

1 Ancient divergence, a crisis of salt and another of ice shaped the evolution
2 of west Mediterranean butterfly *Euchloe tagis*

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4 Running title: Phylogeography of *Euchloe tagis* butterfly

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16

17 **Abstract**

18 The Mediterranean region is an extremely complex hotspot where since the
19 Miocene, extensive geological, habitat and climate changes have taken place,
20 alternating between warm and cold periods. These phenomena have left a toll on species
21 genetic composition and surviving lineages have often adapted locally and diverged
22 until the point of (complete) speciation. In this study we use one of the most enigmatic
23 butterflies to study these phenomena, the Portuguese Dappled-White - *Euchloe tagis*: a
24 West-Mediterranean endemic with fragmented, morphologically differentiated
25 populations whose status have long been disputed. Even its affiliations with other
26 Anthocharidini are largely unresolved. We used mitochondrial and nuclear markers
27 under a phylogenetics and phylogeography framework to evaluate its placement among
28 relatives and population differentiation, reconstructing its evolutionary history. We
29 found this species has a Miocene origin circa 15 Ma, and is nearest to *Euchloe* s.s. and
30 *Elphinstonia*. Its populations show high genetic diversity but all coalesce to 5.3 Ma
31 when European and all but one African populations diverged. Our multiple findings
32 concerning the evolution of a species through a changing narrow habitable area may
33 provide a more general perspective of the solutions species take in order to survive
34 within this hotspot of paramount importance.

35

36 **Keywords:**

37 Biogeography, molecular clock, refugia, genetic divergence, Europe, North Africa,
38 *Euchloe tagis*.

39

MAIN TEXT

40

41 **Introduction**

42 The biogeography of the Western Mediterranean region has been profoundly
43 shaped by dramatic geological and climatic events. Of particular importance, are the
44 Messinian Salinity Crisis (5.96- 5.33 Mya), and Plio-Pleistocene glaciation cycles from
45 2.3 Mya. Repercussions of both events are still detectable deep within the genetic
46 makeup of species and species composition of different areas.

47 Following tectonic movements between the African and European plates at the
48 end of the Miocene, the Mediterranean Sea experienced a significant drop in water level
49 and an almost complete desiccation. This phenomenon, so-called the Messinian Salinity
50 Crisis (MSC), allowed for the formation of land-bridges between major Mediterranean
51 islands and between Europe and North Africa, particularly along the Betico-Rifain
52 sector (Krijgsman *et al.*1999). Land continuity between formerly isolated biomes
53 favoured biotic interchanges, which have first been documented in the fossil record,
54 (mostly from the evaporitic phase of the MSC, 5.59-5.33 Mya , but also slightly earlier
55 as 6.2 Mya for amphibious species (Gibert *et al.* 2013) indicating incipient land bridges.
56 Recently, these interchanges have also been unveiled for an increasing array of species
57 through the analysis of genetic data and the use of molecular clocks indicating such
58 biotic interchanges go back to these times and many lineages split with these geological
59 events. At 5.33 Ma, the sudden opening of the Gibraltar Strait to the Atlantic and
60 refilling of the Mediterranean basin restored the physical discontinuity between Europe
61 and Africa and of biota on both sides of the Strait.

62 With the onset of the first cold stages by the end of the Miocene, which
63 intensified with the Pleistocene glaciations circa 2.3 Mya (Head & Gibbard 2010), the
64 Euro-Mediterranean region gradually changed towards the modern Mediterranean-

65 climate region following a trend of increasing drought (Suc, 1984). The cyclical
66 succession of cold (glacial) and warm (interglacial) stages and the constriction of the
67 whole area by developing ice-capped areas and tundra to the north, while the Sahara
68 Desert acted as a barrier to the south, brought profound changes to its biodiversity. Cold
69 events have progressively eroded warmth-adapted taxa of palaeotropical origin (de Jong,
70 1988; Zagwijn, 1992), while the proximity with Africa and Asia made possible the flow
71 of thermophilic taxa during the shorter-lived warm interglacials. The surviving
72 populations and communities are thus a melting-pot of survivors which successfully
73 coped with such ecological variation, either following suitable conditions or retreating
74 into refugia (Hewitt 1999, 2000; Avise, 2000). However, survival has left a toll in
75 species genetic makeup: dissimilar ecological conditions and interspecific interactions
76 following the interruption of gene-flow with a wider population often lead to genetic
77 drift, local differentiation, adaptation and ultimately trigger the speciation process
78 (Hewitt, 1989, 1996). With climate amelioration, populations formerly in the same
79 gene-pool but then separated by climatically unsuitable areas into refugia often reunite,
80 but may then no longer match in key-morphological or ecological aspects, rendering
81 them two independent biological entities.

82 In the Mediterranean region, the main recognised refugia for biodiversity are the
83 three southern European peninsulas (Balkan, Italian and Iberian), the Maghreb,
84 Mediterranean islands and topographic habitat-islands on large mountain ranges such as
85 the Alps, at the border or outside the current Mediterranean biogeographic region (De
86 Lattin, 1949; Stewart *et al.*, 2010; Schmitt & Varga, 2012). The size and heterogeneity
87 within each of these refugia may itself promote their fragmentation into micro-refugia,
88 with limited contact among population pockets (Paulo *et al.*, 2001; Gómez & Lunt,
89 2006, Feliner, 2011), which may show dissimilar traits (Taberlet *et al.*, 1998; Jansson &
90 Dynesius, 2002; Stewart, 2009). The Atlanto-Mediterranean area acts like one of such

91 important refugia but can be partitioned into smaller sections: the Iberian Peninsula,
92 Mediterranean France and northern Maghreb (Morocco, Algeria, Tunisia). It is the
93 largest and most complex refugium in the Euro-Mediterranean biodiversity hotspot
94 (Myers *et al.*, 2000), and has played a crucial role in the survival of Cenozoic lineages
95 and in the generation of novel adaptations for later interglacial colonisation of central
96 and northern Europe (Rodriguez Sanchez *et al.* 2008).

97 Gene-flow between each of these partitions is often limited by biogeographic
98 barriers, whether mountain ranges (e. g. the Atlas or the Pyrenees), rivers (e.g. the Ebro
99 or the Rhone) or sea. Current phylogeographic knowledge supports the hypothesis of
100 the Maghreb acting mostly as a source for species and lineages (Husemann *et al.*, 2014),
101 even though Iberia is presently the richest partition of the West Mediterranean for: 1)
102 relatively large area, 2) heterogeneous geology with a predominance of basiphilous
103 nutrient-rich soils (Tyler, 2003) and 3) climate and altitudinal heterogeneity. As a result,
104 the European part of this Atlanto-Mediterranean refugium harbours more than 50% of
105 European plant and terrestrial vertebrate species, with endemism rates up to 30%
106 (Araújo *et al.*, 2007; López-López *et al.*, 2011). While it is perceived from fossil and
107 genetic data that many species expanded their ranges northwards into central and
108 northern Europe from the Atlanto-Mediterranean after each deglaciation, termed as
109 “Temperate species”, many others remained stationary near their glacial refugial areas.
110 These are then considered “Thermophilic / Mediterranean”, and are perhaps adapted to
111 warmer conditions, lower altitudes and open habitats.

112 The Portuguese dappled-white butterfly, *Euchloe tagis* (Hübner, 1804) is
113 distributed over both sides of the Western Mediterranean, from Portugal to NW Italy, in
114 Morocco and Algeria (Tennent, 1996; Tolman & Lewington, 1997) in small and
115 fragmented populations. It fits in the concept of thermophilic stationary species, for
116 having remained close to the Mediterranean, while also expressing the Europe-Africa

117 biogeographical connection. Around ten subspecies have been described, owing to a
118 strong, geographically structured wing-shape and pattern differentiation (Olivares
119 Villegas & Back, 2004, Maux & Carsus 2007, Marabuto 2009). *E. tagis* is an ecological
120 specialist, favouring xerothermophilous rocky limestone outcrops on dense
121 Mediterranean shrubland. Larvae are monophagous on species of the genus *Iberis*
122 (Brassicaceae) (Casini, 2001; Olivares & Back, 2004; Marabuto, 2008), which attains
123 its highest diversity in the Iberian Peninsula (Moreno, 1997). Habitats occupied by this
124 butterfly are highly important for conservation, for they are also rich in many other
125 species of concern such as orchids, aromatic herbs, reptiles, bees, snails and especially
126 many narrow endemics. In fact, many of these habitats in Europe fall within the Natura
127 2000 network and the butterfly has been regularly proposed as their flagship and
128 umbrella- species (TARRIER & Delacre, 2008; Marabuto, 2009). *E. tagis* is vulnerable or
129 threatened in central France and Italy because of naturally narrow range (Lafranchis,
130 2000; Casini, 2001), but is endangered in Morocco due to overgrazing (Tennent, 1996),
131 particularly its population in the Middle Atlas, *E. t. atlasica*, which may have already
132 disappeared (MÉRIT & MÉRIT, 2006; TARRIER & Delacre, 2008). In Iberia and France,
133 recurrent habitat loss over human activities is suppressing population viability (Olivares
134 & Back, 2004; Opie/Proserpine, 2009) and its type locality in Portugal, is now lost to
135 urbanisation (Marabuto, 2009). Nevertheless, its current overall European conservation
136 status is still only of Least Concern (van Swaay *et al.*, 2010).

137 The importance of studying *E. tagis* goes beyond its ecological valence,
138 population differentiation, and conservation. Its systematic placement amongst its close
139 relatives has been controversial since a preliminary molecular work using a 326 bp
140 fragment of Cytochrome Oxidase Subunit I (COI) placed *E. tagis* (and *E. pechi*) in their
141 own genus, *Iberochloe*, suggesting a closer relationship to *Anthocharis* than to *Euchloe*
142 s.s. and *Elphinstonia*, whom they had always been associated with (Back *et al.* 2008).

143 The species was never included again in major studies dealing with Pieridae or the
144 Pierinae phylogeny using more comprehensive molecular and/ or morphology data
145 (Braby *et al.* 2006; Wahlberg *et al.* 2014, Cao *et al.* 2016; Ding & Zhang, 2016).
146 Therefore, the biological context in which this species has evolved, and the
147 interpretation of its still poorly known phylogenetic placement makes this a cornerstone
148 species in the understanding of the evolution of the whole tribe and of the
149 differentiation of Mediterranean butterflies.

150 In this work, we use a comprehensive molecular approach, set at deciphering
151 how a) *E. tagis* is framed among its closest relatives, b) how its populations are
152 structured across the geographical space and c) how this relates to current taxonomy.
153 We document patterns of diversity and how and when were these affected by major
154 climatic or geological events. In summary, we aim at understanding how different
155 barriers affect gene-flow among major refugial areas within the West-Mediterranean
156 and the legacy of the different phenomena in the genetic makeup of a butterfly, which is
157 a potential representative of a still poorly studied community.

158

159 **Material and Methods**

160 Sampling and data collection

161 The dataset encompasses 162 newly field-collected specimens, belonging to 19
162 populations and two publicly available sequences of *Euchloe tagis*, totalling 164
163 samples. Field work covered Portugal, Spain, France, Italy and Morocco, including
164 eight of the eleven known taxa within *E.tagis* (Fig. 1). COI sequences of *E. t. atlasica*
165 Rungs, 1950 and *E. t. pechi* (Staudinger, 1885) were obtained from the Barcode of Life
166 Initiative (www.boldsystems.org, Hebert & Ratnasingham, 2007). Other 14 sampled
167 Anthocharidini taxa analysed are: *Zegris pyrothoe* (Eversmann, 1832) & *Z. eupheme*

168 (Esper, 1804); *Anthocharis cardamines* (Linnaeus 1758), *A. belia* (Linnaeus 1767) & *A.*
169 *euphenoides* Staudinger 1869); *Elphnstonia charlonia* (Donzel 1842) & *E. bazae*
170 (Fabiano, 1993), and four *Euchloe* s.s., belonging to four of the six known clades
171 identified in Back *et al.* (2008): *Euchloe belemia* (Esper 1800), *E. creusa* (Doubleday,
172 1847), *E. crameri* Butler 1869 and *E. ausonides* (Lucas, 1852). Finally, the
173 Dismorphinae *Leptidea sinapis* (Linnaeus 1758) and Pierini *Pieris rapae* (Linnaeus
174 1758), were mined from GenBank and used as outgroups. All voucher details,
175 GenBank/ BOLD accession numbers and obtained sequences are listed in
176 Supplementary Table 1.

177

178 DNA extraction and sequencing

179 DNA extraction was performed on two legs of dried or fresh specimens using
180 E.Z.N.A. Tissue DNA kit (Omega bio-tek), following the manufacturer protocols and
181 stored at -20°C. For all individuals, the primer pair LepF/ LepR (Hajibabaei *et al.*, 2005)
182 was used to amplify the 5' fragment of the Cytochrome Oxidase Subunit I (COI)
183 mitochondrial gene, ideally spanning 658 bp. PCR cycling profile for this gene was: 94°
184 C for 1 min, 5 x [94° C/ 30 s, 45° C/ 60 s, 72° C/ 60 s] followed by 30x [94° C/ 60 s, 50°
185 C / 90 s and 72° C / 60 s of extension. These two cycles were finalised by a 5 min
186 extension period at 72° C. 19 samples were also sequenced for nuclear gene EF-1 α
187 using primer pair ef44/ ELF1R (Kim *et al.*, 2010), ideally yielding a fragment of 569 bp.
188 PCR reaction took place according to the following protocol: 94°C / 7 min, 35x [94° C/
189 20 s, 56° C/ 30 s, 72° C/ 40 s] with 72°C/ 7 min extension. Unlike the COI strategy,
190 where every sample was sequenced, individuals to be analysed for nuclear gene EF-1 α
191 were limited to individuals belonging to the most dissimilar COI haplogroups, as long
192 as variation was found. PCR fragments were sequenced using an ABI 310 Automated
193 Sequencer and BigDye terminator sequencing kit (Applied Biosystems).

194

195 Molecular analyses

196 Sequences were edited and aligned using SEQUENCHER v. 4.05 (Gene Codes
197 Corporation) and BIOEDIT v. 7.0.9 (Hall, 1999). We used CONCATENATOR v. 1.1.0
198 (Pina-Martins & Paulo, 2008) for file format conversions and gene concatenation.
199 Haplotype and nucleotide diversity was scored using DNASP v.5.10 (Librado & Rozas,
200 2009) and average pairwise distances calculated in MEGA v.7.0 (Kumar *et al.* 2016).
201 Haplotypes and Median Joining network for COI were recovered using NETWORK
202 (Bandelt *et al.*, 1999).

203 Phylogenetic trees were calculated using a concatenated dataset of (COI+EF-1 α):
204 maximum likelihood (ML) was implemented in RAXML (Stamatakis, 2006) with
205 RAXMLGUI v. 1.3 (Silvestro & Michalak, 2012) according to the ML + thorough
206 bootstrap analysis option and Bayesian Inference analysis (BI) in MRBAYES v. 3.1.2
207 (Ronquist & Huelsenbeck, 2003). After the Bayesian Information Criterion (BIC) in
208 JMODELTEST v. 2.1.7 (Darriba *et al.* 2012) elected GTR +G as the best evolutionary
209 model for each marker, we concatenated datasets (COI+EF-1 α) which were then
210 analysed together. Protocol in MRBAYES included running two *Metropolis Coupled*
211 *Markov Chain Monte Carlo* (MCMCMC) runs with 6M generations and four
212 independent chains (one cold, three hot), sampled every 10k iterations. Convergence
213 was assumed when the average standard deviation of split frequencies fell below 0.01.
214 A majority rule consensus tree was built after a burnin of 20%. Clade support was
215 confirmed by bootstrap analyses (ML) after 1000 pseudo replicates and posterior
216 probability values (BI) after stationarity, checked in TRACER v. 1.7.1 (Rambaut *et al.*
217 2018).

218

219 Divergence time estimates, demography and gene flow

220 We used BEAST v. 1.7.4 (Drummond *et al.*, 2012) to estimate times to most
221 recent common ancestor (TMRCA) for each major node. The analysis was performed
222 using a 23 sequence matrix which includes all outgroups and *E. tagis* clade
223 representative haplotypes. Unavailability of external calibration points, either as
224 biogeographical events or fossils led us to apply a relaxed molecular clock using 1.5% /
225 Ma uncorrected pairwise distance arthropod substitution rate (Quek *et al.*, 2004) for
226 COI and 0.1277% / Ma for EF-1 α , which had been calibrated using the Pieridae fossil
227 record by Braby *et al.* (2006). We used a Speciation: birth-death process tree prior
228 (Gernhard, 2008) and two Markov chains Monte Carlo (MCMC) of 30M generations,
229 sampled at every 3K iterations, conducted and combined with LOGCOMBINER v.
230 1.7.4 (Drummond *et al.*, 2012). TRACER v. 1.7.1 was here used to assess if effective
231 sample sizes (ESS) for all estimated parameters had gone >200 and to decide the burn-
232 in for tree construction. TREEANNOTATOR v. 1.8.0 (Drummond *et al.*, 2012)
233 produced maximum clade credibility trees and the 95% probability density of ages for
234 all nodes. The final Bayesian chronogram shows nodes with 95% confidence bars of
235 lineage differentiation.

236 Analyses of genetic divergence and gene flow among populations were carried
237 out by first implementing a spatial analysis of molecular variance (SAMOVA v. 2.0,
238 Dupanloup *et al.* 2002), followed by analysis of molecular variance (AMOVA) in
239 ARLEQUIN v. 3.5.1.3 (Excoffier & Lischer, 2010). SAMOVA is used to identify
240 maximally genetically diverged (groups of) populations, through the maximization of
241 the fixation index index ϕ_{CT} . This often leads to the identification of barriers to gene-
242 flow within a dataset, and that was a major intention. AMOVA, using the previous
243 SAMOVA-identified k groups, calculates these indices of genetic differentiation, in “k”
244 runs. Pairwise ϕ statistics were calculated and plotted in order to choose the best
245 population arrangements as in Vila *et al.* (2010). Three different descriptors were used:

246 ϕ_{ST} , ϕ_{CT} and ϕ_{SC} , following several combinations possible from the current
247 fragmented distribution of the species.

248 Tajima's D (Tajima, 1989), Fu's F (Fu, 1997) tests were performed using
249 ARLEQUIN to examine the population demographic history of Iberian *E. tagis*
250 populations. In this way, significant negative values of Tajima's D and Fu's F-statistics
251 are considered to be evidence of expanding populations (Aris-Brosou & Excoffier, 1996;
252 Mousset *et al.*, 2004). Mismatch distribution was employed to detect signatures of
253 population expansion through the frequency distribution of the number of pairwise
254 differences between haplotypes taking into account the Sudden Expansion Model
255 (Rogers & Harpending, 1992) to detect and estimate the time of population growth.
256 Values of estimated expansion provided by ARLEQUIN fed graphics of frequency
257 distribution using DNASP.

258

259 **Results**

260

261 From 164 *Euchloe tagis* specimens and 12 outgroup Anthocharidini, we
262 obtained fragments belonging to COI and EF-1 α genes. The final alignment uses 625 bp
263 of COI for all samples and 581 bp of EF-1 α for 19 specimens in all outgroup species
264 and major *E. tagis* COI lineage representatives. COI diversity was very high in *E. tagis*,
265 with 52 haplotypes recovered (21 containing a single specimen). Sequences are A-T
266 rich, and no gaps or stop codons were detected, suggesting fully functional mtDNA
267 copies and not pseudogenes, *Wolbachia* or numts. Haplotype major clade geographic
268 distribution across the sampled populations is provided in Table 1 and Figure 1. While
269 EF1- α was effective distinguishing all taxa at species-level, effectively resolving
270 interspecific phylogenetic relationships, it was unable to discriminate lineages within *E.*
271 *tagis*, with only two, non geographically structured haplotypes differentiated by one

272 SNP. Therefore only seven *E. tagis* individuals were sequenced for EF1- α and used in
273 tree-building.

274

275 Phylogenetics and divergence-time estimates

276 Anthocharidini

277 COI and EF-1 α datasets were concatenated, producing a 1206 bp long alignment.
278 ML and BI analyses broadly agree on topology, including all *E. tagis* populations into a
279 fully supported monophyletic clade among the Palaearctic Anthocharidini genera, which
280 clusters into five clades, corresponding to genera *Zegris*, *Anthocharis*, *Elphinstonia*,
281 *Euchloe* s.s. and *Euchloe tagis* (Figure 2). Still, relationships among these are not well
282 resolved except that of between *Zegris* and *Anthocharis*, which consistently score as
283 sister genera. *E. tagis* stands in an isolated position from the remaining taxa.

284 Bayesian analysis of divergence times implemented in BEAST (Figure 5 Figure
285 2a's topology. It places the most recent common ancestor (MRCA) of all genera of
286 Holarctic Anthocharidini back at more than 22 Mya (node A) and of the highly
287 supported *Anthocharis* + *Zegris* clade at 17 Ma (node D). All the remaining *Euchloe*,
288 *Elphinstonia* and *E. tagis* share a common ancestor 19.3 Mya (node B), soon before *E.*
289 *tagis* split from *Elphinstonia* (17.1 Mya, C) and long before *Euchloe belemia* split from
290 the remaining studied *Euchloe* (13 Mya, E). The MRCA of *Zegris* at 12.2 Ma may
291 represent the true coalescence of all extant members in this genus, as the only other
292 species, *Z. fausti* Christoph, 1877 is sister to *Z. eupheme* (Back 2012). Such is probably
293 not the case with the 6.4 Ma of *Anthocharis* (I), which includes more species in Asia
294 and North America. The narrow Spanish endemic *Elphinstonia bazae* and North
295 African *E. charlonia* converge in node J with an age of 5.7 Ma, which is within
296 approximately the same divergence time-frame of other north African / European pairs:
297 *Anthocharis belia*/ *A. euphenoides* and *E. tagis* clades T1+T2/ T3-9 would have
298 diverged (nodes K and L: 4.8-4.6 Ma).

299

300

301 *Euchloe tagis*

302 Within the *E. tagis* clade, both the phylogenetic tree and the haplotype network
303 methods (Fig. 3) aggregate intra-specific diversity into eight / nine distinct and
304 moderately supported clades or haplogroups which originate from a common central
305 node and are geographically segregated. T1 and T2 comprise singletons from eastern
306 Algeria and the Middle Atlas of Morocco, respectively and form a monophylum yet
307 they are distant by 11 COI SNPs. This haplogroup is differentiated from the remaining
308 *E. tagis* stock by a minimum of 15 mutation steps.

309 Clades T3-T9 are consistently differentiated geographically and genetically from
310 each other, but their strict pairwise relationship is poorly supported, except that of
311 between sister T8 and T9. T3 appears to be the first one splitting, encompassing four
312 poorly differentiated haplotypes from Tuscany, corresponding to *E. t. calvensis* (Casini
313 1993, 1996). Also from Italy, clades T4 (Italian Alps, *E. t. piemonti*) and T5 (Apuan
314 Alps) have an unresolved relationship. The single haplotype in Clade T6 was recovered
315 from the Rif of Morocco, corresponding to *E. t. reisseri* (Back & Reissinger 1989) and
316 diverges from T7 (French populations, *E. t. bellezina*) by five mutational steps. Only the
317 COI substitution in position 375 is shared between T7 and geographically closest T3
318 and T4. At the crown of the phylogeny, clades T8 and T9 are reciprocally monophyletic
319 and endemic to Iberia (Figure 1), the most diverse. T8 has a ‘star-like’ pattern with a
320 most widespread, common haplotype (H8) and 11 lower derived ones. This clade is
321 widespread in the Iberian south and east but is replaced in the west and north by T9
322 which spans 28 haplotypes in a more diffuse star-pattern, where some interior
323 haplotypes show increased geographical spread and frequency (e.g. H24, H30).

324 All currently known *E. tagis* lineages, from Algeria to Italy, coalesce at MSC
325 age 4.58 Mya (node L, 95% HPD: 2.57- 7.75). An initial split between an African

326 (T1+T2) and a fundamentally European group (T3-9) is verified in node L. Earliest
 327 European population splits (Italian clades T3, T4) are contemporaneous with that of
 328 between T1 and T2 in the Maghreb (nodes O, N: 1.9- 2.1 Mya). Divergence of Iberian
 329 clades T8 and T9 and that of T5, T6 and T7 (Apuan Alps, France and Rif) are more
 330 recent, at the 0.7-0.9 Mya interval (Figure 5).

331

332 Measures of genetic diversity

333 Measures of genetic diversity (Table 1) indicate that among the well sampled
 334 populations, those of southern Portugal (Algarve, Milfontes) and the Rif of Morocco are
 335 the least diverse, with one haplotype each. Madrid province (ES) and Arrábida (PT) are
 336 at the opposite end of the spectrum (11 and 10, haplotypes respectively). There is a clear
 337 geographic haplogroup segregation: marginal populations in France, Italy and Morocco
 338 have private haplogroups and in Iberia, clades T8 and T9 sum 40 haplotypes. In Iberia,
 339 H08 and H24 are widespread but the majority are however of narrow distribution (e. g.
 340 H12, H22 and H23 in Adiça and H09 from Gibraltar).

341 Iberia harbours the greatest haplotype diversity ($h=0.9395$), where Madrid and
 342 Burgos populations score the highest values ($h=0.6$) whereas nucleotide diversity (π), is
 343 elevated in Morocco because of the presence of two very divergent lineages
 344 ($\pi=0.02003$). *E. t. castellana* (central and northeastern Iberia) and *E. t. alhajarae* (Sierra
 345 Morena range s), are the most diverse infraspecific taxa, in both haplotype and
 346 nucleotide diversity.

347 Anthocharidini uncorrected pairwise COI genetic distances (Supplementary
 348 Table S2) range in the 7.8% between *Zegris* species; an average of 4.7% among the
 349 *Anthocharis*; 6.75% among *Euchloe* s.s. and 4.96% between *E. bazae* and *E. charlonia*.
 350 In *E. tagis*, variation ranges between 0.213% in France to 0.646% among the 28 Iberian
 351 haplotypes in clade T9. but is greatest between western Iberia and Algeria (T1 vs T9:
 352 3.691%), and 2.8-3.6% between T1+T2 (1.76% divergent) and all other clades.

353 The Elongation factor 1- α gene fragment successfully discriminates all genera,
354 species-groups and pairs of closely related species, but has no intraspecific structure in
355 *E. tagis*, even from different continents. Genetic distance between *E. tagis* and other
356 recognised genera goes from 3.41% (vs *Euchloe* s. s.) to 5.04% vs *Anthocharis*. While
357 the most distant taxa are *Elphinstonia* and *Zegrís*, at 6.17% (Supplementary Table S3).

358 SAMOVA analysis performed with $k=2-20$, yield a best estimate of population
359 structure when $k=13$ or $k=14$ (Fig. 4). With these values, the difference between F_{CT} and
360 F_{SC} is maximal before the latter reaches a negative value. SAMOVA's optimal $k=14$
361 'populations' is achieved by clustering Gibraltar+Baza, Algarve+Milfontes,
362 Arrábida+Candeeiros, and Estremoz+Ronda+Granada+Madrid+Burgos. For $k=13$,
363 Monegros is clustered with Arrábida+Candeeiros. These results are largely at par with
364 previous analyses using other methods, namely the progressive exclusion of North
365 Africa first, then Italian and only then all other populations. AMOVA results are
366 summarized in Table 2.

367 Mismatch distribution analyses, and Fu's F statistics yield largely concordant
368 information evidencing past demographic or spatial expansions in clades T8 and T9:
369 Occurring earlier and involving a larger population effective in the latter than the former.
370 Conversely, Tajima's D values were non-significant at $p=0.05$. (Table S4, Figure S1).

371

372 **Discussion**

373

374 Phylogenetic placement of *Euchloe tagis*

375 Molecular analyses deliver *Euchloe tagis* as one of five, well supported
376 monophyletic lineages of Palearctic Anthocharidini. However, low support values do
377 not resolve its sister group, either the remaining *Euchloe* or *Elphinstonia*. On the other
378 hand, *Zegrís* and *Anthocharis* are well supported sister genera under all methods of

379 analysis, which challenges some previous approaches on the Anthocharidini placing *E.*
380 *tagis* with *Anthocharis* (Back *et al.* 2008) or *Zegris* with *Euchloe* (Wiemers *et al.* 2020),
381 although these studies suffer from lack of gene representativeness in *Euchloe tagis*
382 (only COI was available) and even in various species of *Euchloe*, *Zegris* and
383 *Anthocharis*. However, the grouping of *Zegris* and *Anthocharis* is morphologically
384 supported in sharing a strong sexual dimorphism, characterised by erythropterin
385 deposition at the male forewing tip (Vukusic & Chittka, 2013), absent in *Euchloe* s.l.
386 and *Elphinstonia*. High sequence divergence between *E. tagis* and *Euchloe* s. s. and
387 apparent clustering with *Anthocharis* was the main reason invoked by Back *et al.* (2008),
388 for the creation of genus *Iberochloe*. This study took deep taxonomic considerations
389 from only 326 bp. of the hyper-variable COI, which are unsupported by current analyses.
390 These authors further support such arrangement on early instar morphology and
391 univoltine cycle but all these characters better reflect a phylogenetic continuum instead
392 of a cluster of exclusive traits particular to *E. tagis* (Opler 1968; Opler 1974).

393 For now, even if our analysis fails to deliver clade sistership to *E. tagis*, it
394 provides a step forward in improving on the analyses of both Back *et al.* (2008)
395 (molecular coverage and data analyses) and Wiemers *et al.* (2020) (clade segregation,
396 gene coverage of key species, exclusion of *Zegris* and *Anthocharis*). A conservative
397 approach recognising this phylogenetic continuum uniting *Euchloe* s.s., *Elphinstonia*
398 and the *E. tagis* (*Iberochloe*) and yet acknowledging the elevated genetic divergence to
399 other Anthocharidini at >7.6% COI and >3.4% EF1- α , considers these units as
400 subgenera under a broad concept of *Euchloe*, a view already shared by Klots (1930,
401 1933) and Bridges (1988). Conversely, the same approach applied to the *Zegris* +
402 *Anthocharis* clade does not hold the same support, in spite of similar genetic distances,
403 as these genera differ by a number of morphological and ecological discrete differences
404 (Klots 1930, 1933, Back *et al.* 2012).

405

406 *Euchloe tagis* genetic diversity

407 *Euchloe tagis* sampling covered all major populations and most infraspecific
408 taxa, likely accurately representing its geographic and genetic diversity. *E. t. pechi* and
409 *E. t. atlasica*, suffer from political and conservation problems such as overgrazing, and
410 habitat loss and may already be extinct (TARRIER & DELACRE, 2008; TARRIER, 2011) .
411 Therefore, only the few publicly available COI sequences in GenBank/ BOLD and no
412 EF1- α fragment were used.

413 EF1- α is monomorphic in *E. tagis*, even among geographically distant
414 populations, but COI diversity is very informative. Altogether, 52 COI haplotypes
415 among 164 specimens make *E. tagis* one of the most diverse West-Mediterranean
416 Lepidoptera, only matched by the day-flying moth *Zygaena transalpina hippocrepidis*
417 (Von Reumont *et al.*, 2012: 46 haplotypes / 166 specimens). Other butterfly studies
418 based on the same marker but covering wider areas, such as the Western Palaearctic,
419 yield similar raw values: *Polyommatus icarus* (Talavera *et al.*, 2013: 44 haplotypes /
420 110 specimens); *Erebia triaria* (Vila *et al.*, 2005: 46 haplotypes / 126 specimens) and
421 *Parnassius mnemosyne* from Central and Eastern Europe (Gratton *et al.* 2008: 53
422 haplotypes / 225 specimens). A possible explanation lies in the long term survival of
423 small rather isolated populations with limited contact, preventing overlap and lineage
424 substitution.

425

426

427 Phylogeography

428 The Europe - Africa divide.

429 SAMOVA and phylogenetic analyses indicate the most significant splits among
430 *E. tagis* lineages to have occurred between southern Maghreb T1+T2 and the remaining

431 (T3-T9) clades. More detailed analyses reveal populations outside Iberia to be highly
 432 diverged but genetically impoverished (Table 1). Molecular-clock estimates (Fig 5)
 433 suggest an initial post-MSC split into a north African and a European population ~5
 434 Mya (Fig. 4, node L). The species further diversified in the European continent during
 435 the Pleistocene, and recolonised extreme northern Africa into the Rif Mountains, much
 436 more recently (node Q, <1 Mya). Other studied pairs of putative sister taxa occurring on
 437 both sides of the Gibraltar Strait agree with a Messinian-age vicariant event (6-4 Ma):
 438 *Anthocharis euphenoides* & *A. belia* (node K) and *Elphinstonia bazae* & *E. charlonia*
 439 (node J), likely the end of the MSC triggering isolation between these populations. Other
 440 butterflies conform to the same pattern like *Pararge aegeria* (Weingartner *et al.*, 2006),
 441 *Melanargia occitanica* & *M. ines* (Nazari *et al.*, 2009), and *Zerynthia rumina* (Nazari &
 442 Sperling, 2007). However, none of these cases suggest a recolonisation of northern
 443 Africa from Europe.

444 Divergence-time estimates and genetic distance among *E. tagis* populations
 445 suggest a strong support for a North African clade uniting *E. pechi* (T1) and *E. t.*
 446 *atlasica* (T2), (Figures 2, 3, 5), which would render *E. tagis* paraphyletic according to
 447 previous taxonomic assumptions if *E. pechi* was to be considered a species (Back *et al.*
 448 2008). At nearly opposite sides of the Maghreb, T1 and T2 are separated by more than
 449 600 km (Fig 1), their divergence of 1.76% translates into a MRCA circa 1.93 Mya ago
 450 (Figure 4: node O), between the onset of the first Pleistocene glaciations (Head &
 451 Gibbard, 2010), and the Tiglian interglacial (Pross & Klotz, 2002), when a drought
 452 period impacted the south Mediterranean (Hughes *et al.*, 2011). Such North African
 453 population disruption may have occurred after isolation by distance and along the
 454 biogeographic barrier imposed by the Moulouya drainage in eastern Morocco, which
 455 affected many different taxa (Álvarez *et al.*, 2000; Paulo *et al.*, 2008; Linares, 2011). *E.*
 456 *t. atlasica* (T2) in the Middle Atlas is morphologically closer to western Iberia
 457 populations but has never been considered alongside *E. t. pechi*, as different from *E.*

458 *tagis*. Considering all Maghrebian and European populations as conspecific is the only
459 way of retaining *E. tagis* as a monophyletic species, supported by shared life-history,
460 larval and pupal morphology, ecology, and a variable yet characteristic wing-pattern, as
461 well as successful common-garden crossing of European and Algerian taxa (Back,
462 1984).

463

464

465 Into Italy, across the Alps

466 In Europe, our results strongly support an early clade divergence of T3 and T4 at
467 2.1 Ma (Figure 4, node N, Figure 5), east of the Alps. These haplogroups may not be
468 reciprocally-monophyletic, yet their mutual divergence (0.8-1.4%) in COI is lower than
469 the 1.60-1.92% relative to other European lineages. Among Italian populations, Clade
470 T3 corresponding to *E. t. calvensis* the '*colline metallifere*' of Tuscany. It is replaced
471 by T5 in the Apuan Alps and T4 (*E. t. piemonti*) in the Maritime Alps. Italian *E. tagis*
472 populations are notable for their particularly fragmented distribution over limestone or
473 serpentine outcrops (Casini, 2001). A hypothesis for their origin and structure lies in
474 their long-term Pleistocene isolation, when cooling of the peri-Alpine area, and periodic
475 retreat into topographically suitable refugia kept them systematically isolated from
476 those west of the Alps. *E. t. calvensis* as the furthest away from the Alps south of river
477 Arno, became the best defined both morphologically (Casini, 2001) and genetically (this
478 work). To the north, Clades T4 & T5 appear less diverged from the remaining European
479 populations (Figure 4).

480 Italian populations, while likely survived in the region for long, may never have
481 experienced large population sizes due to an ever contracting and expanding Alpine
482 ecoregion. Showing little, if any gene-flow between habitat-islands, they support the
483 existence of refugia outside Mediterranean peninsulas, particularly relevant near
484 topographically complex areas such as the Alps (Schönswetter *et al.* 2005; Schmitt &

485 Varga, 2012; Kuhne *et al.* 2017). Furthermore, the availability of both geologically
486 suitable geology and foodplant presence further south in the Italian Peninsula (Pignati,
487 1982), but not the butterfly, suggests this species never expanded further into the Italian
488 mainland, unlike its Atlanto-Mediterranean relative *Anthocharis euphenoides*, which
489 reached into Calabria (Bollino *et al.*, 1996). These two species thus likely followed a
490 dispersal route from the west rather than from the south, unlike *Melanargia* butterflies
491 (Habel *et al.*, 2008, 2010).

492

493

494 France and the Rif

495 French *E. t. bellezina*, is represented in the analysis by T7, comprising three
496 poorly differentiated haplotypes. COI genetic distance to nearby Italian populations is
497 higher than expected (Table S2), given their phenotype similarity. Instead, T7 clusters
498 with T6 from Morocco, diverging by 0.91% (whilst >0.96% to other clades, Table S2),
499 corresponding to a MRCA 0.25 Mya (Fig. 5). Recent connection between T6 and T7 is
500 difficult to support because of extreme geographical disconnection and contrasting
501 wing-pattern differences but genetically, T6 and T7 are less differentiated than Iberian
502 clades T8 and T9 (1.42%, Table S2) are to each other. Homoplasmy may be invoked for
503 this connection, but also the former existence of a common ancestor population in the
504 south and east of Iberia, breaking up (by vicariance or dispersal) into current positions.
505 While it involves the potential extinction (or no sampling) of this putative ancestral
506 lineage in Iberia during the last 0.71 Ky and its generalised replacement by the current
507 Iberian lineages it would explain the relatively low genetic diversity of SE Iberian
508 populations: modern replacements belonging to T8 and T9. French and Rif populations,
509 then isolated from Iberia across the Pyrenees and Gibraltar strait were conversely not
510 replaced by other Iberian lineages. COI mtDNA data favours this chain of events at the

511 expense of homoplasy. Over time and late Pleistocene glacial cycles, both populations
512 evolved in very distinct refugia, in extreme southeast France and the Rif mountains.

513 Cast-away from Iberia, both populations were met with founder-effect, genetic
514 erosion and demographic constraints leading to startlingly different phenotypes. In
515 France the existence of a Provençal refuge is widely recognised (Magri, 2008;
516 Svenning *et al.*, 2008; Schmitt & Varga, 2012), and the resilience of a suitable biotope
517 through glacial cycles is supported by the local occurrence of other Atlanto-
518 Mediterranean butterflies: *Melanargia occitanica* (Esper, 1793), *Tomares ballus*
519 (Fabricius 1787), *Zerynthia rumina* (Linnaeus, 1758), *Anthocharis euphenoides*
520 Staudinger 1869, *Glaucopsyche melanops* (Boisduval 1829) and *Muschampia baetica*
521 (Rambur, 1839) (Opie/Proserpine, 2009). The potential habitable area of this refuge is
522 however much wider than in the Rif, especially during interglacials allowing for larger
523 effective population sizes and current higher genetic diversity in *E. tagis*. On the other
524 hand, the Rif mountain chain despite being buffered from climate extremes (Gasse &
525 Fontes, 1992) and successfully acting as both glacial and interglacial refugium for
526 different species (Linares, 2011; Husemann *et al.*, 2014), has a potentially much smaller
527 range for the species, explaining the evolution and apparently low mtDNA diversity of
528 *E. t. reisseri*. Finally, the adaptation and restriction of T6 to these cold Rif mountain
529 summits, in the extreme northern Africa, and nearest to the European continent suggests
530 a relatively recent (glacial) dispersal event from Europe (Figure 5, node Q).

531

532 Iberian Peninsula melting pot

533 Iberia is the only territory where two different haplogroups occur and where *E.*
534 *tagis* presents the greatest genetic diversity. T8 and T9 differ on average by 1.42%
535 (Table S2, supplementary information) and share a great overlapping area. The
536 existence of two Iberian lineages was first suggested by Back *et al* (2008) and Dinca *et*
537 *al* (2017) but not their sympatry. According to the molecular clock and elevated

538 nucleotide diversity (π), all extant Iberian diversity is of local long - lasting presence,
539 coalescing to circa 0.7 M y a, but appears not to have been greatly eroded, as
540 demonstrated by the existence of many private haplotypes (Table 1).

541 A partial overlap in the distribution of the two mtDNA lineages is a novel find,
542 that masks the straightforward attribution of populations to putative allopatric glacial
543 refugia. Nevertheless, the predominance of T9 in the extreme western populations,
544 while T8 is more diverse in southern Iberia, may be interpreted in two ways: 1) Initial
545 glacial allopatric population isolation, followed by expansion and colonisation of
546 suitable areas, fixing one of the mtDNA forms in each geographical area or 2) Clade
547 evolution in sympatry or peripatry, followed by local clade replacement due to genetic
548 drift under climatic extremes.

549 Assuming niche conservatism, Iberian refugial areas for *E. tagis* would have to
550 be hold a Mediterranean climate, high-pH soils and foodplant. Using an elevated
551 richness in the foodplant genus *Iberis* (Brassicaceae), which is most diverse in Iberia
552 (Moreno, 1997), as an acceptable proxy for long-term habitat suitability (Carrión *et al.*
553 2008) alongside the distribution of private and the presumably more ancestral (internal)
554 haplotypes in the network (Fig. 3), possible suitable areas throughout the Pleistocene
555 may be identified in the southeast (Betic cordillera, Tertiary eastern Meseta), in
556 southwestern Portugal and northern Andalucía in edapho-xerophilous outcrops, and even
557 areas subject to a Föhn effect (Ambert, 1984).

558 Clade structure in T8 indicates differentiated evolutionary history respective to
559 T9. T8 variation revolves around a central, most common and widespread haplotype H8.
560 This clade is spread throughout inland Iberia, but haplotype diversity is highest in the
561 south, especially around Sierra Morena ranges (Aracena, Adiça, Estremoz), and Madrid
562 The former area could have acted as a centre of divergence within a putative refugium
563 located in southern Iberia, the western part of the Betic range and Gibraltar or just
564 western Sierra Morena. Clade T8 is mostly associated with taxa *E. t. alhajarae* and *E. t.*

565 *granadensis* but accounts for nearly a third of the samples of *E. t. castilliana*.
566 Demographically, its simple structure indicates a fairly recent expansion (Figure S1).

567 T9 is more complex and diverse, showing several high frequency haplotypes
568 (Figure 5), which denotes the existence of a less centralised occurrence. Diversity,
569 current distribution and demographic analyses (Table S4, Figure S1) indicate always
570 wider population numbers, translating into several potential refugia in southern
571 (Estremoz, Adiça) or north-eastern Iberia (Burgos, Monegros), due to the presence of
572 both derived (H19-23) and basal haplotypes (H17, H18) in both areas. Some diverse
573 populations composed of mainly crown-haplotypes (Arrábida and Madrid) are likely the
574 result of relatively recent range expansions. Unlike T8, we dismiss a role of extreme
575 southern Iberia in the evolution of this clade as all southern specimens (Betics, Algarve)
576 belong to widespread H30 and H24. T9 encompasses all sampled populations of *E. t.*
577 *tagis* and a variable part of *E. t. castilliana*, *E. t. alhajarae* and *E. t. granadensis*.

578

579 **Conclusions**

580

581 The Portuguese Dappled white butterfly provides an example of an ecological
582 specialist surviving in a relatively restricted geographical area through extreme geologic
583 and climate changes, for an extended period of time. In this study, we show that it
584 represents the sole survivor of an early divergence within the Anthocharidini (23-12
585 Mya), yet current data was unable to resolve whether *Euchloe* s.s. or *Elphinstonia* is the
586 closest *E. tagis* relative. In view of this, as a taxonomic outcome of this study we
587 recommend to keep *Euchloe*, *Elphinstonia* and *Iberochloe* as subgenera of *Euchloe*.

588 With the available *E. tagis* molecular dataset we were able to infer many aspects
589 of its genetic structure, such as the relationship among populations and their pattern of
590 differentiation and hotspots of genetic richness. Furthermore we were able to estimate
591 times of divergence in Europe and Africa, or from Europe to Africa and back, and

592 allocate these divergences to particular geologic and climatic events in the West-
593 Mediterranean.

594 The main pattern of Messinian population divergence within our focal species
595 was further supported by the one found in other co-occurring Anthocharidini inhabiting
596 the same region. Therefore, an hypothesis of a biogeographic pattern arises, involving
597 an initial Africa vs. Europe split likely of MSC age, followed by Pleistocene
598 diversification and expansion. This expansion was then impacted by the gateway-effect
599 imposed by further barriers to gene-flow (i. e. mountains and drainages) at specific
600 times: glacials in the north or interglacials in the south, which also imposed erosion on
601 the generated diversity, especially in peripheral areas. This biogeographic pattern of
602 continuous adaptation to climate and habitat change for Mediterranean species is of
603 utter importance for understanding the partitioned West-Mediterranean refugium.

604 Finally, *E. tagis*' intricate mtDNA genetic structure and diversity highlight the
605 importance of studying poorly known Mediterranean species. It also emphasizes the
606 need to conserve even smaller populations, at the expense of losing important
607 evolutionary solutions for survival in a fast changing world.

608

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623

Table 1 - Measures of genetic diversity within *Euchloe tagis* associated with geographical origin or taxon. This comprises all 164 samples from the 17 populations under study, organised according to haplotypes. Columns correspond to populations studied / subspecific taxa (see table S1), the number of specimens and haplotypes scored and genetic diversity indices using COI. Under the Haplotype column, a haplotype in bold is private to the given population. Numbers and letters in the first column correspond to Fig. 1 coding.

Population / taxon	N	N Hap.	N private Hap.	Haplotypes (n)	Haplotype diversity (h)	Nucleotide diversity (π)
Iberia	127	40	40	H06 - H44, H51	0.9395 +/- 0.0100	0.00884 +/- 0.00028
1 Candeciros	8	5	2	H24(1), H30(3), H31(1) , H32(1) , H42(2)	0.8571 +/- 0.1083	0.00229 +/- 0.00177
2 Arrábida	16	10	4	H19(1), H25(1) , H26(1), H30(4), H33(3) , H34(2) , H37(1), H39(1) , H42(1), H51(1)	0.9167 +/- 0.0493	0.00331 +/- 0.00219
3 Milfontes	4	1	0	H24(4)	0.0000 +/- 0.0000	0
4 Algarve	4	1	0	H24(4)	0.0000 +/- 0.0000	0
5 Estremoz	20	8	2	H08(4), H10(1) , H16(1), H19(8), H20(1) , H21(1), H24(1), H37(3)	0.8053 +/- 0.0699	0.00731 +/- 0.00419
6 Adiça	13	4	3	H12(7) , H21(1), H22(4) , H23(1)	0.6538 +/- 0.1060	0.00870 +/- 0.00504
7 Aracena	9	5	3	H13(1), H14(2) , H15(3) , H16(1), H35(2)	0.8611 +/- 0.0872	0.00889 +/- 0.00535
8 Ronda	3	3	0	H08(1), H13(1), H30(1)	1.0000 +/- 0.2722	0.00960 +/- 0.00784
9 Granada	3	2	0	H08(1), H30(2)	0.6667 +/- 0.3143	0.00853 +/- 0.00704
10 Baza	8	3	1	H07(1) , H08(6), H30(1)	0.4643 +/- 0.2000	0.00354 +/- 0.00248
11 Gibraltar	6	2	1	H08(4), H09(2)	0.5333 +/- 0.1721	0.00085 +/- 0.00094
12 Madrid	18	11	7	H08(3), H11(3) , H13(1), H24(3), H28(1) , H29(1) , H30(1), H36(1) , H38(1) , H43(2) , H44(1)	0.9346 +/- 0.0352	0.00914 +/- 0.00514
13 Burgos	6	5	3	H06(1) , H17(1) , H24(2), H26(1), H41(1)	0.9333 +/- 0.1217	0.00533 +/- 0.00367
14 Monegros	9	6	2	H18(1), H24(3), H27(1), H30(2), H40(1), H41(1)	0.8889 +/- 0.0910	0.00364 +/- 0.00250
France	9	3	3	H01 to H03		
15 PACA/ Languedoc	9	3	3	H01(5) , H02(3) , H03(1)	0.6389 +/- 0.1258	0.00116 +/- 0.00107
Italy	24	6	6	H05, H46 to H50	0.6703 +/- 0.0870	0.00495 +/- 0.00297
16 Maritime Alps	3	1	1	H49(3)	0	0
17 Apuane Alps	1	1	1	H50(1)	1.0000 +/- 0.0000	0
18 Toscana	20	4	4	H05(13) , H46(5) , H47(1) , H48(1)	0.5368 +/- 0.1042	0.00150 +/- 0.00120
Maghreb	4	3	3	H04, H45, H52	0.6667 +/- 0.3140	0.02003 +/- 0.00944
19 Rif	2	1	1	H04(2)	0.0000 +/- 0.0000	0
20 Middle Atlas	1	1	1	H45(1)	1.0000 +/- 0.0000	0
21 Algeria	1	1	1	H52(1)	1.0000 +/- 0.0000	0
Subspecies						
A <i>E. t. pechi</i>	1	1	1		1.0000 +/- 0.0000	0
B <i>E. t. atlasica</i>	1	1	1		1.0000 +/- 0.0000	0
C <i>E. t. reisseri</i>	2	1	1		0	0
D <i>E. t. calvensis</i>	20	4	4		0.537 +/- 0.104	0.00150 +/- 0.00120
E <i>E. t. piemonti</i>	3	1	1		0	0
F <i>E. t. bellezina</i>	9	3	3		0.639 +/- 0.126	0.00121 +/- 0.00032
G <i>E. t. granadensis</i>	20	5	2		0.591 +/- 0.118	0.00440 +/- 0.00150
H <i>E. t. alhajarae</i>	42	15	10		0.918 +/- 0.021	0.00978 +/- 0.00049
I <i>E. t. castellana</i>	33	18	14		0.926 +/- 0.031	0.00774 +/- 0.00073
J <i>E. t. tagis</i>	32	13	7		0.871 +/- 0.039	0.00317 +/- 0.00205
Species	164	52	52		0.9541 +/- 0.0067	0.01179 +/- 0.00613

629 **Table 2** Analysis of molecular variance (AMOVA) in *Euchloe tagis* population clusters. Fixation indices
 630 and percentage variation among populations explained by these are presented. These obey criteria
 631 concerning grouping with SAMOVA (1-4), 5) main geographic units or 6) subspecific taxa grouping. For
 632 population assignme nt to subspecies refer to Supplementary Table 1. Optimal values associated with
 633 K=13/14 are enhanced in bold.

	Group	ϕ_{ST}	ϕ_{SC}	ϕ_{CT}	% var.
1	SAMOVA's K=2	0,86029	0,65416	0,59603	59.60
2	SAMOVA's K=5	0,79615	0,52333	0,57236	57.23
3	SAMOVA's K=13	0,63091	0,62308	0,02078	2.07
4	SAMOVA's K=14	0,62651	0,61938	0,01874	1.87
5	[Iberia] [North Africa] [France] [Italy]	0.72702	0.43365	0.51800	51.80
6	subspecies	0.62833	0.22383	0.52114	52.11

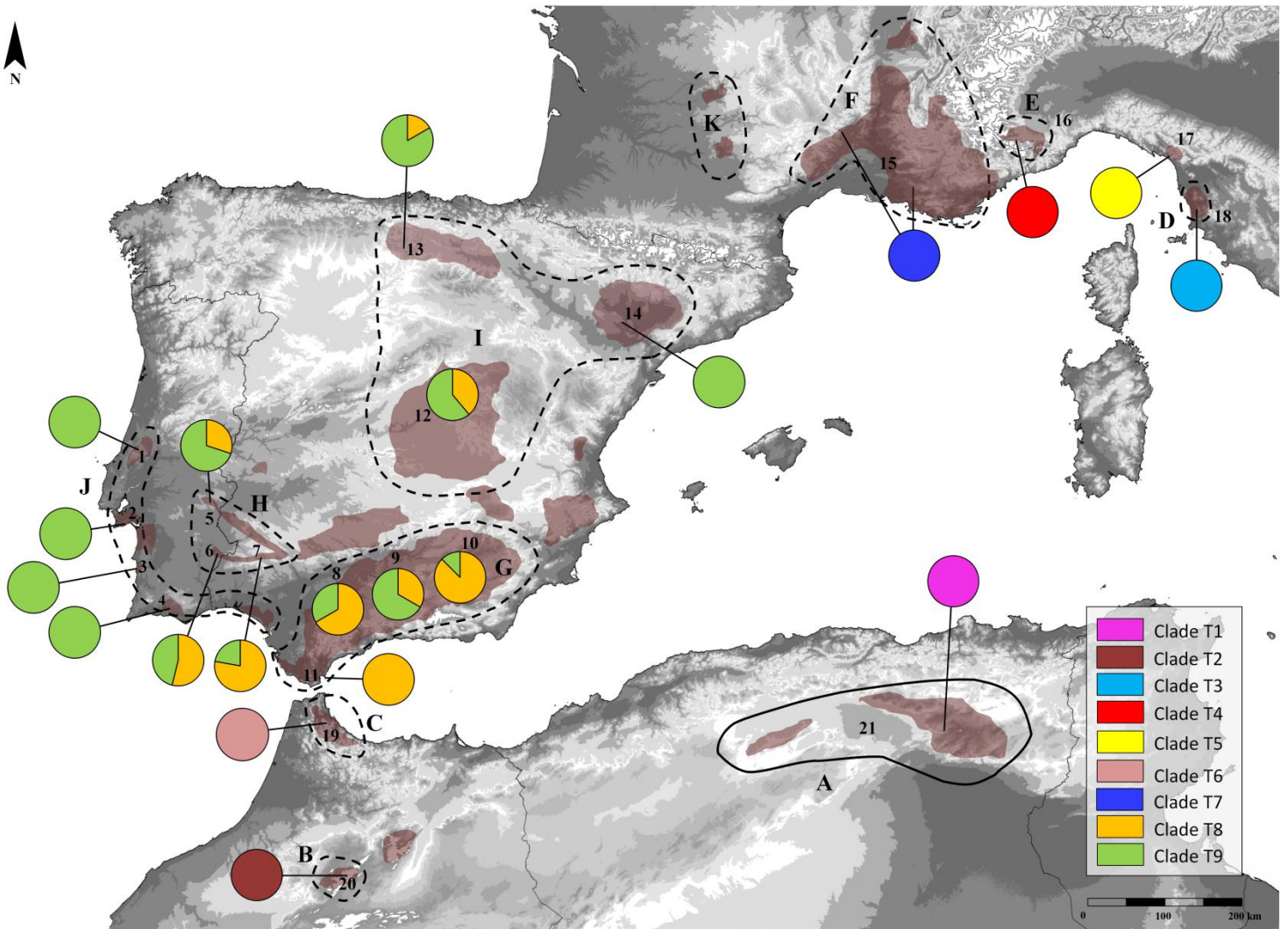
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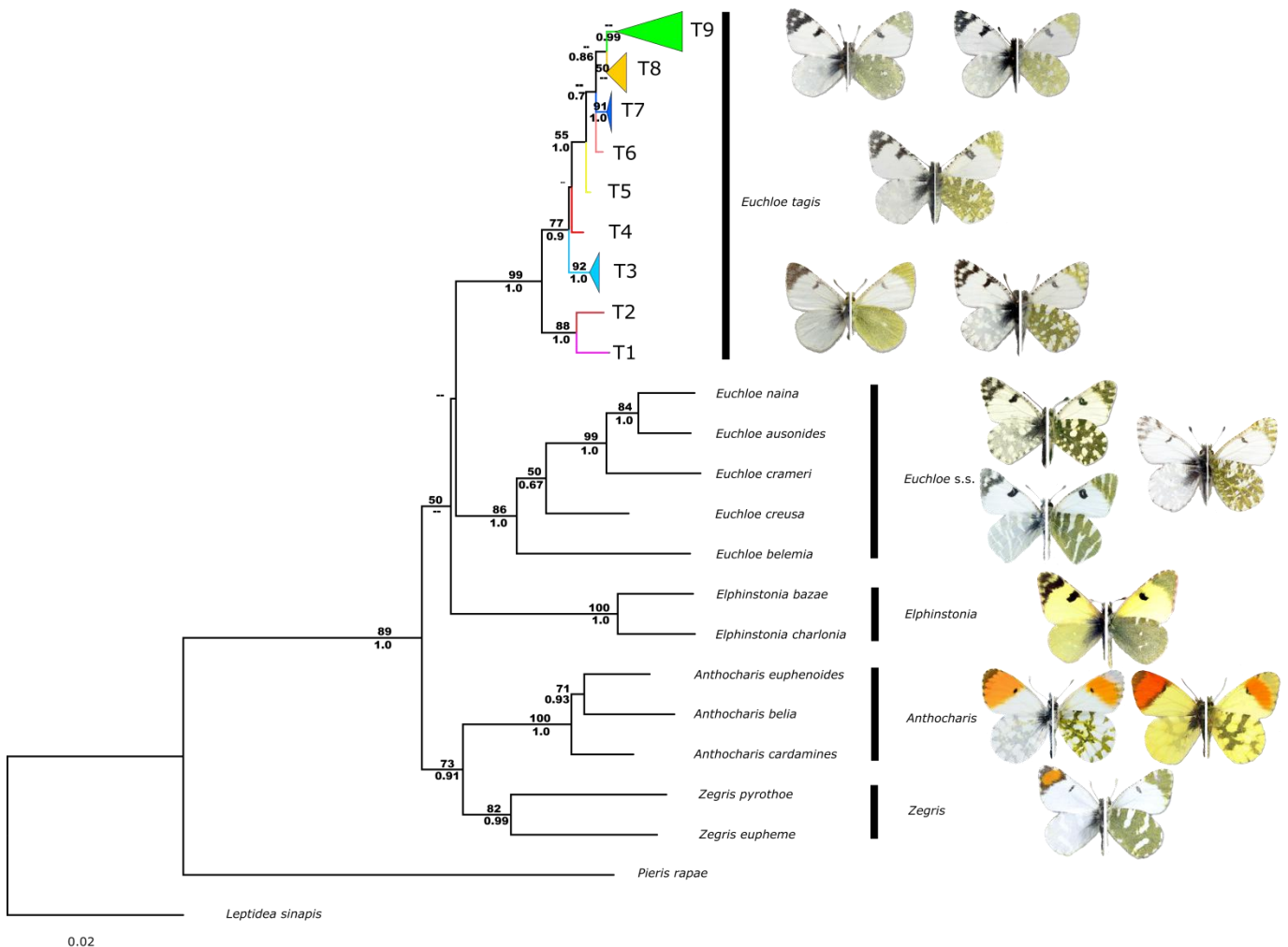
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641 **Figure 1** - Geographical distribution of *Euchloe tagis* butterfly and its subspecific taxa (letters A-K).

642 Genetic composition of a population is represented as the proportion of each major mtDNA genetic

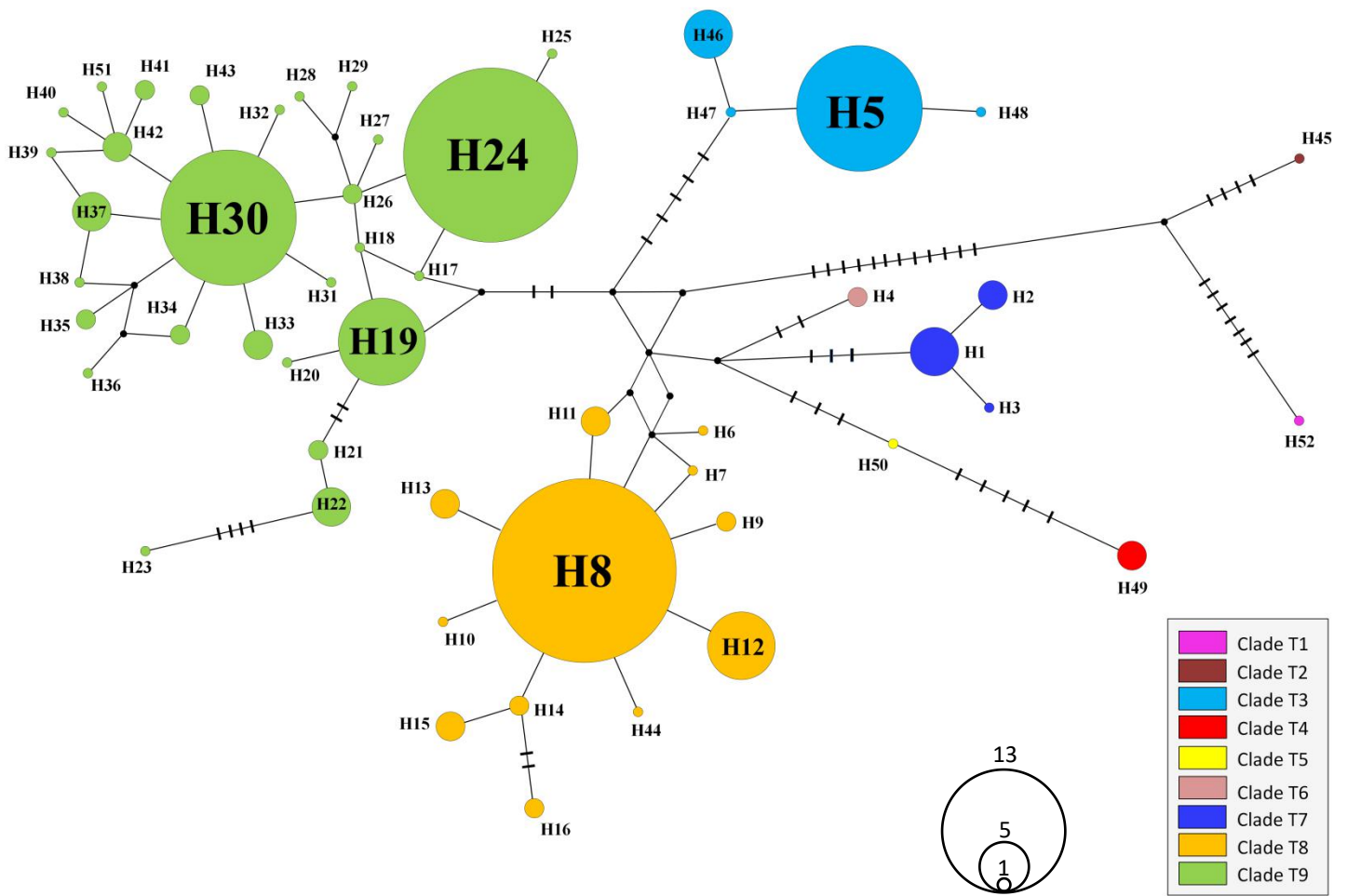
643 cluster. A- *E. t. pechi*; B- *E. t. atlasica*; C- *E. t. reisseri*; D- *E. t. calvensis*; E- *E. t. piemonti*; F- *E. t.*644 *bellezina*; G- *E. t. granadensis*; H- *E. t. alhajarae*; I- *E. t. castiliana*; J- *E. t. tagis*. *E. t. aveyronensis* from

645 France (K) is not represented in the genetic analysis.



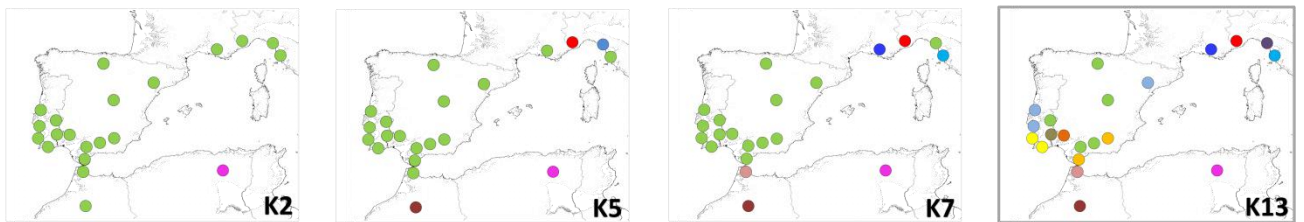
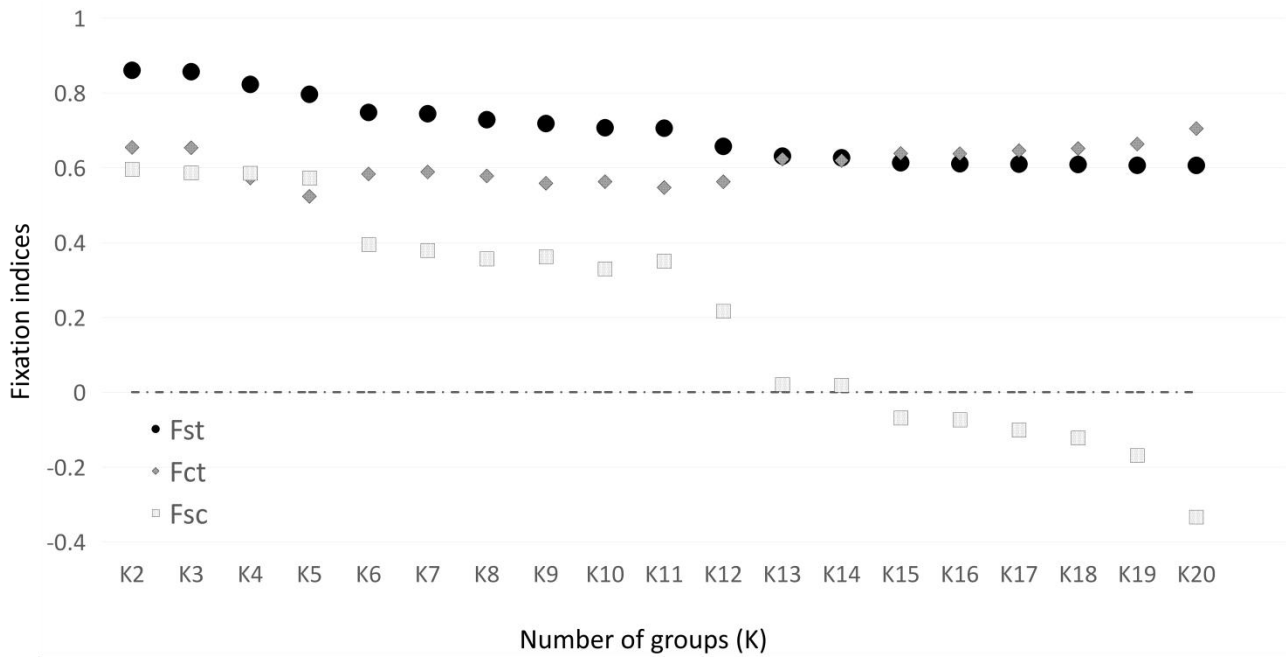
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648 **Figure 2** - Maximum likelihood phylogenetic species tree based on concatenated COI+EF1-a (1206 bp).
 649 Values above and below branches correspond to ML bootstrap values (only >50% are shown) and
 650 Bayesian posterior probabilities, respectively. Illustrated butterflies from top to bottom: *Euchloe tagis*
 651 *tagis* [Arrábida]; *E. tagis alhajarae* [Estremoz], *E. tagis tagis* [Candeeiros]; *E. tagis pechi* [Algeria]; *E.*
 652 *tagis bellezina* [France]; *E. crameri* [Portugal]; *Euchloe creusa* [Russia]; *Euchloe belemia* [Morocco];
 653 *Euchloe charlonia* [Morocco]; *Anthocharis cardamines* [France]; *A. euphenoides* [Spain]; *Zegrus*
 654 *pyrothoe* [Kazakhstan].
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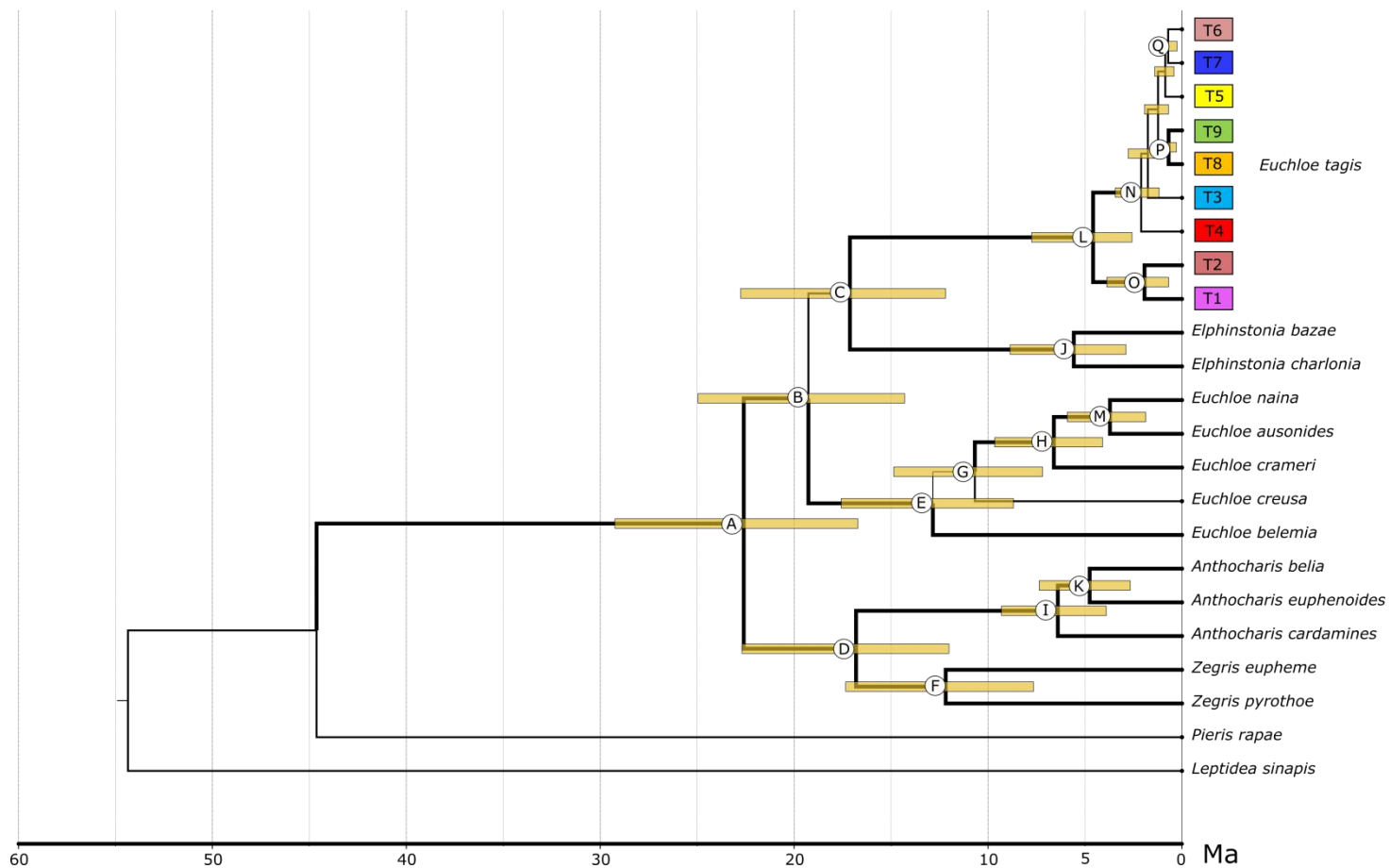
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Figure 3 - Median-joining haplotype network of *Euchloe tagis* for COI (625 bp), comprising all available 164 sequences. Circle size is proportional to sample size.



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Figure 4 Mitochondrial COI population structure of *E. tagis*. Population clusters obtained with SAMOVA from a K=2-20 number of groups. Fixation indices values (Fst=total differentiation among populations; Fct = among groups differentiation; Fsc = among populations within groups differentiation) obtained with AMOVA implemented in ARLEQUIN. Map inserts are the geographical translation of some clusters. Optimal clustering is k=13 or 14.



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698 **Figure 5** Bayesian chronogram with estimated molecular divergence times of the concatenated dataset
 699 (1206 bp) among the Anthocharidini. Clade colours and denomination are upon Figures 1 and 2. 95%
 700 confidence bars are shown at each node. Thicker branches correspond to PP > 0.8. Inset: Estimated
 701 TMRCAs (in millions of years ago, Mya). Mean time estimates and 95% confidence
 702 intervals inferred using Birth Death speciation model and a Relaxed Molecular Clock.

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703 **References**

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