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Relationships of the haematophagous marine snail *Colubraria* (Rachiglossa: Colubrariidae), within the neogastropod phylogenetic framework

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The gastropod genus *Colubraria* includes marine shallow-water species from tropical, subtropical, and temperate rocky coral environments. At least six species are known to feed on fish blood. Although there is general consensus in placing *Colubraria* in the Neogastropoda, the actual relationships and the systematic position of *Colubraria* and related genera are unknown. This is partly the consequence of the lack of a clear phylogenetic framework for the Neogastropoda. This study attempts to propose a phylogenetic framework for the Neogastropoda, by testing: (1) a preliminary phylogenetic arrangement for a large number of recognized neogastropod families; (2) the position of *Colubraria* within the neogastropods; and (3) the relationships of *Colubraria* within one of the major neogastropod lineages. We used two different molecular data sets. The first set included representatives of at least 14 neogastropod families, for points (1) and (2), and was based on mitochondrial (*16S*, *12S*, and *cytochrome oxidase subunit I*, *COI*) and nuclear (*28S*) DNA sequences, giving a total of 3443 aligned positions. The second data set, for point (3), included 30 buccinoid sequences from mitochondrial *16S*, giving a total of 1029 aligned positions. We also studied the anatomy of the type species of *Colubraria* and compared it with other neogastropods within the new phylogenetic framework. The results included the first phylogeny of the neogastropod based on 50% of the recognized families. This clearly indicated that the nematoglossan Cancellariidae represent a basal offshoot of the monophyletic Neogastropoda, and that the toxoglossan Conoidea are the sister group to the Rachiglossa. Within the Rachiglossa, a colubrariid clade, worthy of family ranking, showed clear buccinoid affinities. Most of the anatomy of *Colubraria* is congruent with a buccinoid model. The peculiar anatomical features that do not conform to the buccinoid model seem to be related to the evolution of haematophagous feeding.

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ADDITIONAL KEYWORDS: Bayesian inference – Buccinoidea – Cancellarioidea – Conoidea – Maximum Likelihood – Muricoidea – Neogastropoda – phylogeny – radula – systematics.

INTRODUCTION

The gastropod genus *Colubraria* Schumacher, 1817 includes two dozen marine shallow-water species that inhabit rocky and coral environments in tropical, subtropical, and temperate seas. Most of them (14) occur in the Indo-West Pacific province (Parth, 1992). The unusual feeding habit of the species of this genus was observed only recently. In fact, they use a long proboscis to feed on the blood of parrot-fishes (Scaridae), and occasionally on other fish (Johnson, Johnson & Jazwinski, 1995; Bouchet & Perrine, 1996; M. Oliverio, pers. observ.; Fig. 1). A considerable number of species are involved in this association: *Colubraria tortuosa* (Reeve, 1844), *Colubraria nitidula* (Sowerby, 1833), *Colubraria muricata* (Lightfoot, 1786), *Colubraria obscura* Reeve, 1844, *Colubraria castanea* Kuroda in Habe, 1961, and *Colubraria reticulata* (de Blainville, 1826). Haematophagous parasitism on fishes has been reported for species belonging to two other neogastropod families: Marginellidae (Kosuge, 1986; Bouchet, 1989) and Cancellariidae (O'Sullivan, McConnaughey & Huber, 1987).

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Figure 1. *Colubraria muricata* (Lightfoot, 1786), the type species of the genus. A, *Colubraria muricata* feeding on a *Siganus* sp. at Santo Island (Palliculo Bay; depth, 11 m; photo S. Schiaparelli). B, the cephalic region of *C. muricata* from Santo Island. C, shells of *C. muricata* from the Philippines (photo G. & P. Poppe).

Colubraria is generally placed in the family Colubrariidae with some related genera, such as *Bartschia* Rehder, 1943, *Iredalula* Finlay, 1926, *Kanamarua* Kuroda, 1951, *Metula* Adams, 1853, and *Ratifusus* Iredale, 1929. The anatomy of the group is still largely unknown, with the exception of a single anatomical study by Ponder (1968), on *Ratifusus reticulatus* (A. Adams, 1855) [= *Ratifusus mestayerae* (Iredale, 1915)], and *Iredalula striata* (Hutton, 1873). Generally, the empty shells of *Colubraria* are not uncommon, and are easily collected in shallow waters and from beaches; conversely, live specimens are very difficult to collect, given their mostly nocturnal activity, and their habitat in crevices or caves (where they await for fishes going to rest). The composition, the systematic position, and the phylogenetic affinities of the group remain unclear. Dall (1904) suggested that *Colubraria* should be included in the Rachiglossa, the neogastropod group including muricoids (rock shells) and buccinoids (whelks). This view eventually changed: various authors proposed it should be placed outside the neogastropods, generally in the family Ranellidae (e.g. Wenz, 1941; Kuroda & Habe, 1952). More recently, Dall's original idea has been revived, but a number of different taxonomic placements within the rachiglossans have been proposed over time. Some authors have regarded the colubrariids as a family (Ponder, 1973; Taylor, Morris & Taylor, 1980; Kantor, 1996; Bouchet & Rocroi, 2005), whereas others preferred to give them a subfamilial status, thus including them in either the Buccinidae (Cernohorsky, 1971) or the Fasciolariidae (Vaught, 1989; Millard, 1996, 2004). Ponder & Warén (1988) regarded Colubrariidae as a synonym of Buccininae, clearly following Beu & Maxwell (1987), who placed *Colubraria* and related genera in the buccinid subfamily Pisaniinae, thus denying them even subfamilial status.

This confusing pattern is partly driven by the lack of a clear phylogenetic (and thus systematic) framework for the entire Neogastropoda (Ponder *et al.*, 2008). In fact, the use of morphological characters alone (either from the shell or from the anatomy of soft parts) for defining phylogenetic affinities is severely complicated, in neogastropods, by the strong tendency for parallel evolution of anatomical features. The frequent reliance on a single organ system further hampers the recovery of resolution. In buccinoids, moreover, the uniformity of the foregut further reduces the number of available taxonomic characters, resulting in different schemes, often based merely on different opinions (Kantor, 2003).

In this work we aimed to propose a neogastropod phylogenetic framework for *Colubraria*, based on

molecular data, and to test: (1) a preliminary phylogenetic arrangement for a large number of recognized neogastropod families, and (2) the position of *Colubraria* within the neogastropods, by assessing its relationships with one of the major neogastropod lineages. Thereafter, we enlarged the taxonomic coverage within the selected major neogastropod group, to test the hypothesis that *Colubraria* may represent an independent lineage worthy of family ranking. We used molecular data sets derived from mitochondrial (*16S*, *12S*, and *cytochrome oxidase subunit I*, *COI*) and nuclear (*28S*) DNA sequences. We also studied the anatomy of the type species of *Colubraria*, and of some other species, and discussed the morphological data in the phylogenetic context. This study will also provide an evolutionary framework for ongoing projects on the physiology and biochemistry of the haematophagy in *Colubraria* and other neogastropods.

MATERIAL AND METHODS

Taxon sampling and collection

The material for the present study was either provided by museums or colleagues, or was collected during sampling trips and expeditions to the West Pacific (Philippines, Panglao, 2004 and 2005; Vanuatu, Santo, 2006), the Gulf of Panama (2006), the Mediterranean Sea, and other localities (see Table 1 for details). All specimens of *C. muricata* were collected alive by SCUBA diving at night, in small caves, and were found either partly or entirely buried in the sand on the bottom.

A number of species have been included in the combined data sets for the molecular phylogenetic analysis as representatives of different neogastropod (sub)families (NEO data set). The Cancellariidae are represented by the Cancellariinae and the Plesiotritoninae. The superfamily Conoidea (= Toxoglossa) is represented by the Conidae and the Turridae. We included, as representative of the rachiglossate neogastropods, members of the Muricidae, Olividae, Pseudolividae, Mitridae, Volutomitridae, Costellariidae, Ptychatractidae, Melongenidae, Nassariidae, Buccinulidae, and Buccinidae. Therefore, this data set comprised representatives of at least 14 neogastropod families, which is ~50% of the 28–30 currently recognized families (Bouchet & Rocroi, 2005). The cowry *Cypraea cervinetta* Kiener, 1843 and the periwinkle *Littorina saxatilis* (Olivi, 1792) were chosen as caenogastropod outgroups. We did not aim to test the monophyly of the Neogastropoda molecularly in the present work: we assumed that the morphological support for neogastropod monophyly was sufficient for the present time. This is the reason why we did not take in problematic taxa, such as Tonnoidea or Velutinoidea (see *Discussion*), or potentially closer outgroups, such as the Epitoniidae.

As this analysis clearly indicated buccinid affinities for *Colubraria*, to detail its position within the buccinoideans, we enlarged our *16S* data set by adding most of the sequences used by Hayashi (2005). In this analysis, *Cancellaria cooperi* Gabb, 1865 was used as an outgroup, and a total of 20 Buccinidae, five Nassariidae, three Melongenidae, and two Fasciolariidae, were included (BUC data set).

The voucher specimens of most samples are stored at Muséum national d'Histoire naturelle (MNHN), Paris, whereas either specimens from the same lots or tissue samples of the vouchers are also stored at "La Sapienza" Rome University, Dipartimento di Biologia Animale e dell'Uomo (DBAU), Rome.

Morphology

Five specimens of *C. muricata* were studied in total (Table 2). The three specimens from the Philippines (two males and one female) were used for DNA extraction and dissection; the two specimens from Vanuatu were used for dissection (male) and serial sections (female). Specimens were relaxed in $MgCl_2$ solution, isotonic with seawater. Most specimens could not be removed from the shell without cracking the shell with a vice. Specimens were fixed in 10% formalin or in Bouin, and were then transferred to 75% ethanol. A fragment of the foot was always cut and preserved in 100% ethanol for DNA extraction.

Four specimens were manually dissected under a stereomicroscope, and drawings were made with a camera lucida. One female specimen was dehydrated, embedded in paraffin, and then serially sectioned at a thickness of 7 μm. The sections were stained with Mayer's haemalum and alcoholic eosin, or Mayer's haemalum-eosin-Blue Alcian. The radulae were cleaned in liquid bleach, air-dried, coated with gold, and examined using a JEOL scanning electron microscope. Additionally, specimens of *Colubraria buitendijki* Bayer, 1933, *C. nitidula* (Sowerby, 1833), *C. obscura*, *Colubraria reticulata* (Blainville, 1826) and *Colubraria tenera* Gray, 1839, were dissected to provide a preliminary comparison with the type species and a rough estimation of the possible variation in some characters.

DNA extraction, PCR, cloning, and sequencing

The total DNA was extracted following a standard phenol/chloroform/ethanol protocol (Hillis *et al.*, 1990) with slight modification, as previously described by Oliverio & Mariottini (2001). The QiAmp Extraction Kit (Qiagen, http://www.qiagen.com) was used for the

Table 1. Species included in the present work, for the anatomical study and to compile the molecular NEO-dataset, with collecting data, voucher registration numbers, and EMBL accession numbers. For the other buccinoid taxa used to compile the BUC-dataset, see Hayashi (2005). Provisional classification of the buccinoid family-level taxa is based on Vaught (1989) with few modifications (Buccinulidae ranked as family, according to Harasewych & Kantor, 1999). BAU: Dept of Animal and Human Biology, Rome; MNHN, Muséum National d'Histoire Naturelle, Paris; NMSA: Natal Museum, Pietermaritzburg; ZSM: Zoologishe Staatssammlung, München.

Family	species	locality	Voucher number	EMBL accession number					Notes and References
				12S	$tRNA^{Val}$	16S	COI	28S	
Littorinidae	*Littorina saxatilis* Olivi, 1792	Not available	–	AJ132137	AJ132137	AJ132137	AJ132137	–	Wilding *et al.*, 1999
Cypraeidae	*Cypraea cervinetta* Kiener, 1843	Venado (Panama), 8.89 N 79.59 W, intertidal	BAU00799	FM999072	–	FM999103	FM999155	FM999134	original sequences
Cancellariidae	*Cancellaria cancellata* Linné, 1767	Off Malaga (Spain), 40–50 m	BAU00224	FM999074	FM999181	FM999105	FM999157	FM999136	original sequences
	Plesiotriton vivus Habe & Okutani, 1981	Bohol/Sulu seas sill (Philippines), PANGLAO-2005, st CP2359, 8.83 N 123.58 W, 437–476 m	MNHN IM-2007-32123	FM999075	FM999182	FM999106	FM999158	FM999137	original sequences
	Cancellaria cooperi Gabb, 1865	Off La Jolla (California, USA), 40 m	MNHN IM-2009-4611 BAU00797	FM999073	FM999180	FM999104	FM999156	FM999135	original sequences
Conidae	*Conus ximenes* Gray, 1839	Venado (Panama), 8.89 N 79.59 W, intertidal	BAU00245	FM999076	FM999183	FM999107	FM999159	FM999138	original sequences
Turridae	*Aforia magnifica* (Strebel, 1908)	Weddell Sea, PS61/ 125-1: 62.58 S 55.67 W	ZSM 20021143 BAU00786	FM999077	FM999184	FM999108	FM999160	FM999139	original sequences
	Lophiotoma cerithiformis Powell, 1964	Philippines	–	DQ284754	DQ284754	DQ284754	DQ284754	–	Bandyopadhyay *et al.*, 2006
	Polystira picta (Reeve , 1843)	Las Perlas Is. (Panama), 8.79 N 79.26 W, 50 m	MNHN IM-2009-4612 BAU00260	FM999078	FM999185	FM999109	–	FM999140	original sequences
Muricidae	*Nucella lapillus* Linnè, 1758	Portobello (UK), 55.95 N 3.10 W, intertidal	MNHN IM-2009-4617 BAU00187	FM999088	FM999195	FM999119	FM999169	FM999146	original sequences

	Cronia sp.	Tolo Channel, Hong Kong, 22.45 N 114.26 E, 1 m depth	MNHN IM-2009-5118 BAU00619	FN391982	FM999196	FM999120	FM999170	–		original sequences
	Stramonita haemastoma Linné, 1767	S. Marinella (Italy), 42.03 N 11.90 E, intertidal	BAU00696	FM999090	FM999197	FM999121	FM999171	–		original sequences
	Drupella cornus 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	FM999198	FM999122	–		FM999147	original sequences
	Siratus beauii Fischer & Bernardi, 1857	Guadalupa, 16.35 N 61.64 W	BAU00183	FM999086	FM999193	FM999117	FM999167	–		original sequences
	Muricanthus radix Gmelin, 1798	Venado (Panama), 8.89 N 79.59 W, intertidal	MNHN IM-2009-4618 BAU00297	FM999087	FM999194	FM999118	FM999168	–		original sequences
Buccinulidae	*Paraeuthria plumbea* (Philippi, 1841)	Ushuaia (Argentina), 54.78 S 68.23 W, intertidal	MNHN IM-2009-4613 BAU00697	FM999095	FM999202	FM999126	FM999174	–		original sequences
Buccinidae	*Neobuccinum eatoni* (Smith, 1875)	Terra Nova Bay (Antarctic), 74.69 S 164.12 E	MNHN IM-2009-4614 BAU00785	FM999096	FM999203	FM999127	–		FM999149	original sequences
	Pisania striata Gmelin, 1791	Salina Is. (Italy), 38.58 N 14.80 E, intertidal	BAU00698	FM999097	FM999204	FM999128	FM999175	–		original sequences
Nassariidae	*Ilyanassa obsoleta* (Say, 1822)	Not available	–	DQ238598	DQ238598	DQ238598	DQ238598	–		Simison *et al.*, 2006
	Nassarius pagodus (Reeve, 1844)	Las Perlas Is. (Panama), 8.74 N 79.20 W, 50 m	MNHN IM-2009-4620 BAU00237	FM999094	FM999201	FM999125	FM999173	–		original sequences

Table 1. *Continued*

Family	Species	Locality	Voucher number	EMBL accession number					Notes and references
				12S	$tRNA^{Val}$	*16S*	*COI*	*28S*	
Melongenidae	*Melongena patula* (Broderip & Sowerby, 1829)	Venado (Panama), 8.89 N 79.59 W, intertidal	MNHN IM-2009-4621 BAU00794	FM999093	FM999200	FM999124	FM999172	FM999148	original sequences
	Volema myristica (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	FM999199	FM999123	–	–	original sequences
Olividae	*Oliva spicata* (Röding 1798)	Las Perlas (Panama), 8.53 N 79.09 W, 20–22 m	MNHN IM-2009-4616 BAU00278	FM999083	FM999190	FM999114	FM999165	FM999144	original sequences
	Olivella volutella (Lamarck, 1811)	Venado (Panama), 8.89 N 79.59 W, intertidal	MNHN IM-2009-4615 BAU00241	FM999082	FM999189	FM999113	FM999164	FM999143	original sequences
Pseudolividae	*Sylvanocochlis ancilla* (Hanley, 1859)	SW of Mossel Bay, Agulhas Bank, Western Cape (South Africa), 81 m	NMSA E5279	FM999084	FM999191	FM999115	–	–	original sequences
Mitridae	*Mitra lens*	Panama City (Panama), 8.95 N 79.53 W, intertidal	BAU00800	FM999079	FM999186	FM999110	FM999161	FM999141	
Costellariidae	*Vexillum plicarium* (Linné, 1758)	Panglao Is., Tangibilaran-Panglao Channel (Philippines), PANGLAO-2004, st. R67, 9.64 N 123.86 E, 3–3.5 m	MNHN IM-2009-4603 BAU00207	FM999081	FM999188	FM999112	FM999163	FM999142	original sequences
Volutomitridae	*Microvoluta* 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	FM999187	FM999111	FM999162	–	original sequences

Ptychatractidae	*Latiromitra* 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	FM999192	FM999116	FM999166	FM999145	original sequences
Colubrariidae	*Colubraria muricata* Lightfoot, 1786	Panglao Is., Doljo Point (Philippines,), PANGLAO-2004, st. R51, 9.59/9.60 N 123.72/123.74 E, 2–52 m	MNHN IM-2009-4604 BAU00629	FM999099	FM999206	FM999130	FM999177	FM999151	Male; original sequences
	Colubraria muricata Lightfoot, 1786	Bohol Is., Cortes Takot (Philippines), PANGLAO-2004, st. R43, 9.69 N 123.82 E, 3–41 m	MNHN IM-2009-4605 BAU00628	–	–	–	–	–	Female
	Colubraria muricata Lightfoot, 1786	Panglao Is., Doljo Point (Philippines,), PANGLAO-2004, st. R51, 9.59/9.60 N 123.72/123.74 E, 2–52 m	MNHN IM-2009-4606 BAU00634	–	–	–	–	–	Male
	Colubraria buitendijki (Bayer, 1933)	off Dog Point, N Zululand (South Africa): 27.11 S 32.86 E, 50 m. Dredged by R/V *Meiring Naudé*	NMSA D7070	–	–	–	–	–	Male
	Colubraria nitidula (Sowerby, 1833)	Panglao Is., Doljo Point (Philippines,), PANGLAO-2004, st. R51, 9.59/9.60 N 123.72/123.74 E, 2–52 m	MNHN IM-2009-4607 BAU00630						Female

Table 1. *Continued*

Family	Species	Locality	Voucher number	EMBL accession number					Notes and references
				12S	$tRNA^{Val}$	*16S*	*COI*	*28S*	
	Colubraria nitidula (Sowerby, 1833)	Bohol Is., Cortes Takot (Philippines), PANGLAO-2004, st. R43, 9.69 N 123.82 E, 3–41 m	MNHN IM-2009-4623 BAU00801	FM999102	–	FM999133	FM999179	FM999154	Female; original sequences
	Colubraria tenera (Gray, 1839)	SE of Sheffield beach (South Africa), 29.56 S 31.78 E, 180 m. Dredged by R/V *Meiring Naudè*	NMSA E4601	–	–	–	–	–	Female
	Colubraria reticulata (de Blainville, 1826)	Porto Cesareo (Italy), 40.25 N 17.89 E, intertidal	BAU00792	FM999100	FM999207	FM999131	FM999178	FM999152	original sequences
	Colubraria obscura Reeve, 1844	Panglao Is., House Reef (Philippines) PANGLAO-2004, st. R8, 9.54 N 123.77 E, 4–24 m	MNHN IM-2009-4608 BAU00627	FM999101	–	FM999132	–	FM999153	Female: original sequences
	Metula amosi Vanatta, 1913	Las Perlas Is. (Panama), 8.82 N 79.45 W, 31–31.2 m	BAU00244	FM999098	FM999205	FM999129	FM999176	FM999150	original sequences

Table 2. Partitions in each data set with their total length in the alignment, the portions included in the analysed data set (the range of the obtained sequences are given in parentheses), and the best-fit models and parameters estimated for the partitions within the two data sets (NEO and BUC).

Data set	Partition	Total bp	Included bp	Model	Base frequencies	Substitution rates	*I*
NEO	*12S*	669 (363–600)	549	TrN + I + G	$\pi_A = 0.4291$ $\pi_C = 0.0930$ $\pi_G = 0.1142$ $\pi_T = 0.3638$	$r(A\rightarrow C) = 1.0000$ $r(A\rightarrow G) = 7.6989$ $r(A\rightarrow T) = 1.0000$ $r(C\rightarrow G) = 1.0000$ $r(C\rightarrow T) = 13.0678$ $r(G\rightarrow T) = 1.0000$	0.3397
	$tRNA^{Val}$	72 (37–72)	72	K81uf + G	$\pi_A = 0.4549$ $\pi_C = 0.0763$ $\pi_G = 0.1018$ $\pi_T = 0.3669$	$r(A\rightarrow C) = 1.0000$ $r(A\rightarrow G) = 5.5576$ $r(A\rightarrow T) = 0.1719$ $r(C\rightarrow G) = 0.1719$ $r(C\rightarrow T) = 5.5576$ $r(G\rightarrow T) = 1.0000$	0
	16S	1460 (428–1363)	976	TrN + I + G	$\pi_A = 0.3894$ $\pi_C = 0.1077$ $\pi_G = 0.1302$ $\pi_T = 0.3727$	$r(A\rightarrow C) = 1.0000$ $r(A\rightarrow G) = 7.3626$ $r(A\rightarrow T) = 1.0000$ $r(C\rightarrow G) = 1.0000$ $r(C\rightarrow T) = 9.8978$ $r(G\rightarrow T) = 1.0000$	0.2589
	28S	1514 (1485–1514)	1458	GTR + I + G	$\pi_A = 0.2049$ $\pi_C = 0.2762$ $\pi_G = 0.3329$ $\pi_T = 0.1861$	$r(A\rightarrow C) = 0.9765$ $r(A\rightarrow G) = 1.6285$ $r(A\rightarrow T) = 1.2531$ $r(C\rightarrow G) = 0.5975$ $r(C\rightarrow T) = 7.1597$ $r(G\rightarrow T) = 1.0000$	0.6455
	COI	638 (597–632)	388	GTR + I + G	$\pi_A = 0.3323$ $\pi_C = 0.1012$ $\pi_G = 0.1296$ $\pi_T = 0.4368$	$r(A\rightarrow C) = 0.9194$ $r(A\rightarrow G) = 15.6736$ $r(A\rightarrow T) = 0.5959$ $r(C\rightarrow G) = 4.6156$ $r(C\rightarrow T) = 37.7789$ $r(G\rightarrow T) = 1.0000$	0.3899
BUC	*16S*-B	1436 (532–1458)	1029	GTR + I + G	$\pi_A = 0.3980$ $\pi_C = 0.0904$ $\pi_G = 0.1330$ $\pi_T = 0.3786$	$r(A\rightarrow C) = 2.1226$ $r(A\rightarrow G) = 9.6129$ $r(A\rightarrow T) = 1.2532$ $r(C\rightarrow G) = 1.3726$ $r(C\rightarrow T) = 15.8479$ $r(G\rightarrow T) = 1.0000$	0.3766

extraction of DNA from difficult samples, following the manufacturer's instructions.

Three mitochondrial fragments were PCR amplified: (1) domains II and III of the *12S* ribosomal DNA (rDNA) (550 bp), using primers from Oliverio & Mariottini (2001); (2) the whole *16S* rDNA gene (1500 bp), using primers from Palumbi *et al.* (1991) and Hayashi (2005); and (3) 700 bp of the *COI* gene, using primers CoxAF and CoxAR (Colgan *et al.*, 2003). The first two fragments constitute a contiguous stretch including the intervening transfer RNA (tRNA) Val (72 bp), which was treated in the analyses as a separate partition. A 1500-bp-long fragment of the *28S* rDNA nuclear gene (corresponding to domains D1–D6) was amplified with primers LSU5 (Littlewood, Curini-Galletti & Herniou, 2000) and LSU 1600 (Williams, Reid & Littlewood, 2003).

Diluted total genomic DNA (3–10 ng) was used in 20 or 25-μL reactions, containing 0.1 μM of forward and reverse PCR primers, 200 mM of each dNTP, a gene-dependent concentration of $MgCl_2$, 1 U of BIOLINE TaqPolymerase, and a 0.1 volume of

BIOLINE buffer 10×. The amplification conditions were as follows (for 30–35 cycles): 94 °C for 30 s, 45–50 °C for 30 s, and 72 °C for 60 s.

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 the manufacturer's instructions (Promega, http://www.promega.com), and was then used to chemically transform *Escherichia coli* JM109 cells. Positive clones were PCR screened for insert size. Then, they were purified using a miniprep kit (Sigma-Aldrich, http://www.sigmaaldrich.com). Purified products (amplicons and clones) were then double-strand sequenced with BigDye v2.0 (Applied Biosystems, http://www.appliedbiosystems.com) using the PCR primers and sequences visualized on an automatic sequencer. Sequencing was performed by Macrogen Inc. (http://www.macrogen.com). Chromatograms were analysed by Geneious Pro 4.0 (Drummond *et al.*, 2008). All sequences have been deposited at the European Molecular Biology Laboratory (EMBL) (see Table 1 for accession numbers).

Phylogenetic analyses

Sequences were aligned using the ClustalW multiple aligment (Thompson, Higgins & Gibson, 1994), as implemented in Geneious 4.0 (Drummond *et al.*, 2008), and ClustalX (Thompson *et al.*, 1997), using the default settings. The accuracy of the alignments was improved by manual editing.

Analysis of the nucleotide sequences was performed using Mega 3.1 (Kumar, Tamura & Nei, 2004) and DAMBE (Xia, 2000; Xia & Xie, 2001). The uncorrected 'p' and the maximum-likelihood (ML) distances between the sequences were calculated. The mutational saturation of the sequences in the data set was tested, plotting uncorrected 'p' pairwise distances, transition (Ts), and transversion (Tv) against an ML distance (Nichols, 2005; Philippe *et al.*, 1994) in DAMBE. The χ^2 test implemented in PAUP* v.4b10 (Swofford, 2002) was used to test for base composition homogeneity among the aligned sequences.

Nucleotide substitution models were selected for each gene separately (Pupko *et al.*, 2002; Jones *et al.*, 2007), using the software Modeltest v.3.7 (Posada & Crandall, 1998) and MrModeltest v.2.2 (Nylander, 2004). The best models estimated by Akaike's information criterion (AIC) for each partition were used in the analysis of ML (Felsenstein, 1981), and in the Bayesian inference (BI; Rannala & Yang, 1996). Subsequently, the combined data set was analysed, keeping the different partitions unlinked both in ML and in BI, and using the substitution model estimated by Modeltest and MrModeltest for each partition, whereas the base frequencies, relative rates of the six substitution types, and model parameters were estimated separately for each partition, during the phylogenetic reconstructions. ML was performed using Treefinder (October 2008 version; Jobb, von Haeseler & Strimmer, 2004; Jobb, 2008); support values of the nodes were estimated in Treefinder, using bootstrap and LR-ELW Edge Support (expected likelihood weights on the local rearrangements; Strimmer & Rambaut, 2002) on 1000 replicates.

The BI was performed to obtain posterior probabilities of branches using the MrBayes v.3.1.2 (Ronquist & Huelsenbeck, 2003), which adopts the Markov Chain Monte Carlo method to sample posterior densities (Yang & Rannala, 1997; Larget & Simon, 1999). A four-chain metropolis-coupled Monte Carlo analysis was run twice in parallel for 10^7 generations, and trees were sampled every 1000 generations, starting after a burn-in of 2 500 000 generations. Stationarity was considered to be reached when the average standard deviation of split frequencies shown in MrBayes was less than 0.01 (Ronquist & Huelsenbeck, 2003). The Bayesian posterior probabilities (BPPs) of a branch were estimated as the percentage of trees (after burn-in) that showed the specific node.

RESULTS

Morphology of *Colubraria muricata*

Head-foot (Figs 1B, 2)

Head small, with a pronounced neck, oriented markedly leftwards. Tentacles long and slender, tapering towards the tip. Eyes small, and placed on swellings near the base of the tentacles. Rhynchostome rounded and minute; sharp snout between the tentacles. Foot quite small, rounded posteriorly, with a propodium divided into two slightly marked flaps; pedal gland detected neither by visual inspection nor in sections. Columellar muscle simple, flat, quite robust, and about half a spire whorl long. In males, large penis, extending backwards from behind the right tentacle. Background colour whitish, with reddish brown areas on the tentacles, the head behind the eyes, the neck, and part of the propodium. Operculum elliptical and thin, corneous, brown (semitransparent brownish in juveniles), smaller than the aperture of the shell, and with a terminal nucleus.

Mantle organs (Fig. 2)

Pallial cavity quite broad and deep. Siphon long, thick, and pronounced. Osphradium occupying about one third of the mantle cavity, bipectinate, with the right filaments about double the length of the left filaments. Osphradial axis broad. Ctenidium long

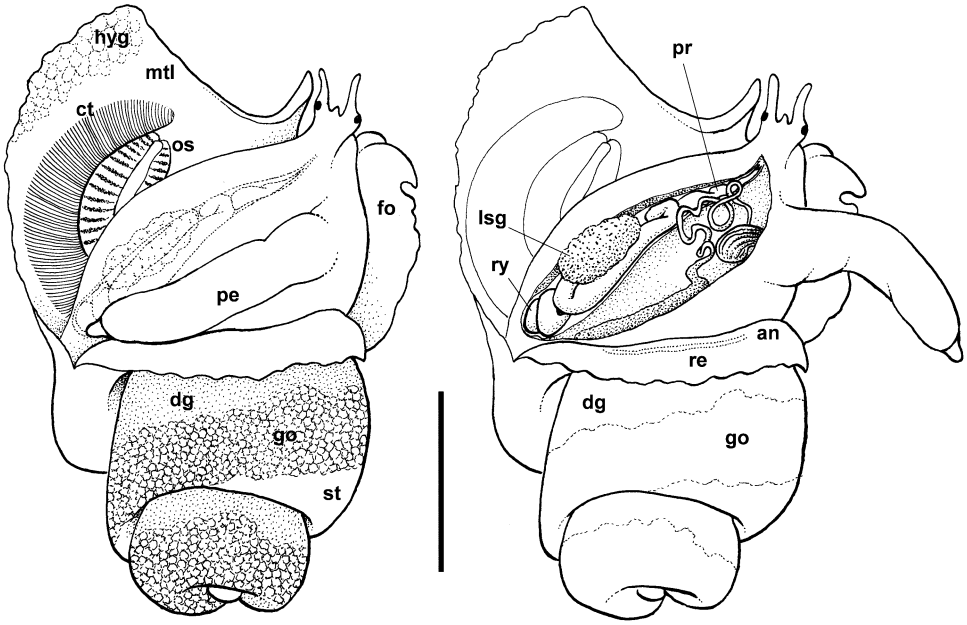

Figure 2. Mantle organs of a dissected male specimen of *Colubraria muricata.* A, mantle dissected medially. B, body wall dissected dorsally. Abbreviations: an, anus; ct, ctenidium; dg, digestive gland; fo, foot; go, gonad; hyg, hypobranchial gland; lsg, left salivary gland; mtl, mantle; os, osphradium; pe, penis; pr, proboscis; re, rectum; ry, rhynchodaeum; st, stomach. Scale bar: 1 cm.

(about twice the osphradium length), narrow, and curved leftwards, with a narrow ctenidial vessel. Gill elements consisting of triangular lamellae. Roof of the pallial cavity occupied by a broad and whitish hypobranchial gland, which produces large quantities of mucus. Rectum extremely thin and narrow, opening in a simple anus in the anterior third of the mantle. Anal gland absent. In females, right side of the mantle occupied by a large pallial oviduct.

Visceral mass and digestive system (Figs 2, 3)

Visceral mass about 3.5 spire whorls long, with the clearly visible stomach covering 1.5 whorls. Kidney whitish, with a single lobe and a branched structure. Heart relatively big (about a quarter of the kidney in volume). Broad renal afferent vessel, with two branches. Anterior aorta wide, running towards the mantle cavity. Proboscis extremely long (when extended, reaching far more than three times the shell length) and thin, with scarcely muscularized walls, and with strong reddish brown pigmentation. Retracted proboscis lying, highly coiled, in the very thin-walled, transparent rhynchodaeum. A pair of powerful proboscis retractor muscles originating ventrally near the base of the proboscis sheath, and attached to the floor of the body haemocoel. Buccal cavity extremely reduced, and placed at the tip of the proboscis. Highly reduced buccal mass, ventral to the buccal cavity, connected to the internal wall of the proboscis by a thin, short muscle (which might be considered a medial odontophore retractor). Radula extremely small (400–500-μm long), rachiglossate, with 70–90 rows. Central tooth multicuspidate, with ten equal cusps, and curved; lateral teeth also multicuspidate, with nine or ten (right) and ten or 11 (left) cusps.

Proboscis artery with quite robust walls, running from the body haemocoel to the tip of the proboscis, flanked by two nervous fibres originating from the left and right cerebral ganglia, respectively. Each fibre ramified, at the proboscis tip, with branches connecting to the internal proboscis wall. Anterior oesophagus thin, and surrounded by relatively reduced circular and longitudinal muscle layers.

The typical gland and valve of Leiblein are absent. However, a small bulge of tissue, where the gland of Leiblein is normally found, seemingly connected to the oesophagus by a short duct, was observed in a single specimen. Salivary glands acinous, whitish, and elliptical: with the left gland covering the rhynchodaeum dorsally, and with the right gland lying beneath the rhynchodaeum. The salivary ducts pass externally to the nerve ring before connecting to the oesophagus, and are lined internally by very long cilia, throughout the entire length (Fig. 3G, H), which are grouped in a single line of tufts. The salivary ducts contact the oesophagus very near their origin, and become embedded in a single structure, with the anterior oesophagus, a couple of nerve fibres, and the proboscis artery. The ducts soon become more

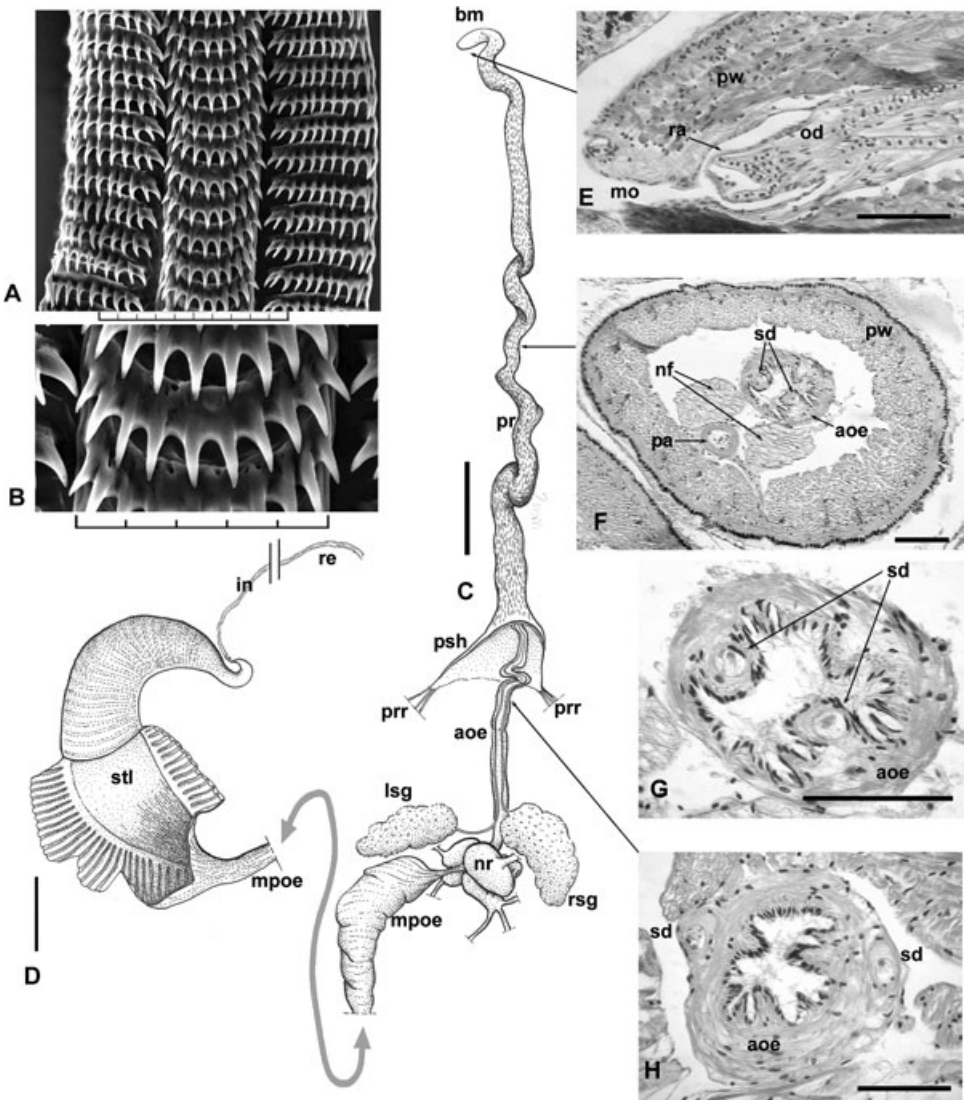

Figure 3. Digestive system of *Colubraria muricata.* A, radula. B, radular central tooth. C, the foregut from the proboscis tip to the mid-posterior oesophagus. D, the dissected stomach. E, longitudinal section of the proboscis tip, showing the buccal mass. F, transversal section of the proboscis. G, enlargement of the anterior oesophagus of F. H, transversal section of the anterior oesophagus at the base of the proboscis. Abbreviations: aoe, anterior oesophagus; bm, buccal mass; in, intestine; lsg, left salivary gland; mo, mouth; mpoe, mid-posterior oesophagus; nf, nerve fibre; nr, nerve ring; od, odontophore; pa, proboscideal artery; pr, proboscis; prr, proboscis retractor muscle; pw, proboscis wall; ra, radula; re, rectum; rsg, right salivary gland; sd, salivary gland duct; stl, stomach lumen. Scale bars: A, 50 μm; B, 25 μm; C, D, 10 mm; E–H, 100 μm.

deeply embedded in the oesophageal wall, and run from this position to their opening in the buccal cavity, at the tip of proboscis. The accessory salivary glands are absent. The oesophagus becomes extremely thick and broad, with internal transverse folds, immediately after its passage through the nerve ring. The oesophagus is internally lined by a glandular epithelium, in which at least two different cell types can be identified: one type is ciliated and filled with fine eosinophilic granules; the second type is characterized by a mucous basophilic cytoplasm. Both cell types are of considerable size (20–40 μm in diameter). This general appearance is maintained until the opening of the oesophagus into the stomach, which hampers the distinction between the mid and the posterior oesophagus. In the absence of other topographical references, we will use the term mid-posterior oesophagus to describe the section that runs posteriorly to the nerve ring. The terminal tract, entering the visceral mass and opening into the

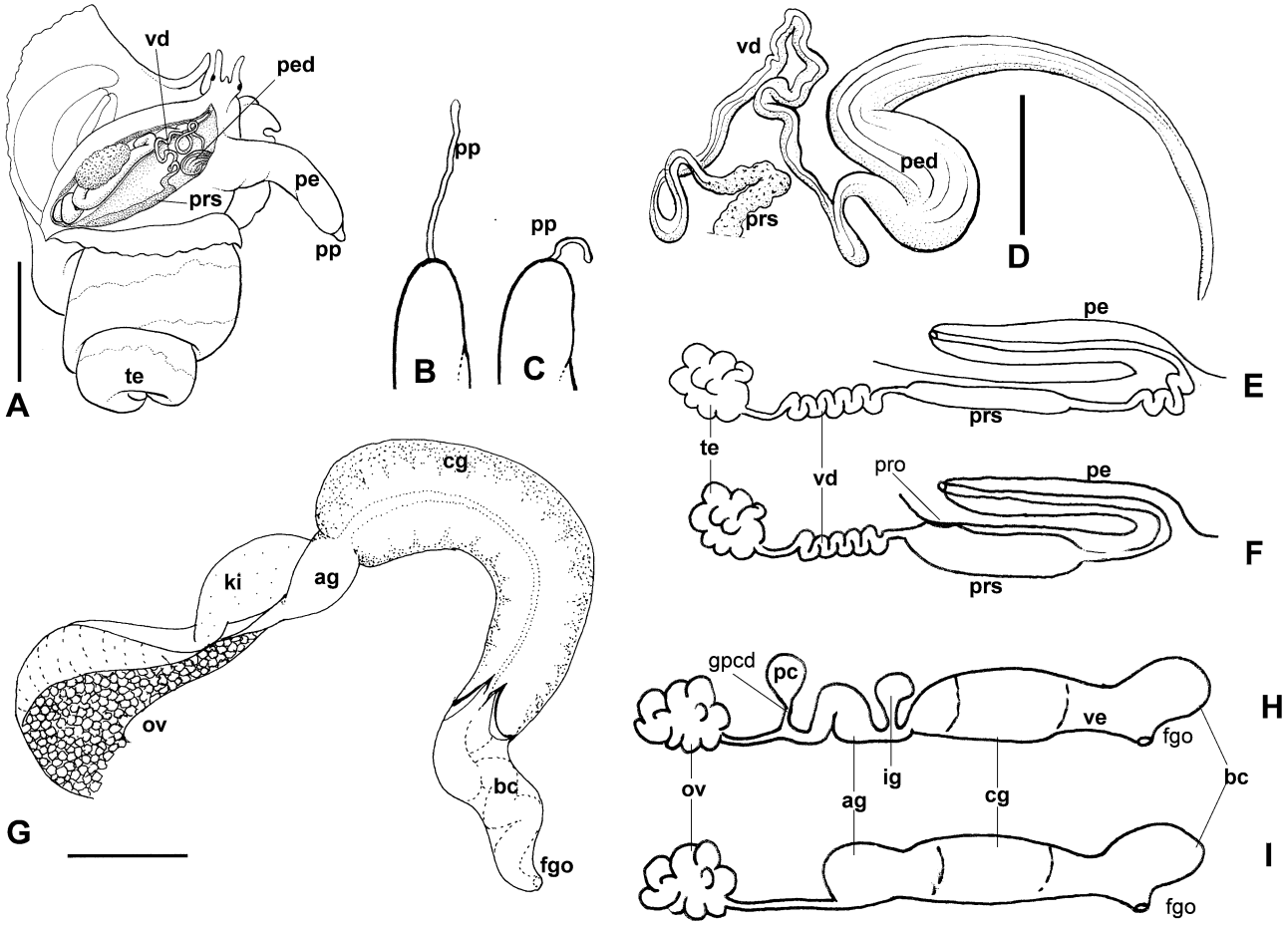

Figure 4. Reproductive system of *Colubraria muricata.* A, a male with the body wall dissected dorsally. B, distal penis with papilla penialis of *Colubraria tenera.* C, distal penis with papilla penialis of *Colubraria reticulata.* D, distal portion of the spermiduct. E, semischematic drawings of the male reproductive system in *C. muricata*. F, semischematic drawings of the male reproductive system in some neogastropods [e.g. *Nucella canaliculata* (Duclos, 1832) or *Anachis lyrata* (Sowerby, 1832); modified after Houston (1976) and deMaintenon (1999)]. H, semischematic drawings of the female reproductive system in some neogastropods [e.g. *Buccinum undatum* Linnaeus, 1758, or *Nucella emarginata* (Deshayes, 1839); modifiend after Houston (1976) and deMaintenon (1999)]. I, semischematic drawings of the female reproductive system in *C. muricata*. Abbreviations: ag, albumen gland; bc, bursa copulatrix; cg, capsule gland; fgo, female genital opening; gpcd, gonopericardial duct; ig, ingesting gland; ki, kidney; ov, ovarium; pc, pericardium; pe, penis; ped, penial duct pp, papilla penialis; pro, prostatic opening in the mantle cavity; prs, prostate; te, testis; vd, vas deferens; ve, vestibulum. Scale bars: A, 1 cm; D, G, 1 mm.

stomach, is very short, quite robust, and broader than the anterior oesophagus.

The stomach is particularly long, extending for about 1.5 visceral whorls, and is crescent shaped. Gastric lumen markedly reduced, resulting in a flattened stomach. Oesophageal opening located at the anterior end of the stomach, with the intestinal opening located at its posterior end. Gastric walls extremely thin. Stomach scarcely differentiated internally, with only a few recognizable features. Well-defined transverse folds particularly developed on the surface of the dorsal wall, and partially covered by the longitudinal oesophageal ridges near to the oesophageal aperture. Folds extending from a ciliated sulcus placed on the left side of the stomach. The single opening of the digestive gland is located in the posterior part of the stomach, where the intestine begins. The intestine runs along the ventral side of the visceral spire, and is extremely thin, with a small and barely detectable anal aperture. In some specimens examined, the rectum was more discernible because of the presence of dark, spherical faecal pellets.

Reproductive systems (Fig. 4)

The testis and visceral spermiduct was not observed in detail; however, the spermiduct appears to be

closed for its entire length, with no evidence of a gonopericardial connection. The pallial spermiduct runs inside the body wall, along the connection with the mantle. The prostatic section of the spermiduct, which is about two-thirds the length of the pallial cavity, starts shortly after the posterior end of the mantle cavity, as a slightly enlarged, glandular, and thick-walled tube, yet is not differentiated into a separate structure. The duct is thin, with a translucid appearance for the remaining anterior third, and becomes strongly coiled in the area under the penial base. The penial duct is highly muscular, broad near the penial base, and gradually tapers towards the tip. The penis is cylindrical, with a small distal papilla.

In females, the ovary is located in the right and posterior side of the visceral mass, and consists of numerous branched tubules lined with a flat epithelium. The observed specimen was probably at the end of the reproductive season, with several empty follicles coexisting with early immature oocytes. Ovarian follicles leading to a thin-walled, nonciliated coelomic oviduct, running anteriorly along the ventral side of the visceral mass. No evidence of a gonopericardial connection. Pallial glandular oviduct consisting of a proximal albumen gland that is continuous with the distal capsule gland. In dissected specimens, only a slight topographical separation was detected between the two glands, with no anatomical differentiation evident by visual inspection. Capsule and albumen glands easily discriminated by histology. Albumen gland partially visceral, consisting of large, weakly basophilic cells, and lined by a cubic epithelium with short cilia. Ciliated ventral channel connecting the albumen gland with the capsule gland; open to the lumen of the capsule gland throughout its whole length. Capsule gland oval, elongated in outline, and divided in different regions. The principal region consists of two lateral lobes that are yellowish and granulated in dissected specimens. The glandular cells of these areas produce acidophilic and eosinophilic secretion granules. Two lobes on each side of the ventral channel (not detectable in dissected specimens) produce strongly acidophilic secretions. The distal part of the pallial oviduct is continuous with the glandular structures, is barely distinguishable from them by dissection, and gradually becomes free of the mantle edge, before finally protruding inside the mantle cavity. The ciliated ventral channel continues in a strongly muscular U-shaped tube, i.e. the bursa copulatrix, with the convex side oriented dorsally, and a very narrow lumen. Bursa copulatrix proximally lined by a ciliated epithelium in continuity with the ventral channel; distally, i.e. in the direction of the female opening, gradually substituted by nonciliated columnar cells with a granular cytoplasm. Masses of unoriented spermatozoa found in the bursa copulatrix. Muscular vagina opening externally with a female aperture, and bordered by small swellings.

Remarks on other Colubraria species

The specimens of other species of *Colubraria* are remarkably similar to *C. muricata*, both in the external morphology of the soft parts and in the gross anatomy, as observed in manual dissection. In the external morphology, some variations in the colour pattern were observed that may have some taxonomic value at the species level. In the gross anatomy, the most evident differences, beside the dimensions, were observed in the shape of the penis, which showed a bulbous distal papilla in *C. muricata*, a short filamentous papilla in *C. reticulata*, and an extremely long filamentous papilla in *C. tenera* (about half the length of the penis) (Fig. 4B, C), and in the presence of a pedal gland in females of *C. nitidula* and *C. obscura*, but not observed in *C. muricata*.

Molecular phylogeny

No bias was detected in base composition across all sites at each of the partitions analysed in both data sets (NEO and BUC). The best-fitting models and parameters estimated for each partition and data set are shown in Table 2. No significant incongruence was revealed among the different partitions in the NEO data set, according to a partition homeogeneity test performed in PAUP* ($P = 0.02$). The NEO data set comprised 4353 nucleotide positions, 716 of which were considered to be uncertainly aligned, and were thus excluded from subsequent analysis. The third codon position of the *COI* fragment was also excluded, as the saturation analysis revealed high saturation levels at this position. Of the 3443 positions included, 2066 were constant, 379 variable positions were parsimony uninformative, and 998 variable positions were parsimony informative. The aligned BUC data set comprised 1436 characters, 407 of which were excluded from subsequent analysis because they were not reliably aligned. Of the 1029 included characters, 500 were constant and 128 variable characters were parsimony uninformative, whereas 401 characters were parsimony informative.

The topologies recovered by ML and BI on the NEO data set did not differ substantially (internal relationships of the buccinoideans, and lack of bootstrap support at some nodes in the ML). The BI topology is shown in Fig. 5. The neogastropods resulted as monophyletic, with strong support. The Cancellariidae also resulted as monophyletic, and are positioned as the sister group to the remaining neogastropods, with strong support. The Conoidea (= Toxoglossa) were shown as monophyletic, and as the sister group to the Rachiglossa. The rachiglossan clade included the first

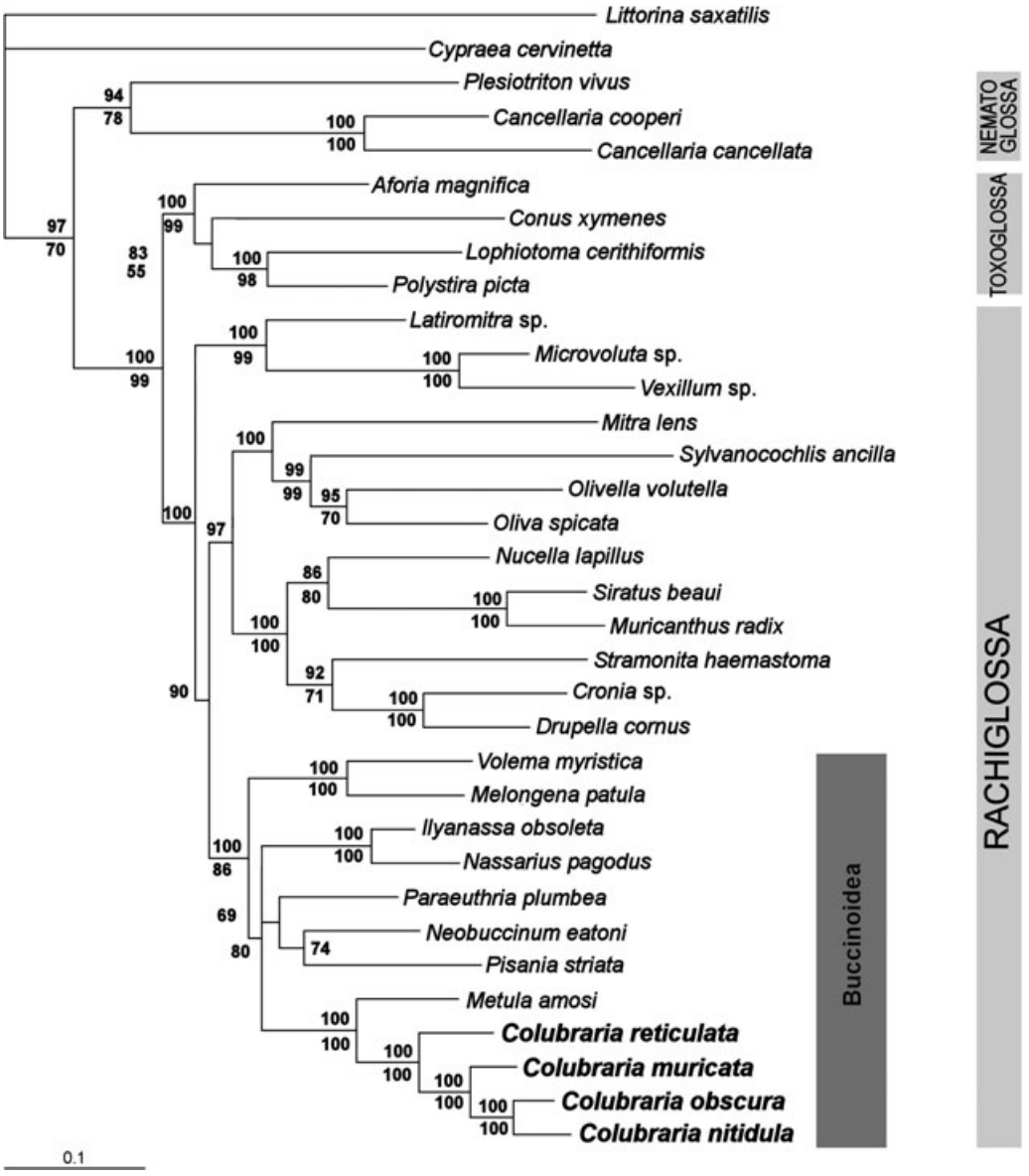

Figure 5. Phylogenetic relationships among the species in the NEO data set (see text). Topology derived after a Bayesian inference. Numbers above the branches are the Bayesian posterior support values; numbers below the branches are the bootstrap support values from a maximum-likelihood (ML) analysis.

offshoot, consisting of the ptychatractine Turbinellidae, the Costellariidae, and the Volutomitridae. Then two major clades were recognizable: (1) a buccinoid clade; (2) a group formed by the Muricidae (with a strongly supported monophyly) and a clade of Mitridae + Olividae + Pseudolividae. In the latter clade, *Mitra lens* Wood, 1828 was the sister to the olivoids ((Olivinae + Olivellinae) + Pseudolividae). In the buccinoid clade the Melongenidae were in a basal position, and were the sister group to a clade with internal unresolved relationships, including the Nassariidae, the Buccinidae, and a well-supported 'colubrariid' clade, with *Metula amosi* Vanatta, 1913 as the sister to the species of *Colubraria*.

Also, only minor differences in the relationships of terminal taxa were detected between the ML and BI analyses of the BUC data set. In this *16S*-based phylogeny (Fig. 6), a basal position is occupied by the Melongenidae. Then a colubrariid clade, also including *M. amosi*, was the sister group to the remaining buccinoids. The Buccinidae were classed as polyphyletic, with Nassariidae and Fasciolariidae, both classed as monophyletic, and with *Buccinulum* (Buccinulidae) nested inside the buccinids. *Penion*,

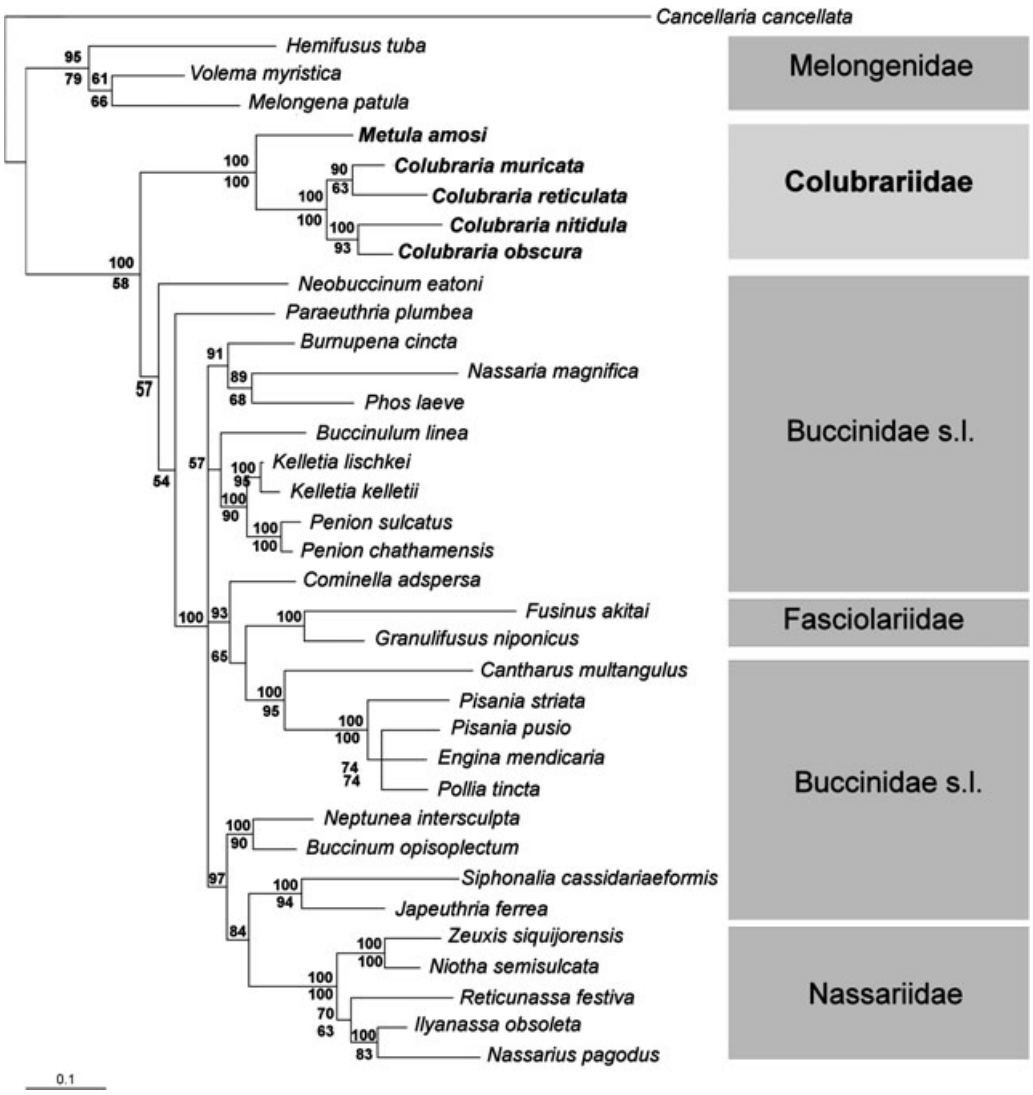

Figure 6. Phylogenetic relationships among the species in the BUC data set (see text). Topology derived after a Bayesian inference. Numbers above the branches are the Bayesian posterior support values; numbers below the branches are the bootstrap support values from a maximum-likelihood (ML) analysis.

Kelletia, and *Buccinulum* clustered together, whereas *Paraeuthria* yielded a basal position in this buccinoid clade, albeit that the basal relationships of buccinoids had only very weak support. The Pisaniinae were the only clade corresponding to a traditional subfamily, although it showed some relationships with the two fasciolariids. The Buccininae, as currently conceived, were clearly polyphyletic, with the included species [*Neptunea intersculpta* (Sowerby, 1899), *Buccinum opisoplectum* Dall, 1907, *Burnupena cincta* (Röding, 1798), and *Neobuccinum eatoni* (Smith, 1875)] scattered in the topology obtained. Also, the monophyly of Photinae as commonly conceived was not confirmed in our analysis: *Nassaria* and *Phos* clustered with *Burnupena*, a genus usually included in the Buccininae (Vaught, 1989), whereas *Cominella* was the sister to the Pisaniinae + Fasciolariidae clade. Relationships among the clades Buccininae + Nassariidae, *Cominella* + Fasciolariidae + Pisaniinae, *Burnupena* + Photinae, and Buccinulidae were not resolved.

DISCUSSION

NEOGASTROPOD PHYLOGENETIC FRAMEWORK

Even though the taxonomic coverage in the present analysis is not complete, this is the first study taking ito account 50% of the family-level diversity of the neogastropods, and the relationships above this level were mostly resolved with strong support. Therefore,

it is possible to outline a provisional phylogenetic framework for the group.

In our molecular phylogeny, the Neogastropoda assayed were monophyletic with respect to the selected caenogastropod outgroups, *Littorina saxatilis* and *Cypraea cervinetta*. The monophyly of Neogastropoda has been strongly supported by morphological analyses (e.g. Strong, 2003; Ponder *et al.*, 2008: 362, fig. 13.16) and by combined morphological and molecular analyses (Ponder *et al.*, 2008: 364 fig. 13.17), yet molecular analyses have often contradicted it (Ponder *et al.*, 2008, and references therein). The tonnoideans (which often made neogastropods polyphyletic in several molecular phylogenies, as the velutinoidean Triviidae and Velutinidae sometimes did; e.g. Hayashi, 2005; Colgan *et al.*, 2007: 727, fig. 3) have not been included in the present analysis. Admittedly, the present data set and analyses do not significantly change the situation, and the monophyly of the Neogastropoda with respect to these problematic taxa remains to be tested by further molecular data.

Of note, the three main groups commonly recognized in the Neogastropoda (Nematoglossa, Toxoglossa, and Rachiglossa) were also classed as monophyletic, with strong support values. The nematoglossan Cancellariidae are here suggested to be the sister group of the other neogastropods, in agreement with some of the hypotheses based on anatomical characters (Kantor, 1996, 2002; Strong, 2003). The monophyly of the Toxoglossa, the species of which share the venom apparatus as a complex feature, has not been discussed here (Taylor, Kantor & Sysoev, 1993), but was recently confirmed on a molecular basis (Puillandre *et al.*, 2008). In our phylogeny the toxoglossans were the sister group to the rachiglossan clade. This result is in agreement with Kantor's hypothesis based on the foregut arrangement, particularly the basal placement of the buccal mass shown by most Toxoglossans, which is sometimes considered to be the plesiomorphic condition for the neogastropods (Kantor, 1996).

Within the rachiglossan radiation, a basal clade comprised members of three 'volutoid' families: Ptychatractidae, Volutomitridae, and Costellariidae. The affinity of Ptychatractidae with volutids or costellariids has been already suggested (Thiele, 1929; Cernohorsky, 1966, 1970), whereas a more recent hypothesis ranked *Latiromitra* and allied genera as a subfamily of the Turbinellidae (Bouchet & Warén, 1985; Bouchet & Kantor, 2000). Remarkably, our phylogeny did not support a close phylogenetic relationship between Costellariidae and Mitridae, that have long been considerd as members of the same family, and are collectively called 'miter shells' (e.g. Thiele, 1929; Cernohorsky, 1966). Instead, *M. lens* was consistenly grouped with Olividae and Pseudolividae, in a sister clade to the monophyletic Muricidae. The splitting of the miter shells into different families was first suggested by Ponder (1972).

The buccinoideans are commonly regarded as a monophyletic group that share some morphological features (Ponder, 1973; Kantor, 1996, 2003; Harasewych *et al.*, 1997), and comprising up to seven families: Buccinidae, Melongeniidae, Columbellidae, Nassariidae, Fasciolariidae, Buccinulidae, and Colubrariidae. In our phylogeny, the monophyly of a buccinoid clade seems to be confirmed, and a derived position within the neogastropods is suggested for them, in agreement with previous morphological (Kantor, 2002, 2003) and molecular (*16S*; Hayashi, 2005) studies. In our analysis, the Melongenidae was placed as the most basal buccinoid family, followed by the colubrariid clade. The other buccinoidean families (Buccinulidae, Nassariidae, and Fasciolariidae) analysed in our BUC data set were nested inside the radiation of the Buccinidae *s.s.*, although relationships at this level did not receive strong support (neither BPP nor bootstrap). The paraphyly of the Buccinidae was also evident in Hayashi's (2005) phylogeny, but with a slightly different placement of the Nassariidae (see below). In fact, the Fasciolariidae showed affinities with Pisaniinae in Hayashi's ML phylogeny, as in our analysis of the BUC data set, in both cases with weak support values. A particularly close relationship between the Buccinidae and Fasciolariidae has already been proposed on a morphological basis (Kosyan, Modica & Oliverio, 2009). The species of Nassariidae clustered with *Phos* and *Nassaria* (Photinae) in Hayashi's ML phylogeny, whereas from our results a relationship with some of the Buccininae is suggested. The Buccininae included in our analysis were not monophyletic, particularly because of the placement (albeit weakly supported) of *N. eatoni* as external to other buccinid taxa. *Neptunea* and *Buccinum*, which unquestionably belong to Buccininae, clustered with *Japeuthria* + *Siphonalia* and the assayed Nassariidae. The subfamily designation for *Japeuthria* and *Siphonalia* has been questioned previously (see Hayashi, 2005, and references therein): our phylogeny indicates that these two genera could belong to the Buccininae, supporting Vaught (1989) and in agreement with the radular similarity between *Buccinum* and *Japeuthria* (Cernohorsky, 1971).

The studied species of *Colubraria* showed clear buccinoid affinities, and formed a well-supported clade with *Metula*. This colubrariid clade is thus clearly identified with strong support as a separate lineage, not nested inside the Buccinidae, and this phylogenetic scheme supports the validity of the

family Colubrariidae, in agreement with Ponder (1968, 1973) and Bouchet & Rocroi (2005), including *Metula*.

The anatomy of *Colubraria* in the phylogenetic context

Some of the anatomical features observed in *Colubraria* are typically buccinoid, such as the absence of the accessory salivary glands and the anal gland, in agreement with the evidence from the molecular phylogeny. Nevertheless, numerous aspects are divergent from a typical buccinoid model. The same situation was evidenced by Ponder (1968) for *Ratifusus* and *Iredalula*. *Colubraria* possesses a long proboscis, as do most buccinoids, with an apical buccal mass protruding into the buccal cavity. The supposed lack of a radula in *Colubraria* (Cernohorsky, 1971; Ponder, 1973; Oliverio, 1993) raised the question of how the snail could have access to the blood vessels of prey. In fact, specimens of *Colubraria* have been observed feeding from previous injuries on the skin of fishes, or in areas where the epithelium is particularly thin, such as the gills, the anus, or the orbit (Johnson *et al.*, 1995; Bouchet & Perrine, 1996; M. Oliverio and M.V. Modica, pers. observ.), but have also been observed to access the epidermal vessels in previously intact areas (M. Oliverio and M.V. Modica, pers. observ.). It is clear that the highly reduced radula reported here can act by scraping at the skin of the fish, thereby making vessels accessible, and we have observed circular scars on the skin of the prey fish. The radula of *C. muricata* is very peculiar, and presents important differences when compared with other described colubrariids. In fact, in *Metula* (Bouchet, 1988), as well as in *R. reticulatus* and *I. striata* (Ponder, 1968), the central tooth is flattened and tricuspidate, and the lateral teeth present a narrow basal plate also with two or three cusps. In *Colubraria*, both the central and the lateral teeth are multicuspidate (with about ten cusps each); the curved central tooth resembles that of the genus *Retifusus* (Buccinidae Colinae; A. Kosyan, pers. comm.), or that of Nassariidae, whereas the lateral teeth are similar to those of the Fasciolariidae.

The two nervous fibres branching at the proboscis tip are possibly involved in the individuation of the superficial blood vessels of the prey. The presence of a robust proboscis artery suggests that it may be involved in the mechanism of the eversion, which is particularly rapid. The folding of the proboscis inside the rhynchodaeum is a feature shared with *I. striata* and *R. reticulatus* (Ponder, 1968), but is absent in *Metula fusiformis* Clench & Aguayo, 1941 (Harasewych, 1990). Anyway, this characteristic is not unique to Colubrariidae. It has been observed in various species of the buccinid genus *Ancistrolepis* Dall, 1895 (Kantor, 1988), and is widespread in the toxoglossans (despite the fact that they possess a different proboscis type). The proboscis can also be coiled together with the rhynchodaeum in the body haemocoel, as seen in Melongeninae (Kosyan & Kantor, 2004) and in Turbinellidae (Ponder, 1973; Medinskaya, Harasewych & Kantor, 1996). In all these cases, the functional mechanism of folding is still unknown. This feature seems to have evolved independently several times in the various neogastropod families, in a homoplastic way.

Other interesting features concern the proboscis retractor muscles. In fact, in neogastropods the eversion of the proboscis is usually accompanied by that of the posterior part of the rhynchodaeum. This mechanism allows the proboscis to attain a greater length. The extent of the evertible part is very variable, and is anatomically determined by the point of insertion of the proboscis retractors on the rhynchodaeal walls. In the buccinoideans, there are generally numerous proboscis retractors attached externally to the central part of the rhynchodaeal wall, and these shift anteriorly when the proboscis is retracted. Thus, buccinoideans can usually evert the posterior half of the rhynchodaeum, which is of considerable length. The same situation was observed in *I. striata* (Ponder, 1968). Conversely, in *Colubraria* (as well as in *R. reticulatus*), only one pair of retractors is present. They originate from the proboscis base and attach to the floor of the body haemocoel. This condition permits the eversion of the rhynchodaeum only for a very short basal tract, if any. A similar arrangement was described in the Melongeninae (Kosyan & Kantor, 2004), for which it was suggested that such retractors might be involved in regulating the proboscis length. The muscularization of the proboscis is scarce. This observation, along with the extremely thin anterior oesophagus (which certainly can not act as a pump), suggests a passive mechanism of blood consumption from the fish. This is also supported by the observation of the blood flow throughout the proboscis during feeding, which seems to pulse in time with heartbeat of the fish (http://neogastropodtol.org/movies/C_muricata_Blood%20close-up.mov).

The salivary gland ducts in *R. reticulatus* and *I. striata* are only free for a short tract of their length, becoming embedded in the oesophageal walls at the level of the valve of Leiblein (Ponder, 1968). This arrangement is the most common for the Neogastropoda, but is atypical for buccinoids, in which the ducts usually run outside the oesophageal walls, entering them near the buccal cavity. A typical buccinoid arrangement was reported for *M. fusiformis* (Harasewych, 1990). The ducts in *Colubraria* enter the oesophageal walls close to the proboscis base, but intimately adhere to the oesophagus from their

beginning. In dissected specimens it was impossible to distinguish this situation from a true passage inside the oesophageal walls. It can be supposed that a similar arrangement is also present in *R. reticulatus* and *I. striata*. This feature was also described for a number of Melongenidae [*Pugilina pugilina* (Born, 1778), *Hemifusus ternatanus* (Gmelin, 1791), and *Volema pyrum* (Gmelin, 1791); Kosyan & Kantor, 2004]. Anyway, the functional significance of this characteristic remains unknown. We can only speculate that the presence of such a compact arrangement might simplify the eversion, especially in animals with a particularly long proboscis.

The absence of the gland and valve of Leiblein is quite common in the buccinoideans: various degrees of reduction have been reported in Buccinidae, Nassariidae, Fasciolaridae, and Columbellidae (Ponder, 1973, and references therein; Kantor, 2002), whereas the Melongeninae completely lack both structures (Kosyan & Kantor, 2004). In *Colubraria*, it is possible that the reduction/absence of these structures might be related to the extraordinary development and glandularization of the mid-posterior oesophagus. This characteristic has also been observed in *R. reticulatus* and *I. striata* (Ponder, 1968), whereas in buccinoids even the dorsal glandular folds are generally lost in this region. This is also confirmed by the situation observed in *M. fusiformis*, in which the gland and valve of Leiblein are well developed (Harasewych, 1990), but the dorsal glandular folds are retained, from which (assuming *Metula* is basal to the colubrariid clade) the glandular mid-posterior oesophagus of *Colubraria* might have evolved.

The stomach is highly variable in buccinoids, and was recently proposed as a source of potentially informative taxonomic characters by Kantor (2003); nevertheless, its organization, as observed in *Colubraria*, is very unlike that of other neogastropods. All the internal features commonly recognized in the neogastropods are completely absent. We argue that the remarkable simplification of this structure is explained by the lesser digestive effort required to process liquid food. It is noteworthy that *M. fusiformis*, which is reported to be a carrion feeder (Harasewych, 1990), has a simple U-shaped stomach (Harasewych, 1990). Scavenging is often a good preadaptation to haematophagy, and in the case of the colubrariid clade, the basal position of *Metula* would be congruent with such a pattern.

The reproductive systems of the buccinoideans are less well known than the alimentary system; however, some features observed in *Colubraria* are shared with *M. fusiformis*, such as the narrow prostate gland, and, in the female, a muscular bursa copulatrix.

The female reproductive system is very variable in the neogastropods, as has already been reported by Fretter (1941) and Houston (1976). The organization observed in *C. muricata*, with the albumen gland in continuity with the capsule gland, has been reported in some muricids [e.g. *Ocenebra japonica* (Dunker, 1869) and *Urosalpinx cinerea* (Say, 1822); Houston 1976) as well as in some columbellids (see deMaintenon, 1999). In *Colubraria*, a separate ingesting gland is missing (as in some columbellids; Marcus & Marcus, 1962), and sperm ingestion takes place in the bursa copulatrix. However, the presence/absence of a gonopericardial duct, a receptaculum seminis, or an ingestion gland are features shown in mosaic combinations by different neogastropod groups. Admittedly, knowledge on the variation of this system in the Neogastropoda is still far from being useful for a wide-range comparison.

A similar lack of adequate knowledge on the variation of the male reproductive system hampers an evaluation of the phylogenetic value of the observed features in *Colubraria*. For instance, a coiled and muscular anterior spermiduct as observed in *C. muricata* was already described in the nassariid *Nassarius incrassatus* (Ström, 1768) and in several columbellids (Houston, 1976; deMaintenon, 1999).

A number of recent studies seemed to indicate that the variability observed in several neogastropod families is too high to make generalizations at the family level. Instead, reproductive systems have provided many useful taxonomic characters below the family level (e.g. Marcus & Marcus, 1962; Richter & Luque, 2002). Thus, even if denser taxonomic sampling within the Neogastropoda facilitates the re-evaluation of the apparently homoplastic condition of some features, it is probable that some of the characters observed in *Colubraria* (e.g. the partially visceral albumen gland, the coiling of the distal spermiduct, the shape of the penial papilla etc.) will prove taxonomically and phylogenetically informative within the colubrariid clade.

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