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Molecular analysis of *Muticaria syracusana* and *M. neuteboomi* from Southeastern Sicily, Italy (Gastropoda, Pulmonata, Clausiliidae).

M. Stella Colomba[1]*, Armando Gregorini[1], Fabio Liberto[2], Agatino Reitano[3], Salvatore Giglio[4] & Ignazio Sparacio[5]

[1]Università di Urbino, DiSTeVA, via Maggetti 22 (loc. Sasso), 61029 Urbino, Italy. [2]Strada Provinciale Cefalù-Gibilmanna n° 93, 90015 Cefalù, Italy. [3]Via Gravina 77, 95030 Tremestieri Etneo, Italy. [4]Contrada Settefrati, 90015 Cefalù, Italy. [5]Via E. Notarbartolo 54 int. 13, 90145 Palermo, Italy. *Email. mariastella.colomba@uniurb.it

ABSTRACT The genus *Muticaria* Lindholm, 1925, is currently distributed either in Southeastern Sicily or in the Maltese islands and comprises the species *M. syracusana* (Philippi, 1836), *M. neuteboomi* Beckmann, 1990 and *M. macrostoma* (Cantraine, 1835). For the first time, we report a molecular study on the topotypicous populations of *M. syracusana* and *M. neuteboomi* carried out on fragments of the ribosomal 16S rDNA subunit and the cytochrome oxydase I (COI) mitochondrial genes by Neighbour Joining, Maximum Likelihood, Maximum Parsimony and Bayesian Inference algorithms. Our results revealed the existence of nucleotide-sequence divergence (Dxy: 5% for 16S rDNA and 12% for COI sequences) between the two taxa.

KEY WORDS Door snails; *Muticaria*; Clausiliidae; Molecular taxonomy; 16S rDNA; COI.

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INTRODUCTION

The genus *Muticaria* Lindholm, 1925, currently attributed to the subfamily Alopiinae A. J. Wagner, 1913, tribe Medorini H. Nordsieck, 1997 (Nordsieck, 1997; Nordsieck, 2007; Bank, 2010), is distributed in Southeastern Sicily and Maltese islands (except Filfla) and shows morphological affinities with the genera *Leucostigma* A. J. Wagner, 1919, widespread in the Apennine Italy, and *Lampedusa* O. Boettger, 1877 occurring in the Pelagie (Sicily) and Maltese islands. Thake (1985; Giusti et al., 1995) suggested that the group might have originated from ancestral Alopiinae that entered Apennine Italy from the Balcans and, successively, during the Messinian salinity crisis (Upper Tertiary), colonized Sicily and the Maltese islands. Afterwards, when the sea water re-invaded the Mediterranean basin with the so-called "Zanclean flood" (Pliocene) these molluscs separated in many populations which diverged and differentiated in the genera *Muticaria, Leucostigma* and *Lampedusa*. Within *Muticaria*, further differentiations at both specific and subspecific levels occurred during glacial and interglacial periods (Pleistocene) when sensitive fluctuations of the sea level went into a cycle of emersions and immersions of land bridges connecting the Sicilian mainland with the Maltese region.

At present, *Muticaria* comprises three species, *M. syracusana* (Philippi, 1836), *M. neuteboomi* Beckmann, 1990 and *M. macrostoma* (Cantraine, 1835), this latter with a few subspecific taxa (Nordsieck, 2007; Bank, 2010): *M. macrostoma macrostoma* reported for Gozo, Comino, Cominotto and Malta; *M. macrostoma scalaris* (L. Pfeiffer, 1850) inhabiting a very limited area on the northwestern coast of Malta (Tal-Blata, Mistra Bay); *M. macrostoma oscitans* (Charpentier, 1852) reported for Gozo and Malta; and *M. macrostoma mamotica* (Gulia, 1861) occurring in a very limited area on the Munxar side of Xlendi Valley in Gozo (Beckmann & Gittenberger, 1987; Schembri, 2003; Nor-

dsieck, 2007; Bank, 2010). Moreover, interestingly there are a few populations which show a shell ribbing intermediate between *macrostoma* and *oscitans*, suggesting that a possible hybridization of the two might have occurred (see Giusti et al., 1995).

M. syracusana locus typicus Siracusa (Philippi, 1836), and *M. neuteboomi* described for Cava d'Ispica, (Modica, Ragusa province) (Beckmann, 1990) are quite widespread in Southeastern Sicily. Giusti et al. (1995) reported *M. neuteboomi* as a local variation of *M. syracusana* (foreseeing Badino and colleagues' results – which remained unpublished – on allozymic polymorphism within the genus *Muticaria*), but a few years later, *M. neuteboomi* was recognized valid species and since then this statement has not been denied (Nordsieck, 1997; 2007; Bank, 2010). Recently, a preliminary molecular study on 16S rDNA partial sequences (Gregorini et al., 2008) revealed that the two taxa show some differences at genetic level. In this study, continuation of previous work, we carried out a more detailed molecular analysis on several *M. syracusana* (from Roman amphitheatre, Siracusa) and *M. neuteboomi* (from Cava d'Ispica, Ragusa) specimens by a comparative investigation of the mitochondrial 16S rDNA and cytochrome oxidase I (COI) partial sequences, in order to improve current taxonomic knowledge and assess the level of differentiation between the two species from a molecular point of view.

MATERIALS AND METHODS

Sample collection, DNA extraction, Amplification and Sequencing

A total of ten specimens (five *M. syracusana* and five *M. neuteboomi;* collection sites: nearby the Roman amphitheatre of Siracusa and Cava d'Ispica, respectively, SE Sicily) were analysed. Samples were stored separately at -20 °C in test tubes. For each individual, the entire animal was used for total DNA extraction (by Wizard Genomic DNA Purification Kit, Promega). Additional specimens belonging to the same batch were stored as vouchers in the laboratory of Cytogenetics and Molecular Biology (University of Urbino, via Maggetti 22). Present study was based on a comparative analysis of 16S rDNA and COI partial sequences, frequently used as genetic markers in the investigation of evolutionary processes at the specific level.

Fragments of 16S rDNA sequences (297 bp) were amplified by a couple of primers (MED16F: 5'-actgtgcaaaggtagcatcc-3; 'MED16R: 5'-ccaacatcgaggtcacaa-3') designed on alignments of several homologous sequences of Clausiliidae species downloaded from GenBank database. Whereas for COI fragments (660 bp), the universal primers LCO1490: 5'-ggtcaacaaatcataaagatattgg-3' and HCO2198: 5'-taaacttcagggtgaccaaaaaatca-3' were employed according to Folmer et al. (1994) with slight modifications. PCR thermal cycles were as follows: 95 °C for 5 min; 95 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min (35 cycles); 72 °C for 5 min. To remove primers and unincorporated nucleotides, the amplified products were purified by the Wizard SV gel and PCR Clean-up kit (Promega). Sequencing of the purified PCR products was carried out using automated DNA sequencers at Eurofins MWG Operon (Germany). GenBank accession numbers for sequences generated in this study are HQ696866-HQ696869.

Phylogenetic analyses

Sequences were visualized with BioEdit Sequence Alignment Editor 7 (Hall, 1999), aligned with the ClustalW option included in this software and double checked by eye. Standard measures of nucleotide polymorphism were computed using MEGA 4 (Tamura et al., 2007). Phylogenetic analyses were conducted in MEGA 4, PhyML 3.0 (Guindon & Gascuel, 2003) and MrBayes 3.1.2 (Huelsenbeck & Ronquist, 2001), the latter two available at http://www.phylogeny.fr/version2_cgi/one_task.cgi?task_type=phyml and http://www.phylogeny.fr/version2_cgi/one_task.cgi?task_type=mrbayes (see Dereeper et al., 2008). To address the phylogenetic relationships among taxa, many different analytical methods were used: Maximum Likelihood (ML), Neighbour Joining (NJ), Maximum Parsimony (MP) and Bayesian Inference (BI). For Maximum Likelihood analyses, the most appropriate model of DNA substitution was selected by Findmodel (available via http://www.hiv.lanl.gov/cgi-bin/findmodel/findmodel.cgi), a web implementation of Modeltest by Posada & Crandall (1998). Models were as follows:

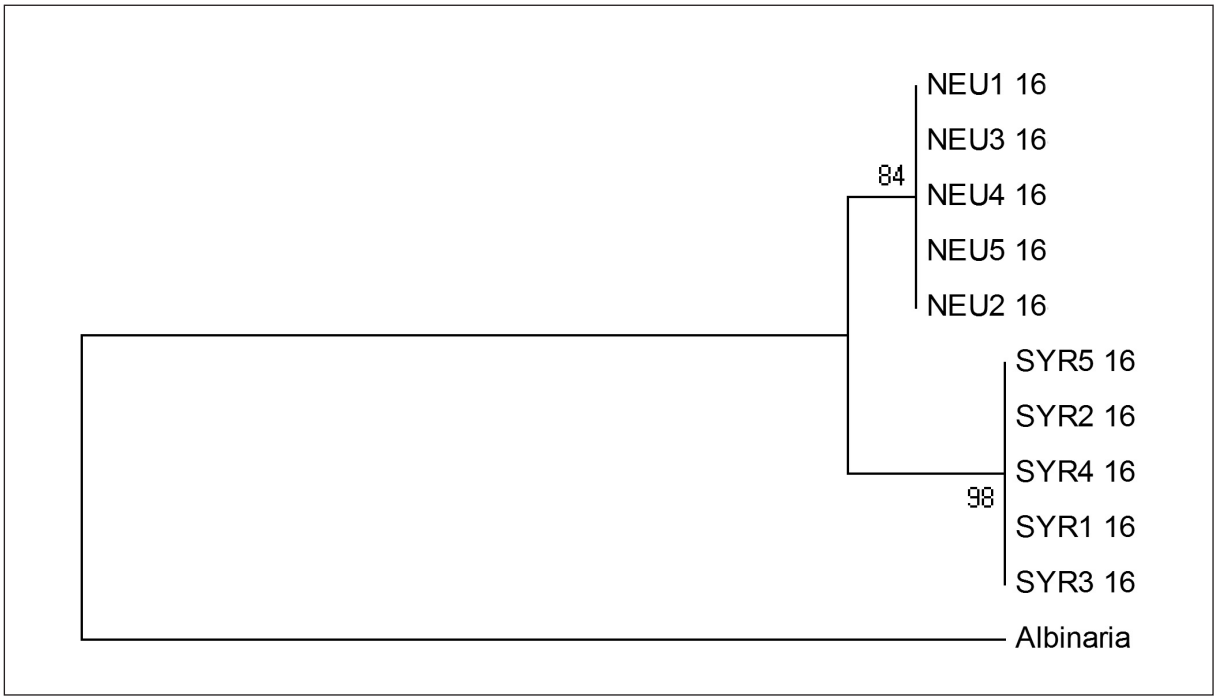


Figure 1. Neighbour Joining bootstrap consensus tree showing a possible reconstruction of evolutionary relationships of the analysed taxa based on mitochondrial 16S rDNA partial sequences. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed; bootstrap values are reported near the branches. The rate variation among sites was modeled with a gamma distribution (shape parameter = 0.57). All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). *Albinaria caerulea* has been employed as outgroup to root the trees (legend, NEU = *M. neuteboomi* SYR = *M. syracusana*).

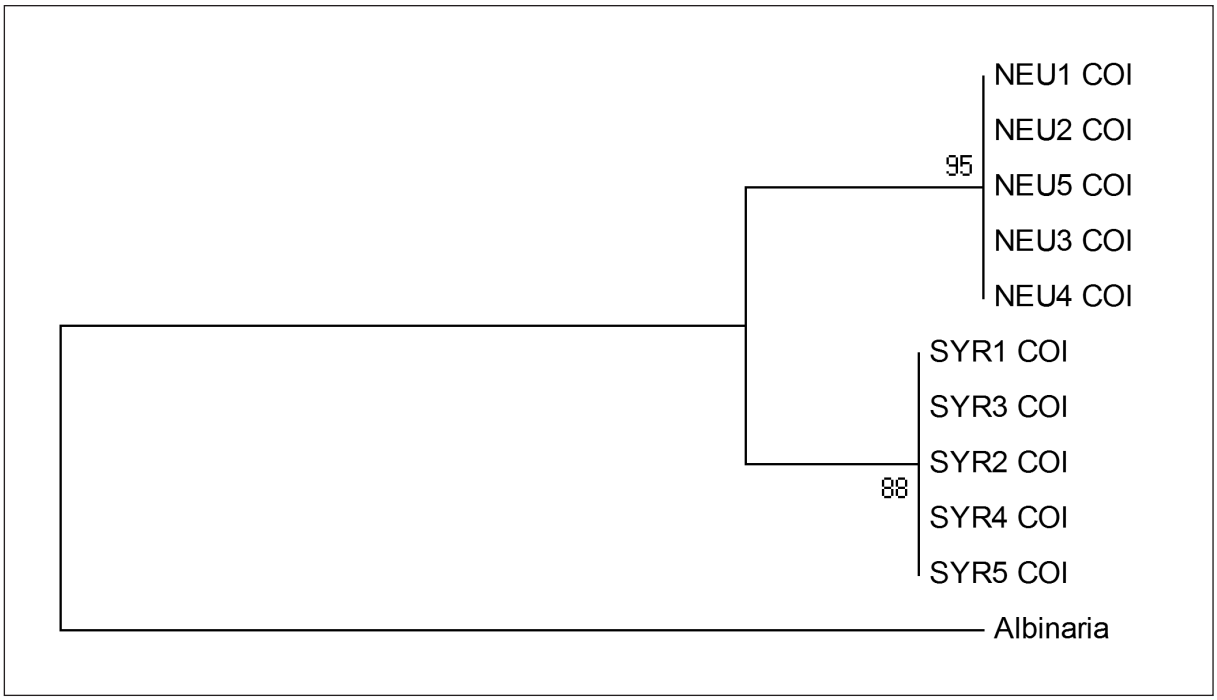


Figure 2. Neighbour Joining bootstrap consensus tree showing a possible reconstruction of evolutionary relationships of the analysed taxa based on mitochondrial COI partial sequences. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed; bootstrap values are reported near the branches. The rate variation among sites was modeled with a gamma distribution (shape parameter = 0.15). All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). *Albinaria caerulea* has been employed as outgroup to root the trees (legend, NEU = *M. neuteboomi* SYR = *M. syracusana*).

- for 16S rDNA data: K81uf (Unequal-frequency Kimura 3-parameter) plus gamma distribution, with an alpha parameter of 0.57;

- for COI data: TVM (Transversion Model) plus gamma distribution, with an alpha parameter of 0.15;

Neighbour Joining trees were constructed treating gaps as missing data. MP trees were obtained using the Close-Neighbour-Interchange (CNI) algorithm with search level 3 in which the initial trees were achieved with the random addition of sequences (10 replicates). All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). Characters were assigned equal weights. MP trees were collapsed to obtain a 50% majority rule consensus tree. Support for the internodes was assessed by bootstrap percentages (BP) (1,000 resampling steps for NJ and MP; 100 replicates for ML). For Bayesian analyses, the number of substitution types was fixed to 6, with a standard (4by4) model of nucleotide substitution and a rate variation across sites fixed to gamma. Four Markov Chain Monte Carlo (MCMC) chains were run for 100,000 generations, sampling every 10 generations; from the 10,000 trees found the first 1,000 were discarded as "burn-in". Finally, a 50% majority rule consensus tree was constructed. All phylogenetic trees were rooted using homologous nucleotide sequences of *Albinaria caerulea* (Deshayes, 1835) (Pulmonata, Mollusca, Clausiliidae) (GenBank IDs: DQ665343, NC_001761).

RESULTS AND DISCUSSION

In spite of the algorithm (NJ, MP, ML and BI) employed, all phylogenetic reconstructions revealed that the two taxa were clearly separated. Moreover, evolutionary trees showed highly congruent topologies that agreed on clustering and branching patterns supported by high bootstrap values. Since analyses produced almost identical results not changing any of the interpretation, only the phylogenetic NJ consensus trees are displayed as an example. As shown in figures 1 and 2, one cluster comprises only *M. syracusana*, whereas the second one includes just *M. neuteboomi* specimens. These findings not only confirmed data previously reported for 16S rDNA (Gregorini et al., 2008), but also corroborated the validity of the two species. In fact, nucleotide distances (5% for 16S rDNA and 12% for COI sequences) suggest strong isolation and strong divergence which may justify considering the two taxa under investigation as different (phylogenetic) species.

From a morphological point of view, *M. neuteboomi* differs from *M. syracusana* for shell morphology showing more rounded whorls, more closely spaced ribs (Figs. 3-4) and the clausilium shorter, narrower and sharper (Figs. 5-6), and for relationship between principalis and upper palatalis plica (Beckmann, 1990; Giusti et al., 1995; Reitano et al., 2009). In particular, *M. syracusana* has principal plica very short and fused to upper palatal plica, while *M. neuteboomi* has principal plica independent of upper palatal plica (Figs. 7-10). According to Beckmann (1990), the relationship between principalis and upper plica reported for *M. neuteboomi* might be considered plesiomorphic with respect to that observed in *M. syracusana*.

Gregorini et al. (2008) also provided a contribute to better define the distribution area of both species revealing that *M. syracusana* occurs only along the entire coast embracing Siracusa province, whereas *M. neuteboomi* inhabits a broader area including Siracusa, Ragusa, Caltanissetta and Catania provinces, and generally occurs at higher altitudes than those reported for *M. syracusana*. By a comparison of morphological data, *M. neuteboomi* populations, a part from the exception of the topotypicous one (employed in this study) show a marked variability of shell morphology which, at least in some cases, is very similar to that observed in *M. syracusana* (Figs. 11-14).

Hence, combining previously results with all data described herein, we suggest, that *M. syracusana* (with limited distribution area and a quite homogeneous shell morphology) might have originated more recently than *M. neuteboomi* (with a much wider distribution area and heterogenous shell morphology).

Anyway, further and more detailed analyses on other *Muticaria* populations are still in progress in order to better define molecular, morphological and ecological features of this highly interesting group.

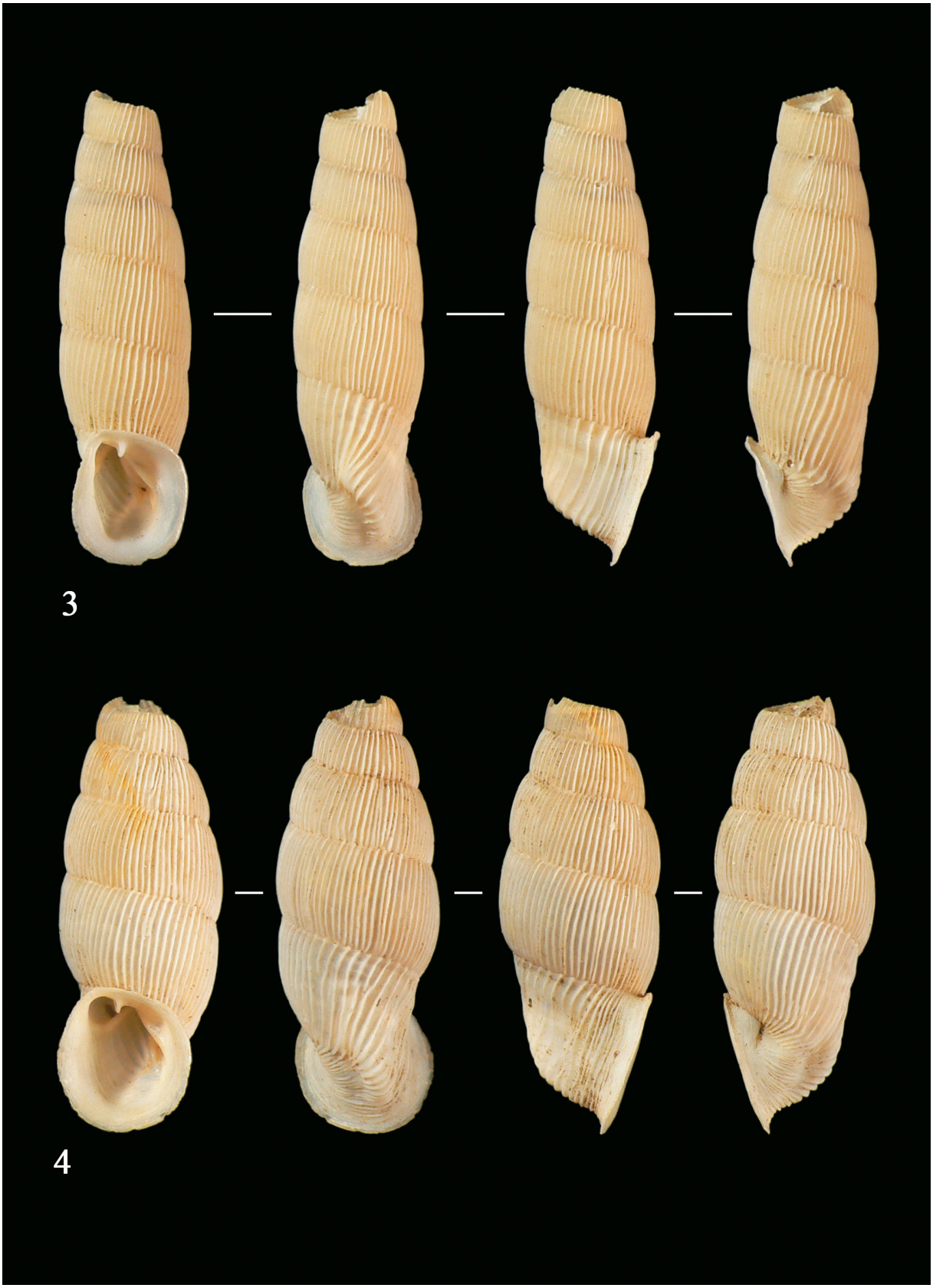


Figure 3. *Muticaria syracusana.* Siracusa, Teatro Romano, 19 m, 37°04'28" N, 15°16'45" E, legit A. Reitano (coll. Liberto); h: 14.5 m; D: 4 mm. – Figure 4. *Muticaria neuteboomi.* Ragusa, Cava d'Ispica, 340 m, 36°51'11" N, 14°50'14" E, legit A. Reitano (coll. Liberto); h: 11.9 mm; D: 4.3 mm.

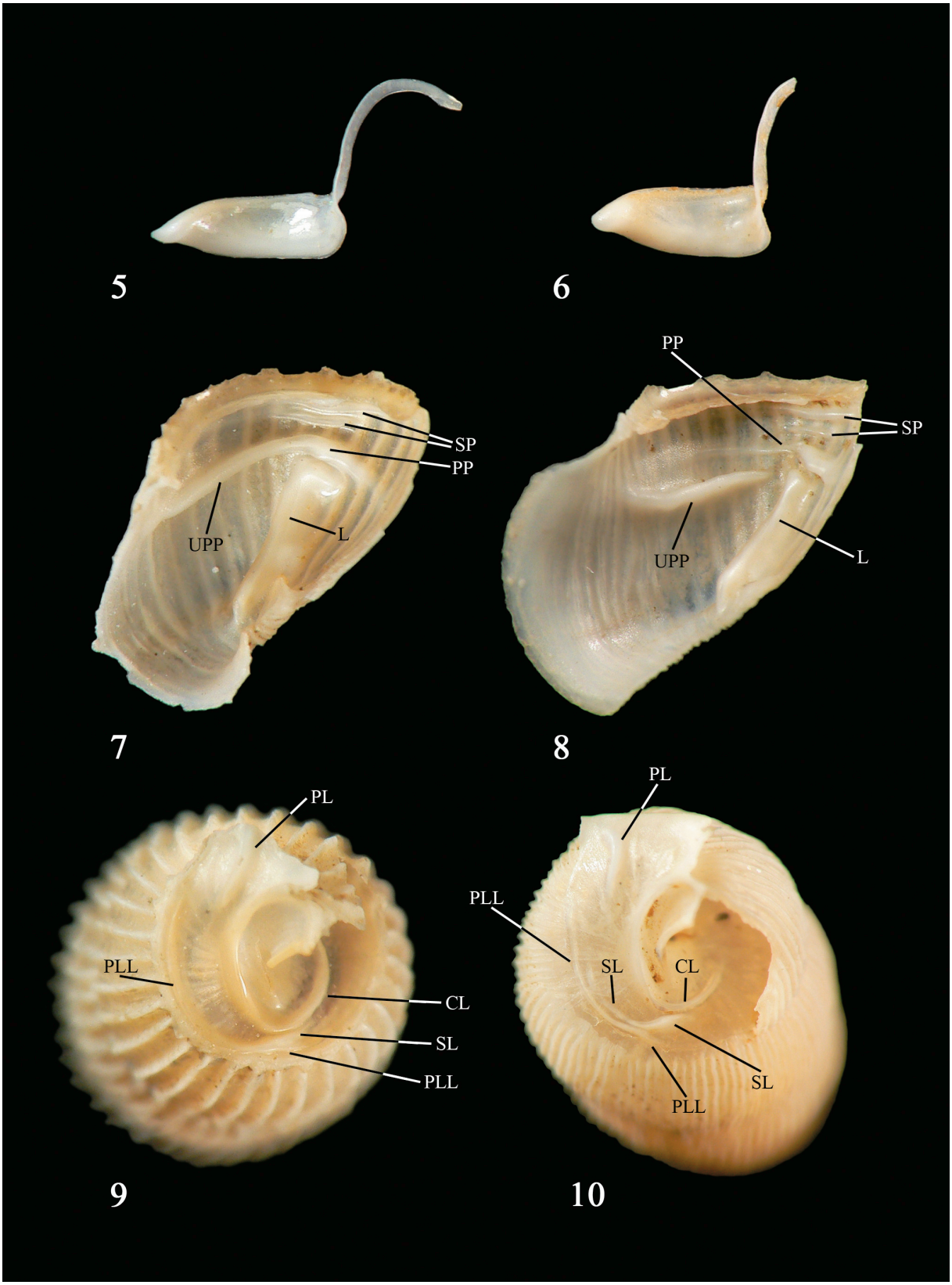
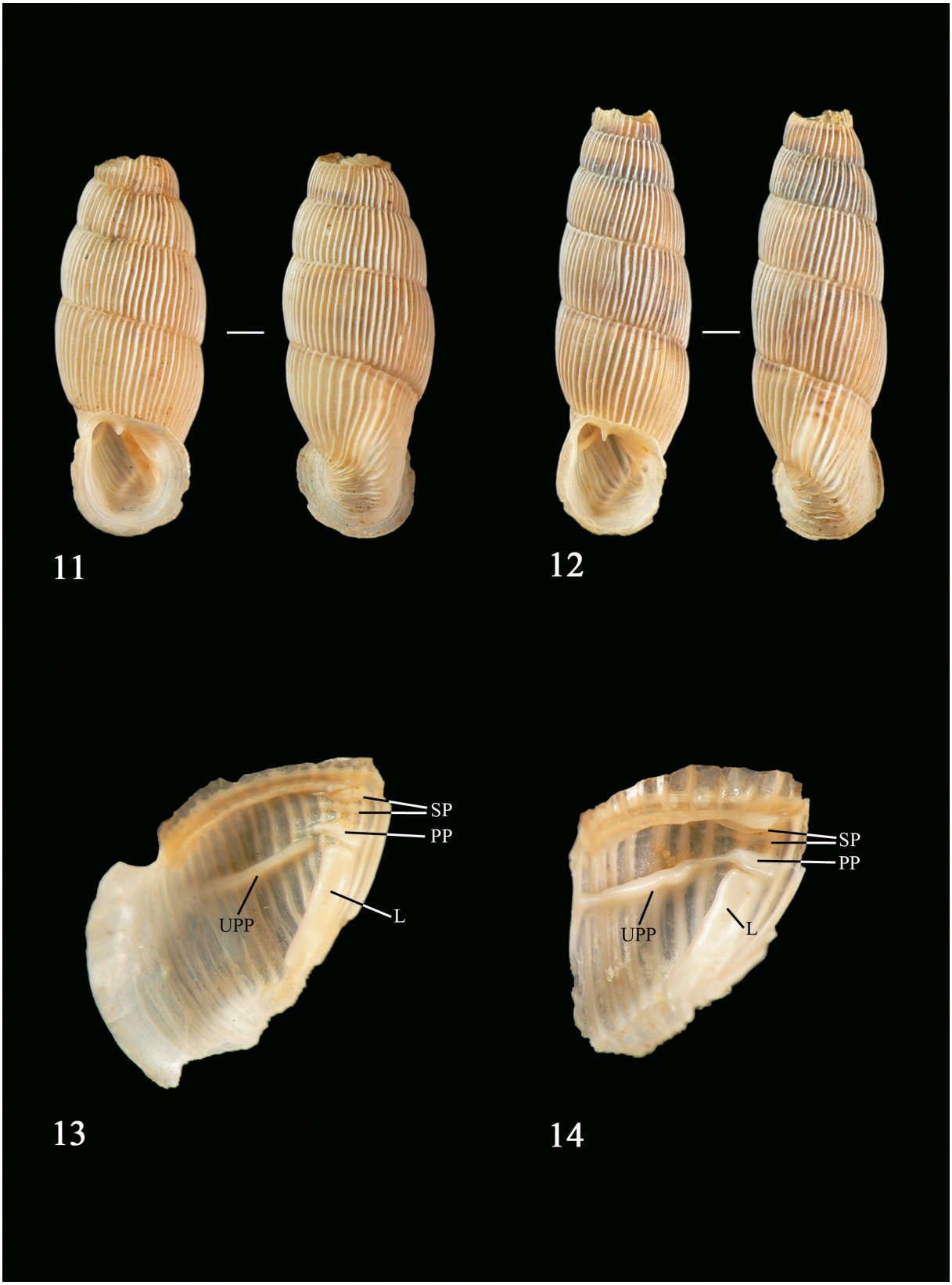


Figure 5. Clausilium of *Muticaria syracusana*, length 2.46 mm. – Figure 6. Clausilium of *Muticaria neuteboomi*, length 2.48 mm. – Figure 7. Parietum of *Muticaria syracusana.* Legend: CL = columellar lamella; L = lunella; PL = parietal lamella; PLL = parallel lamella; PP = principal plica; SL = spiral lamella; SP = sutural plica; UPP = upper palatal plica; – Figure 8. Parietum of *Muticaria neuteboomi.* – Figure 9. Palatum of *Muticaria neuteboomi.* – Figure 10. Palatum of *Muticaria syracusana.*



Figures 11-14. Variability of the shells and palatum of *M. neuteboomi* from Siracusa, Sortino, Pantalica, 265 m, 37°08'28" N, 15°01'54"E, legit A. Reitano (coll. Liberto). Figure 11. h:11.5; D: 4.3; Figure 12. h: 13.7; D: 4.3 mm.

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