

1 **ORIGINAL ARTICLE**

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3 **Phylogeography and molecular phylogeny of Macaronesian island *Tarphius* (Coleoptera:**
4 ***Zopheridae*): why so few species in the Azores?**

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18 **running head**: Phylogenetics and diversification of Azorean *Tarphius*

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20

21 **ABSTRACT**

22 **Aim** We used a phylogenetic framework to examine island colonization and predictions pertaining to
23 differentiation within Macaronesian *Tarphius* (Insecta, Coleoptera, Zopheridae), and explain the
24 paucity of endemics in the Azores compared with other Macaronesian archipelagos. Specifically, we
25 test whether low diversity in the Azores could be due to recent colonization (phylogenetic lineage
26 youth), cryptic speciation (distinct phylogenetic entities within species), or the geological youth of the
27 archipelago.

28 **Location** Macaronesian archipelagos (Azores, Madeira and the Canary Islands), North Portugal and
29 Morocco.

30 **Methods** Phylogenetic analyses of mitochondrial and nuclear genes of *Tarphius* beetles of the Azores,
31 other Macaronesian islands and neighbouring continental areas were used to investigate the origin of
32 island biodiversity and to compare patterns of colonization and differentiation. A comparative
33 nucleotide substitution rate test was used to select the appropriate substitution rate to infer clade
34 divergence times.

35 **Results** Madeiran and Canarian *Tarphius* species were found to be more closely related to each other,
36 while Azorean taxa grouped separately. Azorean taxa showed concordance between species and
37 phylogenetic clades, except for species that occur on multiple islands, which segregated by island of
38 origin. Divergence time estimates revealed that Azorean *Tarphius* are an old group and that the most
39 recent intra-island speciation event on Santa Maria, the oldest island, occurred between 3.7 and 6.1
40 Ma.

41 **Main conclusions** Our phylogenetic approach provides new evidence to understand the
42 impoverishment of Azorean endemics: (1) *Tarphius* have had a long evolutionary history within the
43 Azores, which does not support the hypothesis of fewer radiation events due to recent colonization; (2)
44 the current taxonomy of Azorean *Tarphius* does not reflect common ancestry and cryptic speciation is
45 responsible for the underestimation of endemics; (3) intra-island differentiation in the Azores was
46 found only in the oldest island, supporting the idea that geological youth of the archipelago limits the
47 number of endemics; and (4) the lack of evidence for recent intra-island diversification in Santa Maria

48 could also explain the paucity of Azorean endemics. Phylogenetic reconstructions of other species-
49 rich taxa that occur on multiple Macaronesian archipelagos will reveal whether our conclusions are
50 taxon specific, or of a more general nature.

51 **Keywords**

52 **Azorean enigma, Coleoptera, cryptic diversification, COI, COII, elongation factor 1 α , island**
53 **biogeography, island endemics, Macaronesia, speciation.**

54

55 **INTRODUCTION**

56 The Macaronesian islands (Cape Verde, Canary Islands, Salvage Islands, Madeira and the Azores)
57 host a diverse and distinct biota and are recognized as an important component of the Mediterranean
58 biodiversity hotspot (Myers *et al.*, 2000). Among these archipelagos, the Azores (Fig. 1) is
59 comparatively depauperate, with fewer single-island endemic species, particularly in comparison to
60 Madeira and the Canary Islands (Borges *et al.*, 2008a; Arechavaleta *et al.*, 2010; Borges *et al.*, 2010a),
61 a phenomenon that has been referred to as the "Azorean enigma" (Carine & Schaefer, 2010).
62 Arthropods represent approximately 60% of the endemic species found in the Azores (Borges *et al.*,
63 2010b) and recent studies have focused on this group to investigate why the number of endemic
64 species in the Azores is reduced compared with other Macaronesian archipelagos, testing for
65 correlations between the number of endemics and archipelago/island isolation, area, geological age,
66 habitat diversity and native habitat destruction (Borges & Brown, 1999; Borges & Hortal, 2009;
67 Cardoso *et al.*, 2010; Triantis *et al.*, 2010a,b). However, although a phylogenetic framework has been
68 incorporated in the analyses of the Azorean flora (e.g. Carine & Schaefer, 2010; Schaefer *et al.* 2011),
69 such an approach has not been adopted for arthropods. Phylogenetic studies of Azorean taxa and
70 Macaronesian relatives are few, involve single taxa and/or limited sampling of islands and mainland
71 specimens. Most rely on single markers or short DNA sequences, and do not explicitly address the
72 question of why there are fewer endemics in the Azores compared with the other Macaronesian islands
73 (Cesaroni *et al.*, 2002; Fujaco *et al.*, 2003; Montiel *et al.*, 2008).

74 The genus *Tarphius* Erichson, 1848 (Insecta, Coleoptera, Zopheridae) is one of the arthropod
75 groups with the highest number of endemic species in the Azores, with eight endemic species
76 described (Oromí *et al.*, 2010a) and an undescribed new species from São Miguel (P. Borges, pers.
77 obs.). Six species are single island endemics (*T. wollastoni* Crotch, 1867, *T. acuminatus* Gillerfors,
78 1986, *T. pomboi* Borges, 1991, *T. serranoi* Borges, 1991, *T. rufonodulosus* Israelson, 1984 and the
79 undescribed species from São Miguel), and the other three species (*T. tornvalli* Gillerfors, 1985, *T.*
80 *depressus* Gillerfors, 1985 and *T. azoricus* Gillerfors, 1991) inhabit two, three and six islands
81 respectively (Fig. 1). The genus *Tarphius* also occurs in Madeira, the Canary Islands, North Africa, the

82 Iberian Peninsula, Italy, South America and the Himalayas (Franz, 1967; Dajoz, 1977; Ślipiński,
83 1985), but the majority of species (86%) are endemic to the Macaronesian islands. *Tarphius* are
84 apterous, fungivorous beetles with cryptic life styles, typically associated with the native forest of the
85 Macaronesian islands (Borges, 1992; Emerson & Oromí, 2005). As is the case for other beetle genera
86 (e.g. *Drouetius/Laparocerus*, *Calacalles/Acalles*, *Calathus*, *Trechus*), there are fewer *Tarphius* species
87 endemic to the Azores compared to neighbouring archipelagos: Azores 9, Madeira 23 and Canaries 31
88 (Borges *et al.*, 2008b; Oromí *et al.*, 2010a,b; P.A.V. Borges, pers. obs.; A. Machado Carrillo,
89 Universidade de La Laguna, Tenerife, Spain, pers. obs.). Therefore *Tarphius* represents a good system
90 to investigate, within a phylogenetic framework, the causes of impoverished endemism within the
91 Azores.

92 Previous studies on Macaronesian *Tarphius* using phylogenetic approaches and mitochondrial
93 markers (Emerson & Oromí, 2005) focused on species of the Canary Islands, including only a few taxa
94 from Madeira, a single species from the Azores and no mainland relatives. Here we expand on the
95 genetic and geographic sampling of Emerson & Oromí (2005) by employing nuclear (elongation factor
96 1 alpha – EF1 α) and mitochondrial (cytochrome *c* oxidase subunit I – COI, leucine specific transfer
97 RNA – tRNA_{Leu}, cytochrome *c* oxidase subunit II – COII) sequences to infer phylogenetic
98 relationships among *Tarphius* species from all Macaronesian islands and the closest continental areas
99 of North Africa and Iberia. Special emphasis is given to the Azores, which comprise nine islands (Fig.
100 1) characterized by a rich and diverse volcanic history (Forjaz, 1998; Morton *et al.*, 1998; Nunes,
101 2000), with subaerial geological ages ranging from 0.25 to 8.12 Ma (França *et al.*, 2003). Although it
102 has a total area of 2324 km², approximately half of the Azores (62%) is very recent in origin, less than
103 1 Ma (Triantis *et al.*, 2010b). The archipelago spans 615 km, and the closest land masses are Madeira
104 and the Iberian Peninsula, at 840 km and 1584 km, respectively (Fig. 1).

105 Our sampling represents one of the most comprehensive phylogeographic and phylogenetic
106 studies of a species-rich invertebrate genus in the Macaronesian islands that includes the Azores,
107 allowing us to evaluate the Azorean enigma (see Carine & Schaffer, 2010) by testing the following
108 predictions: (1) due to their geographic proximity, Azorean lineages are more closely related to

109 European species, and Madeiran and Canarian lineages are more closely related to African species;
110 (2) Azorean taxa are monophyletic; (3) intra-island speciation has had a more important role in
111 shaping patterns of genetic diversity of Canarian island *Tarphius* on older islands (Emerson & Oromí,
112 2005). We predict intra-island speciation to be the main mechanistic explanation for species diversity
113 on the older Azorean islands (Santa Maria and São Miguel); (4) we predict that the oldest island of
114 Santa Maria was the first colonized, and contains the most divergent lineages; (5) given the geographic
115 distances among Azorean islands, low vagility of *Tarphius* and the presence of similar morphospecies
116 on different islands, we predict endemic taxa have to have been underestimated because of cryptic
117 speciation; and (6) given the geographic isolation of the Azores, the relatively young age of much of
118 its area, and the intense volcanic activity in the archipelago, we predict Azorean lineages to be of
119 recent origin, much younger than the maximum geological age of the archipelago (8.12 Ma).

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121

122 **MATERIALS AND METHODS**

123 **Sample collection**

124 Collecting effort was focused on the Azorean and Madeiran archipelagos, mainland Portugal and
125 North Africa (Morocco). *Tarphius* species were collected between 1999 and 2009 using a combination
126 of pitfall trapping, manual searching in specific habitats, Berlese funnel extraction from leaf litter, and
127 canopy beating. Specimens were stored individually in absolute ethanol or acetone and refrigerated
128 prior to DNA extraction. Species identification was performed by P.A.V. Borges and A. Serrano, and
129 voucher specimens were deposited in the Dalberto Teixeira Pombo Arthropod Collection (DCA,
130 University of the Azores, Portugal). Additional specimens from the Canary Islands and Madeira were
131 collected during a previous study (Emerson & Oromí, 2005).

132

133 **DNA extraction, PCR amplification and sequencing**

134 Total genomic DNA was extracted using the DNeasy Tissue Kit (Qiagen Inc., Valencia, CA, USA). A
135 mtDNA fragment of approximately 1500 bp, comprising part of the COI gene, the tRNA_{Leu} gene and

136 the complete COII gene was amplified via polymerase chain reaction (PCR) using a combination of
137 primers described in the literature and their modifications (Simon *et al.*, 1994; Emerson *et al.*, 2000;
138 Whiting, 2002). Amplification of approximately 950 bp of the nuclear EF1 α gene was accomplished
139 using a combination of primers designed specifically for *Tarphius* beetles. For details on DNA
140 extraction and PCR amplification see Appendix S1 in Supporting Information. PCR products were
141 purified using the QIAquick PCR Purification Kit (Qiagen Inc., Valencia, CA, USA) and cycle-
142 sequenced in both directions with the PCR amplification primers and internal primers. Sequencing
143 reactions were either performed using the ABI PRISM BigDye Terminator chemistry and separated by
144 electrophoresis on an ABI PRISM DNA automated sequencer (Applied Biosystems, Foster City, CA,
145 USA) or using the CEQ DTCS chemistry and run on a CEQ 2000 Genetic Analysis System (Beckman
146 Coulter Inc., Brea, CA, USA).

147

148 **Phylogenetic analyses**

149 Sequence trace chromatograms were assembled, edited and aligned for both mtDNA and nuclear data
150 sets with the software SEQUENCHER 4.8 (Gene Code Corporation, Ann Arbor, MI, USA). Sequence
151 alignments were verified by eye and edited using MACCLADE v4.08 (Maddison & Maddison, 2005)
152 and all sequences obtained for each partition were collapsed to unique sequences using
153 DNACOLLAPSER (Villesen, 2007). MtDNA sequences for all Canarian *Tarphius* species, some
154 Madeiran species and the outgroup *Pseudotarphius lewisii* were obtained from GenBank (see
155 Appendix S2).

156 The mtDNA and EF1 α data matrices were examined for incongruence length differences (ILD)
157 (Mickey & Farris, 1981) using the partition homogeneity test (Farris *et al.*, 1995a,b) as
158 implemented in PAUP* v4.0b10 (Swofford, 2002). Maximum likelihood (ML) and Bayesian inference
159 (BI) methods were used to reconstruct phylogenetic relationships based on mtDNA and nuclear
160 sequence data separately using *Pseudotarphius lewisii* as the outgroup and treating gaps and nuclear
161 heterozygote positions as missing data. Mitochondrial sequences were analysed as a single fragment,
162 partitioned according to gene, and partitioned according to codon position for the protein-coding

163 fragments. The nuclear data were analysed as a single fragment, partitioned into exon and intron
164 fragments, and partitioned according to codon position for the exonic regions. The program
165 jMODELTEST v0.1.1 (Posada, 2008) was used to select the model of evolution that best explains the
166 nucleotide variation within each partition, excluding the outgroup and using the Akaike information
167 criterion (AIC) (Posada & Buckley, 2004). BI analyses were performed with MRBAYES v3.1.2
168 (Ronquist & Huelsenbeck, 2003) and for each data set and partition scheme, two independent analyses
169 starting from different random trees were run in parallel for five million generations, with four
170 simultaneous Markov chains, and sampling every 100th generation. The average standard deviation of
171 split frequencies was used as a convergence diagnostic, and the first 25% of samples were discarded as
172 burn-in. Each analysis was repeated to test the robustness of the inferred phylogenetic relationships by
173 comparing the topology and log likelihood scores of the consensus trees obtained in each analysis. The
174 software RAxML v7.2.8 (Stamatakis, 2006; Stamatakis *et al.*, 2008), run on the Cipres Portal v2.2
175 (Miller *et al.*, 2009), was used for ML phylogenetic inferences. Settings for ML searches were as
176 follow: random seed value specified for the initial parsimony inference and for rapid bootstrapping,
177 rapid bootstrapping conducted while searching for the best score ML tree (1000 iterations), and
178 GTRGAMMA used both for the bootstrapping phase and final tree (all other parameters using default
179 values).

180

181 **Comparative substitution rate and estimation of divergence time analyses**

182 We combine intrinsic calibration points (island geological ages) with a molecular clock calibration
183 derived from other studies using a Bayesian approach to infer divergence ages. Because accelerated
184 rates of molecular evolution have been reported for groups that have radiated extensively on islands
185 (Chiba, 1999), we performed a comparative rate analysis (Cicconardi *et al.*, 2010) to investigate
186 whether *Tarphius* may be evolving at an anomalous rate compared to other Coleoptera and to choose
187 an adequate rate for dating divergence events. Forty-four COII sequences representing the Coleopteran
188 suborders Adephaga (20), Archostemata (1), Polyphaga (21) and Myxophaga (2) and two Diplura
189 sequences (outgroups) were downloaded from GenBank (see Appendix S3) and added to an alignment

190 of 49 *Tarphius* sequences representing all sampled species and clades. Coding sequences were
191 aligned at the amino acid level (retro-aligned) using REVTRANS 1.4 (Wernersson & Pedersen, 2003),
192 with a final matrix of 675 bp. BEAUTI v1.6.2 was used to generate the input file for the Bayesian
193 Markov chain Monte Carlo (MCMC) analysis of molecular sequences implemented in BEAST v1.6.2
194 (Drummond & Rambaut, 2007). The analysis was run assuming an uncorrelated lognormal relaxed
195 molecular clock (Drummond *et al.*, 2006) with the average number of substitutions per site per million
196 years across the whole tree set to 1 (meanRate constrained under normal distribution, mean = 1, SD =
197 0.01), with individual branch rates unconstrained. Other priors included a Yule speciation process and
198 enforced monophyly of the ingroup, each suborder, infraorder, superfamily, family and genus, to limit
199 tree search space. BEAST runs were performed on the High Performance Computing Cluster supported
200 by the Research Computing Service at University of East Anglia, UK. The analysis was run twice for
201 10 million generations, sampling every 100th generation, and TRACER v1.5 (Rambaut & Drummond,
202 2009a) was used to inspect for stationarity; both runs were combined in LOGCOMBINER v1.6.2
203 (Rambaut & Drummond, 2009b) with burn-in set to 10%. A maximum clade credibility tree was
204 obtained with TREEANNOTATOR v1.6.2 (Rambaut & Drummond, 2009c) and branch specific rates and
205 lengths were visualized with FIGTREE v1.3.1 (Rambaut, 2009). Rates for the groups of interest were
206 calculated as the average of rates for all branches related to the specific group weighted by branch
207 length.

208 Approximate divergence times within Macaronesian island *Tarphius* were estimated with
209 BEAST v1.6.2 (Drummond & Rambaut, 2007) based on the mitochondrial protein-coding sequence data
210 set (COI and COII, excluding the intervening tRNA_{Leu}) and using *T. kiesenwetteri* (from northern
211 Portugal) as the outgroup. Analyses were performed using an uncorrelated lognormal relaxed
212 molecular clock (Drummond *et al.*, 2006) with unconstrained rates for individual branches and
213 applying a mean substitution rate estimate derived from the comparative rate analysis of 2.1% pairwise
214 divergence. Additional calibration was provided by subaerial ages of Macaronesian islands and eight
215 nodes in the phylogeny were constrained under an uniform distribution to be no older than the age of a
216 specific island represented within the clade, thus allowing for the possibility that colonization could

217 have occurred any time since island emergence until the present. Age constraints were applied to
218 nodes with high support (posterior probability ≥ 95 , bootstrap ≥ 90) and to Canary Island taxa (nodes
219 not shown) that have been used for calibration in a previous study (nodes B, C, E and F, Fig. 3 in
220 Emerson & Oromí, 2005). Nodes II, III, VI and VII (Fig. 2) were constrained to be no older than the
221 oldest island represented within the clade: Madeira plus Canary Islands (clade II) ≤ 14 Ma (Porto
222 Santo); Azores (clade III) ≤ 8.12 Ma (Santa Maria); *T. azoricus* plus *T. tornvalli* (clade VI) ≤ 4.01 Ma
223 (São Miguel); and *T. azoricus* plus *T. tornvalli* from the Central group of Azorean islands (clade VII) \leq
224 3.52 Ma (Terceira). Ten independent analyses, consisting of 10 million generations each and sampling
225 every 1000th generations, were performed assuming a Yule speciation process tree prior and selecting
226 the general nucleotide substitution model parameters (GTR+I+G) suggested by jMODELTEST v0.1.1
227 (Posada, 2008). Runs were checked for convergence to a stationary distribution and combined as
228 described above. To estimate time range for intra-island diversification events in the Azores, Azorean
229 taxa were limited to *Tarphius* lineages endemic to Santa Maria, the oldest and only island in the
230 archipelago where there is evidence of intra-island speciation. Age estimation analyses were also
231 performed considering codon positions to investigate the effect of different partition schemes on
232 divergence time estimates.

233

234

235 RESULTS

236 Sample collection

237 All species of Azorean *Tarphius*, except for *T. acuminatus* and one undescribed species from São
238 Miguel were collected. *Tarphius* were collected for the first time on São Jorge and Faial islands (*T.*
239 *azoricus* group), and the species *T. depressus* was collected for the first time on Pico and São Miguel
240 islands. Azorean *Tarphius* species were typically found in the native humid evergreen broadleaf laurel
241 forest (laurisilva), but were also collected on introduced plants, such as, *Acacia* spp. and *Cryptomeria*
242 *japonica*. Specimens of *Tarphius* were also collected in Madeira and Porto Santo islands, in the north
243 of Portugal, and a new species in the High Atlas of Morocco (species not yet described).

244

245 **Phylogenetic analyses**

246 MtDNA and EF1 α sequence data was obtained for ninety-eight specimens of *Tarphius*: 68 from the
247 Azores and 30 from Madeira, Porto Santo, North Portugal and Morocco (unique sequences deposited
248 in GenBank - **accession numbers XXX to XXX** see Appendix S2). The mtDNA alignment consisted
249 of 1383 bp, comprising 642 bp of the COI gene, with a terminal 3 bp deletion in all continental
250 *Tarphius* species, 64 bp of the tRNA_{Leu} gene, with a 1 bp deletion in the outgroup, and 677 bp of the
251 COII gene. The EF1 α alignment consisted of 920 bp, comprising two exonic regions totalling 709 bp
252 intervened by an intron of 211 aligned nucleotide positions, ranging in sequence length from 163 to
253 204 bp. The final data sets consisted of 136 and 109 unique sequence for the mtDNA and EF1 α
254 markers, respectively. With the exception of *Tarphius azoricus* and *T. tornvalli* of Pico and *T. azoricus*
255 of São Jorge, sequences were only shared by conspecific individuals from the same collection site. The
256 ILLD test indicated significant incongruence between the mitochondrial and nuclear sequence matrixes
257 (100 replicates, $P = 0.01$) and so data sets were analysed separately. For both data sets the nucleotide
258 model of evolution that best fits the data, as suggested by the AIC in jMODELTEST, is the general time-
259 reversible model assuming a proportion of invariable sites and a gamma distributed rate heterogeneity
260 (GTR+I+G). This model was used for the parameterization of the ML and BI searches.

261 For both markers, the topologies of the ML and BI phylogenetic trees are similar, regardless of
262 the partition scheme used, with minor terminal differences related to the placement of conspecific
263 sequences. BI reconstructions from replicate runs show the same topology and very similar clade
264 support and likelihood scores. Figures 2 and 3 represent the BI trees obtained for both gene regions
265 with no partitioning of the sequence data. Both phylogenetic methodologies based on either of the
266 molecular markers provided maximum support for the monophyly of *Tarphius* from the Azores, and
267 group taxa from Madeira and the Canary Islands together and place these as the closest relatives to the
268 Azorean taxa. Other clades consistently recovered for the Azorean taxa include: *T. rufonodulosus*
269 (Santa Maria), *T. serranoi* (Santa Maria), *T. pomboi* (Santa Maria), *T. depressus* (Santa Maria), *T.*
270 *wollastoni* (Flores), *T. azoricus* (Terceira) and *T. azoricus* + *T. tornvalli* (São Miguel). Another

271 common feature for both markers is that within the Azores, lineages from the oldest island Santa
272 Maria (*T. rufonodulosus*, *T. serranoi* and *T. pomboi*) are sister taxa to lineages found on the younger
273 Azorean islands (Figs 2 & 3). Specifically, in the mtDNA tree, for each major clade, lineages found on
274 the oldest island within that particular clade show the deepest divergences and are sister taxa to the
275 remaining lineages (Fig. 2). For example, in the *T. azoricus* + *T. tornvalli* clade, lineages from São
276 Miguel (4.01 Ma) are sister taxa to the lineages found on the younger islands of Terceira (3.52 Ma),
277 Faial (0.73 Ma), São Jorge (0.55 Ma) and Pico (0.25 Ma), and in the *T. azoricus* + *T. tornvalli* clade of
278 the central group of islands (Fig. 1), lineages from the oldest island (Terceira) are sister taxa to
279 lineages found on the younger islands of Faial, São Jorge and Pico (Fig. 2).

280 When compared to the EF1 α tree (Fig. 3), the mtDNA tree (Fig. 2) provides finer resolution of
281 the phylogenetic relationships within Azorean *Tarphius*, which is to be expected based on the faster
282 rate of molecular evolution of the latter marker. In particular, the mtDNA tree groups most taxa
283 according to morphospecies identity. The exceptions to this pattern involve species that occur on
284 multiple islands, such as *T. azoricus*, *T. tornvalli* and *T. depressus*, where individuals group by island,
285 rather than by morphospecies. The mitochondrial DNA tree also reveals that the species assemblages
286 of Pico and São Miguel islands are not monophyletic, suggesting at least two colonization events to
287 each of the islands by *T. azoricus* + *T. tornvalli* and *T. depressus* lineages.

288

289 **Comparative substitution rate and estimation of divergence time analyses**

290 The weighted average substitution rates and their standard deviations for the different Coleoptera
291 lineages and for *Tarphius* are presented in Fig. 4. The *Tarphius* specific rate in the comparative
292 substitution rate analysis falls within the second quartile, revealing *Tarphius* to have been evolving at a
293 "typical" coleopteran rate. Therefore, a 2.1% pairwise divergence per million years, the median value
294 of the range of substitution rates reported for Coleoptera (0.7-3.5%), was used for the estimation of
295 *Tarphius* divergence times.

296 The two partition schemes used to estimate divergence times (no partition and partition by
297 codon position) produced concordant results. The estimated times to the most recent common ancestor

298 (TMRCA) obtained for several nodes of interest on the mtDNA phylogeny (Fig. 2) are presented in
299 Table 1. The mean time of divergence estimated for *Tarphius* of the Macaronesian islands (ingroup)
300 was approximately 21 Ma, and 7 Ma for *Tarphius* endemic to the Azores. Within the Azores,
301 colonization of the central group of islands (Fig. 1) by *T. azoricus* + *T. tornvalli* lineages (node VI) and
302 *T. depressus* (node V) were estimated to have occurred approximately 3.7 and 2.9 Ma, respectively
303 (Table 1), and species that colonized the western islands (Flores, Fig. 1) were estimated to have
304 diverged around 3.3 Ma (Table 1, node IV). Estimates for divergence events within Santa Maria, the
305 oldest Azorean island and the only one in the archipelago where there is evidence for intra-island
306 speciation, reveal that: (1) the first divergence event (*T. rufonodulosus* from other lineages) occurred
307 approximately 7.3 Ma (95% HPD interval: 6.27-8.12 Ma); (2) the divergence of *T. serranoi* from other
308 lineages occurred approximately 6.5 Ma (95% HPD interval: 5.28-7.63 Ma); and (3) the most recent
309 divergence event in Santa Maria (*T. pomboi* and *T. depressus*) occurred approximately 4.9 Ma (95%
310 HPD interval: 3.70-6.14 Ma).

311

312

313 **DISCUSSION**

314 **Colonization of the Macaronesian islands and the Azores**

315 Colonization of the Macaronesian islands is estimated to have occurred around 21 Ma, when
316 divergence gave rise to a lineage that ultimately colonized the Azores approximately 7 Ma. The
317 estimated time of colonization to the Macaronesian islands corresponds to the age of Fuerteventura (21
318 Ma, Canary Islands), the oldest currently emergent island among the focal archipelagos. Although
319 older Macaronesian islands that are now submerged may have been available for colonization,
320 possibly as early as 60 million years ago (Fernández-Palacios *et al.*, 2011), our data suggests that
321 *Tarphius* lineages that survived to the present started diverging from their continental relatives about
322 the time that Fuerteventura emerged. Both phylogenetic trees reveal island *Tarphius* to be more closely
323 related to each other than to continental taxa, suggesting a single colonization event, however we
324 cannot completely rule out the possibility that the record of multiple arrivals to the Macaronesian

325 islands may have been erased by continental extinctions (Emerson, 2002). Contemporary
326 continental *Tarphius* species are potentially relicts of a more diverse and abundant fauna that inhabited
327 laurel forests covering most of Western Europe and North Africa during the Tertiary. Thus it remains
328 possible that multiple colonizations of the Macaronesian islands may have occurred, but with the
329 signal for this having been erased by the subsequent extinction of continental taxa following the
330 disappearance of laurel forest. Likewise, limiting sampling of extant continental species may lead to an
331 underestimation of the number of colonization events to the Macaronesian islands (Emerson, 2002).

332 Europe, Africa and other Macaronesian islands are potential sources of colonists to the Azores
333 (Lindroth, 1960; Sundings, 1979; Borges & Serrano, 1993) but sequence data reveals that the closest
334 relatives to the Azorean *Tarphius* are from Madeira and the Canary Islands. The inferred relationships
335 may have been different if, as mentioned above, unsampled and extinct taxa were considered.
336 However, the close relationship between Azorean and other island *Tarphius* species is also supported
337 by morphological evidence, as *Tarphius* species of the Azores have been assigned to the subgenus
338 *Atlantotarphius*, with representatives in the Canary Islands and Madeira, while *Tarphius* species from
339 North Africa and Iberia have been placed in the subgenus *Tarphius* s.str. (Franz, 1967). This suggests
340 that the Azores were colonized by stepping-stone dispersal via islands closer to the continental areas,
341 and not directly from continental areas themselves, a common colonization pattern for oceanic islands.
342 Within the Azores the trend is that lineages found on the oldest islands show the deepest divergences
343 and are sister taxa to the remaining lineages (Figs 2 & 3), supporting the idea that as new islands
344 emerged their colonization occurred from older islands within the archipelago, a pattern consistent
345 with the progression rule (Funk & Wagner, 1995).

346 The Azores is the most isolated of the Macaronesian archipelagos, and land area has been
347 limited until recently. The oldest island emerged 8.12 Ma, the second oldest island emerged only 4
348 million years later, and until about 1 Ma the total area of the Azores was only 62% of its current area
349 (Triantis *et al.*, 2010b). The Azorean *Tarphius* species form a monophyletic clade, which argues for a
350 single colonization event. Studies on other native Azorean taxa, including birds, butterflies, bats,
351 mosses and other plants, also conclude that they result from single colonization events, supporting the

352 hypothesis of low colonization frequency of the archipelago (Marshall & Baker, 1999; Cesaroni *et*
353 *al.*, 2002; Salgueiro *et al.*, 2004; Werner *et al.*, 2009; Ferreira *et al.*, 2011). Isolation and small area
354 may then operate as powerful filters limiting the number of propagules that arrive to the Azores, and
355 ultimately the number of taxa endemic to the archipelago. Additionally, small population sizes and
356 cataclysmic volcanic events on several of the Azorean islands (Serralheiro & Madeira, 1993; Nunes,
357 2000) may have led to extinctions of ancestral taxa (Sousa, 1985; Borges, 1992). However, our results
358 clearly suggest ancient colonization as the mean divergence time estimates for *Tarphius* endemic to
359 the Azores is *c.* 7 Ma. Further, we find that Santa Maria, the oldest Azorean island (8.12 Ma), was the
360 first to be colonized and that colonization occurred soon after its emergence. The genus *Tarphius* has
361 had a long evolutionary history in the Azores and the failure of *Tarphius* to diversify more is not due
362 to the recent arrival of ancestral stock. A similar result showing no support for the lineage age
363 hypothesis for Azorean taxa (Wallace, 1881; Briggs, 1966) has also been reported for the plant genus
364 *Pericallis* (Carine & Schaefer, 2010).

365

366 **Diversification and extinction in the Azores**

367 The Azores have had a history of active volcanism (Forjaz, 1998; Morton *et al.*, 1998; Nunes, 2000)
368 which may have played an important role in shaping patterns of genetic diversity. Namely, habitat
369 destruction, creation of barriers to dispersal and consequent population isolation due to volcanic
370 activity may have promoted intra-island speciation (Carson *et al.*, 1990; Vandergast *et al.*, 2004;
371 Emerson, 2008). Given that *Tarphius* beetles are flightless and some of the Azorean islands are
372 characterized by relatively large inter-island distances, dispersal among islands is likely to be limited,
373 and *in situ* speciation is expected to be the dominant mechanism for the origin of new species within
374 islands. However, intra-island speciation in the Azores has only been inferred on Santa Maria,
375 corresponding to the first diversification events within the archipelago. What might have promoted
376 intra-island differentiation within Santa Maria is not clear. One potential explanation is vicariance
377 followed by species range expansion and overlap, as *Tarphius* species on Santa Maria occur in
378 sympatry. Santa Maria is the oldest Azorean island (8.12 Ma) and thus may be the only island where

379 there has been sufficient time for intra-island speciation to have occurred. Therefore, the geological
380 youth of most of the Azores and the consequent lack of time and opportunity for intra-island speciation
381 could contribute to the low number of endemics in the archipelago. However, although in the Canary
382 Islands intra-island speciation is responsible for many of the endemic *Tarphius* species found on all the
383 older islands (Gran Canaria 14-16 Ma; La Gomera 12 Ma; Tenerife 11.6 Ma), divergences times are
384 very recent, suggesting that island age may not be a key factor in intra-island speciation (Emerson &
385 Oromí, 2005). This pattern of ancient lineages showing recent species diversification has been
386 observed for other endemic invertebrates of the Canary Islands (Emerson & Oromí, 2005; Contreras-
387 Díaz *et al.*, 2007). In contrast, there is no evidence for relatively recent speciation events on the oldest
388 Azorean island of Santa Maria (8.12 Ma). Multiple species have originated within Santa Maria but the
389 most recent speciation event was estimated to have happened between 3.6 and 6.1 Ma (split of *T.*
390 *pomboi* and *T. depressus*). The last major eruptive phase in Santa Maria island dates from about 5.5
391 Ma (Serralheiro & Madeira, 1993) and may have contributed to these speciation patterns. Finally,
392 Santa Maria is one of the islands most severely affected by human activities and only a small patch of
393 9 ha of very disturbed native forest persists (Gaspar *et al.*, 2008). Consequently, recent extinctions due
394 to habitat destruction may have led to species loss on Santa Maria, but it seems improbable that such
395 effects would have selectively targeted *Tarphius* taxa that are the products of recent speciation events.
396 Consequently, the absence of species that have originated within the last few million years on Santa
397 Maria is puzzling.

398 Despite the low vagility of *Tarphius*, it is clear that inter-island colonization within the Azores
399 has played an important role in generating diversity and that it is associated with the most recent
400 divergence events. The importance of inter-island colonization in shaping Azorean *Tarphius* diversity
401 is supported by the presence of distinct but closely related clades of the same species on different
402 islands (e.g. *T. azoricus* and *T. depressus*), and by the fact that island species assemblages are not
403 monophyletic (e.g. Pico and São Miguel islands, Fig. 2). Inter-island colonization and isolation on
404 different islands have also been acknowledged as important mechanisms responsible for the evolution
405 of Azorean endemic land snails (Van Riel *et al.*, 2003, 2005; Jordaens *et al.*, 2009).

406 The paucity of species in the Azores could be exaggerated if the current number of *Tarphius*
407 species described based on traditional taxonomy is an underestimate due to the presence of cryptic
408 species. The phylogenetic relationships among taxa endemic to the Azores strongly support the
409 taxonomic entities *T. serranoi*, *T. pomboi*, *T. wollastoni* and *T. rufonodulosus* (single-island
410 endemics), as all specimens belonging to these species form monophyletic groups with high clade
411 support (Figs 2 & 3). For species that occur on more than one island (*T. azoricus*, *T. tornvalli* and *T.*
412 *depressus*), phylogenetic trees show that they mostly segregate by island of origin, and represent
413 lineages that have been diverging in allopatry. If taxa accepted by traditional taxonomy and distinct
414 phylogenetic lineages are combined, the total number of Azorean endemic *Tarphius* would be
415 increased to 17, 14 taxa sampled plus three not included in this study (*T. azoricus* of Flores, *T.*
416 *acuminatus* of Pico, and one undescribed species of São Miguel). This figure is much closer to the
417 numbers of endemic *Tarphius* species reported for the other Macaronesian archipelagos, as Madeira
418 and the Canary Islands are represented by 23 and 31 species respectively.

419 At the time of their discovery, the Azores were almost entirely covered by native forest which
420 was reduced to less than 3% of its original extent in less than 600 years (Gaspar *et al.*, 2008), the most
421 dramatic destruction of native habitat recorded for the Macaronesian islands (Cardoso *et al.*, 2010;
422 Triantis *et al.*, 2010a). Island *Tarphius* species are intimately associated with the native forest and the
423 extensive deforestation in the Azores may have led to the extinction of some *Tarphius* lineages.
424 Although the lack of fossil data does not allow us to test the hypothesis that more species of *Tarphius*
425 may have existed in the Azores before human establishment, we cannot disregard the possibility of
426 recent extinction also contributing to a low number of endemic species in the Azores.

427

428

429 CONCLUSIONS

430 The biota of the Azores is depauperate compared with that of other Macaronesian archipelagos. This
431 lower biodiversity may result from a combination of geological youth, isolation, small area, small
432 population sizes, lack of habitat diversity, and the massive recent destruction of native forest. Our

433 study provides a complementary approach to species presence/richness based studies for exploring
434 hypotheses concerning the limited diversity of endemic taxa in the Azores. Based on phylogenetic
435 reconstructions *in situ* diversification within a monophyletic assemblage derived from a single
436 colonisation event appears to account for the origin of *Tarphius* species endemic to the Macaronesian
437 islands. However, intra-island diversification, known to be an important mechanism for differentiation
438 in older islands (Emerson & Oromí, 2005), has only been demonstrated for *Tarphius* species of Santa
439 Maria, the oldest island in the archipelago. On the remaining Azorean islands, multiple species on the
440 same island are the result of multiple colonization events. Therefore, island geological youth may be
441 an impediment for intra-island speciation and thus limit the number of endemic taxa found in the
442 Azores. Estimation of divergence times revealed that, as expected based on island age, the oldest
443 divergence events in the Azores occurred in Santa Maria. However, speciation events on Santa Maria
444 have not been detected at least since 3.6 Ma, which contrasts with the range of *in situ* diversification
445 events extending to recent times within older islands of the Canaries (Emerson & Oromí, 2005). This
446 intriguing phenomenon of an intra-island speciation hiatus on the oldest Azorean island when the
447 island reached approximately middle age may also contribute to the smaller number of Azorean
448 endemics. Molecular phylogenetic data also support the possibility that cryptic speciation accounts in
449 part for the paucity of endemics in the Azores, and that the current taxonomy of Azorean *Tarphius*
450 species should be revised. Our data provide evidence that although most Azorean *Tarphius* species are
451 the result of recent divergent events, *Tarphius* has had a long evolutionary history in the Azores, which
452 is not consistent with the hypothesis that the lack of endemics is a consequence of the archipelago
453 having been colonized only recently. The reconstruction of phylogenetic relationships for other
454 species-rich taxa on multiple Macaronesian archipelagos, including the Azores, will facilitate testing
455 whether our conclusions based on *Tarphius* are group specific, or can be applied in general to explain
456 the paucity of Azorean endemics.

457 **ACKNOWLEDGEMENTS**

458 We are indebted to A. Serrano (CBA, FCUL, Portugal) for his assistance with species determination,
459 to F. Pereira, A. Amorim, D. Amorim, J. Hortal, A. Valverde, A. Santos, M. Gomes, H. Fernandes, P.
460 Cardoso, I. Silva, S. Ribeiro, A. Serrano, C. Aguiar, C. Rego and M. Boieiro for their help with
461 specimens collection, and to P. Oromí and G. Gillerfors for providing specimens from entomological
462 collections. We thank J. Hortal, K. Triantis, F. Rigal and D. Rabosky for discussion and comments.
463 GMB-INRB provided access to their lab facilities; and S. Ho, C. Ruiz and F. Cicconardi provided
464 advice with the comparative rate analyses. Analyses with the software BEAST were carried out on the
465 High Performance Computing Cluster supported by the Research Computing Service at the University
466 of East Anglia, UK. This study was supported by grants 17.01-080203 (DRRFlorestais, Azores,
467 Portugal), M2.1.2/I/017/2007 (DRCT, Azores, Portugal), PDCT/BIA-BDE/59202/2004 and
468 PTDC/BIA-BEC/104571/2008 (FCT, Portugal), and a graduate student research grant from the
469 Department of Ecology and Evolutionary Biology, University of California, Los Angeles. I.R. Amorim
470 was supported by BD/5016/95 and BPD/29578/2006 grants (FCT, Portugal).

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678 Siphonaptera. *Zoologica Scripta*, **31**, 93-104.
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680 **SUPPORTING INFORMATION**

681

682 Additional Supporting Information may be found in the online version of this article:

683

684 **Appendix S1** Detailed DNA extraction and amplification methods.

685 **Appendix S2** Sequences used in the phylogenetic reconstructions.

686 **Appendix S3** Sequences used in the comparative substitution rate test.

687

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692

693 **BIOSKETCH**

694 **Isabel R. Amorim** is a post-doctoral fellow at the University of the Azores and her main research
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698

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701 and ecosystems (<http://cita.angra.uac.pt/biodiversidade/>).

702

703 Author contributions: I.R.A., P.A.V.B., R.K.W. and B.C.E. conceived the ideas; I.R.A., B.C.E. and
704 P.A.V.B. collected the data; I.R.A. and B.C.E. analysed the data; and I.R.A., B.C.E. and P.A.V.B. led
705 the writing.

706

708 **TABLES**

709 **Table 1** Estimated times of the most recent common ancestor (TMRCA) for endemic *Tarphius* beetles
 710 of the Macaronesian islands based on mitochondrial protein-coding sequences (COI + COII). Eight
 711 age constraints were used as priors. Nodes labelled with letters are from Fig. 3 in Emerson & Oromí,
 712 2005. Mean value and the 95% highest posterior density (HPD) intervals are presented for tree nodes
 713 (see Fig. 2) in million of years ago (Ma). See text for details.

714

tree node	clade	prior	mean TMRCA (Ma)	95% HPD interval (Ma)
I	Macaronesian islands		20.63	15.94-25.78
II	Madeira + Canary Islands	≤ 14.00	13.52	12.65-14.00
III	Azores	≤ 8.12	7.42	6.38-8.12
IV	<i>T. wollastoni</i> + <i>T. depressus</i>		3.33	2.24-4.54
V	<i>T. depressus</i> (São Miguel + Pico)		2.90	1.81-4.11
VI	<i>T. azoricus</i> + <i>T. tornvalli</i>	≤ 4.01	3.65	3.10-4.01
VII	<i>T. azoricus</i> + <i>T. tornvalli</i> (Azores Central island group)	≤ 3.52	2.93	2.31-3.52
VIII	<i>T. azoricus</i> + <i>T. tornvalli</i> (Faial + São Jorge + Pico)		1.55	0.93-2.26
B	<i>T. setosus</i> (El Hierro + La Gomera)	≤ 1.00	0.86	0.64-1.00
C	<i>T. quadratus</i>	≤ 2.00	1.32	0.74-2.00
E	El Hierro + La Gomera	≤ 1.00	0.90	0.74-1.00
F	La Palma + La Gomera	≤ 2.00	1.11	0.50-1.85

715

716

717 **FIGURES**

718 **Figure 1** Location of the study. Island subaerial ages in million of years ago (Ma) and *Tarphius*
719 species distributions for the Azores. Taxa in grey are new records and taxa in brackets were not
720 sampled. Inset – Macaronesian archipelagos with ages of emergent islands presented as a
721 range.

722

723 **Figure 2** Bayesian inference tree for *Tarphius* beetles of the Macaronesian islands, North Portugal and
724 Morocco based on mitochondrial DNA (COI, tRNA_{Leu} and COII). Clade credibility values are
725 shown for nodes with a posterior probability ≥ 0.95 and bootstrap support ≥ 0.70 in that order.
726 Clades I–VII relate to divergent time estimates.

727

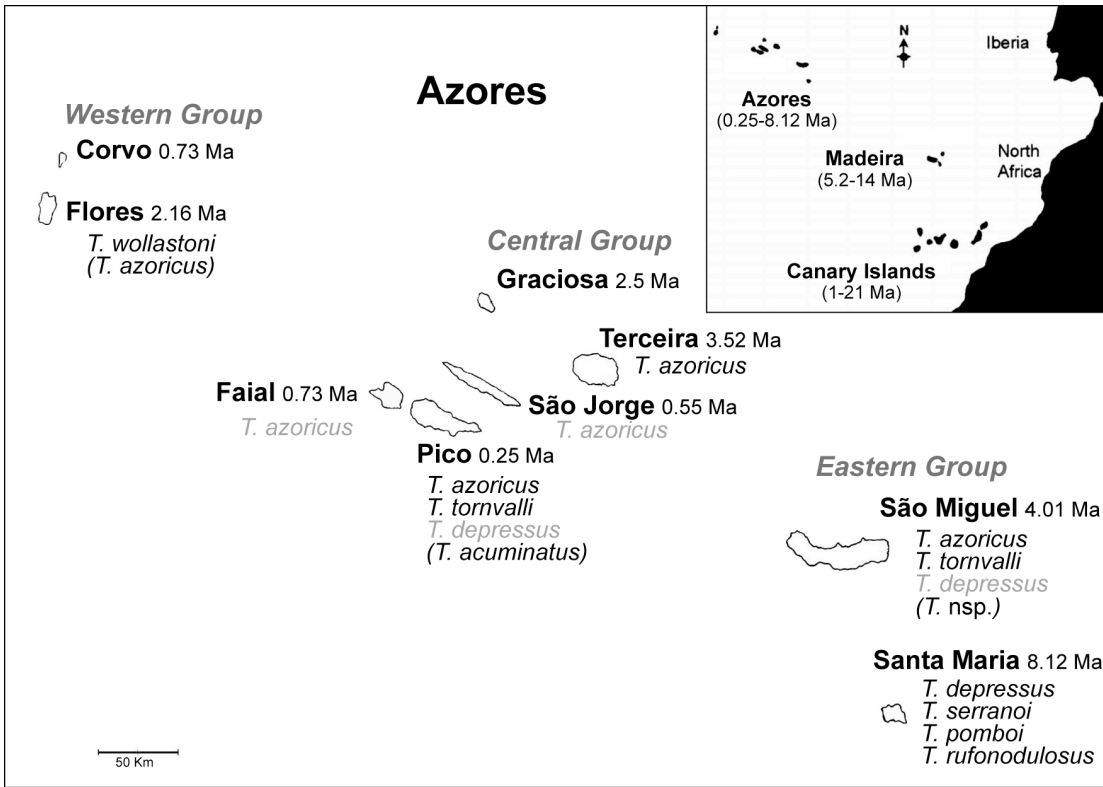
728 **Figure 3** Bayesian inference tree for *Tarphius* beetles of the Macaronesian islands, North Portugal and
729 Morocco based on nuclear DNA (EF1 α). Clade credibility values are shown for nodes with a
730 posterior probability ≥ 0.95 and bootstrap support ≥ 0.70 in that order.

731

732 **Figure 4** Weighted average relative nucleotide substitution rate for mtDNA (COII) of diverse
733 Coleoptera groups and the genus *Tarphius*. Diplura was used as an outgroup. Black bars
734 represent standard deviation.

735

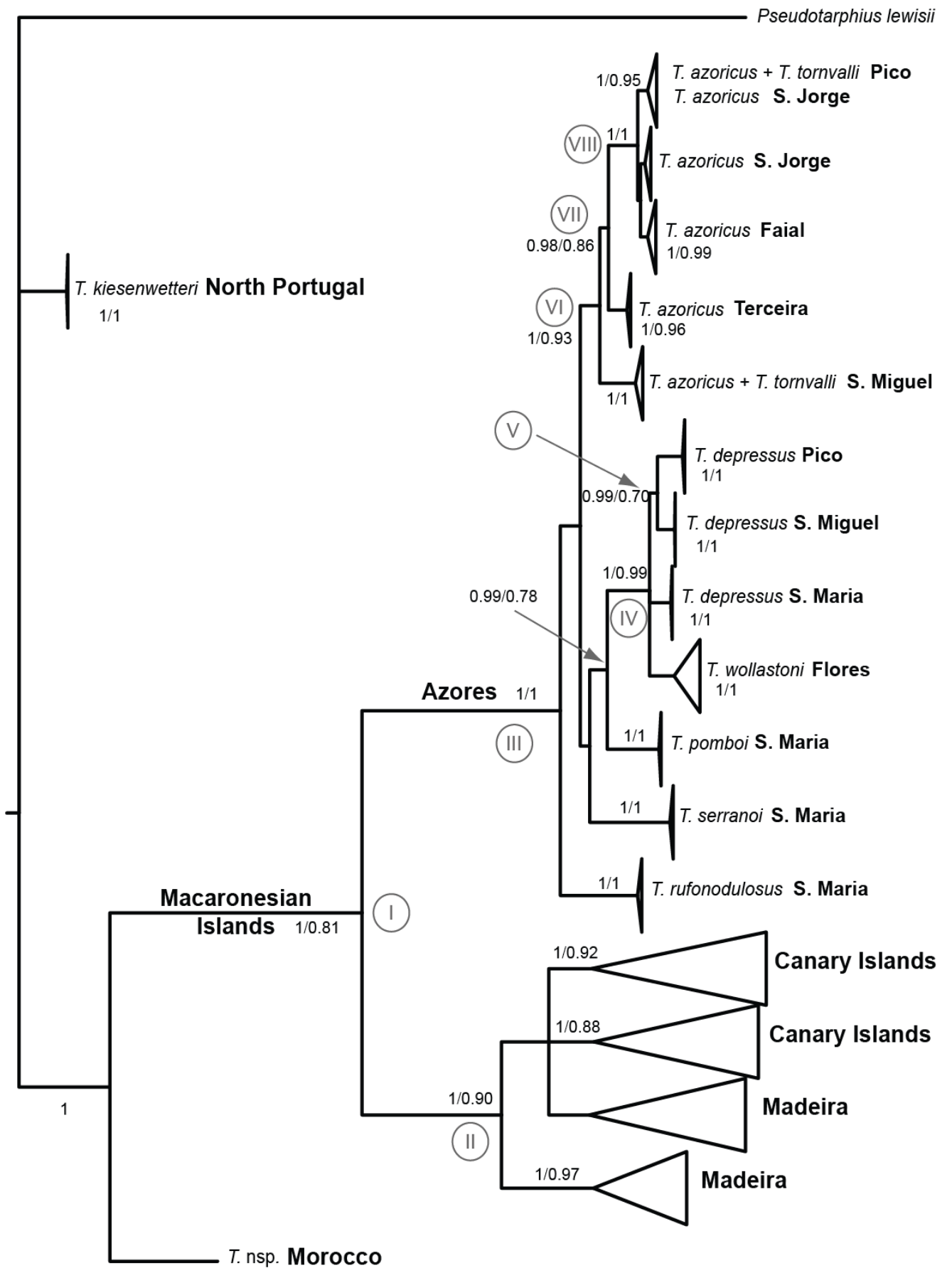
736 Fig 1



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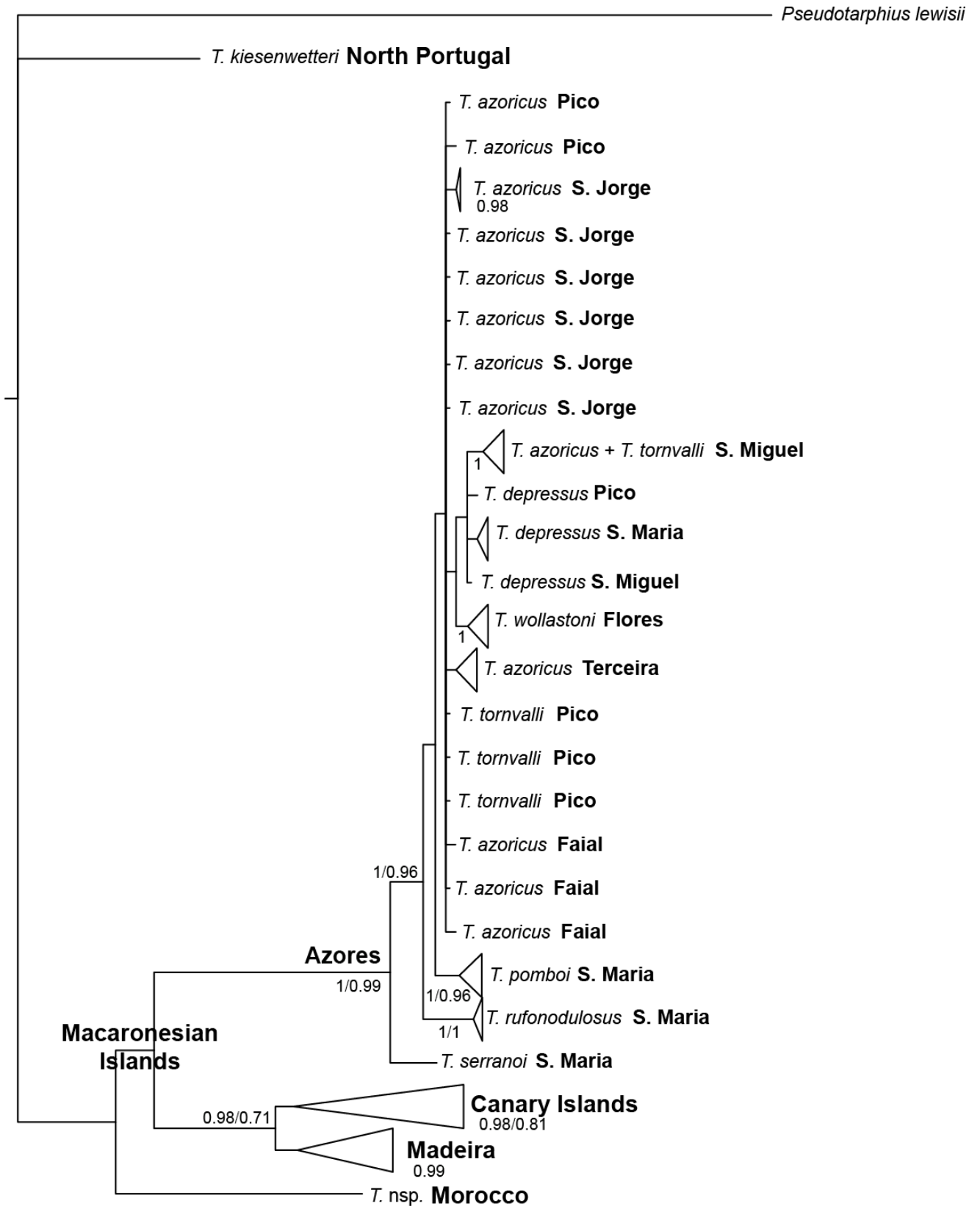
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739 Fig 2



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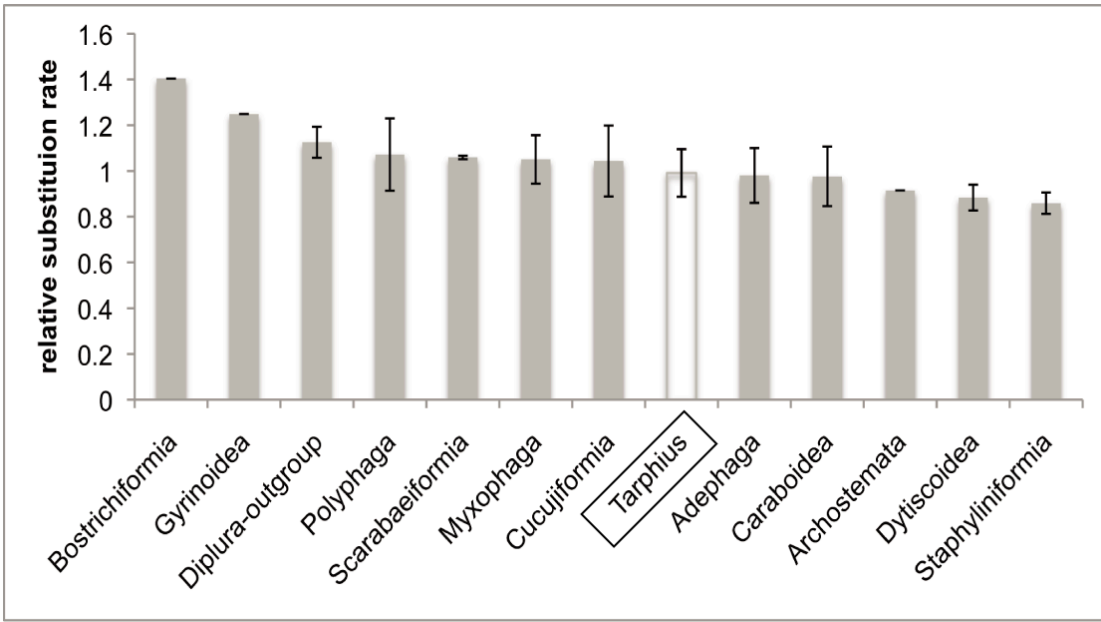
Fig 3



742

743

744 Fig 4



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