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The effect of the Messinian Salinity Crisis on the early diversification of the Tettigettalna cicadas

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9   **Title**

10   The effect of the Messinian Salinity Crisis on the early diversification of the *Tettigettalna*
11   cicadas

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19   Early diversification of *Tettigettalna*

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36   The current distribution patterns of many Mediterranean species are often a consequence of

37   large and impactful past geoclimatic events, such as the Messinian Salinity Crisis (MSC) and the

38   Quaternary glacial cycles. Cicadas are flying insects with poor dispersal ability, which have

39   experienced intense local differentiation in the Mediterranean, where the genus *Tettigettalna*

40   has surfaced as a biogeographic model. The genus includes 10 species with species-specific

41   calling songs but identical morphology. All *Tettigettalna* species are restricted to Southern

42   Iberia, with the exception of *T. estrellae* (northwest Iberia), the widespread *T. argentata*

43   (mainly Iberia, France and Italy) and *T. afroamissa* (Morocco). With an expanded genetic

44   dataset involving nuclear (*EF1α*) and mitochondrial (5' and 3' *COI* and *ATP*) loci, we

45   reconstructed the phylogeny of the genus and estimated divergence dates for *Tettigettalna*

46   species under a Bayesian framework. Phylogeny with the new mitochondrial dataset was in

47   agreement with previous studies, whereas the nuclear *EF1α* supported *T. josei* and *T.*

48   *afroamissa* as monophyletic clades but lacked resolution to resolve the remaining taxa. Some

49   sister taxa share mitochondrial haplotypes, hinting for incomplete lineage sorting. Estimates of

50   divergence time settled *T. josei* as the earliest diverging lineage, likely as a pre- or early-MSC

51   event. As for the origin of *T. afroamissa* in Morocco, though time estimates could not entirely

52   rule out post-MSC dispersal, the most likely scenario points to isolation of African *Tettigettalna*

53   after the reopening of the strait of Gibraltar. The Pleistocene glaciations that followed likely

54   impacted on the diversification of the remaining species of the genus in southern Iberia

55   refugia.

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63   molecular dating.

64

65   1. **INTRODUCTION**

66

67   The complex historical biogeography of Western Mediterranean has been extensively studied

68   for many plant and animal taxa, where two major geo-climatic events have been often evoked

69   to explain current patterns of distribution and molecular diversity: the Pleistocene Ice Ages

70   (<2.6 Ma) and the Messinian Salinity Crisis (MSC) at the end of the Miocene, 5.97-5.33 Ma

71   (Gómez & Lunt, 2007; Hewitt, 2000, 1999; Marabuto et al., 2020; Médail & Diadema, 2009;

72   Schmitt, 2007, Trájer et al., 2021).

73   The MSC was triggered by tectonic movements that isolated the Mediterranean Sea from the

74   Atlantic Ocean and accompanied by climatic changes that led to several series of evaporation

75   cycles, progressively lowering the sea-level to its nearly complete desiccation (Krijgsman et al.,

76   2018, 1999; Manzi et al., 2013). Extensive land connections were formed between Europe and

77   North Africa during this process (see Fig. 1), functioning as land bridges for fauna and flora

78   exchange among the two continents (Gibert et al., 2013; Husemann et al., 2014). These

79   connections were suddenly interrupted when the Mediterranean basin was refilled through

80   the Gibraltar Strait corridor, at 5.33 Ma (Blanc, 2002). This event marked the beginning of the

81   Pliocene and created a sea barrier for poorly dispersive biota and triggered differentiation on

82   either side of the strait, contributing to the high endemism observed today in this biodiversity

83   hotspot (Lavergne et al., 2013; Médail & Diadema, 2009; Puissant & Sueur, 2010).

84   By late Pliocene, the Iberian Peninsula had already acquired its modern coastal configuration

85   (Elez et al., 2016; Jolivet et al., 2006), with the closest distance between Europe and Africa

86   found at the Gibraltar Strait (14 km, Fig. 1C). In the following period, the Pleistocene, the

87   Northern Hemisphere was subject to several glacial cycles which strongly affected the

88   distribution and survival of biota (Hewitt, 2000, 1999). The heterogeneity in topography and

89   habitats within the Iberian Peninsula and Maghreb enabled the survival of previously

90   widespread lineages into a plethora of local refugia (Tzedakis, 2009), supporting the concept of

91   "refugia within refugia" (Fig. 1D, Gómez & Lunt, 2007; Feliner, 2011; Martínez-Freiría et al,

92   2020; Miraldo et al., 2011; Petit, 2003). Nevertheless, overseas dispersal could have been

93   facilitated under glacial maxima by a significant drop in sea levels, which were estimated to

94   have reached over 100 m lower than today (Dumitru et al., 2021; Rohling et al., 2014). Post-

95   MSC overseas dispersal to either side of Gibraltar has been suggested for several taxa, such as

96   bellflowers (García-Aloy et al., 2017), lizards (Mendes et al., 2017), newts (Veith et al. 2004),

97   butterflies (Habel et al., 2011; Marabuto et al., 2020), beetles (Mas-Peinado et al., 2021) and

98   spittlebugs (Rodrigues et al., 2014). In some cases, however, divergence time estimates place

99   lineage split events between Europe and Africa much before the reopening of the Strait of

100 Gibraltar (see for e.g. Mendes et al., 2017; Paulo et al., 2008).

101 Cicadas (Hemiptera: Cicadidae) are a worldwide varied group of insects with over 3,000

102 species (Sanborn, 2014), characterized by low dispersal and long egg-to-adult life cycles (see

103 Table S2 in Simon et al., 2022), with 500 species belonging to the largest tribe Cicadettini

104 (Marshall et al., 2016). The Cicadettini are thought to have an Australasian origin, with two

105 smaller centers of diversity in the Mediterranean and South Africa (Marshall et al., 2016).

106 Cicadas spend multiple years underground as nymphs, contrasting with their ephemeral adult

107 winged stage above ground, generally lasting up to a few weeks in which they seek to mate

108 and lay their eggs. The male uses a unique calling mechanism to attract receptive females.

109 These calls are usually species-specific and recognized as effective for species delimitation

110 (Boulard, 2006). Although several aspects of their biology are still largely unknown, cicadas

111 make interesting models for biogeography, as exemplified by studies from New Zealand,

112 where vicariance appears to have played an important role in their diversification and

113 speciation (Arensburger et al., 2004a; Bator et al., 2021; Buckley & Simon, 2007; Marshall et

114 al., 2008).

115 In the western Mediterranean, cicadas of tribe Cicadettini have recently received some

116 attention regarding their diversity and biogeography (Hertach et al., 2015, 2016; Mendes et al.,

117 2014; Nunes et al., 2014a; Puissant & Sueur, 2010). The genus *Tettigettalna* was created in

118 2010 to accommodate nine morphologically similar species of Cicadettini with distinct calling

119 songs (Puissant & Sueur, 2010), with all but one restricted to the Iberian Peninsula (see Fig.

120 1E). The only species extending its range beyond the Pyrenees is *T. argentata*, reaching

121 southern France, Italy, western Slovenia and southern Switzerland (Gogala & Gogala, 1999;

122 Hertach & Nagel, 2013; Puissant & Sueur, 2010). The biogeographic setting of the genus

123 changed with the recent discovery of *T. afroamissa*, a new species found in northern Morocco

124 and the first *Tettigettalna* to be reported out of Europe (Costa et al., 2017). Ecologically and

125 morphologically analogous to the widespread *T. argentata*, this new species is allopatric to all

126 other *Tettigettalna*, has a distinctive calling song and is genetically well differentiated (Costa et

127 al., 2017). The discovery of this species led to an important question: How and when did *T.*

128 *afroamissa* get into North Africa?

129 Sequence data available thus far to infer the phylogeny of *Tettigettalna spp.* is based on a

130 single mitochondrial gene, the cytochrome c oxidase I (Costa et al., 2017; Nunes et al., 2014a).

131 Both *Tettigettalna josei* and *T. afroamissa* stand out as the earliest diverging species of the

132 genus, but single-gene analysis proved as insufficient to resolve which *Tettigettalna* species

133 diverged in the first place (Costa et al., 2017). Mitochondrial genes evolve faster than nuclear

134 ones and often tell a biased story based on a maternally inheritance only (Ballard & Whitlock,

135 2004; Rubinoff & Holland, 2005; Shaw, 2002). Therefore, we use an extended dataset in this

136 study, with mitochondrial and nuclear gene coverage, and add molecular age dating

137 calibration to unfold the evolutionary history of early *Tettigettalna* diversification and find the

138 best explanation for current distribution of *T. afroamissa* in North Africa.

139 Three biogeographical scenarios can be hypothesized: The first is an overseas dispersal in

140 either direction, resulting in the splitting of the European and Moroccan lineages before the

141 onset of the MSC at 5.9 Ma. The second scenario, that of vicariance, postulates that a large

142 population existed across the land connection during the MSC, which was divided with the

143 opening of the Gibraltar Strait at the end of the MSC (5.33 Ma). The third scenario places

144 overseas dispersal of *Tettigettalna* from Europe to Africa as a post-MSC event, after 5.3 Ma,

145 which should have happened most likely during the Pleistocene glacial maxima, when sea level

146 was remarkably lower. To determine which of these scenarios is the most likely, we

147 reconstructed the phylogenetic relationships of *Tettigettalna* using two methods for species-

148 tree reconstruction within a Bayesian framework: gene concatenation and multispecies

149 coalescence (Heled & Drummond, 2010; Lambert et al., 2015; Tonini et al., 2015). The results

150 of both methods are discussed under these biogeographical hypotheses to determine the most

151 parsimonious scenario explaining *Tettigettalna* spp. distribution.

152

153 **2. MATERIALS AND METHODS**

154

155 **2.1 *Sampling, DNA extraction and sequencing***

156

157 Sampling from previous *Tettigettalna* phylogenies by Nunes et al. (2014a) and Costa et al.

158 (2017) was extended with the collection of 41 new specimens in the Iberian Peninsula. Cicadas

159 were collected by hand or sweeping net and assigned to species according to their male calling

160 song. GPS data was recorded at each capture site and a front leg was removed and preserved

161 in 100% ethanol for genetic analysis (Fig. 2; Table S1, Supp. Info.). Dry specimens are stored at

162 the Department of Animal Biology of the Faculty of Sciences, University of Lisbon, Portugal.

163 Legs from the four Italian specimens of *T. argentata* included in this dataset were provided by

164 Thomas Hertach from his own collection.

165 Genomic DNA was isolated with the DNeasy Blood & Tissue Kit (Qiagen). Four gene fragments

166 were sequenced, with a total of 2504 base pairs (Table 1): (i) COI-Lep: 5' region of the

167 cytochrome C oxidase I (COI) mitochondrial gene; (ii) COI-CTL: 3' region of the cytochrome C

168 oxidase I (COI) mitochondrial gene; (iii) ATP: mitochondrial locus comprising tRNA-Asp gene (

169 complete sequence), ATPase subunit 8 gene (complete coding sequence) and ATPase subunit 6

170 gene (partial sequence) and (iv) EF-1α: nuclear locus of Elongation Factor 1α comprising exon2

171 (partial coding sequence), intron2 (complete sequence), exon3 (complete sequence), intron3 (

172 complete sequence) and exon4 (partial coding sequence). Amplification of each locus by

173 polymerase chain reaction (PCR) was performed in a total volume of 20 µl containing 1xPCR

174 buffer (Promega), 0.6 U Taq polymerase (Promega), 2.8 mM $MgCl_2$, 0.10 mM dNTPs and 0.4

175 µM of each primer (see Table S2, Supp Info. for primers sequences and sources). The standard

176 cycling conditions used were 94ºC for 3 min, 35 x (30 s at 94º C, 30 s at the specific annealing

177 temperature as in Table S2 and 30 s at 72ºC) followed by a final elongation step at 72ºC for 10

178 min. PCR products were purified with Sureclean (Bioline) following the manufacturer

179 instructions. Purified fragments were sequenced with Big Dye Terminator v.3.1 (Applied

180 Biosystems) on Macrogen or Beckman Coulter Genomics facilities.

181 Sequences were edited in Sequencher v4.0.5 (Gene Codes Co.) to correct noisy and ambiguous

182 base calling. Mitochondrial sequences were checked for stop codons in DnaSP v5.10 (Librado &

183 Rozas, 2009). Sequences of nuclear EF-1α found to be heterozygous in length were

184 reconstructed as in Flot et al. (2006). Haplotype phase was inferred with PHASE v2.1.1

185 (Stephens et al., 2001) with default settings and ran for 1000 iterations (ambiguities were

186 assigned as N if phase probability was < 0.70). A total of 262 new sequences were generated

187 by this study and deposited in GenBank (Table S1). This dataset was combined with 107

188 sequences of COI-Lep from *Tettigettalna* generated by previous works (see Table S1; Costa et

189   al., 2017; Nunes et al., 2014a; Simões et al., 2014). All the 148 *Tettigettalna spp* specimens of

190   the dataset were represented by COI-Lep sequences, whilst 61 were successfully sequenced

191   for nuclear EF-1α and 45 were sequenced for all four DNA fragments (Table S1).

192

193   **2.2 *Phylogenetic analysis***

194

195   Sequence alignments were carried out with MAFFT v7.273 (Katoh & Standley, 2013). Site

196   substitution saturation was tested in DAMBE (Xia et al., 2003; Xia & Xie, 2001) for each codon

197   position at coding sequences and found to be non-significant (p-value>0.05) for all

198   mitochondrial loci. Conversion of files from FASTA to NEXUS or PHYLIP format as well as gene

199   concatenation   were   performed   with   TriFusion   (available   at

200   https://github.com/ODiogoSilva/TriFusion). Maximum likelihood (ML) trees were obtained by

201   assigning each separate locus dataset a GTRCAT model, 1000 replicates and a rapid bootstrap

202   analysis (-x) on RAxML-HPC v.8 (Stamatakis, 2014). For the Bayesian inference (BI), each

203   dataset was partitioned into loci subsets and coding sequences were further partitioned into

204   codon positions. These partitions were subsequently tested and assigned an evolution model

205   on PartitionFinder v2 (Lanfear et al., 2016) under the corrected Akaike information criterion

206   (AICc), with a greedy search parameter. Bayesian inference trees were generated on MrBayes

207   v3.2.6 (Ronquist et al., 2012). Each dataset was assigned with two independent runs with four

208   chains, $5x10^7$ generations with burn-in set to the initial 25% trees and the evolution models

209   previously selected with PartitionFinder2. Parameter's convergence was checked in TRACER

210   and if confluence was not attained, runs were assigned additional $5x10^7$ generations and

211   checked again for confluence. *Hilaphura varipes*, *Cicada orni* and *Cicada barbara* were chosen

212   as outgroups (Table S1). All analyses were run as implemented on the CIPRES Science Gateway

213   (Miller   et   al.,   2010).   Output   trees   were   visualized   in   FigTree

214   (http://tree.bio.ed.ac.uk/software/figtree/) and imaged in Inkscape.

215

216   **2.3 *General approach for BEAST model optimization***

217

218   BEAST v.1.8.4 (Drummond et al., 2012) was used to estimate divergence times as implemented

219   in the CIPRES Science Gateway. Input files for BEAST analysis were initially assembled on

220   BEAUti (a package of BEAST) and then visually inspected or adjusted manually for substitution

221   site models not implemented on BEAUti. Initial substitution site models were the same used

222   for the BI tree analysis, *i.e* selected under an AICc criterion on PartitionFinder v2. Site models

223   with low ESS values would be discarded and the next best site model, ranked under AICc,

224   would be selected. After optimization, the selected site models were: TIM1e for the COI-CTL,

225   COI-LEP and ATP unlinked partitions; HKY+G for EF-1α exon and HKY for EF-1α intron. Tree

226   models were linked, with a Yule process prior, for the three mitochondrial partitions and for

227   the EF-1α partitions. Clock models were linked for the COI partitions (COI-LEP + COI-CTL).

228   Because the parameter "ucld.stdev" of the EF-1α exon partition abutted 0 on preliminary runs,

229   the clock model was changed from "uncorrelated relaxed" to a "strict clock with a lognormal

230   distribution". The remaining partitions were assigned an uncorrelated relaxed clock, with a

231   lognormal distribution with "mean in real space" checked.

232   Fossil records of Cicadidae are scarce worldwide (reviewed by Moulds, 2018) and none is

233   adequate for clock calibration for *Tettigettalna* cicadas. Clock rate estimates for COI and EF-1α

234   follow the ones of Marshall et al. (2016) set for phylogenetic analysis of Cicadettini with a

235   relaxed clock (analysis K - subclades), in which *Tettigettalna josei* and *T. argentata* were

236   included (see Marshall et al. (2016) Table S3- COI and Table S4 - EF-1α subclade I). The values

237   used were M=0.01172 with S=0.288 for COI partition, M=0.001965 with S=2.0 for EF-1α exon

238   and M=0.0075 with S=2.0 for EF-1α intron.

239   As no calibrations were available for ATP, we assigned an uniform clock prior bound between

240    $1.0x10^{-4}$ and 100 to this gene during preliminary runs, and afterwards inferred with a

241   lognormal clock prior assigned with M=0.0149 and S=2.0. The *Tettigettalna* clade was

242   constrained to be monophyletic, regardless of the outgroup being *Cicada orni*, *Cicada barbara*

243   or *Hilaphura varipes*. The parameter "ucld.stdev" was set to an exponential distribution bound

244   between 0 and 3.33. MCMC chain length was set for $2,5x10^{8}$ iterations logging every 25000th

245   iteration and ran 6-7 times to check for repeatability.

246   Tracer v1.4 was employed to assess convergence and correct mixing of all parameters by

247   visually inspecting the .log files and securing the Effective Sample Size (ESS) of each

248   informative parameter to be over 200. Logcombiner was used to combine the replicate runs

249   with a 10% burnin of each run for the log and tree files.

250

251   **2.3.1 *Time estimates with concatenation model* -** The gene concatenation model relies on a
252   super-matrix of multiple concatenated loci to retrieve a species-tree. One of the shortcomings
253   of this method is that mitochondrial loci generally have more variable positions which often
254   mask the phylogenetic signal provided by nuclear loci, thus losing much of its resolution,
255   especially on deeper-level phylogenies (Rubinoff & Holland, 2005).

256   To infer divergence time, we selected a subset of 38 *Tettigettalna* samples that represented all
257   relevant branches of the phylogeny: two samples per species/lineage were selected, each
258   corresponding to the most ancestral and most recently derived haplotypes. All samples in this
259   subset were sequenced for all four loci (except for *T. afroamissa*, which couldn't be
260   successfully sequenced for COI-CTL). To implement the concatenated model in BEAST, each
261   locus was defined as a separate, unlinked site model and clock models were assigned as
262   previously stated in the general approach. Tree models were linked across nuclear and
263   mitochondrial loci, on BEAUti. The input file for this analysis can be accessed in the
264   Supplementary File: beast_input.xml.

265

266   **2.3.2 *Time estimates with multispecies coalescence model* -** Multispecies coalescence models
267   are often considered as superior to concatenation by differently weight – and unlink – nuclear
268   and mitochondrial loci, thus accounting for sources of gene and species-tree mismatch, such as
269   incomplete lineage sorting (Heled et al., 2013; Lanier & Knowles, 2015; Mccormack et al.,
270   2010). For time estimates with the multispecies coalescent model, *BEAST (Heled and
271   Drummond, 2010) was used instead, which is an extension package of BEAST v.1.8.4
272   (Drummond et al., 2012). *BEAST enables the user to provide information on the ploidy of the
273   locus – *i.e* differentially weighing the mutations that occur in the slower-evolving nuclear loci
274   and the faster-evolving mitochondrial loci. Preliminary runs with the full mitochondrial and
275   nuclear datasets (148 individuals) mixed poorly, and Tracer showed low convergence among
276   most parameters, resulting in very low effective sample sizes, probably due to the lack of
277   resolution of the only nuclear gene included. Since nuclear EF-1α only resolves *T. josei* and *T.*
278   *afroamissa* as monophyletic clades, leaving the remainder of the *Tettigettalna* as a large
279   polytomy, we decided it was best to place these under a single monophyletic entity, which was
280   named "Core *Tettigettalna''* (*i.e. T. argentata, T. estrellae, T. mariae, T. aneabi, T. defauti, T.*

281 *armandi, T. helianthemi* and *T. boulardi*). Model optimization was rapidly obtained with this

282 reduced trait set *(T. josei, T. afroamissa* and Core *Tettigettalna*). The final dataset for the

283 *BEAST analysis included 5 partitions: COI-CTL, COI-LEP, ATP, EF-1α intron and EF-1α exon and

284 the input file can be accessed in the Supplementary File: star_beast_input.xml.

285 Densitree was used to represent the distribution and topology of the large number of

286 generated trees, and to retrieve clade support. Time to the most recent common ancestor

287 (tMRCA) boxplots were generated in R Studio.

288

289 **3. RESULTS**

290

291 ***3.1 Single and concatenated gene phylogenies***

292

293 Phylogenetic trees constructed for each mitochondrial locus with BI and ML are broadly

294 concordant and successfully retrieve most song-delimited species (Figures S1 and S2, Supp.

295 Info.). While mitochondrial loci efficiently reconstruct more recent relationships, all fail

296 individually to reconstruct the deep nodes amongst *Tettigettalna*, having low support values,

297 particularly the ones involving *T. josei* and *T. afroamissa*. Their relationship remains unclear

298 and even conflicting in single-gene analyses.

299 When concatenating all three mitochondrial loci, most clades defined with COI-Lep in previous

300 studies were here retrieved with both BI (Fig. 3a) and ML (Fig. S3) analyses. The concatenated

301 tree provides good support for considering *T. josei* as sister to all other *Tettigettalna,* in both BI

302 (1 pp, Fig. 3a) and ML (99% BS, Fig. S3), followed by *T. afroamissa* and the remainder of the

303 *Tettigettalna* (0.98 pp; <70% BS).

304 As in Nunes et al. (2014a), an unresolved clade in the concatenated tree joins *T. argentata*, *T.*

305 *aneabi* and *T. mariae* (BI 1pp; ML 99% BS). Regarding *T. heliathemi*, the apparent polyphyly of

306 this species was confirmed in this study, where samples of subspecies *T. h. galantei* from

307 Western Sierra Nevada, defined as type II by Nunes *et al*. (2014a), group with *T. boulardi* (BI

308 1pp). The remainder of *T. h. galantei*, referred as type I by Nunes et al. (2014a), form a well-

309 supported sister clade to *T. h. helianthemi*, as it would be expected from their song differences

310 (BI 1pp, ML 99% BS).

311 The enlarged sampling enabled the resolution of a standing *T. defauti* and *T. armandi* polytomy

312 (1 pp, Fig. 3a; 92% BS, Fig. S3). The new dataset also revealed additional lineages with marked

313 geographical structure: Sierra Nevada and Ronda & Zagra populations of *T. defauti* (0.89 pp,

314 Fig. 3a; 77% BS, Fig. S3) and Jerez and Gibraltar populations of *T. armandi* (1 pp, Fig. 3a; 94%

315 BS, Fig.S3).

316 Sequence data from the nuclear gene EF-1α has about five times fewer parsimony-informative

317 sites than mitochondrial loci (Table 1) and 39 out of 63 sequenced individuals were

318 heterozygous for indel length (length of indels varied between 1 and 4 bases). Indels were

319 coded as N for phylogenetic analyses. Their distribution among taxa was random, without

320 diagnostic value. Both BI and ML phylogenetic reconstruction with nuclear gene EF-1α have

321 low resolution (Fig 3b and Fig. S4). This locus fully retrieved *T. afroamissa* as well as *T. josei* as

322 monophyletic taxa, whereas the remainder of the specimens form a large and weakly

323 supported polytomy. Nevertheless, it is noteworthy that among *T. helianthemi*, while

324 mitochondrial DNA clearly defines two lineages corresponding to the two acoustically defined

325 subspecies (*T. h. helianthemi* and *T. h. galantei* Type I) and a third diverging lineage of *T. h.*

326 *galantei* (Type II) clustering with specimens of *T. boulardi* (Fig. 3a), such genetic structure is not

327 supported by EF-1α, where *T. boulardi* groups with some specimens of the subspecies *T. h.*

328 *helianthemi* instead (Fig. 3b). Samples Thh645 and Thh238 of *T. h. helianthemi* actually share

329 the same EF-1α haplotype with *T. boulardi* samples, signaling incomplete lineage sorting

330 among these parapatric species in southern Spain.

331

332 **3.2 *Divergence time with concatenation method***

333

334 Divergence time estimates for *Tettigettalna* nodes using BEAST are summarized in Table 2 and

335 Fig. 4. The concatenated species-tree is congruent with both the BI and ML trees of

336 mitochondrial datasets, apart from the basal relationships recovered within the core

337 *Tettigettalna*. BEAST concatenated species-tree confirms *T. josei* (from southwestern Iberia) as

338 the earliest diverging taxon (node A), between 9.6 and 2.8 Ma, with a mean age estimate of

339   5.9 Ma. The divergence of Moroccan *T. afroamissa* (node B) was estimated between 7.8 and

340   2.3 Ma, with a mean age estimate of 4.8, after the reopening of the Strait of Gibraltar. The

341   remaining taxa were arranged in two main subclades (node C) with a mean age estimate of 3.4

342   Ma, during late Pliocene. One subclade comprises *T. estrellae*, *T. boulardi*, *T. helianthemi*

343   *galantei* Type II, *T. defauti* and *T. armandi* (BS=1). *Tettigettalna estrellae* remains *incertae-sedis*

344   within this subclade. Relationships between *T. defauti* and *T. armandi,* and between *T. boulardi*

345   and *T. h. galantei* Type II remain well supported (both BS=1). Estimates place divergence of *T.*

346   *defauti* and *T. armandi* (node F) between 2.9 and 0.8 Ma and divergence of *T. boulardi* from *T.*

347   *h. galantei* Type II (node G) between 2.1 and 0.5 Ma, both during the Pleistocene.

348   The second subclade (node H, BS=0.92) comprises the *T. argentata* species complex (*T.*

349   *argentata, T. mariae, T. aneabi*) together with its putative sister taxon *T. helianthemi*, including

350   its two recognized subspecies (*T. h. helianthemi* and *T. h. galantei* Type I). Both groups within

351   this subclade are well resolved (BS=1), but *T. argentata*, *T. mariae* or *T. aneabi* remain

352   polyphyletic. Divergence time estimates within subclade H (nodes I and J) are recent, during

353   the Pleistocene, between 2.5 and 0.6 Ma.

354

3.3 Divergence time with coalescent method

356

357   To make a *bona fide* estimation of the chain of events of the diversification of the

358   *Tettigettalna*, we estimated tMRCA of the clades definable by the nuclear dataset: *T. josei*, *T.*

359   *afroamissa* and Core *Tettigettalna.* Age estimates are summarized in Table 3. Because we were

360   working with a reduced number of ingroup taxa, we could ponder all the three possible

361   phylogenetic relationship scenarios within the *Tettigettalna* clade (see introduction). The

362   probabilities of such sub-clades are presented in Table 3 and Fig. 5. Of the three likely

363   phylogenetic relationship scenarios (Fig. 5), the one with *T. josei* as sister to all other species in

364   the genus has the highest bootstrap support (81.59%, Table 3), against the remaining which

365   have a combined reduced probability (<20%). The mean age estimate for the divergence of *T.*

366   *josei* is 6.9 Ma, before the MSC, though the 95% highest probability density intervals are wide

367   (Table 3). The tMRCA for the *T. afroamissa* – Core *Tettigettalna* split was estimated between

368 9.5 and 2.0 Ma, with a mean age estimate of 5.3 Ma, coinciding with the reopening of the

369 Strait of Gibraltar and the end of the MSC (Fig. 6).

370

4.   DISCUSSION

372

4.1 *Phylogeny of Tettigettalna*

374

375 With this study, we used an extended set of mitochondrial sequences and obtained the first

376 nuclear data to investigate the evolution of the Mediterranean genus *Tettigettalna*. These

377 small-sized and colour-cryptic cicadas occur at fairly low density, being very difficult to sample

378 without the use of acoustic location. Hence, and because females are mute and seldom seen,

379 the dataset is composed of males only. The inclusion of nuclear sequences is particularly

380 relevant to corroborate patterns found with mitochondrial data and discard potential bias in

381 male dispersal. As expected, nuclear EF-1α had much lower diversity than mitochondrial

382 genes and lacked resolution in *Tettigettalna,* though without conflicting with mitochondrial

383 phylogenies.

384 Regarding the order of lineage splitting among *Tettigettalna*, our study gives support to *T. josei*

385 as sister to the remaining extant members of the genus, consistent with morphological and

386 acoustic differentiation observed for this species (Mendes et al., 2014). Nonetheless, the other

387 two phylogenetic scenarios in the species coalescent tree (i.e subclades *T. josei* – Core

388 *Tettigettalna* and *T. afroamissa* – *T. josei*) have some degree of support (8 – 9 %). Species *T.*

389 *afroamissa* and *T. argentata* are amongst the most genetically distant taxa in the phylogeny,

390 even though they share a similar calling song pattern. *Tettigettalna* has no parallel with any

391 other cicada genus occurring in Iberia, being the most species rich, with species defined mainly

392 by song characterization (Puissant & Sueur, 2010). Such diversity of songs within *Tettigettalna*

393 has evolved without significant morphological divergence, not even at genitalia morphology,

394 often a key trait to distinguish between closely related species, as it may prevent successful

395 mating of heterospecific couples (Knowles et al., 2016; Langerhans et al., 2016). A few song

396 operational taxonomic units within *Tettigettaln*a remain polyphyletic with the new sequence

397   data as in Nunes et al. (2014a) and Costa et al. (2017), namely the widespread *T. argentata*

398   with cryptic *T. mariae* and *T. aneabi,* which present partially overlapping distribution ranges

399   (Nunes et. al, 2014b). Insect songs are genetically inherited but the genes ruling them remain

400   poorly known, in particular for cicadas (Fujisawa et al. 2018; Sueur, 2006; Xu & Shaw, 2019,

401   2021). Neutral genetic markers fail to differentiate among such close species, either because

402   divergence among their songs is too recent for complete lineage sorting or episodes of

403   introgression on secondary contact have eroded such divergence, or very likely both. Similar

404   cases of polyphyly among cryptic species of Cicadettinni have been detected in *Cicadetta* of

405   Italy and Greece (Hertach et al., 2015, 2016; Wade et al., 2015), and in *Kikihia* and *Maoricicada*

406   of New Zealand (Buckley et al. 2006; Marshall et al., 2008), where molecular phylogeny failed

407   to recover some acoustically defined taxa.

408   Only a few nuclear genes have been sequenced thus far to investigate phylogenetic

409   relationships among cicadas worldwide, being EF-1α the most extensively used and

410   informative (Arensburger et al., 2004b; Banker et al., 2017; Buckley & Simon, 2007; Hill et al.

411   2021; Lee & Hill, 2010; Marshal et al., 2008, 2016, 2018; Owen et al., 2017; Price et al., 2019;

412   Simon et al., 2019). Nuclear genes have been shown as insufficient to resolve the phylogeny of

413   cryptic cicada species where hybridization is suspected, even when multiple genes were used

414   (Banker et al., 2017; Buckley & Simon, 2007; Wade et al., 2015). A genomic approach and a

415   fine-scale sampling at contact zones would be preferable to overcome single-gene

416   shortcomings and will certainly help to disentangle introgression events and incomplete

417   lineage sorting among *T. argentata*, *T. mariae* and *T. aneabi.*

418   Another incongruence between acoustics and genetics found within the *T. helianthemi* ssp.

419   remains unexplained, as *T. h. galantei* Type II, though sharing the same calling song with *T. h.*

420   *galantei* Type I, is remarkably different at mitochondrial data. *T. h. galantei* Type II has a

421   parapatric distribution with Type I (they were not overlapping) with no obvious breaks in

422   habitat features to justify such level of genetic divergence. Amplification of nuclear

423   mitochondrial DNA sequences (NUMTs) could be a reason for this pattern of divergence i.e,

424   mutations at the primer biding sites that would bias amplification towards nuclear copies of

425   COI at this particular taxon (Song et al., 2008). This seems unlikely since the same phylogenetic

426   pattern was recovered for all three independently amplified fragments of mitochondrial DNA

427   (COI-LEP, COI-CTL and ATP). Further studies with phylogenomic data should bring some light to

428   the roots to this mismatch.

429

4.2   Biogeographic scenarios

431

Time estimates inferred with the concatenated model and the multispecies coalescence model for the oldest splits within *Tettigettalna* have both very wide confidence intervals (12-2 Ma), with which we cannot confidently exclude any of the three scenarios. However, if considering mean ages, both methods place the divergence of *T. josei* as a likely pre-MSC or early MSC event, probably before the land-bridge was fully established (mean ages of 5.9 and 6.9 Ma, respectively). As for the divergence of the Moroccan *T. afroamissa* from the remainder of the European *Tettigettalna,* mean age estimates place this event as a likely post-MSC event (4.8 and 5.3 MA, respectively). Divergence time estimates are sensitive to assumptions used in clock calibration, hence a considerable degree of uncertainty is inevitably associated to inferred dates (Carruthers & Scotland, 2020), particularly among cicadas, where no accurate fossil calibration can be used. When dating divergence events among Cicadettini, Marshall et al. (2016) tested many assumptions and attempted to correct inflation when using literature based values from other arthropods. Even though relaxed clock models accommodate more uncertainty about the substitution rate, configuring analyses such that they adequately account for uncertainty remains a challenge (Carruthers & Scotland, 2020).

When coupling divergence-time estimates with the tree topology, our most parsimonious biogeographic reconstruction has a chain of events scenario involving three phases (Fig. 6). First, an ancestral *Tettigettalna* population was likely present across the southern Iberian Massif before or during early Messinian (Fig 6A). Then, in the second phase, the ancestor of *T. josei* became geographically or ecologically disconnected from the remainder of the *Tettigettalna* in the southwest, where today is southern Portugal (Fig. 6B). This divergence is concurrent with the closing of the Baetic and Guadalhorce corridors, 7.3 Ma and 6.8 Ma, respectively, and the formation of the Guadalquivir Basin (Martin et al., 2001; 2009). Presently there is little recorded evidence for the role of the Guadalquivir Sea basin as a biogeographical barrier within the Iberian Peninsula, but it has been implied in the divergence of two Iberian subspecies of fire salamander, though more recently, during the early Pliocene (Antunes et al., 2018; García-París et al., 1998). The progressive uplift of the Baetic basement basin led to the formation of the Eurafrican land connection (Schoorl and Veldkamp, 2003), enabling the main

460 *Tettigettalna* ancestral population to migrate southwards, into Africa (Fig. 6B). Finally, in the

461 third phase, the reopening of the Strait of Gibraltar at 5.33 Ma likely worked as an effective

462 barrier to gene flow between the ancestor of *T. afroamissa* in Africa and "core" *Tettigettalna*

463 *in* southeastern Iberia (Fig. 6C). Examples of rupture of genetic and biological intercontinental

464 continuity with the end of MSC are well known among western Mediterranean lineages

465 occurring in Iberia and/or the Maghreb. To cite only some of the most remarkable, the re-

466 opening of the Strait of Gibraltar isolated midwife toads *Alytes maurus* from *Alytes dickhilleni*

467 and *Alytes muletensis* on opposite sides of the Mediterranean (Martínez-Solano et al., 2004),

468 and the same happened to trapdoor spiders *Ummidia* sp. 'Tarifa' and *U. aedificatoria* (Opatova

469 et al., 2016) and to Anthocharidini butterflies (Marabuto et al., 2020).

470 The lower 95% HPD for the divergence of *T. afroamissa* points to less than 2.5 Ma, although a

471 founder event resulting from post-MSC long dispersal to Africa seems very unlikely. Adult

472 cicadas are short-lived (1-6 weeks) and need to feed daily on plant sap, turning long travelling

473 overseas without feeding sources on the way into a risky endeavor. Dispersal in cicadas seems

474 to occurs mostly in a step-wise fashion, with travel distances reported as not exceeding 1 km

475 for large-body *Quesada gigas* (Andrade et al., 2020), 500 m for *Cicada orni* (Simões & Quartau,

476 2007) or 150 m for *Magicicada spp.* (Karban, 1981). Even in a scenario where dispersal across

477 Gibraltar was mediated by extreme winds or other stochastic events (Mas-Peinado et al., 2021;

478 Osozawa et al., 2021), the likelihood of survival and successful settling afterwards is low. Some

479 accidental human-mediated introductions of cicada nymphs have been occasionally reported,

480 likely via potted plants trading (Hertach & Nagel, 2013; Hill et al., 2005; Osozawa et al., 2021;

481 Pons et al., 2021), but this hypothesis can be safely discarded for *T. afroamissa*, as it was found

482 in remote and nearly untouched habitats of Morocco (Costa et al., 2017). Adding to this, the

483 deep level of divergence of *T. afroamissa* from Iberian *Tettigettalna* is not compatible with an

484 anthropogenic founder event.

485

486 *4.3*   ***The effect of Pleistocene climate changes***

487

488 After the MSC, with *T. afroamissa* isolated in the Maghreb, and *T. josei* in SW Iberia, the chain

489 of events therein is however less well supported. Nevertheless, it seems to be closely tied to

490 the Pleistocene climate changes, since age estimates inferred with the concatenated model for

491 species within "core" *Tettigettalna* resulted in upper 95% HPD that do not predate 2.9 Ma,

492 well after the reopening of the Strait of Gibraltar. Splits are estimated to have occurred in the

493 early to mid-Pleistocene (Fig 4: nodes F, G, I and J), during the Gelasian and Calabrian periods

494 (2.58 – 0.781 Ma), coinciding with the acceleration of glacial conditions and the retreat of

495 populations into more favorable areas, especially the thermophilic ones (Gómez & Lunt, 2007).

496 Given the heightened species richness of this area, complex geography and climatic stability

497 over long periods of time (Manzano et al., 2017), it is conceivable that the most recent

498 common ancestor for most modern *Tettigettalna* could have inhabited the southern portion of

499 the Iberian Peninsula, and especially the Baetic area. Nowadays, *Tettigettalna* spp. in southern

500 Iberia have parapatric or partially overlapping distributions and it is likely that finer-scale

501 sampling would detect hybrid zones between sister taxa, as detected for Cicadettini cicadas of

502 the genus *Kikihia* (Marshall et al., 2008, 2011; Banker et al., 2017). The genus *Kikihia* provides a

503 remarkable example of species-rich lineages that experienced rapid evolutionary radiation of

504 song-defined cryptic species during the Pleistocene, as mountain-building isolated populations

505 and affected habitat structure in New Zealand (Marshall et al., 2008; Marshall et al., 2011).

506 This process might be analogous to what could have happened with *Tettigettalna* in southern

507 Iberia, at a smaller geographical scale.

508 Among extant Iberian "core" *Tettigettalna*, only *T. estrellae* seems not to be affiliated with the

509 Baetic southern hotspot of diversity. Instead, the NW Iberian distribution of *T. estrellae* seems

510 to gravitate around a putative glacial refugium represented by the Douro valley or mountains

511 around it, as seen for other ectotherms such as newts (Martínez-Solano et al., 2006), lizards

512 (Miraldo et al. 2011; Paulo et al., 2001) and vipers (Martínez-Freiría et al., 2020). Likewise, the

513 northern clade of widespread *T. argentata* probably expanded from some uncertain refugia,

514 up and beyond the Iberian Peninsula. Haplotypes found in France and Italy seem to be the

515 most derived, but further sampling and genomic data is necessary to investigate the

516 phylogeography of *T. argentata* in its full range. We found no consistent differences in ecology

517 or morphology compared to other *Tettigettalna* spp. that could explain *T. argentata's* success

518 in expanding northwards (Mendes et al., 2014; Puissant & Sueur, 2010; Nunes et al., 2014b).

519 Perhaps tolerance to temperate climate, adaptation to a wider range of plant hosts or less

520 competition with other *Tettigettalna* while expanding from southern refugia could be

521 hypotheses to explore in the future.

522 Several other cicada species share a similar trans-Mediterranean distribution as *Tettigettalna*.

523 Genera such as *Cicada*, *Tibicina*, *Euryphara* or *Pseudotettigetta* have species on both sides of

524 the Strait of Gibraltar, but the North-African counterparts remain poorly studied (Pinto-Juma

525 et al, 2009; Puissant & Sueur, 2010; Sueur et al, 2007). We hope that future studies may lessen

526 the knowledge gap between the two continents and investigate the underlying causes for their

527 current patterns of distribution.

528

Credit authorship contribution statement

530 Study design: GJC, VLN, OSP, PCS; Sampling: VLN, EM, RM, PP, JMB, TH; DNA isolation and
531 sequencing: VLN; Data analyses: GJC, VLN, DNS; Writing: GJC, VLN. All authors contributed to
532 review the final manuscript.

533

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544

Declaration of Competing Interest

546 The authors declare that they have no known competing financial interests or personal

547 relationships that could have appeared to influence the work reported in this paper.

548

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6   550
7
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10   552   **Figure S2.** Single locus Maximum likelihood trees for mitochondrial loci.
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12   553   **Figure S3.** Maximum likelihood tree for concatenated mitochondrial loci.
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14   554   **Figure S4.** Maximum likelihood tree for the nuclear locus EF1-α.
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18   556   **Table S1.** Specimens included in phylogenetic analyses, with collection points, codes, GPS
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20   557   coordinates and GenBank accession numbers.
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22   558   **Table S2.** Primers and annealing temperature used to amplify each locus.
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24   559   **SuppInfo_beast_input.xml** input file used for BEAST analysis.
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26   560   **SuppInfo_star_beast_input.xml** input file used for *BEAST analysis.
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908

909   **Legends of figures**

910   **Figure 1.** Major geological events of the Western Mediterranean, Pleistocenic glacial refugia

911   and *Tettigettalna* spp. distributions. Panels A – D show a schematic of the evolution of the

912   West Mediterranean region from the Tortonian to the Late Pleistocene. A – Mid Tortonian,

913   depicting the three Eurafrican corridors that later closed, between 7.8 to 6.0 Ma. B – Late

914   Messinian, during the Salinity Crisis an extensive land bridge formed between Iberia and North

915   Africa. Arrow points to the Guadalquivir basin, a large saltwater basin. C – Early Pliocene, land

916   bridge is now disrupted, and the Guadalquivir basin has almost retreated. D – Late Pleistocene,

917   during the period when sea level was the lowest, according to Rohling et al. (2014), approx.

918   150 m lower. No land bridges are present during this period. Putative Pleistocenic glacial

919   refugia of the Western Mediterranean inferred for flora (Médail & Diadema 2009) in green,

920   and terrestrial fauna and flora (Gómez & Lunt 2007) shown with broken lines. E – Present day

921   *Tettigettalna* spp. distributions in orange, according to Puissant & Sueur (2010), Simões et al.,

922 (2014), Nunes et al. (2014b) and Costa *et al.* (2017). Legend: 1–*T. estrellae*; 2–*T. josei*; 3–*T.*
923 *mariae*; 4–*T. armandi*; 5–*T. aneabi*; 6–*T. defauti*; 7– *T. helianthemi helianthemi*; 8–*T. h.*
924 *galantei*; 9–*T. boulardi*; 10–*T. afroamissa*. Species' distributions in brown overlap with those of
925 other species. The distribution of *T. argentata* is not shown as it is widespread across several
926 European countries and the Iberian Peninsula with exception of the Baetic ranges in
927 southeastern Iberia. Scale bar equals 100 km.

928 **Figure 2.** Sampling of *Tettigettalna* spp. Circles indicate same-species collection points. Due to
929 the volume of sampling from the Southern Iberian Peninsula, the smaller box below shows
930 additional sampling points for other species annotated for that area. Legend: 1–*T. estrellae*; 2–
931 *T. josei*; 3–*T. mariae*; 4–*T. armandi*; 5–*T. aneabi*; 6–*T. defauti*; 7– *T. helianthemi helianthemi*;
932 8–*T. h. galantei*; 9–*T. boulardi*; 10–*T. afroamissa*; 11A –*T. argentata* South Clade; 11B – *T.*
933 *argentata* North Clade; 11C – *T. argentata* Central Clade; 11D – *T. argentata* Catalonia Clade.

934 **Figure 3.** Bayesian phylogenetic trees for the concatenated mitochondrial loci (A) and nuclear
935 EF-1α (b). Posterior probabilities > 0.90 are shown next to branch nodes. Scale bar represents
936 the number of estimated changes per branch length. *H. varipes* (Hva608), *C. barbara* (Cba203)
937 and *C. orni* (Cor298) were set as outgroup. Root length was truncated for imaging purposes.

938 **Figure 4.** *Tettigettalna* species tree with concatenation model as output of BEAST. Posterior
939 probabilities >0.9 are shown next to each node. Node bars A-J illustrate the 95% HPD interval
940 (age estimates for each node are listed in Table 2). Vertical gray shading under the timescale
941 bar refers to two past geological events: the Messinian Salinity Crisis and the Pleistocene Ice
942 ages. To illustrate song diversity within the target genus, oscillograms are shown next to each
943 taxon.

944 **Figure 5.** Age estimate boxplots of the possible nodes by the multispecies coalescent species-
945 tree with *BEAST. The first boxplot plots the age estimates of the basal node of *Tettigettalna*,
946 with the remainder plotting a different topology (TAF=*T. afroamissa*, TJO= *T. josei*, TCO= "core"
947 *Tettigettalna*).

948 **Figure 6.** DensiTree output of the Bayesian inference species tree of *Tettigettalna* with the
949 partitioned unlinked mtCOI and nuEF-1α dataset. The consensus trees are shown by the bold
950 blue line. Uncertainty of node heights and topology is shown by the transparent green, purple
951 and red lines. Core *Tettigettalna* refers to the clade composed of the remainder of the

952 *Tettigettalna* (see methods for explanation). Scale bar indicates Ma. The broken lines A-C refer

953 to key moments in time illustrated in the left panes. A) Mid-Tortonian (~10-8 Ma) when the

954 ancestral population of the *Tettigettalna* occurred in the southern Iberian Peninsula; the

955 broken line marks the separation of the *T. josei* lineage from the main ancestral population. B)

956 Late Messinian, during the Salinity Crisis, when the main population disperses to North Africa,

957 via the formed land bridge; the broken line indicates the rupture caused by the opening of the

958 Gibraltar Strait by end of the Messinian (5.33 Ma). C) Early Pliocene (~4 Ma), showing the three

959 lineages: *T. josei* in Southwestern Iberia; *T. afroamissa* in Morocco and the remainder of the

960 European *Tettigettalna* lineage which would later diverge into all other species. In the lower

961 left corner, a female of the Moroccan species *T. afroamissa* is shown.

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Table 1. Loci sequenced for *Tettigettalna*, with information about sequence length, number of
individuals sequenced (N), number of haplotypes, number of variable sites (V) and number of
parsimony-informative sites (P)*.

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Locus name	Locus Size (*bp*)	N	Haplotypes	V	P
COI-Lep mtDNA	581	148	83	208	175
COI-CTL mtDNA	683	59	41	106	76
ATP mtDNA	668	55	42	211	162
EF-1α nuDNA	561	63	59*	82*	30

965   *including indels

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975 **Table 2.** Mean age estimates in million years ago (Ma) and 95% highest posterior density (HPD)
976 intervals for nodes A to J, as in Fig. 4, according to BEAST analysis (concatenation model).

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Node	Mean ± Std Error	95% HPD Interval
A	$5.9191 \pm 9.18 \times 10^{-3}$	2.8488 - 9.6307
B	$4.8203 \pm 7.48 \times 10^{-3}$	2.2826 - 7.8374
C	$3.4142 \pm 5.26 \times 10^{-3}$	1.6048 - 5.5004
D	$2.7216 \pm 4.21 \times 10^{-3}$	1.3082 - 4.4336
E	$2.5574 \pm 4.00 \times 10^{-3}$	1.2458 - 4.1976
F	$1.8258 \pm 2.84 \times 10^{-3}$	0.8167 - 2.9201
G	$1.2287 \pm 2.15 \times 10^{-3}$	0.4944 - 2.1052
H	$2.9220 \pm 4.60 \times 10^{-3}$	1.3616 - 4.7695
I	$1.5040 \pm 2.43 \times 10^{-3}$	0.6722 - 2.5244
J	$1.2858 \pm 2.05 \times 10^{-3}$	0.5653 - 2.0989

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981 **Table 3.** Mean age estimates in million years ago (Ma) and 95% highest probability density
982 intervals of tMRCA as in Fig.5 according to *BEAST analysis (multispecies coalescent model).
983 Clade support is given in percentage of trees post-burnin that support that topology.

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Split	Mean ± Std Error	95% HPD Interval	Support
Tettigettalna spp	7.039 ± 0.080	2.638 -12.274	95.12%
T. afroamissa / T. josei	6.952 ± 0.080	2.429×10^{-3} - 12.41	9.00%
T. afroamissa / "Core" *Tettigettalna*	5.308 ± 0.054	2.047 - 9.565	81.59%
T. josei / "Core" *Tettigettalna*	6.965 ± 0.080	2.548 - 12.297	8.40%

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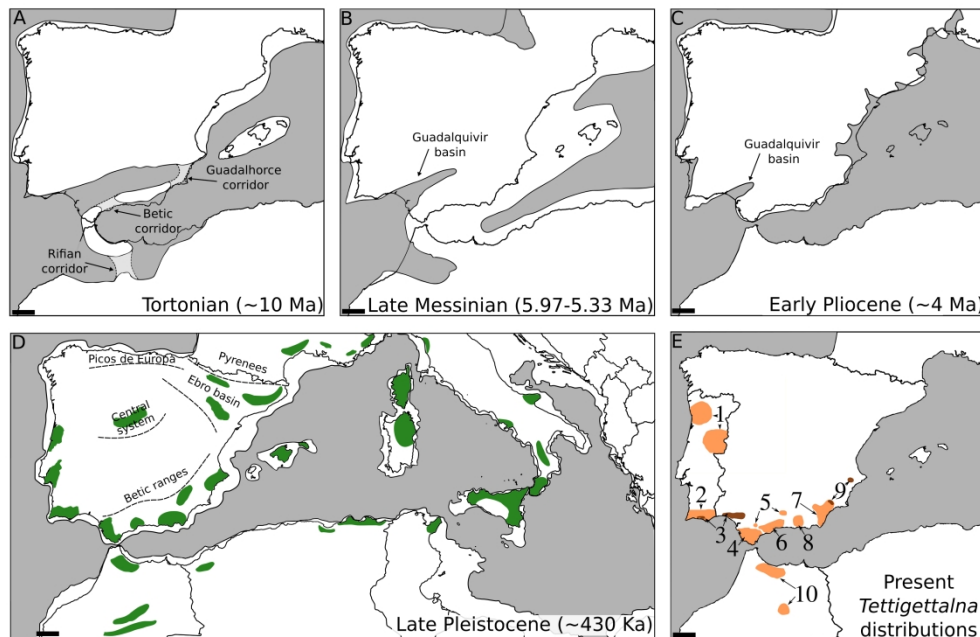
Figure 1. Major geological events of the Western Mediterranean, Pleistocenic glacial refugia and *Tettigettalna* spp. distributions. Panels A – D show a schematic of the evolution of the West Mediterranean region from the Tortonian to the Late Pleistocene. A – Mid Tortonian, depicting the three Eurafrican corridors that later closed, between 7.8 to 6.0 Ma. B – Late Messinian, during the Salinity Crisis an extensive land bridge formed between Iberia and North Africa. Arrow points to the Guadalquivir basin, a large saltwater basin. C – Early Pliocene, land bridge is now disrupted, and the Guadalquivir basin has almost retreated. D – Late Pleistocene, during the period when sea level was lowest, according to Rohling et al. (2014), approx. 150 m lower. No land bridges are present during this period. Putative Pleistocenic glacial refugia of the Western Mediterranean inferred for flora (Médail & Diadema 2009) in green, and terrestrial fauna and flora (Gómez & Lunt 2007) shown with broken lines. E – Present day *Tettigettalna* spp. distributions in orange, according to Puissant & Sueur (2010), Simões et al., (2014), Nunes et al. (2014b) and Costa et al. (2017). Legend: 1–*T. estrellae*; 2–*T. josei*; 3–*T. mariae*; 4–*T. armandi*; 5–*T. aneabi*; 6–*T. defauti*; 7– *T. helianthemi helianthemi*; 8–*T. h. galantei*; 9–*T. boulardi*; 10–*T. afroamissa*. Species' distributions in brown overlap with those of other species. The distribution of *T. argentata* is not shown as it is widespread across several European countries and the Iberian Peninsula with exception of the Baetic ranges in southeastern Iberia. Scale bar equals 100 km.

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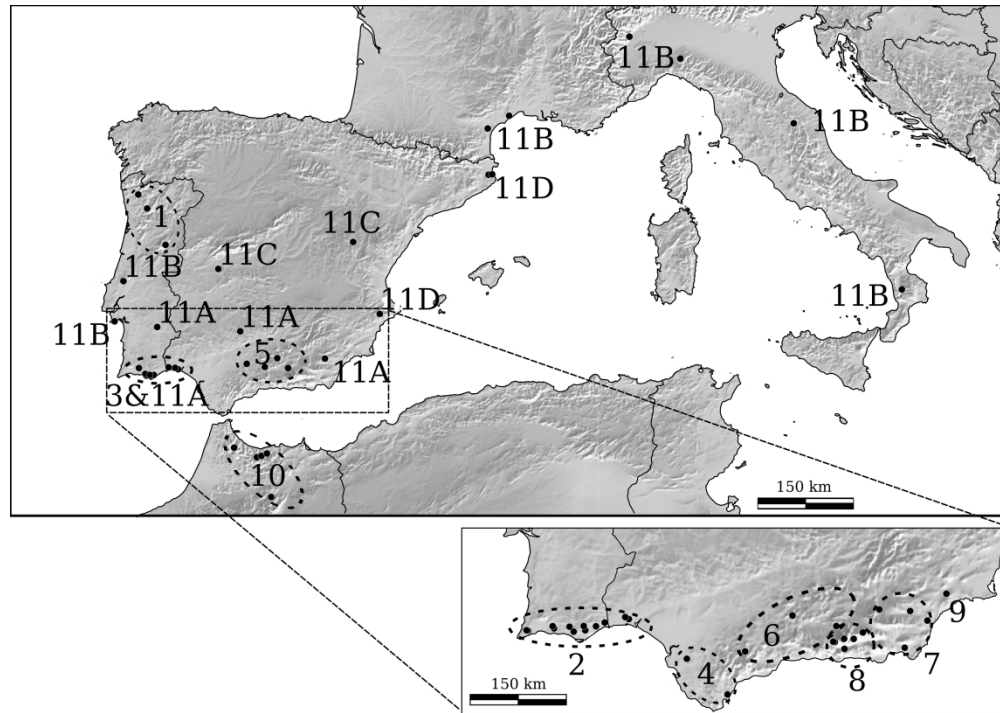
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Figure 2. Sampling of *Tettigettalna* spp. Circles indicate same-species collection points. Due to the volume of sampling from the Southern Iberian Peninsula, the smaller box below shows additional sampling points for other species annotated for that area. Legend: 1–*T. estrellae*; 2–*T. josei*; 3–*T. mariae*; 4–*T. armandi*; 5–*T. aneabi*; 6–*T. defauti*; 7– *T. helianthemi helianthemi*; 8–*T. h. galantei*; 9–*T. boulardi*; 10–*T. afroamissa*; 11A –*T. argentata* South Clade; 11B – *T. argentata* North Clade; 11C – *T. argentata* Central Clade; 11D – *T. argentata* Catalonia Clade.

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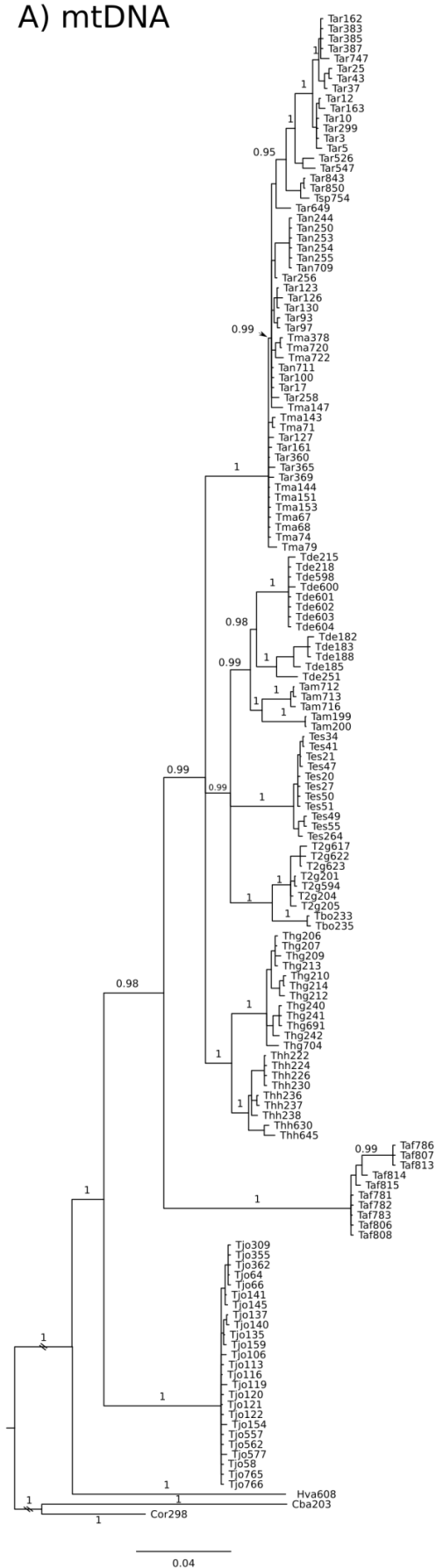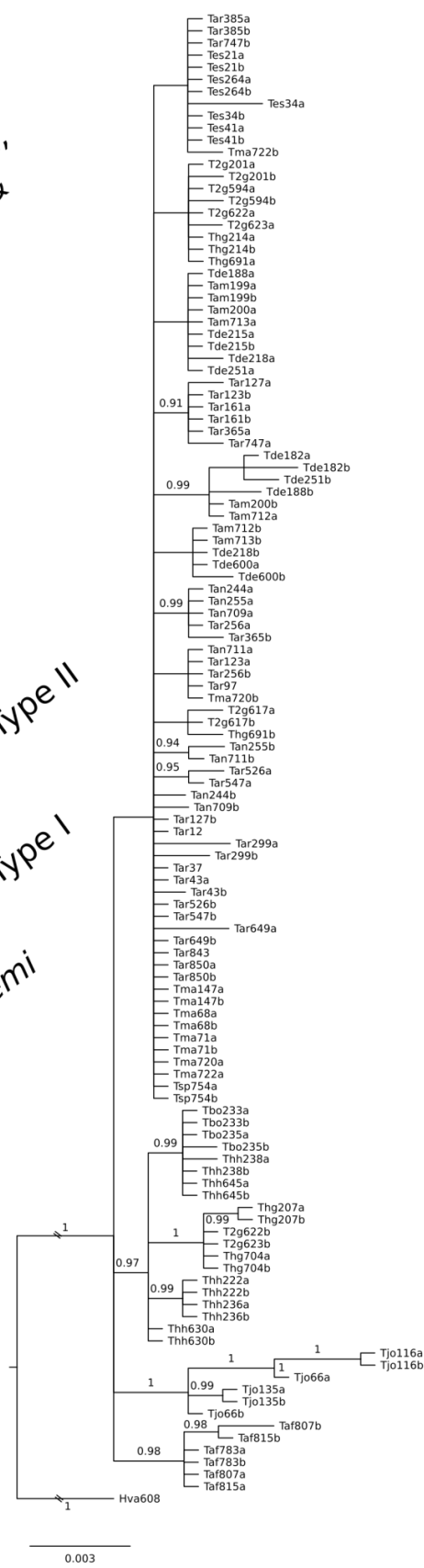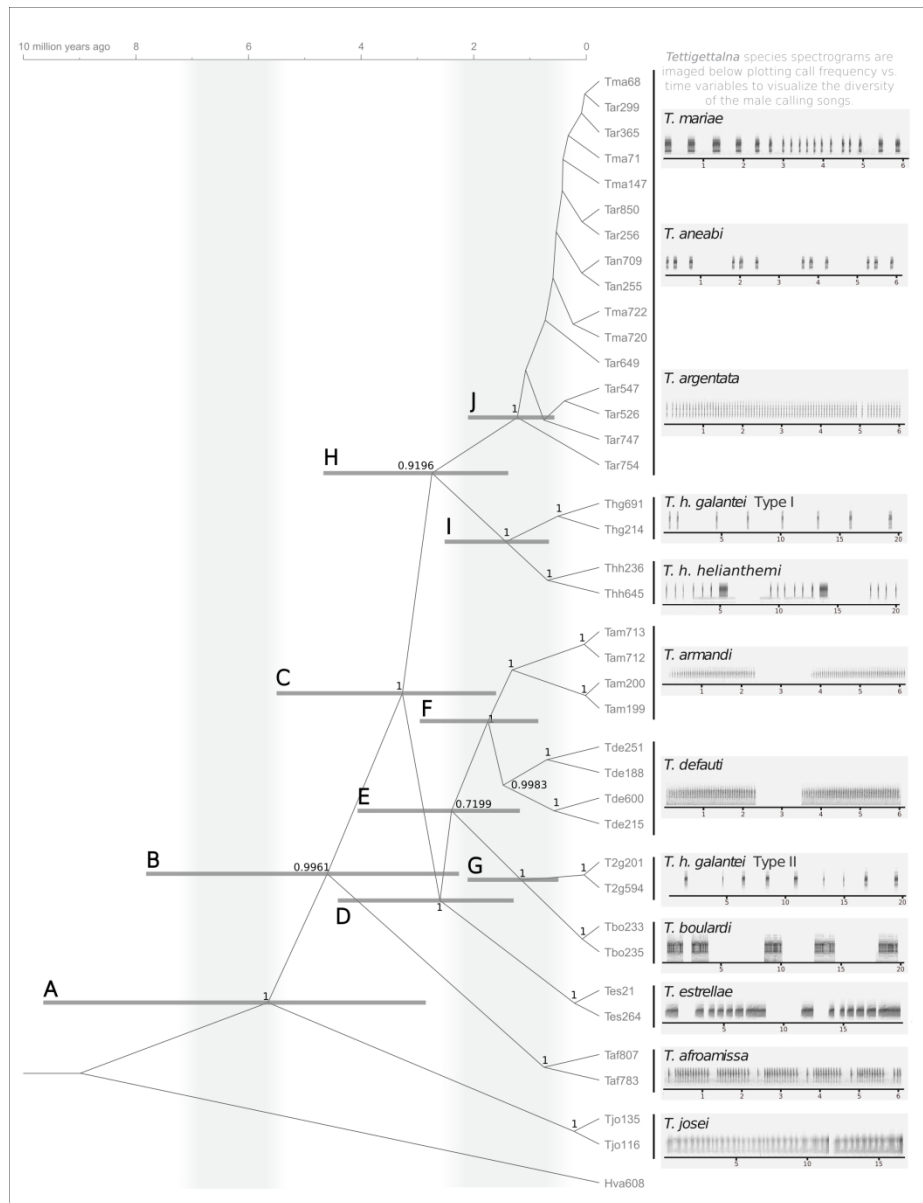
Figure 4. *Tettigettalna* species tree with concatenation model as output of BEAST. Posterior probabilities >0.9 are shown next to each node. Node bars A-J illustrate the 95% HPD interval (age estimates for each node are listed in Table 2). Vertical gray shading under the timescale bar refers to two past geological events: the Messinian Salinity Crisis and the Pleistocene Ice ages. To illustrate song diversity within the target genus, oscillograms are shown next to each taxon.

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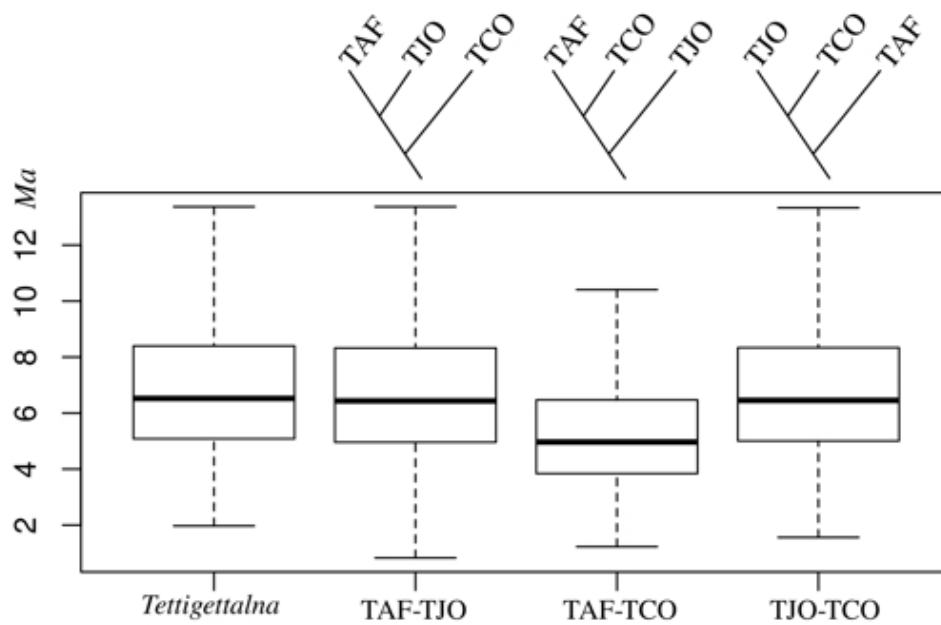
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Figure 5. Age estimate boxplots of the possible nodes by the multispecies coalescent species-tree with *BEAST. The first boxplot plots the age estimates of the basal node of *Tettigettalna*, with the remainder plotting a different topology (TAF=*T. afroamissa*, TJO= *T. josei*, TCO= "core" *Tettigettalna*).

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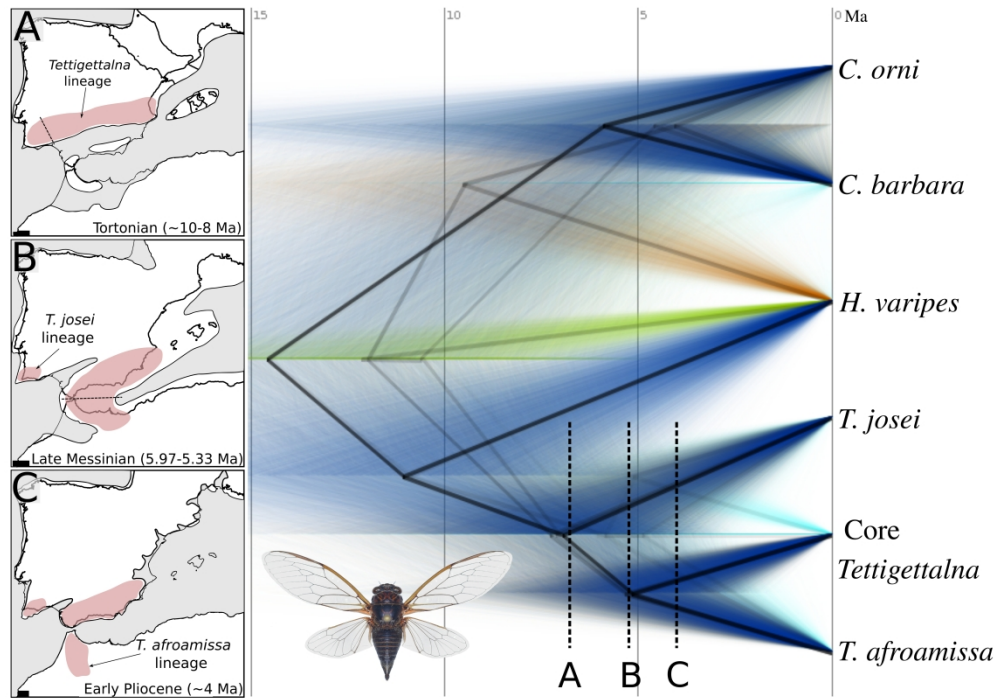
Figure 6. DensiTree output of the Bayesian inference species tree of *Tettigettalna* with the partitioned unlinked mtCOI and nuEF-1α dataset. The consensus trees are shown by the bold blue line. Uncertainty of node heights and topology is shown by the transparent green, purple and red lines. Core *Tettigettalna* refers to the clade composed of the remainder of the *Tettigettalna* (see methods for explanation). Scale bar indicates Ma. The broken lines A-C refer to key moments in time illustrated in the left panes. A) Mid-Tortonian (~10-8 Ma) when the ancestral population of the *Tettigettalna* occurred in the southern Iberian Peninsula; the broken line marks the separation of the *T. josei* lineage from the main ancestral population. B) Late Messinian, during the Salinity Crisis, when the main population disperses to North Africa, via the formed land bridge; the broken line indicates the rupture caused by the opening of the Gibraltar Strait by end of the Messinian (5.33 Ma). C) Early Pliocene (~4 Ma), showing the three lineages: *T. josei* in Southwestern Iberia; *T. afroamissa* in Morocco and the remainder of the European *Tettigettalna* lineage which would later diverge into all other species. In the lower left corner, a female of the Moroccan species *T. afroamissa* is shown.

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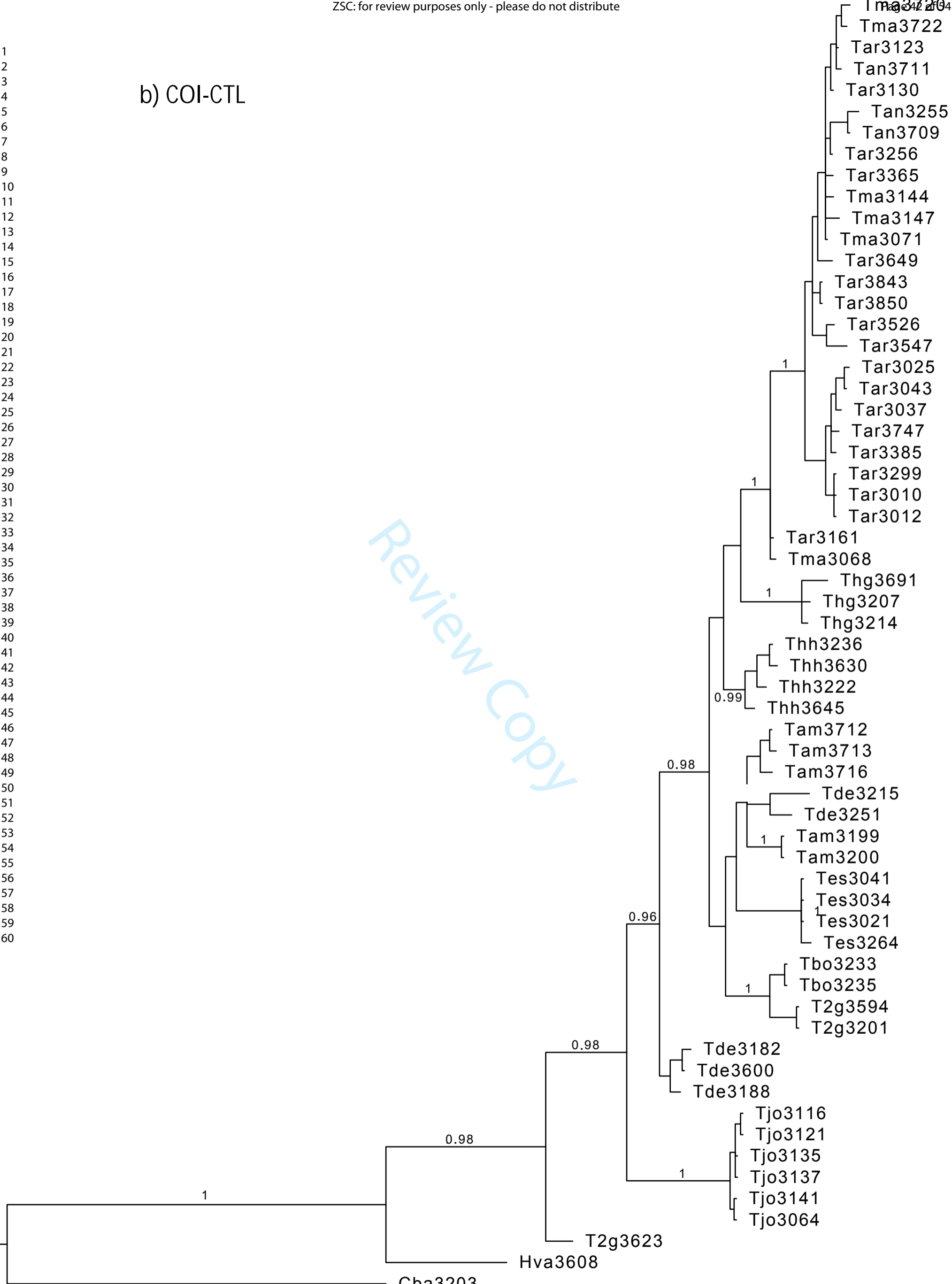
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57 **Figure S1.** Single locus Bayesian trees for mitochondrial loci : COI-LEP (a), COI-CTL (b)
58 and ATP (c).   ZSC submitted manuscript
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b) COI-CTL


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c) ATP

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a) COI-LEP

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Figure S2. Single locus Maximum likelihood trees for mitochondrial loci: a) COI-LEP, b) COI-CTL and c) ATP.

b) COI-CTL

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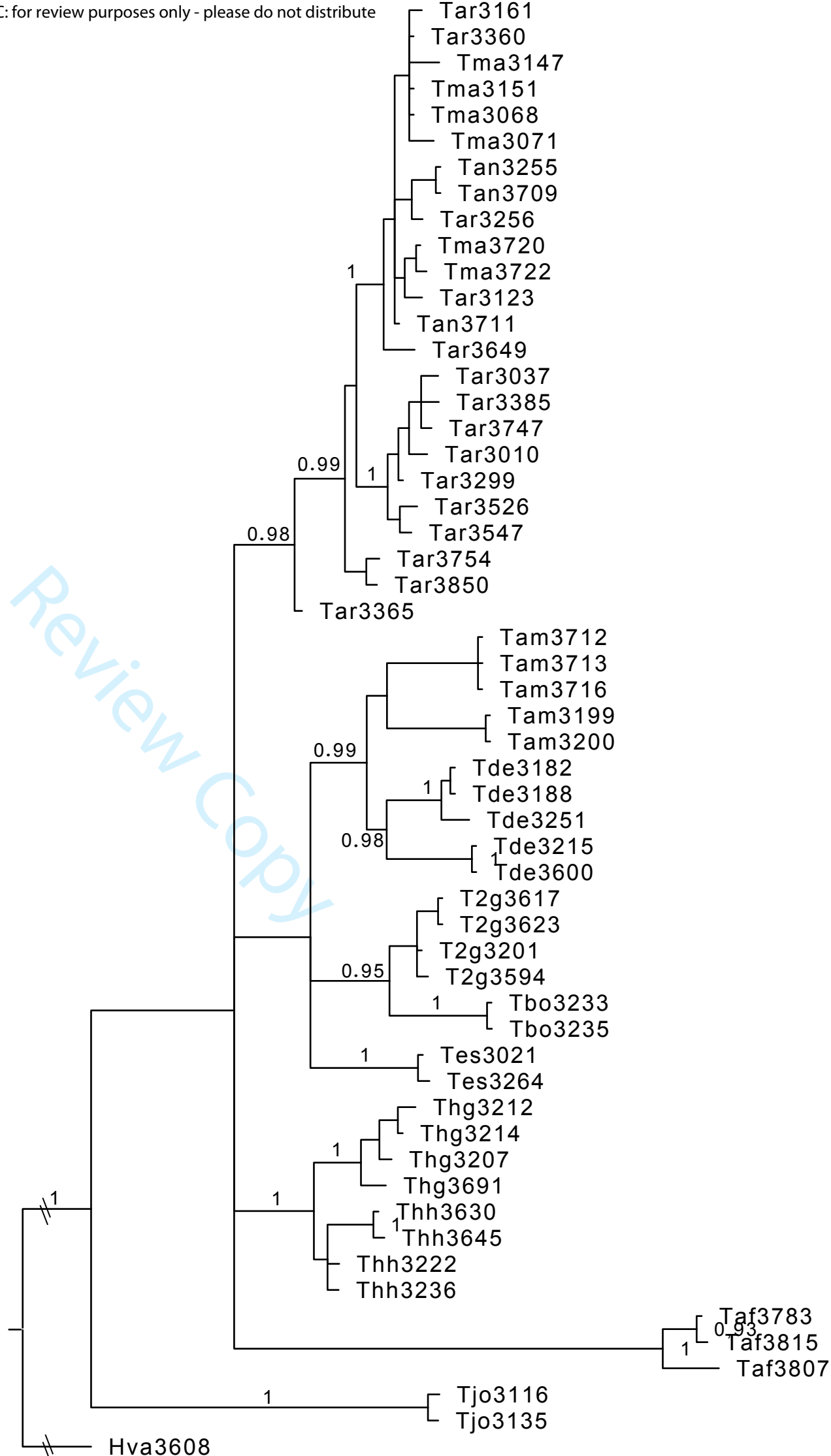69
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Mitochondrial genes
ML tree

Review Copy

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D) Elongation Factor 1-α

Figure S4. Maximum likelihood tree for the nuclear locus Elongation Factor 1-α.

Table S1. Specimens included in phylogenetic analyses, with codes, collection points, GPS coordinates and GenBank accession numbers. For nuclear EF1-α, when specimens were heterozygous, both haplotype accession numbers are listed, separated by a hyphen. Accession numbers starting with "M" or "ON" (marked in bold) were sequenced for this study. The remaining come from previous studies and are marked with [a]/[b]/[c], corresponding to Nunes *et al.* 2014a, Simões *et al.* 2014 and Costa *et al.* 2017, respectively.

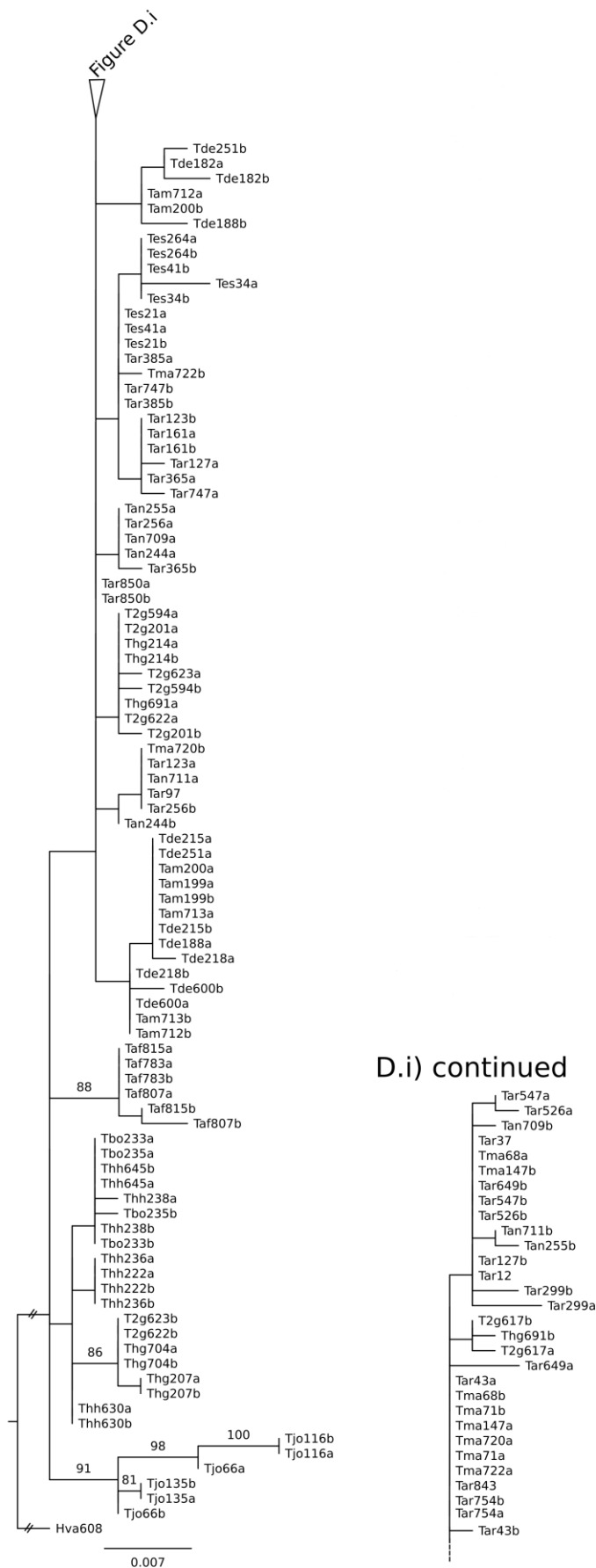| Taxa	Code	Country	Locality	GPS coordinates	COI-Lep	COI-CTL	ATP	EF1-α
T. afroamissa	Taf781	Morocco	Chefchaouane	35.184, -5.224	KX582158 [c]			
T. afroamissa	Taf782	Morocco	Chefchaouane	35.184, -5.224	KX582159 [c]			
T. afroamissa	Taf783	Morocco	Chefchaouane	35.184, -5.224	KX582160 [c]		**MG918037**	**MG978212**
T. afroamissa	Taf786	Morocco	Afouzar	33.871, -4.029	KX582161 [c]			
T. afroamissa	Taf806	Morocco	Bni Hadifa	35.030, -4.164	KX582162 [c]			
T. afroamissa	Taf807	Morocco	Bni Hadifa	35.030, -4.164	KX582163 [c]		**MG918038**	**MG978213-4**
T. afroamissa	Taf808	Morocco	Bni Hadifa	35.030, -4.164	KX582164 [c]			
T. afroamissa	Taf813	Morocco	Targuist	34.965, -4.344	KX582165 [c]			
T. afroamissa	Taf814	Morocco	Tizi Tchen	34.929, -4.492	KX582166 [c]			
T. afroamissa	Taf815	Morocco	Tizi Tchen	34.929, -4.492	KX582167 [c]		**MG918039**	**MG978215-6**
T. josei	Tjo106	Portugal	Porches	37.136, -8.385	KC807272 [a]			
T. josei	Tjo113	Portugal	Porches	37.136, -8.385	KF977493 [b]			
T. josei	Tjo116	Portugal	Lagoa	37.136, -8.385	KC807271 [a]	**MG918021**	**MG918080**	**MG978292**
T. josei	Tjo119	Portugal	Budens	37.079, -8.837	KF977491 [b]			
T. josei	Tjo120	Portugal	Budens	37.073, -8.812	KC807267 [a]			
T. josei	Tjo121	Portugal	Budens	37.073, -8.812	KC807268 [a]	**MG918022**		
T. josei	Tjo122	Portugal	Budens	37.073, -8.812	KF977492 [b]			
T. josei	Tjo135	Portugal	Castro Marim	37.186, -7.484	KC807270 [a]	**MG918023**	**MG918081**	**MG978293**
T. josei	Tjo137	Portugal	Castro Marim	37.186, -7.484	KF977502 [b]	**MG918024**		
T. josei	Tjo140	Portugal	Castro Marim	37.186, -7.484	KC807269 [a]			
T. josei	Tjo141	Portugal	Moncarapacho	37.078, -7.821	KF977499 [b]	**MG918025**		
T. josei	Tjo145	Portugal	S. Brás de Alportel	37.137, -7.848	KF977498 [b]			
T. josei	Tjo154	Portugal	Moncarapacho	37.078, -7.821	KF977500 [b]			
T. josei	Tjo159	Portugal	Tavira	37.134, -7.635	KF977501 [b]			
T. josei	Tjo309	Portugal	Quinta do Lago	37.060, -8.021	KF977495 [b]			
T. josei	Tjo355	Portugal	Quinta do Lago	37.060, -8.021	KF977496 [b]			
T. josei	Tjo362	Portugal	Quinta do Lago	37.060, -8.021	KF977497 [b]			
T. josei	Tjo557	Spain	Cartaya	37.261, -7.129	KF977503 [b]			
T. josei	Tjo562	Spain	Cartaya	37.261, -7.129	KF977504 [b]			
T. josei	Tjo577	Spain	Cartaya	37.234, -7.066	KF977505 [b]			

Species	Code	Country	Location	Coordinates				
T. josei	Tjo58	Portugal	Vale Judeu	37.128, -8.093	KC807273 [a]			
T. josei	Tjo64	Portugal	Vale Judeu	37.128, -8.093	KC807274 [a]	MG918020		
T. josei	Tjo66	Portugal	Vale Judeu	37.128, -8.093	KF977494 [b]			MG978294-5
T. josei	Tjo765	Portugal	Armação de Pêra	37.105, -8.361	MG905079			
T. josei	Tjo766	Portugal	Armação de Pêra	37.105, -8.361	MG905080			
T. estrellae	Tes21	Portugal	Braga	41.582, -8.321	KC807263 [a]	MG918006	MG918070	MG978275
T. estrellae	Tes20	Portugal	Braga	41.582, -8.321	MG905075			
T. estrellae	Tes27	Portugal	Braga	41.582, -8.321	KC807261 [a]			
T. estrellae	Tes34	Portugal	Braga	41.582, -8.321	MG905076	MG918007		MG978277-8
T. estrellae	Tes41	Portugal	Braga	41.582, -8.321	KC807264 [a]	MG918009		MG978279-80
T. estrellae	Tes47	Portugal	Amarante	41.243, -8.034	KC807262 [a]			
T. estrellae	Tes49	Portugal	Amarante	41.243, -8.034	MG905077			
T. estrellae	Tes50	Portugal	Amarante	41.244, -8.034	KC807260 [a]			
T. estrellae	Tes51	Portugal	Amarante	41.243, -8.034	KC807259 [a]			
T. estrellae	Tes55	Portugal	Amarante	41.243, -8.034	KC807266 [a]			
T. estrellae	Tes264	Portugal	Serra Estrela	40.355, -7.440	KC807265 [a]	MG918008	MG918071	MG978276
T. h. galantei type 1	Thg206	Spain	Capileira, Sierra Nevada	36.957, -3.353	KC807285 [a]			
T. h. galantei type 1	Thg207	Spain	Capileira, Sierra Nevada	36.957, -3.353	KC807282 [a]	MG918011	MG918072	MG978281
T. h. galantei type 1	Thg209	Spain	Capileira, Sierra Nevada	36.956, -3.347	KC807289 [a]			
T. h. galantei type 1	Thg210	Spain	Capileira, Sierra Nevada	36.956, -3.347	KC807291 [a]			
T. h. galantei type 1	Thg212	Spain	Capileira, Sierra Nevada	36.956, -3.347	KC807284 [a]		MG918073	
T. h. galantei type 1	Thg213	Spain	Capileira, Sierra Nevada	36.963, -3.341	KC807290 [a]			
T. h. galantei type 1	Thg214	Spain	Capileira, Sierra Nevada	36.963, -3.341	KC807286 [a]	MG918012	MG918074	MG978282
T. h. galantei type 1	Thg240	Spain	Laroles, Sierra Nevada	37.049, -3.017	KC807287 [a]			
T. h. galantei type 1	Thg241	Spain	Laroles, Sierra Nevada	37.049, -3.017	KC807283 [a]			
T. h. galantei type 1	Thg242	Spain	Laroles, Sierra Nevada	37.049, -3.017	KC807288 [a]			
T. h. galantei type 1	Thg691	Spain	Narila, Sierra Nevada	36.960, -3.175	ON470143	MG918015	MG918075	MG978283-4
T. h. galantei type 1	Thg704	Spain	Rubite	36.822, -3.335	MG905078			MG978285
T. h. helianthemi	Thh222	Spain	Cabo da Gata	36.838, -2.293	KC807292 [a]	MG918016	MG918076	MG978286
T. h. helianthemi	Thh224	Spain	Cabo da Gata	36.838, -2.293	KC807296 [a]			
T. h. helianthemi	Thh226	Spain	Cabo da Gata	36.838, -2.293	KC807294 [a]			
T. h. helianthemi	Thh230	Spain	Cabo da Gata	36.838, -2.293	KC807297 [a]			
T. h. helianthemi	Thh236	Spain	Vera	37.213, -1.900	KC807295 [a]	MG918017	MG918077	MG978287
T. h. helianthemi	Thh237	Spain	Vera	37.213, -1.900	KC807293 [a]			
T. h. helianthemi	Thh238	Spain	Vera	37.213, -1.900	KC807298 [a]			MG978288-9
T. h. helianthemi	Thh630	Spain	Sierra Filabres, north slope	37.366, -2.732	ON470142	MG918018	MG918078	MG978290

T. h. helianthemi	Thh645	Spain	Cantoria, Sierra Filabres	37.345, -2.199	ON470141	MG918019	MG918079	MG978291
T. h. galantei type 2	T2g201	Spain	Lanjarón, Sierra Nevada	36.923, -3.531	KC807279 [a]	MG918010	MG918033	MG978202-3
T. h. galantei type 2	T2g204	Spain	Lanjarón, Sierra Nevada	36.916, -3.504	KC807281 [a]			
T. h. galantei type 2	T2g205	Spain	Lanjarón, Sierra Nevada	36.916, -3.504	KC807280 [a]			
T. h. galantei type 2	T2g594	Spain	W Lanjarón, Sierra Nevada,	36.923, -3.531	MH248134	MG918013	MG918034	MG978204-5
T. h. galantei type 2	T2g617	Spain	Pinos Genil, Sierra Nevada	37.138, -3.476	MH248135		MG918035	MG978206-7
T. h. galantei type 2	T2g622	Spain	Pinos Genil, Sierra Nevada	37.138, -3.476	MG905049			MG978208-9
T. h. galantei type 2	T2g623	Spain	Pinos Genil, Sierra Nevada	37.138, -3.476	ON470144	MG918014	MG918036	MG978210-11
T. boulardi	Tbo233	Spain	Campico de los López, Murcia	37.583, -1.571	KC807276 [a]	MG917999	MG918063	MG978261
T. boulardi	Tbo235	Spain	Campico de los López, Murcia	37.583, -1.571	KC807275 [a]	MG918000	MG918064	MG978262-3
T. armandi	Tam199	Spain	Gibraltar	36.188, -5.359	KC807277 [a]	MG917973	MG918040	MG978217
T. armandi	Tam200	Spain	Gibraltar	36.188, -5.359	KC807278 [a]	MG917974	MG918041	MG978218-9
T. armandi	Tam712	Spain	Estella del Marques	36.685, -6.063	MG905050	MG917975	MG918042	MG978220-1
T. armandi	Tam713	Spain	Estella del Marques	36.685, -6.063	MG905051	MG917976	MG918043	MG978222-3
T. armandi	Tam716	Spain	Estella del Marques	36.685, -6.063	MG905052	MG917977	MG918044	
T. defauti	Tde182	Spain	Puerto del Viento, Ronda	36.787, -5.053	KC807305 [a]	MG918001	MG918065	MG978264-5
T. defauti	Tde183	Spain	Puerto del Viento, Ronda	36.787, -5.053	KC807307 [a]			
T. defauti	Tde185	Spain	Puerto del Viento, Ronda	36.787, -5.053	KC807309 [a]			
T. defauti	Tde188	Spain	Puerto del Viento, Ronda	36.787, -5.053	KC807308 [a]	MG918002	MG918066	MG978266-7
T. defauti	Tde215	Spain	Sierra Nevada	37.138, -3.468	KC807310 [a]	MG918003	MG918067	MG978268
T. defauti	Tde218	Spain	Sierra Nevada	37.138, -3.468	KC807304 [a]			MG978269-70
T. defauti	Tde251	Spain	Zagra	37.283, -4.234	KC807306 [a]	MG918004	MG918068	MG978271-2
T. defauti	Tde598	Spain	Sierra Nevada	37.138, -3.476	MG905069			
T. defauti	Tde600	Spain	Sierra Nevada	37.138, -3.476	MG905070	MG918005	MG918069	MG978273-4
T. defauti	Tde601	Spain	Sierra Nevada	37.138, -3.476	MG905071			
T. defauti	Tde602	Spain	Sierra Nevada	37.138, -3.476	MG905072			
T. defauti	Tde603	Spain	Sierra Nevada	37.138, -3.476	MG905073			
T. defauti	Tde604	Spain	Sierra Nevada	37.138, -3.476	MG905074			
T. aneabi	Tan244	Spain	Granada	37.256, -3.482	KC807300 [a]			MG978224-5
T. aneabi	Tan250	Spain	Zagra	37.283, -4.234	KC807301 [a]			
T. aneabi	Tan253	Spain	Zagra	37.283, -4.234	KC807303 [a]			
T. aneabi	Tan254	Spain	Zagra	37.283, -4.234	KC807302 [a]			
T. aneabi	Tan255	Spain	Zagra	37.283, -4.234	KC807299 [a]	MG917978	MG918045	MG978226-7
T. aneabi	Tan709	Spain	Frailes	37.508, -3.832	MG905053	MG917979	MG918046	MG978228-9
T. aneabi	Tan711	Spain	Estepa	37.366, -4.818	MG905054	MG917980	MG918047	MG978230-1
T. mariae	Tma143	Portugal	Vale do Lobo	37.061, -8.061	KC807253 [a]			

Species/clade	ID	Country	Locality	Coordinates				
T. mariae	Tma144	Portugal	Vale do Lobo	37.061, -8.061	KC807249 [a]	MG918028		
T. mariae	Tma147	Portugal	Vale do Lobo	37.061, -8.061	KC807255 [a]	MG918029	MG918082	MG978296-7
T. mariae	Tma151	Portugal	Vale do Lobo	37.061, -8.061	KC807250 [a]		MG918083	
T. mariae	Tma153	Portugal	Vale do Lobo	37.061, -8.061	KC807257 [a]			
T. mariae	Tma378	Spain	Cartaya	37.262, -7.130	MG905083			
T. mariae	Tma67	Portugal	Vale Judeu	37.106, -8.095	KC807258 [a]			
T. mariae	Tma68	Portugal	Vale Judeu	37.106, -8.095	KC807251 [a]	MG918026	MG918084	MG978298-9
T. mariae	Tma71	Portugal	Vale Judeu	37.106, -8.095	KC807254 [a]	MG918027	MG918085	MG978300
T. mariae	Tma720	Spain	Huelva	37.226, -7.035	MG905082	MG918030	MG918086	MG978301-2
T. mariae	Tma722	Spain	Huelva	37.226, -7.035	MG905083	MG918031	MG918087	MG978303-4
T. mariae	Tma74	Portugal	Vale Judeu	37.106, -8.095	KC807252 [a]			
T. mariae	Tma79	Portugal	Vale Judeu	37.106, -8.095	KC807256 [a]			
T. argentata North clade	Tar3	Portugal	Sesimbra	38.447, -9.086	KC807243 [a]			
T. argentata North clade	Tar5	Portugal	Sesimbra	38.443, -9.089	KC807245 [a]			
T. argentata North clade	Tar10	Portugal	Sesimbra	38.443, -9.089	KC807244 [a]	MG917981	MG918048	
T. argentata North clade	Tar12	Portugal	Sesimbra	38.445, -9.091	MG905055	MG917982		MG978232
T. argentata North clade	Tar25	Portugal	Braga	41.582, -8.321	KC807230 [a]	MG917983		
T. argentata North clade	Tar37	Portugal	Braga	41.582, -8.321	MG905061	MG917984	MG918055	MG978245
T. argentata North clade	Tar43	Portugal	Braga	41.582, -8.321	KC807229 [a]	MG917985		MG978247-8
T. argentata North clade	Tar162	France	Bouzigues	43.455, 3.657	KC807233 [a]			
T. argentata North clade	Tar163	France	Narbonne	43.155, 2.964	KC807234 [a]			
T. argentata North clade	Tar299	Portugal	Serra d'Aire & Candeeiros	39.456, -8.800	MG905058	MG917990	MG918052	MG978241-42
T. argentata North clade	Tar383	Italy	Benne, Piedmont	45.281, 7.541	KC807237 [a]			
T. argentata North clade	Tar385	Italy	Serradica, Marche	43.278, 12.847	KC807236 [a]	MG917992	MG918056	MG978246
T. argentata North clade	Tar387	Italy	Cella, Lombardy	44.780, 9.187	KC807235 [a]			
T. argentata North clade	Tar747	Italy	Pietrafitta, Calabria	39.249, 16.340	MG905064	MG917996	MG918060	MG978255-6
T. argentata South clade	Tar17	Portugal	Portel	38.303, -7.709	KC807238 [a]			
T. argentata South clade	Tar93	Portugal	Portel	38.303, -7.709	KC807239 [a]			
T. argentata South clade	Tar97	Portugal	Portel	38.303, -7.709	MG905068			MG978260
T. argentata South clade	Tar100	Portugal	Portel	38.303, -7.709	KC807248 [a]			
T. argentata South clade	Tar123	Portugal	S. Bartolomeu de Messines	37.257, -8.297	KC807240 [a]	MG917986	MG918049	MG978233-4
T. argentata South clade	Tar126	Portugal	S. Bartolomeu de Messines	37.257, -8.297	KC807242 [a]			
T. argentata South clade	Tar127	Portugal	S. Bartolomeu de Messines	37.257, -8.297	MG905056			MG978235-6
T. argentata South clade	Tar130	Portugal	S. Bartolomeu de Messines	37.257, -8.297	KC807241 [a]	MG917987		
T. argentata South clade	Tar161	Portugal	Moncarapacho	37.078, -7.821	MG905057	MG917988	MG918050	MG978238
T. argentata South clade	Tar256	Spain	Espiel	38.194, -5.027	KC807232 [a]	MG917989	MG918051	MG978239-40

T. argentata South clade	Tar258	Spain	Espiel	38.194, -5.027	KC807231 [a]		
T. argentata South clade	Tar360	Portugal	Mata Lobo	37.080, -7.949	**MG905059**		**MG918053**
T. argentata South clade	Tar365	Spain	Ayamonte	37.276, -7.342	KC807246 [a]	**MG917991**	**MG918054**	**MG978243-44**
T. argentata South clade	Tar369	Spain	Ayamonte	37.276, -7.342	KC807247 [a]		
T. argentata South clade	Tar649	Spain	Oria	37.497, -2.292	**MG905060**	**MG917995**	**MG918059**	**MG978253-4**
T. argentata Central clade	Tar526	Spain	Almaraz	39.760, -5.735	**MG905062**	**MG917993**	**MG918057**	**MG978249-50**
T. argentata Central clade	Tar547	Spain	Albarracín	40.425, -1.381	**MG905063**	**MG917994**	**MG918058**	**MG978251-2**
T. argentata Catalonia clade	Tar843	Spain	Girona	42.057, 2.991	**MG905066**	**MG917997**		**MG978258**
T. argentata Catalonia clade	Tar850	Spain	Catalonia	42.069, 3.107	**MG905067**	**MG917998**	**MG918062**	**MG978259**
T. argentata Catalonia clade	Tar754	Spain	Alicante	38.634, -0.523	**MG905065**		**MG918061**	**MG978257**
Cicada barbara	Cba203	Spain	Lanjarón, Sierra Nevada	36.916, -3.504	KC807317 [a]	**MG917971**		
Cicada orni	Cor298	Spain	Serra d'Aire & Candeeiros	39.455, -8.752	KC807318 [a]		
Hilaphura varipes	Hva608	Spain	Pinos Genil, Sierra Nevada	37.138, -3.476	KX582168 [c]	**MG917972**	**MG918032**	**MG978201**
Maoricicada cassiope	Mcass14	New Zealand	-	-			

Table S2. Primers and annealing temperature used to amplify each locus.

Gene	Primers	Primer sequence (from 5' to 3')	References	Product length (bp)	$T_{annealing}$ (ºC)
Mitochondrial loci					
Cytochrome oxidase I (COI-Lep) 5' region	LepF	ATT CAA CCA ATC ATA AAG ATA TTG G	Hajibabaei *et al.* (2006)	650	45
	LepR	TAA ACT TCT GGA TGT CCA AAA AAT CA	Hajibabaei *et al.* (2006)		
Cytochrome oxidase I (COI-CTL) 3' region	C1-J-2195	TTG ATT TTT TGG TCA TCC AGA AGT	Simon *et al.* (1994)	850	53
	TL2-N-3014	TCC AAT GCA CTA ATC TGC CAT ATT A	Simon *et al.* (1994)		
ATP synthetase A6/A8	TK-J-3799_for	GGC TGA AAG TAA GTA ATG GTC TCT	Buckley *et al.* (2001)	800	57
	A6A8_rev	ATG RCC AGC AAT TAT ATT AGC TG	modified from Marshall *et al.*(2008)		
Nuclear loci					
Elongation factor 1α	EF1a-97_for	ACG CCC CTG GAC ATA GAG AT	Buckley *et al.* (2006)	600	60
	EF1a-189_rev	CAA CCT GAG ATT GGC ACA AA	Buckley *et al.* (2006)		