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RESEARCH ARTICLE

The EU protected taxon *Morimus funereus* Mulsant, 1862 (Coleoptera: Cerambycidae) and its western Palaearctic allies: systematics and conservation outcomes

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Abstract *Morimus funereus* is a large longhorn beetle included in the European Habitats Directive and in previous releases of the IUCN red list. It represents a flagship species of old-growth forest saproxylic communities in E and SE Europe. The morphologically based taxonomy of W Palaearctic *Morimus* is rather unstable due to high phenetic intrapopulational and geographic variability and different authors have attempted to recognise one to five different taxa of specific/subspecific rank. No previous molecular data are available for the genus *Morimus*. Here, for the first time, a molecular approach based on *COI* and *ITS2* gene sequences was applied in European and Anatolian *Morimus* specimens. The genetic variability among Euro-Anatolian *Morimus* populations and the geographical structure suggest that they can not be ascribed to the currently accepted five W Palaearctic *Morimus* species and may actually represent a single, genetically and morphologically variable biological species (*M. asper*), highlighting the necessity of an extended taxonomical revision. In light of these results, a phylogeographical hypothesis of postglacial colonisation of the central Mediterranean area has been developed and the consequences of this new taxonomic arrangement regarding conservation strategies for "*Morimus funereus*" and allied taxa in Europe and Turkey are discussed.

Keywords Longhorns beetles · COI and ITS2 · Invertebrate conservation · Postglacial phylogeography · European Habitats Directive · IUCN red list

Introduction

The reliable identification of populations is considered a fundamental issue in conservation biology for the purpose of avoiding erroneous decisions and efficiently targeting conservation efforts and resources (Haig 1998; Soltis and Gitzendanner 1999; Vink et al. 2003; Rubinoff and Sperling 2004; Audisio et al. 2009; Morrison et al. 2009; Frankham 2010; Todisco et al. 2010).

Morimus funereus Mulsant, 1862 [alternatively treated as a subspecies of *M. asper* (Sulzer 1776); see Sama (2002) and Sama and Löbl (2010)] is a longhorn beetle found in deciduous broad-leaved forests of southeastern Europe. This large, showy wingless species predominantly inhabits old-growth forests and is part of the saproxylic fauna [sensu Speight (1989)], but it can also be collected on stumps or logs that are artificially released into forests.

This taxon is listed in Annex II of Habitats Directive 92/43/CEE among species requiring the designation of special conservation areas. In the last Habitats Directive

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Article 17 Report, covering the period from 2001 to 2006 (Commission of the European Communities 2009), the conservation status of *M. funereus* in Alpine and Continental regions was categorised as favourable (but was reported as unknown for Austria) based on distribution and habitat quality, whereas it was listed as unknown in Pannonian and Mediterranean regions. In 1996, this taxon was also listed as Vulnerable in the IUCN red list (A1c: facing a high risk of extinction in the wild in the medium-term future; World Conservation Monitoring Centre (WCNC) 1996), because of an estimated population reduction of at least 20 % over the last 10 years due to a decline in its area of occupancy, extent of occurrence and/or the quality of its habitat (IUCN Red List Categories and Criteria v2.3, 1994). However, *M. funereus* was recently excluded from the European Red List of Saproxylic Beetles (Nieto and Alexander 2010), mainly because the unstable taxonomy of this beetle, strongly hindered data collection and conservation monitoring.

According to Sama and Löbl (2010), six taxa of specific/subspecific rank could be currently accepted within the *Morimus* genus from the Palaearctic region: *Morimus asper asper* (C and W Europe), *M. asper funereus* Mulsant, 1862 (E and SE Europe), *M. asper verecundus* (Faldermann, 1836) (N Turkey to the Caucasus and NW Iran), *M. assamensis* Breuning, 1936 (S China), *M. lethalis* Thomson, 1857 (S China) and *M. orientalis* Reitter, 1894 (W Turkey), and seven additional species recorded from the Oriental region (www.lamiinae.org for the list of these species). Additionally, among the W Palaearctic species, correct identification of specimens is complicated by the presence of so-called "transitional" forms between *M. funereus* + *M. orientalis* and *M. asper* + *M. verecundus*, which are usually recognised as *M. ganglbaueri* Reitter 1894, based on the available identification keys (Reitter 1894; Dajoz 1976).

The genus *Morimus* itself is clearly in need of a comprehensive revision, since no extensive morphological/geographical data on the genus are available. In fact, all the revisions have been conducted only on elytral patterns and often on restricted samples and areas (Dajoz 1976; Sama and Löbl 2010; Simonetta 1989). It is fairly evident that only the five W Palaearctic taxa should be retained in *Morimus* and all the other taxa are likely to be transferred to other related but distinct Lamiinae genera (Sama and Löbl 2010).

Moreover, some of the most commonly used morphological characters delimiting species boundaries in beetles (e.g., the shape of aedeagus and ovipositor, sexual characters on ventrites) are not useful for discriminating among the five putative W Palaearctic *Morimus* taxa, which represents a fundamental problem for conservation. In fact, only a few weak characters are currently used to identify these taxa: (i) the distribution of elytral granulosity and the size of each granule; and (ii) the elytral colour pattern, chiefly with respect to the shape and size of dark elytral spots (Dajoz 1976). However, the combined intra- and inter-population variability of these characters does not allow clear-cut species boundaries to be established and recognised, despite the fact that the differences in their patterns are frequently related to the geographic origin of the examined specimens (Sama personal communication).

Thus, given the unresolved taxonomic status of W Palaearctic *Morimus*, we aimed to evaluate the genetic diversity within and among the *Morimus* populations from the W Palaearctic region using maternal and biparentally hereditable unlinked molecular markers (COI and ITS2) which have been successfully applied in not-previously genetically studied taxa and proven to be effective in many cases in tracing species boundaries (Li et al. 2010) or to unveil genetically differentiated groups that could be considered either distinct biological entities and taxonomic units or populations of the same species. On the basis of the observed phylogeographic structure, we also discussed the role of historical factors in shaping the current patterns of the distribution of these taxa. We, finally, evaluated the implications of our findings for the conservation of these taxa and of their primary habitat (old-growth forests) with reference to the IUCN Red List and the European Habitats Directive.

Materials and methods

Sampling

A total of 65 adult specimens of *Morimus* ssp. were collected by hand at the end of the reproductive period in a total of 32 localities (Fig. 1a and Supplementary material 1) from Italy (12 localities; $N = 14$), Slovenia (4 localities; $N = 8$), Croatia (5 localities; $N = 13$), Montenegro (5 localities; $N = 15$), Greece (2 localities; $N = 2$), Turkey (4 localities; $N = 13$) and Iran (1 locality; $N = 1$). *Morimus* specimens were put through morphological identification by specialists using characters (genitalia ad elytral pattern) and dichotomic keys provided by Reitter (1894), Dajoz (1976) and Sama and Löbl (2010). Furthermore, images of the elytral sculpture (e.g., dimension and density of tubercles) and microsetosity (being responsible for the colour pattern, well visible without magnification) were taken with a environmental scanning electron microscope (ESEM, Hitachi TM1000; data not shown). None of the analysed characters was distinctive (Sama and Cerretti, personal communications). Therefore, on the basis of this morphological preliminary analysis was not possible to assign our specimens to the taxa described in literature and all of the individuals were labelled as *Morimus* sp. Details on the sampling localities are reported in Table 1, Fig. 1a, and

Supplementary material 1. In Italy (Visso, Marche), we collected a representative specimen of *Herophila tristis* (Linnaeus, 1767) (Lamiinae), a morphologically closely related species (Sama 1991, 2008), and used it as an outgroup. All the specimens were preserved in sterile plastic vials containing 100 % ethanol for molecular analysis.

DNA extraction, amplification and sequencing

DNA was extracted from dissected metafemoral muscles following the salting out procedure described by Aljanabi and Martinez (1997). Two markers were amplified: a fragment of the mitochondrial cytochrome C oxidase subunit I (*COI*) gene and the second internal transcribed spacer (ITS2) of nuclear ribosomal DNA. PCR was conducted in a final volume of 25 μL, containing 1 μL of DNA, 0.5 U of Taq polymerase (Platinum® Taq DNA Polymerase, Invitrogen, Life Technologies, US), 0.8 pmol of each primer, 1 mM each dNTP (Invitrogen) and 5 μL of 10× buffer + 1.5 mM $MgCl_2$. We used an MJ MINI Personal Thermal Cycler (BIO-RAD Laboratories, US) to perform PCR amplifications. The obtained PCR products were purified with the Charge Switch® PCR Clean-Up Kit (Invitrogen) and sent to an external sequencing service (BMR Genomics, Padua, Italy).

COI

A partial fragment of approximately 800 bp at the 3′ terminus of the mtDNA *COI* gene was amplified and sequenced for all of the 65 *Morimus* individuals and

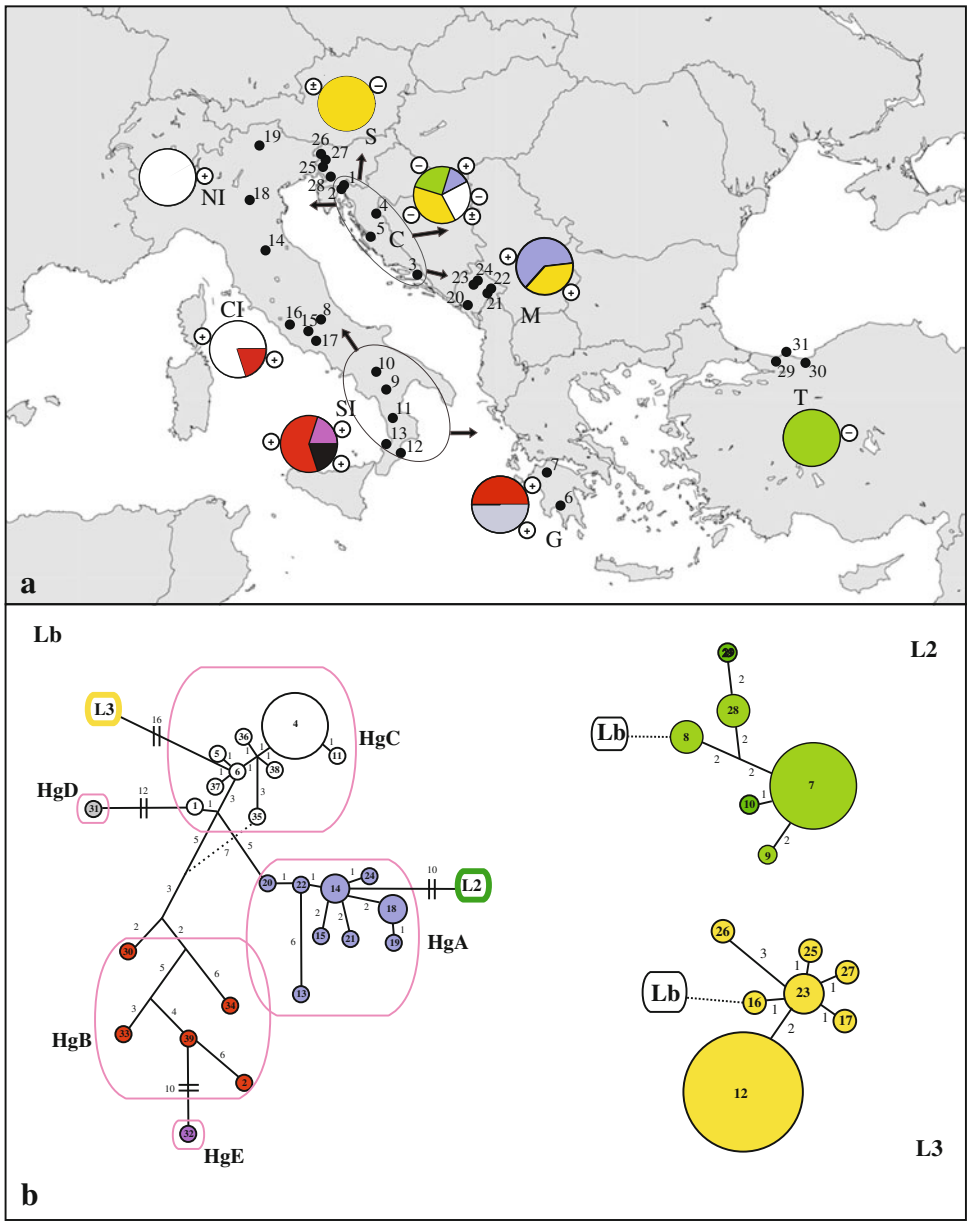

Fig. 1 **a** Map representing the study area. The map reports the sampling localities (*black dots*, the numbers refer to the localities reported in Supplementary Material 1); the distributions and frequencies of the lineages (L1–L3) and haplogroups of the basal lineage (HgA–HgE) of *Morimus sp.* for each samples region, indicated by the acronyms (*NI* northern Italy, *SI* southern Italy, *CI* central Italy, *S* Slovenia, *C* Croatia, *M* Montenegro, *G* Greece, *T* Turkey). The distribution of the ITS2 character in the areas, for each lineage, is indicated by (−) symbol for genotype-a, (+) symbol for genotype-b and (±) symbol for heterozygous individuals (see text for details). The *circles* indicate the putative glacial refugia for *Morimus* and the *arrows* the direction of postglacial colonization routes. **b** Parsimony networks performed on the lineages Lb (*left*), L2 (*right up*) and L3 (*right bottom*) separately. The *thin rectangle* delimited the Hgs of the basal lineage (Lb), the *bold rectangle* indicate the position of L2 and L3 in respect to the Lb

Table 1 Trapping locality, number of individuals per locality (N), number of haplotypes for each locality (Nh), total number of mutations (Nm)

	N	Nh	Nm	π	*h*	Tajima's D
COI						
Central Italy	5	5	22	22	1.000	−0.81456
Croatia	13	8	44	42	0.859	0.45585
Greece	2	2	14	14	1.000	-
Montenegro	15	13	28	28	0.981	1.09329
Northern Italy	4	4	7	7	1.000	−0.38921
Southern Italy	6	5	44	44	0.933	0.39693
Slovenia	8	2	5	5	0.250	−1.59524
Turkey	12	4	8	8	0.561	−0.95564
Pooled sample	65	39	91	86	0.959	−0.37331*
ITS2						
Central Italy	5	2	1			
Croatia	15	3	4			
Greece	2	2	1			
Montenegro	15	3	5			
Northern Italy	5	3	1			
Southern Italy	5	1	0			
Slovenia	8	3	4			
Turkey	6	2	10			
Pooled sample	61	12	20			

In addition, for the COI dataset, are reported nucleotide (π) and haplotype (*h*) diversity and the neutrality test results (Tajima's D) for each area and for the pooled sample. An asterisk next to the test value indicates statistical significance ($p < 0.05$). Calculations based on a sequence length of 765 bp

Herophila tristis using the universal primers C1-J-2183 and TL2-N-3014 (Simon et al. 1994). Both forward and reverse primers were used to sequence the fragment in double strand. PCR was conducted under the following amplification conditions: 94 °C denaturation (30 s), 59.5 °C annealing (1 min) and 72 °C extension (30 s) for 33 cycles, followed by a 7 min elongation step at 72 °C.

The obtained sequences were edited and aligned using Geneious v4.8.3 (Biomatter). Phylogenetic trees were produced using maximum parsimony (MP), Bayesian inference (BY) and maximum likelihood (ML) approaches. The MP analysis was run in PAUP v4.0 beta10 (Swofford 2001). Bootstrap re-sampling was carried out with a heuristic search producing 1,000 replicates, each with of 100 random taxa added as sub-replicates, applying tree bisection-reconnection branch swapping and saving 10 trees per replicate. A generalised time-reversible model with a proportion of invariable sites and heterogeneous substitution rates following a gamma distribution (GTR + I + G, Rodriguez et al. 1990) was selected by MrModelTest (Nylander et al. 2004) using the AICc criterion. Under the selected substitution model, both BY and ML phylogenetic reconstructions were performed. BY inference was carried out with MrBayes v3.2.1 (Huelsenbeck and Ronquist 2001) by running 1,000,000 generations, with Markov chains sampled every 1,000 generations. A burn-in of 10 % was applied and the remaining trees were used to compute a 50 % majority rule consensus tree and posterior probabilities. ML inference was carried out with PAUP, with the same tree-building algorithm used for MP analysis. For both of the BY and ML tree topologies, the node supports were determined by running 1,000 bootstrap re-sampling replicates. The genetic divergence between the lineages was estimated using MEGA v5.05 (Tamura et al. 2011).

The standard molecular diversity index, the number of polymorphic sites, the number of haplotypes and the nucleotide (π) and haplotype (*h*) diversities were calculated with DNAsp v.5 (Librado and Rozas 2009) for the pooled samples and for individuals grouped based on their geographic origin. Tajima's *D* statistic (Tajima 1989) was used to test the expansion of the geographical populations under the hypothesis of neutral evolution.

Parsimony networks of COI haplotypes were reconstructed with TCS v1.21 (Clement et al. 2000), in default settings, considering gaps as fifth state of mutation and calculating connection limits at 90 %, to describe the relationships within/among lineages indicated by the phylogenetic analyses. The estimates of genetic differentiation among lineages were assessed by calculating pairwise *Fst* values and testing their significance with 1,000 permutations using Arlequin v3.5.x (Excoffier and Lischer 2010).

ITS2

The entire ITS2 sequence was amplified in a subset of 57 individuals using the primers ITS-3d and ITS-4r (Oliverio and Mariottini 2001). The thermal cycling parameters used in these amplifications were as follows: 95 °C for 5 min, followed by 33 cycles of 94 °C for 1 min, 55 °C for 30 s and 72 °C for 1 min, with the final elongation step extended to 7 min. The PCR amplifications were carried out in a 25 μL volume containing 1 μL of DNA, 1.25 U of Taq DNA polymerase (Bioline, London, UK), 0.8 pmol of each primer, 1 mM each dNTP (Invitrogen) and 5 μL of 10× buffer + 1.5 mM $MgCl_2$. The obtained PCR products were purified with ExoSAP-IT enzymatic reactions (USB Corporation 2000) and sequenced in double strand, using the primer ITS-4r and a primer specifically designed for *Morimus* ITS2 sequences: ITS-1f (5′-TTGCGCGTCAA CTTGTGAAC-3′). The obtained sequences were inspected for double peaks, edited and aligned using Geneious v4.8.3 (Biomatter).

The Mfold web server (http://mfold.rna.albany.edu/?q=mfold) was employed to fold the ITS2 sequences to examine the position of the insertion identified between

nucleotide positions 142 and 147 (see "Results") in the ITS2 secondary structure.

Results

COI

The 65 COI sequences from the collected *Morimus* specimens and the single sequence from *H. tristis* resulted in an alignment of 765 bp. Thirty-nine *Morimus* spp. haplotypes sequences were deposited in GenBank under accession numbers KC592216-KC592254.

Most of the haplotypes (36 out of 39) were present at single localities, whereas the three remaining were shared between Croatia and central Italy (Mor_04), Croatia, Montenegro and Slovenia (Mor_12), and Croatia and Montenegro (Mor_23; Table 1; Supplementary material 1).

The sequences showed an overall haplotype diversity (*h*) of 0.959 and a nucleotide diversity (π) of 0.0226 (Table 1). The highest diversity was found in southern Italy ($\pi = 0.027$), whereas slightly lower but comparable π values were detected in central and northern Italy, Croatia, Greece and Montenegro (π values of 0.0124, 0.0085, 0.0206, 0.0185, and 0.0143, respectively). Slovenia and Turkey exhibited the lowest diversity values ($\pi = 0.0016$ and $\pi = 0.0026$, respectively) (Table 1). The highest *h* values, ranging from 0.859 to 1.000, were observed in Croatia, Greece, Italy and Montenegro. An intermediate *h* value was found in Turkey (0.561), and the lowest in the Slovenian sample (0.250). The geographic areas with the greatest number of haplotypes were Croatia and Montenegro, which exhibited 8 and 13 haplotypes, respectively.

The topologies of the phylogenetic trees obtained using the three specified approaches (BY, ML and MP) were comparable. The Bayesian consensus tree topology with posterior probability and the additional bootstrap values from the ML and MP analyses are reported in Fig. 2 (only posterior probability and bootstrap values exceeding 70 % are shown). Most of the terminal clades were strongly supported by the bootstrap values at their interior nodes (posterior probability values >0.98; bootstrap values >95 %) and the relationships between the terminal branches were maintained in all topologies. However, the basal nodes defining the relationships among the main lineages were poorly supported by the bootstrap values (<50 %). Four principal lineages are distinguishable (designated as L1, L2, L3, and Lb in Fig. 2), showing only a partial correspondence with the geographical origin of the sampled *Morimus* individuals and are defined as follows. L1 lineage, supported by the highest node values, consists of 2 individuals from southern Italy. L2 (BY p.p. = 1, MP boot. = 98 and ML boot. = 98) groups all individuals from Turkey and 3 individuals from Croatia (Biokovo Mountains, see Table 1; Supplementary material 1); L3 lineage (BY p.p. = 1, MP boot. = 99 and ML boot. = 100) appears to be more heterogeneous, as it groups individuals from Croatia, Montenegro and Slovenia. Finally, we defined a "basal lineage" (Lb) which, although not monophyletic and including clades not strongly supported by all analyses (BY p.p. between 1 and 0.73 but with bootstrap values <70 % in both the ML and MP analyses), was worth to be considered as an "operational" group. The Lb lineage is, in fact, constituted by closely (less than six parsimony steps in the reconstructed network, see below) and geographically-related haplotypes included in five haplogroups (HgA–HgE) all having a relatively basal position in the COI phylogenetic tree. The genetic distances between the lineages show low values, varying between 2.5 % and 3.6 % (L1 vs. Lb = 2.5 %; L1 vs. L2 = 3.2 %; L1 vs. L3 = 3.6 %; L3 vs. L2 = 2.9; L3 vs. Lb = 2.9; Lb vs. L2 = 2.5). The geographic distribution of lineages and haplogroups and the relative frequencies are plotted on the map of the study area (Fig. 1a, color pattern following Fig. 2). The relationships among haplogroups and haplotypes constituting the Lb lineage (Fig. 1b) (that, as mentioned above, is composed by closely-related haplotypes distant less than six parsimony steps) are shown in Fig. 1b. In particular, HgA groups all haplotypes from Montenegro and a single haplotype from Croatia (Mor_13). HgB includes haplotypes from Greece and central and southern Italy separated by several steps each (ranging from 2 to 6); a single haplotype (Mor_32) related to this haplogroup, but separated by 10 parsimony steps, can be considered representative of a separate haplogroup (HgE). HgC consists of haplotypes from Croatia and northern and central Italy; from this group, haplogroup HgD was derived (12 steps), which consists of a single haplotype (Mor_31). The geographic distribution and the relative frequencies of the lineages and the haplogroups are plotted on the map of the study area in Fig. 1a and represented with the corresponding colours (Fig. 2). The COI parsimony network also shows the relationships among the lineages and haplogroups described above. The most closely related groups are L2–HgA (10 steps) and L30–HgC (16 steps). In the Lb lineage, HgC–HgA (8 steps) and HgC–HgB (13 steps from Mor_30, the most closely related haplotype) are strictly connected. HgD and HgE, as noted above, are derived from HgC (12 steps) and HgB (10 steps), respectively.

The values for Tajima's *D* index are all non-significant (except for the pooled sample), albeit negative, as expected for a recent population expansion (Table 1). Positive values for Tajima's *D* were obtained only for populations from Croatia and Montenegro, suggesting a recent bottleneck. The *Fst* values computed between groups from

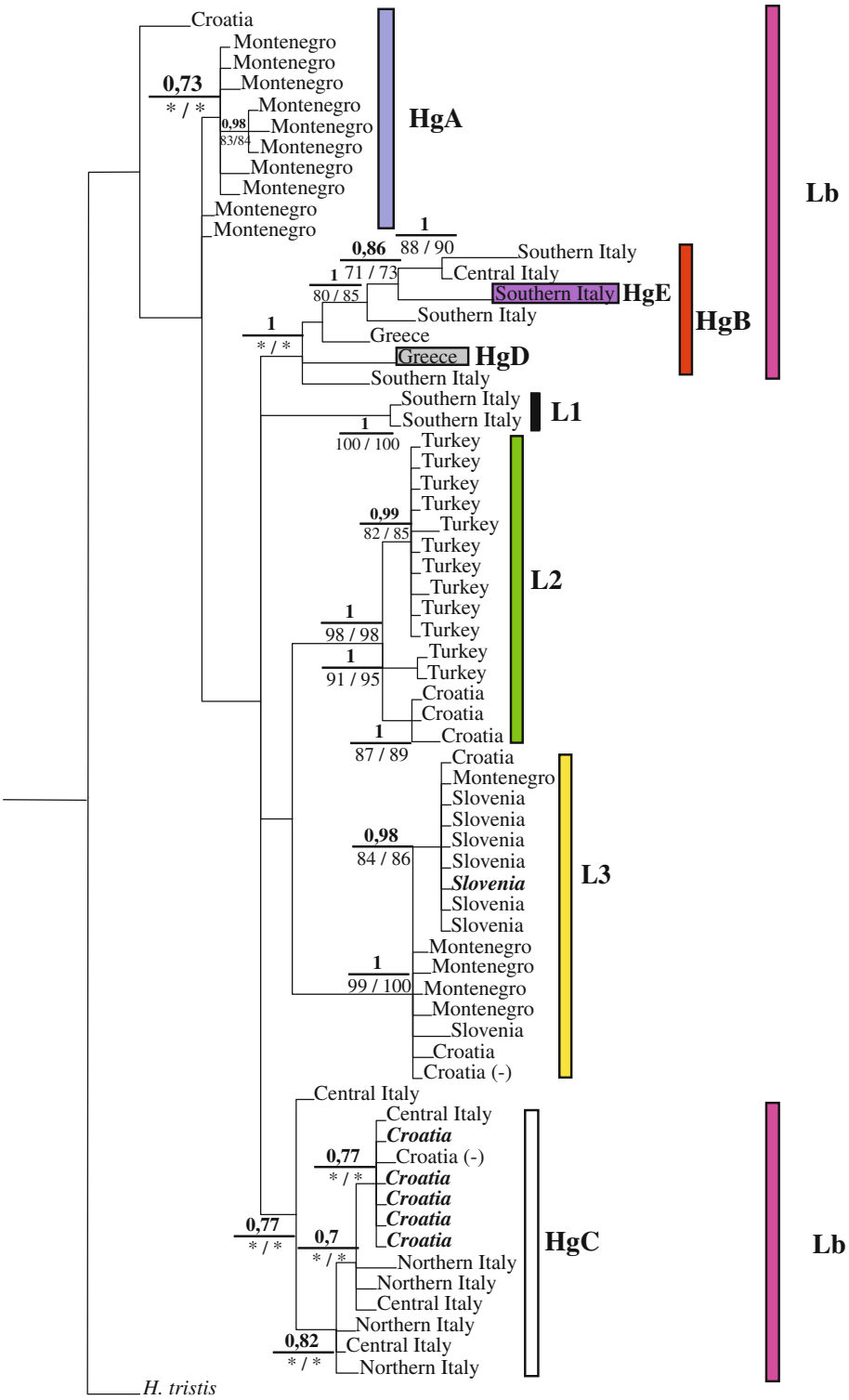

Fig. 2 Bayesian tree topology of the entire dataset. *Herophila tristis* was used as outgroup. Posterior probability (*up the line*) and bootstrap value of MP and ML (*down the line*) major of 70 % are reported at nodes. *Asterisks* indicate nodes not supported in the correspondent MP or ML topology. The *vertical rectangles* highlight the major lineages (L) and haplogroups (Hg). The *two horizontal rectangles* highlight the minor Hgs (D and E). For all the ITS2 genotypes see the map in Fig. 1a. Individuals in *italic/bold* are the ones that retain ITS2 character in heterozygous state and the symbol (−) indicates the individuals with "minus" state included in "plus" lineage

different geographic regions indicated that overall, the Turkish and Slovenian populations are the most differentiated in our sample. In particular, the Turkish population diverges significantly from the populations from Greece ($Fst = 0.817$), northern Italy ($Fst = 0.869$, $p < 0.05$) and central Italy ($Fst = 0.777$, $p < 0.05$) (less so from southern Italy, $Fst = 0.655$, $p < 0.05$). Similarly, the Slovenian population is highly diverged from those of Turkey ($Fst = 0.924$, $p < 0.05$, indicating a nearly complete isolation of these two populations), Greece ($Fst = 0.858$, $p < 0.05$), northern Italy ($Fst = 0.908$, $p < 0.05$) and central Italy ($Fst = 0.795$, $p < 0.05$) (less so from southern

Italy, $Fst = 0.624$, $p < 0.05$). By contrast, the lowest Fst estimates ($Fst \leq 0.503$, $p < 0.05$, excluding Turkey and Slovenia) were observed comparing gene pools from Croatia and Montenegro with all other groups, whereas no significant differentiation was found when the Greek population was compared with those from Italy, Croatia and Montenegro.

ITS2

The ITS2 sequence dataset obtained from 57 *Morimus* specimens consisted of 438 bp of aligned positions (Table 1), which have been deposited in GenBank under Accession Nos. KC592159-KC592215 (Table 1; Supplementary material 1). A total of 12 genotypes ($h = 0.606$ and $\pi = 0.0351$) were observed in our sample (Fig. 3a), which were differentiated from each other by 1–9 nucleotide substitutions overall. The presence or absence of a 6 bp deletion in the ITS2 sequence allowed us to define two genotypes, designated genotype-a and genotype-b (see Table 1 of Supplementary material 1 for details). Genotype-a was characterised by the presence of a 6 bp deletion (and, thus, labelled with a minus symbol (−) when plotted on the geographic map and phylogenetic tree in Figs. 1a and 2) between positions 142 and 147 (following the numbering of our alignment) and included 5 genotypes: MOR-ITS2-1a, shared by 2 individuals from Slovenia and 9 from Croatia (tot = 11); MOR-ITS2-2a, found in 5 individuals from Turkey; MOR-ITS2-3a and MOR-ITS2-4a, which were present in the 2 sampled individuals from Greece; and MOR-ITS2-5a (also characterised by 3 deletions at positions 76, 77, and 139), found in 1 specimen from Turkey. Genotype-b was characterised by the presence of a 'TGGTTC' motif between positions 142 and 147 (and, thus, labelled with a plus symbol (+) when plotted on the geographic map and phylogenetic tree) and grouped 7 genotypes (Fig. 3a): MOR-ITS2-1b, the most widespread genotype ($N = 35$), which was found in 11 individuals from Italy, 5 from Slovenia, 13 from Montenegro, 5 from Croatia and the single *Morimus* specimen collected in Iran; MOR-ITS2-2b, found in 3 Italian individuals (from Lombardy, E. Romagna and Latium); MOR-ITS2-3b, detected in 1 Italian specimen (Lombardy); MOR-ITS2-4b, observed in 1 individual from Slovenia (Goriška); MOR-ITS2-5b and MOR-ITS2-6b, which were carried by 2 individuals from Montenegro (Nikšić and Cetinje, respectively); and finally, MOR-ITS2-7b, which was exclusive to 1 individual from Croatia (Učka). It is worth noting that 4 individuals from Croatia (Učka Mountains) and 1 from Slovenia (Goriška) retained heterozygosis for the 6 bp deletion in the ITS2 marker and showed a MOR-ITS2-1a/1b genotype (because the sequences of these five heterozygous individuals were phased and included in our database, we obtained a total of 62 ITS2 sequences). The inspection and comparison of the secondary structures of the *Morimus* ITS2 sequences (Fig. 3b) showed that the 'TGGTTC' motif found in genotype-b was partially integrated in a loop and is therefore situated in one of the least constrained (and most prone to accumulate substitutions) portions of the ITS2 molecule.

Discussion

Systematics and phylogeography

This study represents the first molecular investigation of the longhorn beetles of the genus *Morimus*. The intriguing taxonomic issues that have emerged due to the pronounced morphological variability in this genus have lead to the necessity of obtaining better insight into its systematics. The relatively recent molecular approaches applied to insects have been found to be extremely useful for the definition of species boundaries and to ascertain the validity of morphological characters that are traditionally considered diagnostic (Wiens 2007).

The phylogenetic tree obtained for *Morimus* sp. shows features that are typical of a recent and rapid evolution history, as expected from COI data (Ballard and Rand 2005; Simon et al. 2006) and demonstrated by the low support of the basal nodes. However, high values are observed at terminal nodes and indicate three well-supported clades (L1–L3) and the presence of a basal lineage (Lb). The haplogroups only partially follow the geographical distribution since, in most cases, an admixture of haplotypes from different areas is evident. It then follows that none of the clades could be consistent with the species attribution provided in literature merely basing this definition on the geographic distribution described for the hypothesized sub-species (sensu Reitter 1894). Besides, even considering the debatable possibility to provide distance-based species delimitations, the low genetic distance values associated with the clades fall in a range of variability that, for species with similar vagility, it is not considered supportive for species distinction (Nakamine and Takeda 2008; Woodcock et al. 2007). In Lamiinae (Nakamine and Takeda 2008), for example, an average value of genetic divergence between four lineages equal to 4.53 % was not considered by the authors supportive for defining the observed lineages as species. About less closely related and not-vagile taxa than Lamiinae, the genetic distance values that are consistent with species boundaries definition are largely variable, ranging, for instance, from 5.92 to 6.38 % between species and 1.3–1.99 % between subspecies in the genus *Cicindela* (Woodcock et al. 2007) and 3.06 % of mean intra-specific distance in genus

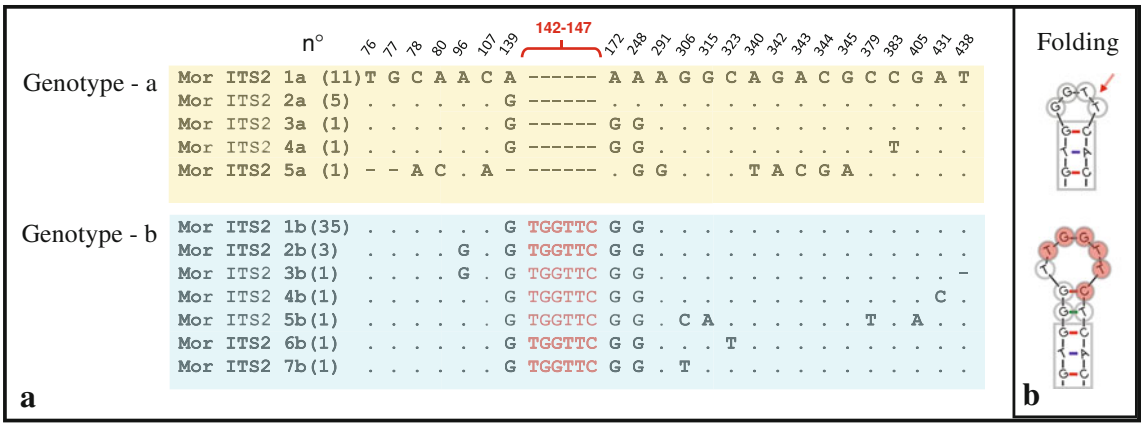

Fig. 3 **a** ITS2 alignment of genotype-a (*up shaded rectangle*) and genotype-b (*bottom shaded rectangle*). **b** folding example for the correspondent genotype. The *arrow* indicate the position of nucleotides insertion in the loop

Agronum (Raupach et al. 2010). Finally, although the issue is controversial, genetic distances provided by our data are below or at the boundaries of what some molecular systematists have considered as a reasonable COI sequence divergence threshold for insect species delimitation (~3 %), and well far below if a more conservative 2 % cut-off value (as for vertebrates, see Duckett and Kjer 2003; Hebert et al. 2003, 2004; Moritz and Cicero 2004) is considered. Similarly, when using the ITS2 marker, *Morimus* specimens sampled from distant localities shared similar or identical ITS2 genotypes, thus not consistently supporting the possible presence of *Morimus* sub-species having different geographic distributions. Furthermore, we also observed that these genotypes were related to two widespread ITS2 variants (genotype-a and -b) characterized by a 6 bp insertion/deletion. This character plausibly originated by a single mutational event very early in *Morimus* gene pool, but the presence of heterozygous individuals for these two ITS2 variants further suggests the absence of gene flow barriers within *Morimus* sp. from a relatively long time.

Our results further discourage the use of the currently accepted diagnostic criteria (Dajoz 1976) which are not reliable for the distinction of the five W Palaearctic *Morimus* species and that, therefore, do not provide a valid instrument for species identification. Taxonomic experts on the W Palaearctic species of *Morimus* have most likely been strongly influenced by the geographic origin of the collected specimens. For instance, the above-mentioned "transitional forms" were identified as *M. asper* if collected in the Apennines and as *M. ganglbaueri* if collected in the Balkans. Despite this taxonomic uncertainty, no study has previously attempted to assess the reliability of the diagnostic criteria using more recent and complementary techniques, even though these are the criteria that are currently adopted to designate the conservation status of *M. funereus* at both the European (Nieto and Alexander 2010) and international (World Conservation Monitoring Centre 1996) levels.

COI sequence data evidenced a rather strong population genetic structuring for our *Morimus* sp. sample. In fact, we identified three main and distinct lineages (L1, L2, and L3, in southern Italy, Turkey, Croatia and the Balkans) and the additional basal haplogroups assemblage (Lb) in the Balkans (HgC and HgA), northern and central Italy (HgC), southern Italy and Greece (HgB) (Fig. 2). The Croatian populations were the most variable ($\pi = 2.1$ %), presenting haplotypes belonging to three different lineages (L1, L2, and L3), whereas the Turkish, Slovenian and northern Italian populations are less diverse and are confined to single lineages (L2, L3) and HgC, respectively. The ITS2 sequences are polymorphic, corresponding to the "minus" (Croatia, Slovenia and Turkey), "heterozygous" (Slovenia and Croatia) and "plus" (all the other genotypes) states of the character data (Fig. 3a, b).

Overall, both molecular markers indicated that the Turkish and Slovenian groups are the most genetically differentiated. The *Fst* values computed for the COI sequences suggested that these populations are nearly completely isolated from each other and are the most differentiated from all other populations. This finding is clearly indicated by the position and the shape of L2 and L3 in the network (Fig. 1b), which are distinct from the basal clade and show a marked star-like pattern. Moreover, the negative Tajima's *D* values indicate that these populations have undergone recent expansion, possibly following a bottleneck likely caused by habitat fragmentation. However, the populations from Croatia and Montenegro show the opposite pattern regarding their *Fst* and Tajima's *D* values and are spread among the haplogroups of Lb, showing no significant differentiation from all other groups. The same positive value of Tajima's *D* is found in southern Italy, indicating a stable population (i.e., lack of recent demographic expansion).

Although the available sequence data do not allow us to draw a plausible scenario, these genetic results provide

some interesting clues regarding the possible spread of the genus *Morimus* in Europe and allow us to formulate some hypotheses that may contribute to lead further investigation and help in future sampling strategies of this genus. We assume that the origin of the genus is in the Oriental region (Sama and Löbl 2010; Nakamine and Takeda 2008). Our findings show that the Croatian and southern Italian populations show haplotypes that are included in almost all of the described COI lineages and Croatia, in addition, shows insertion/deletion polymorphisms in the ITS2 marker. Our data suggest two different potential scenarios: (i) the Croatian and southern Italian populations may represent the oldest population, which still retain most of the genetic variability inherited from Oriental *Morimus* ancestors and these geographic areas could therefore be considered the cores of *Morimus* diffusion in the central Mediterranean area; alternatively, (ii) Croatia could be considered a site of multiple events of colonisation, from the northern Alps and from the Anatolian region, whereas the populations from southern Italy and Greece might be associated with a separate wave of colonisation.

The second scenario, however, seems unlikely as the most haplotypically diverse areas should usually represent the source of colonisation (Hewitt 1996). We, therefore, consider the first hypothesis to be more reasonable because it could be consistent with both the genetic results of our study and the current knowledge regarding the route of postglacial colonisation of Europe from *refugia*, as described for several species of animals and plants (Hewitt 1999, 2003, 2004), including one of the host tree species for the larval development of *Morimus*, the European beech (*Fagus sylvatica* L.) (Magri et al. 2006; Magri 2008).

More specifically, the COI data indicate the presence of the greatest number of haplotypes in Croatia and Montenegro. However, whereas in Montenegro all these haplotypes belong to the same lineage and can, therefore, be ascribed to a single event of colonisation by a related pool of haplotypes, in Croatia, the haplotypes belong to several different lineages, indicating that this is an area of elevated diversity, perhaps representing the source of diffusion for the *Morimus* lineages in the Mediterranean area. The obtained *h* values are generally high, while the π values are generally low. This pattern is in accord with the hypothesis that the observed genetic diversification among the mtDNA lineages did not occur in situ but preceded the arrival of colonising populations. A similar hypothesis can be put forth for southern Italy. Moreover, the described genetic features are appropriate for populations from a *refugium* area and the Balkans and southern Italy have been demonstrated to have acted as glacial *refugia* for several species (Hewitt 1999; Ribera and Vogler 2004; Dapporto et al. 2011; Fattorini and Ulrich 2012). A decrease in diversity and lineages sharing is expected for areas secondarily colonised from a *refugium*. From this perspective, we can hypothesise that *Morimus* from the Balkans underwent colonisation in a northward direction, reaching Slovenia (L3), the Alps and northern Italy (HgC) and southward to Montenegro (HgA and L3) and Turkey (L2). From southern Italy, it is expected that a northward expansion occurred and that a contact was established with a population from the Balkans, as shown by the coexistence in central Italy of both HgB and HgC (Fig. 1a). The presence of a shared haplogroup (HgB) between the Balkan peninsula and southern Italy could derive from secondary and more recent human-mediated transport of beetles (i.e., timber trading) from Italy (as observed in other Cerambycids, Cocquempot and Lindelöw 2010; similar situations are known in other insects, see Hewitt 1999; Dapporto and Dennis 2009; Fattorini and Ulrich 2012). The very small sample from Greece does not allow us to put forth a hypothesis, but a larger sample is required to highlight the genetic variability for this area with more accuracy.

The polymorphic ITS2 sequences observed in Croatia support this hypothesis and are useful in determining the polarisation of the colonisation events. The Iranian individual (the closest locality to the genus origin) shows the "plus" character, which can therefore be considered the ancestral condition. Then, individuals with this insertion would have colonised the Balkans. In Croatia, the 6 bp indel in the ITS is would have arisen in a single mutational event, as supported by the extant polymorphism. Subsequently, the beetles would have undergone colonisation northward to Slovenia ("plus" and "Hz") and northern and central Italy ("plus") and southward to Montenegro ("plus") and Turkey ("minus"). The available data cannot conclusively demonstrate that the expansion followed the spread of this mutation, but it is highly likely, as it is not parsimonious to postulate that the same 6 bp indel arose in distant localities through independent events. In central and southern Italy and Greece, the "ancestral" condition, i.e., the insertion, was observed, suggesting a separate colonisation event from the East.

The preference of *Morimus* sp. for beech forests in Europe is commonly accepted (Romero-Samper and Bahillo 1993), representing the inheritance of a past, more strictly monophagous habit, despite the fact that this genus is not only associated with beech forests at present, e.g., being found in association with *Populus* spp. *Quercus* spp., *Fraxinus* sp. and *Pinus* spp. This change in alimentary preference is due to the retreat of *Morimus* species from the mountain zone to the foothills, concurrently acquiring more polyphagous preferences. As previously highlighted by Romero-Samper and Bahillo (1993), *Morimus* likely underwent a postglacial dispersion that was considerably parallel to that of *Fagus sylvatica* from *refugia* areas following the cooling at the end of the last interglacial period (Guiot et al. 1989). A very intriguing point for future

studies on more extended sample could be lead a parallel analysis between *Morimus* and the very detailed data available on beech postglacial colonisations pattern in the Mediterranean area (Magri et al. 2006 and Magri 2008).

Conservation considerations

If it is reasonable to group together the five *Morimus* species sensu Reitter (1894) into a single species, this would imply delisting and re-evaluation of *M. funereus* as a valid taxon in the Habitats Directive. As no diagnostic morphological characters are available to reliably identify *M. funereus* as a separate species, it remains problematic defining the geographic boundaries of this unit for practical conservation purposes. Accordingly, we believe that the available estimates of the distribution of *M. funereus* (e.g., van Helsdingen et al. 1996) are likely unreliable and suffer from a lack of diagnostic criteria and the unresolved taxonomic status of the genus.

Further taxonomic research on *Morimus* populations, using a multitude of morphological, genetic and ecological traits, might indeed reveal that some are sufficiently distinct to be recognised as ecologically significant units (ESU; Ryder 1986; Waples 1991). However, this possibility would require assessing the degree of genetic and/or ecological exchangeability (Crandall et al. 2000) among putatively distinct *Morimus* population units. Unfortunately, due to the paucity of bionomical and ecological data on *M. asper* and its local populations (and/or "forms"), it is currently not possible to delineate a definitive conservation strategy for this species. While more thorough datasets are collected to definitively assess the taxonomic status of this genus, we suggest that the current protected status of *M. funereus* should be extended to the whole *M. asper* species, including all of its local populations, as this strategy would represent the most effective way to ensure its conservation while extending conservation benefits to its primary habitat and the saproxylic beetle communities therein. In fact, similar to all of the *M. asper* populations across Europe, habitat loss and degradation, due to degraded environmental quality and reduction of forest cover due to changes in human land use, represent the major threats to all saproxylic beetles (Buse et al. 2007; Lindhe and Lindelöw 2004; Ranius and Jansson 2000).

Conclusions

We hypothesize that the origin of the genus *Morimus* is in the Oriental region and its expansion brought the genus to Europe, and specifically to the Mediterranean basin in relatively recent times. The distribution of lineages and haplogroups in the genus indicated the existence of at least two nuclei of dispersion for *Morimus* in the central Mediterranean, the Balkans and southern Italy and it can be hypothesised that these areas could have acted as *refugia* for *Morimus* during the last glacial period. From these sources *Morimus* colonised the central Mediterranean area. The presence of individuals that are heterozygous for the nuclear marker we examined, is a clear indication that individuals from this polymorphic area are (or have been) interfertile. The elytral spot pattern-based species descriptions provided in the literature indicate the presence in this area of three species: *M. funereus*, *M. asper* and *M. ganglbaueri*. We detected no relationship between elytral spot patterns and ITS2 characters, but conversely, we found evidence of interfertility. Future studies addressed to better clarify the taxonomic issues in this group should consider inbreeding experiments (Dojnov et al. 2012) in parallel with the evaluation of morphological characters.

As for other saproxylic beetles, the ecology and biology of *Morimus* species makes them a difficult group to study. The habitat loss and restriction to old forests observed for this group, in addition to the long life span and development of larvae and adults result in serious sampling constraints for these beetles. Nevertheless, although preliminary in this direction, our results provide new insights regarding the *Morimus asper* complex. Genetic data coupled with less invasive investigations (e.g., Geometric Morphometrics analyses on the specimens collections or inbreeding experiments) are fundamental for determining the most important features of the species complex and focusing further sampling to better address systematic and taxonomic issues. These kinds of investigations are essential not only to extend our knowledge on this genus, but to better define an optimal conservation strategy.

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