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Evolutionary relationships within European Monochamus (Coleoptera: Cerambycidae) highlight the role of altitude in species delineation

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15 highlight the role of altitude in species delineation.

16

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31

32 **Abstract.**

33 Phylogenetic relationships within the European *Monochamus* (Coleoptera: Cerambycidae)
34 remain understudied despite their increasing importance in the Pine Wood Nematode spread
35 in Europe. To clarify the delimitation and the evolutionary history of the two main European
36 *Monochamus* species, *M. galloprovincialis* and *M. sutor* as well as their sub-species, a
37 comparative study using morphological, molecular and biogeographic criteria was
38 conducted. Four morphological characters, including a newly described one on the male genitalia,
39 separated the two species. Additionally, molecular data revealed twelve and two single
40 nucleotide polymorphisms in the COI and 28S respectively, supporting species segregation.
41 In contrast, incongruence between morphological and genetic results did not allow
42 discriminating the sub-species of *M. galloprovincialis* and *M. sutor*, even though
43 mitochondrial DNA revealed intraspecific differentiation, mostly consenting to a multiple
44 refugia origin. Within species variability was explained to a large extent by biogeography i.e.
45 altitude, climate. These different ecological adaptations within beetle species together with
46 potential climate change impact increase the risk of spreading the nematode across Europe to
47 novel conifer hosts and challenge the European biosecurity.

48

49 **Keywords.** biogeography - genitalia - integrative taxonomy - *Monochamus* morphology -
50 mitochondrial DNA – nuclear DNA – PWN vector.

51

52 **Introduction**

53 The coexistence of closely related taxa is an issue of fundamental interest in evolutionary
54 biology and accurate taxonomy is crucial for such evolution studies as well as biodiversity,
55 ecology and conservation studies (Agapow *et al.*, 2004; Cracraft, 2002; Mace, 2004).

56 Nevertheless, much argument seems to surround species concept and lower taxa delimitation
57 (De Queiroz, 2007). Lacking standardized operational criteria to delimit them, several authors
58 have stressed the importance of Integrative Taxonomy, i.e. a multidisciplinary approach to
59 separate species (De Queiroz, 2007; Fujita *et al.*, 2012; Heethoff *et al.*, 2011; Roe & Sperling,
60 2007b; Schlick-Steiner *et al.*, 2010). Even though monophyly usually supports species
61 separation, discordance has been observed between phylogenies based on morphological
62 versus molecular markers (Wiens & Penkrot, 2002) but also between molecular markers
63 (mitochondrial versus nuclear DNA) (Shaw, 2002). The mitochondrial DNA and especially
64 the cytochrome oxidase subunit I (COI) gene, as proposed by Hebert's *et al.* barcode (2003a;
65 2003b), has a number of advantages for the distinction of species but collaboration with
66 traditional taxonomy is necessary (DeSalle *et al.*, 2005; Lipscomb *et al.*, 2003; Mallet &
67 Willmott, 2003; Seberg *et al.*, 2003; Taylor & Harris, 2012; Will & Rubinoff, 2004). Thus
68 studies attempting to define species boundaries, particularly in cases of recent speciation
69 events or cryptic species, need the consensus of numerous independent criteria.

70 *Monochamus* (Coleoptera, Cerambycidae) is a worldwide distributed genus that has
71 drawn attention due to its association with the transmission to conifers of the highly
72 pathogenic Pine Wood Nematode (PWN) *Bursaphelenchus xylophilus* Steiner & Buhrer, 1934
73 (Nickle, 1970). Five species of the genus *Monochamus* have been described in Europe, i.e. *M.*
74 *galloprovincialis* (Olivier, 1795), *M. sutor* (Linnaeus, 1758), *M. saltuarius* (Gebler, 1830), *M.*
75 *sartor* (Fabricius, 1787) and *M. urussovi* (Fischer, 1806); all attacking conifers and mainly
76 *Pinus* and *Picea* species. European *Monochamus* species are described in the literature as
77 closely related species further separated into sub-species (Cesari *et al.*, 2005). Phylogenetic
78 relationships within the European *Monochamus* remain understudied despite the increased
79 importance of such clarification since the discovery of the invasive PWN in Portugal
80 (Fonseca *et al.*, 2012; Mota *et al.*, 1999; Rodrigues, 2008) and more recently in Spain
81 (Abelleira *et al.*, 2011; Robertson *et al.*, 2011). Of the five European *Monochamus* species,
82 only *M. galloprovincialis* has been proved to vector the nematode in Europe so far (Sousa *et*
83 *al.*, 2001). *M. sutor* is absent from Portugal while it is present in Spain and in the rest of

84 Europe, at higher altitudes than *M. galloprovincialis* (Hellrigl, 1971; Sama, 2002; Sama,
85 2008; Villiers, 1978; Vives, 2000). Due to the progression of the nematode infestation and its
86 recent discovery at the Spanish border with Portugal, *M. sutor* is the closest co-vectoring
87 candidate in Europe (see geographic distribution Fig. 1) and increases the undeniable risk of
88 PWD expansion to the continent.

89 Up to now, the taxonomic status of *Monochamus* species is based on morphological
90 features; however some controversy remains due to consistent variability of these characters
91 both within species and between sister-species. Taxonomic uncertainties still remain
92 especially within the highly polymorphic *M. galloprovincialis* species, with the dark
93 specimens easily mistaken for its sister-species *M. sutor* or at the sub-species where the
94 confusions are the most frequent. Hellrigl (1971), Sama (2002; 2008), Tomminen and
95 Leppänen (1991), Villiers (1978) and Vives (2000) have morphologically and ecologically
96 described these two *Monochamus* species and their sub-taxa (Table 1). It is generally
97 admitted that *M. g. galloprovincialis* is found in Southwest Europe and North Africa while *M.*
98 *g. pistor* (Germar, 1818) occurs in northern, central and eastern Europe. *M. g. pistor* (Germar,
99 1818) is also present in southern Europe (central Spain, southern France and the French Alps)
100 but in these cases higher elevations compensate the cooler conditions required by this sub-
101 species (Vives, 2000). In his description of *M. g. galloprovincialis* specimens from North
102 Africa, Sama (2008), mentions four specimens identical to the *M. g. pistor* holotype from
103 Slovenia. Likewise, within *M. sutor*, it is unclear whether *M. s. sutor* and *M. s. pellio*
104 represent morphologically different forms or sub-species with distinct geographic
105 distribution. Moreover, further clarification is needed on their geographic distribution since
106 the limits of their sympatry or syntopy are not clearly defined (Sama, 2002; 2008) (Fig.1).

107 We consider the clarification of the phylogenetic relationships of *M. galloprovincialis*
108 and *M. sutor* fundamental for future investigation on integrating pest management programs.
109 Although the European species of the genus *Monochamus*, and mainly *M. galloprovincialis*,
110 have been subject of numerous studies on their biology and association with PWN (Akbulut,
111 2009; Akbulut & Stamps, 2012; Akbulut *et al.*, 2008; Koutroumpa *et al.*, 2009b; Koutroumpa

112 *et al.*, 2008b; Naves, 2007; Naves *et al.*, 2007; Naves *et al.*, 2006a; Naves *et al.*, 2006b; Sousa
113 *et al.*, 2001; Sousa *et al.*, 2002), literature reports on taxonomy and geographic distribution of
114 Palearctic *Monochamus* species are scarce (Hellrigl, 1971; Sama, 2002; Sama, 2008;
115 Tomminen & Leppänen, 1991; Villiers, 1978; Vives, 2000). With the exception of one
116 molecular phylogenetic study on the European *Monochamus* (Cesari *et al.*, 2005), the genetic
117 variability at the intra- and interspecific level is unknown in Europe.

118 We focus our study on *M. galloprovincialis* - *M. sutor* as the most expanded
119 European *Monochamus* species, and investigate their relationships with the sub-species *M. g.*
120 *pistor* and *M. s. pellio*. Under the assumption of a morphological delimitation we use
121 molecular characters to examine the level of differentiation between and within the two sister
122 species *M. galloprovincialis* and *M. sutor*. We applied a multi-marker approach on samples
123 mainly collected from their southern European distribution, which represents the area under
124 direct risk of natural invasion by the PWN (Robinet *et al.*, 2011) and where the delimitation
125 of the different sub-species is even more complex (Vives, 2000). France is a key area and thus
126 occupies a core position in this study due both to the occurrence of the two *Monochamus*
127 species and their sub-species, and to its strategic position for the natural dissemination of the
128 nematode from Portugal and Spain to the rest of Europe. A broad range of elevations and
129 potential host trees was considered, as they offer potential environmental factors hiding
130 cryptic taxa within these species. The results highlight new morphological characters in
131 *Monochamus* male genitalia and provide essential knowledge on European *Monochamus*
132 genetic delimitation in combination with their ecological requirements.

133

134 **Materials and methods**

135

136 *Monochamus* sampling

137

138 From June to September 2003 and 2004, specimens of *Monochamus* were sampled either by
139 cross van traps (Ibeas *et al.*, 2007; Koutroumpa, 2007; Koutroumpa *et al.*, 2008a) or by field

140 collection. A total of 150 specimens of *M. galloprovincialis* were collected at 32 locations in
141 natural pine stands in seven European countries plus Morocco (see details in table 2). In
142 addition, 50 specimens of *M. sutor* were sampled from 17 locations originating from natural
143 populations of *Pinus* species and *Picea abies* (L. H. Karst. 1881) in four countries in Europe.
144 A sampling effort was conducted in the French Mountains, following an elevation gradient
145 (up to 1700m) suspected to house cryptic forms of the studied species. Insects were stored at -
146 80°C or in absolute ethanol.

147

148 *Morphological features*

149

150 Seventeen morphological features in total, seven binary and ten multi-states, including eight
151 external characters and seven internal characters on male genitalia, were analyzed (see online
152 supporting information 1). Seven were chosen based on previous systematic and
153 morphological studies on *Monochamus* species and other Coleoptera (Ahrens, 2005; Bense,
154 1995; Hellrigl, 1971; Sama, 2008; Sanmartin & Martin-Piera, 2003; Takami & Suzuki, 2005;
155 Tomminen & Leppänen, 1991; Villiers, 1978; Vives, 2000). Within the main characters
156 usually used to differentiate *M. galloprovincialis* from *M. sutor*, one can underline the form of
157 the scutellum and the color of the pubescence on the elytra (online supporting information 1,
158 5 and 6) (Table 1). Tomminen and Leppänen (1991) published a difference on the shape of
159 the median lobe of the male genitalia of the two species (Table 1). In *M. galloprovincialis*
160 lower taxa, *M. g. galloprovincialis* has brown-red legs and antennae while *M. g. pistor* has
161 black ones (online supporting information 1). According to Vives (2000) and Villiers (1978)
162 *M. g. pistor* is smaller than *M. g. galloprovincialis* and has less pubescence on the elytra of
163 yellow-white color (online supporting information 1). Based on these generally accepted
164 characters and considering the distribution of *M. g. pistor* at higher altitude, latitudes and
165 longitudes in Europe (north and central-east european distribution according to Hellrigl 1971,
166 Sama 2002 and 2008 and Tomminen and Leppänen 1991), we consider individuals from nine
167 populations in our sampling (Table 2) potentially belonging to the *M. g. pistor* sub-species.

168 *M. sutor* individuals from Austria were recognized as the *M. s. pellio* by their collectors
169 (Table 2). Furthermore, nine new morphological features were included in our analysis
170 (online supporting information 1- h, i and k-q).

171 A total of 60 specimens of *M. galloprovincialis* and 46 of *M. sutor* were analyzed for
172 the majority of the morphological features. The other samples (e.g. larvae or seriously
173 damaged specimens) could not be considered due to lacking or destroyed morphological
174 characters. Because of no obvious variability, none of the characters studied on the female
175 genitalia are mentioned here. Male genitalia were prepared using a modified version of the
176 Chatzimanolis (2005) protocol (see also Jeannel, 1955 and Dupuis, 2005). Pictures of all
177 morphological characters studied were analyzed using Image J 1.32j (Rasband). As it was
178 impossible to show all characters for all individuals studied a representative sample is shown
179 in the online supporting information 2 to 6.

180

181 *Mitochondrial and nuclear DNA sequences*

182

183 DNA was extracted from one or two legs to allow subsequent morphological observations
184 while reducing contamination from organisms like nematodes. Tissue from the last abdominal
185 segment was used for larval DNA extraction. Body parts and DNA extracts were kept as
186 vouchers in the entomological collection at the URZF at INRA-Orléans in France. Extraction
187 and isolation of genomic DNA were performed using the GenElute Mammalian Genomic
188 DNA miniprep kit (Sigma-Aldrich, St Quentin, France) at a final elution of 100 µl.

189 Polymerase Chain Reactions (PCRs) were conducted using the Sigma Red Taq
190 package. The primers used were C1-J-2183a and TL2-N-3014 (Simon *et al.*, 1994) for the
191 COI gene and D1-F and D3-R (Lopez-Vaamonde *et al.*, 2001) for the D2 region of 28S rDNA
192 gene. The annealing temperatures were 48°C for the COI gene, and 57°C for the 28S rDNA.
193 Amplification of 35 cycles was completed and PCR products were then purified using
194 GenElute PCR Clean-Up kit (Sigma-Aldrich).

195 Sequencing was performed using the BigDye Terminator v3.1 sequencing kit
 196 (Applied Biosystems, Courtaboeuf, France), on both strands. Sequencing reactions were
 197 purified using Sephadex G-50 (Amersham; Sigma-Aldrich) and detected with an ABI 3100
 198 automatic sequencer. The sequences obtained for each gene were aligned using CLUSTAL W
 199 version 1.4 (Thompson *et al.*, 1994) as implemented in BioEdit version 4.7.8.

200 A fragment of 792 bp of the mitochondrial COI gene was sequenced for all 199
 201 collected individuals, 1 to 11 individuals per population. This fragment corresponds to base
 202 pair positions 441-1319 of the *Drosophila yakuba* reference sequence (Simon *et al.*, 1994).
 203 Owing to co-amplification of nuclear paralogs, i. e. *numts* (Koutroumpa *et al.*, 2009a), 55 *M.*
 204 *galloprovincialis* COI sequences with ambiguities were withdrawn from the analysis. Three
 205 sequences of *M. galloprovincialis* and one of *M. sutor* from Italy, published by Cesari *et al.*
 206 (2005) were added to the dataset (GenBank ID: AY260835-37 and AY260843, respectively).
 207 Finally, 76 *M. galloprovincialis* specimens originating from 35 European and one Moroccan
 208 populations, as well as all 50 *M. sutor* individuals were used for the intra- and interspecific
 209 mtDNA analysis (for more details see Tables 2 and 3). Most of them were sampled in France
 210 with 21 locations (53 specimens) sampled for *M. galloprovincialis* and 13 locations for *M.*
 211 *sutor* (32 specimens). The sampling sites, host tree species, altitudes and date of capture of
 212 each *Monochamus* specimen are summarized in Table 2.

213 In addition, 878 bp of the D2 domain of the 28S rDNA were sequenced for 21 *M.*
 214 *galloprovincialis* and 11 *M. sutor* individuals, including their two sub-species (see Tables 2
 215 and 3 for details). The individuals were chosen according to ambiguous morphological
 216 features detected previously within *M. galloprovincialis* or related to the two mtDNA
 217 sequence clusters observed in *M. sutor*.

218

219 *Data analysis*

220

221 *Phylogenetic analysis.* Phylogenetic analyses were performed with PAUP*4b10 (Swofford,
 222 2000) for each gene independently (COI and the D2 region of 28S rDNA). COI sequences

223 were used for the phylogenetic analysis and subsequently compared to the morphological
224 characters and the 28S sequences. The congener species *M. alternatus* (Hope, 1842) was used
225 as outgroup. Trees were reconstructed using both maximum parsimony (MP) and maximum
226 likelihood (ML). MODELTEST version 3.7 (Posada & Crandall, 1998) was used to select the
227 substitution model that best describes the data. Following the likelihood ratio test
228 (Felsenstein, 1988), the most appropriate model of nucleotide substitution was determined as
229 well as the proportion of invariant sites, and γ -shape parameter. The hypothesis of a
230 molecular clock was also tested. For MP trees, we used a heuristic search with a simple
231 stepwise addition of sequences and tree bisection-reconnection (TBR) branch-swapping
232 option as implemented in PAUP*4b10. Support values for MP trees were estimated with 1000
233 bootstrap replicates. Uncorrected “p” genetic distances were computed using PAUP*4b10.

234 The Incongruence Length Difference (ILD) test was used to test congruence between
235 molecular (COI) and morphological data sets and was completed for 1000 iterations. D2 was
236 not included in this analysis because of lack of variability and therefore, low phylogenetic
237 resolution. This method, developed by Farris *et al.* (1994) and implemented within PAUP* as
238 partition homogeneity test, determines whether combining data sets is appropriate for further
239 phylogenetic analyses. A MP tree was also reconstructed using the 15 morphological
240 characters and a bootstrap procedure of 1000 iterations was completed using PAUP*. We
241 used MacClade v.4.06 (Madison & Madison, 2000) under constraints of species or species
242 lower taxa monophyly, to optimize morphological characters on molecular topologies. We
243 tested the significance of likelihood differences among constrained *vs* unconstrained
244 topologies using the Shimodaira – Hasegawa (S-H) test (Shimodaira & Hasegawa, 1999) as
245 implemented in PAUP* (1000 replicates).

246
247 *Population genetic analysis.* Mitochondrial DNA haplotype network was performed using
248 TCS version 1.21 (Clement *et al.*, 2000) for both species *M. galloprovincialis* and *M. sutor*
249 independently.

250 Analysis of Molecular Variance (AMOVA; Excoffier *et al.*, 2005) was used to
251 partition molecular variance into different hierarchical levels in *M. galloprovincialis*
252 populations in France using ARLEQUIN 3.11. Specimens were grouped (see Table 2) either
253 according to (1) the elevation > 800 m or < 800 m (800 m proposed by the literature as the
254 highest elevation that *M. g. galloprovincialis* occurs); (2) the geographic origin, based on
255 geographic distances and climatic conditions and (3) the host tree species (Table 2).

256

257 Results

258

259 *Between-species analysis*

260

261 *Morphological features.* Of the seventeen characters analyzed, including external and internal
262 features, only four were diagnostic at the species level (see Fig. 2 and online supporting
263 information 1, diagnostic characters c, k, l, and o). So far, these four characters have never
264 been used (or described) to separate the two species (Hellrigl, 1971; Sama, 2002; Sama, 2008;
265 Tomminen & Leppänen, 1991; Villiers, 1978; Vives, 2000). More precisely, *M.*
266 *galloprovincialis* individuals had patches of colored bristle that formed one to three large
267 stripes on their elytra while for *M. sutor* individuals, bristle formed points all over their elytra
268 (character 3), *M. sutor* sclerotization degree of the lateral styli of the male genitalia was
269 stronger than for *M. galloprovincialis* (character 11) and their color was red for the first one
270 and dark brown for the second one (character 12). The most unambiguous character that can
271 separate the two species was the copulatory piece in the aedeagus (character 15). This very
272 small structure attached to the aedeagus has a very different shape between the two species.
273 We present it for each species in Fig. 2 (for complete photo library see the online supporting
274 information 2 and 3). We found no variability between *M. galloprovincialis* and *M. sutor* on
275 the median lobe (Tomminen & Leppänen, 1991), besides the sclerotization degree (online
276 supporting information 1 and 4), and therefore we will no further refer to this character. The
277 scutellum shape is more variable for *M. galloprovincialis* than for *M. sutor*. *M. sutor* adults

278 have always a nude line in the middle of the scutellum. *M. galloprovincialis* usually has V
279 form scutellum but many individuals have a nude line in the middle of the scutellum as *M.*
280 *sutor* adults (online supporting information 5). In addition to the overlap observed at the
281 shape, the color of the scutellum bristle also overlaps between the two species. Matrix of all
282 the morphological characters used is presented in the online supporting information 7.

283

284 *Sequence analysis.* The alignment of the 76 and 50 partial COI sequences of *M.*
285 *galloprovincialis* and *M. sutor*, respectively, revealed 48 SNPs (no deletions nor insertions),
286 12 of which appear as diagnostic mutations separating the two species. The alignment of the
287 32 nuclear gene sequences also separated *M. galloprovincialis* from *M. sutor*, by the presence
288 of one diagnostic transitions and the deletion of two nucleotides in *M. sutor*.

289

290 *Genetic distances.* Uncorrected nucleotide pairwise distances between *M. galloprovincialis*
291 and *M. sutor* ranged from 0.023 to 0.037 (mean 0.031) for the COI gene and no overlap was
292 observed between intraspecific and interspecific pairwise distances (Fig. 3). The 28S genetic
293 pairwise distances between the two species corresponded to 0.0023 and reached 0.007 when
294 considering *M. galloprovincialis* or *M. sutor* versus the outgroup species, *M. alternatus*.

295

296 *Phylogenetic analysis.* The ILD test applied between mtDNA and morphology data sets
297 revealed significant conflict (P=0.001). Consequently, COI and morphology were analyzed
298 separately.

299 MP analysis of COI resulted in 28 equally most parsimonious trees with a length of
300 121 steps, consistency index (CI) 0.7851 and retention index (RI) 0.9795. The transversal
301 distance model (TVM + I + G) with gamma shape parameter 0.7868 and proportion of
302 invariable sites of 0.7336 was selected as the best model explaining the data. Both MP and
303 ML consensus phylogenetic reconstructions showed a monophyletic clade for each of the two
304 species, *M. galloprovincialis* (clade A) and *M. sutor* (clade B) (only MP tree is shown, Fig.
305 4b). *M. galloprovincialis* monophyly was supported by low bootstrap values contrary to *M.*

306 *sutor*, but the S-H test was not significant when *M. galloprovincialis* and *M. sutor* were
307 constrained to be monophyletic. The D2 domain of 28S rDNA also supported the monophyly
308 of the two species with comparable bootstrap values to the COI analyses. MP tree is shown in
309 Fig. 4c.

310 MP analysis of seventeen variable morphological characters yielded 100 most
311 parsimonious trees with a length of 112 steps (CI=0.277, RC=0.233). The resulting topology
312 was poorly resolved with no bootstrap values above 50 (Fig. 4a). However, *M. sutor* appeared
313 as monophyletic while *M. galloprovincialis* as polyphyletic. The optimization of
314 morphological characters onto the COI MP topology identified the four synapomorphies
315 corresponding to the four diagnostic morphological characters described above, each of them
316 separating clearly *M. galloprovincialis* from *M. sutor* (one of the four morphological
317 characters, i.e. the elytra bristle, is shown in Fig. 4b).

318

319 *Within-species analysis*

320

321 *Morphological features.* The morphological characters proposed in the literature as being
322 diagnostic at the intraspecific level between *M. g. galloprovincialis* and *M. g. pistori* (Hellrigl,
323 1971; Sama, 2002; Sama, 2008; Tomminen & Leppänen, 1991; Villiers, 1978; Vives, 2000),
324 appear confusing and ambiguous (Table 1 and online supporting information 4-6). Several
325 individuals showed intermediate features and none of the characters mentioned in the
326 literature, i.e. legs, antennae and elytra bristle color, was shown to be diagnostic for sub-taxa
327 identification.

328 By contrast, no morphological variability was observed within *M. sutor*, a fact also reflected
329 by absence of description of its sub-species *M. s. sutor* and *M. s. pellico* in the literature
330 (Hellrigl, 1971; Sama, 2002; Tomminen & Leppänen, 1991; Villiers, 1978; Vives, 2000). To
331 our knowledge, only the geographic distribution is mentioned as difference between these
332 sub-species.

333

334 *Sequence analysis.* The final alignment of the *M. galloprovincialis* COI sequences consisted
 335 of 792 bp, with a total of 10 (1.26%) polymorphic nucleotides, all parsimony informative.
 336 Nine different haplotypes were identified (Table 3) and are submitted to GenBank under
 337 accession numbers EU556544, 52, 58, 69, 73, 74, 88, 94 and 96.

338 On the same final alignment of 792 bp, 29 (3.28%) polymorphic nucleotides of which 18 are
 339 parsimoniously informative have been detected in *M. sutor* COI sequences. Sixteen different
 340 haplotypes were identified (Table 3) and are submitted to GenBank under accession numbers
 341 EU556670-82, X, X and AY260843.

342 Both species *M. galloprovincialis* and *M. sutor* showed no intraspecific variation in the D2
 343 region of 28S rDNA, each species showing one allele (submitted to GenBank under accession
 344 numbers XXX and YYY respectively).

345 *Genetic distances.* *M. galloprovincialis* uncorrected p genetic distances ranged between 0 and
 346 0.009. Interestingly, higher genetic distances were observed within *M. g. galloprovincialis*
 347 specimens, 0.009 (Iberian and Moroccan specimens *versus* the rest of the European
 348 specimens) than between *M. g. galloprovincialis* and *M. g. pistor*, 0.005.

349 Within *M. s. sutor* genetic distances ranged between 0 and 0.019. Specimens from Greece had
 350 lower genetic distances, 0.017, compared to the putative *M. s. pellio* sub-species than to the
 351 rest of the *M. s. sutor* in our sampling, 0.019.

352

353 *Phylogenetic reconstruction and haplotype demographic history.* Within *M. galloprovincialis*,
 354 phylogenetic structure was weak and displayed very poor bootstrap values. However, as
 355 confirmed on the haplotype network (Fig. 5), two major haplotypes, GI 8 and GI 9, clustered
 356 together. Interestingly, individuals belonging to the GI 9 haplotype were all (except 1)
 357 sampled at elevations between 800 m to 1350 m at different localities in the Alps (mostly
 358 individuals previously characterized as the *pistor* sub-species), as well as in the Massif
 359 Central and the Pyrenees Orientales (Fig. 6). GI 8 was the most widespread haplotype shared
 360 mainly between French lowland populations and one population in Austria (GpAuCa) as well

361 as one on the Rodopi Mountain in northeastern Greece (GGrF). Individuals from the Iberian
 362 Peninsula and Morocco revealed unique haplotypes, respectively.

363 In order to check for spatial structure (by region or altitude) and/or host effect on our
 364 data, *M. galloprovincialis* populations collected in France were grouped adequately (for the
 365 different groups see Table 2 and material and methods) and were analyzed by AMOVA. The
 366 results on the COI sequences are given in Table 4. Interestingly, the analysis of the molecular
 367 variance showed that significant partition of total genetic variation accounted between the
 368 altitudinal groups (24%, $p < 0.01$). However, no significant variation was observed between the
 369 groups made by region in France or host species. In the three different clusterings, the
 370 variances among and within populations were significant.

371 Within *M. sutor*, the two major subclades observed in the MP tree, (B1 and B2
 372 haplotype groups, Fig. 4) were distant by up to 12 mutational steps on the haplotype network
 373 (Fig. 5). Subclade B1 was well supported (bootstrap value 82) and was represented by two
 374 dominant haplotypes, SI 3 and SI 7, while B2 was supported by lower bootstrap value (59)
 375 and had one major haplotype, SI 15. A contact zone between these two divergent subclades
 376 was observed in southwestern Alps populations (Fig. 6 populations SuSeyM, SuSeyF and
 377 SuAdr). Interestingly, the two haplotypes, SI 7 and SI 15, are the largest of each subclade and
 378 were only found in this geographic region. Individuals identified as *M. s. pello* (SI 12 and SI
 379 13 from Austria) grouped in the B2 clade together with the SI 15 haplotype from the
 380 Northeast Alps and the most northern French alpine haplotypes found near the Swiss border,
 381 namely the SI 11, 10 and 14 (Fig. 6). Therefore, the most northern haplotypes in our study
 382 cluster together. Most individuals from Greece shared the same dominant haplotype (SI 3).
 383 All Greek individuals grouped in the B1 clade together with haplotypes from populations
 384 sampled in the most southern part of our sampling in France, the southern French Alps and
 385 the Pyrenees. More precisely, the two haplotypes SI 4 and SI 8 group individuals from the
 386 French Alps (SuSam) and Massif Central (SuLG) with the Pyrenean individuals SuPra and
 387 SuMon, respectively. Both haplotypes were positioned in subclade B1 with the previously
 388 mentioned southern most haplotypes.

389

390 **Discussion**

391

392 *Species delineation – multiple lines of evidence*

393 *M. galloprovincialis* – *M. sutor* species complex comprises a taxonomically challenging

394 assemblage of recently diverged species with ongoing speciation. These species are

395 interesting models for studies on biodiversity and species conservation concepts for two

396 reasons. First because of the specific environmental requirements found in our study within

397 these species lineages, i.e. altitude, and second because of the difficulty in finding adequate

398 screening tools to clarify present taxonomic and phylogenetic confusion within each species.

399 An important confirmation from this non-tidal data, showing high intraspecific variability,

400 was the need of using integrated data from multiple independent sources. Using the

401 integrative taxonomy approach we gain accuracy in solving the taxonomic impediment while

402 revealing the evolutionary relations between the different taxa (Fujita *et al.*, 2012; Heethoff *et*

403 *al.*, 2011; Schlick-Steiner *et al.*, 2010). In a recent review, Schlick-Steiner *et al.* (2010)

404 claimed that there is no silver-bullet discipline and that several disciplines are needed to guard

405 against single-discipline failure. In our study we attempted a more rigorous delimitation

406 between and within *Monochamus* that would be crucial information regarding the monitoring

407 of the PWN in Europe.

408

409 *Morphological evidence.* Even though numerous characters have been proposed to distinguish

410 *Monochamus* species (Pershing & Linit, 1985), scutellum form and elytra color have been so

411 far the main morphological characters used for their identification (Hellrigl, 1971; Sama,

412 2002; Sama, 2008; Tomminen & Leppänen, 1991; Villiers, 1978; Vives, 2000). We showed

413 in our study that these characters are extremely variable within the species. The

414 polymorphism observed within *M. galloprovincialis* in contrast to the monomorphic *M. sutor*

415 is clearly visualized on the phylogenetic reconstruction using the morphological characters.

416 Most of these characters do not support a monophyletic group for *M. galloprovincialis*

417 specimens. They do not seem to have reached fixation and are difficult to interpret, especially
418 for old and badly preserved samples. Nevertheless, four morphological characters, including
419 genitalia, do allow an accurate delimitation of these two species. In contrast to the
420 *Monochamus* male genitalia description from Tomminen and Leppänen (1991), the
421 differences reported in our paper allow the unambiguous identification of the two species. We
422 describe a new male morphological character, the copulatory piece, which due to its position
423 and difference in shape must be an important character for mating. This finding reinforces the
424 hypothesis that the two species would not hybridize in sympatric area, even though no
425 difference was found between the females. We have observed aggressive behavior, i.e.
426 mutilation of antennae and legs, of *M. galloprovincialis* females towards *M. sutor* males
427 attempting to copulate (Rougon personal observation), but this statement should be confirmed
428 by crossing experiments. Regarding our results, only four morphological characters seem
429 valid to confirm the species status in further studies, i.e. the elytra bristle pattern (for a first
430 and quick identification in the field) and the 3 characters on the male genitalia (structure of
431 the copulatory piece and overall sclerotization and color).

432
433 *Molecular evidence.* Our molecular data confirm the close relationship of *M.*
434 *galloprovincialis* and *M. sutor*, already considered as sister species in the phylogenetic
435 analyses of the *Monochamus* by Cesari *et al.* (2005). COI mitochondrial gene and 28S nuclear
436 genes allow an accurate delimitation of the two species. However, the genetic distances
437 observed between *M. galloprovincialis* and *M. sutor* were quite low for both markers in
438 comparison to those separating other Coleopteran species (Farrell, 2001; Kerdelhué *et al.*,
439 2002; Monaghan *et al.*, 2005). They were close to the 3% mtDNA sequence divergence, a
440 threshold usually designed in species-level identification (Hebert *et al.*, 2003b). This
441 underlies the pitfall to associate standard threshold value for species boundaries, suggesting
442 that delineation of species using the COI gene barcode distances is fairly subjective (DeSalle
443 *et al.*, 2005; Taylor & Harris, 2012). The interspecific divergence values found in our data are
444 usually observed in intraspecific lineages. Similarly, nuclear genetic distances correspond

445 mostly to intraspecific distances (see also Auger-Rozenberg *et al.*, 2006; Duan *et al.*, 2004;
446 Lopez-Vaamonde *et al.*, 2001), indicating that 28S has also evolved much slower in this
447 group. However, when the mtDNA genetic distances are plotted on a histogram they show an
448 interspecific distribution. Moreover, these results are comparable with the genetic distances
449 found so far for other *Monochamus* species (Cesari *et al.*, 2005; Kawai *et al.*, 2006) as well as
450 those observed in other intrageneric analyses in Coleoptera (Clark *et al.*, 2001). In this study,
451 the COI diagnostic SNPs were not found in the barcoding region proposed for species
452 delimitation, i.e. 600 bp at the 5' end of this gene (Hebert *et al.*, 2003b). Instead, its extension
453 (C1-J-2183a and TL2-N-3014) in the 3' end was significantly more variable and better
454 reflected sequence divergence within and between species, as suggested by Roe and Sperling
455 (2007a). Introduction of the barcoding theory by Hebert *et al.* (2003b) meant to give a
456 universal solution to the need for stabilized taxonomic criteria but recent studies prove that it
457 should be interpreted with caution and in combination with additional data sources like
458 morphology and geography or, when these are not available, other gene regions and ecology
459 should be included (DeSalle *et al.*, 2005; Roux-Morabito *et al.*, 2008; Taylor & Harris, 2012).
460 Considering the genetic distances observed between *M. galloprovincialis* and *M. sutor*,
461 although we are aware of the lack of such estimations, we could roughly date their separation
462 to 1.35 to 2 million years ago (mya), which would correspond to the beginning of the
463 Pleistocene. This estimation was calculated according to the molecular clock for Coleopteran
464 mitochondrial genes, calibrated from other Cerambycidae, the genus *Tetraopes*, for which
465 1.5% genetic divergence is equivalent to one mya (Farrell, 2001). Speciation of these two
466 species is thus likely to be associated to pre-Quaternary events, during which the area of
467 distribution was repeatedly covered by ice or affected by local climate change (Zhang *et al.*,
468 2001). These episodes of expansions and contractions into refugia would have likely
469 patterned the genetic distribution of the species. Divergence in their ecological adaptation
470 may have fostered distinct vertical and horizontal inter- and intra- specific distribution.

471

472 *Sub-taxa differentiation*

473

474 In the literature, separation of *M. galloprovincialis* and *M. sutor* into two sub-species is based
475 on morphological and eco-geographical criteria. In this study we aimed to compare these
476 criteria with molecular data.

477

478 *Morphology versus genetics.* *M. galloprovincialis* displayed extreme variability in the
479 morphological features which did not allow to accurately differentiate *M. g. galloprovincialis*
480 and *M. g. pistor*. The color of the legs and antennas, used so far as the main characteristic to
481 separate *M. g. pistor* from *M. g. galloprovincialis* (Hellrigl, 1971; Sama, 2002; Sama, 2008;

482 Tomminen & Leppänen, 1991; Villiers, 1978; Vives, 2000), is represented by a palette of
483 light red to dark brown and black colors. Such phenotypic plasticity is observed in other
484 insect species and could reflect adaptation to intra- and inter-specific signaling and/or
485 environmental needs like camouflage and thermoregulation (Tuomaala *et al.*, 2012). We

486 could assume that northern individuals would be selected for higher melanization degree as
487 proposed for *M. pistor*, and southern ones would be selected against as it has been observed in
488 other cases (Trullas *et al.*, 2007). In Sama 2008 this character was confusing for Moroccan
489 specimen characterization. Four of those specimens look like the *M. g. pistor* holotype from

490 Slovenia even though Morocco is out of the geographic distribution range of this taxon.
491 Similarly, characters such as the body size and the elytra bristle density and color, appear very
492 variable in the whole geographic distribution range of the species (Sama, 2008). The
493 combination of all these characters makes the data even more contradictory and confusing.

494 The extreme inconsistency between the morphological characters emphasizes the risk of error
495 when using such characters to delineate *M. g. pistor* from *M. g. galloprovincialis*.

496 This great variability in morphological features is followed by high genetic divergence but no
497 correlation could be found between the phenotypes' and the haplotypes' structure within our
498 sampling borders. Furthermore, none of the morphological characters separately was
499 consistent with the revealed genetic haplotypes. Consequently, we cannot speculate on the

500 existence of the sub-species proposed in the literature and we propose the use of the term sub-
501 forms in order to designate specimens with intermediate morphology.

502

503 *Ecological traits' significance versus morphology and genetics.*

504 More than being associated to morphological features, *M. galloprovincialis* genetic structure
505 seems to be shaped by ecological requirements. European *Monochamus* species are known to
506 be ecologically and geographically subdivided, with most species occurring at high latitudes
507 and/or moderate to elevated altitudes (Hellrigl, 1971). *M. galloprovincialis* is unique in that
508 sense since it is the only species with a Mediterranean origin that occurs mainly in lowland
509 areas to low elevations (Hellrigl, 1971). In a previous evolutionary study on the genus
510 *Monochamus*, Cesari *et al.* (2005) indicated a more primitive condition of the Eurasiatic sub-
511 alpine species *Monochamus saltuarius* (Gebler, 1830) with respect to the studied taxa.
512 Furthermore, while *M. sutor* as *M. saltuarius* infest the trunk of weakened spruces and pines,
513 *M. galloprovincialis* is restricted to branches of pines (Hellrigl, 1971; Starzyk & Hilszczanski,
514 1997). This shift to branches would have prevented competition between the two sister
515 species and suggests that *M. galloprovincialis* is a photophilic taxa with a higher tolerance to
516 dryness. It is tempting to assume that this particular ecological requirement may have fostered
517 *M. galloprovincialis* to spread all over southern countries, with distinct differentiation
518 patterns regarding the climatic conditions of the colonized area and the occurrence of putative
519 host tree species.

520 Our study reveals a mitochondrial lineage that is likely to be adapted to particular elevations
521 in France (between 800 m and 1444 m). This lineage is represented by haplotype GI 9,
522 occurring in the southwestern Alps as well as in other mountain systems, i.e. the western
523 Massif Central and the Pyrenees in the South. This altitudinal structure is also shown in the
524 AMOVA analyses. Furthermore, even though *M. galloprovincialis* has been reported to occur
525 mainly in lowland or in regions of intermediate elevation, we sampled some individuals at
526 altitude of up to 1444 m, as previously described in the Alps by Rougon (1975). According to
527 the literature, *M. g. pistor* is mainly located in Eastern Europe but some specimens have been

528 reported at high elevations in central Spain, southern France and the French Alps (Vives,
529 2000). It is difficult to assess if the altitudinal lineage found in our study could be associated
530 to *M. g. pistor* since no morphological evidence could confirm this hypothesis but supports a
531 primary effect of vicariance on the genetic structure of these two ecologically divergent
532 lineages, probably due to distinct refugia and recolonization paths during and after the
533 glaciations. However, the existence of a *M. galloprovincialis* ecotype adapted at higher
534 elevation revealed reduced gene flow between the lowland populations and this lineage. So
535 far, only unfavorable climatic conditions seem to stop the proliferation and the symptoms
536 caused by the nematode, as it has been observed in Japan at the Hokkaido and Honshu
537 islands, even though the insects and the pine-hosts are indigenous to this area (Mamiya, 1984;
538 Shoda-Kagaya, 2007). To spread beyond Spain, the nematode would have to overcome a
539 physical barrier, the Pyrenean Mountains. Altitudinal and temperature barriers in the Pyrenees
540 could break down the proliferation of the PWN as it has already been observed at the Ohu
541 Mountain in Tohoku in Japan where *M. alternatus* populations have been found isolated at the
542 two sides of this mountain (Shoda-Kagaya, 2007).

543 The European situation is quite different from the Asian one; in that sense a vectoring relay
544 could be possible with *M. sutor*, which occurs in elevated areas. The similarities between
545 *Monochamus* species around the world are tremendous (Akbulut & Stamps, 2012;
546 Koutroumpa *et al.*, 2009b; Koutroumpa *et al.*, 2008b). They share similar life histories, even
547 though thousands miles of distance, similar survivorship from egg to adult and similar
548 emergence dynamics. All *Monochamus* species vectoring the PWN (for details on the PWN
549 vectors see Akbulut and Stamps, 2012) feed and oviposit on various conifer host species
550 under adequate conditions (stressed trees) and in our study no genetic variability within the
551 species could be attributed to the host range. Furthermore, we found *M. sutor* on the same
552 host as *M. galloprovincialis*. Even though no study records *M. sutor* as a PWN vector so far,
553 sympatry and common hosts between the two species could be a deadly combination for
554 conifers susceptible to the PWN at high altitude and proliferation of the PWN beyond the
555 Pyrenean barrier,

556 Our molecular study revealed a northeast/southwest geographical pattern of *M. sutor*
557 haplotype distribution, which fits with the two sub-species previously described in the
558 literature. Our molecular analysis revealed that *M. sutor* separates into two well-supported
559 distinct subclades on the COI MP tree and higher genetic distances separate these distinct
560 lineages which may be attributed to the two sub-species, *M. s. sutor* and *M. s. pellio*.
561 However, no morphological features could be associated to this variability and the two
562 lineages co-occur in southwestern Alps. Therefore, populations originating from the western
563 Alps include both forms (both major haplotypes) in sympatry, thus hybridization should not
564 be excluded at this suture zone, even though the two haplotypes remain distant. The Austrian
565 individuals (*M. s. pellio*) belong to the B1 clade including the northern Alps populations. We
566 hypothesize, according to the geographic distribution, that this clade belongs to the *M. s.*
567 *pellio* (Hellrigl, 1971), whereas the B2 clade likely corresponds to the *M. s. sutor*. Maximum
568 diversity on the area of the French Alps is common to other insects like the mountain
569 caddisfly *Drusus discolor* (Rambur, 1842) (Pauls *et al.*, 2006). Especially the western part of
570 the French Alps is often considered an important hybridization zone for many species, besides
571 a suture zone for different lineages (Barilani *et al.*, 2007; Deschamps-Cottin *et al.*, 2000;
572 Flanagan *et al.*, 1999; Godoy *et al.*, 2004; Hewitt, 1999; Taberlet *et al.*, 1998; Wojcik *et al.*,
573 2002). Similar distinct pattern of differentiation between closely related species has already
574 been observed within the alpine *Erebia melampus/sudetica* (Staudinger, 1861) species
575 complex (Haubrich & Schmitt, 2007). Pleistocene climate cycles have also acted on the
576 diversification of the alpine *Nebria* species, characterized by altitudinal zonation and habitat
577 preferences (Schoville *et al.*, 2012).

578

579

580

581 **Conclusion**

582 In contrast to what is currently believed, *M. galloprovincialis* and *M. sutor* are not clearly
583 subdivided in the cited sub-species but rather to multiple intermediate morphological forms.

584 The origin of this high morphological variability and important gene flow is found in the life
585 traits of these species as well as movements due to human forestry activity and world wide
586 wood trade. The different lineages of these species are hardly delimited with the markers
587 used. A more fine scale analysis of the dispersion with highly polymorphic microsatellites
588 markers has been initiated in order to specify the genetic structure and dispersal patterns of
589 these two sister species. These studies will be applied on a larger sampling and at higher
590 elevations, especially in the French Alps and Pyrenees, which could be crucial areas for the
591 surveillance of the PWN infestation.

592

593 Database linking and Accession numbers

594 GenBank ID: AY260835, AY260837 and AY260843

595 EU556544, EU556552, EU556558, EU556569, EU556573, EU556574, EU556588,
596 EU556594 and EU556596

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599

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610

611

612 **Contribution of authors:**

613 G.Ro. and F.Li. designed the study; F.Ko. conducted the laboratory work; C. Be. helped in the
 614 laboratory work. D. Ro. helped for the taxonomic task. F.Ko. and G.Ro. analyzed the data and
 615 together with C. Be. drafted the manuscript; all authors carried out the sampling, read,
 616 discussed and approved the final version of the paper.

617

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900 Figure captions

901

902 **Fig. 1** PWN infested areas (stars) and *M. galloprovincialis* vs *M. sutor* geographic distribution in
 903 Europe (lines). Combination map of the CABI Crop Protection Compendium data and observations of
 904 Hellrigl (1971), Sama (2002) and Vives (2000). A) Yellow line encircles the regions of *M.*
 905 *galloprovincialis* geographic distribution. B) Blue line encircles the regions of *M. sutor* geographic
 906 distribution.

907

908 **Fig. 2** Differences on elytra and male genitalia of the two species *M. galloprovincialis* (top) and *M.*
 909 *sutor* (bottom): Three stripes on elytra characterize *M. galloprovincialis* and points all over elytra
 910 characterize *M. sutor*. The lateral styli are less sclerotinized (A) with brown setae (B) for *M.*
 911 *galloprovincialis* and more sclerotinized with red setae for *M. sutor*. The form of the sclerotinized part
 912 of the copulatory piece (C) has an elongated thin shape for *M. galloprovincialis* and is heart shaped for
 913 *M. sutor*

914

915 **Fig. 3** Frequency distribution of intraspecific and interspecific (congeneric) genetic divergence in
 916 *Monochamus*. Intraspecific distances correspond A) to *M. galloprovincialis* COI sequences, B) to *M.*
 917 *sutor* COI sequences

918

919 **Fig. 4** A) Consensus MP tree for *M. galloprovincialis* and *M. sutor* species using 15 morphological
 920 characters. No BootStrap values were higher than 50% and therefore are not shown.
 921 B) Consensus MP tree for *M. galloprovincialis* and *M. sutor* species using COI sequence data. Only
 922 bootstrap values higher than 50% are shown. In this example, the four different lines, attributed to each
 923 branch of the tree, indicate the four different states of the first morphological character (patches on the
 924 elytra) separating *M. galloprovincialis* from *M. sutor*. Identical patterns were observed for three other
 925 morphological characters (characters 9, 10 and 13 on supplementary data I).
 926 C) Consensus MP tree for *M. galloprovincialis* and *M. sutor* species using 28S sequence data.
 927 Population codes were given in Table 2

928

929 **Fig. 5** COI TCS network showing haplotype distribution. A) *M. galloprovincialis* and B) *M. sutor*.
 930 Color code corresponds to different populations. Population codes were given in Table 2 and circle size
 931 is proportionate to the number of individuals having each haplotype. Clades correspond to those of the
 932 MP tree (figure 4)

933

934 **Fig. 6** A) *M. galloprovincialis* COI haplotype geographic distribution. B) Same as (A) but for *M. sutor*.
 935 Dotted delimitations correspond to suture zone and double line shows Pyrenees barrier. Left maps
 936 show haplotype distribution in the Southeast France. Population codes were given in Table 2. Clades
 937 correspond to clades of the MP tree (see figure 4)

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940 **Table 1** *M. galloprovincialis* / *M. sutor* taxon comparison by Hellrigl (1971), Sama (2002), Tomminen
 941 and Leppänen (1991), Villiers (1978) and Vives (2000)
 942

	Host	Distribution	Elevation	Climatic requirements	Morphology
<i>M. g. galloprovincialis</i>	<i>Pinus</i> spp.	North Africa South-west and Central Europe	< 800 m	Mediterranean Warm, dry Branches	a) - V form scutellum - Round apex of the median lobe (male genitalia) b) - Legs and antennae brown-red - Elytra covered grey or red-white bristle - Large body size
<i>M. g. pistor</i>	<i>P. sylvestris</i> <i>P. nigra</i> <i>Picea</i> (occasionally)	North Europe Central and East Europe South Europe (occasionally) Russia North Kazakhstan Causasia Transcaucasia Armenia North Turkey	> 800 m	Cold, humid Branches	Same as a) c) - Legs and antennae black - Elytra rare yellow-white bristle - Small body size
<i>M. s. sutor</i>	<i>Picea abies</i> <i>Picea excelsa</i> <i>Pinus</i> spp.	Middle and east Europe The Pyrenees, Alps Russia North Asia up to Japan		Cold, humid Trunk	d) - II form scutellum - Flattened apex of the median lobe (male genitalia) e) - Legs and antennae black - Elytra black with few white-yellow bristle - Large body size
<i>M. s. pellio</i>	<i>Picea abies</i> <i>Picea excelsa</i> <i>Pinus</i> spp.	East Europe Siberia		Cold, humid Trunk	Same as d) f) - Legs and antennae black - Elytra black without bristle - Large body size

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946 Table 2 Abbreviations of *Monochamus galloprovincialis* and *M. sutor* populations, sampling sites,
947 collectors' names, host trees, date of capture, altitude and geographical coordinates are listed

Population Code (AMOVA geographic group)	Country	Location	Collector	Host species	Date	Altitude (m)	Latitude	Longitude
<i>M. g. galloprovincialis</i>								
GPigA (E)	FRANCE	FD 3 Pignons	B. Germain	<i>P. pinaster</i>	2003	76	48°24' N	02°33' E
GLor (E)	FRANCE	Lorris	G. Roux	<i>P. sylvestris</i>	2003,2004	124	47°53' N	02°30' E
GOle (D)	FRANCE	St Pierre d'Oléron	R. Pelloquint	<i>P. pinaster</i>	2003,2004	7	45°58' N	01°19' W
GHer (D)	FRANCE	Herm (Léon)	J-L. Hautclocq	<i>P. pinaster</i>	2004	65	43°51' N	01°14' W
GPis (D)	FRANCE	Pissos	P. Ninosque	<i>P. pinaster</i>	2004	47	44°18' N	00°46' W
GFar (D)	FRANCE	Fargues	R. Delpont	<i>P. pinaster</i>	2004	123	44°11' N	00°11' E
GHou (D)	FRANCE	Hourtin	J-P. Coste	<i>P. pinaster</i>	2004,2005	18	45°12' N	01°04' W
GSarI (D)	FRANCE	Sare (St Ignace)	C. Van-Meer	<i>P. nigra</i>	2004	172	43°20' N	01°36' W
GSarL (D)	FRANCE	Sare (Lizarieta)	C. Van-Meer	<i>P. nigra</i>	2004	485	43°16' N	01°37' W
GAud (C)	FRANCE	Peyriac de Mer	C. Van-Meer	<i>P. halepensis</i>	2004	226	43°17' N	01°48' E
GCou (C)	FRANCE	Coustouges (66)	H. Brustel	<i>P. nigra</i>	2003	841	42°22' N	02°38' E
GLar (B)	FRANCE	Larzac	J-P. Ansonnaud	<i>P. nigra</i>	2005	800	43°58' N	03°11' E
GCor (B)	FRANCE	St Jean de Cornies	P. Girard	<i>P. halepensis</i>	2003,2004	73	43°44' N	04°00' E
GMey (B)	FRANCE	Meyrargues	J. Hintzy	<i>P. halepensis</i>	2003,2004	320	43°28' N	05°38' E
GEyg (A)	FRANCE	FD Eygues Rosans	P. Fee	<i>P. nigra</i>	2004	1058	44°24' N	05°28' E
GAdr	FRANCE	St André les Alpes	L. Micas	<i>P. sylvestris</i>	2004	1226	43°59' N	06°30' E
GDig	FRANCE	Digne (Les Bains)	L. Micas	<i>P. sylvestris</i>	2005	1216	44°03' N	06°15' E
GPC (I)	PORTUGAL	Comporta	P. Naves	<i>P. pinaster</i>	2003	24	38°22' N	08°45' W
GPL (I)	PORTUGAL	Leiria	G. Roux	<i>P. pinaster</i>	2005	33	39°40' N	08°48' W
GEspM (H)	SPAIN	Murcia	D. Gallego	<i>P. pinaster</i>	2004	173	37°59' N	01°07' W
GMarA (G)	MOROCCO	Atlas mountain	D. Ghaïoule	<i>P. pinaster</i>	2003	475	32°21' N	06°24' W
GGrA (F)	GREECE	Afete (Magnesia)	A. Koutroumpas	<i>P. brutia</i>	2004,2005	5	39°22' N	22°56' E
GGrEr (F)	GREECE	Erythres	A. Koutroumpas	<i>P. brutia</i>	2005	397	38°13' N	23°19' E
GGrB (F)	GREECE	Bilia	A. Koutroumpas	<i>P. brutia</i>	2005	625	38°10' N	23°20' E
GGmo (K)	ITALY	Montefalcone-Pisa	Cesari <i>et al</i> 2005	-	-	101	43°43' N	10°44' N
<i>M. g. pistor</i>								
GpFau (A)	FRANCE	Seyne (Le Faut)	D. Rougon	<i>P. sylvestris</i>	2004	1364	44°19' N	06°24' E
GpAdr (A)	FRANCE	St André les Alpes	D. Rougon	<i>P. sylvestris</i>	2005	1226	43°59' N	06°30' E
GpVer (A)	FRANCE	Verdaches	D. Rougon	<i>P. sylvestris</i>	2005	1444	44°15' N	06°20' E
GLG (B)	FRANCE	Puechagut le Vigan	C. Rulliere	<i>P. nigra</i>	2003,2004	1014	44°01' N	03°34' E
GGrF (F)	GREECE	Frakto, Mt Rodopi	A. Koutroumpas	<i>Picea abies</i>	1997	1200	41°15' N	25°30' E
GPmu (J)	ITALY	Mules-Bolzano	Cesari <i>et al.</i> 2005	-	-	946	46°51' N	11°31' E
GPvs (J)	ITALY	Villa Santina-Udine	Cesari <i>et al.</i> 2005	-	-	374	46°25' N	12°55' E
GpAuCa (J)	AUSTRIA	Carinthia (Austria)	U. Tomitzek	<i>P. sylvestris</i>	2006	775	46°43' N	14°10' E
GpAuSo (J)	AUSTRIA	Sollenau (Austria)	U. Tomitzek	<i>P. sylvestris</i>	2006	269	47°53' N	16°15' E
<i>M. sutor</i>								
SuSam	FRANCE	Samoens	A. Drumont	-	1992	1122	46°05' N	06°44' E
SuCha	FRANCE	Chatel (Près la Joux)	M. Simon	<i>Picea abies</i>	2004	1627	46°14' N	06°48' E
Sulse	FRANCE	Meaudre Isere	L. Micas	-	2005	1457	45°05' N	05°50' E
SuSor	FRANCE	Sorbiers	L. Micas	<i>P. sylvestris</i>	2003	900	44°37' N	05°56' E
SuMed	FRANCE	Meolans-Revel	L. Micas	<i>P. sylvestris</i>	2004	1036	44°24' N	06°31' E
SuSeyC	FRANCE	Seyne (Col Bas)	D. Rougon	-	2005	1162	44°21' N	06°21' E
SuSeyM	FRANCE	Seyne	D. Rougon	<i>P. sylvestris</i>	2003	1600	44°21' N	06°23' E
SuSeyF	FRANCE	Seyne (Le Faut)	D. Rougon	<i>P. sylvestris</i>	2004	1348	44°20' N	06°24' E
SuAdr	FRANCE	St André les Alpes	L. Micas	<i>P. sylvestris</i>	2005	1226	43°59' N	06°30' E
SuDig	FRANCE	Digne (Les Bains)	L. Micas	<i>P. sylvestris</i>	2003	1216	44°03' N	06°15' E
SuLG	FRANCE	Puechagut le Vigan	C. Rulliere	<i>P. nigra</i>	2003	1014	44°01' N	03°34' E
SuMon	FRANCE	Montals	C. Rulliere	<i>Picea abies</i>	2004	700	44°41' N	02°88' E
SuPra	FRANCE	Prades	D. Rougon	-	2005	1505	42°47' N	01°52' E
SuGrE	GREECE	Elatia Mt Rodopi	A. Koutroumpas	<i>P. nigra</i>	2005	1550	41°15' N	25°30' E
SuGrX	GREECE	Elatia Mt Rodopi	A. Koutroumpas	<i>Picea abies</i>	2005	1400	41°15' N	25°30' E
SUVg	ITALY	Val di Genova-Trento	Cesari <i>et al.</i> 2005	-	-	1126	46°10' N	10°40' E
<i>M. s. pello</i>								
SupAuNa	AUSTRIA	Nasswald (Austria)	U. Tomitzek	-	2003,2004	774	47°45' N	15°41' E
<i>M. alternatus</i>								
AJapF	JAPAN	Fukuyama	A. Drumont	<i>P. densiflora</i>	2004	91	34°29' N	133°21' E

948 Letters in parenthesis next to the population code shows the different geo-climatic groups used for the AMOVA analyses. GAdr and GDig were only used for

949 the 28S analyses

950

951 **Table 3** Number of *M. galloprovincialis* and *M. sutor* individuals (all adults except 7 larvae
 952 marked with L) studied in the mitochondrial COI and morphological analyses, haplotypes and
 953 sequence types found in each population

Code	Number of individuals				Haplotype codes
	COI	28S	Morphology		(nb of sequences when more than 1)
			♂	♀	
<i>M. g. galloprovincialis</i>					
GPigA	1	1	-	1	GI 8
GLor	1	2	-	1	GI 8
GOle	3	-	1	2	GI 6, GI 8 (2)
GHer	2	1	1	1	GI 8 (2)
GPis	2	1	1	1	GI 8 (2)
GFar	2	-	1	1	GI 8 (2)
GHou	3	-	1	2	GI 8 (3)
GSarI	5	-	3	1	GI 6, GI 8 (4)
GSarL	3	-	-	2	GI 6, GI 8 (2)
GAud	1	1	1	-	GI 4
GCou	1	1	1	-	GI 9
GLar	1	-	1	-	GI 9
GCor	6	1	2	3	GI 2, GI 6, GI 5, GI 8 (3)
GMey	2	1	1	1	GI 8, GI 9
GEyg	1	1	-	1	GI 2
GAdr	0	1	-	-	-
GDig	0	1	-	-	-
GPC	3	1	-	-	GI 3 (3)
GPL	1	0	1	-	GI 3 (1)
GEspM	3	2	2	1	GI 3 (3)
GMarA	2	-	1	1	GI 1 (2)
GGrEr	4 L	-	-	-	GI 2 (4)
GGrB	3 L	-	-	-	GI 2 (3)
GGrA	4	2	3	1	GI 2 (2), GI 7 (2)
GGmo	1	0	0	0	GI 2
<i>M. g. pistor</i>					
GpFau	2	1	2	-	GI 2 (2)
GpVer	5	-	3	2	GI 9 (5)
GpAdr	5	0	2	3	GI 9 (5)
GLG	3	1	2	1	GI 2, GI 9 (2)
GGrF	1	1	1	-	GI 8
GPmu	1	0	0	0	GI 2
GPvs	1	0	0	0	GI 2
GpAuCa	1	1	1	-	GI 4
GpAuSo	2	-	1	1	GI 8 (2)
<i>M. s. sutor</i>					
SuSor	1	1	1	0	SI 15
SuDig	1	0	1	0	SI 15
SuCha	2	1	1	1	SI 10, SI 11
SuSam	2	-	-	-	SI 4, SI 14
SuAdr	2	0	1	1	SI 7, SI 15
SuLG	1	1	1	0	SI 9

SuMon	1	1	0	1	SI 9
SuIse	1	1	0	1	SI 8
SuMed	2	1	0	2	SI 7(2)
SuSeyF	14	2	10	4	SI 5, SI 7 (6), SI 15 (7)
SuSeyM	7	0	2	3	SI 5, SI 7 (2), SI 15 (4)
SuSeyC	1	0	1	0	SI 6
SuPra	1	1	1	0	SI 4
SuGrE	6	1	5	0	SI 1, SI 2, SI 3 (4)
SuGrX	5	0	4	1	SI 3 (5)
SUvg	1	0	0	0	SI 16
<hr/>					
<i>M. s. pellio</i>					
SupAuNa	2	1	2	0	SI 12, SI 13
<hr/>					
<i>M. alternatus</i>					
AJapF	1	1	1	0	

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Table 4 AMOVA results of *M. galloprovincialis* French populations based on COI sequences

	Source of variation	Variance components	Percentage of variation
Grouping by altitude <800 / >800	Among groups	0.00391Va	23.97**
	Among populations within groups	0.01857Vb	29.00*
	Within populations	0.00196Vc	47.03**
Grouping by region	Among groups	0.16325Va	07.36 NS
	Among populations within groups	0.01075Vb	40.19*
	Within populations	0.00000Vc	52.46***
Grouping by host	Among groups	0.08504Va	17.02 NS
	Among populations within groups	0.00000Vb	34.23***
	Within populations	0.00000Vc	48.75***

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ NS: non significant.

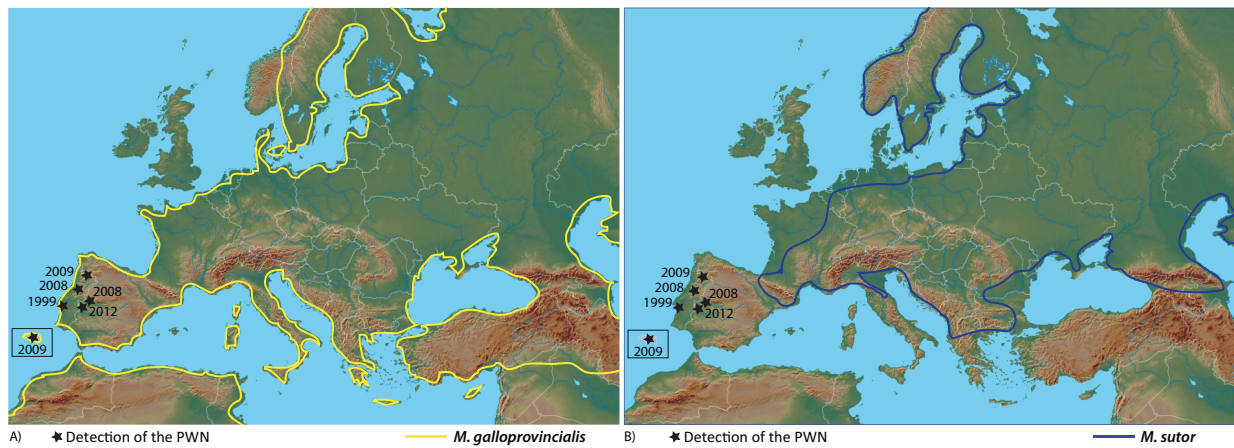
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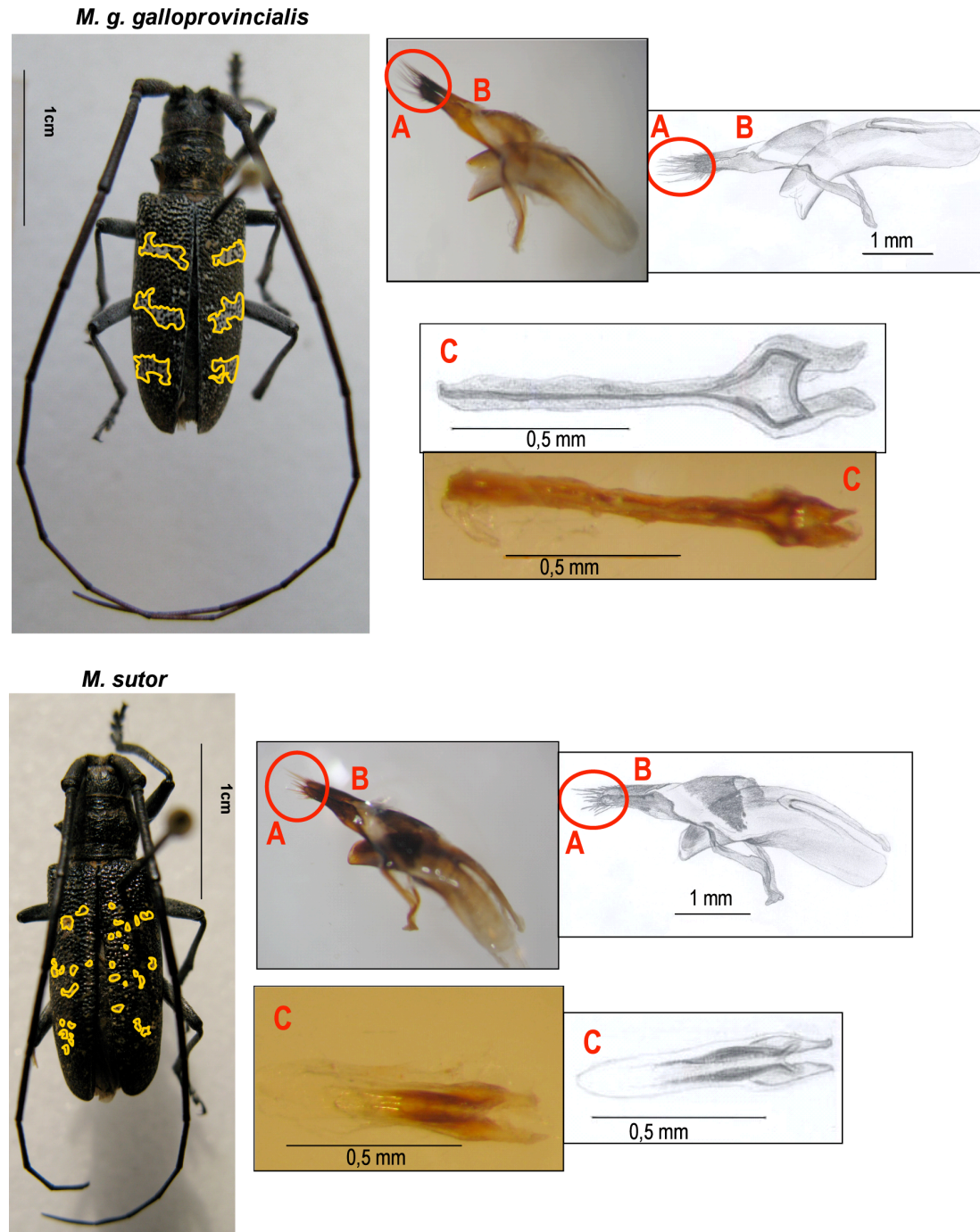
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Fig. 3

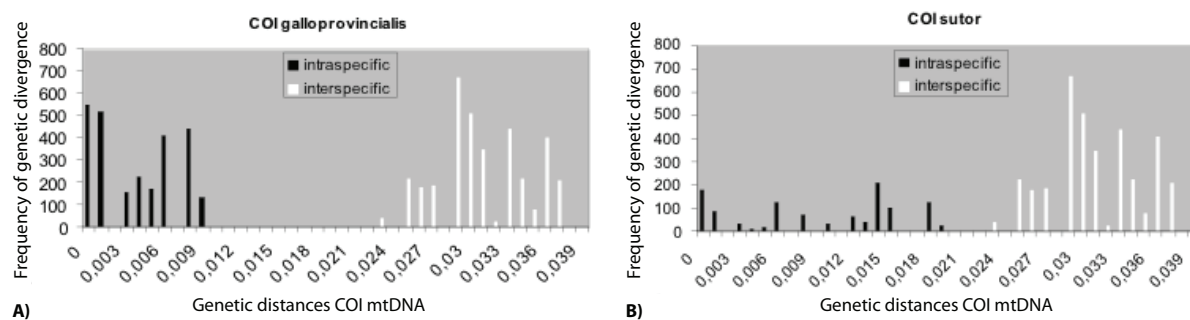


Fig. 4

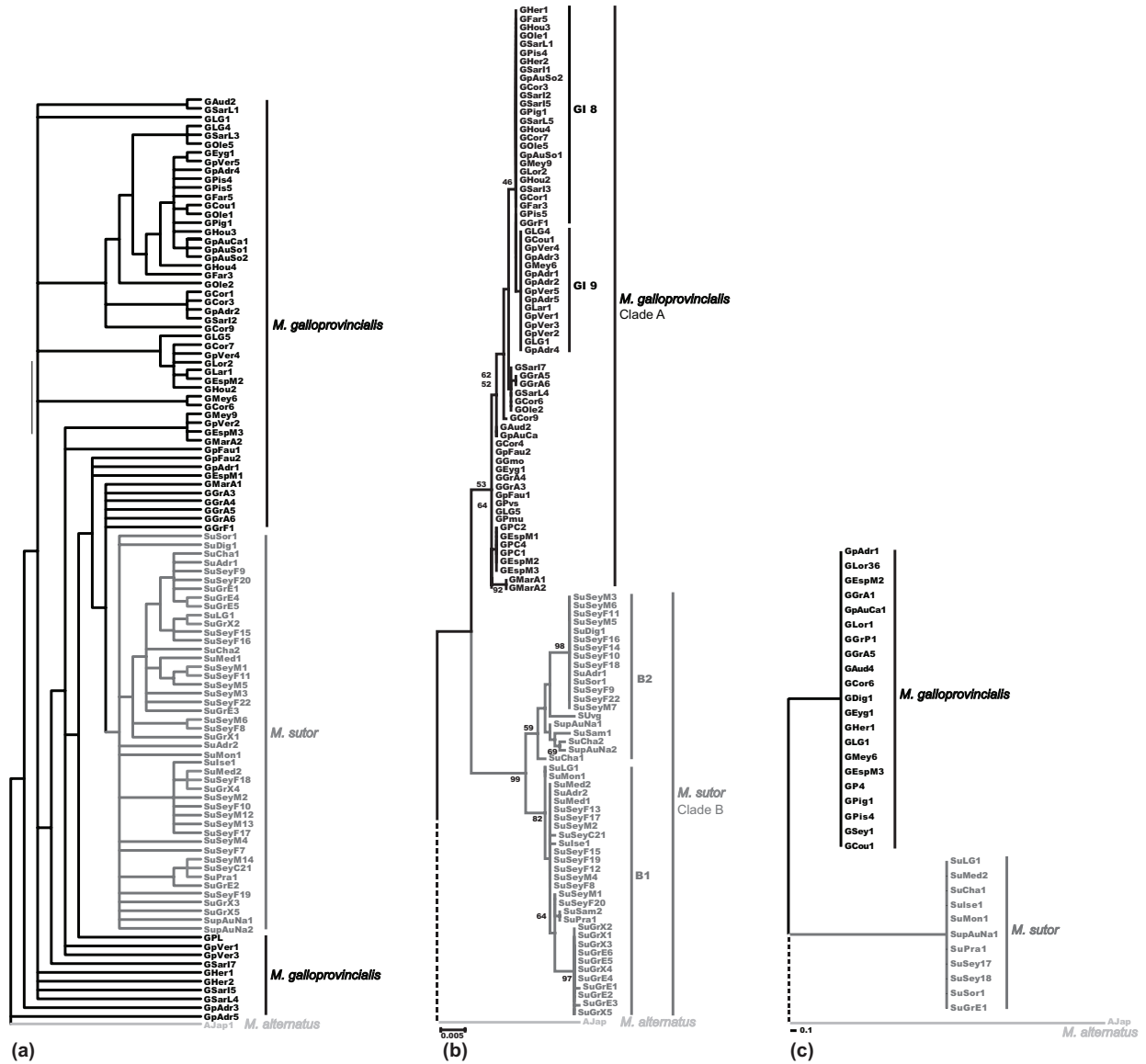


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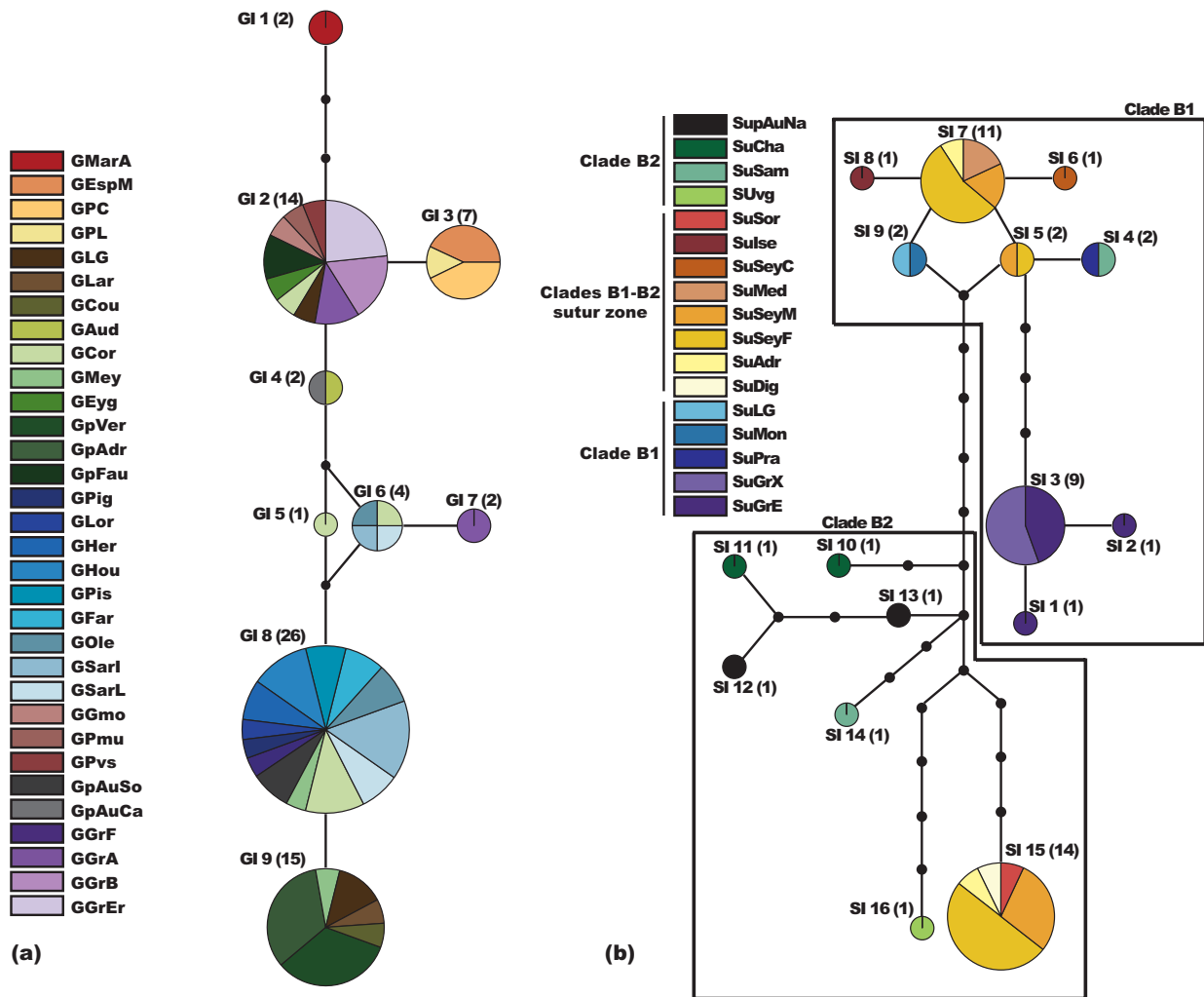
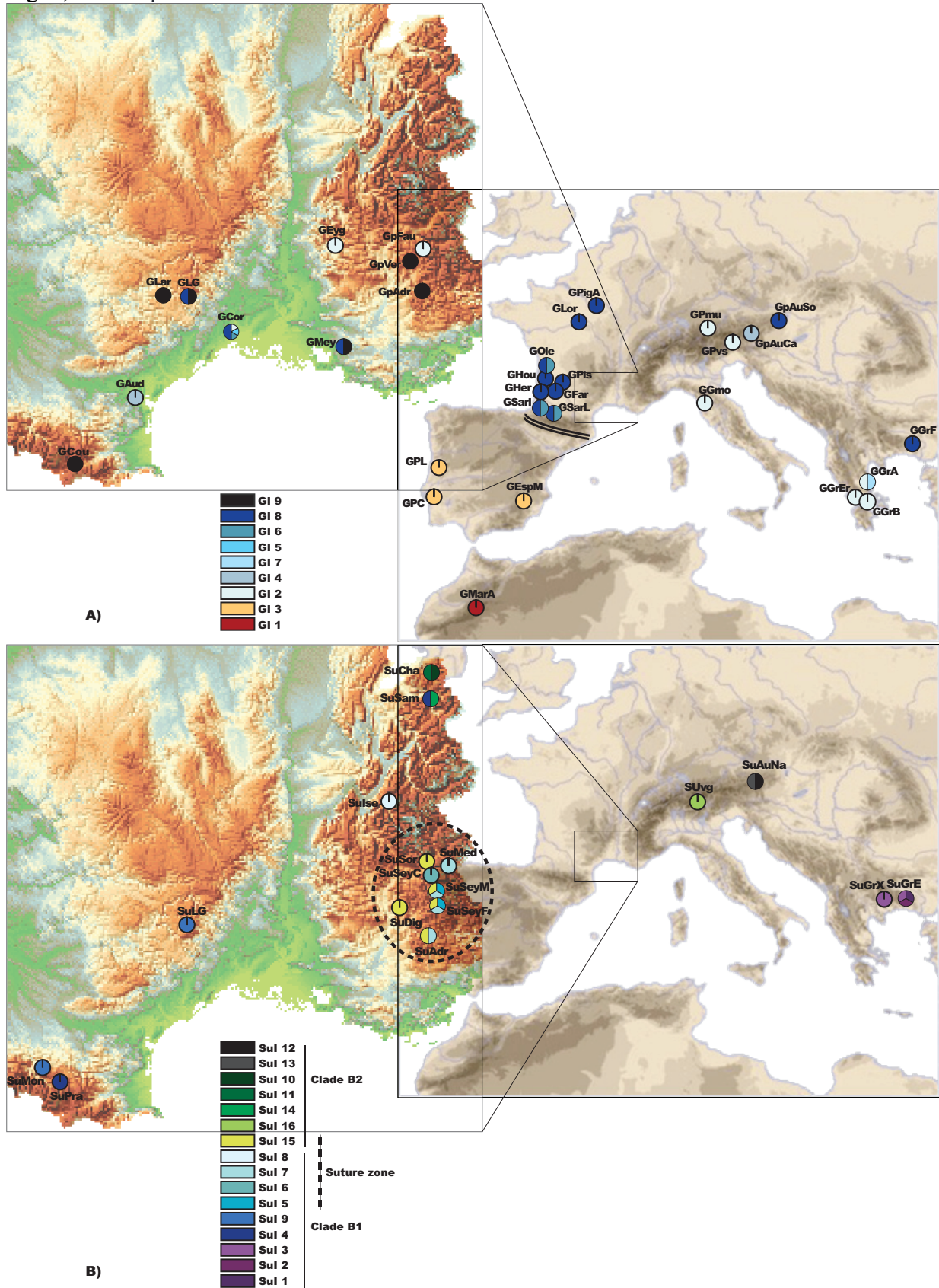


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