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Pleistocene phylogeography of tiger beetle, *Calomera littoralis*, in North-Eastern Mediterranean and Pontic regions inferred from mitochondrial COI gene sequence

Radomir Jaskuła, Tomasz Rewicz, Mateusz Płóciennik, Michał Grabowski

Background. *Calomera littoralis* is a Palearctic species, widely distributed in Europe; inhabiting predominantly its Atlantic, Mediterranean and Black Sea coastlines.

Methods. Its phylogeography on the Balkan Peninsula and on the north-western Black Sea coast was inferred using a 697 bp long portion of the mitochondrial COI gene, amplified from 169 individuals collected on 43 localities.

Results. The results revealed two genetically divergent groups/lineages, the southern one inhabiting both the Balkan Peninsula and the Pontic Region and the northern one found exclusively in the Pontic Region. Species delimitation based on DNA barcoding gap suggested an interspecific level of divergence between these groups. Multivariate analysis of eight male and female morphometric traits detected no difference between the groups, implying they may represent cryptic species. The Bayesian time-calibrated reconstruction of phylogeny suggested that the lineages diverged ca. 2.3 Ma, in early Pleistocene.

Discussion. This might result from contemporary isolation of the Pontic basin from the Mediterranean that broke the continuous strip of coastal habitats inhabited by *C. littoralis*. Demographic analyses indicated that both lineages have been in demographic and spatial expansion since ca. 0.15 Ma. It coincides with the terminal stage of MIS-6, i.e. Wartanian/Saalian glaciation, and beginning of MIS-5e, i.e. Eemian interglacial, during which, due to eustatic sea level rise, a wide connection between Mediterranean and the Pontic basin was re-established. This, along with re-appearance of coastal habitats could initiate north-east expansion of the southern lineage and its secondary contact with the northern one. Isolation of Pontic basin from Mediterranean during the Weichselian glaciation did not have probably any effect on their phylogeography.

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Pleistocene phylogeography of tiger beetle, *Calomera littoralis*, in North-Eastern Mediterranean and Pontic regions inferred from mitochondrial COI gene sequences

Radomir Jaskuła[1]*, Tomasz Rewicz[2], Mateusz Płóciennik[1], Michał Grabowski[1]

[1]Department of Invertebrate Zoology and Hydrobiology, University of Lodz, Banacha 12/16, 90-237 Lodz, Poland

[2]Laboratory of Microscopic Imaging and Specialized Biological Techniques, University of Lodz, Banacha 12/16, Łódź 90-237, Poland

Corresponding author:

Radomir Jaskuła[1]

Ul. Banacha 12/16, 90-237 Łódź, Łódź, Poland

Email address: radekj@biol.uni.lodz.pl

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Abstract

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Key words: Coleoptera – Cicindelidae – ice age – interglacial – sea level changes – population expansion – southern Europe

Introduction

The Eastern Mediterranean including the Pontic area is recognised as one of the major biodiversity and endemism hot spots on a global scale as well as a major glacial refugium in Europe (eg. Myers et al., 2000; Kotlík, Bogutskaya & Ekmekçi, 2004; Blondel et al., 2010). Among others, it is a consequence of complex geological history of the region that was an archipelago and united with rest of the European continent only in Neogene (Pffifner, 2014). On the other side, a shallow epicontinental sea, Paratethys, occupied vast areas of the continent and regressed gradually leaving relics, such as Black, Azov and Caspian Sea (Nahavandi et al., 2013). Local isostatic and eustatic changes of sea level were among superior phenomena shaping local landscapes. For example, there were at least twelve saline water intrusions from the Mediterranean Sea, and eight intrusions from the Caspian Lake to the Black Sea during the last 0.67 million years (Ma) (Badertscher et al., 2011). Inevitably, they played an important role in modelling diversity and distribution patterns for numerous organisms, particularly those inhabiting coastal ecosystems both in Mediterranean and in Pontic area. However, the evidence comes mostly from aquatic, predominantly marine or brackish water, taxa (e.g. Audzijonyte, Daneliya & Vainola, 2006; Neilson & Stepien, 2011). There is a deficiency of studies focusing upon coastal species inhabiting terrestrial habitats in this region (Akin et al., 2010).

Tiger beetles, Cicindelidae Latreille, 1806, seem to be ideal model organisms to test such assumptions. The family, with more than 2600 species, has a worldwide distribution with exception of polar regions and some oceanic islands (Pearson & Cassola 2005). Most species, both in larval and adult stage, prefer various types of sandy areas and are habitat specialists; often inhabiting coastal areas (Pearson & Vogler 2001). Several studies dealt with

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phylogeography of tiger beetles in various regions of the world (eg. Vogler et al., 1993; Cardoso & Vogler, 2005; Woodcock *et al*., 2007), yet so far only few focused on the role of sea level oscillations in their evolutionary history (Vogler & DeSalle, 1993; Sota et al., 2011) or compared the diversity patterns on both, the molecular and morphological, levels (Cardoso, Serrano & Vogler, 2009; Tsuji et al., 2016).

The tiger beetle, *Calomera littoralis* Fabricius 1787, is widely distributed in Palaearctic, from the Iberian Peninsula and Morocco in the west to the Middle Asia and Russian Far East in the east (Putchkov & Matalin, 2003; Serrano, 2013; Jaskuła, 2011, 2015). Generally, it is recognised as euryoecious (Jaskuła, 2011, 2013, 2015). However, in Europe it occupies predominantly the very narrow stretch of Atlantic, Mediterranean and Black Sea coastal habitats (Cassola & Jaskuła, 2004; Franzen, 2006; Jaskuła, 2007a-b; Jaskuła, Pešić & Pavicević, 2005; Serrano, 2013).

Our study aims at (1) exploring and comparing spatial patterns of molecular and morphological diversity of this coastal species in the Mediterranean and Pontic region, (2) interpreting the observed patterns in the context of local paleogeography.

Material and methods

Sample collection and identification

In total, 169 imagines of *Calomera littoralis* were collected with entomological hand net on 43 sites on the Mediterranean coasts of the Balkan Peninsula, Crete and Turkey as well as on the northern and western coast of the Black and Azov Seas, in the years 2009-2012 (Figure 1, Table 1). At a site the material was fixed in 96% ethanol for DNA preservation. Taxonomic identification of the collected material followed Mandl (1981).

DNA extraction, amplification and sequencing

90 Following Hillis et al. (1996) the standard phenol-chloroform method was used to extract DNA
91 from all the collected individuals. Air-dried DNA pellets were eluted in 100 µl of TE buffer,
92 pH 8.00, stored at 4°C until amplification, and subsequently at -20°C for long-term storage.

93 Fragments of mitochondrial cytochrome oxydase subunit I gene (COI), ca. 700 bp long,
94 were amplified using the Jerry and Pat pair of primers (Simon *et al*., 1994). Each PCR reaction
95 was conducted in a total volume of 10 µl and contained DreamTaq Master Mix (1x) Polymerase
96 (ThermoScientific), 200nM of each primer and 1 µl of DNA template. The thermal regime
97 consisted of initial denaturation at 94°C for 2min, followed by 34 cycles of denaturation at 94°C
98 for 30s, annealing at 44°C for 30s, and elongation at 72°C for 60s, completed by a final
99 extension at 72°C for 10min. The amplified products were visualized on 2.0% agarose gels
100 stained with MidoriGreen (Nippon Genetics) to verify the quality of the PCR reactions. Then,
101 the PCR products were chemically cleaned up of dNTPs and primer residues by adding 1U of
102 Exonuclease I (Thermo Scientific) and 2U of FastAP Alkaline Phosphatase (Thermo Scientific)
103 per sample. Sequencing was performed using BigDye sequencing protocol (Applied
104 Biosystems 3730xl) by Macrogen Inc., Korea.

105 ***Molecular data analysis***

106 First, all the obtained sequences were positively verified as *Calomera* DNA using
107 GenBankBLASTn searches (Altschul et al., 1990). Then they were edited and assembled with
108 CLUSTALW algorithm (Chenna et al., 2003) using BIOEDIT© 7.2.5. The resulting alignment was
109 697 bp long with no gaps, and composed of 169 COI sequences. The sequence data and trace
110 files were uploaded to BOLD and subsequently also to GenBank (accession nos KU905171-
111 KU905339).

112 Pairwise Kimura 2-parameter (K2p) distances between sequences were estimated using
113 MEGA 6.2 (Tamura et al., 2013). Haplotypes were retrieved using DNASP v5 (Librado & Rozas,
114 2009). Phylogenetic relationships between the haplotypes were visualised with phylogenetic

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network computed using the neighbour-net algorithm and uncorrected p-distances in SplitsTree ver. 4.13.1 (Huson & Bryant 2006).

To test for presence of distinct operational taxonomic units (OTUs) that may represent potential cryptic species/subspecies in the sequenced pool of individuals we used the Automatic Barcode Gap Discovery (ABGD) procedure (Puillandre et al., 2012). The default value of 0.001 was used as the minimum allowed intraspecific distance. The maximum allowed intraspecific distance was set to P max = 0.03 and 0.06, as both threshold values have been already used in literature to delimit insect species (Hebert et al., 2003; Hebert, Ratnasingham & de Waard, 2003). We applied the K2P model sequence correction, which is a standard for barcode analyses (Hebert et al., 2003). We used primary partitions as a principal for group definition for they are usually stable over a wider range of prior values, minimise the number of false positive (over split species) and are usually close to the number of groups described by taxonomists (Puillandre et al., 2012).

To reveal the temporal framework for the divergence of the two phylogenetic lineages (potential cryptic species) revealed in *Calomera littoralis*, the time calibrated phylogeny was reconstructed in BEAST, version 1.8.1 (Drummond et al., 2012). A COI sequence of *Calomera lugens aphrodisia* Baudi di Selve 1864 from GenBank (acc. no. KC963733) was used as an outgroup. This analysis was performed on a reduced dataset, containing only the most distant haplotypes from each OTU. The HKY model of evolution and coalescent model were set for priors. The strict clock with rate 0.0115, widely used for phylogenetic studies upon insects, was applied for the analyses (Brower, 1994). Five runs of 20M iterations of Markov chain Monte Carlo (MCMC) sampled each 2000 iterations were performed. The runs were examined using Tracer v 1.6 and all sampled parameters achieve sufficient effective sample sizes (ESS>200). Tree files were combined using Log-Combiner 1.8.1 (Drummond et al., 2012), with removal of

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139 the non-stationary 20% burn-in phase. The maximum clade credibility tree was generated using
140 TreeAnnotator 1.8.1 (Drummond et al., 2012).

141 To provide insight into historical demography, i.e. the temporal changes of the effective
142 population size of *Calomera littoralis* in the studied region, we performed Bayesian Skyline
143 Plot (BSP) analysis (Drummond et al., 2005) in BEAST, version 1.8.1 (Drummond et al., 2012).
144 Separate analysis was performed for each of the two phylogenetic lineages revealed in our study
145 (see Results). The Northern Lineage was represented by 84 individuals from 22 localities, while
146 the Southern Lineage was represented by 85 individuals from 32 localities. The HKY+I model
147 of evolution was used as the best fitting model in case of the Eastern Lineage, while TN93+I
148 was used in case of the Western Lineage. Two runs of MCMC, 20M iterations long sampled
149 each 2000 iterations, were performed. In both cases the runs were examined using Tracer v 1.6
150 (Drummond et al., 2012) and all sampled parameters achieved sufficient effective sample sizes
151 (ESS>200).

152 Two models of population expansion, demographic and spatial, were examined using
153 mismatch distribution analysis (Slatkin & Hudson, 1991; Rogers & Harpending, 1992) and
154 Tajima's D neutrality test (Tajima, 1989). Analyses were performed for the COI groups, using
155 Arlequin 3.5.1.3 (Excoffier & Lischer, 2010) with 1000 replicates.

156 ***Morphometric data analysis***

157 To test whether variation of morphometric traits reflects presence of two genetically divergent
158 lineages (potential cryptic species), measurements of eight body parameters (Fig. 2) were taken
159 from all the 69 males and 100 females used previously for the molecular analyses: 1 – right
160 mandible length (RML), 2 – length of head (LH), 3 – width of head (WH), 4 – maximum
161 pronotum width (MPW), 5 – pronotum length (PL), 6 – elytra length (EL), 7 – maximum elytra
162 width (MEW), and 8 – total body length (TBL). The principal component analysis (PCA) was
163 performed separately for each sex (Figure 3, Table 1). To test for significance ($p<0.01$) of

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164 morphological differences (separately for males and females) between the two divergent
165 lineages one-way ANOSIM Pairwise Test was performed. All the above statistical analyses
166 were done with PRIMER 6 software (Clarke & Gorley, 2006).
167

168 **Results**

169 ***Molecular data***

170 A total of 81 haplotypes were identified in the dataset composed of 169 individuals from 43
171 sites from the Mediterranean and the Pontic areas (Table 1). The phylogenetic network
172 illustrating phylogenetic relationships among haplotypes (Fig.4) uncovered presence of two
173 distinct haplotype groups (phylogenetic lineages). The first group, from now on defined as
174 southern lineage, includes 36 haplotypes present all over the studied range including the Balkan
175 Peninsula and the Pontic area. The other group, from now on defined as northern lineage, is
176 composed of 45 haplotypes present exclusively along the north-western coast of the Black Sea.
177 The mean K2p genetic distance between both groups of haplotypes is relatively high (0.039,
178 SD 0.007). Both variants of the ABGD analysis resulted in partitioning of the dataset into two
179 OTUs, that may represent distinct operational taxonomic units – potential cryptic species or
180 subspecies within *Calomera littoralis* in the studied area (Fig. 3A).

181 The Bayesian time-calibrated reconstruction of phylogeny shows that the two lineages
182 split at ca. 2Ma, i.e. in early Pleistocene (Fig. 5A). Results of the BSP analyses showing the
183 temporal changes of the effective population size suggests that both lineages experienced rapid
184 population growth that has started ca. 0.15Ma (Fig. 5B). In both cases, a small decline in
185 effective population size may be observed in most recent times (<0.05 Ma). Results of the
186 mismatch analysis shows that both lineages are currently in the stage of both demographic and
187 spatial expansion (Fig 5C). Interestingly, geographical distribution of both lineages shows that
188 the spatial expansion of southern lineage was efficient enough to spread eastwards into the

Black Sea and colonise effectively the north-western Black Sea coast. The northern lineage has spread only in the Pontic region.

Morphometric data

The results of PCA and ANOSIM revealed no differences in the analysed morphometric traits between the southern and the northern lineages, neither in males nor in females (Fig. 3B). In PCA (Fig. 3B), a very weak gradient (R=0.03) could be seen in case of female body length. Females from the northern lineage clade seem to be slightly larger than those from the southern one (body length; ANOSIM Pairwise Tests p=0.03).

Discussion and conclusions

Cryptic diversity of Calomera littoralis

Known as very important hotspot of biodiversity, endemicity and cryptic diversity (eg. Myers et al., 2000; Kryštufek & Reed, 2004; Huemer & Timossi, 2014; Blondel et al., 2010; Previšić et al., 2014; Caković *et al*., 2015), the southern Europe holds also most diverse tiger beetle fauna in the entire Palearctic realm (Jaskuła, 2011). Presence of cryptic diversity was already pointed out for *Cicindela hybrida* in the Mediterranean (Cardoso, Serrano & Vogler, 2009) as well as for several species of tiger beetles occurring in other part of the world (Vogler & Pearson, 1996; López-López, Hudson & Galián, 2012). Thus, existence of well-defined OTUs within *Calomera littoralis* is not surprising in the studied area. The level of divergence, 0.04 K2p distance, between the northern and the southern lineage is similar as those found between species of tiger beetles in other studies (eg. Cardoso & Vogler, 2005; López-López, Abdul Aziz & Galián, 2015). Interestingly, we could not detect any conclusive morphological differences between the two lineages based on the multivariate analysis of eight morphometric traits. It must be mentioned that three subspecies of *Calomera littoralis*, described on the basis of morphology, were reported from the studied area: *C. l. nemoralis* from all the studied Balkan

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countries, Crete, Moldova, western Ukraine and western Turkey; *C. l. conjunctaepustulata* (Dokhtouroff, 1887) from the Azov Sea area; *C. l. winkleri* (Mandl, 1934) from Crete and the coastal zone of southern Turkey (Werner, 1991; Putchkov & Matalin, 2003; Avgın & Özdikmen, 2007). However, the morphological differences between the subspecies, such as body size, maculation of elytra and shape of aedeagus, are poorly defined and did not allow the identification of the studies material further than to the species level. Unfortunately, we had no opportunity to study the topotypical material – Provence, France, is *locus typicus* for *C. l. nemoralis*, Tibet for *C. l. conjunctaepustulata*, and Cyprus for *C. l. winkleri*. Thus, we cannot exclude a possibility that the two lineages we found in our material overlap with any of the above mentioned subspecies. However, only a further taxonomic revision combining more phenotypic traits, including e.g. cuticle ultrastructure, with several, mitochondrial and nuclear DNA data, could help to resolve this problem. Until such revision is done, we propose to use the tentative name "*Calomera littoralis* complex" for populations from the studied area.

Phylogeography of Calomera littoralis

Occurrence of *C. littoralis* in Europe is restricted predominantly to marine shorelines with sandy beaches and salt marshes as main habitats (e.g. Franzen, 2006; Jaskuła, 2011; Serrano, 2013). In the eastern Mediterranean it is distributed continuously all along the Adriatic and Aegean coasts, Turkish Straits and the Black Sea coastline (Cassola & Jaskuła, 2004; Jaskuła Pešić & Pavicević 2005; Franzen, 2006; Jaskuła, 2007a; Jaskuła 2007b). However, pronounced genetic structure with two divergent operational taxonomic units (OTUs) implies prolonged spatial isolation in the evolutionary past of this species. The observed level of divergence indicates that this isolation initiated an allopatric speciation. Their present distribution i.e. sympatry in the Pontic region reveals secondary contact of the already divergent lineages in this area. The Bayesian time-calibrated reconstruction of phylogeny shows that split between these OTUs begun in early Pleistocene. This coincides with beginning of recurrent glaciations

resulting in eustatic sea level changes and climate aridisation that ever since dominated the global climate and landscape/habitat distribution (Fagan, 2009). In the Mediterranean and in the Pontic region such global effects overlaid and strengthen the local effects of tectonic plate collision leading to Alpine orogeny, i.e. local land uplift and subsidence resulting in isostatic sea level changes, salinity fluctuations from freshwater to fully marine and habitat mosaicism (Stanley & Blanpied, 1980). For example, during that time the connections of Pontic basin to Mediterranean Sea was lost and regained for more than a dozen times (Kerey et al., 2004; Badertscher et al., 2011). A profound impact of these events on the evolution and, hence, distribution of local both aquatic (Audzijonyte, Daneliya & Vainola, 2006; Nahavandi et al., 2013) and terrestrial taxa (eg. Böhme et al., 2007; Ferchaud et al., 2012). We can assume that in case of *C. littoralis*, a halophilic species bound to coastal habitats, sea level fluctuations would significantly affect its distribution. The 2 Ma divergence time for *C. littoralis* OTUs derived from our data coincides with one particular disconnection of the Mediterranean and Pontic basins. At that time, from ca. 2 to ca. 1.5 Ma, the Meothic Sea, one of several predecessors of the Black Sea, turned into the predominantly freshwater Pontos Sea/Lake (Grinevetsky et al., 2015). This surely broke the formerly continuous stretch of coastal habitats connecting the two basins and thus, could be an effective barrier leading to split of *C. littoralis* population into the allopatric southern and northern lineages. Their detailed history is impossible to unravel, yet results of BSP analyses reconstructing past changes in effective population size indicate that both lineages started their demographic expansions at ca. 0.15 Ma. This coincides with the terminal stage of MIS-6, i.e. Wartanian/Saalian glaciation, and beginning of MIS-5e, i.e. Eemian interglacial (Lisiecki & Raymo, 2005; Marks, 2011). The latter was characterized by warmer climate and sea level higher by 6-9 m in comparison to Holocene (Kopp et al., 2009; Dutton & Lambeck, 2012). In result, a wide connection between Mediterranean and the Pontic basin was re-established and the coastal habitats extended again,

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enabling exchange of faunas. Due to deficiency of local studies, it is hard to compare our results to evolutionary history of any other terrestrial taxa in the area. However, a wealth of studies showing very similar spatiotemporal scenario in animal taxa comes from the coastal regions of the Gulf of Mexico and the adjacent Atlantic coast (summarised by Avise, 1992). During Pleistocene, Cuba was connected with a land bridge to the Florida Peninsula what lead to divergence of populations of several terrestrial and aquatic animals, including also a local tiger beetle species *Cicindela dorsalis* Say, 1817 (Vogler & DeSalle, 1993). Interestingly enough, however according to our results both lineages are until now in the stage of demographic and spatial expansion, only the southern one has crossed the present Turkish straits. This asymmetry is hard to explain. Another interesting fact is that the isolation of Pontic basin from Mediterranean during the following Weichselian glaciation did not have probably any effect on the demography and phylogeography of the species. Based on the mitochondrial DNA marker only we cannot also conclude, whether the secondary contact of the divergent lineages effected in hybridization and or introgression. Answering this question requires employment of nuclear marker, what leaves a space for the future studies – much wider in terms of geographic coverage and molecular markers used.

Concluding, we have demonstrated that Pleistocene glaciations and associated sea level changes in the Mediterranean/Pontic region had a profound effect on the genetic diversity and distribution of widely distributed coastal insect species, generating some level of cryptic diversity. Our case study casts more light on the evolutionary relationships between populations of terrestrial animals inhabiting both the Mediterranean and Black Sea shorelines – a phenomenon that is still weakly explored in literature.

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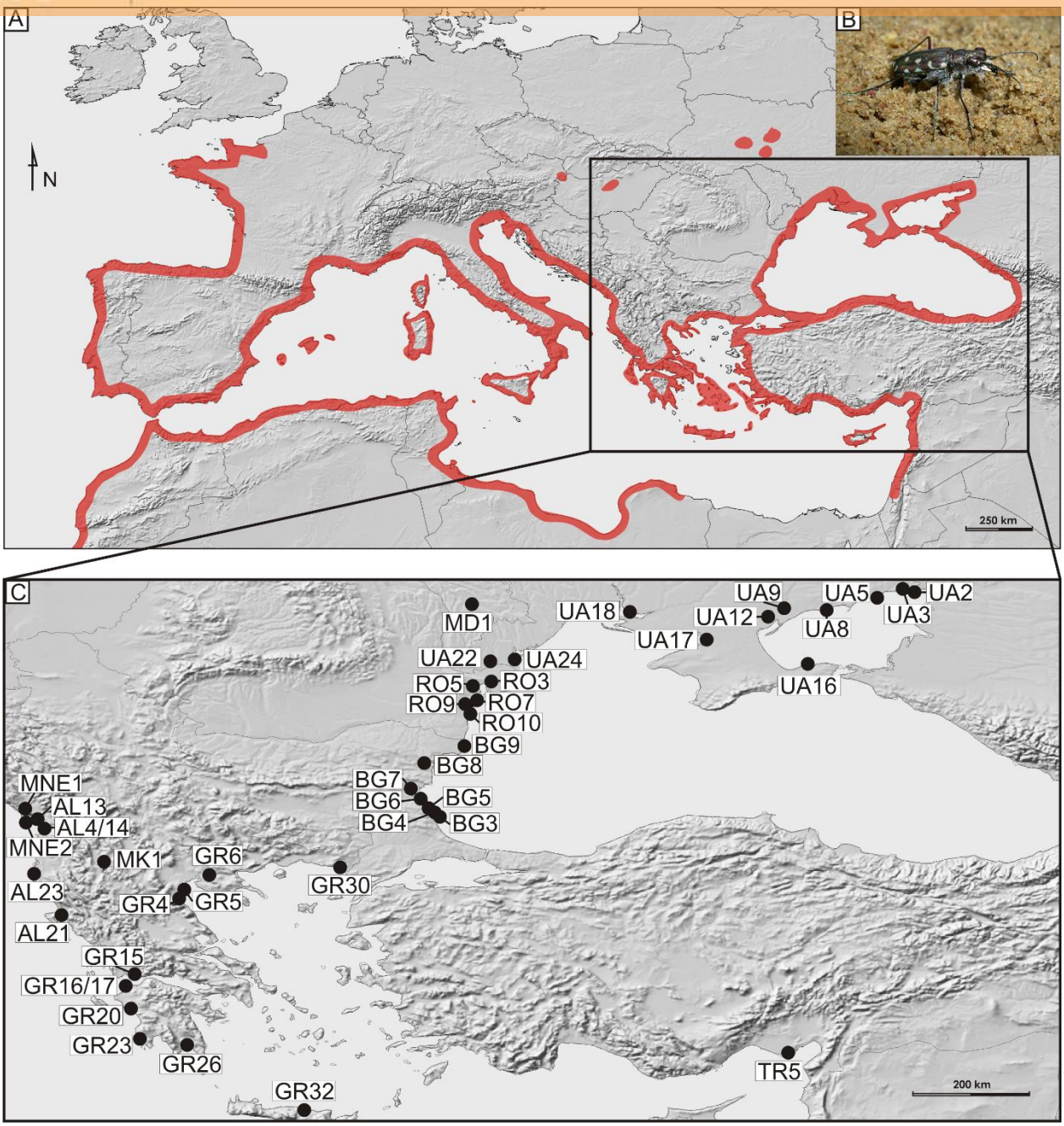

Figure 1. Distribution and sampling of *Calomera littoralis* in Europe. (A) General distribution of *Calomera littoralis* in Europe shown as red-shaded area. (B) Picture of *Calomera littoralis* beetle. (C) Sampling sites in Balkan Peninsula, Black Sea region and Turkey shown as black dots. Localities coded as in Table 1.

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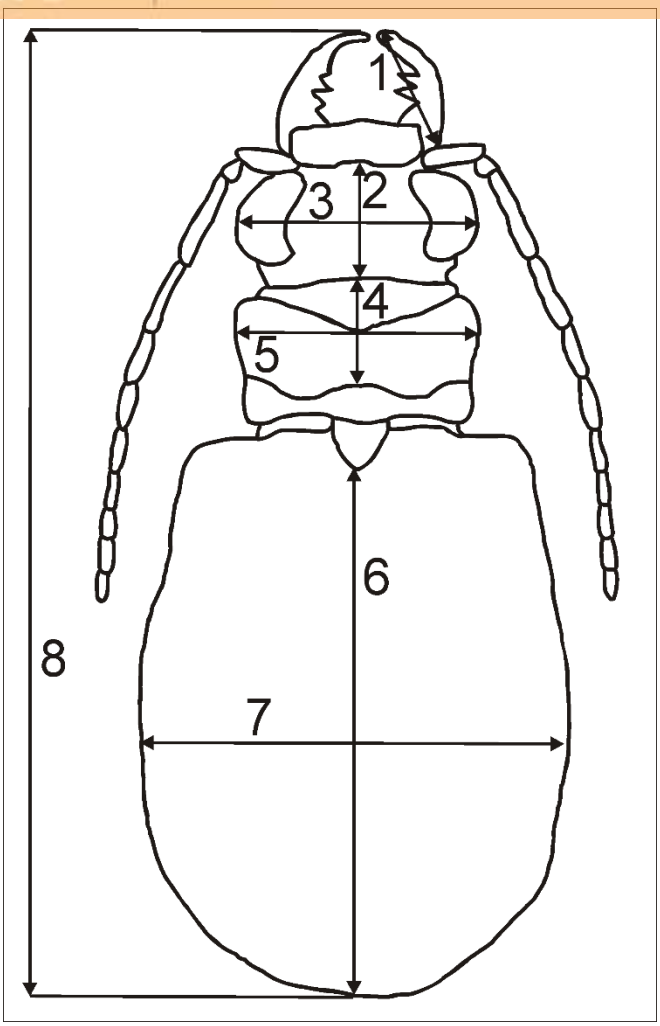

Figure 2. Body parameters measured in *Calomera littoralis.* 1 – RLM – right mandible length, 2 – WH – width of head, 3 – LH – length of head, 4 – MPW – maximum pronotum width, 5 – PL – pronotum length, 6 – EL – elytra length, 7 – MEW – maximum elytra width, 8 – TBL – total body length.

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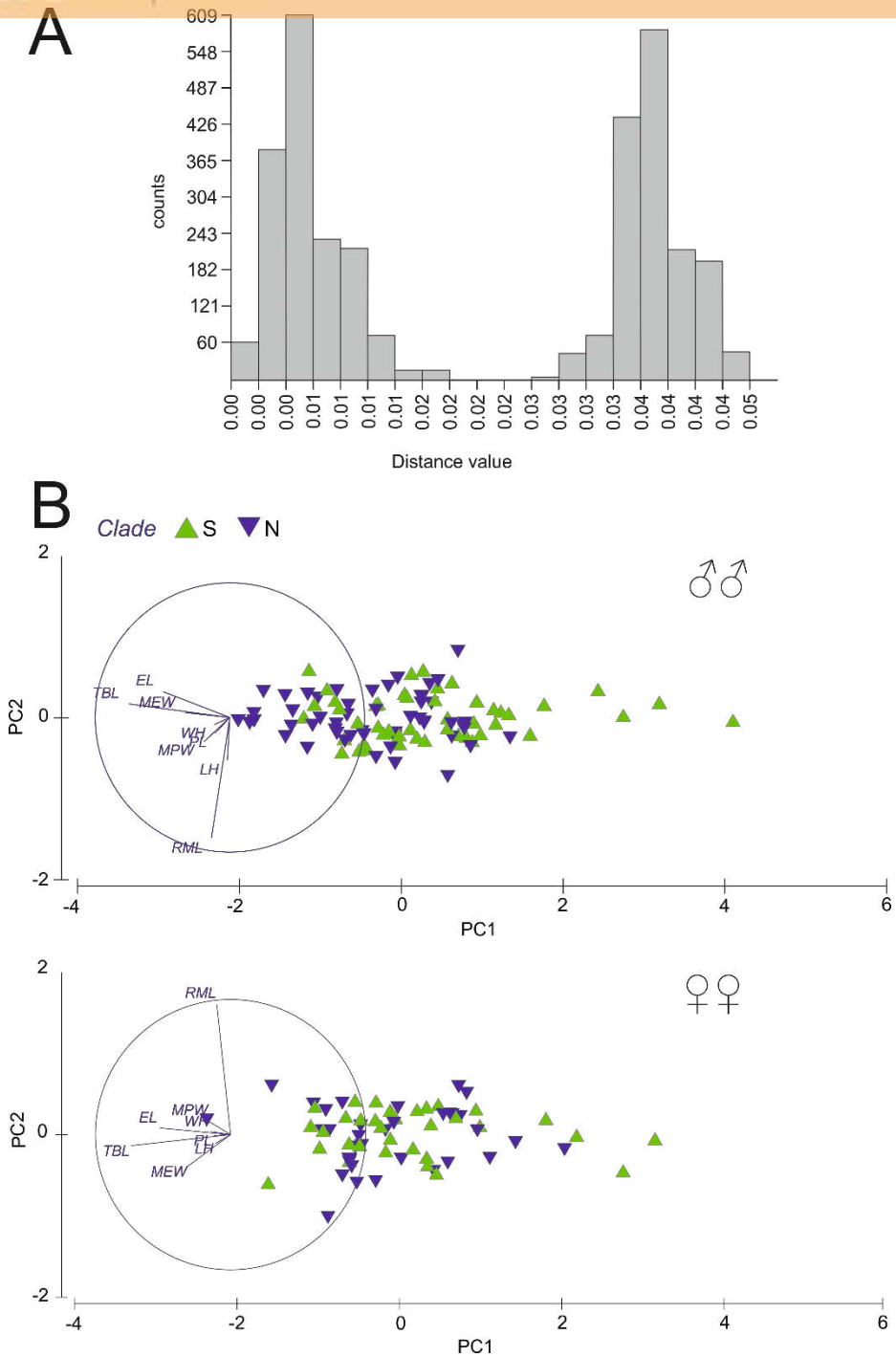

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483 **Figure 3.** (A) Automatic Barcode Gap Discovery (ABGD) analysis of *Calomera littoralis* and
484 (B) Results of Principal Component Analysis performed for investigated specimens on main
485 body dimensions. S – southern lineage, N – northern lineage, RLM – right mandible length,
486 WH – width of head, LH – length of head, MPW – maximum pronotum width, PL – pronotum
487 length, EL – elytra length, MEW – maximum elytra width, TBL – total body length. Both in
488 ABGD and PCA analyses 169 specimens from 43 sites from the Mediterranean and the Pontic
489 areas were used.

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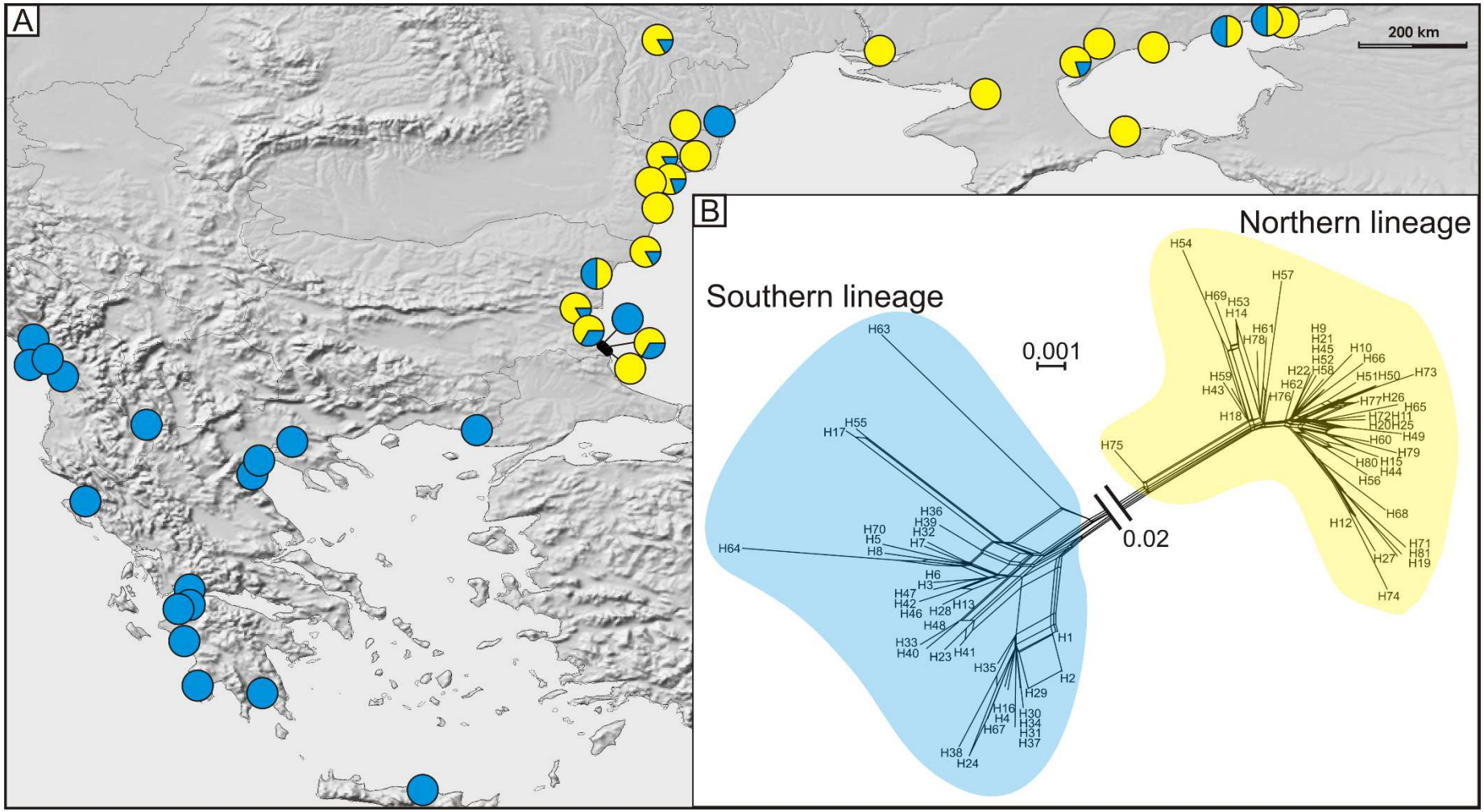

Figure 4. (A) Geographic distribution of haplogroups from southern (blue circles) and northern (yellow circles) lineages (B).

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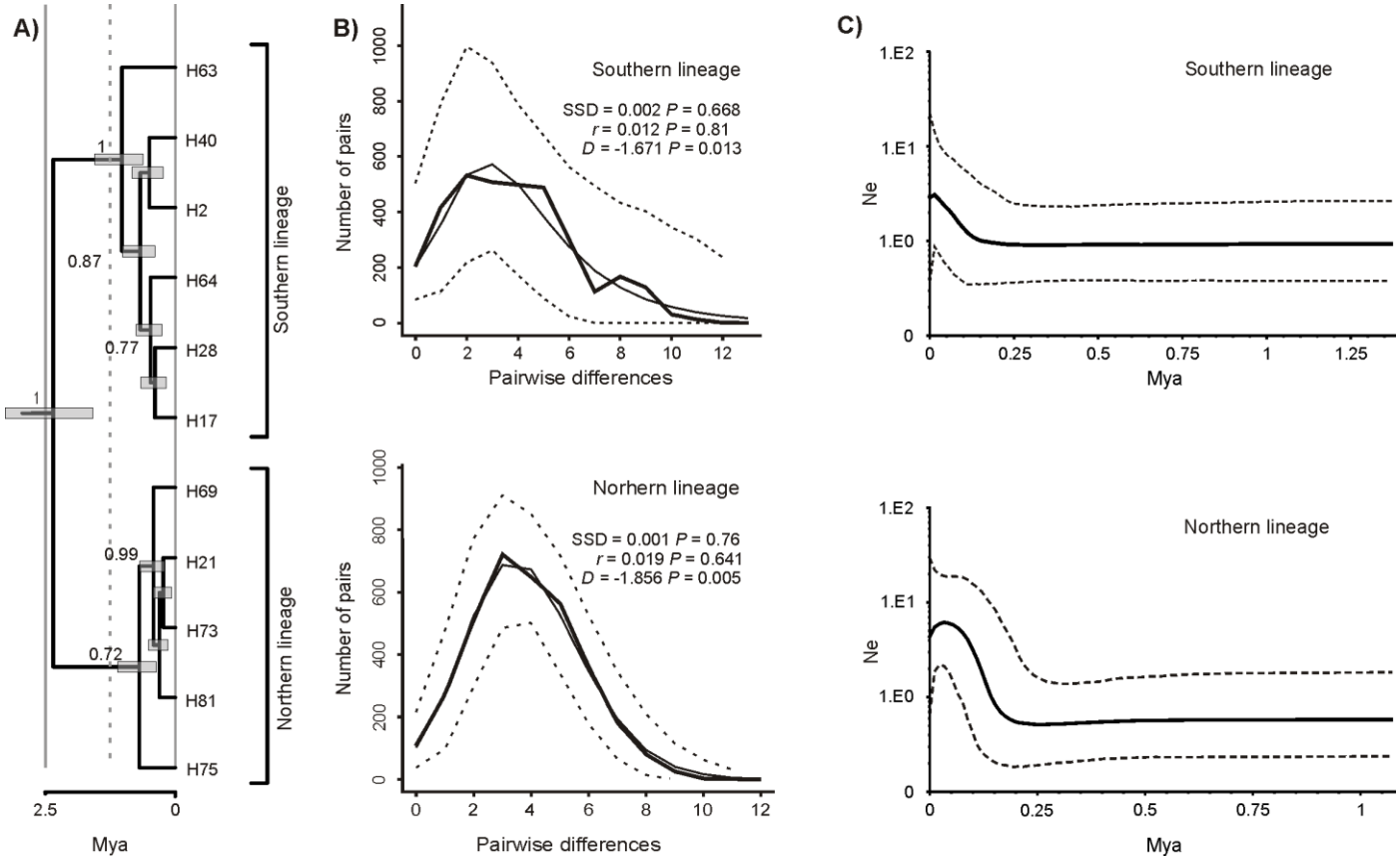

Figure 5. Phylogeny and demography of *Calomera littoralis*. (A) Maximum clade credibility chronogram with a strict molecular clock model inferred from COI sequences. The numbers next to the respective node indicate Bayesian posterior probabilities higher than 0.5. (B) Mismatch plots for southern and northern lineage. Thin solid lines indicate expected frequency under model of population demographic expansion, thick solid lines represent observed frequency, and dashed lines indicate 95% confidence intervals for the observed mismatch. SSD, sum of squared deviation;

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498 r, Harpending's raggedness index; D, Tajima's D. (C) Bayesian skyline plots for southern and northern lineages of *Calomera littoralis*. Solid lines
499 indicate the median posterior effective population size through time; dashed lines indicate the 95% highest posterior density interval for each
500 estimate.

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