

1 **The radiation of alopiine clausiliids in the Sicilian Channel (Central**
2 **Mediterranean): phylogeny, patterns of morphological diversification and**
3 **implications for taxonomy and conservation of *Muticaria* and *Lampedusa*.**

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5 **V. Fiorentino*¹, N. Salomone², P. J. Schembri³, G. Manganelli⁴, F. Giusti⁴**

6 ¹ *University of Potsdam, Unit of Evolutionary Biology/Systematic Zoology Institute of Biochemistry*
7 *and Biology Karl-Liebknecht-Str. 24-25, Potsdam, Germany*

8 ² *University of Siena, Department of Evolutive Biology, Via A. Moro, 2, 53100 Siena, Italy*

9 ³ *University of Malta, Department of Biology, Msida MSD2080, Malta*

10 ⁴ *University of Siena, Department of Environmental Sciences, Via P.A. Mattioli 4, 53100 Siena,*
11 *Italy*

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14 **Running title**

15 Phylogeny and morphological diversification of alopiine clausiliids

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17

18 **Key words**

19 Land snails, clausiliids, island radiation, phylogeny, morphology

20

21 **Abstract**

22 The phylogeny, biogeography and taxonomy of the alopiine clausiliids of the Sicilian
23 Channel, belonging to the genera *Lampedusa* and *Muticaria*, were investigated using morphological
24 (shell characters and anatomy of the reproductive system) and genetic (sequencing of a fragment of
25 the mitochondrial large ribosomal subunit 16S rRNA, and the nuclear internal transcriber spacer 1,
26 ITS-1 rRNA) data. Classically, the genus *Lampedusa* includes three species: *L. imitatrix* and *L.*
27 *melitensis* occurring in circumscribed localities in western Malta and on the islet of Filfla, and *L.*
28 *lopadasae* on Lampedusa and Lampione. The genus *Muticaria* includes two species in south-
29 eastern Sicily (*M. siracusana* and *M. neuteboomi*) and one in the Maltese islands (*M. macrostoma*),
30 which is usually subdivided into four entities based on shell characters (*macrostoma* on Gozo,
31 Comino, Cominotto and central-eastern Malta; *mamotica* in southeastern Gozo; *oscitans* on Gozo
32 and central-western Malta; *scalaris* in northwestern Malta). These have sometimes been considered
33 as subspecies and sometimes as mere morphs.

34 The *Lampedusa* of Lampedusa and Lampione form a well distinct clade from those of the
35 Maltese Islands. The population of Lampione islet is a genetically distinct geographic form that
36 deserves formal taxonomic recognition (as *L. nodulosa* or *L. l. nodulosa*). The *Lampedusa* of Malta
37 are morphologically distinct evolutionary lineages with high levels of genetic divergence and are
38 confirmed as distinct species (*L. imitatrix* and *L. melitensis*).

39 The *Muticaria* constitute a clearly different monophyletic clade divided into three
40 geographical lineages corresponding to the Sicilian, Maltese and Gozitan populations. The Sicilian
41 *Muticaria* form two morphologically and genetically distinguishable subclades that may either be
42 considered subspecies of a polytypic species, or two distinct species. The relationships of Maltese
43 and Gozitan *Muticaria* are complex. Two of the three Maltese morphotypes resulted monophyletic
44 (*oscitans* and *scalaris*) while the other was separated in two lineages (*macrostoma*); however this
45 picture may be biased as only few samples of *macrostoma* were available to study. The Gozitan
46 morphotypes (*macrostoma*, *mamotica* and *oscitans*) were resolved as polyphyletic but with clear
47 molecular evidence of mixing in some cases, indicating possible relatively recent differentiation of
48 the Gozitan *Muticaria* or repetitive secondary contacts between different morphotypes. Definitive
49 taxonomic conclusions from these results are premature. Maltese *Muticaria* could be subdivided
50 into three taxa according to morphological and molecular data (*M. macrostoma* or *M. m.*
51 *macrostoma*, *M. oscitans* or *M. m. oscitans* and *M. scalaris* or *M. m. scalaris*). Gozitan *Muticaria*
52 could be considered a distinct polytypic species (for which the oldest available name is *Muticaria*
53 *mamotica*) subdivided into subspecies showing a morphological range from *macrostoma*-like to
54 *mamotica*-like and *oscitans* like.

55 Only the two Maltese species of *Lampedusa* are legally protected (by the European Union's
56 'Habitats Directive' and Maltese national legislation). The present study has shown that the alopiine
57 clausiliids of the Sicilian Channel constitute a number of genetically and/or morphologically
58 distinct populations that represent important pools of genetic diversity, with, in some cases, a very
59 circumscribed distribution. As such, these populations deserve legal protection and management. It
60 is argued that without formal taxonomic designation, it would be difficult to extend international
61 legal protection to some of the more threatened of these populations.
62

63 Introduction

64 Archipelagos are ideal places for studying evolutionary biology; their limited geographical
65 area makes them “paradigm systems” for understanding the origin of diversity (Mayr, 1963).

66 In the past century, archipelagos have received much attention from conservation biologists
67 since most are inhabited by species-rich groups in fragile equilibria with their environment.
68 Numerous studies have focussed on insular groups of snails, for example, *Partula* in the Indo-
69 Pacific islands (Johnson, Murray & Clarke, 1993; Goodacre & Wade, 2001), *Polymita* in Cuba
70 (Reyes-tur, Fernández & Suárez, 2001), *Napaeus* in the Canary archipelago (Alonso et al., 2006),
71 and leptaxine hygromiids in the Azores islands (Van Riel et al., 2003; Jordaens et al., 2009).

72 Mediterranean archipelagos, such as the Pelagian and Maltese island groups, are also rich in
73 endemic land snails, especially the alopiine clausiliids and the trochoideine hydromiids (Giusti,
74 Manganello & Schembri, 1995). The former, a group of clausiliid snails mostly diversified in the
75 Balkan peninsula and in Crete, occur in the Italian area with four genera: *Leucostigma* and *Medora*
76 in the Apennines, and *Lampedusa* and *Muticaria* in south-eastern Sicily and the Maltese and
77 Pelagian islands (Nordsieck, 2007) (Fig. 1).

78 Most of the recognized taxa of *Lampedusa* and *Muticaria* exhibit allopatric or parapatric
79 distribution patterns. The genus *Lampedusa* includes three species: *L. imitatrix* and *L. melitensis*
80 occurring in two isolated areas in western Malta and on the islet of Filfla (5.2 km south of Malta),
81 and *L. lopadusae* on Lampedusa and Lampione. Qualitative shell and anatomical characters
82 distinguish *L. lopadusae* from *L. imitatrix* and *L. melitensis* (Giusti et al., 1995).

83 The genus *Muticaria* includes two species in south-eastern Sicily (*M. siracusana* and *M.*
84 *neuteboomi*) and one in the Maltese islands (*M. macrostoma*) (Fig. 2). Maltese *Muticaria* are
85 usually subdivided into four entities on the base of shell shape and ribbing; these are sometimes
86 considered as distinct species, sometimes as subspecies, and sometimes as morphs without
87 taxonomic value (Table 1). The four are: *macrostoma* characterized by a conical fusiform shell with
88 strong sparse ribs (Gozo, Comino, Cominotto and central-eastern Malta); *mamotica* characterized
89 by a ventricose shell with slender minute close ribs (Xlendi Valley, near Munxar, southeastern
90 Gozo); *oscitans* characterized by a conical fusiform shell with slender minute close ribs (Gozo and
91 central-western Malta); and *scalaris* characterized by a conical scalariform shell with strong sparse
92 ribs (Mistra Bay, northwestern Malta). Qualitative shell characters distinguish the Maltese
93 *Muticaria* from the Sicilian species but no anatomical feature seems to distinguish the different
94 Maltese forms (Giusti et al., 1995).

95 Interestingly, Thake (1985) and Holyoak (1986) reported an area of hybridization between
96 *L. imitatrix* and *M. macrostoma*. Giusti (1995), re-examining the voucher specimens, demonstrated

97 that some alleged hybrids belonged to *L. imitatrix* or *M. macrostoma*. However, some other
98 specimens were real hybrids between species of *Lampedusa* and *Muticaria* (hybrids occur in a small
99 area of a few tens of square metres, see Giusti et al., 1995 for details).

100 The Pelagian and Maltese archipelagos are located in the Sicilian Channel. Together with
101 the eastern coast of Tunisia and the Hyblean region of Sicily, the Maltese and Pelagian islands are
102 the only currently exposed parts of the Pelagian Block, which is the foreland margin of the African
103 continental plate (Pedley, House & Waugh, 1978; Pedley 1990; Grasso & Pedley 1985; Gatt, 2007).
104 The submerged parts of the Pelagian Block were exposed to form a land bridge or corridor (sensu
105 Simpson, 1940) between northern Africa and Italy, via Sicily, starting ca 5.59 million years BP
106 during the Messinian Salinity Crisis (Hsü, Ryan & Cita, 1973; Krijgsman et al, 1999, Krijgsman,
107 2002; CIESM, 2008), allowing biota from North Africa and the Italian peninsula to colonise the
108 exposed land. Much of the Pelagian Block was gradually and intermittently submerged with the
109 refilling of the Mediterranean at the end of the Miocene ca 5.33 million years BP (Krijgsman et al,
110 1999; Pedley et al., 2007) and it is generally held that Africa and Sicily have not been connected
111 again since, although some evidence has now accumulated for an exchange of fauna between Africa
112 and Sicily via a land bridge after the end of the Messinian Salinity Crisis (Stöck et al, 2008). Faunal
113 exchange would almost certainly have occurred during the Plio-Pleistocene glaciations when the
114 African palaeocoast would have repeatedly approached Sicily during sea level lowstands (Thiede,
115 1978; Rohling et al., 1998) facilitating jump dispersal across the narrow channel that separated the
116 African and Sicilian palaeocoasts, a process that may have been assisted by any islands that were
117 present in the Pleistocene Sicilian Channel acting as stepping stones for dispersal (Flemming et al.,
118 2003); there are several banks in the present day Sicilian Channel that would have become exposed
119 as islands during some of the Plio-Pleistocene marine regressions (British Admiralty, 2005, 2010).

120 The Maltese archipelago, located approximately 100 km from Sicily and 300 km from North
121 Africa, consists of low islands aligned in a NW-SE direction. The three main islands of Malta
122 (245.7 km²), Gozo (67.1 km²) and Comino (2.8 km²) are inhabited, and there are a number of
123 uninhabited islets each less than 10 ha (Schembri, 1997). The islands are composed almost entirely
124 of marine sedimentary rocks, mainly limestones of Oligo-Miocene age (30–5 million years BP)
125 with some minor Quaternary deposits of terrestrial origin (Pedley, House & Waugh, 1976; Pedley,
126 Hughes Clarke & Galea, 2002).

127 The Maltese Islands received their first influx of terrestrial biota during the Messinian when
128 they were part of the Afro-Sicilian corridor. This biota was isolated following refilling of the
129 Mediterranean and remained so during the Pliocene. However, further influxes of biota from Sicily
130 occurred during the Pleistocene marine regressions either when the islands became connected to

131 Hyblean Sicily by actual land bridges during the more extreme sea level lowstands, or by jump
132 dispersal facilitated by a narrowing of the channel between Sicily and the Maltese Islands. With the
133 present bathymetry, the drop in sea-level needed to connect Malta to Sicily is about 155 m but the
134 maximum Pleistocene regression was of 120-130m (Bard, Hamelin & Fairbanks, 1990; Ferland,
135 1995; Rohling et al., 1999), however, tectonic uplift due to crustal rebound during regressions, and
136 sedimentation (Gatt, 2007), may have caused the depth of the Sicilian-Maltese channel to vary in
137 the past; on the other hand a drop of 100m in sea-level would narrow the present day Sicilian-
138 Maltese channel to less than 14km wide (Hunt & Schembri, 1999).

139 In general, the Maltese biota resembles that of Sicily (Francini Corti & Lanza, 1973; Hunt &
140 Schembri, 1999), although it also comprises a number of endemics (Giusti et al., 1995; Schembri,
141 2003). Only few species occur on Malta and North Africa and are not present in Sicily (Giusti et al.,
142 1995; Schembri, 2003), although some of the Maltese endemic, or putatively endemic, species have
143 a North African rather than a European affinity (Schembri, 2003).

144 The islands of Lampedusa and Linosa and the islet of Lampione constitute the Pelagian
145 archipelago which lies on the northern edge of the African continental shelf. Lampedusa and
146 Lampione consist of Oligo-Miocene limestones broadly similar to those of the Maltese group
147 (Grasso & Pedley, 1985; Grasso, Pedley & Reuther, 1985), and like these islands, they arose in the
148 Late Miocene (Torelli et al., 1995; André et al., 2002); Linosa has a volcanic origin. Like the
149 Maltese Islands, the Pelagian archipelago was part of the exposed corridor of land between Africa
150 and Europe during the Messinian, and remained isolated for a long time following the inundation of
151 the Mediterranean at the end of the Messinian Salinity Crisis. However, during Quaternary marine
152 lowstands it was connected to the African continent (Giraudi, 2004), but never to Sicily. The
153 Pelagian biota is on the whole very disharmonic, and includes many endemics (Massa et al., 1995;
154 Caruso, Noto La Diega & Bernini, 2005).

155 Numerous unanswered questions on the biogeography and taxonomy of the alopiine
156 clausiliids of the Sicilian Channel, as well as the need for conserving the rarer members of this
157 complex group of land snails, have prompted the present study. Our analysis includes
158 representatives from nearly all taxa traditionally defined for this geographic area and constitutes a
159 first attempt to build a phylogeny of the alopiinae radiation in the Sicilian Channel on the basis of
160 both morphological and genetic data (shell morphology; sequencing of a fragment of the
161 mitochondrial large ribosomal subunit, 16S rRNA, and the nuclear internal transcriber spacer 1,
162 ITS-1 rRNA).

163 **Material and Methods**

164 SAMPLE COLLECTION

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165 Information on sampling sites and specimens is summarized in Table 1 and Fig. 3.

166 All representatives of alopiine clausiliids reported from southeastern Sicily and the islands
167 of the Sicily Channel were sampled. Since some taxa are endangered endemics, only a limited
168 number of specimens was collected. Thus, whenever possible, alcohol preserved material was used.

169 Analyses were performed on operational groups determined on the basis of taxonomy and
170 geographical distribution. Since genetic analyses (see below) separated robustly the *Lampedusa*
171 specimens of Lampione islet from those of Lampedusa island, we considered this population
172 separately, denoting it with the available name “*nodulosa*”. Consequently, the *Lampedusa*
173 operational groups studied are: *imitatrix*, *lopadusae*, *melitensis* and *nodulosa*. Based on shell
174 ribbing and shape, some populations of *Muticaria* from the Maltese Islands appeared to be
175 intermediate between *macrostoma* and *oscitans* (central-south of Malta) or *mamotica* and *oscitans*
176 (western Gozo). Thus we identified the following *Muticaria* operational groups: *macrostoma*,
177 *macrostoma-oscitans*, *oscitans*, *mamotica*, *mamotica-oscitans*, *scalaris*, *syracusana* and
178 *neuteboomi*.

179 Two different species were chosen as outgroups: *Medora italiana* (Küster, 1847) and
180 *Leucostigma candidescens* (Rossmässler, 1835). Both species are generally thought to be closely
181 related to the ingroup taxa, although their phylogenetic relationships have never been addressed.

182

183 MORPHOLOGICAL DATA

184 Shell measurements were usually taken for ten specimens selected randomly from each
185 locality, for a total of 56 specimens of *Lampedusa* and 470 of *Muticaria*. Only adult shells were
186 used for measuring height (H), width (D) and the number of ribs (NR) on the penultimate whorl
187 (Fig. 4). H and D were measured to the nearest 0.01 mm on shells positioned in apertural view,
188 using an eyepiece micrometer fitted in a light microscope (Wild M5A) (Fig. 4).

189 Genital measurements were recorded for five sexually developed specimens randomly
190 selected from each locality, for a total of 25 specimens of *Lampedusa* and 40 of *Muticaria*.
191 Specimens were dissected under a light microscope (Wild M5A) using fine-pointed watchmaker’s
192 tweezers. Eight linear variables (Fig. 5) were measured on isolated genitalia using an eyepiece
193 micrometer fitted in a light microscope (accurate to 0.01 mm).

194

195 ANALYSIS OF MORPHOLOGICAL DATA

196 Morphological variables were log-transformed to obtain linear relationships, when
197 necessary.

198 Two-way analysis of variance (ANOVA) was performed on shell measurements and number
199 of ribs. The *a posteriori* Tukey test ($\alpha = 0.05$) was used to check group significance. All the
200 analyses were run for the *Muticaria* data set, for the *Lampedusa* data set, and for the two data sets
201 combined.

202 Discriminant Function Analysis (DFA) was then performed considering all measured genital
203 variables. The analysis was run with groups defined *a priori* (“operational groups” and “islands”).
204 With this analysis we assessed which measurements contributed to discrimination of groups defined
205 *a priori*. The sequential chi-square test was used to quantify the extent to which each discriminant
206 function significantly separated groups and structure, and canonical coefficient tables were used to
207 establish the contribution of each measurement to the first two discriminant functions.

208 All calculations were made using R-package version 2.3.0 (R Development Core Team,
209 2006). The STATISTICA 5 (StatSoft Inc., Tusla, USA) package was used to run the DFA analyses.

210

211 DNA EXTRACTION, PCR AND SEQUENCING

212 A total of 11 specimens of *Lampedusa* from five sites and 30 of *Muticaria* from 12 sites
213 were studied. Specimens of *Lampedusa* were representatives of all taxa (except *L. imitatrix gattoi*
214 from Filfla islet); specimens of *Muticaria* were representatives of all operational groups.

215 Total genomic DNA was extracted from foot muscle of fresh or alcohol preserved
216 specimens using the C-TAB buffer (0.1M Tris-HCl pH 8.0, 1.4 M NaCl, 0.02 M EDTA, 2% CTAB,
217 0.2% 2-mercaptoethanol) and subsequent standard phenol-chloroform/ethanol extraction (Hillis et
218 al., 1996).

219 For all the sampled snails, a fragment of the mitochondrial gene encoding for the large
220 ribosomal subunit (16S rDNA) was polymerase chain reaction (PCR) amplified using the primer
221 pair 5'-CGATTTGAACTCAGATCA-3' (Simon et al., 1994) and 5'-
222 GTGCAAAGGTAGCATAATCA -3'(Gantenbein et al., 1999). In addition, the nuclear ribosomal
223 gene cluster encompassing the ribosomal internal transcribed spacer (ITS-1) was sequenced in
224 specimens of the genus *Muticaria*, using primers annealing to flanking regions of the 18S and the
225 5.8S (CS249, 5'-TCGTAACAAGGTTTCCG-3' and DT421, 5'-GCTGCGTTCTTCATCG-3';
226 Schlötterer et al., 1994).

227 All PCR reactions were carried out in a total volume of 50 μ l under the following
228 conditions: 95°C for 20'', 55°-52°C for 30'' and 72°C for 30'' (repeated for 25 cycles), plus a final
229 extension step at 72°C for 5'. Reaction products were isolated on 1% agarose gel, excised under
230 long-wavelength UV light, and purified using a “Nucleospin extract” (Genenco™) column kit. Both
231 strands of the amplified fragments were directly cycle-sequenced using the same amplification

232 primers and the CEQ dye terminator cycle sequencing kit. DNA sequences were then
233 electrophoresed on a CEQ 8000XL (Beckman Coulter™). DNA sequences have been deposited in
234 the GenBank database (see Table 1 for GenBank references - *will be submitted on acceptance*).

235

236 ANALYSIS OF DNA SEQUENCES

237 The 16S rDNA sequences were aligned and checked with Clustal X (version 1.8, Thompson
238 et al. 1997) and easily aligned by eye where necessary. Phylogenetic relationships were conducted
239 on the 16S mitochondrial dataset using Maximum Parsimony and Bayesian inference. Parsimony
240 analyses were performed with PAUP* (version 4.0, Swofford, 2001) using the heuristic search
241 option with equal weighting of all characters (ACCTRAN character-state optimisation, 100 random
242 stepwise additions, TBR branch-swapping algorithm) (Farris, 1970). To assess the robustness of the
243 phylogenetic hypotheses, 1000 bootstrap replicates were performed (Felsenstein, 1985).

244 Prior to Bayesian analysis, we determined an appropriate model of sequence evolution using
245 MRMODELTEST (vers. 2.2, Nylander, 2004). Bayesian analysis was then carried out with MRBAYES
246 (version 3.1, Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003) using the same
247 model as estimated from MRMODELTEST. MRBAYES was run for 2 million generations with a
248 sampling frequency of 100 generations and one cold and three heated Markov chains.

249 Reached stationarity was evaluated by plotting the likelihood scores of sampled trees against
250 generation time. Trees generated before the stationarity phase were discarded as “burn-in” and
251 posterior probability values for each node were calculated based on the remaining sampled trees.

252 The nuclear ITS-1 region was easily aligned by eye using the program BIOEDIT (version 7.0,
253 Hall 1999). Relationships within congeneric populations were then inferred by constructing a
254 median-joining network (Bandelt, Forster & Röhl, 1999) using the program NETWORK 4.1.0
255 (www.fluxus-engineering.com, Fluxus Technology Ltd.).

256

257 **Results**

258 MORPHOLOGICAL ANALYSIS

259 ANOVA performed on H, D, D/H and NR of the shells of *Lampedusa* and *Muticaria*
260 revealed significant differences between groups defined *a priori* (Tables 2-5; Figs. 6-7).

261 ANOVA revealed that NR clearly distinguished the *Lampedusa* of Lampedusa from the
262 *Lampedusa* of Malta and distinguished the *Lampedusa* of Lampione from all the other *Lampedusa*.

263 ANOVA showed that NR in *Muticaria* was also reliable for recognising the forms from each
264 island, excluding the following groups: (1) Maltese *macrostoma* and *scalaris*; (2) Maltese

265 *macrostoma-oscitans* and *neuteboomi*; (3) Maltese *macrostoma-oscitans* and Gozitan *oscitans*; (4)
266 Gozitan *oscitans* and *neuteboomi*; (5) Gozitan *macrostoma-oscitans* and Gozitan *macrostoma*; (6)
267 Gozitan *macrostoma-oscitans* and *neuteboomi*; (7) *oscitans-mamotica* and *mamotica*; (8) *oscitans-*
268 *mamotica* from Gozo and Maltese *oscitans*.

269 DFA on the genital variables of *Lampedusa* revealed that the first discriminant function
270 accounted for 97% of the variance while the second accounted only for 3%. The highest loadings on
271 the first function were DBC (-6.33) and PEP (12.26). ANOVA on DBC and PEP showed significant
272 differences between species. *L. imitatrix* was significantly different from *L. melitensis* and *L.*
273 *lopadusae* for DBC; while *L. lopadusae* was significantly different from *L. imitatrix* and *L.*
274 *melitensis* for PEP. Analyses on ratios of all the variables considered did not result in significant
275 differences among species.

276 DFA on genital variables of *Muticaria* showed that the first discriminant function accounted
277 for 69% of the variance and the second accounted for 28%. The highest loadings on the first
278 function were: PC (- 1.02), DBC (-0.73), P (0.77) and PEP (0.57). ANOVA on these variables did
279 not clearly distinguish species but only some pairs of taxa. In particular, F distinguished *syracusana*
280 and *scalaris*, and P distinguished *syracusana* and *oscitans*.

281

282 SEQUENCE CHARACTERISTICS

283 A total of 413 bp of the mitochondrial 16S rRNA was sequenced. Few indels were found in
284 the alignment. However, removal or inclusion of indels in the phylogenetic analyses (indels were
285 counted as one single mutation each, regardless of size) did not result in significant differences in
286 tree topologies. There were a total of 152 variable characters, 96 of which were parsimony
287 informative.

288 Base composition was homogeneous ($\chi^2 = 29.1$, $df = 120$, $P = 1.0$), but skewed toward a
289 deficiency in guanine (16.9%) and cytosine (13.5%), as expected for mitochondrial genes (Simon et
290 al., 1994).

291 The nuclear dataset was limited to the subset including representatives of *Muticaria*.
292 Alignment of the 30 ITS-1 nuclear sequences resulted in a matrix with 495 nucleotide positions
293 (including gaps), providing 13 different nuclear variants. On the agarose gels, no evidence of intra-
294 individual length variation was observed. There were 14 variable sites, 9 parsimony informative
295 sites and 11 possible insertions or deletions. The distribution of indels seemed to be diagnostic for
296 specific ITS-1 geographic variants, allowing, in particular, distinction between specimens from
297 Sicily and those from Malta and Gozo. Nucleotide composition was skewed towards an increase of
298 G+C (total = 59.1%).

299

300 PHYLOGENETIC RELATIONSHIPS

301 Parsimony analysis from 16S rDNA sequence data produced three equally parsimonious
302 trees (tree length=330, CI= 0.660, RI= 0.861) showing essentially the same topology.

303 The most appropriate model selected by MRMODELTEST was HKY+ Γ +I (Hasegawa, Kishino
304 & Yano, 1985). Plots of the $-\ln$ likelihood scores over generation time showed that stable parameter
305 estimates were obtained after approximately 300 trees (=30.000 generations). Therefore, only trees
306 sampled after this burn-in period were used to determine posterior probabilities of model
307 parameters (bpp), branch lengths and clades and to generate a 50% majority-rule consensus tree
308 with PAUP*. The analysis was repeated several times with the same settings and it always generated
309 similar results.

310 Parsimony and Bayesian analyses produced largely congruent results (Fig. 8), but with some
311 topological differences (Fig. 9). Overall, both methods separated the *Lampedusa* and *Muticaria*
312 haplotypes into two well distinct and supported lineages.

313 Populations grouped in the *Lampedusa* clade were further subdivided into two supported
314 assemblages corresponding to geographic areas. The first subclade grouped specimens from Malta
315 in two distinct lineages, corresponding to *L. melitensis* and *L. imitatrix*. The second subclade
316 grouped all specimens from Lampedusa (*L. lopadusae* / *L. l. lopadusae*) and Lampione islet (*L.*
317 *nodulosa* / *L. lopadusae nodulosa*). Remarkably, the three individuals from Lampione islet were
318 robustly distinguished from those of Lampedusa island.

319 Within representatives of *Muticaria*, both parsimony and Bayesian reconstructions clearly
320 defined three main groups. The first included all specimens from Sicily, resolved into two
321 subclades, one for *M. neuteboomi* (site CI) and the other for *M. syracusana* (site NA). A second
322 lineage included all individuals from Malta. Within this group, all specimens corresponding to
323 *oscitans* (sites MJ, MG and MG) were grouped in a distinct and well supported monophyletic
324 lineage. Specimens of *scalaris* (site MM) were also resolved as a separate monophyletic subgroup,
325 while individuals of *macrostoma* (site MA) were in two distinct lineages characterized by distantly
326 related haplotypes. The third main assemblage included all the specimens from Gozo. The
327 *macrostoma* specimens GM1 and the *mamotica* specimens GWc1 formed a supported and distinct
328 clade. According to the Bayesian analysis, these latter haplotypes (GM1 and GWc1) were
329 unresolved within the *Muticaria* assemblage. All the other specimens from Gozo were grouped in a
330 clade subdivided into two subclades, containing *oscitans* and *mamotica* haplotypes.

331 Relationships between the three main *Muticaria* groups were differently defined by the
332 Bayesian and Maximum Parsimony analyses (Fig. 9). Bayesian reconstructions placed haplotypes
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333 from Gozo as a sister group of the Sicilian + Maltese lineages, but with a scarce support (68%). On
334 the contrary, Parsimony analysis suggested the Sicilian clade as sister group of the Gozo + Malta
335 lineage (81%).

336 The median-joining network from the nuclear data set did not robustly distinguish the
337 *Muticaria* sequences as did mtDNA data, but recognised three major groups: Sicily (sites CI and
338 NA), south-east Malta (MJ, MG and MC) and north-west Malta + Gozo (Fig. 10). Populations
339 from Sicily (CA and NA) showed two distinct nuclear variants close to each other (three mutational
340 steps) and connected to those from southeastern Malta (9 mutational steps). The network was also
341 indicative of a clear split between populations from southern-central-western Malta (MJ, MG and
342 MC) and those from northwestern Malta (MMA and MM) + Gozo (GW, GS and GM).
343 Relationships within this latter group were not well resolved. The two specimens of *macrostoma*
344 from Malta (site MMA) shared the same sequence with an individual from Gozo (GWb2) and were
345 separated by one mutational step from *scalaris* from Malta (site MM). The remaining specimens
346 from Gozo were close to nuclear variants from Malta and showed an overall star-like pattern with
347 two most common nuclear variants shared among different local populations.

348

349 MITOCHONDRIAL SEQUENCE VARIATION

350 As for *Lampedusa*, haplotype sequence divergence (HKY distances) between *L. lopadusae*
351 and the population on Lampione islet was on average 0.03. Higher values were observed between *L.*
352 *melitensis* and *L. imitatrix* from Malta, on average 0.12. Divergence between the Maltese *L.*
353 *imitatrix* and *L. melitensis*, and *L. lopadusae*, was on average 0.20 and 0.22, respectively (similar
354 values with the population from Lampione Islet: 0.23 and 0.24).

355 As for *Muticaria*, haplotype sequence divergence between *M. siracusana* and *M.*
356 *neuteboomi* was on average 0.05. Divergences between Maltese *Muticaria* were on average: 0.07
357 between *macrostoma* and *oscitans*; 0.07 between *macrostoma* and *scalaris*; and 0.068 between
358 *oscitans* and *scalaris*. Divergences between *Muticaria* from Gozo were on average: 0.05 between
359 *macrostoma* and *oscitans*, and 0.03 between *oscitans* and *mamotica*.

360

361 Discussion

362 PHYLOGEOGRAPHIC RELATIONSHIPS

363 Phylogenetic analyses of the mitochondrial data (Fig. 8) clearly revealed that two well
364 distinct evolutionary lineages occur across the Sicily Channel corresponding to the genera
365 *Lampedusa* and *Muticaria* and that three parallel radiations might have occurred in the Maltese

366 archipelago, one involving *Lampedusa*, one the Gozitan *Muticaria*, and the other the Maltese
367 *Muticaria*.

368 These geographically structured lineages suggest that vicariance events may have played a
369 substantial role in the pattern of diversification in this geographic area. This is noticeable within the
370 *Lampedusa* lineage: taxa from Malta (*L. imitatrix* and *L. melitensis*) were well distinct,
371 morphologically and genetically, from those from Lampedusa and Lampione (*L. lopadusae* and *L.*
372 *nodulosa* or *L. l. lopadusae* and *L. l. nodulosa*). The disjunct distribution, the relatively high degree
373 of genetic divergence, and the morphological differences may support an ancient vicariant event for
374 the *Lampedusa* radiation, linked to the separation of the two island complexes (Pelagian and
375 Maltese groups).

376 Phylogenetic analyses revealed a geographical structure within the *Muticaria* lineage as
377 well. The two Sicilian *Muticaria* (*siracusana* and *neuteboomi*), which occur in south-eastern Sicily,
378 constituted a distinct, well supported clade. Despite geographic closeness of the two sites sampled
379 (about 20 km), both mitochondrial and nuclear data revealed a clear genetic distinction, already
380 evidenced by Colomba et al. (2010). These two populations also differ significantly in shell
381 characters although for some characters *neuteboomi* was not significantly different from some
382 Maltese *Muticaria*. The origin of the morphological and genetic divergence of the two south-eastern
383 Sicilian *Muticaria* clades remains unclear. A similar pattern in south-eastern Sicily was also found
384 in cyprinodontid freshwater fishes (Ferrito et al., 2007). It is possible that the geomorphology of
385 the area (a relatively high Cenozoic calcareous plateau deeply separated by incised valleys) could
386 have promoted fragmentation and isolation of populations. In fact, due to the extremely low vagility
387 and a neighbourhood population structure of land snails (Wright, 1946; Schilthuizen & Lombaerts,
388 1994; Fiorentino et al., 2009) rapid genetic and morphological differentiation, even in
389 geographically close populations, is not uncommon (Goodacre 2002; Uit De Weerd, Piel &
390 Gittenberger, 2004; Kameda, Kawakita & Kato, 2007).

391 As for the Maltese *Muticaria*, both *scalaris* and *oscitans* resulted monophyletic, while
392 *macrostoma* was separated in two lineages. The three morphotypes, as well as the intermediate
393 *macrostoma-oscitans* (for which genetic data were not available), were morphologically distinct
394 (*oscitans* by NR; *macrostoma* and *scalaris* by the ratio D/H). Since only few samples of
395 *macrostoma* were available, a fine local sampling is required to study the two *macrostoma* lineages
396 in more detail. As in Sicilian *Muticaria*, the Maltese *Muticaria* also show a pattern of fine
397 morphological and genetic geographical differentiation. In fact, the presence of morphologically
398 significant *macrostoma-oscitans* intermediates may indicate that Maltese *Muticaria* are structured
399 in demes across the island at a very local scale. A future more exhaustive sampling could unravel

400 morphological and genetic microgeographical variability (i.e. many distinct genetic lineages
401 corresponding to different morphotypes or a parapatric pattern between *macrostoma* and *oscitans*,
402 as already supposed by Holyoak, 1986; Giusti et al., 1995).

403 The three Gozitan *Muticaria* morphotypes (*macrostoma*, *mamotica* and *oscitans*) were
404 subdivided in three lineages, but with clear evidence of mixing in three cases: one *mamotica*
405 (GWC1) grouped with *macrostoma*; one *mamotica* (GWB1) grouped with *oscitans*; one *oscitans*
406 (GWA2) grouped with *mamotica*. Morphological analyses supported also this pattern of intermixed
407 clades of the two morphotypes in *mamotica* and *oscitans*. Thus, the *mamotica* - *oscitans* clades
408 showed a wide range of morphological variability, from the *oscitans* morphotype to the *mamotica*
409 morphotype, the causes of which are still unknown (isolation vs. selection). However, it is worth
410 noting that the *oscitans* and *mamotica* samples, which were mixed together in the same clades,
411 came from the same locality. Thus, the two morphotypes are sympatric but genetically not
412 monophyletic. This implies that the *mamotica* morphotypes appeared more than once and may be
413 an adaptation. Morphotype *macrostoma* was also grouped with one sample of *mamotica* according
414 to genetic analysis. Unfortunately, scarcity of *macrostoma* samples does not allow us to clarify this
415 pattern. In general, these results indicate a possible relatively recent differentiation of Gozitan
416 *Muticaria* or repetitive secondary contacts between different morphotypes. The latter hypothesis
417 should be further investigated.

418 Allopatric differentiation seems to be the main mechanism underlying the radiation of the
419 clausiilids across the Sicily Channel, although the sequence of events leading to the spread of the
420 group still remains unclear according to mitochondrial data. The two outgroups used in this study,
421 *Medora albescens* and *Leucostigma candidescens*, were resolved as distantly related taxa, providing
422 no useful information on the origin of the group. Mitochondrial data do not contain sufficient
423 phylogenetic signal to unequivocally infer the radiation within the *Muticaria* lineage, due to low
424 resolution at internal nodes (Bayesian Analysis) and contrasting results between Bayesian and
425 Parsimony reconstructions (Fig. 9). Difficulties in defining deep phylogenetic relationships in a tree
426 are generally related to the effects of early and rapid diversification. Cladogenetic events occurring
427 in close proximity might result in a lack of univocal phylogenetic signals, independent of the
428 marker used (Albertson et al., 1999). However, Median-Joining Network analysis on nuclear ITS-1
429 sequences across *Muticaria* specimens (Fig. 10) and Maximum Parsimony analysis (Fig. 9) showed
430 a closer relationship between haplotypes of Malta and Gozo than the Sicilian ones. Moreover,
431 haplotypes of Gozo are strictly related to those of Malta but not to Sicily. Thus, considering that
432 alopeiine clausiilids are a mainly SE Euro-Mediterranean group (Nordsieck, 2007), colonisation
433 events must have occurred through Sicilian corridors towards Malta and Gozo.

434 The origin of the Gozitan radiation from Malta is confirmed by the analysis of the nuclear
435 ITS1 sequences. In fact, two specimens of *macrostoma* from Malta (site MC) and one *mamotica*
436 from Gozo (site GWb2) shared the same nuclear variant. The most common nuclear variant present
437 on Gozo was only one mutational step away from that found at site MC on Malta. A similar pattern
438 is generally recognized as due to either lineage sorting of ancestral polymorphisms, or to gene flow
439 between different lineages, or a combination of the two, since the two mechanisms are not mutually
440 exclusive (Donnelly et al., 2004; Emerson & Oromí, 2005). In fact, shared haplotypes are randomly
441 maintained in certain populations through incomplete lineage sorting. Rapid and recent radiations
442 are consistent with this scenario since there would have been a short time for sorting of ancestral
443 haplotypes into the descendant taxa. The star-like topology of the nuclear network for sequences
444 from Gozo and the sharing of the same nuclear variant between specimens from Malta and Gozo
445 may support this explanation.

446

447 TAXONOMIC IMPLICATIONS

448 One goal of this study was to determine if *Muticaria* morphotypes previously described as
449 formal taxa represent distinct evolutionary units. In the case of *Muticaria* from Malta, we found
450 evidence supporting the monophyly of morphologically defined taxa such as *macrostoma*, *oscitans*
451 and *scalaris*. *Muticaria* from Gozo, instead, were resolved as polyphyletic. For example, Gozitan
452 specimens sampled at site GW, corresponding morphologically to *oscitans* and *mamotica*, were
453 grouped together in the same subclade.

454 Incongruence between molecular and morphological evidence is not uncommon for land
455 snails and has been repeatedly found even within the clausiliids. Repetitive parallel evolution of
456 shell structures has been described for the clausilial apparatus in species belonging to the genera
457 *Albinaria*, *Isabellaria*, and *Sericata* (Van Moorsel, Dijkstra & Gittenberger, 2000; Uit de Weerd et
458 al., 2004; Uit de Weerd & Gittenberger, 2005). Moreover, a study on *Albinaria* based on
459 mitochondrial data showed that specimens with strikingly different shell morphology (ribbed, semi-
460 ribbed or smooth) and traditionally considered as different subspecies, are characterized by identical
461 or very similar nucleotide sequences (Douris et al., 2007).

462 The phylogenetic results emerging from the present study indicate the need for a taxonomic
463 re-evaluation of the included taxa. This raises the question of defining species boundaries. Several
464 papers published in recent years and based wholly or partly on the same 16S rDNA region indicated
465 that there is considerable variation in genetic distances at intraspecific level. Within other
466 pulmonate species maximal sequence divergences have been reported i.e. 6% in *Cepaea nemoralis*
467 (Davison 2000), 14% in *Euhadra quaesita* (Watanabe & Chiba 2001), 10% in *Partula* spp.

468 (Goodacre 2002), 4.9% in *Candidula unifasciata* (Pfenninger & Posada 2002), and 23% in *Arion*
469 *subfuscus* (Pinceel et al. 2004). For clausiliids, intraspecific values generally lower than 10% have
470 been reported for populations of *Albinaria* spp., while distances between well distinct congeneric
471 species are in the range 11-18% (Douris et al. 1998). However, it is clear that defining species
472 boundaries cannot be reduced to a simple value of sequence divergence and other more valuable
473 factors such as population history, geographical distribution of lineages and the presence of
474 isolating barriers between them, should be taken in consideration.

475 *L. imitatrix* and *L. melitensis* may be considered distinct species, since they co-occur (not
476 sympatrically) on the same island, and are morphologically distinct evolutionary lineages with high
477 levels of genetic divergence (about 11%). On the other hand, the population of Lampione islet may
478 be classified as a geographic form within *L. lopadusae*. The population from Lampione islet,
479 however, is of particular conservation interest. Lampione is a small islet 700 m long by 180 m wide,
480 located about 17 km northwest of Lampedusa. The risk of extinction is presumably high for taxa
481 limited to very small areas such as the clausiliid population on Lampione. We stress that this
482 population represents an important pool of genetic diversity within the Pelagian *Lampedusa*, and
483 would argue for formal taxonomic recognition of this population (as *L. nodulosa* or *L. l. nodulosa*).

484 Considering the *Muticaria* lineage, phylogenetic relationships and observed mtDNA genetic
485 distances would suggest that a definitive assessment is still difficult to achieve. The two entities
486 occurring on Sicily can be considered two distinct taxa as they are morphologically and genetically
487 distinguishable: *M. neuteboomi* and *M. siracusana* or *M. s. neuteboomi* and *M. s. siracusana*.
488 Maltese *Muticaria* could be subdivided into three taxa according to morphological and molecular
489 data (clade support and genetic divergence): *M. macrostoma* or *M. m. macrostoma*, *M. oscitans* or
490 *M. m. oscitans* and *M. scalaris* or *M. m. scalaris*. Gozitan *Muticaria* could be considered a distinct
491 polytypic species (its oldest available name is *Muticaria mamotica*) subdivided into subspecies
492 showing a morphological range from *macrostoma*-like to *mamotica*-like and *oscitans* like.

493

494 The taxonomic setting has important implications for the conservation of the clausiliids of
495 the Sicilian Channel since legislation protecting species is usually based on recognizable taxa and
496 does not normally take into account particular populations or sub-populations.. The *IUCN Red List*
497 *of Threatened Species* designates *Lampedusa melitensis* as ‘Critically Endangered (B1+2c)’,
498 *Lampedusa imitatrix* as ‘Vulnerable (D2)’ and *Muticaria macrostoma* as ‘Lower Risk/near
499 threatened’ under its 1994 ‘IUCN Red List Categories and Criteria version 2.3’ based on
500 assessments made by Schembri (1996). Accession of new member states, including Malta, to the
501 European Union (EU) in 2004 resulted in amendments to the EU’s ‘*Council Directive 92/43/EEC of*
502 *21 May 1992 on the conservation of natural habitats and of wild fauna and flora*’, better known as
23/10/2017

503 the ‘Habitats Directive’, to include amongst many other species, *Lampedusa imitatrix* and
504 *Lampedusa melitensis* in Annexes II and IV. Annex II lists “Animal and plant species of
505 community interest whose conservation requires the designation of Special Areas of Conservation”,
506 while Annex IV lists “Animal and plant species of community interest in need of strict protection”.
507 In Annex II, *Lampedusa melitensis* is further designated a ‘priority species’. The IUCN does not list
508 any Italian species of *Lampedusa* and *Muticaria* and neither are any included in the EU’s ‘Habitats
509 Directive’.

510 Therefore, while the Maltese *Lampedusa* species are adequately protected by international
511 legislation (and also national legislation, since the requirements of the ‘Habitats Directive’ have
512 been transposed to Maltese legislation), none of the other species/subspecies/populations/sub-
513 populations of *Lampedusa* and *Muticaria* are, even if the areas occupied by some of these
514 genetically distinct entities are of a few tens to hundreds of square metres only (see Giusti et al.,
515 1995). Without formal taxonomic designations, it would be difficult to extend international legal
516 protection to some of the more threatened populations, such as the *Lampedusa* of Lampione islet,
517 the ‘*scalaris*’ population of Malta and the ‘*mamotica*’ populations of Gozo. In the interim period
518 until the formal taxonomy of these entities is worked out, one solution may be to designate the more
519 important and circumscribed populations of conservation importance as ‘evolutionarily significant
520 units’ (ESUs) sensu Waples (1991) or as ‘management units’ (MUs) sensu Moritz (1994). ESUs are
521 defined as populations that are reproductively separate from other populations and have unique or
522 different adaptations while MUs are sets of populations that are currently demographically
523 independent and which need to be managed independently of other populations for conservation
524 purposes. Green (2005) recommends recognizing ‘designatable units’ (DUs) where not all
525 populations of a species have the same probability of extinction, and therefore need different
526 management strategies. According to Green (2005), DUs must be distinguishable on the basis of
527 some morphological, genetic or distributional element and must have differing conservation status.
528 It can be argued that the *Lampedusa* of Lampione islet, the ‘*scalaris*’ population of Malta and the
529 ‘*mamotica*’ populations of Gozo qualify as ESUs, MUs and DUs on these criteria, particularly the
530 last, since in this case, populations designated as DUs need not be evolutionary units but are
531 determined by ecology and conservation status (Green, 2005, COSEWIC, 2009).

532 As already underlined (see discussion), since Maltese and Gozitan *Muticaria* were under-
533 sampled, it cannot be ruled out that a more exhaustive sampling in the area could show more
534 lineages or areas of secondary contacts (occurred in the past or still present) where different
535 morphotypes met. Thus, any scenario on the evolution of these clausiliids must be proposed with
536 caution, and tested by further research.

537

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542

543 **References**

- 544 ALBERTSON, R.C., MARKERT, J.A., DANLEY, P.D. & KOCHER, T.D. 1999. Phylogeny of a
545 rapidly evolving clade: the cichlid fishes of Lake Malawi, East Africa. *Proceedings of the*
546 *National Academy of Sciences USA*, **96**(9): 5107-5110.
- 547 ALONSO, M.R., GOODACRE, S.L., EMERSON, B.C., IBÁÑEZ, M., HUTTERER, R. & GROH,
548 K. 2006. Canarian land snail diversity: conflict between anatomical and molecular data on the
549 phylogenetic placement of five new species of *Napaeus* (Gastropoda, Pulmonata, Enidae).
550 *Biological Journal of the Linnean Society*, **89**: 169-187.
- 551 ANDRÉ, J.P., CORNEE, J.J., SAINT MARTIN, J.P., LOPAINTE, P. 2002. Organisation
552 séquentielle de la plate-forme carbonatée messinienne du seuil pélagien à Lampedusa
553 (Méditerranée centrale). *Geodiversitas*, **24**: 625-639.
- 554 BANDELT, H.J., FORSTER, P. & RÖHL, A. 1999. Median-joining networks for inferring
555 intraspecific phylogenies. *Molecular Biology and Evolution*, **16**: 37-48.
- 556 BARD, E., HAMELIN, B & FAIRBANKS, R.G. 1990. U-Th ages obtained by mass spectrometry
557 in corals from Barbados: sea level during the past 130,000 years. *Nature*, **346**: 456-458.
- 558 BRITISH ADMIRALTY 2005. *Chart 3403 Cap Afrique to Misratah, 1:500000*.
- 559 BRITISH ADMIRALTY 2010. *Chart 2124 Isola di Lampedusa to Capo Passero including Malta,*
560 *1:300000*.
- 561 CARUSO, T., NOTO LA DIEGA, R., BERNINI, F. 2005. The effects of spatial scale on the
562 assessment of soil fauna diversity: data from the oribatid mite community of the Pelagian
563 Islands (Sicilian Channel, southern Mediterranean). *Acta Oecologica*, **28**: 23-31.
- 564 CIESM 2008. The Messinian Salinity Crisis from mega-deposits to microbiology - a consensus
565 report. *CIESM Workshop Monographs*, **33**: 1-168.
- 566 COLOMBA, M.S., GREGORINI, A., LIBERTO, F., REITANO, A., GIGLIO, S. & SPARACIO, I.
567 2010. Molecular analysis of *Muticaria syracusana* and *M. neuteboomi* from Southeastern
568 Sicily, Italy (Gastropoda, Pulmonata, Clausiliidae). *Biodiversity Journal*, **1**: 7-14.
- 569 COSEWIC 2009. Guidelines for recognizing Designatable Units [Approved by COSEWIC in
570 November 2009]. Committee on the Status of Endangered Wildlife in Canada

- 571 http://www.cosewic.gc.ca/eng/sct2/sct2_5_e.cfm [Last updated 08 Dec 2010] Downloaded on
572 01 August 2011.
- 573 DAVISON, A. 2000. An east-west distribution of divergent mitochondrial haplotypes in British
574 populations of the landsnail, *Cepaea nemoralis* (Pulmonata). *Biological Journal of the Linnean*
575 *Society*, **70**: 697-706.
- 576 DONNELLY, M.J., PINTO, J., GIROD, R., BESANSKY N.J. & LEHMANN, T., 2004. Revisiting
577 the role of introgression vs shared ancestral polymorphisms as key processes shaping genetic
578 diversity in the recently separated sibling species of the *Anopheles gambiae* complex. *Heredity*,
579 **92**: 61-68.
- 580 DOURIS, V., CAMERON, R.A.D., RODAKIS, G.C., & LECANIDOU, R. 1998. Mitochondrial
581 phylogeography of the land snail *Albinaria* in Crete: long-term geological and short-term
582 vicariance effects. *Evolution*, **52**: 116-125.
- 583 EMERSON, B.C. & OROMÍ, P., 2005. Diversification of the forest beetle genus *Tarphius* on the
584 Canary Islands, and the evolutionary origins of island endemics. *Evolution*, **59**: 586-598.
- 585 FARRIS, J.S. 1970. Methods for computing Wagner trees. *Systematic Zoology*, **18**: 374-385.
- 586 FELSENSTEIN, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap.
587 *Evolution*, **39**: 783-791.
- 588 FERLAND, M.A., ROY, P.S. & MURRAY-WALLACE, C.V. 1995 Glacial lowstand deposits on
589 the outer continental shelf of southeastern Australia. *Quaternary Research*, **44**: 294-299.
- 590 FERRITO, V., MANNINO, M.C., PAPPALARDO, A.M. & TIGANO C., 2007. Morphological
591 variation among populations of *Aphanius fasciatus* Nardo, 1827 (Teleostei, Cyprinodontidae)
592 from the Mediterranean. *Journal of Fish Biology*, **70**: 1-20.
- 593 FIORENTINO, V., CARUSO, T., MANGANELLI, G. & GIUSTI, F., 2009. Population dynamics
594 of an urban population of the land snail *Marmorana serpentina* (Gastropoda: Pulmonata).
595 *Malacologia*, **51**: 201-209.
- 596 FLEMMING, N.C., BAILEY, G.N., COURTILLOT, V., KING, G., LAMBECK, K., RYERSON,
597 F. & VITA-FINZI, C. 2003. *Coastal and marine palaeoenvironments and human dispersal*
598 *points across the Africa-Eurasia boundary*. *Maritime Heritage*. Wessex Institute of
599 Technology, University of Malta, Malta.
- 600 FRANCI CORTI, E. & LANZA, B. 1972. XVIII Congresso della Società Italiana di
601 Biogeografia: note conclusive sulla storia del popolamento animale e vegetale delle isole
602 circumsiciliane. *Lavori della Società Italiana di Biogeografia Nuova Serie*, **3**: 911-918.

- 603 GANTENBEIN, B., FET, V., LARGIADER, C.R. & SCHOLL, A. 1999. First DNA phylogeny of
604 *Euscorpius* Thorell, 1876 (Scorpiones, Euscorpiidae) and its bearing on taxonomy and
605 biogeography of this genus. *Biogeographica (Paris)*, **75**: 49-65.
- 606 GATT, P.A. 2007. Controls on Plio-Quaternary foreland sedimentation in the Region of the Maltese
607 Islands. *Bollettino della Società Geologica Italiana*, **126**: 119-129.
- 608 GIRAUDI C., 2004. The Upper Pleistocene to Holocene sediments on the Mediterranean island of
609 Lampedusa (Italy). *Journal of Quaternary Science*, **19**: 537-547.
- 610 GIUSTI, F., MANGANELLI, G. & SCHEMBRI, P.J. 1995. The non-marine molluscs of the
611 Maltese Islands. *Monografie Museo Regionale di Scienze Naturali (Torino)*, **15**: 1-607.
- 612 GOODACRE, S.L., 2002. Population structure, history and gene flow in a group of closely related
613 land snails: genetic variation in *Partula* from the Society Islands of the Pacific. *Molecular*
614 *Ecology*, **11**: 55-68.
- 615 GOODACRE, S.L. & WADE, C.M. 2001. Molecular evolutionary relationships between partulid
616 land snails of the Pacific. *Proceedings of the Royal Society of London series B*, **268**: 1-8.
- 617 GRASSO, M. & PEDLEY, H.M. 1985. The Pelagian Islands: a new geological interpretation from
618 sedimentological and tectonic studies and its bearing on the evolution of the Central
619 Mediterranean Sea (Pelagian Block). *Geologica Romana*, **24**: 13-44.
- 620 GRASSO, M., PEDLEY, H.M. & REUTHER, C.-D. 1985. The geology of the Pelagian Islands and
621 their structural setting in relation to the Pantelleria rift (Central Mediterranean Sea). *Centro*
622 *[Malta]*, **1** (2): 1-19.
- 623 GREEN, D.M. 2005. Designatable units for status assessment of endangered species. *Conservation*
624 *Biology*, **19**: 1813-1820.
- 625 HALL, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis
626 program for Windows 95/98/NT. *Nucleic Acids Symposium Series*, **41**:95-98.
- 627 HASEGAWA, M., KISHINO, H. & YANO, T. 1985. Dating of the human-ape splitting by a
628 molecular clock of mitochondrial DNA. *Journal of Molecular Evolution*, **21**: 160-174.
- 629 HILLIS, D.M., MABLE, B.K., LARSON, A., DAVIS, S.K. & ZIMMER, E.A. 1996. Nucleic acids
630 IV: sequencing and cloning. In: *Molecular Systematics* (D.M. Hillis, C. Moritz & B.K. Mable,
631 eds), pp. 321-381. Sinauer Associates, Sunderland, MA.
- 632 HOLYOAK, D.T. 1986. Biological species limits and systematics of the Clausiliidae of the Maltese
633 Islands (Gastropoda). *Journal of Conchology*, **32**: 211-220.
- 634 HSÜ, K.L., RYAN, W.B.F. & CITA, M.B. 1973. Late Miocene dessication of the Mediterranean.
635 *Nature*, **242**: 240-244.

- 636 HUELSENBECK, J.P. & RONQUIST, F. 2001. MRBAYES: Bayesian inference of phylogeny.
637 *Bioinformatics*, **17**: 754-755.
- 638 HUNT, C.O. & SCHEMBRI, P.J. 1999. Quaternary environments and biogeography of the Maltese
639 Islands. In: *Facets of Maltese prehistory* (A. Mifsud. & C. Savona Ventura, eds), pp. 41-75. The
640 Prehistoric Society of Malta, Malta.
- 641 JOHNSON, M.S., MURRAY, J. & CLARKE, B. 1993. The ecological genetics and adaptive
642 radiation of *Partula* on Moorea. *Oxford Surveys in Evolutionary Biology*, **9**: 167-238.
- 643 JORDAENS, K., VAN RIEL, P., FRIAS MARTINS, A.M. & BACKELJAU, T. 2009. Speciation
644 on the Azores islands: congruent patterns in shell morphology, genital anatomy, and molecular
645 markers in endemic land snails (Gastropoda, Leptaxinae). *Biological Journal of the Linnean
646 Society*, **97**: 166-176.
- 647 KAMEDA, Y., KAWAKITA, A. & KATO, M., 2007. Cryptic genetic divergence and associated
648 morphological differentiation in the arboreal land snail *Satsuma (Luchuhadra) largillierti*
649 (Camaenidae) endemic to the Ryukyu Archipelago, Japan. *Molecular Phylogenetics and
650 Evolution*, **45**: 519-533.
- 651 KRIJGSMAN, W., 2002. The Mediterranean: the *Mare Nostrum* of Geology. *Earth and Planetary
652 Science Letters*, **205**: 1-12.
- 653 KRIJGSMAN, W., HILGEN, F.J., RAFFI, I., SIERRO, F.J. & WILSON, D.S. 1999. Chronology,
654 causes and progression of the Messinian Salinity Crisis. *Nature*, **400**: 652-655.
- 655 MASSA, B. 1995. Considerazioni conclusive sui popolamenti e sulla loro possibile origine. In:
656 *Arthropoda di Lampedusa, Linosa e Pantelleria (Canale di Sicilia, Mar Mediterraneo)* (R.
657 Massa, ed.), pp. 825-870. *Naturalista Siciliano*, **19** (suppl.).
- 658 MAYR, E. 1963. *Animal Species and Evolution*. Belknap Press, Cambridge.
- 659 MORITZ, C. 1994. Defining 'Evolutionarily Significant Units' for conservation. *Trends in Ecology
660 and Evolution*, **9**: 373-375.
- 661 NORDSIECK, H. 200. Worldwide Door Snails (Clausiliidae), recent and fossil, ConchBooks,
662 Hackenheim, Germany.
- 663 NYLANDER, J.A.A. 2004. *Mrmodeltest v2. Program distributed by the author*. Evolutionary
664 Biology Centre, Uppsala: Uppsala University, Uppsala.
- 665 PEDLEY, H.M. 1990. Syndepositional tectonics affecting Cenozoic and Mesozoic deposition in the
666 Malta and SE Sicily areas (Central Mediterranean) and their bearing on Mesozoic reservoir
667 development in the N Malta offshore region. *Marine and Petroleum Geology*, **7**: 171-180.
- 668 PEDLEY H.M., HOUSE, M.R. & WAUGH, B. 1976. The geology of Malta and Gozo.
669 *Proceedings of the Geologists' Association*, **87**: 325-341.

- 670 PEDLEY, H.M., HOUSE, M.R. & WAUGH, B. 1978. The geology of the Pelagian Block: the
671 Maltese Islands. In: *The ocean basins and margins*. Vol 4B *The western Mediterranean*
672 (A.E.M. Nairn, W.H. Kanes & F.G. Stehli, eds), pp. 417-433. Plenum Press, London.
- 673 PEDLEY, M., HUGHES CLARKE, M. & GALEA, P. 2002. *Limestone isles in a crystal sea. The*
674 *geology of the Maltese Islands*. Publishers Enterprises Group, San Gwann, Malta.
- 675 PEDLEY, H.M., GRASSO, M., MANISCALCO, R. & ESU, D. 2007. The Monte Carrubba
676 Formation (Messinian, Sicily) and its correlatives: new light on basin-wide processes
677 controlling sediment and biota distributions during the Palaeomediterranean-Mediterranean
678 transition. *Palaeogeography, Palaeoclimatology, Palaeoecology*, **253**, 363-384.
- 679 PFENNINGER, M. & POSADA, D., 2002. Phylogeographic history of the land snail *Candidula*
680 *unifasciata* (Helicellinae, Stylommatophora): fragmentation, corridor migration, and secondary
681 contact. *Evolution*, **56**: 1776-1788.
- 682 PINCEEL, J., JORDAENS, K., PFENNINGER, M. & BACKELJAU, T., 2005. Rangewide
683 phylogeography of a terrestrial slug in Europe: evidence for Alpine refugia and rapid
684 colonization after the Pleistocene glaciations. *Molecular Ecology*, **14**: 1133-1150.
- 685 R DEVELOPMENT CORE TEAM 2006. *R: a language and environment for statistical computing*.
686 R Foundation for Statistical Computing, Vienna, Austria (Available at: [http://www.R-](http://www.R-project.org)
687 [project.org](http://www.R-project.org))
- 688 REYES-TUR, B., FERNÁNDEZ, A. & SUÁREZ, M. 2001. Shell colour polymorphism in two
689 populations of *Polymita picta nigrolimbata* from Baracoa, Guantánamo. *Reviews in Biology*,
690 **15**: 75-78.
- 691 ROHLING, E.J., FENTON, M., JORISSEN, F.J., BERTRAND, P., GANSSEN, G., CAULET, J.P.
692 1998. Magnitudes of sea level lowstands of the past 500,000 years. *Nature*, **394**:162-165.
- 693 RONQUIST, F. & HUELSENBECK, J.P. 2003. MrBayes 3: Bayesian phylogenetic inference under
694 mixed models. *Bioinformatics*, **19**, 1572-1574.
- 695 SCHEMBRI, P.J. 1996. *Lampedusa melitensis, Lampedusa imitatrix, Muticaria macrostoma*. In:
696 IUCN 2011. IUCN Red List of Threatened Species. Version 2011.1. <www.iucnredlist.org>.
697 Downloaded on 01 August 2011.
- 698 SCHEMBRI, P.J. 1997. The Maltese Islands: climate, vegetation and landscape. *GeoJournal*, **41**: 115-
699 125.
- 700 SCHEMBRI, P.J. 2003. Current state of knowledge of the Maltese non-marine fauna. In: *Annual*
701 *report and accounts 2003* (Malta Environment and Planning Authority, ed.). pp. 33-65. Malta
702 Environment and Planning Authority, Floriana, Malta.
- 703 SCHILTHUIZEN, M. & M. LOMBAERTS, 1994. Population structure and level of gene flow in
704 *Albinaria corrugata*. *Evolution*, **48**: 577-586.

- 705 SCHLÖTTERER, C., HAUSER, M.T., VON HAESELER, A. & TAUTZ, D. 1994. Comparative
706 evolutionary analysis of rDNA ITS regions in *Drosophila*. *Molecular Biology and Evolution*,
707 **11**: 513-522.
- 708 SIMON, C., FRATI, F., BECKENBACH, A.T., CRESPI, C., LIU, H. & FLOOK, P. 1994.
709 Evolution, weighting and phylogenetic utility of mitochondrial gene sequences and a
710 compilation of conserved polymerase chain reaction primers. *Annals of the Entomological*
711 *Society of America*, **87**: 651-701.
- 712 SIMPSON, G.G. 1940 Mammals and land bridges. *Journal of the Washington Academy of Science*
713 **30**: 137-163.
- 714 STÖCK, M., SICILIA, A., BELFIORE, N.M., BUCKLEY, D., LO BRUTTO, S., LO VALVO, M.,
715 & ARCULEO, M. 2008. Post-Messinian evolutionary relationships across the Sicilian channel:
716 mitochondrial and nuclear markers link a new green toad from Sicily to African relatives. *BMC*
717 *Evolutionary Biology*, **8**: 56 doi:10.1186/1471-2148-8-56.
- 718 SWOFFORD, D.L. 2001. *PAUP*: phylogenetic analysis using parsimony (*and other methods)*,
719 *vers. 4*. Sinauer Associates, Sunderland, MA.
- 720 THAKE, M.A. 1985a. The biogeography of the Maltese islands, illustrated by the Clausiliidae.
721 *Journal of Biogeography*, **12**:269-287.
- 722 THIEDE, J. 1978. A glacial Mediterranean. *Nature*, **276**: 680-683.
- 723 THOMPSON, J.D., GIBSON, T.J., PLEWNIAK, F., JEANMOUGIN, F. & HIGGINS, D.G. 1997.
724 The CLUSTAL X window interface: flexible strategies for multiple sequence alignment aided by
725 quality analysis tools. *Nucleic Acids Research*, **25**: 4876-4882.
- 726 TORELLI, L., GRASSO, M., MAZZOLDI, Q., PEIS, D. & GORI, D. 1995. Cretaceous to Neogene
727 structural evolution of the Lampedusa shelf (Pelagian Sea, Central Mediterranean). *Terra Nova*,
728 **7**: 200-212.
- 729 UIT DE WEERD, D.R. & GITTENBERGER, E., 2005a. Towards a monophyletic genus *Albinaria*
730 (Gastropoda, Pulmonata): the first molecular study into the phylogenetic position of eastern
731 *Albinaria* species. *Zoological Journal of the Linnean Society*, **143**: 531-542.
- 732 UIT DE WEERD, D.R., PIEL, W.H. & GITTENBERGER, E., 2004. Widespread polyphyly among
733 Aloiinae snail genera: when phylogeny mirrors biogeography more closely than morphology.
734 *Molecular Phylogenetics and Evolution*, **33**: 533-548.
- 735 VAN MOORSEL, C.H.M., DIJKSTRA, E.G.M. & GITTENBERGER, E. 2000. Molecular
736 Evidence for repetitive parallel evolution of shell structure in Clausiliidae (Gastropoda,
737 Polmonata). *Molecular Phylogenetics and Evolution*, **17**: 200-208.

- 738 VAN RIEL, P., JORDAENS, K., VERHAGEN, R., FRIAS MARTINS, A.M. & BACKELJAU, T.
739 2003. Genetic differentiation reflects geological history in the Azorean land snail, *Leptaxis*
740 *azorica*. *Heredity* **91**: 239-247.
- 741 WAPLES, R. S. 1991. Pacific salmon, *Oncorhynchus* spp., and the definition of 'species' under the
742 Endangered Species Act. *Marine Fisheries Review*, **53**: 11-22.
- 743 WATANABE, Y. & CHIBA, S., 2001. High within-population mitochondrial DNA variation due to
744 microvicariance and population mixing in the land snail *Euhadra quaesita* (Pulmonata:
745 Bradybaenidae). *Molecular Ecology*, **10**: 2635-2645.
- 746 WRIGHT, S., 1946. Isolation by distance under diverse systems of mating. *Genetics*, **31**: 39-59.
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749 **Captions to figures**

750

751 **Figure 1.** Representatives of the alopiine clausiliids of the Sicilian Channel: from left to right:

752 *Lampedusa lopadusae*, *Lampedusa imitatatrix*, *Lampedusa melitensis*, *Muticaria macrostoma* morph

753 *scalaris*, *Muticaria macrostoma* morph *macrostoma*, *Muticaria macrostoma* morph *oscitans* and

754 *Muticaria macrostoma* morph *mamotica*, according to current taxonomy (Giusti et al., 1995).

755 **Figure 2.** Distribution of *Muticaria* morphs in the Maltese Islands (modified from Thake, 1985).

756 Filled square, *mamotica*; empty square *scalaris*; filled circles *oscitans*; empty circles *macrostoma*;

757 half-filled circles, *macrostoma-oscitans* (population of difficult determination).

758 **Figure 3.** Sampling locations used in this study. See Table 1 for abbreviations.

759 **Figure 4.** Shell shapes and measurements. Abbreviations: D, diameter; H, height.

760 **Figure 5.** Distal genitalia of *Muticaria*: outline and measurements. Abbreviations: BC bursa

761 copulatrix, DBC diverticulum of bursa copulatrix duct, DEP distal epiphallus, P penis, PBCD

762 proximal bursa copulatrix duct, PC penial caecum, PEP proximal epiphallus, V vagina.

763 **Figure 6.** Box plot of the number of ribs in *Lampedusa*.

764 **Figure 7.** Box plot of the number of ribs in *Muticaria*.

765 **Figure 8.** Consensus tree (50% majority rules) from Bayesian analysis based on mitochondrial 16S

766 sequence data. Numbers at nodes represent MP bootstrap values >70% and posterior clades

767 probability of each clade.

768 **Figure 9.** Comparison between consensus trees (50% majority rules) from Bayesian analysis and

769 Maximum Parsimony analysis based on mitochondrial 16S sequence data. Numbers at basal nodes

770 of *Muticaria* clades represent posterior clade probability and MP bootstrap values.

771 **Figure 10.** Median-joining network based on nuclear ITS-1 sequences across the *Muticaria*

772 specimens. Numbers represent variable positions at sequences of the studied species. Diameter of

773 the circles is directly proportional to the frequency and colours refer to geographical origin of the

774 specimens (Red: Malta, Blue: Gozo, Green: Sicily). Codes are as in Table 1.

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777 **Captions to tables.**

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779 **Table 1.** Sampling locations and Genbank accession numbers.

780 **Table 2.** Two-way analysis of variance (ANOVA) of the number of ribs on shells of *Lampedusa*.

781 **Table 3.** Two-way analysis of variance (ANOVA) of the D/H ratio in *Lampedusa*.

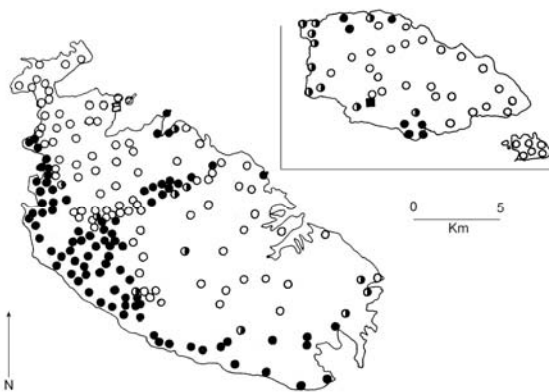
782 **Table 4.** Two-way analysis of variance (ANOVA) of the number of ribs on shells of *Muticaria*.

783 **Table 5.** Two-way analysis of variance (ANOVA) of the D/H ratio in *Muticaria*.

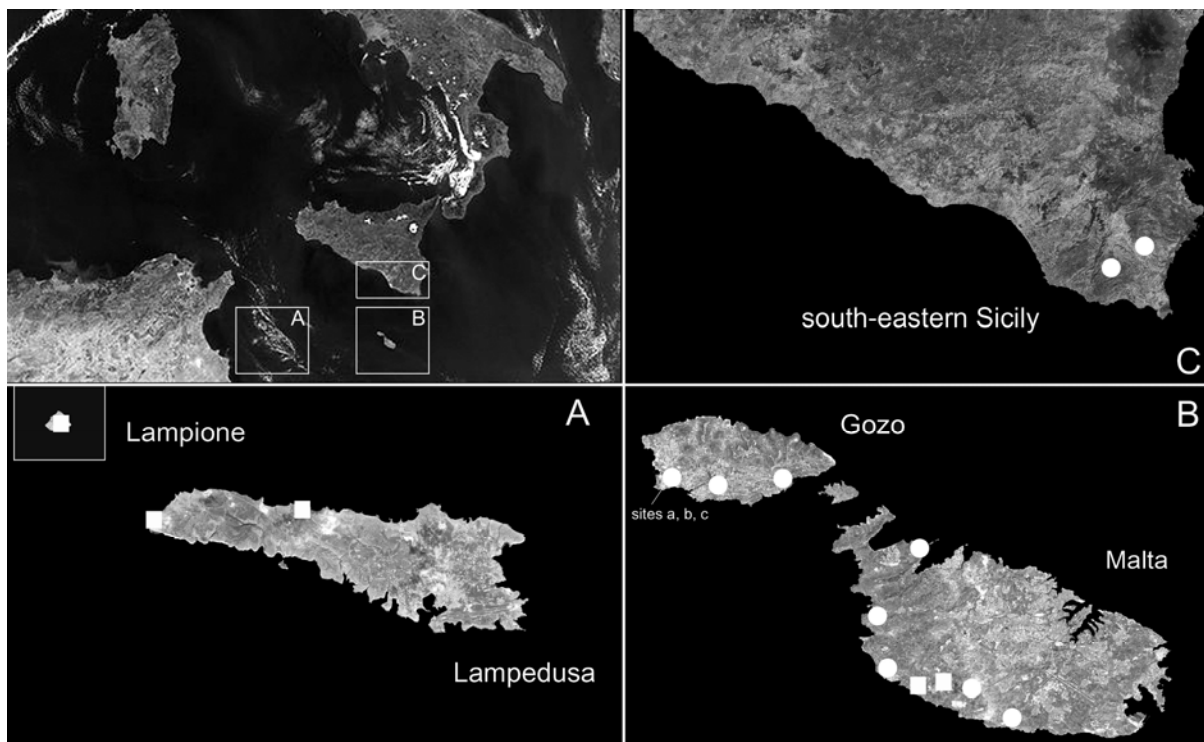
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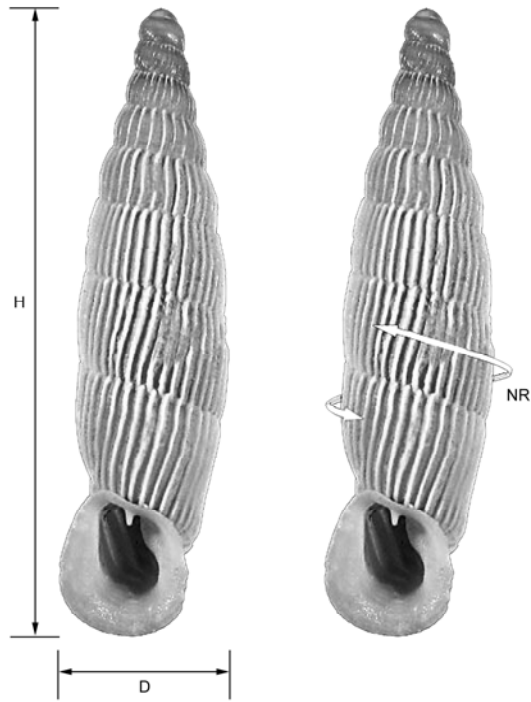
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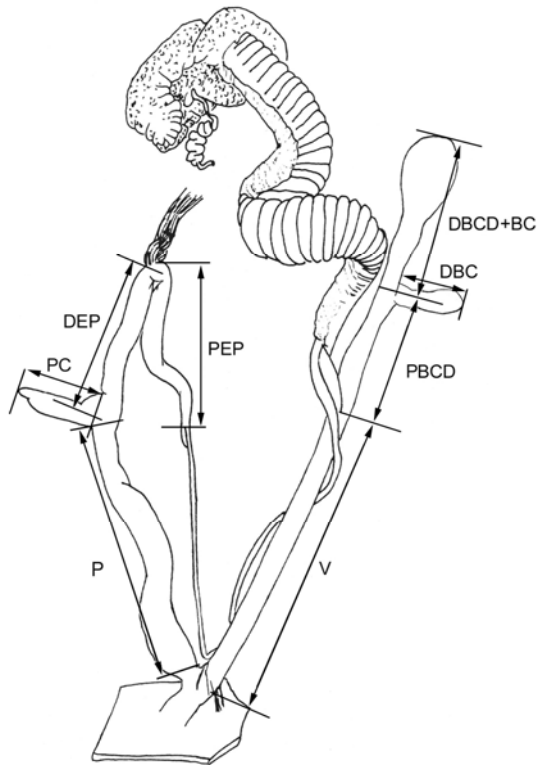
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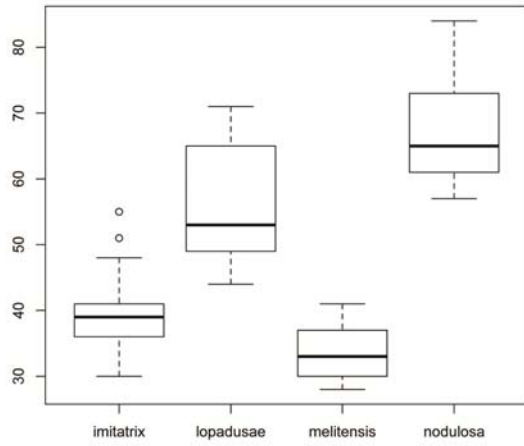
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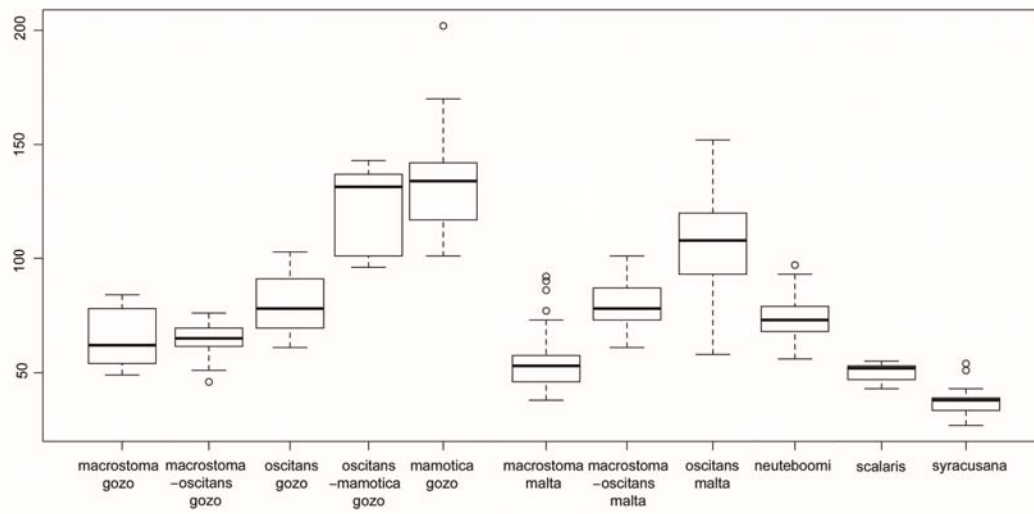
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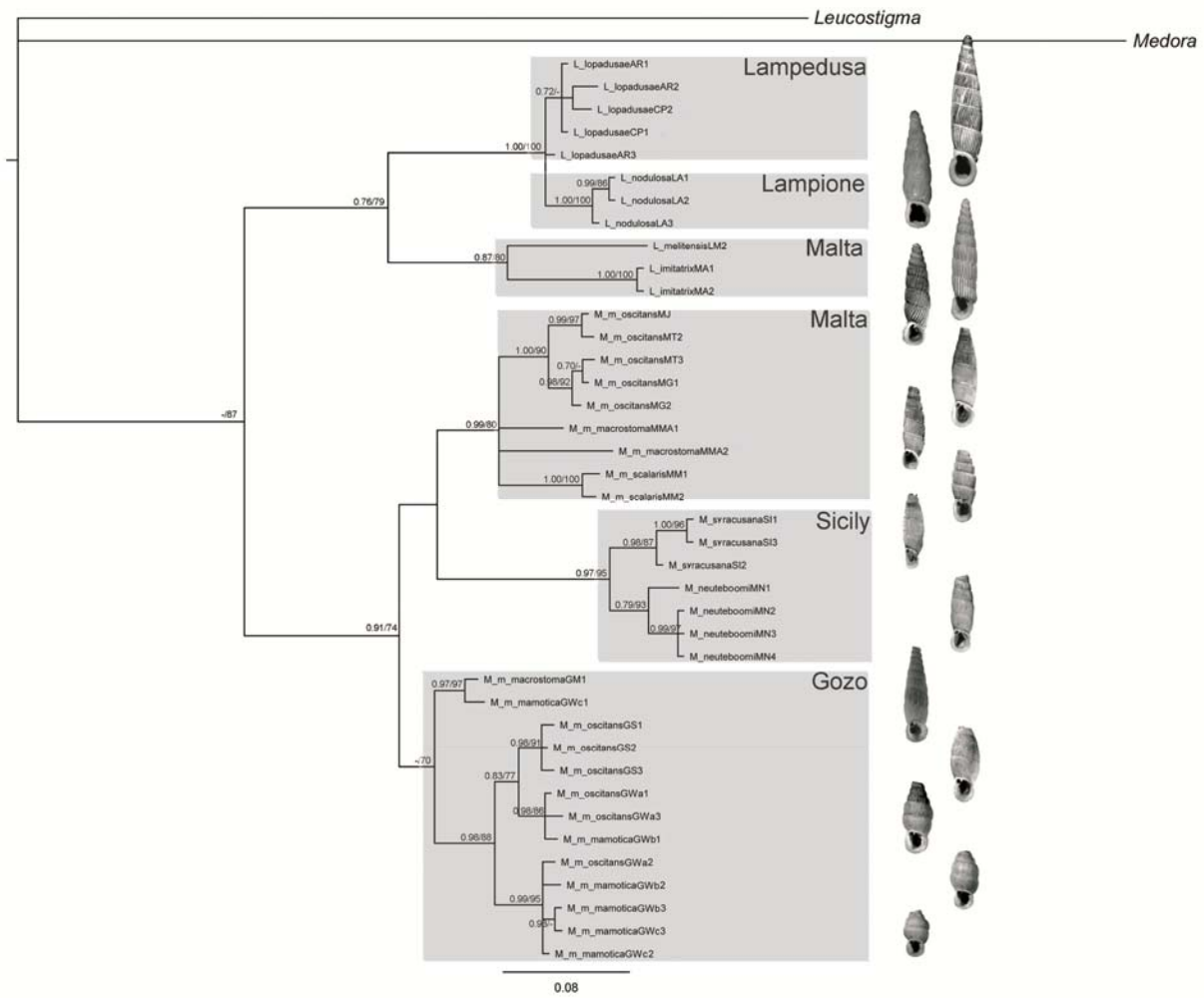
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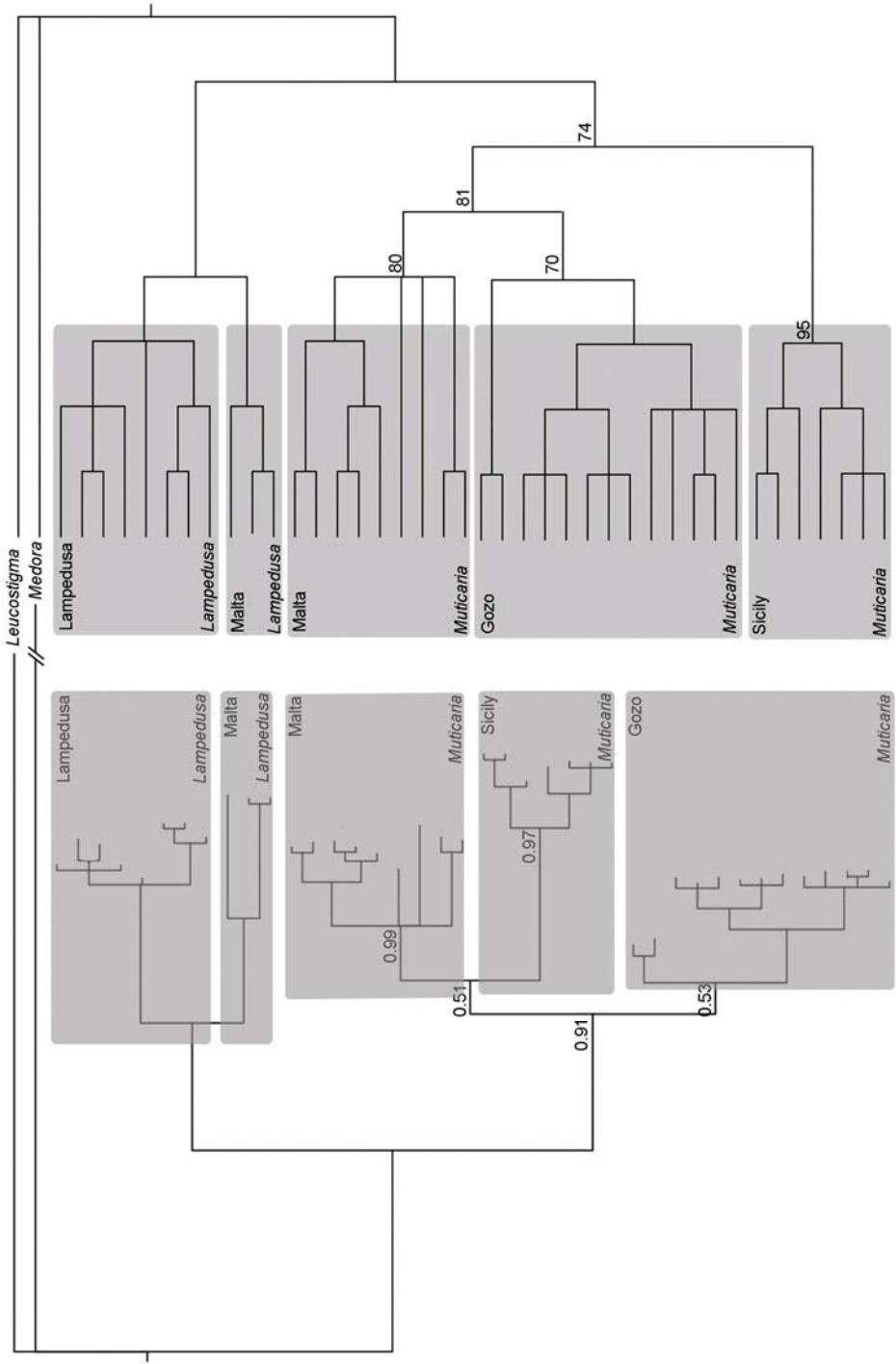
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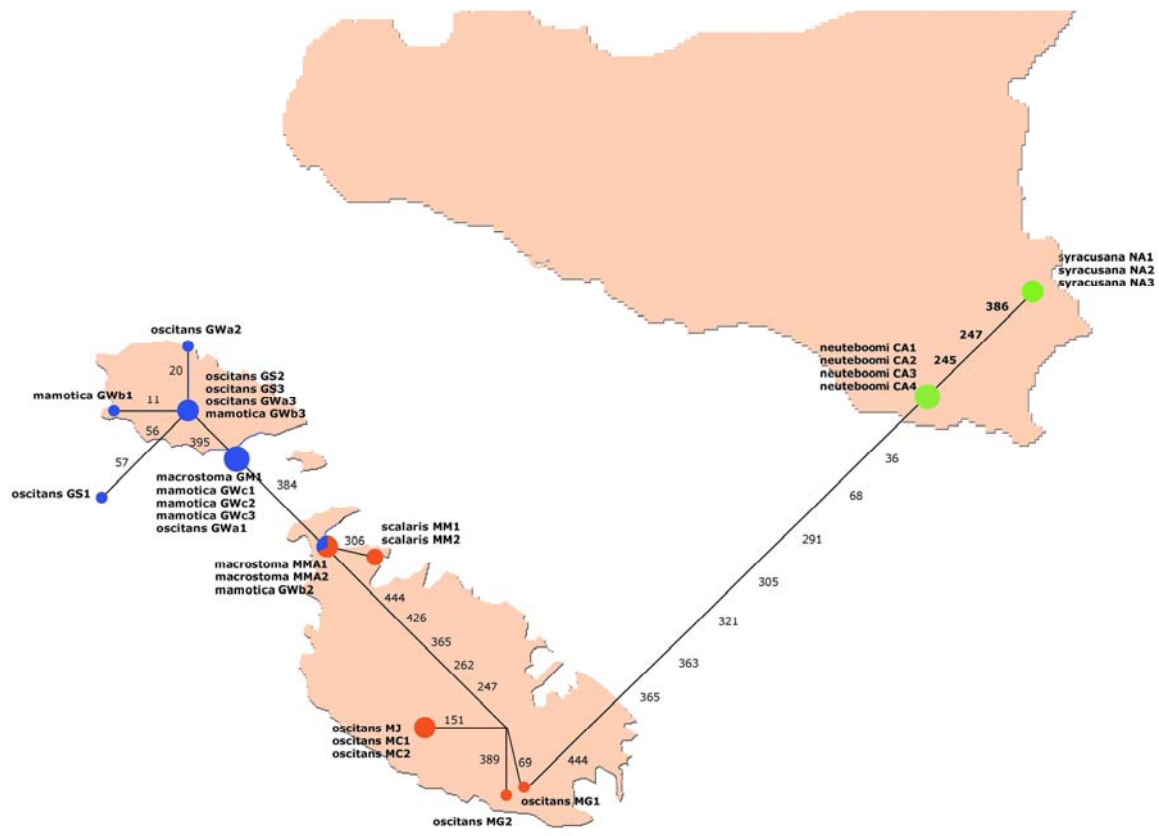
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797 Table 1. Material examined. FGC: F. Giusti collection inventory number, Department of Evolutive Biology, University of Siena. Collectors: AD
 798 Alan Deidun, ET Enrico Talenti, FG Folco Giusti, GM Giuseppe Manganelli, JD Joseph Debono, PJS Patrick J. Schembri, RG Rosario Grasso, SC
 799 Simone Cianfanelli, VF Viviana Fiorentino

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FGC	Current taxonomy (Giusti et al., 1995)	revised taxonomy	Acronym	Locality, collectors and date	Gen bank accession numbers	
MALTA						
35573	<i>Muticaria macrostoma</i> morph <i>oscitans</i>	<i>M. oscitans</i> or <i>M. macrostoma oscitans</i>	MJ	Migra Ferha (car park), AD leg. 11.02.04	16S	ITS-1
35575	<i>Muticaria macrostoma</i> morph <i>oscitans</i>	<i>M. oscitans</i> or <i>M. macrostoma oscitans</i>	MC1	Clapham Junction, AD leg. 11.02.04	16S	ITS-1
35575	<i>Muticaria macrostoma</i> morph <i>oscitans</i>	<i>M. oscitans</i> or <i>M. macrostoma oscitans</i>	MC2	Clapham Junction, AD leg. 11.02.04	16S	ITS-1
35738	<i>Muticaria macrostoma</i> morph <i>oscitans</i>	<i>M. oscitans</i> or <i>M. macrostoma oscitans</i>	MG1	Ghar Lapsi (Wied Hoxt), AD & JD leg. 04.05.04	16S	ITS-1
35738	<i>Muticaria macrostoma</i> morph <i>oscitans</i>	<i>M. oscitans</i> or <i>M. macrostoma oscitans</i>	MG2	Ghar Lapsi (Wied Hoxt), AD & JD leg. 04.05.04	16S	ITS-1
35740	<i>Muticaria macrostoma</i> morph <i>macrostoma</i>	<i>M. macrostoma</i> or <i>M. macrostoma macrostoma</i>	MMA1	Mgarr (San Martin Valley), AD & JD leg. 30.04.04	16S	ITS-1
35740	<i>Muticaria macrostoma</i> morph <i>macrostoma</i>	<i>M. macrostoma</i> or <i>M. macrostoma macrostoma</i>	MMA2	Mgarr (San Martin Valley), AD & JD leg. 30.04.04	16S	ITS-1
35743	<i>Muticaria macrostoma</i> morph <i>scalaris</i>	<i>M. scalaris</i> or <i>M. macrostoma scalaris</i>	MM1	Mistra (Harrieq), AD & JD leg. 30.04.04	16S	ITS-1
35743	<i>Muticaria macrostoma</i> morph <i>scalaris</i>	<i>M. scalaris</i> or <i>M. macrostoma scalaris</i>	MM2	Mistra (Harrieq), AD & JD leg. 30.04.04	16S	ITS-1
33261	<i>Lampedusa melitensis</i>	<i>Lampedusa melitensis</i>	LM	Rdum Il-Maddalena, FG, GM & PJS leg. 27.11.87 (alcohol specimen)	16S	

23/10/2017

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35574	<i>Lampedusa imitatrix</i>	<i>Lampedusa imitatrix</i>	MP1	Il-Qaws on the Migra Ferha plateau, AD leg. 22.02.04	16S	
35574	<i>Lampedusa imitatrix</i>	<i>Lampedusa imitatrix</i>	MP2	Il-Qaws on the Migra Ferha plateau, AD leg. 22.02.04	16S	
GOZO						
35739	<i>Muticaria macrostoma</i> morph <i>oscitans</i>	<i>M. mamotica</i> ssp 2	GS1	Sannat, Ta' Cenc, AD & JD leg. 23.04.04	16S	ITS-1
35739	<i>Muticaria macrostoma</i> morph <i>oscitans</i>	<i>M. mamotica</i> ssp 2	GS2	Sannat, Ta' Cenc, AD & JD leg. 23.04.04	16S	ITS-1
35739	<i>Muticaria macrostoma</i> morph <i>oscitans</i>	<i>M. mamotica</i> ssp 2	GS3	Sannat, Ta' Cenc, AD & JD leg. 23.04.04	16S	ITS-1
35741	<i>Muticaria macrostoma</i> morph <i>macrostoma</i>	<i>M. mamotica</i> ssp 1	GM1	Mgarr Harbour, AD & JD leg. 23.04.04	16S	ITS-1
36440	<i>Muticaria macrostoma</i> morph <i>oscitans</i>	<i>M. mamotica</i> ssp 2	GWa1	Wied ix-Xlendi (II-Fekruna), AD & PJS leg. 05.10.05 (<i>site A</i>)	16S	ITS-1
36440	<i>Muticaria macrostoma</i> morph <i>oscitans</i>	<i>M. mamotica</i> ssp 3	GWa2	Wied ix-Xlendi (II-Fekruna), AD & PJS leg 05.10.05 (<i>site A</i>)	16S	ITS-1
36440	<i>Muticaria macrostoma</i> morph <i>oscitans</i>	<i>M. mamotica</i> ssp 2	GWa3	Wied ix-Xlendi (II-Fekruna), AD & PJS leg 05.10.05 (<i>site A</i>)	16S	ITS-1
36441	<i>Muticaria macrostoma</i> morph <i>mamotica</i>	<i>M. mamotica</i> ssp 2	GWb1	Wied ix-Xlendi (II-Fekruna), AD & PJS leg 05.10.05 (<i>site B</i>)	16S	ITS-1
36441	<i>Muticaria macrostoma</i> morph <i>mamotica</i>	<i>M. mamotica</i> ssp 3	GWb2	Wied ix-Xlendi (II-Fekruna), AD & PJS leg 05.10.05 (<i>site B</i>)	16S	ITS-1
36441	<i>Muticaria macrostoma</i> morph <i>mamotica</i>	<i>M. mamotica</i> ssp 3	GWb3	Wied ix-Xlendi (II-Fekruna), AD & PJS leg 05.10.05 (<i>site B</i>)	16S	ITS-1
36441	<i>Muticaria macrostoma</i> morph <i>mamotica</i>	<i>M. mamotica</i> ssp 1	GWc1	Wied ix-Xlendi (II-Fekruna), AD & PJS leg 05.10.05 (<i>site C</i>)	16S	ITS-1
36439	<i>Muticaria macrostoma</i> morph <i>mamotica</i>	<i>M. mamotica</i> ssp 3	GWc2	Wied ix-Xlendi (II-Fekruna), AD & PJS leg 05.10.05 (<i>site C</i>)	16S	ITS-1
36439	<i>Muticaria macrostoma</i> morph <i>mamotica</i>	<i>M. mamotica</i> ssp 3	GWc3	Wied ix-Xlendi (II-Fekruna), AD & PJS leg 05.10.05 (<i>site C</i>)	16S	ITS-1

SICILY

36478	<i>Muticaria syracusana</i>	<i>M. syracusana</i> or <i>M. syracusana syracusana</i>	NA1	Noto Antica, VF leg. 22.04.04	16S	ITS-1
36478	<i>Muticaria syracusana</i>	<i>M. syracusana</i> or <i>M. syracusana syracusana</i>	NA2	Noto Antica, VF leg. 22.04.04	16S	ITS-1
36478	<i>Muticaria syracusana</i>	<i>M. syracusana</i> or <i>M. syracusana syracusana</i>	NA3	Noto Antica, VF leg. 22.04.04	16S	ITS-1
35854	<i>Muticaria neuteboni</i>	<i>M. neuteboni</i> or <i>M. syracusana neuteboni</i>	CA1	Cava d'Ispica, RG leg. 11.11.04	16S	ITS-1
35854	<i>Muticaria neuteboni</i>	<i>M. neuteboni</i> or <i>M. syracusana neuteboni</i>	CA2	Cava d'Ispica, RG leg. 11.11.04	16S	ITS-1
35854	<i>Muticaria neuteboni</i>	<i>M. neuteboni</i> or <i>M. syracusana neuteboni</i>	CA3	Cava d'Ispica, RG leg. 11.11.04	16S	ITS-1
35854	<i>Muticaria neuteboni</i>	<i>M. neuteboni</i> or <i>M. syracusana neuteboni</i>	CA4	Cava d'Ispica, RG leg. 11.11.04	16S	ITS-1

LAMPEDUSA

39858	<i>Lampedusa lopadusae</i>	<i>L. lopadusae</i> or <i>L. lopadusae lopadusae</i>	AR1	Aria Rossa, SC & ET leg. 10.05.00 (alcohol specimen)	16S	
39858	<i>Lampedusa lopadusae</i>	<i>L. lopadusae</i> or <i>L. lopadusae lopadusae</i>	AR2	Aria Rossa, SC & ET leg. 10.05.00 (alcohol specimen)	16S	
39858	<i>Lampedusa lopadusae</i>	<i>L. lopadusae</i> or <i>L. lopadusae lopadusae</i>	AR3	Aria Rossa, SC & ET leg. 10.05.00 (alcohol specimen)	16S	
39859	<i>Lampedusa lopadusae</i>	<i>L. lopadusae</i> or <i>L. lopadusae lopadusae</i>	CP1	Capo Ponente, SC & ET leg. 10.05.00 (alcohol specimen)	16S	
39859	<i>Lampedusa lopadusae</i>	<i>L. lopadusae</i> or <i>L. lopadusae lopadusae</i>	CP2	Capo Ponente, SC & ET leg. 10.05.00 (alcohol specimen)	16S	

LAMPIONE

9467	<i>Lampedusa lopadusae</i>	<i>L. nodulosa</i> or <i>L. lopadusae nodulosa</i>	LA1	Lampione Island, SC & ET leg. 16.05.00 (alcohol specimen)	16S	
9467	<i>Lampedusa lopadusae</i>	<i>L. nodulosa</i> or <i>L. lopadusae nodulosa</i>	LA2	Lampione Island, SC & ET leg. 16.05.00 (alcohol specimen)	16S	

9467 *Lampedusa lopadusae* *L. nodulosa* or *L. lopadusae nodulosa* **LA3** Lampione Island, SC & ET leg. 16.05.00 (alcohol specimen) 16S

OUTGROUP

28524 *Medora albescens* Gualdo Tadino, GM leg. 13.12.84 (alcohol specimen) 16S

36024 *Leucostigma candidescens* Capri Island, GM leg. 10.12.05 (alcohol specimen) 16S

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803 **Table 2.** Two-way analysis of variance (ANOVA) on numbers of ribs in shell of *Lampedusa*.

Taxa	diff	lwr	upr	p
imitatrix-melitensis	6.51	-0.22	13.25	0.06
lopadusae-melitensis	22.3	14.20	30.39	0
nodulosa-melitensis	33.5	25.40	41.59	0
lopadusae-imitatrix	15.78	9.05	22.52	0
nodulosa-imitatrix	26.98	20.25	33.72	0
nodulosa-lopadusae	11.2	3.10	19.29	0

807 **Table 3.** Two-way analysis of variance (ANOVA) on the ratio D/H in *Lampedusa*.

Taxa	diff	lwr	upr	p
lopadusae-melitensis	0.02847848	-0.0496073	0.10656422	0.77
nodulosa-melitensis	0.03193357	-0.0461522	0.11001931	0.70
imitatrix-melitensis	0.03237076	-0.0326005	0.09734203	0.55
nodulosa-lopadusae	0.00345509	-0.0746307	0.08154083	0.99
imitatrix-lopadusae	0.00389228	-0.061079	0.06886355	0.99
imitatrix-nodulosa	0.00043719	-0.0645341	0.06540845	0.99

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812 **Table 4.** Two-way analysis of variance (ANOVA) on numbers of ribs in shell of Muticaria.

Taxa	diff	lwr	upr	p
scalaris-syracusana	0.30	0.11	0.49	0.00
macrost_malta-syracusana	0.36	0.26	0.46	0.00
macrost_gozo-syracusana	0.53	0.40	0.66	0.00
macrost_oscitans_gozo-syracusana	0.55	0.40	0.70	0.00
neuteboomi-syracusana	0.69	0.54	0.84	0.00
oscitans_gozo-syracusana	0.76	0.62	0.91	0.00
macrost_oscitans_malta-syracusana	0.77	0.63	0.91	0.00
oscitans_malta-syracusana	1.04	0.95	1.14	0.00
oscitans_mamotica_gozo-syracusana	1.19	1.00	1.38	0.00
mamotica_gozo-syracusana	1.29	1.12	1.47	0.00
macrost_malta-scalaris	0.06	-0.12	0.23	1.00
macrost_gozo-scalaris	0.23	0.03	0.43	0.01
macrost_oscitans_gozo-scalaris	0.25	0.04	0.46	0.01
neuteboomi-scalaris	0.39	0.18	0.60	0.00
oscitans_gozo-scalaris	0.46	0.25	0.67	0.00
macrost_oscitans_malta-scalaris	0.47	0.26	0.67	0.00
oscitans_malta-scalaris	0.74	0.56	0.92	0.00
oscitans_mamotica_gozo-scalaris	0.89	0.65	1.13	0.00
mamotica_gozo-scalaris	0.99	0.76	1.22	0.00

macrost_gozo-macrost_malta	0.18	0.06	0.29	0.00
macrost_oscitans_gozo-macrost_malta	0.20	0.06	0.33	0.00
neuteboomi-macrost_malta	0.33	0.20	0.46	0.00
oscitans_gozo-macrost_malta	0.41	0.27	0.54	0.00
macrost_oscitans_malta-macrost_malta	0.41	0.29	0.53	0.00
oscitans_malta-macrost_malta	0.68	0.62	0.75	0.00
oscitans_mamotica_gozo-macrost_malta	0.83	0.65	1.01	0.00
mamotica_gozo-macrost_malta	0.94	0.78	1.10	0.00
macrost_oscitans_gozo-macrost_gozo	0.02	-0.14	0.18	1.00
neuteboomi-macrost_gozo	0.16	0.00	0.31	0.04
oscitans_gozo-macrost_gozo	0.23	0.07	0.39	0.00
macrost_oscitans_malta-macrost_gozo	0.23	0.09	0.38	0.00
oscitans_malta-macrost_gozo	0.51	0.40	0.62	0.00
oscitans_mamotica_gozo-macrost_gozo	0.66	0.46	0.86	0.00
mamotica_gozo-macrost_gozo	0.76	0.58	0.94	0.00
neuteboomi-macrost_oscitans_gozo	0.14	-0.03	0.31	0.23
oscitans_gozo-macrost_oscitans_gozo	0.21	0.04	0.38	0.00
macrost_oscitans_malta-macrost_oscitans_gozo	0.21	0.05	0.38	0.00
oscitans_malta-macrost_oscitans_gozo	0.49	0.36	0.62	0.00
oscitans_mamotica_gozo- macrost_oscitans_gozo	0.64	0.43	0.85	0.00

mamotica_gozo-macroscitans_gozo	0.74	0.55	0.94	0.00
oscitans_gozo-neuteboomi	0.07	-0.10	0.24	0.95
macroscitans_malta-neuteboomi	0.08	-0.08	0.24	0.91
oscitans_malta-neuteboomi	0.35	0.22	0.48	0.00
oscitans_mamotica_gozo-neuteboomi	0.50	0.29	0.71	0.00
mamotica_gozo-neuteboomi	0.60	0.41	0.80	0.00
macroscitans_malta-oscitans_gozo	0.00	-0.16	0.17	1.00
oscitans_malta-oscitans_gozo	0.28	0.15	0.41	0.00
oscitans_mamotica_gozo-oscitans_gozo	0.43	0.22	0.64	0.00
mamotica_gozo-oscitans_gozo	0.53	0.34	0.72	0.00
oscitans_malta-macroscitans_malta	0.27	0.16	0.39	0.00
oscitans_mamotica_gozo- macroscitans_malta	0.42	0.22	0.63	0.00
mamotica_gozo-macroscitans_malta	0.53	0.34	0.71	0.00
oscitans_mamotica_gozo-oscitans_malta	0.15	-0.03	0.32	0.19
mamotica_gozo-oscitans_malta	0.25	0.10	0.41	0.00
mamotica_gozo-oscitans_mamotica_gozo	0.10	-0.12	0.33	0.92

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818 **Table 5.** Two-way analysis of variance (ANOVA) on the ratio D/H in *Muticaria*.

Forms	diff	lwr	upr	p
syracusana-macrosc_malta	0.03	-0.02	0.09	0.71
macrosc_oscitans_malta-macrosc_malta	0.05	-0.01	0.12	0.24
macrosc_gozo-macrosc_malta	0.06	0.00	0.12	0.06
oscitans_gozo-macrosc_malta	0.10	0.02	0.17	0.00
oscitans_malta-macrosc_malta	0.13	0.09	0.16	0.00
macrosc_oscitans_gozo-macrosc_malta	0.14	0.07	0.21	0.00
neuteboomi-macrosc_malta	0.23	0.16	0.30	0.00
scalaris-macrosc_malta	0.26	0.16	0.36	0.00
oscitans_mamotica_gozo-macrosc_malta	0.29	0.19	0.39	0.00
mamotica_gozo-macrosc_malta	0.56	0.47	0.65	0.00
macrosc_oscitans_malta-syracusana	0.02	-0.06	0.10	1.00
macrosc_gozo-syracusana	0.03	-0.04	0.10	0.97
oscitans_gozo-syracusana	0.06	-0.02	0.15	0.36
oscitans_malta-syracusana	0.09	0.04	0.15	0.00
macrosc_oscitans_gozo-syracusana	0.11	0.02	0.19	0.00
neuteboomi-syracusana	0.20	0.11	0.28	0.00
scalaris-syracusana	0.22	0.12	0.33	0.00
oscitans_mamotica_gozo-syracusana	0.25	0.14	0.36	0.00
mamotica_gozo-syracusana	0.52	0.43	0.62	0.00

macrost_gozo-macrost_oscitans_malta	0.01	-0.07	0.09	1.00
oscitans_gozo-macrost_oscitans_malta	0.04	-0.05	0.13	0.94
oscitans_malta-macrost_oscitans_malta	0.07	0.01	0.14	0.02
macrost_oscitans_gozo-macrost_oscitans_malta	0.09	0.00	0.18	0.08
neuteboomi-macrost_oscitans_malta	0.17	0.08	0.26	0.00
scalaris-macrost_oscitans_malta	0.20	0.09	0.32	0.00
oscitans_mamotica_gozo- macrost_oscitans_malta	0.23	0.12	0.34	0.00
mamotica_gozo-macrost_oscitans_malta	0.50	0.40	0.61	0.00
oscitans_gozo-macrost_gozo	0.03	-0.05	0.12	0.98
oscitans_malta-macrost_gozo	0.06	0.00	0.12	0.02
macrost_oscitans_gozo-macrost_gozo	0.08	-0.01	0.17	0.12
neuteboomi-macrost_gozo	0.17	0.08	0.25	0.00
scalaris-macrost_gozo	0.19	0.08	0.31	0.00
oscitans_mamotica_gozo-macrost_gozo	0.22	0.11	0.33	0.00
mamotica_gozo-macrost_gozo	0.50	0.40	0.60	0.00
oscitans_malta-oscitans_gozo	0.03	-0.04	0.10	0.95
macrost_oscitans_gozo-oscitans_gozo	0.05	-0.05	0.14	0.91
neuteboomi-oscitans_gozo	0.13	0.04	0.23	0.00
scalaris-oscitans_gozo	0.16	0.04	0.28	0.00
oscitans_mamotica_gozo-oscitans_gozo	0.19	0.07	0.31	0.00

mamotica_gozo-oscitans_gozo	0.46	0.35	0.57	0.00
macrost_oscitans_gozo-oscitans_malta	0.01	-0.06	0.09	1.00
neuteboomi-oscitans_malta	0.10	0.03	0.17	0.00
scalaris-oscitans_malta	0.13	0.03	0.23	0.00
oscitans_mamotica_gozo-oscitans_malta	0.16	0.06	0.26	0.00
mamotica_gozo-oscitans_malta	0.43	0.34	0.52	0.00
neuteboomi-macrost_oscitans_gozo	0.09	-0.01	0.18	0.10
scalaris-macrost_oscitans_gozo	0.12	0.00	0.23	0.06
oscitans_mamotica_gozo-macrost_oscitans_gozo	0.14	0.03	0.26	0.00
mamotica_gozo-macrost_oscitans_gozo	0.42	0.31	0.53	0.00
scalaris-neuteboomi	0.03	-0.09	0.14	1.00
oscitans_mamotica_gozo-neuteboomi	0.06	-0.06	0.17	0.89
mamotica_gozo-neuteboomi	0.33	0.22	0.44	0.00
oscitans_mamotica_gozo-scalaris	0.03	-0.11	0.16	1.00
mamotica_gozo-scalaris	0.30	0.17	0.43	0.00
mamotica_gozo-oscitans_mamotica_gozo	0.27	0.15	0.40	0.00

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