; not recovered in bayesian analysis), followed by a 'volutoid' clade ($bs=95$ and $BPP=0.99$), comprising Volutomitridae (*Microvoluta* sp.) and Costellariidae (*Vexillum* sp.) plus Ptychatractidae (*Latiromitra* sp.). A clade formed exclusively of Muricidae ($bs=92$ and $BPP=0.97$) was the sister taxon to a clade consisting of the 'buccinoid' families Nassariidae, Buccinidae, and Melongeniidae ($bs=95$ and $BPP=0.95$).

DISCUSSION

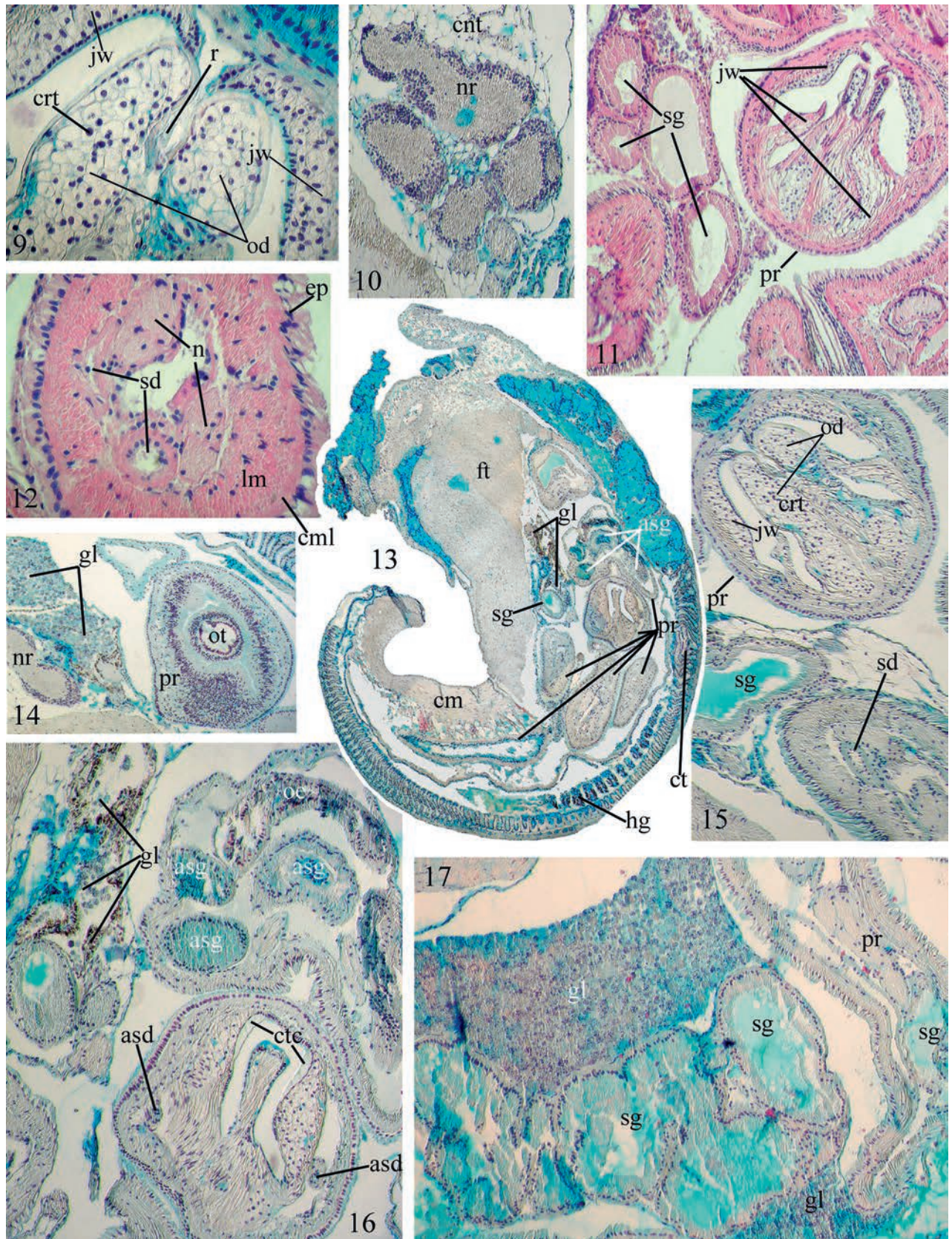
MORPHOLOGY AND ANATOMY: Although *Tritonoharpa* is similar to the Colubrariidae and other neogastropods in the morphology of its head-foot, pallial complex, reproductive and excretory systems, and extremely long, coiled proboscis, it differs in its foregut anatomy. Beu and Maxwell (1987: 7) reported the lack of a radula in *T. antiquata* based on the examination of two specimens (one result admittedly "inconclusive", due to the extreme fragmentation of the specimen). We have observed the presence of a radula in at least three specimens. It is

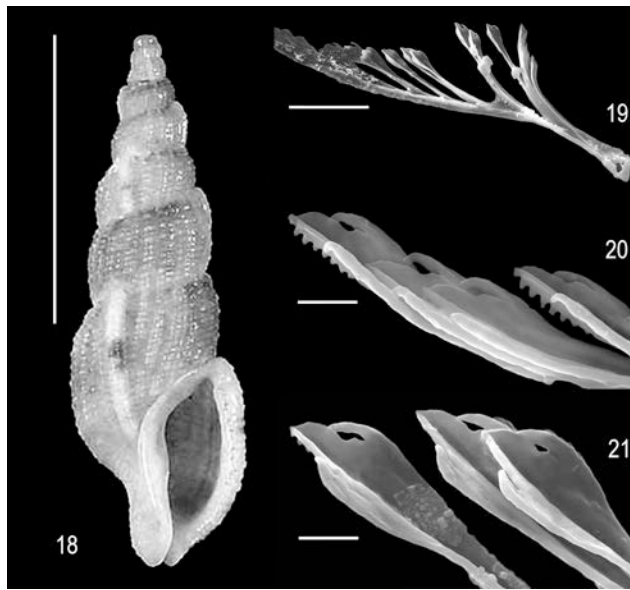
possible that Beu and Maxwell did not recognize a radula due to its extremely reduced size ($< 200 \mu\text{m}$ long). In some cancellariid species the radula may be present or absent (at different stages), as Oliver (1982) reported a radula only in the largest of two specimens of *Nothodmete tumida* Oliver, 1982. The radula of *Tritonoharpa* has the typical nematoglossan structure, and is very similar to those of *Plesiotriton vivus* Habe and Okutani, 1981, and *Africotriton crebriliratus* (G. B. Sowerby III, 1903) (Beu and Maxwell, 1987, pls. 1 a-f and 13 a-d, respectively), comprising a single row of long, narrow, ribbon-like teeth. The peculiar tubular jaw surrounding the odontophore is typical of all Cancellariidae examined so far (Oliver, 1982; Harasewych and Petit, 1984, 1986; Simone and Birman, 2006) and may represent a synapomorphy of the Nematoglossa. Conceivably, the modification and reduction of the nematoglossan radula prompted the formation of protective jaws (**ju** in Figures 9, 15) around the median part of the odontophore (Figure 9, 15, **od**). This innovation was possibly induced by the necessity to either (1) raise the thin and long radular teeth, improving operational efficiency, and/or (2) strengthen the tip of the proboscis, which may be useful for suctorial feeding.

Tritonoharpa antiquata has two pairs of salivary glands. The accessory salivary glands have the typical tubular structure and location as described for other cancellariids (Graham, 1966; Harasewych and Petit, 1982, 1984, 1986). The primary salivary glands are tubular and located in the body haemocoel rather than in the proboscis. Such a position is unusual in cancellariids: it may be explained by the large size of these glands in *Tritonoharpa*, or alternatively it may be a plesiomorphic feature of the neogastropods.

Tritonoharpa antiquata has a large and well developed midgut gland located posterior to the nerve ring, which strongly resembles the gland of Leiblein of other neogastropods in its form and coloration. Although we have not detected any real duct connecting the gland to the esophagus, the only possible connection can be where the tissue of the gland and the esophagus are in contact, i.e. in the anterior portion of the gland, still posterior to the nerve ring. The tissue of this gland appears less structured than in the gland of Leiblein of other neogastropods (e.g., *Nucella lapillus*, Andrews and Thorogood, 2005; A. Richter, personal communication), although it is known that the general appearance of the gland can be related to feeding habits and the physiolog-

Figures 1–8. Anatomy of *Tritonoharpa antiquata*, Santo Is. (Vanuatu) and Aliguay Is. (Philippines). **1–3.** External view of the soft body of a female (BAU00303, Vanuatu). **4.** Head of a female (BAU00269, Philippines). **5.** Anterior section of the proboscis of a female (BAU00301, Vanuatu), dissected dorsally. **6.** Foregut anatomy of a female (BAU00268, Philippines). **7.** Head-foot of a male (BAU00269, Philippines). **8.** Mantle of a female (BAU00268, Philippines). Scale bar – 1 mm. Abbreviations: **aoe**, anterior esophagus; **asd**, accessory salivary duct; **asg**, accessory salivary gland; **bh**, body haemocoel; **bm**, buccal mass; **cg**, capsule gland; **cm**, columellar muscle; **cnt**, connective tissue; **ct**, ctenidium; **dg**, digestive gland; **e**, eye; **fo**, female orifice; **ft**, foot; **gl**, gland of Leiblein; **gon**, gonad; **hd**, head; **hg**, hypobranchial gland; **kd**, kidney; **m**, mouth; **mo**, male orifice; **n**, nerves; **nr**, nerve ring; **odr**, odontophoral retractors; **oe**, esophagus; **os**, osphradium; **ot**, oral tube; **p**, penis; **poe**, posterior esophagus; **pr**, proboscis; **prr**, proboscis retractors; **pw**, proboscis wall; **r**, radula; **s**, siphon; **sd**, salivary duct; **sg**, salivary gland; **sn**, snout; **st**, stomach; **t**, tentacles; **vl**, valve of Leiblein.





Figures 18–21: Shell and radula of *Tritonoharpa antiquata*. **18.** Shell, off Tayud Is., Lilo-an (Cebu, Philippines) (photo courtesy, G. and P. Poppe). **19–21.** Radula, Mactan (Philippines; BAU00269). Scale bars: 10 mm (18), 50 µm (19), 5 µm (20–21).

ical state of the specimens (Andrews and Thorogood, 2005; A. Richter, personal communication). Large globular cells of this gland, with large nuclei and multiple nucleoli and granules in the cytoplasm indicate high secretion activity; the presence of vesicles filled with granules suggests an apocrine secretion mechanism. While the diet of *Tritonoharpa antiquata* is unknown, it is likely that individuals in this species are suctorial, feeding on body fluids as do other cancellarioideans. This conjecture is supported by the extreme modification of the radula, which suggests use for piercing rather than rasping (Oliver, 1982; Petit and Harasewych, 1986), by the tubular nature of the jaw, and by the large stomach resembling that of the haematophagous Colubrariidae (Ponder, 1968; Oliverio and Modica, in press). Furthermore, haematophagy has been already reported for the cancellariine *Cancellaria cooperi* Gabb, 1865 (O'Sullivan et al., 1987), while other cancellariid species have been observed feeding on bivalves (*Trigonostoma scalariformis* (Lamarck, 1822)), sand-dwelling gastropods (*Trigonostoma scalata* (Sowerby, 1832)) and, in aquarium, on fish pieces and squid eggs (Loch, 1987).

Figures 9–17. Histology of *Tritonoharpa antiquata*, Santo Is. (Vanuatu; BAU00302, female). **9.** Cross-section of odontophore and radula. **10.** Nerve ring. **11.** Anterior part of the proboscis with buccal mass and salivary glands, stained with hematoxylin and eosin. **12.** Cross-section through the posterior part of the proboscis with primary salivary ducts and nerves. **13.** General view of the cross-section through the medial region of the last whorl of the animal. **14.** Cross-section of the proboscis at the level of the oral tube and medial part of the midgut gland. **15.** Anterior part of the proboscis with buccal mass and salivary glands, stained with alcian blue. **16.** Cross-section of the proboscis with accessory salivary glands and their ducts. **17.** Longitudinal section through the posterior parts of the midgut gland and salivary glands. Abbreviations: **asd**, accessory salivary duct; **asg**, accessory salivary gland; **cm**, columellar muscle; **cml**, circular muscles; **cnt**, connective tissue; **crt**, odontophoral cartilages; **ct**, ctenidium; **ctc**, cuticle; **ep**, epithelium; **ft**, foot; **gl**, gland of Leiblein; **hg**, hypobranchial gland; **lm**, longitudinal muscles; **lw**, lateral wings of the odontophoral cartilage; **modr**, middle part of the odontophoral cartilage; **n**, nerves; **nr**, nerve ring; **oe**, esophagus; **ot**, oral tube; **pr**, proboscis; **r**, radula; **sd**, salivary duct; **sg**, salivary gland.

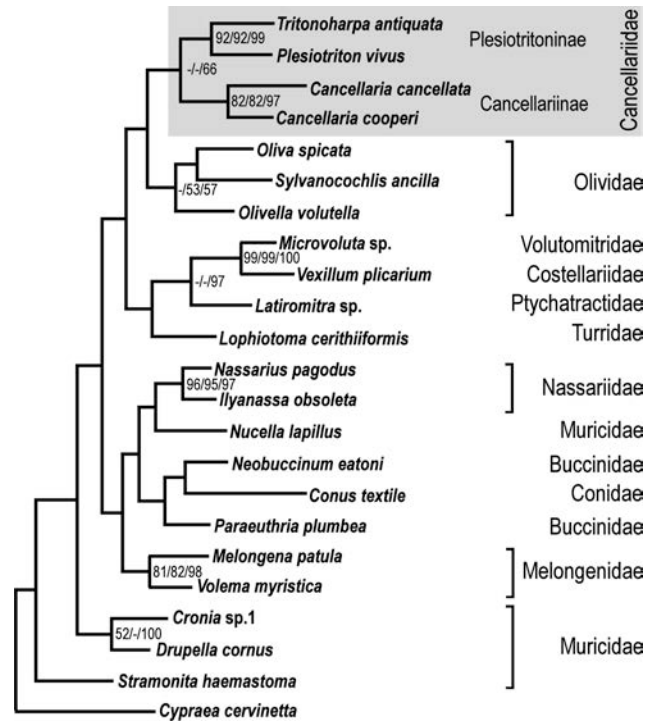


Figure 22: Maximum Parsimony topology obtained for the combined molecular dataset. Numbers at nodes represent Bootstrap values (1000 replicates) in the analysis of the 12S, 16S, and combined datasets, respectively.

During several days of aquarium observations (SANTO 2006 expedition: MO, unpublished), two specimens of *T. antiquata* did not show any feeding activity in the presence of living specimens of various species of fishes.

The peculiar long and spirally convoluted valve of Leiblein, which differs from the pyriform valve of other Neogastropoda, has been also reported in *Plesiotriton vivus* (Kantor and Fedosov, 2009). Its functional significance deserves further investigation.

PHYLOGENY: The MP analyses of each partition and of the combined dataset, produced highly implausible results, particularly as the Rachiglossa, the Muricidae and the Buccinidae all emerged as polyphyletic (Figure 22), yet with a very few nodes with strong bootstrap support. This was probably due to the inclusion in our dataset of some highly divergent sequences (e.g., *Stramonita haemastoma* (Linnaeus, 1767), and *Conus textile* Linnaeus, 1758), a

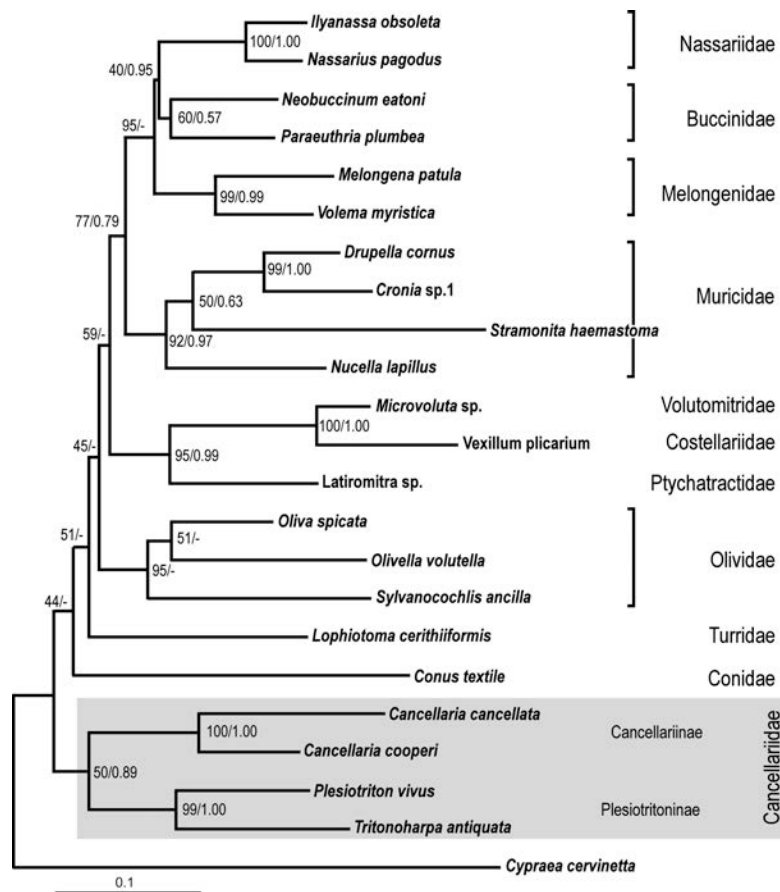


Figure 23: Partitioned Maximum Likelihood topology obtained for the molecular dataset. Numbers at nodes represent Bootstrap values/Bayesian Posterior Probability.

situation in which MP is expected to perform poorly (Felsenstein, 1978; Kim, 1996; Holder and Lewis, 2003). Therefore, MP results will not be described and discussed in details.

The ML and BI phylogenetic analyses of the molecular datasets confirms Beu and Maxwell's placement of *Tritonoharpa* in the Cancellariidae within a plesiotritonine group. It also suggests that cancellariids could be the sister-group to other neogastropods, in agreement with neogastropod phylogenetic hypotheses based on anatomical characters (Kantor, 1996, 2002; Strong, 2003) and larger molecular datasets (Oliverio and Modica, in press).

The presence of a midgut gland resembling (and possibly homologous to) the neogastropod gland of Leiblein in *Tritonoharpa* raises some interesting questions on the evolution of the foregut. In fact, current hypotheses interpret the lack of separation between the midgut gland and esophagus in the cancellariids as indicating that the elongation site is the mid-esophagus. In the rachiglossans the elongation site is the anterior esophagus, causing the detachment of the glandular tissue from the oesophageal walls and the formation of the gland of Leiblein (Ponder, 1974). If further studies on the midgut gland of the Plesiotritoninae (e.g., biochemical charac-

terization of the secretion, exact localization of the connection to the esophagus) will confirm its homology with the neogastropod gland of Leiblein, the possession of a separate gland should be considered as an apomorphy of the Neogastropoda (instead of only of rachiglossans + toxoglossans). It may thus not be the site of elongation of the esophagus that determined the formation of the gland of Leiblein. The presence of glandular band of tissue, and not a separate gland, in other cancellariids (Harasewych and Petit, 1982; 1984; 1986) could be considered as a secondary reduction. Alternatively, either the plesiotritonine midgut gland or the separate glandular tissue of other cancellariids may not be homologous to the true gland of Leiblein. The development of a compensatory glandular region, has already been reported for other neogastropods, where it is associated with a reduced or absent gland of Leiblein (e.g., the glandular mid-posterior esophagus of Colubaridae: Ponder, 1968, 1973; Oliverio and Modica, in press).

The buccal mass is displaced posteriorly from the proboscis tip of cancellarioideans by the length of the oral tube. This condition does not correspond to a basal position (as in the toxoglossans), which has been hypothesized as the plesiomorphic state for the ancestral neogastropod

(Kantor, 1996; 2002). An intermediate and variable condition in the buccal mass position is observed in olivids (Kantor, 1996; 2002), which our ML tree shows to be a basal clade within the rachiglossan radiation (Figure 23).

In our phylogeny, several clades are well supported (Figure 23). In a 'volutoid' clade, comprising *Latiromitra*, *Vexillum*, and *Microvoluta* (members of Ptychactariidae, Costellariidae, and Volutomitridae respectively), at least the first two species exhibit a primitive arrangement of the foregut (Bouchet and Kantor, 2000; Ponder, 1972). Ptychactariids have been recently treated as a separate family (Bouchet and Rocroi, 2005), but they had been included as a subfamily of the Turbinellidae (e.g., Bouchet and Warén, 1985), which are a group displaying remarkable variation among the recognized subfamilies (Ponder, 1974; Kantor and Bouchet, 1997). The placement of *Latiromitra* in our analysis suggests that a 'volutoid' affinity of the ptychactariids may exist, as suggested by, e.g., Thiele (1929) or Cernohorsky (1970). A 'buccinoid' clade is recognizable in a more derived position (including members of the families Nassariidae, Buccinidae, and Melongenidae), sister to a clade constituted exclusively by Muricidae. This result is in agreement with a recent morphology-based phylogenetic hypothesis (Strong, 2003).

It is evident that cancellariids are a key group for understanding neogastropod evolution, although their anatomical disparity is still largely unexplored. As more anatomical data on *Plesiotriton* and other cancellariids become available, a new light could be shed on the evolution of the foregut in Neogastropoda and on the early radiation of the group.

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