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MONOGRAPH

Molecular studies on the genus *Muticaria* Lindholm, 1925 (Pulmonata Clausiliidae) from the Maltese Islands

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ABSTRACT The present study has been carried out with focus on *Muticaria macrostoma* group from the Maltese Islands to characterize and define, from a molecular standpoint, their identity and relationships with topotypical Sicilian *Muticaria* (i.e. *M. syracusana*, *M. neuteboomi*, *M. cyclopica* and *M. brancatoi*). Molecular study included amplification of 16S rDNA (ca. 300 bp) and COI (ca. 700 bp) gene partial sequences which were used for single and combined gene analysis by Bayesian Inference to achieve the phylogenetic reconstructions with the highest posterior probabilities. Obtained results showed that, within *M. macrostoma* group, the taxa *mamotica* and *oscitans* can be elevated to the specific rank, thus bringing to three the Maltese *Muticaria* species, i.e. *M. macrostoma*, *M. mamotica*, and *M. oscitans*; whereas *scalaris* may be considered a subspecies, or even a synonim. Present findings confirmed the validity of the Sicilian species *M. syracusana*, *M. neuteboomi*, *M. cyclopica* and *M. brancatoi*. Furthermore, the populations of the Sicilian and Maltese *Muticaria* seem to belong to two different levels of differentiation. Finally, we have also examined some *Lampedusa* populations but the position of this genus still remains to be clarified. In particular, it is confirmed that *Lampedusa* and *Muticaria* are different genera, but at present, the relations within the *Lampedusa* group need further studies to be analysed in details.

KEY WORDS *Muticaria macrostoma* group; Malta; Phylogenetic reconstructions; 16S rDNA; COI.

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INTRODUCTION

The genus *Muticaria* Lindholm, 1925 (Clausiliidae Alopiinae Medorini) comprises xeroresistant and calcicolous molluscs, distributed in Central-eastern and South-eastern Sicily and Maltese Islands. At present, four *Muticaria* species can be found on the Sicilian territory: *M. syracusana* (Philippi, 1836), *M. neuteboomi* Beckmann, 1990, *M. brancatoi* Colomba, Reitano, Liberto, Giglio, Gregorini et Sparacio, 2012 and *M. cyclopica* Liberto, Reitano, Giglio, Colomba et Sparacio, 2016; while

only *M. macrostoma* Cantraine, 1835 is reported for the Maltese Islands (Bank, 2017; Bank & Neubert, 2019).

As far as concerns the distribution of Sicilian *Muticaria* species, *M. syracusana,* locus typicus Siracusa (Philippi, 1836), occurs along the entire coast embracing Siracusa province; *M. neuteboomi*, locus typicus Cave d'Ispica, Ragusa province (Beckmann, 1990), inhabits a quite broad area including Siracusa, Ragusa, Caltanissetta and Catania provinces, and generally occurs at higher altitudes than those reported for *M. syracusana*; *M. brancatoi* is known only for the localities of description, i.e. Cugnolungo (type locality), Spinagallo, Vallone Moscasanti (Siracusa) (Colomba et al., 2012); and, finally, *M. cyclopica,* type locality Castello di Eurialo (Epipoli, Siracusa), is currently reported only for Epipoli, a hill about 150 m high, very close to the modern city of Siracusa (Liberto et al., 2016).

On the other hand, the Maltese species includes morphologically different populations that have been variously considered different species, or subspecies or simple morphs (see also Holyoak, 1986; Beckmann, 1992; Giusti et al., 1995; Nordsieck, 2007). Currently, one species with four subspecies is accepted (Bank, 2017; Bank & Neubert, 2019):

M. macrostoma macrostoma, locus typicus Malta (Cantraine, 1835), reported for Gozo, Comino, Cominotto and Malta;

M. macrostoma scalaris (L. Pfeiffer, 1850), locus typicus: Malta (L. Pfeiffer, 1850), inhabiting a very limited area on the Northwestern coast of Malta (Tal-Blata, Mistra Bay = St. Paul's Bay);

M. macrostoma oscitans (Charpentier, 1852), locus typicus: Malta (Charpentier, 1852), reported for Gozo and Malta;

M. macrostoma mamotica (Gulia, 1861), locus typicus: "*in insula Gaulos*" (Gulia, 1861), occurring in a very limited area on the Munxar side of Xlendi Valley in Gozo.

Numerous studies have been conducted on Maltese *Muticaria*, especially on the morphology of the shell and the genital organs (see Giusti et al., 1995 and cited bibliography), with often conflicting results on the taxonomic interpretation, also due to the presence of hybridization phenomena between some populations (Giusti et al. 1995; Cilia et al., 2012).

Taking into account available literature data and results obtained in previous studies carried out by this research team (Colomba et al., 2010; 2012; Liberto et al., 2016), we decided to further investigate on this group (Colomba et al., 2017). In particular, we focused on *M. macrostoma* from the Maltese Islands in order to characterize and define, from a molecular standpoint, their taxonomic status and the relationships with Sicilian *Muticaria*. In this work on *Muticaria*, we also included *Lampedusa imitatrix* (O. Boettger, 1879) specimens from Malta to study the relationships between these two genera that share part of the same area in the islands of the Sicilian Channel. Indeed, the genus *Lampedusa* O. Boettger, 1877 is distributed also in the Pelagie Islands (Sicilian Channel) with *L. lopadusae lopadusae* (Calcara, 1846) from Lampedusa and *L. lopadusae nodulosa* (Monterosato, 1892) from Lampione.

Lampedusa and *Muticaria* were also investigated using genetic data (sequencing of a fragment of the mitochondrial large ribosomal subunit 16S rRNA, and the nuclear internal transcriber spacer 1, ITS-1 rRNA) with a study available online as a bioRxiv preprint (Fiorentino et al., 2017, http://dx.doi.org/10.1101/208348).

Our results, compared also with available data, will be employed to achieve a better understanding of the speciation and dispersal phenomena of the populations of these interesting alopiine clausiliids and provide useful indications for their taxonomy.

MATERIAL AND METHODS

Samples and Collection sites

Two to five specimens per each population were employed for the study. Representatives of all subspecies of *M. macrostoma* sampled in different locality on the Maltese Islands, in addition to the topotypical populations of Sicilian *Muticaria* species, were analysed, along with specimens of *Lampedusa imitatrix* from Malta. Data on samples and collection sites, including the acronyms of the different examined populations, localities and GenBank Accession Numbers are reported in Table 1.

As seen in Table 1, in some cases where the specimens initial identification was not certain (ei-

ther at the subspecific or specific level) the samples were labelled separately and differently, i.e. *macrostoma* x *oscitans* (see also acronyms). Each collection site or locality is named in the original languages (i.e. Italian or Maltese).

DNA extraction

Samples were stored separately at -20 °C in test tubes. For each individual, a piece of foot tissue was used for total DNA extraction (by Wizard Genomic DNA Purification Kit, Promega). Voucher specimens were stored in the laboratory of Cytogenetics and Molecular Biology (University of Urbino, via Maggetti 22). Fragments of 16S rDNA (251–297 bp): and COI (529–660 bp) sequences were amplified using the primer pairs: MED16F/R (forward: 5'-ACTGTGCAAAGGTAGCATAA-3'/reverse: 5'-CCAACATCGAGGTCACAA-3') designed by Colomba and LCO_1490/HCO2198 as in Folmer et al. (1994). PCR cycles were as follows: 95 °C for 5 min; 95 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min (30 cycles); 72 °C for 5 min (16S rDNA); 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 (COI). 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 all sequences generated in this study are listed in Table 1. Homologous sequences of *Clausilia bidentata* (Strøm, 1765) (AF012082; JX911288), *Medora garganensis* (A.J. Wagner, 1918) (KC833909; KC853248), *Arianta arbustorum* (Linnaeus, 1758) (JF717810; MF140994) and *Massylea vermiculata* (O.F. Müller,

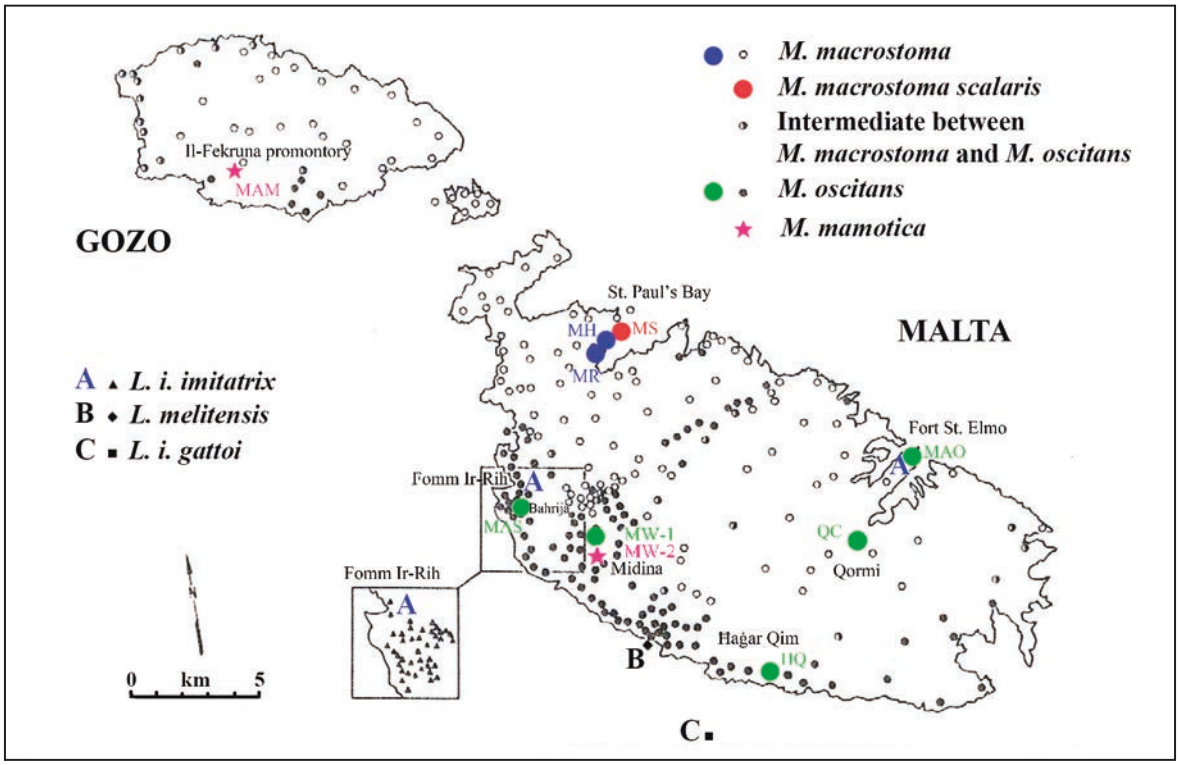

Figure 1. Distribution map of *Muticaria* and *Lampedusa* in the Maltese Islands (Holyoak, 1986 modified): white dots: distribution of *M. macrostoma macrostoma*; blue dots: sampling localities of *M. macrostoma macrostoma*; red dot: distribution and sampling localities of *M. macrostoma scalaris*; white and black dots: distribution of morphologically intermediate populations between *M. macrostoma macrostoma* and *M. macrostoma oscitans*; black dots: distribution of *M. macrostoma oscitans*; green dots: sampling locaties of *M. macrostoma oscitans*; purple star: distribution and sampling locality of *M. macrostoma mamotica*; triangles: distribution of *L. imitatrix imitatrix*; blue A: sampling localities of *L. imitatrix imitatrix*; rhombus: distribution of *L. melitensis*; square: distribution of *L. imitatrix gattoi*.

Taxon (initial classification)	Collection site	Voucher label	Coordinates	Species (revised classification)	GenBank Accession Number
M. m. macrostoma 1	St. Paul's Bay, Mistra, Malta	MH	35°57'26"N 14°23'27"E	*M. macrostoma*	MN395320, MN395321; MN395351, MN395353
M. m. macrostoma 2	St. Paul's Bay, Mistra, Malta	MR	35°57'18"N 14°23'08"N	*M. macrostoma*	MN395322, MN395323; MN395352, MN395354
M. m. mamotica	Il-Fekruna promontory, Munxar, Gozo	MAM	36°01'59"N 14°13'48"E	*M. mamotica*	MN395326, MN395328; MN395357, MN395358
M. m. oscitans	Mdina, Malta	MW2	35°53'09"N 14°24'03"E	*M. mamotica*	MN395329; MN395361
M. m. macrostoma x *oscitans*	Baharija, Rabat, Malta	MAS	35°53'47"N 14°20'50"E	*M. oscitans*	MN395334, MN395332; MN395364, MN395365
M. m. macrostoma 3	Wied il-Kbir, Qormi, Malta	QC	35°51'56"N 14°28'21"E	*M. oscitans*	MN395336, MN395337 MN395367, MN395368
M. m. oscitans	Ħaġar Qim, Qrendi, Malta	HQ	35°49'36"N 14°26'30"E	*M. oscitans*	MN395331, MN395335; MN395363, MN395362
M. m. oscitans	Mdina, Malta	MW1	35°53'09"N 14°24'03"E	*M. oscitans*	MN395333; MN395366
M. m. macrostoma x *oscitans*	Fort St. Elmo, Valletta, Malta	MAO	35°54'07"N 14°31'04"E	*M. oscitans*	MN395330, MN395327; MN395359, MN395360
M. m. scalaris	St. Paul's Bay, Mistra, Malta	MS	35°57'35"N 14°23'41"E	*M. m. scalaris*	MN395324, MN395325; MN395355, MN395356
Lampedusa (*imitatrix*) *imitatrix*	Fomm Ir-Rih, Rabat, Malta	LIM	35°54'11"N 14°20'03"E	*Lampedusa* (*imitatrix*) *imitatrix*	MN395310, MN395313; MN395344, MN395345
Lampedusa (*imitatrix*) *imitatrix*	Fort St. Elmo, Valletta, Malta	LIMS	35°54'07"N 14°31'04"E	*Lampedusa* (*imitatrix*) *imitatrix*	MN395311, MN395312; MN395346, MN395347
M. syracusana	Roman Amphitheatre Siracusa, Italy	SYR	37°04'28"N 15°16'45"E	*M. syracusana*	HQ696868; HQ696869
M. neuteboomi	Cava di Ispica, Ragusa, Italy	NEU	36°51'11"N 14°50'14"E	*M. neuteboomi*	HQ696866; HQ696867
M. brancatoi	Spinagallo, Siracusa, Italy	SPI	37°00'12"N 15°10'50"E	*M. brancatoi*	MN395314, MN395315, MN395316; KC550118, KC550119, KC550120
M. cyclopica	Epipoli, Castello Eurialo, Siracusa, Italy	EPI	37°05'20"N 15°13'49"E	*M. cyclopica*	MN395317, MN395318, MN395319; MN395348, MN395349, MN395350

Table 1. Data on specimens employed for the present study including initial taxonomic classification, sampling localities, voucher labels, coordinates, final revised taxonomic classification and GenBank Accession Numbers.

1774) (JF277389; JF802033) were used as Out-Groups (OGs).

Phylogenetic analyses

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. Genetic distances were assessed as p distances. Gene sequences were analysed by either single or combined analysis. Phylogenetic analyses were conducted in BEAST 1.6.1 (Drummond & Rambaut, 2007) using the *BEAST implementation (Heled & Drummond, 2010). A series of initial runs was performed to optimize priors and runtime parameter choices to obtain effective sampling sizes (ESS) above 500 for all estimated parameters. Parameter estimates were gained from combined log files. The best-fit evolution model of nucleotide substitution resulted in HKY+G for both genes 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 the starting point. Five

single runs were combined with the LogCombiner 1.6.1 implemented in the software package BEAST. Trees from all runs were combined to produce an ultrametric consensus tree using TreeAnnotator 1.6.1. The first 10^5 trees were discarded as burnin. Support for nodes is expressed as posterior probabilities.

RESULTS

Partial sequences of the 16S rDNA and COI molecular markers of all the sampled specimens of *Muticaria* from Maltese Islands were analyzed along with the homologous sequences of the four Sicilian species of *Muticaria* in addition to those of two populations of *Lampedusa imitatrix*.

The cladogram in figure 1, as a result of 16S rDNA sequences analysis, shows three mega-clusters: *Lampedusa imitatrix* (in green), three clusters of the *Maltese Muticaria* taxa (in red), including *M. macrostoma+scalaris*, *M. oscitans* and *M. mamotica*, and four clusters (in blue) of the Sicilian *Muticaria* species.

The obtained phylogenetic tree of the Maltese and Sicilian taxa are clearly distinct, and the high posterior probability values are a confirmation of the reliability of this reconstruction.

As a result of COI sequences analysis, the clusters are exactly the same as those already described, and the output does not change (Fig. 2).

After Combined analyis (Fig. 3) as well, clusters result exactly the same as those described before.

Finally, to add further information on the genetic distance between the examined taxa, genetic distances have also been calculated. In particular, for both 16S rDNA and COI partial sequences, p distances between all the sampled populations (Tables

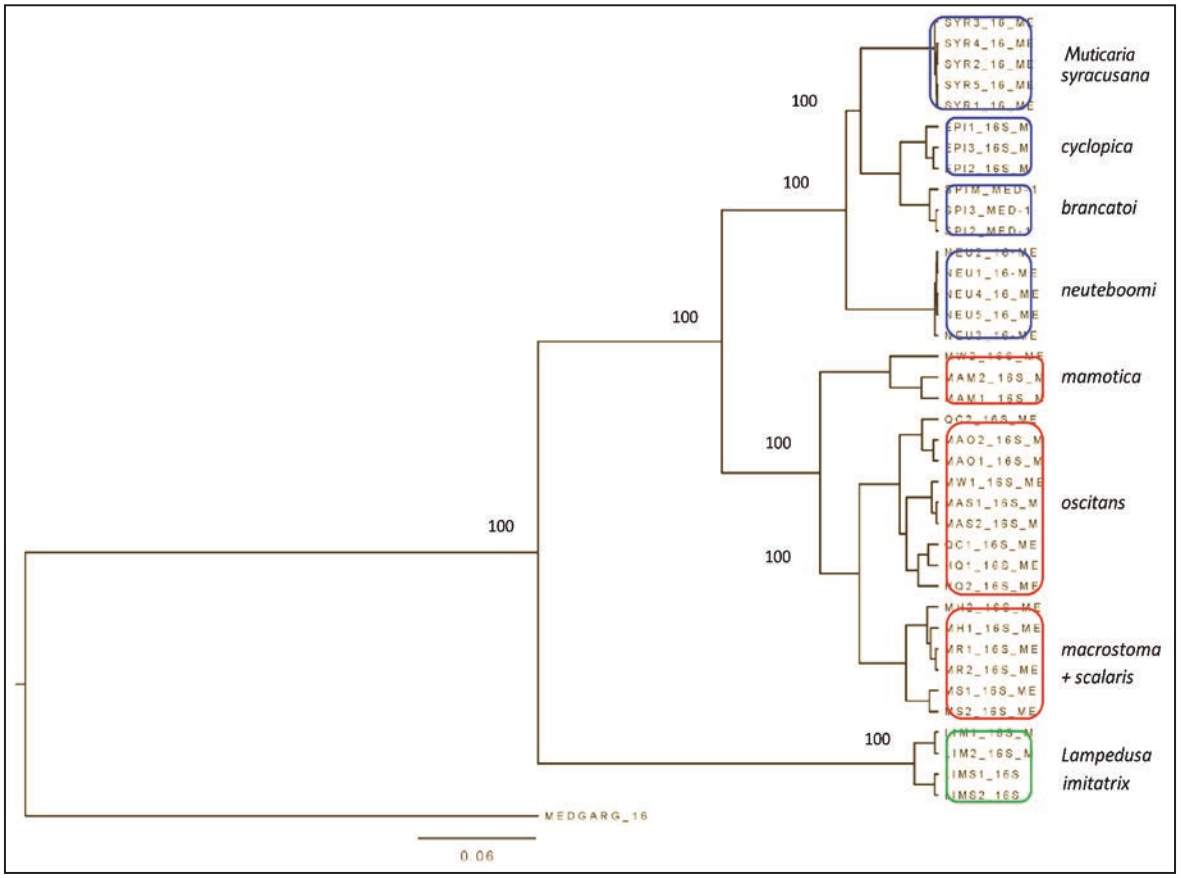

Figure 1. Phylogenetic reconstruction by Bayesian Inference obtained by single gene analysis of 16S rDNA partial sequences. Posterior Probabilities are reported on nodes. Maltese *Muticaria* taxa are shown in red clusters, Sicilian *Muticaria* taxa are shown in blue clusters and *Lampedusa imitatrix* specimens in green clusters.

2, 4) and between the populations grouped by species (Tables 3, 5) were assessed.

DISCUSSION

The results obtained from this work, continuing the studies conducted on the *Muticaria* genus (Colomba et al., 2010, 2012, 2017; Liberto et al., 2016), suggest that the taxa hitherto considered as subspecies of *M. macrostoma* may (with the exception of "*scalaris*") be elevated to the specific rank, on the basis of both the phylogenetic trees topography and genetic p distance values.

In particular, if we consider 16S rDNA p distances >0.2 as delimiting taxa at the genus level and from 0.1 to 0.05 as delimiting taxa at the species rank, it is possible to maintain that, as far as concerns the Maltese taxa:

(i) *M. mamotica* appears very different from *M. macrostoma* (ca. p= 0.103) and *M. oscitans* (p= 0.092);

(ii) *M. oscitans* can be elevated to the species rank with respect to *M. macrostoma* (p= 0.072) and *M. mamotica* (p= 0.092), whereas

(iii) "*scalaris*" seems to fall into *M. macrostoma* (p= 0.032) leaving at the moment unsolved the question of whether to consider it a subspecies or a synonym.

the result of the molecular data for the populations marked with the voucher labels MAO and MAS (apparently hybrid populations, *M. macrostoma* x *oscitans*, see Table 1) is significant of the difficulties that occur in the study of this group by a morphological and anatomical approach only. The shape and the ribs of the shell of these two populations did not allow a sure taxonomic classification. Molecular data indicated that they

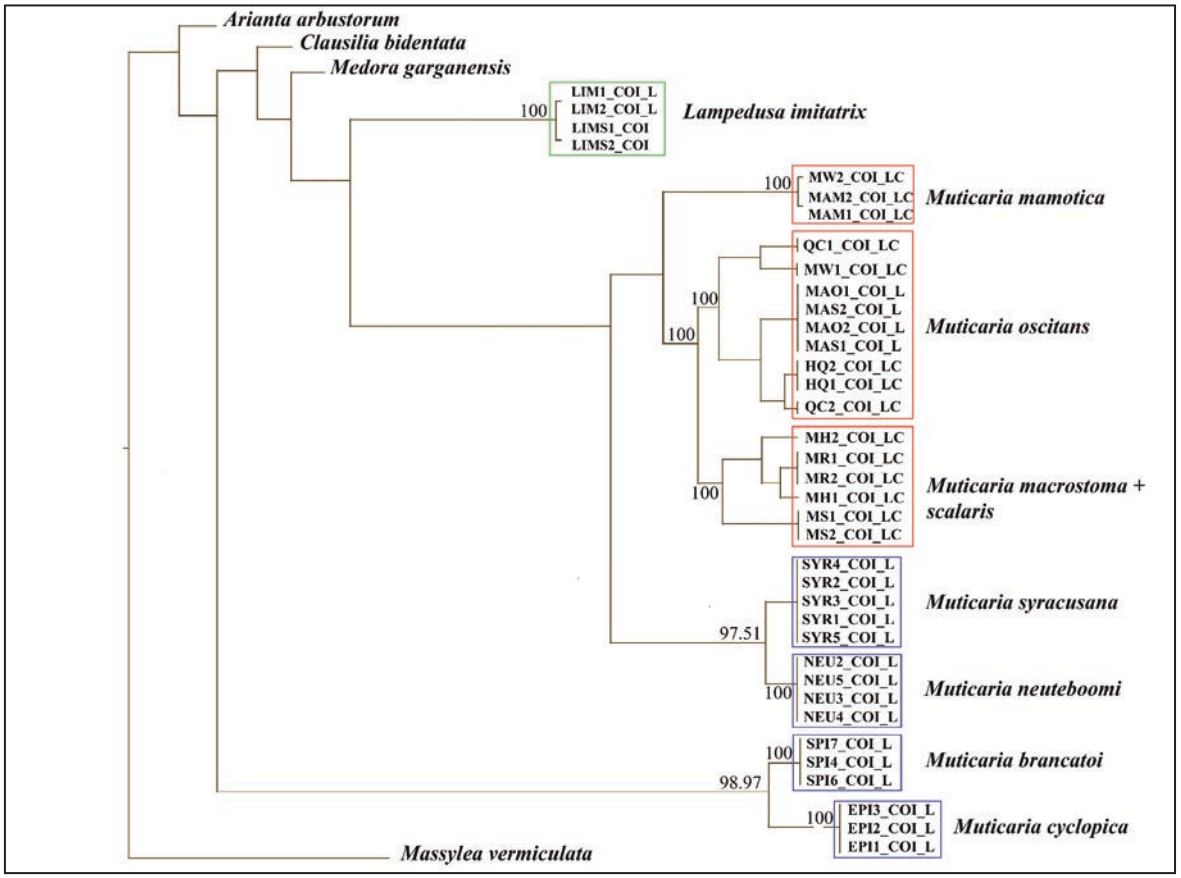

Figure 2. Phylogenetic reconstruction by Bayesian Inference obtained by single gene analysis of COI partial sequences. Maltese *Muticaria* taxa are shown in red clusters, Sicilian *Muticaria* taxa are shown in blue clusters and *Lampedusa imitatrix* specimens in green clusters.

were two populations attributable to *M. oscitans* clade. The molecular diversity of *M. oscitans* (see also Fiorentino et al., 2017) requires further studies.

Sicilian *Muticaria* species are confirmed with *M. syracusana* being clearly distinct from *M. neuteboomi* (p= 0.066) and a little less from *M. brancatoi* (p= 0.046); interestingly, 16 S rDNA p distance between *M. brancatoi* and *M. cyclopica* is quite low (p= 0.021). This is most likely because the two taxa are very similar in the (short) part of the 16S rDNA sequence analyzed, but the topography of the cladogram shows the taxa clearly distinct.

A very similar picture emerges from the analysis of COI p distances. Briefly, from COI amplicons the validity of the Maltese species *M. macrostoma*, *M. mamotica* and *M. oscitans* is confirmed. For the Sicilian species, in addition to *M. syracusana* and *M. neuteboomi*, COI sequences also reveled a significant distance between *M. brancatoi* and *M. cyclopica* (0.626). These molecular data with the known morphological and anatomical differences (Colomba et al., 2012; Liberto et al., 2016) confirm that two different species can be considered.

It should also be noted on all the cladograms examined, how the Sicilian *Muticaria* populations remain distinct from the Maltese ones, clearly indicating two geographical differentiation levels.

Based on the 16S rDNA distances, *Lampedusa* is confirmed as a different genus (p= 0.206) from *Muticaria*. COI distances confirm that they are different genera (*Lampedusa imitatrix* - *Muticaria macrostoma* group p distance= 0.139; *L. imitatrix* - Sicilian *Muticaria* species p distance = 0.261) considering also the known morphological and anatomical differences (Giusti et al., 1995).

The molecular and morphological data obtained

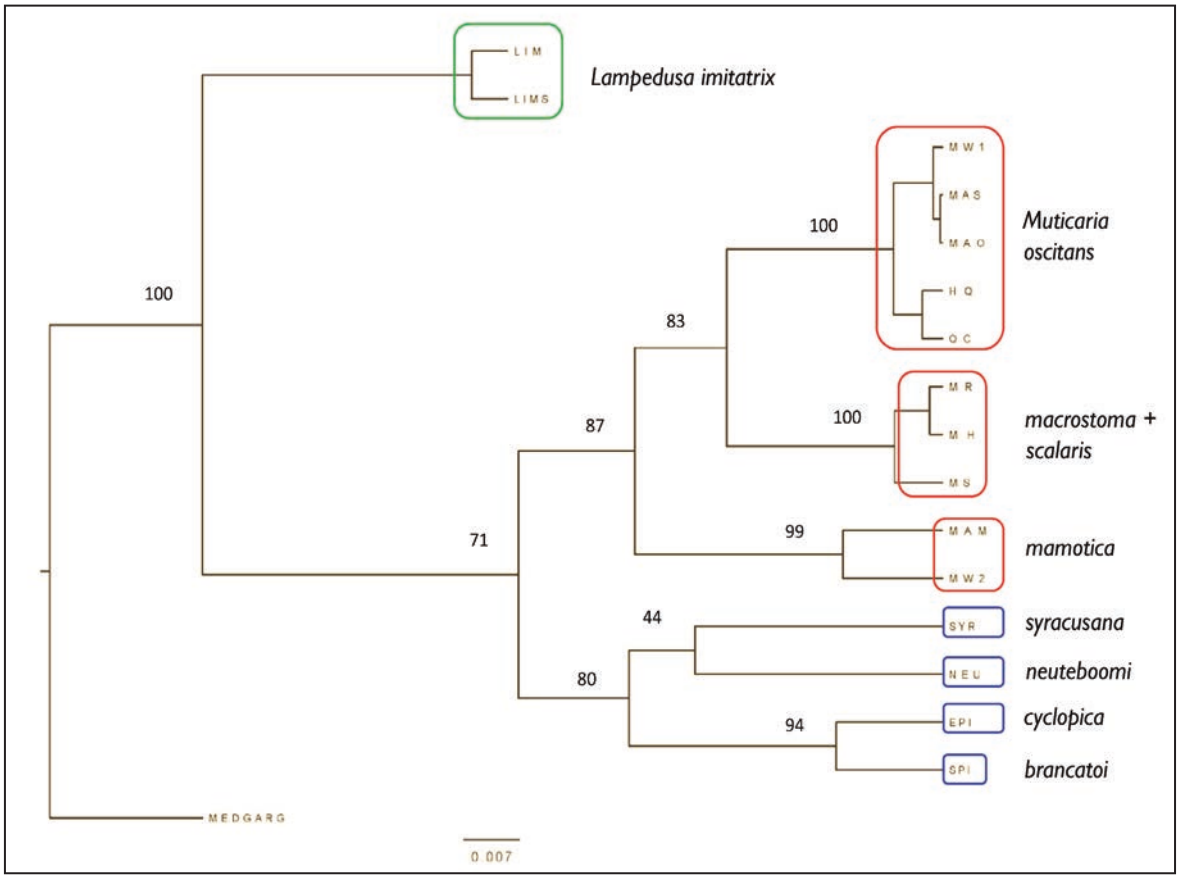

Figure 3. Phylogenetic reconstruction by Bayesian Inference obtained by combined gene analysis of 16S and COI rDNA partial sequences (i.e. concatenated analysis). Posterior Probabilities are reported on nodes. Maltese *Muticaria* taxa are shown in red clusters, Sicilian *Muticaria* taxa are shown in blue clusters and *Lampedusa imitatrix* specimens in green clusters.

	EPI	HQ	LIM	LIMS	MAM	MAO	MAS	MED	MH	MR	MS	MW1	MW2	NEU	QC	SPI	SYR
EPI																	
HQ	0.121																
LIM	0.202	0.171															
LIMS	0.200	0.162	0.018														
MAM	0.117	0.091	0.157	0.155													
MAO	0.130	0.023	0.175	0.165	0.096												
MAS	0.135	0.031	0.166	0.157	0.095	0.037											
MED	0.291	0.277	0.274	0.262	0.288	0.290	0.277										
MH	0.137	0.065	0.175	0.166	0.109	0.070	0.072	0.281									
MR	0.135	0.067	0.174	0.164	0.113	0.072	0.074	0.286	0.009								
MS	0.121	0.061	0.177	0.168	0.105	0.058	0.070	0.290	0.033	0.032							
MW1	0.131	0.028	0.169	0.160	0.091	0.033	0.003	0.281	0.068	0.070	0.067						
MW2	0.115	0.082	0.182	0.172	0.045	0.091	0.093	0.286	0.087	0.089	0.089	0.089					
NEU	0.054	0.108	0.204	0.196	0.120	0.117	0.122	0.279	0.139	0.137	0.123	0.119	0.117				
QC	0.119	0.021	0.173	0.164	0.093	0.019	0.035	0.281	0.077	0.079	0.068	0.031	0.085	0.108			
SPI	0.021	0.123	0.205	0.197	0.127	0.133	0.136	0.278	0.135	0.133	0.132	0.132	0.118	0.058	0.123		
SYR	0.044	0.112	0.198	0.188	0.120	0.124	0.122	0.289	0.132	0.130	0.123	0.119	0.117	0.066	0.114	0.046	

Table 2. p distances between all the sampled populations calculated on 16S rDNA partial sequences (acronyms in Table 1).

	M. cyclopica	M. oscitans	L. imitatrix	M. mamot.	Med. garg.	M. macrost	scalaris	M. neuteb.	M. brancat.	M. syrac.
M.cyclopica										
M. oscitans	0.127									
L. imitatrix	0.201	0.166								
M. mamot.	0.116	0.092	0.163							
Med. garg.	0.291	0.281	0.268	0.287						
M. macrost.	0.136	0.072	0.170	0.103	0.284					
scalaris	0.121	0.065	0.173	0.100	0.290	0.032				
M. neuteb.	0.054	0.115	0.200	0.119	0.279	0.138	0.123			
M. brancat.	0.021	0.129	0.201	0.124	0.278	0.134	0.132	0.058		
M. syrac.	0.044	0.118	0.193	0.119	0.289	0.131	0.123	0.066	0.046	

Table 3. 16S rDNA p distances between all the sampled populations arranged by species.

	EPI	HQ	LIM	LIMS	MAM	MAO	MAS	MED	MH	MR	MS	MW1	MW2	NEU	QC	SPI	SYR
EPI																	
HQ	0.591																
LIM	0.605	0.128															
LIMS	0.609	0.135	0.021														
MAM	0.592	0.088	0.160	0.147													
MAO	0.601	0.033	0.132	0.133	0.100												
MAS	0.600	0.033	0.132	0.134	0.094	0.000											
MED	0.614	0.174	0.187	0.195	0.185	0.181	0.180										
MH	0.595	0.080	0.143	0.150	0.092	0.075	0.074	0.172									
MR	0.595	0.078	0.139	0.146	0.094	0.073	0.072	0.169	0.010								
MS	0.589	0.075	0.141	0.146	0.092	0.070	0.069	0.177	0.017	0.022							
MW1	0.593	0.029	0.134	0.137	0.091	0.003	0.003	0.179	0.071	0.068	0.065						
MW2	0.586	0.084	0.150	0.148	0.051	0.100	0.100	0.177	0.094	0.098	0.095	0.098					
NEU	0.610	0.117	0.160	0.157	0.141	0.125	0.126	0.183	0.124	0.125	0.126	0.122	0.131				
QC	0.598	0.036	0.125	0.123	0.090	0.036	0.036	0.158	0.076	0.072	0.074	0.033	0.088	0.107			
SPI	0.626	0.256	0.265	0.285	0.274	0.254	0.259	0.241	0.260	0.257	0.256	0.257	0.277	0.252	0.242		
SYR	0.614	0.128	0.150	0.149	0.134	0.134	0.134	0.191	0.122	0.120	0.130	0.131	0.134	0.121	0.124	0.254	

Table 4. p distances between all the sampled populations calculated on COI partial sequences (acronyms in Table 1).

	M.cyclopica	M. oscitans	L. imitatrix	M. mamot.	Med. garg.	M. macrost	scalaris	M. neuteb.	M. brancat.	M. syrac.
M.cyclopica										
M. oscitans	0.597									
L. imitatrix	0.607	0.131								
M. mamot.	0.590	0.093	0.152							
Med. garg.	0.614	0.174	0.191	0.182						
M. macrost.	0.595	0.074	0.145	0.094	0.171					
scalaris	0.589	0.071	0.143	0.093	0.177	0.019				
M. neuteb.	0.610	0.119	0.158	0.138	0.183	0.125	0.126			
M. brancat.	0.626	0.253	0.275	0.275	0.241	0.259	0.256	0.252		
M. syrac.	0.614	0.130	0.150	0.134	0.191	0.121	0.130	0.121	0.254	

Table 5. COI p distances between all the sampled populations arranged by species.

by Fiorentino et al. (2017, bioRxiv unpublished preprint) show *Lampedusa* and *Muticaria* as two different genera, and *Muticaria* as a monophyletic clade divided into three geographical lineages (Sicilian, Maltese and Gozitan populations).

CONCLUSIONS

All the above data allow us to draw taxonomic conclusions, even partial, on the *Muticaria* populations examined for their greater knowledge and protection.

In conclusions, our findings suggest that:

(i) within Maltese *Muticaria* is possible to elevate to the specific rank "*mamotica*" and "*oscitans*", thus bringing to three the Maltese *Muticaria* species i.e. *M. macrostoma*, *M. mamotica* and *M. oscitans*; whereas *M. macrostoma scalaris* would remain a subspecies;

(ii) as far as concerns the Sicilian species, *M. syracusana*, *M. neuteboomi*, *M. brancatoi* and *M. cyclopica* are confirmed;

(iii) the Sicilian and Maltese *Muticaria* populations seem to belong to two geographical differentiation levels;

(iv) *Lampedusa* and *Muticaria* are two different genera.

The high level of differentiation found within this group is a consequence both of the complex biogeographical history of this region and of strict connection between the geological (calcareous) nature of the soil these molluscs live in and the scarce vagility of the specimens, leading to island-like distributional patterns characterized by high levels of endemism.

All this requires a greater commitment in the protection and management of these land molluscs and the environments in which they live.

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