

The genus *Ercella* Monterosato, 1894: new molecular evidence (Pulmonata Stylommatophora Helicidae)

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ABSTRACT

In this paper we report on new molecular data (COI sequences) of different and representative populations of *Ercella mazzullii* (De Cristofori et Jan, 1832), *E. cephaloeditana* Giannuzzi-Savelli, Oliva et Sparacio, 2012 and *E. insolida* (Monterosato, 1892) (Pulmonata, Stylommatophora, Helicidae). Present results are compared with those from recent literature and the current knowledge on phylogenetic relationships among Helicidae pulmonate gastropods is reviewed. Obtained results suggest that: i) *Cornu* Born, 1778 and *Cantareus* Risso, 1826 are separate and well distinct from *Helix* Linnaeus, 1758; ii) *Ercella* Monterosato, 1894 is a valid and independent genus rather than a subgenus of *Cornu*; iii) *Cornu aspersum* (O.F. Müller, 1774) is a group of species (i.e. "*aspersum*" group) whose taxonomic status needs to be defined further studies; iv) *Cornu*, *Cantareus* and *Ercella* might belong to the same tribe that, still, remains to be defined.

KEY WORDS

Ercella; Helicidae; mitochondrial markers; phylogenetic reconstruction.

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INTRODUCTION

Colomba et al. (2011) reported on a multidisciplinary study based on genital morphology, DNA analysis, distribution, ecology and fossil records of *Cornu mazzullii* (De Cristofori et Jan, 1832), a species endemic to North-Western Sicily. Obtained results supported the hypothesis that *C. mazzullii* should be attributed to the genus *Ercella* Monterosato, 1894 and that this genus was probably structured in three discrete clades (i.e., the *mazzullii* group) recognized as species including: (i) the populations living in Monte Pellegrino (Palermo)

and nearby mountains, *E. mazzullii* s. str., (ii) the endemic population of Cefalù, La Rocca, *E. cephaloeditana* Giannuzzi-Savelli, Oliva et Sparacio, 2012, and (iii) the populations living in the mountains of Trapani surroundings, *E. insolida* (Monterosato, 1892).

Based on the phylogenetic reconstruction obtained by the multigenic analysis of nuclear (ITS2) and mitochondrial (16S rDNA, 12S rDNA) molecular markers, Colomba et al. (2011) strongly suggested that the genus *Ercella* should be kept distinct from the closely related genera *Cornu* Born, 1778 and *Cantareus* Risso, 1826. In the

same paper, this hypothesis was also corroborated by the analysis of several 16S rDNA partial sequences downloaded from GenBank for other genera representatives of Western Palaearctic Helicidae taxa; noteworthy, the phylogenetic tree topology clearly showed *Cornu* and *Cantareus* distinct from *Helix* Linnaeus, 1758 (see Colomba et al., 2011, fig. 42).

Cornu Born, 1778 (type species: *Cornu copiae* Born, 1778) was reintroduced as distinct genus by Waldén (1976) with *Cryptomphalus* De Charpentier, 1837 (type species: *Cryptomphalus aspersum* O.F. Müller, 1774) as junior synonym; it was sometimes considered as subgenus of *Helix* Linnaeus, 1758 (type species: *Helix pomatia* Linnaeus, 1758) and sometimes as a distinct genus. The description of *Cornu copiae* was based on a teratological specimen of "*Helix*" *aspersa*; due to different interpretations of the Article 1.3.2 of the Code, a request for conservation of the name *Cornu* is still pending a ruling of the International Commission on Zoological Nomenclature.

Cantareus Risso, 1826 (type species: *Cantareus apertus* Born, 1778) was sometimes considered as subgenus of *Helix* and sometimes as a distinct genus.

Schileyko (1978) was the first one who described the internal structure of male sexual organs of "*Helix*" *aspersa* characterized by a penial papilla and a prominent semicircular fold in the distal part of the penis (see also Nordsieck, 2013). Because of these anatomical differences, the Author attributed this species to the genus *Cryptomphalus*.

Giusti et al. (1995) showed a close similarity between genitalia of "*Helix*" *aperta* and "*Helix*" *aspersa* and, therefore, attributed these two species to the same genus, *Cantareus*, morphologically well distinct from *Helix*. Moreover, they reported that *Helix* has a real penial papilla inside the penis and, distally, an accessory penial papilla, whereas *Cantareus* shows a system of a real penial papilla, a false penial papilla and, distally, an "annular pad".

Neubert & Bank (2006) mainly confirmed these morphological differences and concluded in considering *Cornu* and *Cantareus* as related but distinct genera. One year later, similar observations were reported by Alonso & Ibáñez (2007).

At the same time, findings of scientific studies based on molecular data were in line with the taxo-

nomic frame showing *Helix* distinct from *Cantareus* and *Cornu*, the latter two considered the same genus (Manganelli et al., 2005; Koene & Schulenburg, 2005; Wade et al., 2006, 2007).

Nevertheless, despite all these anatomical and molecular evidence, recently Welter-Schultes et al. (2011) and Welter-Schultes & Audibert (2012) considered *Cornu* and *Cantareus* to belong to the genus *Helix*. Bank (2012) argued that such a systematic position is wrong, and, above all, it does not take into account a number of studies (cited above) suggesting a taxonomic choice closer to the real affinities among these taxa. Welter-Schultes et al. (2012), however, reaffirmed their beliefs and, besides, Welter-Schultes (2012) reported *Ercella* as synonym of *Helix*.

Nordsieck (2013), reviewing the papers, published in the last decades, dealing with anatomical and molecular data, concluded, in summary, that: "*According to genital morphology and DNA analysis, "Helix" aspersa and relatives are not more related to Helix than Eobania and other genera of the Helicinae [...] These species must therefore be generically separated from Helix. The shell and the genital differences, especially those of the penis (Giusti et al. 1995, Neubert & Bank 2006, Colomba et al. 2011), are sufficient for the generic separation of Cantareus and Cornu (or Cryptomphalus, if the name Cornu is not valid because of Art. 1.3.2 ICZN, cf. Giusti et al. 1995: 491). Ercella is regarded as a subgenus of Cornu instead of a genus, because it is more closely related to Cornu than to Cantareus*".

More recently, detailed molecular genetics studies (Korábek et al., 2014; 2015; Razkin et al., 2015) confirmed *Cornu* and *Cantareus* as two distinct genera forming a group with no sign of a close relationship with *Helix*. In addition, *Ercella* DNA sequences, when included in such analysis (see Korábek et al., 2015), confirmed this item, in line with Colomba et al. (2011).

At present there seems to be broad agreement in considering *Cornu* and *Cantareus* distinct genera, while on the position of *Ercella* opinions are still diverging. In order to be able to further test the "genus hypothesis" (*Ercella* as a distinct genus, Colomba et al., 2011) versus the "subgenus hypothesis" (*Ercella* as a *Cornu* subgenus, Nordsieck, 2013), we performed an additional molecular analysis to characterize and define even better, from a

molecular standpoint, the identity and reliability of *Ercetella*.

In particular, phylogenetic relationships among taxa under study were analysed by comparing partial sequences of the gene encoding for the cytochrome oxidase subunit I (COI) - which is one of the most commonly used mitochondrial markers in molecular evolution and molecular phylogeny. Besides, to provide a little contribute in shedding some more light on Helicidae systematics, the analysis was extended to hundreds of specimens of the family Helicidae whose COI sequences were downloaded from GenBank database. A similar analysis was carried out including 16S rDNA partial sequences of the same taxa. Molecular analyses have been performed either with single (16S or COI) or combined (16S+COI) molecular datasets.

MATERIAL AND METHODS

Specimens and Collection sites

For each population, 2-5 sicilian *Ercetella* specimens were analysed. Please note that each locality and/or collection site is named in the original language (Italian). Collected samples were identified and [labelled] as follows: *Ercetella insolida* (from Trapani province: Custonaci, Trapani [CU], M.te Cofano, Trapani [COF]; San Vito lo Capo: cala Mancina, Trapani [SV]); *Ercetella mazzullii* (from W-Palermo surroundings: M.te Pellegrino [MP]; Sferracavallo, Palermo [CMS]; Carini: M.te Columbrina, Palermo [COL]; Cinisi: M.te Pecoraro, Palermo [PEC]); *Ercetella cephaloeditana* from Cefalù: la Rocca, Palermo [CM]; *Cornu aspersum* (= *H. aspersa*) [CA] from Cefalù, Palermo, Sicily; and *Cantareus apertus* [CAP] from Cefalù, Palermo, Sicily and Assoro, Enna, Sicily.

DNA extraction, amplification and sequencing

Samples were stored separately at -20 °C in test tubes. Of each individual, a piece of foot tissue was used for total DNA extraction (by Wizard Genomic DNA Purification Kit, Promega). COI fragments (581-663 bp) were amplified using LCO_1490 (5'-GGTCAACAATCATAAAGATATTGG-3') and HCO_2198 (5'-TAAACTTCAGGGTGACCAAATCA-3')

(Folmer et al., 1994). PCR cycles were as follows: 95°C for 5 min; 95°C for 1 min, 42°C for 1 min, 72°C for 1 min (35 cycles); 72°C for 5 min. To remove primers and unincorporated nucleotides, amplified products were purified with the Wizard SV gel and PCR Clean-up kit (Promega). Sequencing of purified PCR products was carried out using automated DNA sequencers at Eurofins MWG Operon (Germany). All COI sequences generated in this study were uploaded in GenBank (accession numbers: KR921883-KR921914).

Phylogenetic analyses

The analysis was conducted on two partial gene sequences: COI and 16S rDNA, integrating our data with those obtained from GenBank database. In particular, in addition to the sequences obtained from specimens tested directly in this study (KR921883-KR921914), to further expand the analysis and refine its resolving power, we included 16S rDNA sequences of *Ercetella mazzullii*, *E. insolida*, *E. cephaloeditana*, *Cornu aspersum* and *Cantareus apertus* previously generated by our research group (GQ402393-GQ402396, GQ402398-GQ402402, GQ402403-GQ402405, GQ402407-GQ402409, GQ402410-GQ402411, GQ402412-GQ402414, GQ402417-GQ402419, GQ402420-GQ402422, GQ402387-GQ402389, GQ402390-GQ402392, see Colomba et al., 2011), joined to both COI and 16S rDNA sequences downloaded from GenBank of the following taxa: *Eobania vermiculata* (O.F. Müller, 1774) (KJ458509, KJ458510, KJ458511, JF277395, JF277393, JF277391), *Theba geminata* Mousson, 1857 (KJ458559, HM034468), *T. subdentata* (Férussac, 1821) (KJ458562, HM034496), *T. pisana* (O.F. Müller, 1774) (KJ458561, JX911311), *T. andalusica* Gittenberger et Ripken, 1987 (KJ458558, KF582631), *Murella muralis* (O.F. Müller, 1774) (GU391399, JX827154), *Helix lucorum* Linnaeus, 1758 (AF126144, GU784803), *Helix pomatia* Linnaeus, 1758 (AF208297, JX911304), *Helix secernenda* Rossmässler 1847 (KP072386, KP072387, KP072388, KP072086, KP072087, KP072088), *Helix vladika* Kobelt, 1898 (KP072303, KF823104), *Helix melanostoma* Draparnaud 1801 (KJ458524, KP072107), *Iberus gualtierianus* (Linnaeus, 1758) (AY928605, AY928606, DQ822123, DQ822165, DQ822166, AY546285), *Hemicycla*

bidentalis (Lamarck, 1822) (KJ458528, HM147180), *Pseudotachea splendida* (Draparnaud, 1801) (KJ458552, AY546292), *Levantina caesareana* (Mousson, 1854) (KP072332, KP072181) *Otala lactea* (O.F. Müller, 1774) (AY937264, AY937263), *O. punctata* (O.F. Müller, 1774) (JF717823, JF717824, KJ458545, JF717805, JF717806, JF717807), *Helix aspersa* (AF126139, AF126135, AF126134, AF126140, AF126136, JN701926, JN701927, GU598217, AY546283, HQ203051, HQ203052, JX911287), *Cantareus apertus* (KJ458491, JX911286). Finally, *Limax maximus* Linnaeus, 1758 (Family Limacidae) (KF894386), *L. cinereoniger* Wolf, 1803 (KF894380), *Limacus flavus* (Linnaeus, 1758) (FJ896815), *Muticaria syracusana* (Philippi, 1836) (Family Clausiliidae) (HQ696868, AY425597) and *M. neuteboomi* Beckmann, 1990 (HQ696866, HQ696867) were employed as outgroups.

All sequences were visualized with BioEdit Sequence Alignment Editor 7 (Hall, 1999), aligned with the ClustalW option included in this software and refined by eye. As far as concerns single (COI or 16S rDNA) molecular data sets, phylogenetic analyses were conducted in MEGA 5 (Tamura et al., 2011) by Maximum Likelihood algorithm. Substitution models, selected according to the “Find Best DNA model” option included in the software, were: HKY+G (COI) and GTR+G (16S rDNA); support for the internodes was assessed by bootstrap percentages (BP) (1000 replicates). For the combined (COI+16S rDNA) datasets, phylogenetic analyses were conducted in BEAST 1.6.1 (Drummond & Rambaut, 2007) using the *BEAST implementation (Heled & Drummond, 2010). A series of initial runs were performed to optimize priors and runtime parameter choice to obtain effective sampling sizes (ESS) above 500 for all estimated parameters. The best-fit evolution models of nucleotide substitution were: HKY+G (COI) and GTR+G (16S rDNA) with empirical base composition; the Yule Process tree prior for mitochondrial data with piecewise linear population size model was applied with a UPGMA-generated tree as starting point. Trees from all runs were combined to produce an ultrametric consensus tree using TreeAnnotator 1.6.1. The first 10^3 trees were discarded as burnin. Support for nodes was expressed as posterior probabilities.

RESULTS AND DISCUSSION

COI and 16S rDNA consensus trees and the multi-genic (COI+16S rDNA) tree included 69 molecular sequences, each. Obtained results allowed to make a few observations of some interest. In particular, COI consensus tree (Fig. 1), showed three separate clusters for (A) *Ercetella* (discussed in detail below), (B) *Cantareus apertus* and (C) *Cornu aspersum* clearly distinct. Similarly, (D) *Eobania vermiculata*, (E) *Levantina caesareana*, (F) *Helix* spp. (including several species), (G) *Otala* spp. (*O. punctata* and *O. lactea*), (H) *Murella muralis*, (I) *Hemicycla bidentalis*, *Pseudotachea splendida*, *Iberus gualtierianus* and (L) *Theba* spp. (*T. geminata*, *T. subdentata*, *T. pisana*, *T. andalusica*) are separated. With regard to *Ercetella*, the three taxa are clearly distinct and separated as *E. insolida* (SV1-SV3, CU4-CU5, COF2-COF4, from Trapani province), *E. mazzullii* (CMS1-CMS5, COL1-COL3, PEC1-PEC3, MP1-MP3, comprising specimens sampled on M.te Pellegrino and the nearby mountains of surroundings of Palermo), and *E. cephaloeditana* (CM1-CM4, from Cefalù, La Rocca).

The 16S rDNA consensus tree topology (Fig. 2) is similar to that shown in figure 1. In fact, also in this case, *Ercetella* is clearly distinct and well structured in three taxa, *Ercetella insolida*, *E. cephaloeditana* and *E. mazzullii*. Once again, it is confirmed a distinction between the (closely related) genera *Ercetella*, *Cornu* and *Cantareus*; based on 16S rDNA sequences analysis, *Ercetella* appears closer to *Cornu*, while in the COI tree *Cornu* is closer to *Cantareus*.

Mean molecular distances among the three taxa of *Ercetella* (assessed by the maximum composite likelihood method), range from nearly 6 to 10% (16S rDNA) and about 4 to 7.5% (COI). These values, despite the issues of using mean molecular distances (see Meier et al., 2008), nevertheless, compared with those reported for other species, including Pulmonata (e.g. Hebert et al., 2003a, 2003b; Steinke et al., 2005; Nekola et al., 2009) can, in our opinion, justify the separation of *Ercetella* populations into three species.

Genetic distances between different species within various animal groups, especially invertebrates, are variable (see for example Meier et al., 2008 and references therein). This is because they

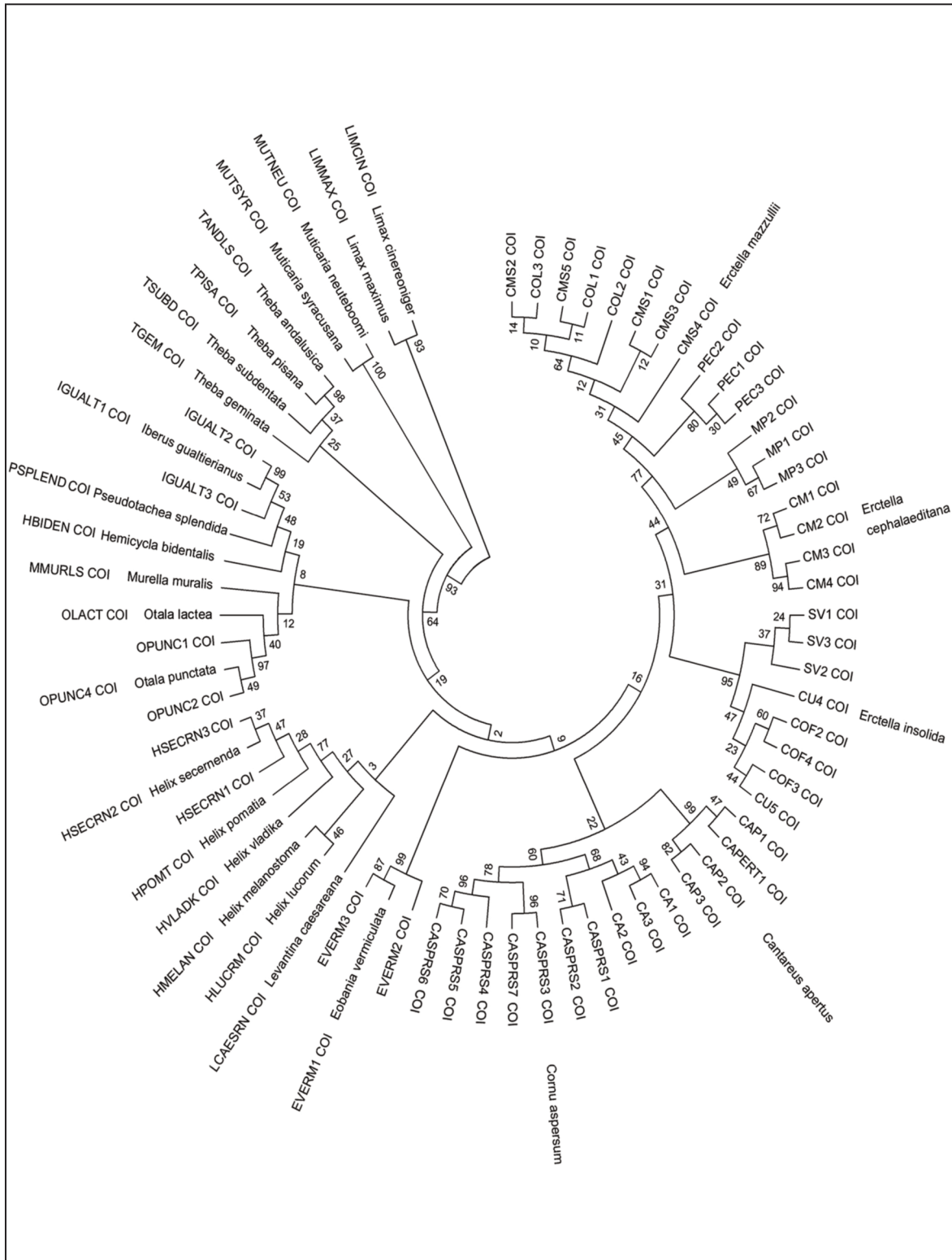


Figure 1. COI consensus tree. The evolutionary history was inferred by using the Maximum Likelihood method based on HKY model. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. A discrete Gamma distribution was used to model evolutionary rate differences among sites [5 categories (+G, parameter = 0.6175)].

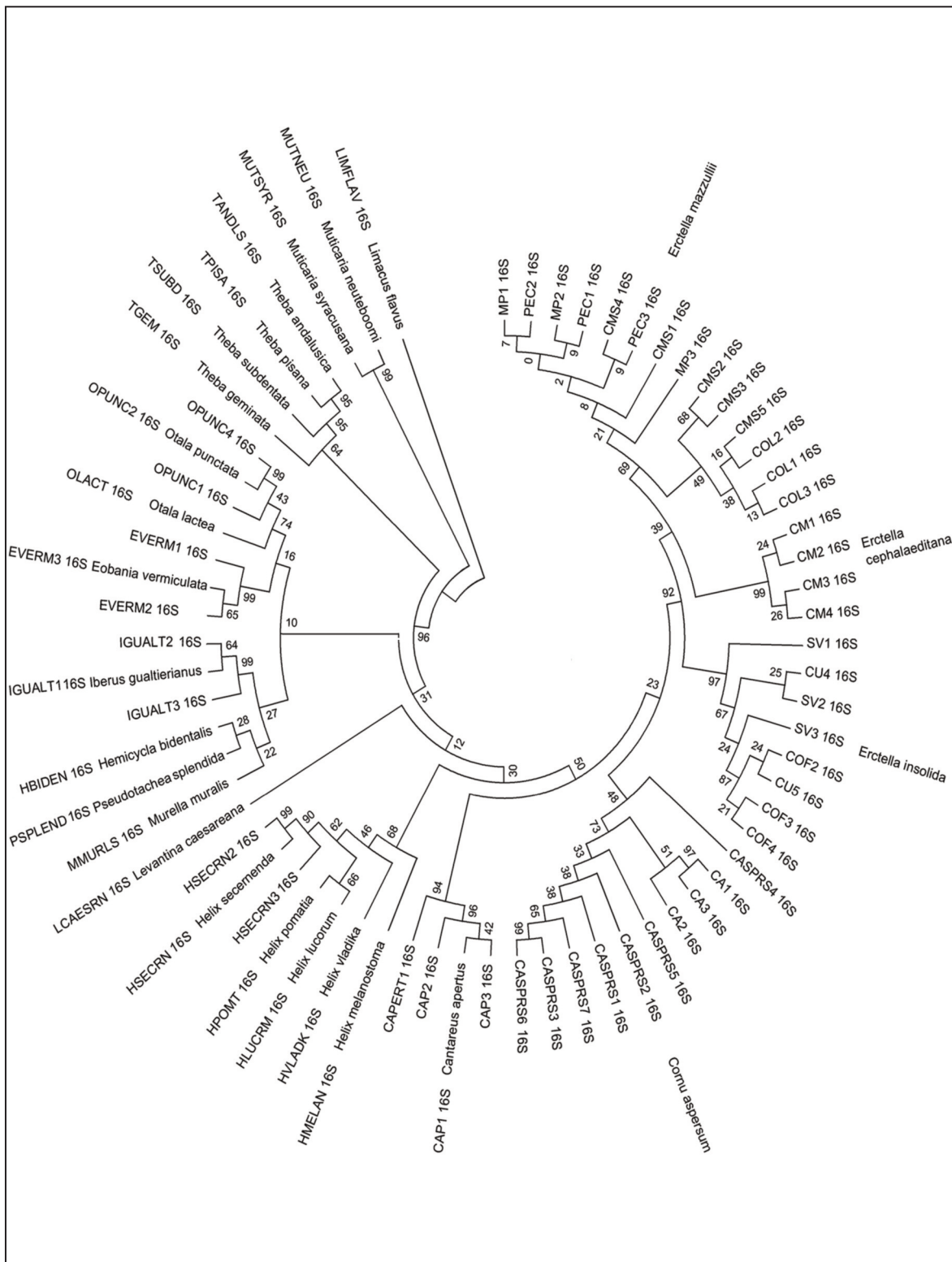


Figure 2. 16S rDNA consensus tree. The evolutionary history was inferred by using the Maximum Likelihood method based on GTR model. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. A discrete Gamma distribution was used to model evolutionary rate differences among sites [5 categories (+G, parameter = 0.7920)].

are function of different parameters among which: different rates of nucleotide substitution, different types of environmental pressure, or different types of mutation which the nucleotide sequences are subject to, which at times (e.g. for retro-mutations) cannot be detected a-posteriori. Moreover, generally speaking, genetic distances per se are not sufficient to discriminate between different species and, for pulmonates, a few cases have been documented where distances turned out to be misleading, necessitating to be integrated with additional data (see Davison et al., 2009; Sauer & Hausdorf, 2012).

In *Ercella*, molecular data combined with other significant data such as morphological, biological, ecological and paleontological features allow us to consider it a genus with three different species, endemic to northwestern Sicily (Liberto et al., 2010; Colomba et al., 2011).

Concatenated-gene analysis was better resolved than single-gene analysis and thus represents, probably, more accurately present relationships among taxa. It resulted in a tree topology (Fig. 3) which is quite superimposable to that of the ML trees (Figs. 1, 2) and, for the most part, in line with a recent review of the molecular phylogeny of the Western Palearctic Helicoidea by Razkin et al. (2015). In particular, it is visible the group including *Eobania vermiculata*, *Otala lactea* and *O. punctata* (tribe Otalini, in pink), the group including *Iberus gualtierianus*, *Pseudotachea splendida* and *Hemicycla bidentalis* (Allognathini, in red), *Theba* species (Thebini, in yellow), and several species of *Helix* and *Levantina caesareana* (Helicini, in lilac).

In the concatenated-gene analysis, *Ercella* and *Cornu*, considered two distinct genera, are sister groups.

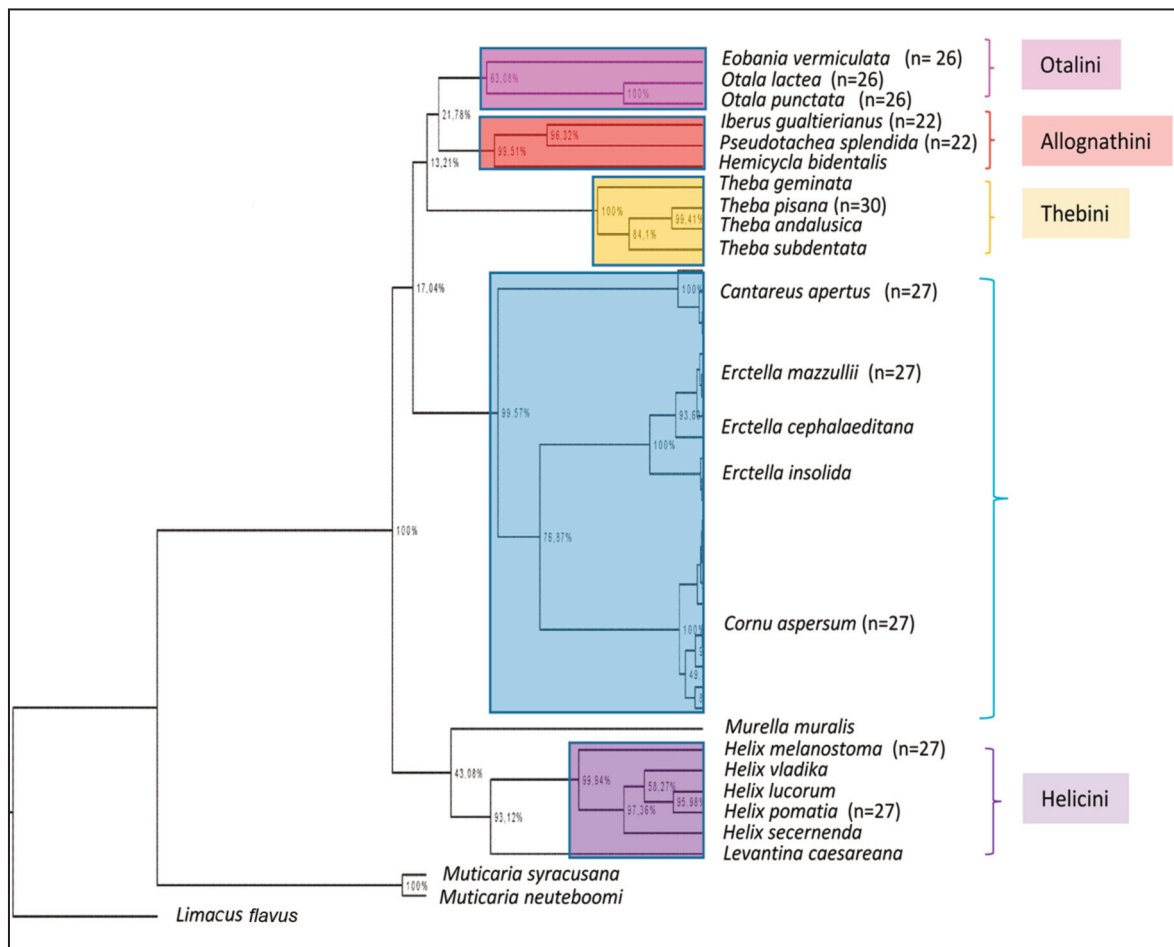


Figure 3. Phylogenetic annotated tree based on Bayesian inference analysis of the concatenated data set including 16S rRNA and COI sequences. Numbers correspond to BI posterior probabilities (in %).

Regarding relationships within the group *Cantareus-Ercella-Cornu* our data differ from Razkin et al. (2015). In fact, while for *Ercella* it is not possible to make a comparison because the Authors did not include this taxon in their analysis, on the other hand, in our tree, neither *Cornu* nor *Cantareus* can be considered Otalini, rather belonging to a distinct cluster (attributable to the "tribe" level) including *Ercella*.

Therefore, although *Cornu* and *Cantareus* show a certain degree of affinity particularly with *Eobania* for genitalia architecture (see Giusti et al., 1995) and share with Otalini similar biogeographic, ecological and evolutionary items typical of Western Mediterranean areas where these terrestrial molluscs differentiated (see Colomba et al., 2011), nevertheless, the consideration of *Cornu*, *Cantareus* and *Ercella* as a separate tribe, which still remains to be defined, is suggested. Furthermore, *Cornu*, *Cantareus* and *Ercella* share the same chromosome number ($n = 27$) (Vitturi et al., 1982; Vitturi et al., 2005) (see Fig. 3), while *Eobania* and other Otalini examined up to now have $n = 26$ (Burch, 1965; Thiriou-Quévieux, 2003). Finally, Otalini show in genital organs a relatively little dart sac and well-developed digit-like appendages, *Cornu-Cantareus-Ercella*, instead, show a massive dart sac and two groups of digitiform glands with short base and numerous and short digit-like appendages.

On the other hand, the separation between *Cornu-Cantareus-Ercella* and *Helix* is supported by: (i) the different geographical distribution of the genera: *Cornu* and *Cantareus* are widespread in North Africa and Southern Europe, with *Ercella* endemic to Northwestern Sicily, while *Helix* is mainly distributed in Central and Eastern Europe and, to a lesser extent, North Africa; (ii) the different morphology of genital organs (Schileyko, 1978; Giusti et al., 1995; Neubert & Bank, 2006; Alonso & Ibáñez, 2007) showing in *Cornu-Cantareus-Ercella* a different form of dart sac and of digitiform glands (see above); and (iii) molecular data (see Korábek et al., 2015 and quotes therein).

Comparing the three phylogenetic trees an interesting consideration about *Cornu aspersum* can be made. In fact, in line with other studies (Guiller et al., 2001; Guiller & Madec, 2010), in our study as well, this taxon seems to be not a single species but rather a species group (ie "*aspersum*" group)

showing a taxonomic situation more complex and heterogeneous than previously hypothesized within its area of origin and diversification (Southern Italy, Sicily and NW Africa). This result is further confirmed by personal unpublished morphological and molecular data of numerous Italian, Maltese and North African *C. aspersum* populations.

Finally, the position of *Murella muralis* remains to be clarified. In fact, it is not only different in all phylogenetic trees but, above all, discordant with what reported in other papers. This issue, which is beyond the aim of the present paper, requires further study and investigation, possibly increasing the number of specimens (joining to sequences downloaded from the database also sequences obtained from new samples collected directly in the field), increasing the number of genes analyzed and, above all, including in the analysis other taxa representatives of subfamilies more closely related to Murellinae, such as Ariantinae.

Overall, present results correspond well to several previous molecular studies carried out by nuclear and mitochondrial markers (Koene & Schulenburg, 2005; Colomba et al., 2011; Korábek et al., 2014; Razkin et al., 2015) and confirm that *Ercella* species lie always outside the clusters of *Cornu* and *Cantareus*.

CONCLUSIONS

New molecular evidence provided in this study suggested also several comments on *Ercella* closely related genera. Hence, on this basis, despite the difficulties that the argument implies, some conclusions can be drawn.

The groups comprising *Cornu-Cantareus-Ercella* on one hand, and *Helix* on the other hand, appear separate and distinct from each other. In line with most of the papers reporting on anatomical and molecular characteristics observed in these animals, there seems to be no evidence that "*aperta*", "*aspersa*" and / or "*mazzullii*" may belong to the genus *Helix*.

Considering *Cornu* and *Cantareus* as Otalini, as assumed by Razkin et al. (2015) is not confirmed in our analysis. However, as mentioned above, the issue certainly needs further study in view of their aforementioned anatomical and biogeographical affinities.

We suggest considering *Cornu*, *Cantareus* and *Erctella* as related but distinct genera belonging to independent lineages; as hypothesized, they might be included into a new tribe (between Otalini and Helicini).

Cornu aspersum complex is in need of a thorough taxonomic revision in its area of origin.

Finally, it is appropriate to reiterate that our decision to consider *Erctella* a distinct genus including three different species (Colomba et al., 2011) was not only made on the basis of some, although important, molecular evidence, but also by the analysis of many other data that allowed us to assign to the various *Erctella* populations morphological, biological, paleontological and biogeographical peculiar characters, amplified by the particular distribution of the taxon, endemic to Northwestern Sicily. In this regard it is worth remembering that in the characterization of a taxon, at different levels, while gathering as many informations as possible (including morphological, ecological, molecular data, etc ...) is necessary, taxonomic reconstructions obtained with a methodology not always correspond to the ones obtained with another method (see Schileyko, 2013); for *Erctella*, instead, all (numerous) data are consistent with the hypothesis of differentiating it from other (similar, closely related) genera.

So that it seems appropriate to conclude with the words reported by A. Schmidt (1868) who claimed that, in taxonomy : “*Künstliche Systeme entstehen durch consequentes Geltendmachen eines einzelnen Principis*” [“the application of a single criterion produces artificial classifications”].

In more contemporary terms, we could say with Poins et al. (2014): “*Molecular phylogenetics is an irreplaceable tool for taxonomists, but interpretation of the results must be based on clear taxonomic concepts corroborated by all available resources - that is, the primary reference, the subsequent taxonomic literature and the type specimens of the organisms of interest. Otherwise, molecular phylogenetics can cause confusion with detrimental consequences to follow-up studies (e.g. ecological and evolutionary)*”.

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