1 **Population genomics of *Bombus terrestris* reveals high but unstructured genetic diversity in a potential**

2 **glacial refugium**

3 Sara E. Silva[1], Sofia G. Seabra[1], Luísa G. Carvalheiro[1,2], Vera L. Nunes[1], Eduardo Marabuto[1], Raquel

4 Mendes[1], Ana S. B. Rodrigues[1], Francisco Pina-Martins[1], Selçuk Yurtsever[3], Telma G. Laurentino[4], Elisabete

5 Figueiredo[5], Maria T. Rebelo[6], Octávio S. Paulo[1]

6

7 [1] Centre for Ecology, Evolution and Environmental Changes (cE3c), Departamento de Biologia Animal,

8 Faculdade de Ciências, Universidade de Lisboa, 1749-016 Lisboa, Portugal.

9 [2] Department of Ecology, Universidade Federal de Goiás, Goiânia, Brazil.

10 [3] Biology Department, Science Faculty, Trakya University, Edirne, Turkey.

11 [4] Zoological Institute, University of Basel, Vesalgasse 1, 4051 Basel, Switzerland.

12 [5] Linking Landscape, Environment, Agriculture and Food (LEAF), Instituto Superior de Agronomia,

13 Universidade de Lisboa, Tapada da Ajuda, Lisboa, Portugal.

14 [6] Centre for Environmental and Marine Research (CESAM), Departamento de Biologia Animal, Faculdade

15 de Ciências, Universidade de Lisboa, Lisboa, Portugal.

16

17 **Running title**

18 Population genomics of an Iberian bumblebee.

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Abstract

Ongoing climate change is expected to cause temperature rise and reduction of precipitation levels in the Mediterranean region, which might cause changes in many species' distribution. These effects negatively impact species' gene pools, decreasing genetic variability and adaptive potential. Here we use mitochondrial DNA and RADseq to analyse population genetic structure and genetic diversity of the bumblebee species *Bombus terrestris* (subspecies *B. terrestris lusitanicus*), in the Iberian Peninsula. While this subspecies shows a panmictic pattern of population structure across Iberia and beyond, we found differentiation between subspecies *lusitanicus* and subspecies *africanus*, likely caused by the existence of barriers to gene flow between Iberia and North Africa. Furthermore, results revealed that the Iberian Peninsula harbours a large fraction of *B. terrestris* intraspecific genetic variation, with the highest number of mitochondrial haplotypes found when compared to any other region in Europe studied so far, suggesting a potential glacial *refugium* role for the Iberian Peninsula. Our findings strengthen the idea that Iberia is a very important source of diversity for the global genetic pool of this species, as rare alleles may play a role in population resilience against human or climate-mediated changes.

Introduction

As a consequence of several human-driven environmental changes (e.g. habitat loss, agricultural intensification, use of pesticides, the introduction of new parasites and climate change), insect pollinators have purportedly declined dramatically in recent decades (reviewed in Potts *et al.*, 2010). Moreover, the massive use of managed and commercial bees for crop pollination and honey production (e.g. *Apis mellifera*, *Bombus terrestris*) facilitate the introduction and spread of diseases and parasites (Goulson, 2010), and interfere with the genetic composition of natural populations through hybridization (Jaffé *et al.*, 2016; Seabra *et al.*, 2019).

Bumblebees (*Bombus* spp.) have been particularly affected worldwide by the above mentioned problems. Several species have endured dramatic changes in their distribution or became locally extinct in developed regions, such as central and western Europe and North America (Goulson *et al.*, 2008; Bommarco *et al.*, 2012). Although several studies have reported high levels of gene flow among European populations (Woodard *et al.*, 2015; Lecocq *et al.*, 2017), some species have experienced a decline on genetic diversity at a local scale (Woodard *et al.*, 2015), which might increase the risk of inbreeding and hinder population's ability to cope with environmental change (Goulson *et al.*, 2008; Maebe *et al.*, 2015).

Populations that have persisted in glacial refugia are expected to show higher levels of intraspecific genetic variation comparing to populations outside these regions, due to long-term population persistence and isolation (Hewitt, 1999). The Iberian Peninsula was one of the largest Mediterranean refugia during the Quaternary (2.6 Mya – present) glaciations, and is also at the southernmost latitude limit (rear edge) for many continental species ranges (Hewitt, 2000; Arias *et al.*, 2006). Therefore, many species in Iberia present geographically structured genetic lineages (Miraldo *et al.*, 2011; Rodrigues *et al.*, 2014; Chávez-Galarza *et al.*, 2015). Additionally, the proximity of Iberia to Africa, especially at the Strait of Gibraltar and with episodic bridges between the two continents, enabled occasional dispersal of the more vagile organisms, particularly during periods of glacial southern contraction or of postglacial northern expansion (Pinto *et al.*, 2013). However, according to Rasmont *et al.* (2015), the Iberian Peninsula is expected to experience major reductions of bumblebee suitable climatic conditions within the forthcoming decades, alongside other southern European regions. Intensive land-use regimes and degradation of semi-natural areas might be already impacting species richness patterns for many taxa across the peninsula (Martins *et al.*, 2014; Newbold *et al.*, 2015). Moreover, the use of commercial bumblebees for crop pollination, which is a

88 common practice in several parts of the peninsula, may negatively impact native natural populations through

89 pathogen spillover and introgression of maladaptive alleles (Murray *et al.*, 2013; Seabra *et al.*, 2019).

90   The buff-tailed bumblebee *Bombus terrestris* (Linnaeus, 1758) (Hymenoptera, Apidae) presents a wide

91 distribution in the West Palearctic region (Rasmont *et al.*, 2008), and has been deliberately introduced as a

92 crop pollinator into several areas worldwide (Ings *et al.*, 2005; Goulson, 2010). Nine subspecies were

93 described based on morphology, particularly in coat colour variation, with additional differences in

94 behaviour, phenology, physiological traits and resistance to parasites (see Figure 1; Rasmont *et al.*, 2008).

95 Mitochondrial and microsatellite variation studies have shown a clear differentiation of northern African and

96 islander *B. terrestris* populations from European mainland ones, with no differentiation amongst the latter. In

97 fact, mainland populations are largely homogeneous with nearly panmixia patterns (Estoup *et al.*, 1996;

98 Widmer *et al.*, 1998; Lecocq *et al.*, 2013b; 2016; Woodard *et al.*, 2015). Genetic cohesiveness found across

99 broad geographical scales has been attributed to the absence of effective barriers to gene flow and

100 bumblebees' great dispersal capability (Estoup *et al.*, 1996; Woodard *et al.*, 2015; Lecocq *et al.*, 2016). Flight

101 radius of *Bombus terrestris* males, for example, varies between 2.6 and 9.9 km (Kraus *et al.*, 2009). Queen

102 dispersal in *B. terrestris* is unknown but estimates for *Bombus pascuorum* (Scopoli, 1763) and *Bombus*

103 *lapidarius* (Linnaeus, 1758) queens show they are able to disperse by at least three and five km, respectively,

104 during their lifetime (Lepais *et al.*, 2010). In the case of *B. terrestris,* two hypotheses have been suggested to

105 explain the genetic homogeneity in European mainland: (1) a recent population expansion from a single

106 glacial refuge, although low sampling efforts hinder definite conclusions (Lecocq *et al.,* 2016); (2) the

107 erosion of past population structure due to genetic homogenisation linked to translocations of commercial *B.*

108 *terrestris*, but this is unlikely since the same pattern of genetic homogenisation was found in the early 1990s,

109 when bumblebee commercialization was barely a practice (Estoup *et al.*, 1996). Unfortunately, studies on *B.*

110 *terrestris* largely undersampled the Iberian Peninsula (see Estoup *et al.* (1996), Widmer *et al.* (1998),

111 Moreira *et al.* (2015) and Lecocq *et al.* (2016)), despite the importance of this area as a potential glacial

112 refuge for the species and its present location at the south-western edge of *B. terrestris* mainland distribution.

113   To address this gap, we explore the role of the Iberian Peninsula in the differentiation dynamics of *B.*

114 *terrestris*, by assessing if (1) Iberian *B. terrestris lusitanicus* (Krüger, 1956) is panmictic with the rest of the

115 *B. terrestris*' distribution, and (2) if Iberian populations of *B. terrestris* harbour standing genetic variation in

116 order to adapt to the currently changing environment. We will address these questions by determining: (i) the

117   level of genetic differentiation between *B. t. lusitanicus* and other recognised subspecies of *B. terrestris*; (ii)

118   the population genetic structure within the Iberian Peninsula and (iii) the levels of genetic diversity within *B.*

119   *t. lusitanicus*. We contrast the use of mitochondrial Cytochrome Oxidase I (COI) marker, which has been

120   commonly used to study inter- and intraspecific relationships in bumblebees (Lecocq *et al.*, 2013a, 2016;

121   Dellicour *et al.*, 2015; Moreira *et al.*, 2015), with a novel genome-wide dataset of restriction site-associated

122   DNA sequencing (RADseq). This method readily provides thousands of SNPs and has proven to be effective

123   in biogeography, adaptation, association and conservation studies, even when individual and population

124   sampling is limited (Lozier, 2014; Woodard *et al.*, 2015; Lozier & Zayed, 2016). With RADseq we obtained

125   the first comprehensive dataset of nuclear loci from *B. t. lusitanicus* and evaluated its population structure at

126   a finer scale resolution. This is the first step to investigate the spatial patterns of population structure and

127   genetic diversity on a bumblebee species within the Iberian Peninsula and to identify the main priorities for

128   future research on the Iberian bumblebees' conservation, evolution and environmental adaptation.

129

130   **Material and Methods**

131   **Sampling and DNA extraction**

132   A total of 198 individuals of *B. t. lusitanicus* were collected from six regions within the Iberian

133   Peninsula, covering most of *B. t. lusitanicus*' Iberian distribution and habitat heterogeneity. To minimize the

134   probability of sampling individuals from the same colony, individuals were caught from locations separated

135   by at least one km, within each region, whenever possible (Figure 1B; Table S1, Supporting Information).

136   Additional samples from other subspecies of *B. terrestris* were collected: one *B. t. terrestris* from

137   Switzerland, from Germany and from France, one *B. t. dalmatinus* from Turkey, one *B. t. audax* from Great

138   Britain and four *B. t. africanus* from Morocco (Figure 1A; Table S1, Supporting Information). We focused on

139   diploid individuals (females, mainly workers) for the genetic analyses, so that we would better capture the

140   genetic variation in the populations. The only exception was one male from France, from where we did not

141   collect females.

142   For the genetic analyses, *B. t. lusitanicus*' samples were grouped according to the six defined regions,

143   while samples from other subspecies were grouped according to the country where they were collected

144   (Table S1, Supporting Information). Thirteen females from closely related species were collected to serve as

145   outgroup: one individual of *Bombus hortorum* (Linnaeus, 1761), three of *B. lapidarius*, three of *Bombus*

146 *lucorum* (Linnaeus, 1761), four of *B. pascuorum* and two of *Bombus ruderatus* (Fabricius, 1775). Samples

147 were preserved for DNA extraction either in absolute ethanol and stored at -20ºC, or dried and preserved at -

148 80ºC. Total genomic DNA was extracted from fore and mid legs, the head, and for smaller individuals, a

149 portion of the thorax was also used. DNA was isolated with the DNeasy Blood & Tissue extraction kit

150 (Qiagen), following the manufacturer's standard protocol. To maximize DNA yield, some samples were

151 eluted in lower volume of buffer AE (minimum of 60 μl), and the eluted volume was transferred again to the

152 silica column of the kit for a second elution and incubation times with buffer AE were extended up to 30

153 min.

154

155 **COI amplification, RAD libraries construction and sequencing**

156 For all samples, a fragment from the mitochondrial *Cytochrome Oxidase I* gene (COI) was amplified by

157 Polymerase Chain Reaction (PCR) with the primer set LepF/LepR (Hajibabaei *et al.*, 2006). PCR

158 amplifications were carried out in 20 μL volumes containing approximately 10-45 ng of template DNA, 1x

159 reaction buffer, 1.8 mM of MgCl2, 1.0 mM dNTPs, 0.04 units of *GoTaq Flexi* DNA polymerase and 0.4 μM

160 of each primer. The thermocycling profile consisted of one cycle of one min at 94ºC, five cycles of 30 s at

161 94ºC, one min at 45ºC, and one min at 72ºC, followed by 30 cycles of one min at 94ºC, one min and 30 s at

162 50ºC, and one min at 72ºC, with a final step of five min at 72ºC. All PCR products were purified with

163 SureClean (Bioline) purification kit and sequenced in the forward direction on an ABI3730XL by Macrogen

164 Europe. DNA sequences were quality controlled with *Sequencher* version 4.0.5 (Gene Codes Corporation).

165 A subset of 55 individuals, including 37 individuals of *B t. lusitanicus* from the six defined Iberian

166 regions, five individuals from other *B. terrestris* subspecies and 13 individuals from outgroup species (see

167 Figure 1A and B; Table S1, Supporting Information) was used for RAD sequencing analyses. RADseq

168 libraries for Illumina paired-end sequencing were prepared following the protocol by Etter *et al.* (2011)

169 available at https://www.wiki.ed.ac.uk/display/RADSequencing/Home, with some minor modifications as

170 reported in Seabra *et al.* (2019). We used the restriction enzyme *Pst*I-HF (New England Biolabs).

171 Sequencing took place on a Illumina HiSeq 2000/2005 at Edinburgh Genomics, Ashworth Laboratories. The

172 55 individuals were run together with other 53 samples from another study (Seabra *et al.*, 2019), over two

173 lanes.

174

COI data analysis

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176   Mitochondrial COI sequences obtained in this study were deposited in GenBank (Acession numbers

177   MN652675 – MN652877; Table S1a, Supporting Information). We also included 17 sequences from our

178   previous study (Seabra *et al.*, 2019) and we followed the designation of haplotypes in that same study (Table

179   S1a, Supporting Information). In order to extend our *B. terrestris* COI dataset (207 sequences) into the

180   species' geographical range, we downloaded from GenBank five additional sets of COI sequences from

181   previous studies, namely Coppée (2010), Williams *et al.* (2012a, b), Moreira *et al.* (2015) and Schmidt *et al.*

182   (2015) (see Figure 1; see Table S1b, Supporting Information, for GenBank accession numbers). In this way, a

183   total of 233 COI sequences were added to our dataset, totalizing 441 sequences. Sequences related to

184   commercially reared or introduced populations were not considered.

185   COI dataset of *B. terrestris* was aligned in *MAFFT* version 7.271 (Katoh & Standley, 2013) using default

186   settings. The final alignment was checked for accuracy and sequences were trimmed to the same length (597

187   bp) to eliminate missing data, using *BioEdit* version 7.2.5 (Hall, 1999). Median-joining haplotype networks

188   were constructed in *PopART* version 1.7 (Bandelt *et al.*, 1999; Leigh & Bryant, 2015), in order to visualise

189   the relationship among *B. terrestris* haplotypes. We used *Arlequin* version 3.5.2.2 (Excoffier & Lischer,

190   2010) and only the individuals from the Iberian Peninsula to perform a standard analysis of molecular

191   variance (AMOVA) with 10,000 permutation steps and calculate haplotype (h) and nucleotide (π) diversities.

192   We also calculated haplotype and nucleotide diversities for the individuals of the remaining *B. terrestris*

193   distribution range in order to compare with the Iberian Peninsula. File format conversion for *PopART* and

194   *Arlequin* were performed using *PGDSpider* version 2.1.0.3 (Lischer & Excoffier, 2012).

195

RADseq data analysis

196

197   RADseq data obtained in this study are available at Sequence Read Archive (PRJNA578045). All

198   console commands used for RADseq data filtering, SNP discovery and subsequent analyses are available in

199   Appendix 2. Quality control of the RADseq raw read data was performed using *FastQC* version 0.11.3

200   (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). The script *process_radtags* implemented in

201   *Stacks* version 1.29 (Catchen *et al.*, 2013) was used (with default settings) to remove low quality (with a

202   phred quality score below 33) and unidentifiable reads as well as to demultiplex the data. *Bowtie2* version

203   2.1.0 (Langmead & Salzberg, 2012) was used to trim the last three bases from the 3' end of each read before

204   alignment, as this region is richer in low-quality base calls, and to align each sample's reads to the assembled

205   reference genome of *B. terrestris* (NCBI Assembly GCA_000214255.1) with the "sensitive" option.

206   *SAMtools* version 0.1.19 (Li *et al.*, 2009) was used to remove low quality alignments (mapping quality below

207   20) and any unmapped reads.

208   After data filtering, RAD loci were identified and SNPs called using the *Stacks* pipeline 1.45 (Catchen *et*

209   *al.*, 2013). In order to minimize the impact of differences among taxa in the number of SNPs obtained and

210   the amount of missing data, three datasets were created from the initial 55 individuals for different analyses

211   (see Table S1, Supporting Information): (1) BT_OUT: includes all the studied subspecies (all *B. terrestris*) as

212   well as the outgroup species; (2) BT_SSP: includes all *B. terrestris* subspecies and does not include the

213   outgroup species; (3) BT_BTL: includes the Iberian *B. t. lusitanicus* only.

214   Preliminary tests were carried out to identify optimal *Stacks* parameters (Appendix 1, Figures S3, S4, S5,

215   Supporting Information). In *pstacks,* minimum stack depth was set to six; in *cstacks,* maximum number of

216   mismatches allowed when building catalog loci was two; in *populations,* individuals were grouped into

217   putative locations based on geographical region (see Table S1, Supporting Information) and one random SNP

218   per RAD locus was used to avoid confounding signals of linkage disequilibrium. SNPs were only retained if

219   they were present in 50% of individuals in at least n-1 (where n is the number of geographic regions) in all

220   datasets. To test for differences among outputs with different randomly selected SNPs, module *populations*

221   was run several times, but no significant differences were obtained in the final results (data not shown).

222   Finally, we performed an additional filtering step to remove loci with minor allele frequencies <0.05 and

223   more than 25% of missing data across all samples using *VCFtools* version 0.1.15 (Danecek *et al.*, 2011).

224   *VCFtools* was also used to obtain the mean coverage per site per individual. The resulting SNP datasets were

225   then used in subsequent population genomic and phylogeographic analyses: dataset BT_OUT to check for

226   differentiation between *B. terrestris* and outgroup species; dataset BT_SSP to determine differentiation

227   between *B. t. lusitanicus* and the other subspecies; dataset BT_BTL to determine population genetic structure

228   and genetic diversity of *B. t. lusitanicus* within the Iberian Peninsula.

229   Principal component analyses (PCA) of the three RADseq datasets were performed using the package

230   SNPRelate version 1.12.0 (Szulkin *et al.*, 2016) as implemented in the *R* script *snp_pca_static.R*

231   (https://github.com/CoBiG2/RAD_Tools) as of commit "bb2fc45". To test for differentiation between *B. t.*

232   *lusitanicus* and the other subspecies and within *B. t. lusitanicus*, clustering analyses of population structure

233 were performed using the datasets BT_SSP and BT_BTL and *MavericK* version 1.0.4 (Verity & Nichols,

234 2016). As the model used by *MavericK* assumes that markers are neutral (Verity & Nichols, 2016), we first

235 performed an outlier analysis of both datasets to identify and remove non-neutral markers, using both

236 *BayeScan* version 2.1 (Foll & Gaggiotti, 2008) and *SelEstim* version 1.1.7 (Vitalis *et al.*, 2014). *BayeScan*

237 was run using a matrix of SNP genotypes, with prior odds for the neutral model turned to five and assuming

238 a detection threshold of 0.05. The remaining parameters were set to default values. Plots and convergence

239 were checked using the *R* script *plot_R.r* available within the *BayeScan* package, and the package CODA

240 version 0.19-1 (Plummer *et al.*, 2006). *SelEstim* was run after randomizing the reference allele for each locus

241 (using the *R* script *SelEstim.R* available within *SelEstim* package) and using 50 pilot runs of 1 K length,

242 followed by a main run of 1 M length with a burnin of 100 K, a thinning interval of 20, and a detection

243 threshold of 0.01. The *R* script *SelEstim.R* was also used to obtain the list of outliers and check for

244 convergence. The total number of outliers identified by *BayeScan* and *SelEstim* were removed from the

245 datasets using the *Python* script *outlier_removal.py* (https://github.com/CoBiG2/RAD_Tools) as of commit

246 "ba731f2". Datasets with only neutral markers were analysed using *MavericK* version 1.0.4 (Verity &

247 Nichols, 2016), wrapped under *Structure_threader* version 1.2.4 (Pina-Martins *et al.*, 2017) for values of "K"

248 between one and five for dataset BT_SSP, and values between one and four for the dataset BT_BTL. We first

249 performed a single "pilot" run of 5,000 iterations, with a burnin of 500 using an admixture model, a free

250 alpha parameter of one with a standard deviation of the normal proposed distribution of 0.10 and

251 "thermodynamic integration" (TI) turned off. Posterior median and posterior standard deviation of alpha

252 were obtained from the "pilot" run and used in a "tuned" run as parameters for the admixture model as

253 follows: alpha was set to 10 times the posterior median and alphaPropSD to five times the posterior SD. This

254 "tuned" run was comprised of five runs of 10,000 iterations, with a burnin of 2000, with TI turned on and

255 another set of 20 rungs of 10,000 samples with a burnin of 2000. The most suitable value of "K" was

256 calculated for both datasets using the TI method. The R script *MavericK1.0_functions.R* available within the

257 *MavericK* package was used to produce diagnostic plots in order to check for convergence and

258 autocorrelation, and the Qmatrix plots.

259   Finally, a locus-by-locus AMOVA was performed in *Arlequin* with RADseq dataset BT_BTL, which

260 includes all the SNPs, in order to examine the variance within and among geographical regions, and

261 significance was calculated using 10,000 permutation steps. Genome-wide measures of genetic diversity,

262 including per-SNP nucleotide diversity (π_{SNP}), and the mean per-individual observed and expected

263 heterozygosities (H_O and H_E) were calculated using *VCFtools* and the same dataset.

264   File format conversions for *BayeScan*, *MavericK* and *Arlequin* were performed using *PGDSpider*

265 whereas file format conversion for *SelEstim* was performed with the bash script *GESTE2SelEstim.sh* (https://

266 github.com/Telpidus/omics_tools) as of commit "f74f66b".

267

Results

269   The final alignment of the mitochondrial COI dataset included no indels, and consisted of a total of 26

270 variable sites, of which nine were parsimony-informative. A total of 16 haplotypes were found (Table S1a

271 and S1b, Supporting Information; Figure 2).

272   After Illumina sequencing of RAD libraries for 55 individuals, we obtained an average of 7.7 M paired

273 end reads of 125 bp, per individual. Of those, an average of 7.4 M were retained after filtering with

274 *process_radtags*, representing approximately 8.18% of the genome sequenced. Quality scores of the retained

275 reads ranged from 36 to 37, with a GC content of ~40%. An average of 52.69% of the quality-filtered reads

276 aligned to the *B. terrestris* genome. Of the 55 sequenced individuals, nine were excluded from further

277 analyses (six *B. t. lusitanicus* from four of the Iberian defined regions and three representatives of the

278 outgroup species) due to lower mean coverage per individual (≤12x). Missing data of the remaining samples

279 averaged 5.4%. Information concerning the output of RADseq filtering steps for each sample (number of

280 initial and mapped reads, coverage, datasets at which samples belong and missing data) are provided in Table

281 S1 (Supporting Information). Final individual counts and statistics per dataset after filtering were as follows:

282 BT_OUT dataset comprises 46 individuals and 5,357 SNPs (aprox. 22.7 markers/ Mb) with a mean coverage

283 of 57.7X per site, per individual; BT_SSP dataset comprises 36 individuals and 10,765 SNPs (aprox. 45.6

284 markers/ Mb) with a mean coverage of 52.3X per site, per individual; BT_BTL dataset comprises 31

285 individuals and 11,369 SNPs (aprox. 48.2 markers/ Mb) with a mean coverage of 56.6X per site, per

286 individual.

287

Differentiation of *Bombus terrestris lusitanicus*

289   Seven of the 16 *B. t. lusitanicus'* COI haplotypes are present in the Iberian Peninsula. The most common

290 haplotype (H1) is common to the entire Peninsula and to the remaining European mainland regions analysed

291   whereas H2 is shared with central Europe (Switzerland) and Ireland (Figure 2). The other five haplotypes

292   found in Iberia are exclusive to this peninsula (Figure 2). No shared haplotypes were detected between the

293   Iberian Peninsula and North Africa. Haplotypes H9 and H16 are exclusive of North Africa and H14 is shared

294   between this region and Italy. Some haplotypes found in Great Britain, Sardinia, Corsica and Canary Islands

295   are also exclusive from these regions (Figure 2A). Most of the haplotypes differ from H1 in only one or two

296   mutational steps, with the exception of the haplotypes found only in islands or in North Africa, the

297   haplotypes from Sardinia and Morocco being the most differentiated (Figure 2C).

298   PCA of RADseq dataset BT_OUT revealed a clear separation between *B. terrestris* and the outgroup

299   species, with the exception of *B. lucorum* which is the closest to *B. terrestris* (Figure 3A). PCA using dataset

300   BT_SSP, with *B. terrestris* samples only, revealed a very narrow separation between samples from the

301   Iberian Peninsula and those from Great Britain and Germany across EV2 (EV2 explains 3.08% of the

302   variance). On the other hand, individuals from Morocco show a greater separation across EV1 from the

303   remaining samples (EV1 explains 10.14% of the variance; Figure 3B). Outlier tests of dataset BT_SSP

304   revealed that a total of 44 SNPs were non-neutral: 43 SNPs (0.40% of the total SNPs) when using *SelEstim*,

305   and two SNPs (0.02% of the total SNPs) when using *BayeScan* (one SNP was identified by both softwares).

306   Clustering analyses of the dataset BT_SSP with non-neutral loci removed, using *MavericK*, determined the

307   existence of two groups ($K = 2$) as the most likely scenario (Figure 3C). These two groups correspond to i)

308   the individuals from the Iberian Peninsula, Great Britain and Germany, and ii) individuals from Morocco,

309   which is consistent with what was observed in the PCA (Figure 3B).

310

311   **Iberian populations genetic structure**

312   The most common COI haplotype H1 is present in 95% of the *B. terrestris* sampled in the Iberian

313   Peninsula. H2 is present in three samples from IP-NO and IP-WE regions, while H3 is only present in two

314   samples from southern locations, one IP-SE and another from IP-SW, respectively. All the remaining Iberian

315   COI haplotypes (H4-H7) are represented by a single sample (Figure 2B). The Iberian region with the highest

316   haplotype diversity is IP-SE with five haplotypes (Figure 2B and Table S2, Supporting Information). The

317   haplotype network did not show a structured phylogeographic pattern, with the most common haplotype

318   being shared among geographically distant regions, and the less frequent and unique haplotypes being

319   closely related to H1, in a 'star-like' configuration (Figure 2C).

320   PCA of RADseq dataset BT_BTL showed no clear separation between any Iberian geographic regions

321   (EV1 and EV2 only explain 3.75% and 3.67% of the variation, respectively; Figure 3D). The most

322   segregated individuals are BTL_075 (IP-NW) and BTL_136 (IP-SE) along EV1, and BTL_306 (IP-WE)

323   along EV2. Missing data values for these individuals does not explain their separation from the remaining

324   (3.7%, 5.7% and 10.6% respectively), and their COI haplotype is the most common one, H1. Outlier tests of

325   dataset BT_BTL revealed a total of 31 non neutral SNPs: 29 (0.26% of the total SNPs) when using *SelEstim*,

326   and two SNPs (0.02% of the total SNPs) when using *BayeScan*. Clustering analyses of the dataset BT_BTL

327   with non-neutral loci removed, using *MavericK*, determined the existence of one group (K = 1) as the most

328   likely scenario (data not shown), which is concordant with the PCA results.

329

330   **Genetic diversity**

331   AMOVA results revealed an absence of genetic structure for *B. terrestris* COI dataset, suggesting that the

332   overall source of variation was within geographical regions instead of among these (Table 1). Measures for

333   haplotype (*h*) and nucleotide (π) diversities calculated using *B. terrestris* COI dataset for the total Iberian

334   Peninsula were 0.08470 and 0.00018, respectively (Table 2). More than 43% (7 in 16) of the haplotypes

335   found were present in the Iberian Peninsula, and 31% (5 in 16) were exclusive of this area and no other

336   European region showed such a high number of haplotypes. It is important to note, however, that sample

337   sizes differ substantially (Figure 2 and Table 2).

338   AMOVA results using the RADseq dataset BT_BTL mirror those of the COI dataset, with an absence of

339   genetic structure, indicating that the overall source of variation is within and not among geographical regions

340   (Table 1). Measures of per-SNP nucleotide diversity and mean per-individual observed and expected

341   heterozygosities for the total Iberian Peninsula were 0.2780, 0.2326 and 0.2773, respectively (Table 2).

342

343   **Discussion**

344   We conducted a population genetic study, with mitochondrial and nuclear genome-wide markers, to

345   measure differentiation of *B. t. lusitanicus* from other *B. terrestris'* subspecies, and to investigate this

346   subspecies' population structure and genetic diversity within the Iberian Peninsula. We found no evident

347   differentiation pattern on mitochondrial DNA between *B. t. lusitanicus* and the other European mainland

348   subspecies *B. t. terrestris*, *B. t. dalmatinus* and *B. t. calabricus*. On the other hand, we found a clear

349   differentiation of North African *B. t. africanus* from the remaining subspecies, including the geographically

350   close *B. t. lusitanicus*, which is in accordance with previous studies (Coppée, 2010; Lecocq *et al.*, 2016) and

351   confirmed here with samples from southern Iberia, where any evidence of admixture with *B. t. africanus*

352   would be more likely to occur. Considering the genetic diversity of *B. terrestris* at the species level, it is

353   homogeneous across mainland populations, while subspecies from the islands and North Africa appear to be

354   more differentiated, although with evidence of some admixture, particularly between the British and

355   continental populations (as previously reported by Moreira *et al.*, 2015). The presence of the same haplotype

356   in both Algeria and central Italy could also indicate some admixture in this region. Our study corroborates

357   those of Estoup *et al.* (1996), Widmer *et al.* (1998), Moreira *et al.* (2015) and Lecocq *et al.* (2016) based on

358   mitochondrial and microsatellite markers. Our genome-wide analyses with RAD sequencing show very

359   slight distinction of *B. t. lusitanicus* from *B. t. terrrestris* (Germany) and *B. t. audax* (Great Britain) samples

360   in the PCA, although not supported by *Maverick* results, but corroborates the clear differentiation from *B. t.*

361   *africanus*. However, larger sample sizes of *B. t. terrestris* and *B. t. audax* are needed to better evaluate this

362   small differentiation at the genomic level.

363   The lack of differentiation of *B. t. lusitanicus* from other European mainland subspecies can be explained

364   by: (1) a common origin with subsequent local differentiation, which is supported by the star-like pattern in

365   the mitochondrial COI network with rarer haplotypes deriving from a single ancestral haplotype (H1); (2)

366   high dispersal ability of these insects across large distances (Kraus *et al.*, 2009; Lepais *et al.*, 2010) and

367   extensive mountain ranges such as the Pyrenees; or (3) erosion of genetic differentiation caused by

368   hybridization with commercial hives from allochthonous origin which are used in several areas in Europe for

369   crop pollination (commercial stocks used in the Iberian Peninsula include mostly subspecies *B. t. terrestris*

370   and *B. t. dalmatinus* (Lecocq *et al.*, 2016; Velthuis & van Doorn, 2006)). Putative hybrids with commercial

371   hives in the western Iberian Peninsula were already detected (Seabra *et al.*, 2019), but a widespread genetic

372   erosion is not expected because the transfer of colonies of this species across Europe for crop pollination is a

373   relatively recent phenomena (Estoup, 1996).

374   On the other hand, the differentiation found between *B. t. lusitanicus* and *B. t. africanus* suggests that

375   gene flow between the Iberian Peninsula and North Africa is much lower than to elsewhere in mainland

376   Europe. The number of accumulated differences on mitochondrial DNA also suggests these two subspecies

377   probably started to diverge earlier than the others. The Mediterranean sea thus seem to be an effective barrier

to gene flow, even though the two continents are geographically very close at the Strait of Gibraltar (< 15 km at the closest point) and despite the good dispersal capability of bumblebees. According to the information retrieved from http://www.atlashymenoptera.net/, *B. terrestris* is currently present right up to the coast on both sides of the Strait. Both Moreira *et al.* (2015) and Estoup *et al.* (1996) reported evidence of *B. terrestris* migrating over sea, across the English Channel and between the Isle of Man and Ireland, though at recognisably very low rates. Also other bumblebee species, such as *Bombus jonellus* (Kirby, 1802) was found to be able to disperse over sea barriers up to 30 km (Darvill *et al.*, 2010). As bumblebees are known to disperse such large distances, two hypothesis might explain this result: 1) migration is conditioned by the prevailing wind conditions, characterised by strong winds from easterly or westerly directions, which are known to have an important role in dispersion patterns of several species (e.g. moth *Cornifrons ulceratalis* (Dantart *et al.*, 2009); 2) migration occurs between both continents, but local differences in environmental conditions, and/or sexual selection, may be acting against migrants and preventing effective gene flow. The Strait of Gibraltar seems to hinder the dispersal of other flying species between North Africa and Iberian Peninsula (e.g. the butterfly *Pararge aegeria* (Weingartner *et al.*, 2006)) while acted as a route of dispersal for others, mainly during lower sea level periods (e.g. the Iberian honey bee *Apis mellifera iberiensis* (Chávez-Galarza *et al.*, 2015).

We did not find population genetic structure within *B. t. lusitanicus* across the Iberian Peninsula, contrarily to what was reported for the Iberian honey bee *A. m. iberiensis* (Chávez-Galarza *et al.*, 2015). In this latter species, two highly divergent genetic lineages are observed which form a northeastern-southeastern cline, better explained by secondary contacts between divergent populations from distinct and isolated glacial refugia (Chávez-Galarza *et al.*, 2015). The panmictic pattern of *B. t. lusitanicus* within Iberia is likely due to *B. terrestris*' long-distance flights capability, coupled with the absence of effective geographical barriers to its dispersal. Long-distance flights of queens and males contribute towards regular gene flow and were suggested to be sufficient to maintain genetic cohesion of common bumblebees' species over large areas (Lepais *et al.*, 2010). Also, the fact that *B. terrestris* is a short-tongued generalist bumblebee (Chapman *et al.*, 2003), having a large foraging range (Walther-Hellwig & Frankl, 2000), probably increases its capacity to find suitable habitats under a variety of conditions. In addition, this species is tolerant to a broad range of climates (Penado *et al.*, 2016), from Mediterranean beaches under high temperatures to high mountains of crio-oromediterranean regimes. Thus, individuals are more capable to disperse and occupy a

407 vast majority of habitats when compared to other species, contributing to the observed large-scale

408 connectivity. The already referred hypothesis of a recent population expansion from a single periglacial

409 refuge in the Iberian Peninsula could also explain not only the observed pattern of homogeneity but also the

410 star-like pattern in the mitochondrial COI network. This refuge may have existed in the Iberian Peninsula or

411 elsewhere in Europe (as also referred by Estoup *et al.*, 1996), as the most common haplotype H1 is

412 widespread across the continent. Nonetheless, some rarer haplotypes could have evolved in a smaller refuge

413 in the Betic ranges of southern Spain ('refugia within refugia' paradigm of Gómez & Lunt (2007)), which

414 might explain the higher genetic diversity found in mitochondrial DNA for the region IP-SE (Figure 2B;

415 Table S2). This region is characterized by semi-arid low-lands drastically contrasting with steep changes in

416 vegetation and climate along an altitudinal cline. It is considered as a hotspot for Mediterranean biodiversity,

417 harbouring many endemic species or lineages, and so the high genetic diversity found in this region (five

418 haplotypes from a total of 10 in continental Europe) is not unexpected (Hewitt, 2011; Nunes *et al.*, 2014).

419 The hypothesis of admixture between this region and North Africa is unlikely because there are no

420 haplotypes shared between both regions as referred before. The use of commercial hives of *B. terrestris* for

421 crop pollination in Southeast of Spain (IP- SE), and in IP-SW and IP-WE, is a common practice (Cejas *et al.*,

422 2018). In these regions, commercial bumblebees have been found foraging outside greenhouses and on

423 natural habitats, and introgression between commercial and native bumblebees was detected (Cejas *et al.*,

424 2018, 2019; Seabra *et al.*, 2019; Trillo *et al.*, 2019). Thus some of the variation found in IP-SE could have

425 been artificially introduced. We found one COI haplotype (H3) in regions IP-SE and IP-SW which is also

426 relatively common in commercial stocks and in individuals collected from greenhouse areas investigated by

427 Seabra *et al.* (2019). This haplotype was found in two specimens: one collected from about 300 km from the

428 area where Cejas *et al.* (2018, 2019) detected potential hybrids between commercial and native populations,

429 based on morphological and mitochondrial 16S data; one collected from about 30 km from the area where

430 Seabra *et al.* (2019) also detected potential introgression between both and escaped individuals, based on

431 RADseq data. None of the other unique haplotypes from IP_SE were found within the commercial samples

432 analysed by Seabra *et al.* (2019). Moreira *et al.* (2015) found that commercially reared populations were

433 differentiated from the majority of the wild populations from Ireland, having the highest number of private

434 microsatellite alleles. Thus the introduction of non-native specimens can lead to changes in the genetic

435 structure of the native ones, and ultimately, increase the risk of displacement and the consequent loss of rare

436 beneficial alleles, especially in populations with low genetic diversity. In this way, we cannot underestimate

437 the potential impact of the use of allochthonous commercial bumblebees for local crop pollination,

438 independent of its origin.

439   The extended sampling in the Iberian Peninsula revealed this region as one of the richest in genetic

440 diversity for *B. terrestris*, with the highest number of mitochondrial COI haplotypes than any other region in

441 Europe studied so far (though some mainland regions remain undersampled). Iberia seems to be an important

442 source of diversity for the global genetic pool of this species, as rare alleles may play a role in population

443 resilience against human or climate-mediated changes (Barret & Schluter, 2007), especially at the extremes

444 of the species range. Since this is the first study evaluating population genetic diversity in *B. terrestris* with

445 RADseq, we were not able to compare our results with other regions in Europe regarding the diversity at the

446 genomic level. When comparing with North American *Bombus* species (Lozier, 2014; Jackson *et al.,* 2018),

447 despite the different RADseq markers used, *B. t. lusitanicus* from the Iberian Peninsula showed similar or

448 higher diversity values, even when comparing with *Bombus impatiens* Cresson, 1863, a common species in

449 eastern North America.

450   Further ecological studies comparing habitats, phenology and phenotypic characteristics of *B. terrestris*

451 from the south of the peninsula with those from North Africa could help to describe barriers to dispersion

452 and to gene flow within this species in more detail. Also, the absence of population genetic structure will

453 facilitate the analyses of the adaptive potential of *B. t. lusitanicus* to environmental changes within the

454 Iberian Peninsula, by finding adaptive genetic diversity and by modelling species response to future land-use

455 and/or climatic changes. Ecological and genetic studies focusing in arid regions or in other Mediterranean

456 peninsulas are also needed, in order to understand how environmental change is affecting natural populations

457 of *B. terrestris*. It could also help to evaluate impacts of global warming on crop pollination efficiency of

458 commercial hives at the extremes of this species range and in economically important regions.

459

471

Author's contributions

473   SES, SGS, LGC and OSP designed the study. SES, VLN, RM, ASB, EM, SY, TGL, EF, JM, MTR and

474   OSP were responsible for sampling. SES, SGS and VLN were responsible for DNA extraction and

475   mitochondrial DNA amplification. VLN constructed RAD libraries. SES and SGS performed the

476   bioinformatic analyses, with important contributions from VLN, FPM and OSP. SES wrote the manuscript

477   with contributions from all the other authors.

478

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662

663 **Figures and Tables**

664 **Figure 1.** Sampling locations of samples used in this study. A) Geographic distribution of each *B. terrestris*'

665 subspecies, according to Lecocq *et al.* (2016), here represented by different colours; geographic location of

666 collected samples of *B. t. terrestris, B. t. dalmatinus, B. t. audax* and *B. t. africanus*, as well as of COI

667 sequences (from previous studies of Coppée (2010), Williams *et al.* (2012a, b), Moreira *et al.* (2015) and

668 Schmidt *et al.* (2015)) downloaded from GenBank. B) Sampling locations of collected samples of *B. t.*

669 *lusitanicus* across the Iberian Peninsula (IP-NW: Iberian Peninsula, Northwest; IP-WE: Iberian Peninsula,

670 West; IP-SW: Iberian Peninsula, Southwest; IP-NO: Iberian Peninsula, North; IP-CE: Iberian Peninsula,

671 Centre; IP-SE: Iberian Peninsula, Southeast). B is a zoom-in of the black square in A. Sampling locations are

672 represented as follows: orange dots – samples used for COI analyses; yellow stars – samples used for both

673 COI and RAD analyses; black dots – COI sequences downloaded from GenBank.

674

675 **Figure 2.** Geographic distribution and frequency of mtDNA COI haplotypes. A) Map of haplotype

676 frequencies for *B. terrestris* across Europe and North Africa. B) Map of haplotype frequencies for each

677 Iberian geographic region. B is a zoom-in of the black square in A. C) Median-joining network representing

678 relationship among haplotypes, coloured by haplotype. Sequences from Coppée (2010), Williams *et al.*

679 (2012a, b), Moreira *et al.* (2015) and Schmidt *et al.* (2015) were also included. The size of the pie charts in

680 A, B and of the circles in C is in proportion to the haplotype frequencies. Each haplotype is represented by

681 the respective colour and designation (H1-H9 and H11-H17).

682

683 **Figure 3.** Principal components (PCA) and *MavericK* analyses of RADseq data. A) PCA of dataset

684 BT_OUT, comparing *B. terrestris* with other species; B) PCA of dataset BT_SSP comparing *B. t. lusitanicus*

685 samples with samples of other *B. terrestris'* subspecies; C) *MavericK* clustering plot of dataset BT_SSP, for

686 K=2; D) PCA of dataset BT_BTL comparing *B. t. lusitanicus* among Iberian geographical regions. In

687 *MavericK* results, estimated membership of each individual to each cluster are shown by vertical bars with

688 the clusters represented by different shades of grey.

689

690 **Table 1.** Results of the analyses of molecular variance (AMOVA) considering mitochondrial DNA COI data

691 and RADseq data.

692

693 **Table 2.** The sample size and diversity indices across *B. t. lusitanicus* samples from the Iberian Peninsula

694 considering mitochondrial DNA COI data and RADseq data, along with data for other populations of *B.*

695 *terrestris* or for other species (from Lozier, 2014 and Jackson *et al.*, 2018). Note that for RADseq markers, it

696 was also indicated the restriction enzyme used and the number of SNPs obtained.

697

698 **Supporting Information**

699

Table S1. List of samples used in this study: **Table S1a:** List of collected samples; **Table S1b:** List of samples with sequences downloaded from GenBank.

702

Table S2. The sample size and diversity indices across *B. t. lusitanicus* samples by Iberian region considering mitochondrial DNA COI data and RADseq dataset BT_BTL.

705

Appendix 1. Preliminary tests to identify optimal *Stacks* parameters: **Figure S1:** Results of parameter tests for the *Stacks* module *pstacks*; **Figure S2:** results of parameter tests for the *Stacks* module *ctsacks* and *sstacks*; **Figure S3:** results of parameter tests for the *Stacks* module *populations*.

709

Appendix 2. List of command line commands used for RADseq dataset analyses.

Figure 1
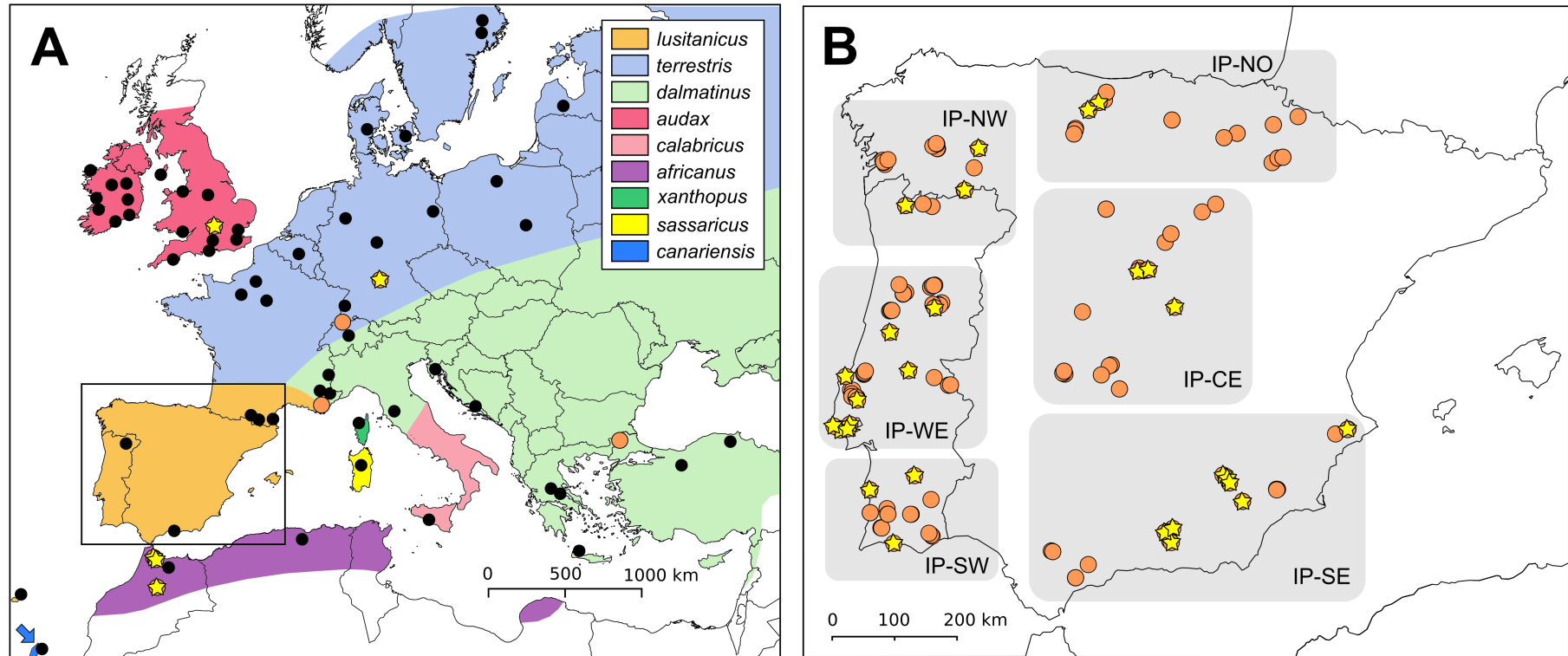
Figure 2
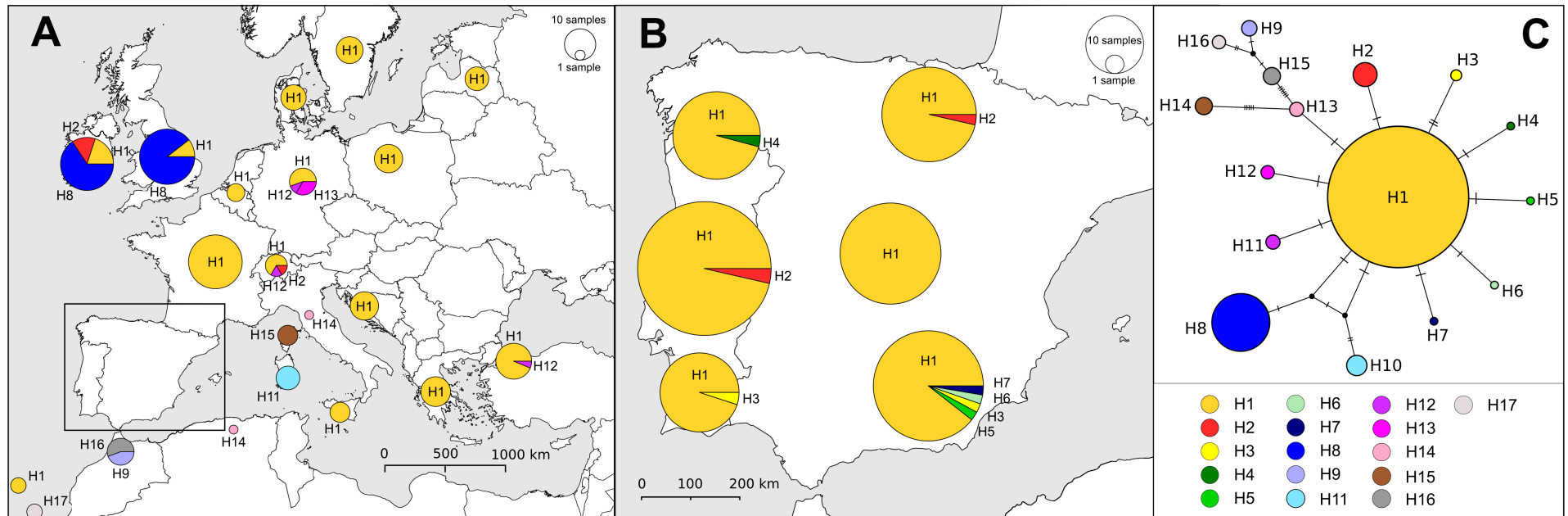
Figure 3
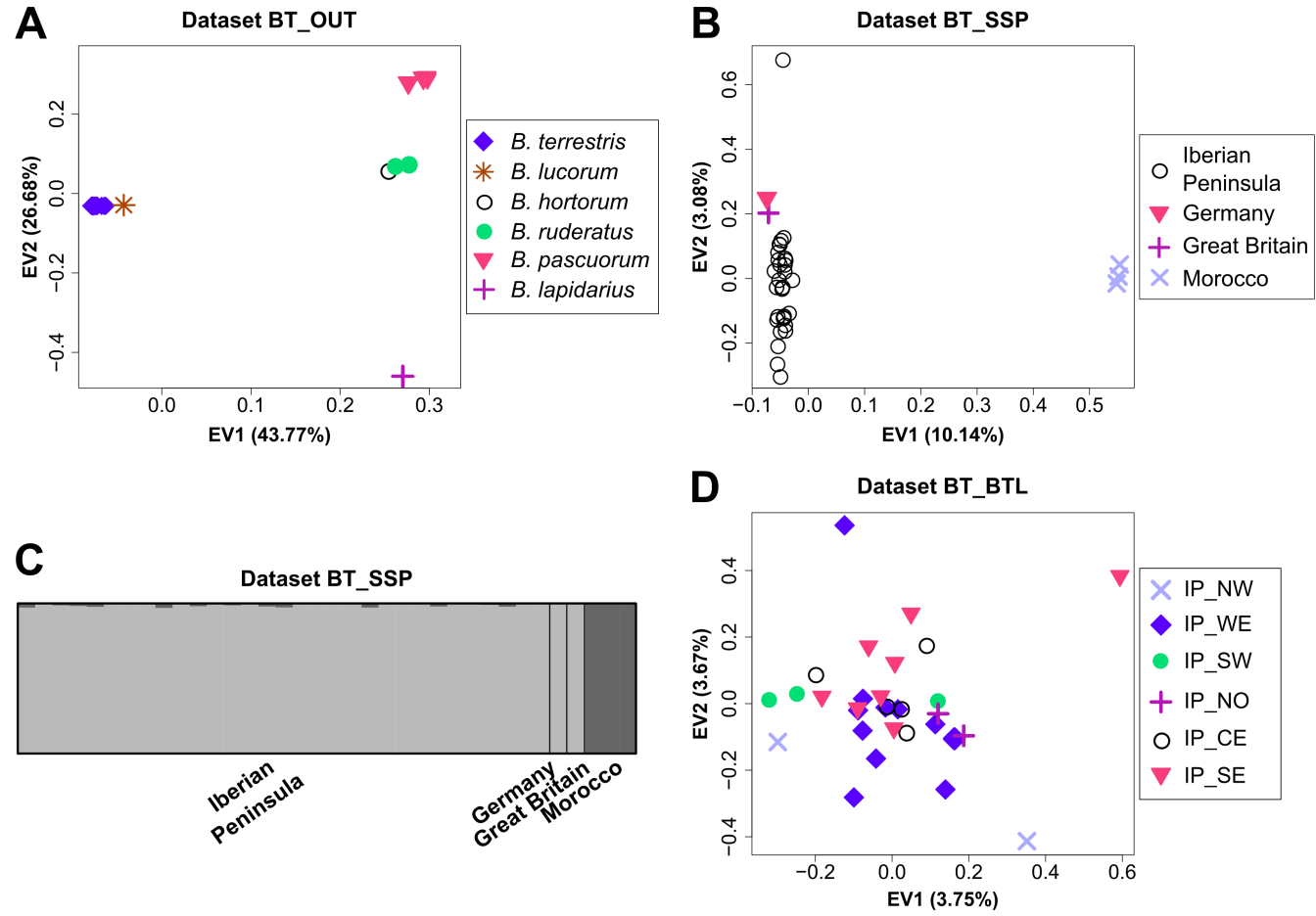
Table 1

DNA marker	Source of variation	Sum of squares	Variation components	Percentage of variation
COI	Within geographical regions	19.62	0.06	100.03
	Among geographical regions	0.27	-0.00002	-0.03
RAD seq	Within geographical regions	79454.05	1532.05	97.81
	Among geographical regions	9121.49	34.29	2.19

Table 2

Species	Geographic region	Reference	Sample size	DNA marker	h (nº of haplotypes)	π	π_{SNP}	H_O	H_E
B. t. lusitanicus	Iberian Peninsula	this study	208	COI	0.08470 (7 hap.)	0.00018	-	-	-
Bombus terrestris	Europe (continental)	this study	340	COI	0.09740 (10 hap.)	0.00034	-	-	-
Bombus terrestris	Europe (continental + islands) & north Africa	this study	441	COI	0.39900 (16 hap.)	0.00209	-	-	-
B. t. lusitanicus	Iberian Peninsula	this study	31 ♀	RAD seq, PstI (10,938 SNPs)	-	-	0.278	0.233	0.277
Bombus impatiens	eastern U.S.A.	Lozier *et al.* (2014)	24 ♀	RAD seq, SgrAI (2,387 – 9,148 SNPs)	-	-	0.136 – 0.289	n.d.	n.d.
Bombus pensylvanicus	eastern U.S.A.	Lozier *et al.* (2014)	24 ♀	RAD seq, SgrAI (3,240 – 9,376 SNPs)	-	-	0.135 – 0.276	n.d.	n.d.
Bombus bifarius	mountain regions of western U.S.A.	Jackson *et al.* (2018)	383 ♀	RAD seq, PstI (598 – 37,474 SNPs)	-	-	0.122 – 0.140	n.d.	n.d.
Bombus vosnesenskii	mountain regions of western U.S.A.	Jackson *et al.* (2018)	587 ♀	RAD seq, PstI (356 – 18,700 SNPs)	-	-	0.105 – 0.116	n.d.	n.d.

h, haplotype diversity; π, nucleotide diversity; π_{SNP}, per-SNP nucleotide diversity; H_O, mean per-individual observed heterozygosity; H_E, mean per-individual expected heterozygosity. "-" indicates "non-applicable"; "n.d." indicates "no data".

Table S1a. List of collected samples with information concerning: sampling (taxa, sex, country, number of samples, geographic groups, origin, date of collection and respective collectors); outputs of mitochondrial COI analyses (haplotypes, mapped reads, coverage, datasets at which belongs and missing data).

B. t. lusitanicus

Sample Code	Taxa	Sex	n	Country / Island	Geographic region	Sampling Location	Latitude	Longitude	Date of collection	Collector(s)	mtDNA analysis	COI haplotype	GenBank accession no.
BOM_GEH_002	*B. t. lusitanicus*	F	1	Portugal	**IP-NW** Iberian Peninsula, northwest (n=24)	Montalegre	41.72247	-7.68883	03/07/2017	S.E. Silva & R. Mendes	x	H1	MN652675
BOM_GEH_003	*B. t. lusitanicus*	F	1	Portugal		Montalegre	41.72247	-7.68883	03/07/2017	S.E. Silva & R. Mendes	x	H1	MN652676
BOM_GEH_006	*B. t. lusitanicus*	F	1	Portugal		Montalegre, Parafita	41.75589	-7.84843	03/07/2017	S.E. Silva & R. Mendes	x	H1	MN652677
BOM_GEH_009	*B. t. lusitanicus*	F	1	Portugal		Montalegre, Parafita	41.75589	-7.84843	03/07/2017	S.E. Silva & R. Mendes	x	H1	MN652678
BOM_GEH_028	*B. t. lusitanicus*	F	1	Portugal		P.N. Peneda-Gerês, Vilar da Veiga	41.73505	-8.159281	15/05/2014	T.G. Laurentino & O.S. Paulo	x	H1	MN652679
BTL_064	*B. t. lusitanicus*	F	1	Portugal		P.N. Peneda-Gerês, Vilar da Veiga	41.73505	-8.159281	15/05/2014	T.G. Laurentino & O.S. Paulo	x	H1	MN652680
BTL_065	*B. t. lusitanicus*	F	1	Portugal		P.N. Peneda-Gerês, Vilar da Veiga	41.73505	-8.159281	15/05/2014	T.G. Laurentino & O.S. Paulo	x	H1	MN652681
BTL_060	*B. t. lusitanicus*	F	1	Portugal		P.N. Montesinho, Pinheiro Novo	41.935667	-7.114667	13/05/2014	T.G. Laurentino & O.S. Paulo	x	H1	MN652682
BTL_061	*B. t. lusitanicus*	F	1	Portugal		P.N. Montesinho, Pinheiro Novo	41.935667	-7.114667	13/05/2014	T.G. Laurentino & O.S. Paulo	x	H1	MN652683
BTL_062	*B. t. lusitanicus*	F	1	Portugal		P.N. Montesinho, Pinheiro Novo	41.937944	-7.108444	13/05/2014	T.G. Laurentino & O.S. Paulo	x	H1	MN652684
BOM_GEL_002	*B. t. lusitanicus*	F	1	Spain		Pontevedra, Pazos de Borbén	42.277028	-8.525778	02/07/2016	S.E. Silva, R. Mendes & M.J. Dores	x	H1	MN652685
BOM_GEL_010	*B. t. lusitanicus*	F	1	Spain		Pontevedra, Pazos de Borbén	42.302028	-8.546667	02/07/2016	S.E. Silva, R. Mendes & M.J. Dores	x	H1	MN652686
BOM_GEL_016	*B. t. lusitanicus*	F	1	Spain		Pontevedra, Soutomaior	42.331472	-8.563556	02/07/2016	S.E. Silva, R. Mendes & M.J. Dores	x	H1	MN652687
BOM_GEL_021	*B. t. lusitanicus*	F	1	Spain		Pontevedra, Soutomaior	42.308389	-8.5495	02/07/2016	S.E. Silva, R. Mendes & M.J. Dores	x	H1	MN652688
BOM_GEL_026	*B. t. lusitanicus*	F	1	Spain		Pontevedra, Soutomaior	42.339139	-8.471333	02/07/2016	S.E. Silva, R. Mendes & M.J. Dores	x	H1	MN652689
BOM_MLL_001	*B. t. lusitanicus*	F	1	Spain		Monforte de Lemos, Canabal	42.48316	-7.59038	04/07/2017	S.E. Silva & R. Mendes	x	H1	MN652690
BOM_MLL_003	*B. t. lusitanicus*	F	1	Spain		Monforte de Lemos, Pantón	42.48619	-7.59861	04/07/2017	S.E. Silva & R. Mendes	x	H1	MN652691
BOM_MLL_010	*B. t. lusitanicus*	F	1	Spain		Monforte de Lemos, Pantón	42.51736	-7.67458	04/07/2017	S.E. Silva, R. Mendes & M.J. Dores	x	H1	MN652692
BOM_MLL_011	*B. t. lusitanicus*	F	1	Spain		Lugo, Monforte de Lemos	42.54425	-7.603667	03/07/2016	S.E. Silva, R. Mendes & M.J. Dores	x	H1	MN652693
BOM_MLL_026	*B. t. lusitanicus*	F	1	Spain		Lugo, Monforte de Lemos	42.549833	-7.612667	03/07/2016	S.E. Silva, R. Mendes & M.J. Dores	x	H1	MN652694
BTL_075	*B. t. lusitanicus*	F	1	Spain		Ourense, Serra da Enciña da Lastra	42.486833	-6.860333	12/05/2014	E. Marabuto	x	H1	MN652695
BTL_076	*B. t. lusitanicus*	F	1	Spain		Ourense, Serra da Enciña da Lastra	42.486833	-6.860333	12/05/2014	E. Marabuto	x	H1	MN652696
BOM_MLH_010	*B. t. lusitanicus*	F	1	Spain		Ourense, A Veiga	42.232389	-6.941028	03/07/2016	S.E. Silva, R. Mendes & M.J. Dores	x	H1	MN652697
BOM_MLH_016	*B. t. lusitanicus*	F	1	Spain		Ourense, Serra da Enciña da Lastra	42.486833	-6.860333	12/05/2014	E. Marabuto	x	H4	MN652698
BOM_SMH_003	*B. t. lusitanicus*	F	1	Portugal	**IP-WE** Iberian Peninsula, west (n=56)	Serra de São Mamede	39.310361	-7.380139	23/04/2017	S.E. Silva	x	H1	MN652699
BOM_SMH_004	*B. t. lusitanicus*	F	1	Portugal		Serra de São Mamede	39.310361	-7.380139	23/04/2017	S.E. Silva	x	H1	MN652700
BOM_SMH_005	*B. t. lusitanicus*	F	1	Portugal		Serra de São Mamede	39.308694	-7.393694	23/04/2017	S.E. Silva	x	H1	MN652701
BOM_SMH_006	*B. t. lusitanicus*	F	1	Portugal		Serra de São Mamede	39.308694	-7.393694	23/04/2017	S.E. Silva	x	H1	MN652702
BOM_SMH_013	*B. t. lusitanicus*	F	1	Portugal		Serra de São Mamede	39.312944	-7.359917	19/06/2016	S.E. Silva	x	H1	MN652703
BOM_SML_001	*B. t. lusitanicus*	F	1	Portugal		Portalegre, Gaféte	39.412861	-7.651167	23/04/2017	S.E. Silva	x	H1	MN652704
BTL_306	*B. t. lusitanicus*	F	1	Portugal		Abrantes, Mouriscas	39.508733	-8.103575	08/04/2012	E. Figueiredo	x	H1	MN652705
BTL_048	*B. t. lusitanicus*	F	1	Portugal		Coimbra, Larça	40.327167	-8.409417	05/05/2014	E. Marabuto	x	H1	MN652706
BTL_050	*B. t. lusitanicus*	F	1	Portugal		Coimbra, Rabaçal	40.031833	-8.435833	05/05/2014	E. Marabuto	x	H1	MN652707
BOM_CML_001	*B. t. lusitanicus*	F	1	Portugal		Mealhada, Pampilhosa do Botão	40.322861	-8.441917	21/05/2017	S.E. Silva	x	H1	MN652708
BOM_CML_002	*B. t. lusitanicus*	F	1	Portugal		Mealhada, Pampilhosa do Botão	40.331306	-8.423778	21/05/2017	S.E. Silva	x	H1	MN652709
BOM_CML_003	*B. t. lusitanicus*	F	1	Portugal		Mealhada, Pampilhosa do Botão	40.331306	-8.423778	21/05/2017	S.E. Silva	x	H1	MN652710
BOM_CML_006	*B. t. lusitanicus*	F	1	Portugal		Mealhada, Pampilhosa do Botão	40.328694	-8.40275	21/05/2017	S.E. Silva	x	H1	MN652711
BOM_CML_007	*B. t. lusitanicus*	F	1	Portugal		Mealhada, Pampilhosa do Botão	40.328694	-8.40275	21/05/2017	S.E. Silva	x	H1	MN652712
BOM_CMH_006	*B. t. lusitanicus*	F	1	Portugal		Serra do Caramulo	40.573694	-8.157056	21/05/2017	S.E. Silva	x	H1	MN652713
BOM_CMH_009	*B. t. lusitanicus*	F	1	Portugal		Serra do Caramulo	40.554528	-8.195139	21/05/2017	S.E. Silva	x	H1	MN652714
BOM_CMH_011	*B. t. lusitanicus*	F	1	Portugal		Serra do Caramulo	40.554528	-8.195139	21/05/2017	S.E. Silva	x	H1	MN652715
BOM_CMH_012	*B. t. lusitanicus*	F	1	Portugal		Serra do Caramulo	40.548806	-8.202083	21/05/2017	S.E. Silva	x	H1	MN652716
BOM_CMH_015	*B. t. lusitanicus*	F	1	Portugal		Serra do Caramulo	40.554167	-8.198556	01/07/2016	S.E. Silva, R. Mendes & M.J. Dores	x	H1	MN652717
BTL_316	*B. t. lusitanicus*	F	1	Portugal		Oliveira de Frades, Reigoso	40.67725	-8.274169	16/06/2013	E. Figueiredo	x	H1	MN652718
BOM_SEL_001	*B. t. lusitanicus*	F	1	Portugal		Penalva do Castelo	40.644139	-7.713361	04/07/2016	S.E. Silva, R. Mendes & M.J. Dores	x	H1	MN652719
BOM_SEL_002	*B. t. lusitanicus*	F	1	Portugal		Penalva do Castelo	40.644139	-7.713361	04/07/2016	S.E. Silva, R. Mendes & M.J. Dores	x	H2	MN652720
BOM_SEL_008	*B. t. lusitanicus*	F	1	Portugal		Penalva do Castelo	40.663583	-7.683472	04/07/2016	S.E. Silva, R. Mendes & M.J. Dores	x	H1	MN652721
BOM_SEL_013	*B. t. lusitanicus*	F	1	Portugal		Penalva do Castelo	40.671056	-7.641194	04/07/2016	S.E. Silva, R. Mendes & M.J. Dores	x	H1	MN652722
BOM_SEL_026	*B. t. lusitanicus*	F	1	Portugal		Penalva do Castelo	40.665444	-7.657806	04/07/2016	S.E. Silva, R. Mendes & M.J. Dores	x	H1	MN652723
BTL_203	*B. t. lusitanicus*	F	1	Portugal		Serra da Estrela	40.431731	-7.51575	11/06/2014	T.G. Laurentino	x	H1	MN652724
BTL_213	*B. t. lusitanicus*	F	1	Portugal		Serra da Estrela	40.365167	-7.642167	11/06/2014	E. Marabuto	x	H1	MN652725

						Location	Lat	Long	Date	Collector		Hap	Accession
BTL_217	*B. t. lusitanicus*	F	1	Portugal		Serra da Estrela	40.410833	-7.671833	11/06/2014	E. Marabuto	x	H1	MN652726
BTL_220	*B. t. lusitanicus*	F	1	Portugal		Serra da Estrela	40.365167	-7.642167	11/06/2014	E. Marabuto	x	H1	MN652727
BOM_SEH_002	*B. t. lusitanicus*	F	1	Portugal		Serra da Estrela	40.408528	-7.663583	04/07/2016	S.E. Silva, R. Mendes & M.J. Dores	x	H1	MN652728
BOM_SEH_007	*B. t. lusitanicus*	F	1	Portugal		Serra da Estrela	40.406306	-7.566139	04/07/2016	S.E. Silva, R. Mendes & M.J. Dores	x	H1	MN652729
BOM_SEH_010	*B. t. lusitanicus*	F	1	Portugal		Serra da Estrela	40.398917	-7.552556	04/07/2016	S.E. Silva, R. Mendes & M.J. Dores	x	H1	MN652730
BOM_SEH_027	*B. t. lusitanicus*	F	1	Portugal		Serra da Estrela	40.410833	-7.671833	11/06/2014	E. Marabuto	x	H1	MN652731
BOM_SEH_029	*B. t. lusitanicus*	F	1	Portugal		Serra da Estrela	40.365167	-7.642167	11/06/2014	E. Marabuto	x	H1	MN652732
BTL_001	*B. t. lusitanicus*	F	1	Portugal		Oeiras, Lage	38.709267	-9.311717	10/04/2014	E. Marabuto	x	H1	MHO18622
BTL_297	*B. t. lusitanicus*	F	1	Portugal		Oeiras	38.690789	-9.313989	25/07/2014	V.L. Nunes	x	H1	MHO18657
BTL_021	*B. t. lusitanicus*	F	1	Portugal		Cascais	38.743	-9.4307	27/04/2014	E. Marabuto	x	H1	MHO18623
BTL_037	*B. t. lusitanicus*	F	1	Portugal		Lisboa, near the airport	38.768117	-9.148017	01/05/2014	V.L. Nunes	x	H1	MHO18626
BTL_059	*B. t. lusitanicus*	F	1	Portugal		Lisboa, near the airport	38.768117	-9.148017	13/05/2014	V.L. Nunes	x	H1	MHO18627
BTL_324	*B. t. lusitanicus*	F	1	Portugal		Lisboa, Tapada da Ajuda	38.707639	-9.182306	13/04/2014	E. Figueiredo	x	H1	MHO18660
BTL_278	*B. t. lusitanicus*	F	1	Portugal		Bombarral, Portela	39.244861	-9.134778	17/07/2014	O.S. Paulo, S.E. Silva & A.S. Rodrigues	x	H1	MHO18652
BTL_281	*B. t. lusitanicus*	F	1	Portugal		Cadaval	39.240472	-9.099444	17/07/2014	O.S. Paulo, S.E. Silva & A.S. Rodrigues	x	H1	MHO18653
BTL_282	*B. t. lusitanicus*	F	1	Portugal		Cadaval, Vilar	39.188861	-9.112944	17/07/2014	O.S. Paulo, S.E. Silva & A.S. Rodrigues	x	H1	MHO18654
BTL_283	*B. t. lusitanicus*	F	1	Portugal		Alenquer, Vila Verde dos Francos	39.153389	-9.112583	17/07/2014	O.S. Paulo, S.E. Silva & A.S. Rodrigues	x	H1	MHO18655
BTL_288	*B. t. lusitanicus*	F	1	Portugal		Alenquer, Vila Verde dos Francos	39.153389	-9.112583	17/07/2014	O.S. Paulo, S.E. Silva & A.S. Rodrigues	x	H1	MHO18656
BOM_SAL_002	*B. t. lusitanicus*	F	1	Portugal		Alenquer, Abrigada	39.136833	-9.019722	16/04/2017	S.E. Silva	x	H1	MN652733
BOM_SAL_003	*B. t. lusitanicus*	F	1	Portugal		Alenquer, Abrigada	39.136833	-9.019722	16/04/2017	S.E. Silva	x	H1	MN652734
BOM_SAL_004	*B. t. lusitanicus*	F	1	Portugal		Alenquer, Abrigada	39.143333	-9.038556	16/04/2017	S.E. Silva	x	H1	MN652735
BOM_SAL_005	*B. t. lusitanicus*	F	1	Portugal		Alenquer, Abrigada	39.143333	-9.038556	16/04/2017	S.E. Silva	x	H1	MN652736
BOM_SAL_018	*B. t. lusitanicus*	F	1	Portugal		Alenquer, Ota	39.10375	-9.0115	26/06/2016	S.E. Silva	x	H1	MN652737
BTL_630	*B. t. lusitanicus*	F	1	Portugal		Foz do Arelho	39.429139	-9.223472	11/06/2014	B. Costa	x	H1	MHO18630
BOM_SAH_006	*B. t. lusitanicus*	F	1	Portugal		Serra de Aire e Candeeiros	39.457528	-8.900222	26/06/2016	S.E. Silva	x	H2	MN652738
BOM_SAH_008	*B. t. lusitanicus*	F	1	Portugal		Serra de Aire e Candeeiros	39.471139	-8.904333	26/06/2016	S.E. Silva	x	H1	MN652739
BOM_SAH_009	*B. t. lusitanicus*	F	1	Portugal		Serra de Aire e Candeeiros	39.471139	-8.904333	26/06/2016	S.E. Silva	x	H1	MN652740
BOM_SAH_013	*B. t. lusitanicus*	F	1	Portugal		Serra de Aire e Candeeiros	39.478861	-8.888917	26/06/2016	S.E. Silva	x	H1	MN652741
BOM_SAH_014	*B. t. lusitanicus*	F	1	Portugal		Serra de Aire e Candeeiros	39.503083	-8.872111	26/06/2016	S.E. Silva	x	H1	MN652742
BOM_MOH_001	*B. t. lusitanicus*	F	1	Portugal	**IP-SW**	Serra de Monchique	37.321593	-8.5957798	18/03/2017	S.E. Silva, A.S.B. Rodrigues & O.S. Paulo	x	H1	MN652743
BOM_MOH_002	*B. t. lusitanicus*	F	1	Portugal		Serra de Monchique	37.321593	-8.5957798	18/03/2017	S.E. Silva, A.S.B. Rodrigues & O.S. Paulo	x	H1	MN652744
BOM_MOH_008	*B. t. lusitanicus*	F	1	Portugal	Iberian	Serra de Monchique	37.317333	-8.583278	18/03/2017	S.E. Silva, A.S.B. Rodrigues & O.S. Paulo	x	H1	MN652745
BTL_200	*B. t. lusitanicus*	F	1	Portugal	Peninsula,	Silves, Armação de Pêra	37.102358	-8.368486	08/06/2014	V.L. Nunes	x	H1	MN652746
BTL_356	*B. t. lusitanicus*	F	1	Portugal	southwest	Sines, Porto Covo	37.854286	-8.793353	16/06/2014	E. Figueiredo	x	H1	MHO18670
BTL_357	*B. t. lusitanicus*	F	1	Portugal		Sines, Porto Covo	37.854286	-8.793353	16/06/2014	E. Figueiredo	x	H3	MHO18671
BTL_358	*B. t. lusitanicus*	F	1	Portugal	(n=20)	Sines, Porto Covo	37.854286	-8.793353	16/06/2014	E. Figueiredo	x	H1	MHO18672
BTL_346	*B. t. lusitanicus*	F	1	Portugal		Odemira, Zambujeira do Mar	37.534159	-8.785925	16/06/2014	E. Figueiredo	x	H1	MN652747
BTL_347	*B. t. lusitanicus*	F	1	Portugal		Odemira, Zambujeira do Mar	37.534159	-8.785925	16/06/2014	E. Figueiredo	x	H1	MN652748
BTL_349	*B. t. lusitanicus*	F	1	Portugal		Odemira, Zambujeira do Mar	37.534159	-8.785925	16/06/2014	E. Figueiredo	x	H1	MN652749
BOM_MOL_001	*B. t. lusitanicus*	F	1	Portugal		Odemira, Luzianes	37.5911472	-8.488128	18/03/2017	S.E. Silva, A.S.B. Rodrigues & O.S. Paulo	x	H1	MN652750
BOM_MOL_005	*B. t. lusitanicus*	F	1	Portugal		Odemira, Azinhaga do Calvário	37.514778	-8.476028	18/03/2017	S.E. Silva, A.S.B. Rodrigues & O.S. Paulo	x	H1	MN652751
BOM_MOL_007	*B. t. lusitanicus*	F	1	Portugal		Odemira, Santa Clara a Velha	37.513111	-8.471389	18/03/2017	S.E. Silva, A.S.B. Rodrigues & O.S. Paulo	x	H1	MN652752
BOM_CAH_002	*B. t. lusitanicus*	F	1	Portugal		Serra do Caldeirão, Alcaria do Cume	37.204217	-7.687183	22/05/2016	S.E. Silva, A.S.B. Rodrigues & O.S. Paulo	x	H1	MN652753
BOM_CAH_004	*B. t. lusitanicus*	F	1	Portugal		Serra do Caldeirão, Alcaria do Cume	37.219033	-7.705183	22/05/2016	S.E. Silva, A.S.B. Rodrigues & O.S. Paulo	x	H1	MN652754
BOM_CAH_006	*B. t. lusitanicus*	F	1	Portugal		Serra do Caldeirão, Alcaria do Cume	37.244667	-7.742817	22/05/2016	S.E. Silva, A.S.B. Rodrigues & O.S. Paulo	x	H1	MN652755
BOM_CAL_001	*B. t. lusitanicus*	F	1	Portugal		Mértola, Corte de Gafo de Cima	37.7194141	-7.7067368	19/03/2017	S.E. Silva, A.S.B. Rodrigues & O.S. Paulo	x	H1	MN652756
BOM_CAL_003	*B. t. lusitanicus*	F	1	Portugal		Almodôvar	37.5124	-8.057394	19/03/2017	S.E. Silva, A.S.B. Rodrigues & O.S. Paulo	x	H1	MN652757
BOM_CAL_009	*B. t. lusitanicus*	F	1	Portugal		Almodôvar	37.5127963	-8.0692471	19/03/2017	S.E. Silva, A.S.B. Rodrigues & O.S. Paulo	x	H1	MN652758
BTL_235	*B. t. lusitanicus*	F	1	Portugal		Beja, Beringel	38.055417	-7.9973	08/07/2014	R. Mendes	x	H1	MN652759
BOM_PIL_001	*B. t. lusitanicus*	F	1	Spain	**IP-NO**	Navarra, Caparroso	42.300444	-1.650056	11/06/2017	S.E. Silva, R. Mendes & A.S.B. Rodrigues	x	H1	MN652760
BOM_PIL_008	*B. t. lusitanicus*	F	1	Spain		Navarra, Mélida	42.360944	-1.546083	11/06/2017	S.E. Silva, R. Mendes & A.S.B. Rodrigues	x	H1	MN652761
BOM_PIL_009	*B. t. lusitanicus*	F	1	Spain	Iberian	Navarra, Mélida	42.360944	-1.546083	11/06/2017	S.E. Silva, R. Mendes & A.S.B. Rodrigues	x	H1	MN652762
BOM_PIL_011	*B. t. lusitanicus*	F	1	Spain	Peninsula,	Navarra, Carcastillo	42.371722	-1.457694	11/06/2017	S.E. Silva, R. Mendes & A.S.B. Rodrigues	x	H1	MN652763
BOM_PIL_012	*B. t. lusitanicus*	F	1	Spain	north	Navarra, Carcastillo	42.371722	-1.457694	11/06/2017	S.E. Silva, R. Mendes & A.S.B. Rodrigues	x	H1	MN652764
BOM_PIH_021	*B. t. lusitanicus*	F	1	Spain		Navarra, Abaurregaina	42.902528	-1.187833	12/06/2017	S.E. Silva, R. Mendes & A.S.B. Rodrigues	x	H1	MN652765
BOM_NAV_002	*B. t. lusitanicus*	F	1	Spain		Navarra, Pamplona	42.797	-1.627778	29/06/2014	E. Figueiredo	x	H1	MN652766
BOM_NAV_004	*B. t. lusitanicus*	F	1	Spain	(n=28)	Navarra, Pamplona	42.797	-1.627778	29/06/2014	E. Figueiredo	x	H1	MN652767
BTL_077	*B. t. lusitanicus*	?	1	Spain		Cantabria, Vega de Liébana	43.120833	-4.626	14/05/2014	E. Marabuto	x	H1	MN652768
BTL_078	*B. t. lusitanicus*	F	1	Spain		Cantabria, Vega de Liébana	43.120833	-4.626	14/05/2014	E. Marabuto	x	H1	MN652769
BTL_092	*B. t. lusitanicus*	F	1	Spain		Cantabria, Vega de Liébana	43.086333	-4.7245	14/05/2014	E. Marabuto	x	H1	MN652770

						Locality	Lat	Lon	Date	Collector			
BTL_094	*B. t. lusitanicus*	F	1	Spain		Cantabria, Vega de Liébana	43.086333	-4.7245	14/05/2014	E. Marabuto	x	H1	MN652771
BTL_099	*B. t. lusitanicus*	F	1	Spain		Cantabria, Vega de Liébana	43.086333	-4.7245	14/05/2014	E. Marabuto	x	H1	MN652772
BOM_CTL_002	*B. t. lusitanicus*	F	1	Spain		Navarra, Mendaza	42.684667	-2.270778	12/06/2017	S.E. Silva, R. Mendes & A.S.B. Rodrigues	x	H1	MN652773
BOM_CTL_003	*B. t. lusitanicus*	F	1	Spain		Navarra, Mendaza	42.684667	-2.270778	12/06/2017	S.E. Silva, R. Mendes & A.S.B. Rodrigues	x	H1	MN652774
BOM_CTL_010	*B. t. lusitanicus*	F	1	Spain		Álava, Bernedo	42.627278	-2.504111	12/06/2017	S.E. Silva, R. Mendes & A.S.B. Rodrigues	x	H1	MN652775
BOM_CTH_002	*B. t. lusitanicus*	F	1	Spain		Burgos, Merindad de Cuesta	42.858028	-3.4275	13/06/2017	S.E. Silva, R. Mendes & A.S.B. Rodrigues	x	H1	MN652776
BOM_CTH_003	*B. t. lusitanicus*	F	1	Spain		Burgos, Merindad de Cuesta	42.858028	-3.4275	13/06/2017	S.E. Silva, R. Mendes & A.S.B. Rodrigues	x	H1	MN652777
BOM_PEH_001	*B. t. lusitanicus*	F	1	Spain		Cantabria, Allende	43.21725	-4.593722	13/06/2017	S.E. Silva, R. Mendes & A.S.B. Rodrigues	x	H1	MN652778
BOM_PEH_002	*B. t. lusitanicus*	F	1	Spain		Cantabria, Allende	43.21725	-4.593722	13/06/2017	S.E. Silva, R. Mendes & A.S.B. Rodrigues	x	H1	MN652779
BOM_PEH_003	*B. t. lusitanicus*	F	1	Spain		Cantabria, Allende	43.21725	-4.593722	13/06/2017	S.E. Silva, R. Mendes & A.S.B. Rodrigues	x	H1	MN652780
BOM_PEH_010	*B. t. lusitanicus*	F	1	Spain		Léon, Boca de Huérgano	42.984167	-4.907944	13/06/2017	S.E. Silva, R. Mendes & A.S.B. Rodrigues	x	H1	MN652781
BOM_PEH_013	*B. t. lusitanicus*	F	1	Spain		Léon, Boca de Huérgano	42.984167	-4.907944	13/06/2017	S.E. Silva, R. Mendes & A.S.B. Rodrigues	x	H1	MN652782
BOM_PEL_002	*B. t. lusitanicus*	F	1	Spain		Léon, Cistierna	42.748556	-5.140139	13/06/2017	S.E. Silva, R. Mendes & A.S.B. Rodrigues	x	H1	MN652783
BOM_PEL_006	*B. t. lusitanicus*	F	1	Spain		Léon, Cubillas de Rueda	42.7285	-5.1455	13/06/2017	S.E. Silva, R. Mendes & A.S.B. Rodrigues	x	H1	MN652784
BOM_PEL_007	*B. t. lusitanicus*	F	1	Spain		Léon, Cubillas de Rueda	42.7285	-5.1455	13/06/2017	S.E. Silva, R. Mendes & A.S.B. Rodrigues	x	H2	MN652785
BOM_PEL_010	*B. t. lusitanicus*	F	1	Spain		Léon, Cubillas de Rueda	42.676	-5.169167	13/06/2017	S.E. Silva, R. Mendes & A.S.B. Rodrigues	x	H1	MN652786
BOM_PEL_011	*B. t. lusitanicus*	F	1	Spain		Léon, Cubillas de Rueda	42.676	-5.169167	13/06/2017	S.E. Silva, R. Mendes & A.S.B. Rodrigues	x	H1	MN652787
BOM_GDH_001	*B. t. lusitanicus*	F	1	Spain	**IP-CE** Iberian Peninsula, centre (n=32)	Ávila, Sierra de Gredos, Cuevas de Valle	40.309167	-5.016778	10/06/2017	S.E. Silva, R. Mendes & A.S.B. Rodrigues	x	H1	MN652788
BOM_TOL_001	*B. t. lusitanicus*	F	1	Spain		Ciudad Real, Santa Quiteria	39.257099	-4.3604809	03/04/2017	S.E. Silva, R. Mendes & O.S. Paulo	x	H1	MN652789
BOM_TOL_002	*B. t. lusitanicus*	F	1	Spain		Ciudad Real, Santa Quiteria	39.257099	-4.3604809	03/04/2017	S.E. Silva, R. Mendes & O.S. Paulo	x	H1	MN652790
BOM_TOH_010	*B. t. lusitanicus*	F	1	Spain		Toledo, Montes de Toledo, Hontanar	39.586946	-4.506851	03/04/2017	S.E. Silva, R. Mendes & O.S. Paulo	x	H1	MN652791
BOM_TOH_011	*B. t. lusitanicus*	F	1	Spain		Toledo, Montes de Toledo, Hontanar	39.586946	-4.506851	03/04/2017	S.E. Silva, R. Mendes & O.S. Paulo	x	H1	MN652792
BOM_TOH_015	*B. t. lusitanicus*	F	1	Spain		Toledo, Montes de Toledo, Hontanar	39.5865000001	-4.507	15/06/2016	S.E. Silva, R. Mendes & E. Marabuto	x	H1	MN652793
BOM_TOH_025	*B. t. lusitanicus*	F	1	Spain		Toledo, Montes de Toledo, Hontanar	39.5752237423	-4.5410353884	15/06/2016	S.E. Silva, R. Mendes & E. Marabuto	x	H1	MN652794
BOM_TOH_028	*B. t. lusitanicus*	F	1	Spain		Toledo, Montes de Toledo, Valdeazores	39.4500492018	-4.6827687569	15/06/2016	S.E. Silva, R. Mendes & E. Marabuto	x	H1	MN652795
BOM_GUH_003	*B. t. lusitanicus*	F	1	Spain		Cáceres, Sierra de Guadalupe, Guadalupe	39.4615341	-5.3295201	04/04/2017	S.E. Silva, R. Mendes & O.S. Paulo	x	H1	MN652796
BOM_GUH_004	*B. t. lusitanicus*	F	1	Spain		Cáceres, Sierra de Guadalupe, Cañamero	39.4725	-5.362778	04/04/2017	S.E. Silva, R. Mendes & O.S. Paulo	x	H1	MN652797
BOM_GUH_006	*B. t. lusitanicus*	F	1	Spain		Cáceres, Sierra de Guadalupe, Cañamero	39.476167	-5.36725	04/04/2017	S.E. Silva, R. Mendes & O.S. Paulo	x	H1	MN652798
BOM_GUH_016	*B. t. lusitanicus*	F	1	Spain		Cáceres, Sierra de Guadalupe, Alía	39.500917	-5.345	04/04/2017	S.E. Silva, R. Mendes & O.S. Paulo	x	H1	MN652799
BOM_GUH_017	*B. t. lusitanicus*	F	1	Spain		Cáceres, Sierra de Guadalupe, Alía	39.500917	-5.345	04/04/2017	S.E. Silva, R. Mendes & O.S. Paulo	x	H1	MN652800
BTL_150	*B. t. lusitanicus*	F	1	Spain		Madrid, Loeches	40.371944	-3.379861	26/05/2014	V.L. Nunes, T.G. Laurentino & E. Marabuto	x	H1	MN652801
BTL_144	*B. t. lusitanicus*	F	1	Spain		Madrid, Sierra de Guadarrama, Rascafría	40.878583	-3.847806	26/05/2014	V.L. Nunes, T.G. Laurentino & E. Marabuto	x	H1	MN652802
BTL_145	*B. t. lusitanicus*	F	1	Spain		Madrid, Sierra de Guadarrama, Rascafría	40.878583	-3.847806	26/05/2014	V.L. Nunes, T.G. Laurentino & E. Marabuto	x	H1	MN652803
BTL_148	*B. t. lusitanicus*	F	1	Spain		Madrid, Sierra de Guadarrama, Rascafría	40.878583	-3.847806	26/05/2014	V.L. Nunes, T.G. Laurentino & E. Marabuto	x	H1	MN652804
BTL_301	*B. t. lusitanicus*	F	1	Spain		Madrid, Sierra de Guadarrama, Real Sitio de San Ildefonso	40.900481	-4.009089	13/08/2014	V.L. Nunes, T.G. Laurentino & E. Marabuto	x	H1	MN652805
BOM_GRH_002	*B. t. lusitanicus*	F	1	Spain		Madrid, Sierra de Guadarrama, Real Sitio de San Ildefonso	40.861	-4.027333	11/06/2017	S.E. Silva, R. Mendes & A.S.B. Rodrigues	x	H1	MN652806
BOM_GRH_004	*B. t. lusitanicus*	F	1	Spain		Madrid, Sierra de Guadarrama, Real Sitio de San Ildefonso	40.861	-4.027333	11/06/2017	S.E. Silva, R. Mendes & A.S.B. Rodrigues	x	H1	MN652807
BOM_GRH_008	*B. t. lusitanicus*	F	1	Spain		Madrid, Sierra de Guadarrama, Rascafría	40.872	-3.885	11/06/2017	S.E. Silva, R. Mendes & A.S.B. Rodrigues	x	H1	MN652808
BOM_GRH_009	*B. t. lusitanicus*	F	1	Spain		Madrid, Sierra de Guadarrama, Rascafría	40.872	-3.885	11/06/2017	S.E. Silva, R. Mendes & A.S.B. Rodrigues	x	H1	MN652809
BOM_GRH_023	*B. t. lusitanicus*	F	1	Spain		Madrid, Sierra de Guadarrama, Rascafría	40.878583	-3.847806	26/05/2014	V.L. Nunes, T.G. Laurentino & E. Marabuto	x	H1	MN652810
BOM_GRL_002	*B. t. lusitanicus*	F	1	Spain		Segovia, Cerezo de Arriba	41.245	-3.548722	11/06/2017	S.E. Silva, R. Mendes & A.S.B. Rodrigues	x	H1	MN652811
BOM_GRL_003	*B. t. lusitanicus*	F	1	Spain		Segovia, Ribota	41.358972	-3.44675	11/06/2017	S.E. Silva, R. Mendes & A.S.B. Rodrigues	x	H1	MN652812
BOM_GRL_004	*B. t. lusitanicus*	F	1	Spain		Segovia, Ribota	41.358972	-3.44675	11/06/2017	S.E. Silva, R. Mendes & A.S.B. Rodrigues	x	H1	MN652813
BOM_GRL_009	*B. t. lusitanicus*	F	1	Spain		Soria, Valdealvillo	41.649472	-2.891194	11/06/2017	S.E. Silva, R. Mendes & A.S.B. Rodrigues	x	H1	MN652814
BOM_GRL_010	*B. t. lusitanicus*	F	1	Spain		Soria, Villaciervos	41.752611	-2.651472	11/06/2017	S.E. Silva, R. Mendes & A.S.B. Rodrigues	x	H1	MN652815
BTL_086	*B. t. lusitanicus*	F	1	Spain		Valladolid, Castronuevo de Esgueva	41.686667	-4.597	16/05/2014	E. Marabuto	x	H1	MN652816
BTL_089	*B. t. lusitanicus*	F	1	Spain		Valladolid, Castronuevo de Esgueva	41.686667	-4.597	16/05/2014	E. Marabuto	x	H1	MN652817
BTL_090	*B. t. lusitanicus*	F	1	Spain		Valladolid, Castronuevo de Esgueva	41.686667	-4.597	16/05/2014	E. Marabuto	x	H1	MN652818
BTL_097	*B. t. lusitanicus*	F	1	Spain		Valladolid, Castronuevo de Esgueva	41.686667	-4.597	16/05/2014	E. Marabuto	x	H1	MN652819
BOM_ESH_003	*B. t. lusitanicus*	F	1	Spain	**IP-SE** Iberian Peninsula, southeast (n=38)	Murcia, Sierra España	37.8627000005	-1.55323	12/06/2016	S.E. Silva, R. Mendes & E. Marabuto	x	H1	MN652820
BOM_ESH_010	*B. t. lusitanicus*	F	1	Spain		Murcia, Sierra España	37.8650027781	-1.5713306989	12/06/2016	S.E. Silva, R. Mendes & E. Marabuto	x	H1	MN652821
BOM_ESH_013	*B. t. lusitanicus*	F	1	Spain		Murcia, Sierra España	37.85508	-1.568043	11/07/2017	S.E. Silva & R. Mendes	x	H3	MN652822
BTL_115	*B. t. lusitanicus*	F	1	Spain		Granada, Puebla de Don Fadrique	37.953222	-2.408	28/05/2014	V.L. Nunes, T.G. Laurentino & E. Marabuto	x	H1	MN652823
BTL_118	*B. t. lusitanicus*	F	1	Spain		Granada, Puebla de Don Fadrique	38.042361	-2.472611	28/05/2014	V.L. Nunes, T.G. Laurentino & E. Marabuto	x	H1	MN652824
BTL_121	*B. t. lusitanicus*	F	1	Spain		Granada, Puebla de Don Fadrique	37.953222	-2.408	28/05/2014	V.L. Nunes, T.G. Laurentino & E. Marabuto	x	H1	MN652825
BTL_123	*B. t. lusitanicus*	F	1	Spain		Granada, Puebla de Don Fadrique	38.064417	-2.5215	28/05/2014	V.L. Nunes, T.G. Laurentino & E. Marabuto	x	H1	MN652826
BTL_124	*B. t. lusitanicus*	F	1	Spain		Granada, Puebla de Don Fadrique	38.042361	-2.472611	28/05/2014	V.L. Nunes, T.G. Laurentino & E. Marabuto	x	H1	MN652827
BTL_125	*B. t. lusitanicus*	F	1	Spain		Granada, Puebla de Don Fadrique	37.953222	-2.408	28/05/2014	V.L. Nunes, T.G. Laurentino & E. Marabuto	x	H5	MN652828
BTL_109	*B. t. lusitanicus*	F	1	Spain		Almería, Sierra María-Los Vélez	37.694417	-2.174639	28/05/2014	V.L. Nunes, T.G. Laurentino & E. Marabuto	x	H1	MN652829
BTL_110	*B. t. lusitanicus*	F	1	Spain		Almería, Sierra María-Los Vélez	37.694417	-2.174639	28/05/2014	V.L. Nunes, T.G. Laurentino & E. Marabuto	x	H1	MN652830

Sample Code	Taxa	Sex	n	Country / Island	Geographic region	Sampling Location	Latitude	Longitude	Date of collection	Collector(s)	mtDNA analysis	COI haplotype	GenBank accession no.
BTL_112	*B. t. lusitanicus*	F	1	Spain		Almería, Sierra María-Los Vélez	37.694417	-2.174639	28/05/2014	V.L. Nunes, T.G. Laurentino & E. Marabuto	x	H1	MN652831
BTL_136	*B. t. lusitanicus*	F	1	Spain		Alicante, Serra de Serrella-Barranc Fort	38.697333	-0.309361	27/05/2014	V.L. Nunes, T.G. Laurentino & E. Marabuto	x	H1	MN652832
BTL_138	*B. t. lusitanicus*	F	1	Spain		Alicante, Serra de Serrella-Barranc Fort	38.697333	-0.309361	27/05/2014	V.L. Nunes, T.G. Laurentino & E. Marabuto	x	H1	MN652833
BTL_141	*B. t. lusitanicus*	F	1	Spain		Alicante, Serra de Serrella-Barranc Fort	38.697333	-0.309361	27/05/2014	V.L. Nunes, T.G. Laurentino & E. Marabuto	x	H1	MN652834
BOM_ALH_001	*B. t. lusitanicus*	F	1	Spain		Alicante, Ibi	38.6356000015	-0.5228	13/06/2016	S.E. Silva, R. Mendes & E. Marabuto	x	H6	MN652835
BOM_ALH_003	*B. t. lusitanicus*	F	1	Spain		Alicante, Serra de Serrella-Barranc Fort	38.7025000015	-0.3031000003	13/06/2016	S.E. Silva, R. Mendes & E. Marabuto	x	H1	MN652836
BOM_ALH_009	*B. t. lusitanicus*	F	1	Spain		Alicante, Serra de Serrella-Barranc Fort	38.697333	-0.309361	27/05/2014	V.L. Nunes, T.G. Laurentino & E. Marabuto	x	H1	MN652837
BOM_ALH_016	*B. t. lusitanicus*	F	1	Spain		Alicante, Serra de Serrella-Barranc Fort	38.697333	-0.309361	27/05/2014	V.L. Nunes, T.G. Laurentino & E. Marabuto	x	H1	MN652838
BOM_ROL_001	*B. t. lusitanicus*	F	1	Spain		Sevilla, Montellano	36.993333	-5.569361	01/04/2017	S.E. Silva, R. Mendes & O.S. Paulo	x	H1	MN652839
BOM_ROL_002	*B. t. lusitanicus*	F	1	Spain		Sevilla, Montellano	36.993333	-5.569361	01/04/2017	S.E. Silva, R. Mendes & O.S. Paulo	x	H1	MN652840
BOM_ROL_008	*B. t. lusitanicus*	F	1	Spain		Sevilla, Montellano	36.984194	-5.545111	01/04/2017	S.E. Silva, R. Mendes & O.S. Paulo	x	H1	MN652841
BOM_ROL_009	*B. t. lusitanicus*	F	1	Spain		Sevilla, Montellano	36.984194	-5.545111	01/04/2017	S.E. Silva, R. Mendes & O.S. Paulo	x	H1	MN652842
BOM_ROH_001	*B. t. lusitanicus*	F	1	Spain		Málaga, Serranía de Ronda, Pujerra	36.612167	-5.140722	01/04/2017	S.E. Silva, R. Mendes & O.S. Paulo	x	H1	MN652843
BOM_ROH_002	*B. t. lusitanicus*	F	1	Spain		Málaga, Serranía de Ronda, Pujerra	36.612167	-5.140722	01/04/2017	S.E. Silva, R. Mendes & O.S. Paulo	x	H7	MN652844
BOM_ROH_003	*B. t. lusitanicus*	F	1	Spain		Málaga, Serranía de Ronda, Pujerra	36.612778	-5.139194	01/04/2017	S.E. Silva, R. Mendes & O.S. Paulo	x	H1	MN652845
BOM_ROH_004	*B. t. lusitanicus*	F	1	Spain		Málaga, Serranía de Ronda, Pujerra	36.612778	-5.139194	01/04/2017	S.E. Silva, R. Mendes & O.S. Paulo	x	H1	MN652846
BOM_ROH_014	*B. t. lusitanicus*	F	1	Spain		Málaga, El Burgo	36.8001999999	-4.9138	08/06/2016	S.E. Silva, R. Mendes & E. Marabuto	x	H1	MN652847
BTL_185	*B. t. lusitanicus*	F	1	Spain		Granada, Sierra Nevada, El Molinillo	37.310833	-3.413889	29/05/2014	V.L. Nunes, T.G. Laurentino & E. Marabuto	x	H1	MN652848
BTL_191	*B. t. lusitanicus*	F	1	Spain		Granada, Sierra Nevada, Alfacar	37.258889	-3.549167	29/05/2014	V.L. Nunes, T.G. Laurentino & E. Marabuto	x	H1	MN652849
BTL_197	*B. t. lusitanicus*	F	1	Spain		Granada, Sierra Nevada, Alfacar	37.258889	-3.549167	29/05/2014	V.L. Nunes, T.G. Laurentino & E. Marabuto	x	H1	MN652850
BTL_168	*B. t. lusitanicus*	F	1	Spain		Granada, Sierra Nevada, Monachil	37.139167	-3.467778	29/05/2014	V.L. Nunes, T.G. Laurentino & E. Marabuto	x	H1	MN652851
BTL_180	*B. t. lusitanicus*	F	1	Spain		Granada, Sierra Nevada, Monachil	37.139167	-3.467778	29/05/2014	V.L. Nunes, T.G. Laurentino & E. Marabuto	x	H1	MN652852
BOM_NEH_006	*B. t. lusitanicus*	F	1	Spain		Granada, Sierra Nevada, Alfacar	37.2592	-3.54825	09/06/2016	S.E. Silva, R. Mendes & E. Marabuto	x	H1	MN652853
BOM_NEH_007	*B. t. lusitanicus*	F	1	Spain		Granada, Sierra Nevada, Alfacar	37.2592	-3.54825	09/06/2016	S.E. Silva, R. Mendes & E. Marabuto	x	H1	MN652854
BOM_NEH_011	*B. t. lusitanicus*	F	1	Spain		Granada, Sierra Nevada, Alfacar	37.2660500741	-3.5265	09/06/2016	S.E. Silva, R. Mendes & E. Marabuto	x	H1	MN652855
BOM_NEH_013	*B. t. lusitanicus*	F	1	Spain		Granada, Sierra Nevada, Monachil	37.1130500004	-3.4397	09/06/2016	S.E. Silva, R. Mendes & E. Marabuto	x	H1	MN652856
BOM_NEH_038	*B. t. lusitanicus*	F	1	Spain		Granada, Sierra Nevada, Alfacar	37.258889	-3.549167	29/05/2014	V.L. Nunes, T.G. Laurentino & E. Marabuto	x	H1	MN652857

Other Subspecies

Sample Code	Taxa	Sex	n	Country / Island	Geographic region	Sampling Location	Latitude	Longitude	Date of collection	Collector(s)	mtDNA analysis	COI haplotype	GenBank accession no.
BOM_SWI_003	*B. t. terrestris*	F	1	Switzerland	Switzerland	Basel	--	--	08/2016	T.G. Laurentino	x	H2	MN652858
BT_046	*B. t. terrestris*	F	1	Germany	Germany	Rothenburg ob der Tauber	49.374933	10.17695	08/05/2014	V.L. Nunes	x	H1	MN652859
BT_029	*B. t. terrestris*	M	1	France	France	Sainte-Anastasie-sur-Issole,	43.3555	6.141	23/04/2014	E. Marabuto	x	H1	MN652860
BOM_TUR_001	*B. t. dalmatinus*	F	1	Turkey	Turkey	Tekirdag, Ganos Mountains	--	--	06/2016	S. Yurtsever	x	H1	MN652861
BTX_395	*B. t. audax*	F	1	Great Britain	England	P. Hill near Oxford	--	--		T.G. Laurentino	x	H8	MN652862
BTA_258	*B. t. africanus*	F	1	Morocco		Chefchaouen	35.182969	-5.222889	15/07/2014	V.L. Nunes, T.G. Laurentino & E. Marabuto	x	H9	MN652863
BTA_261	*B. t. africanus*	F	1	Morocco	Morocco	Chefchaouen	35.182969	-5.222889	15/07/2014	V.L. Nunes, T.G. Laurentino & E. Marabuto	x	H9	MN652864
BTA_262	*B. t. africanus*	F	1	Morocco	(n=4)	Azrou	33.406333	-5.2035	16/07/2014	V.L. Nunes, T.G. Laurentino & E. Marabuto	x	H9	MN652865
BTA_104	*B. t. africanus*	F	1	Morocco		Chefchaouen	35.104	-5.139417	18/07/2014	V.L. Nunes, T.G. Laurentino & E. Marabuto	x	H9	MN652866

Outgroup

Sample Code	Taxa	Sex	n	Country / Island	Geographic region	Sampling Location	Latitude	Longitude	Date of collection	Collector(s)	mtDNA analysis	COI haplotype	GenBank accession no.
BHO_067	*B. hortorum*	F	1	Portugal		P.N. Peneda-Gerês, Vilar da Veiga	41.73505	-8.159281	14/05/2014	T.G. Laurentino & O.S. Paulo	x	-	MN652867
BLA_205	*B. lapidarius*	F	1	Portugal		Serra da Estrela	40.427972	-7.654022	10/06/2014	T.G. Laurentino	x	-	MN652868
BLA_212	*B. lapidarius*	F	1	Portugal		Serra da Estrela	40.462928	-7.513517	11/06/2014	T.G. Laurentino	x	-	MN652869
BLA_069	*B. lapidarius*	F	1	Spain		Cantabria, Vega de Liébana	43.086333	-4.7245	14/05/2014	E. Marabuto	x	-	MN652870
BTL_084	*B. lucorum*	F	1	Spain		Burgos, Barbadillo de Herreros	42.136667	-3.1935	15/05/2014	E. Marabuto	x	-	MHO18628
BTL_103	*B. lucorum*	F	1	Spain	Outgroup	Léon, Sierra de Ancares	42.871333	-6.452083	12/05/2014	E. Marabuto	x	-	MN652871
BTL_146	*B. lucorum*	F	1	Spain	(n=13)	Madrid, Sierra de Guadarrama, Rascafría	40.878583	-3.847806	26/05/2014	V.L. Nunes, T.G. Laurentino & E. Marabuto	x	-	MHO18629
BPA_229	*B. pascuorum*	F	1	Portugal		Mafra, Sobral da Abelheira	--	--	28/06/2014	O.S. Paulo, S.E. Silva & A.S. Rodrigues	x	-	MN652872
BPA_068	*B. pascuorum*	F	1	Spain		Cantabria, Vega de Liébana	43.086333	-4.7245	14/05/2014	E. Marabuto	x	-	MN652873
BPA_133	*B. pascuorum*	F	1	Spain		Alicante, Serra de Serrella-Barranc Fort	38.690556	-0.287111	27/05/2014	V.L. Nunes, T.G. Laurentino & E. Marabuto	x	-	MN652874
BPA_152	*B. pascuorum*	F	1	Spain		Madrid, Sierra de Guadarrama, Miraflores de La Sierra	40.800278	-3.756667	26/05/2014	V.L. Nunes, T.G. Laurentino & E. Marabuto	x	-	MN652875

| BHO_223 | *B. ruderatus* | F | 1 | Portugal | | Torres Vedras, Coutada | -- | -- | 28/06/2014 | O.S. Paulo, S.E. Silva & A.S. Rodrigues | x | - | MN652876 |
| BHO_153 | *B. ruderatus* | F | 1 | Spain | | Madrid, Sierra de Guadarrama, Miraflores de La Sierra | 40.800278 | -3.756667 | 26/05/2014 | V.L. Nunes, T.G. Laurentino & E. Marabuto | x | - | MN652877 |

RAD analysis	RAD sequencing lane	Barcode	Total reads	Retained reads (Process_ Radtags)	Mapped reads properly paired (Bowtie2)	% of mapped reads	Number of loci (Stacks)	Mean Coverage (Stacks)	Samples of dataset BT_OUT	Samples of dataset BT_SSP	Samples of dataset BT_BTL	Frequency of missing data, dataset BT_OUT (VCFTools)	Frequency of missing data, dataset BT_SSP (VCFTools)	Frequency of missing data, dataset BT_BTL (VCFTools)
x	Lane 2	TTAAT	8109844	7734007	4605896	59.6	207938	17.92	x	x	x	0.00545171	0.0243446	0.0313586
removed	Lane 2	TGGTT	236766	120102	10810	9.0	747	8.77						
x	Lane 2	ATTAG	7315526	7127626	4316556	60.6	200433	17.62	x	x	x	0.0103193	0.0309482	0.0373926
x	Lane 2	AGGAC	4660438	4538676	2643980	58.3	136442	15.69	x	x	x	0.0564642	0.10408	0.105595
x	Lane 2	AGAGT	11736928	11350762	6998274	61.7	282072	19.6	x	x	x	0.00564642	0.0222748	0.0210276
x	Lane 2	AAGGG	5382908	5200161	3092488	59.5	148671	16.53	x	x	x	0.036215	0.0824956	0.0830133

x	Lane 1	CCGGT	7346304	7111056	4258414	59.9	150611	19.65	x	x	x	0.00778816	0.0283856	0.0291644
x	Lane 1	CAGTC	8489710	8257735	5231910	63.4	159071	21.98	x	x	x	0.00584112	0.0172482	0.0149936
removed	Lane 1	GTCAC	1968792	1872510	1055220	56.4	57562	12.11						
x	Lane 1	CTCTT	9251988	8770867	4913908	56.0	166760	20.57	x	x	x	0.00428349	0.0214863	0.0206619
x	Lane 1	TCTCT	15162584	14508026	8652690	59.6	208682	27.55	x	x	x	0.00175234	0.00818056	0.00895959
x	Lane 1	TATAC	3328796	3176285	1883466	59.3	74807	15.79	x	x	x	0.0630841	0.111275	0.109435
x	Lane 1	TGTGG	16898876	16338434	9724544	59.5	233263	27.95	x	x	x	0.00097352	0.00857481	0.00895959
x	Lane 1	CAACT	10468942	10070127	6041328	60.0	197828	21.49	x	x	x	0.00506231	0.015277	0.0152679
x	Lane 1	TTTTA	14787532	14167207	8373646	59.1	202131	27.48	x	x	x	0.00311526	0.008082	0.00722253
x	Lane 2	AAAAA	29499926	28594514	17801252	62.3	624304	22.68	x	x	x	0.00194704	0.00965898	0.00950814
removed	Lane 2	TCAGA	1399906	1244947	636054	51.1	41413	11.61						
x	Lane 2	TCGAG	4576352	4431323	2658398	60.0	134666	15.82	x	x	x	0.0609424	0.105855	0.109618
x	Lane 2	TGACC	7852030	7596370	4624002	60.9	211407	17.89	x	x	x	0.0132399	0.0361719	0.0326385
removed	Lane 2	ACGTA	337128	286528	152578	53.3	11331	9.29						
x	Lane 2	AACCC	13519934	13186619	8314690	63.1	325446	20.19	x	x	x	0.00097352	0.00798344	0.00987383

x	Lane 2	AATTT	3466684	3201722	1756018	54.8	100208	14	x	x	x	0.138629	0.214567	0.224538
x	Lane 2	CAGTC	7963274	7719129	4557522	59.0	210993	17.74	x	x	x	0.0109034	0.0351863	0.0339184
x	Lane 2	CAACT	4352780	3997355	2198672	55.0	117516	14.95	x	x	x	0.0858645	0.149024	0.15149
x	Lane 2	CCAAC	8593044	8391856	5424226	64.6	229334	18.62	x	x	x	0.0107087	0.0249359	0.0232218
x	Lane 2	ACCAT	12321256	11796388	7165928	60.7	300695	19.57	x	x	x	0.00311526	0.0156712	0.0136222
x	Lane 2	ACTGC	10753424	10501149	6708582	63.9	277030	19.33	x	x	x	0.00584112	0.0189237	0.0165478
x	Lane 2	TTCCG	7266592	7040446	4228904	60.1	195504	17.63	x	x	x	0.024338	0.0508575	0.0457122
x	Lane 2	TGTGG	9003316	8749325	5598210	64.0	239485	18.62	x	x	x	0.0122664	0.024246	0.0235875
x	Lane 2	TTTTA	3092968	2861849	1641620	57.4	92469	13.84	x	x	x	0.163162	0.237138	0.244012
removed	Lane 2	TGCAA	531410	428555	200060	46.7	13988	10.18						

RAD analysis	RAD sequencing lane	Barcode	Total reads	Retained reads (Process_Radtags)	Mapped reads properly paired (Bowtie2)	% of mapped reads	Number of loci (Stacks)	Mean Coverage per individual (Stacks)	Samples of dataset BT_OUT	Samples of dataset BT_SSP	Samples of dataset BT_BTL	Frequency of missing data, dataset BT_OUT (VCFTools)	Frequency of missing data, dataset BT_SSP (VCFTools)	Frequency of missing data, dataset BT_BTL (VCFTools)
x	Lane 2	GGCCT	6162856	5893962	3516654	59.7	170923	16.7	x	x	x	0.0272586	0.0613049	0.0571402
removed	Lane 2	GCTAA	1227456	1127861	658826	58.4	44981	10.83						
x	Lane 2	GCCGG	4709680	4580094	2737926	59.8	138878	15.87	x	x	x	0.0521807	0.0958999	0.0989212
x	Lane 2	TCCTC	12811682	12436736	7682970	61.8	302233	20.02	x	x	x	0.0155763	0.0408043	0.0417809
x	Lane 2	TATAC	2652990	2545204	1452420	57.1	85674	13.38	x	x	x	0.196262	0.266805	0.282319
x	Lane 2	TCTCT	5151776	4804789	2861534	59.6	144522	15.92	x	x	x	0.0488707	0.0885078	0.0911501

RAD analysis	RAD sequencing lane	Barcode	Total reads	Retained reads (Process_Radtags)	Mapped reads properly paired (Bowtie2)	% of mapped reads	Number of loci (Stacks)	Mean Coverage per individual (Stacks)	Samples of dataset BT_OUT	Samples of dataset BT_SSP	Samples of dataset BT_BTL	Frequency of missing data, dataset BT_OUT (VCFTools)	Frequency of missing data, dataset BT_SSP (VCFTools)	Frequency of missing data, dataset BT_BTL (VCFTools)
x	Lane 2	AGCTG	6885900	6683291	4088484	61.2	188241	17.73	x	x		0.0132399	0.0342007	
x	Lane 1	TTCCG	6060962	5863768	3366584	57.4	117490	18.99	x	x		0.00584112	0.0308496	
x	Lane 1	CCCCA	7457604	7241194	4306960	59.5	136690	20.94	x	x		0.00837227	0.0230633	
x	Lane 2	AGTCA	14513666	14223980	7884990	55.4	313539	20.05	x	x		0.00292056	0.0150798	
x	Lane 2	GACTA	7370284	7201198	4162876	57.8	198189	17.32	x	x		0.0144081	0.0381431	

RAD analysis	RAD sequencing lane	Barcode	Total reads	Retained reads (Process_Radtags)	Mapped reads properly paired (Bowtie2)	% of mapped reads	Number of loci (Stacks)	Mean Coverage per individual (Stacks)	Samples of dataset BT_OUT	Samples of dataset BT_SSP	Samples of dataset BT_BTL	Frequency of missing data, dataset BT_OUT (VCFTools)	Frequency of missing data, dataset BT_SSP (VCFTools)	Frequency of missing data, dataset BT_BTL (VCFTools)
x	Lane 2	CCCCA	6654410	6373456	1857064	29.1	94848	16.91	x			0.03602		
x	Lane 1	TCCTC	9630278	9237789	3797870	41.1	129379	21.73	x			0.122079		
x	Lane 2	CTCTT	7673874	7385489	3059096	41.4	138893	18.26	x			0.127531		
x	Lane 2	CTTCC	9673764	9393983	3976006	42.3	177966	19.07	x			0.120911		
x	Lane 1	GGCCT	5369388	4996943	2764028	55.3	100492	17.97	x			0.0216121		
removed	Lane 2	GTCAC	623266	527856	263090	49.8	18635	9.97						
removed	Lane 1	TGCAA	1606326	1471245	821262	55.8	44092	12.09						
removed	Lane 1	TACGT	2535204	2160906	743172	34.4	39070	11.54						
x	Lane 2	CGTAT	6366508	4132680	1413486	34.2	77692	15.43	x			0.178349		
x	Lane 2	CGCGC	6155140	5978181	2104318	35.2	107737	16.99	x			0.132788		
x	Lane 2	CCTTG	12943046	12573453	4313748	34.3	181112	20.31	x			0.118769		

| x | Lane 2 | CACAG | 22264514 | 21811529 | 7183830 | 32.9 | 275076 | 22.88 | x | | 0.0149922 | | |
| x | Lane 2 | CATGA | 7015302 | 6827666 | 2053094 | 30.1 | 107354 | 17.18 | x | | 0.0428349 | | |

Table S1b. List of samples with sequences downloaded from GenBank, with information concerning: sampling (taxa, sex, country, number of samples, origin, date of collection and bibliographic reference); outputs of mitochondrial COI analyses (haplotypes and GenBank accession numbers).

Taxa	Sex	n	Country / Island	Sampling Location	Latitude	Longitude	Date of collection	Collector(s)	mtDNA analysis	COI haplotype	GenBank accession no.
B. terrestris	F	5	Ireland	Wexford	52°20020 N	6°27036 W	01/07/2007	Moreira *et al.* (2015)	x	HA / H8	KP670306
B. terrestris	F	5	Ireland	Cork	51°53052 N	8°28015 W	01/07/2007	Moreira *et al.* (2015)	x	HA / H8	KP670306
B. terrestris	F	4	Ireland	Donegal	54°39016 N	8°06038 W	01/07/2007	Moreira *et al.* (2015)	x	HA / H8	KP670306
B. terrestris	F	1	Ireland	Donegal	54°39016 N	8°06038 W	01/07/2007	Moreira *et al.* (2015)	x	HB / H1	KP670307
B. terrestris	F	2	Ireland	Galway	53°16025 N	9°03006 W	01/07/2007	Moreira *et al.* (2015)	x	HA / H8	KP670306
B. terrestris	F	3	Ireland	Galway	53°16025 N	9°03006 W	01/07/2007	Moreira *et al.* (2015)	x	HB / H1	KP670307
B. terrestris	F	2	Ireland	Meath	53°37052 N	6°47027 W	01/06/2007	Moreira *et al.* (2015)	x	HA / H8	KP670306
B. terrestris	F	3	Ireland	Meath	53°37052 N	6°47027 W	01/06/2007	Moreira *et al.* (2015)	x	HB / H1	KP670307
B. terrestris	F	5	Ireland	Kildare	53°09035 N	6°54032 W	01/06/2007	Moreira *et al.* (2015)	x	HA / H8	KP670306
B. terrestris	F	5	Great Britain	North Wales, Rhyl	53°19010 N	3°29019 W	01/07/2007	Moreira *et al.* (2015)	x	HA / H8	KP670306
B. terrestris	F	3	Great Britain	South Wales, Lampeter	51°18052 N	3°10049 W	01/07/2007	Moreira *et al.* (2015)	x	HA / H8	KP670306
B. terrestris	F	2	Great Britain	South Wales, Lampeter	51°18052 N	3°10049 W	01/07/2007	Moreira *et al.* (2015)	x	HB / H1	KP670307
B. terrestris	F	2	Isle of Man	Isle of Man	54°14009 N	4°32053 W	01/08/2007	Moreira *et al.* (2015)	x	HA / H8	KP670306
B. terrestris	F	1	Isle of Man	Isle of Man	54°14009 N	4°32053 W	01/08/2007	Moreira *et al.* (2015)	x	HB / H1	KP670307
B. terrestris	F	5	Great Britain	England, Oxford	51°45008 N	1°15020 W	01/07/2007	Moreira *et al.* (2015)	x	HA / H8	KP670306
B. terrestris	F	4	Great Britain	England, Dover	51°07046 N	1°18040 W	01/08/2007	Moreira *et al.* (2015)	x	HA / H8	KP670306
B. terrestris	F	1	Great Britain	England, Dover	51°07046 N	1°18040 W	01/08/2007	Moreira *et al.* (2015)	x	HB / H1	KP670307
B. terrestris	F	5	Greece	Macedonia	40°45000 N	22°53059 E	01/07/2007	Moreira *et al.* (2015)	x	HB / H1	KP670307
B. terrestris	F	5	Portugal	Bragança	41°48025 N	6°45033 W	01/05/2007	Moreira *et al.* (2015)	x	HB / H1	KP670307
B. terrestris	F	5	Croatia	Pula	44°52010 N	13°50028 E	01/03/2007	Moreira *et al.* (2015)	x	HB / H1	KP670307
B. terrestris	F	5	Croatia	Zadar	44°01046 N	17°48000 E	01/03/2007	Moreira *et al.* (2015)	x	HB / H1	KP670307
B. terrestris	F	5	France	Samer	44°38021 N	1°44042 E	01/07/2007	Moreira *et al.* (2015)	x	HB / H1	KP670307
B. terrestris	F	5	France	Beauvais	49°25045 N	2°04051 E	01/07/2007	Moreira *et al.* (2015)	x	HB / H1	KP670307
B. terrestris	F	5	France	Grenoble	45°11039 N	5°43053 E	01/05/2007	Moreira *et al.* (2015)	x	HB / H1	KP670307
B. terrestris	F	5	Poland	Grudziadz	54°20038 N	18°39056 E	01/06/2007	Moreira *et al.* (2015)	x	HB / H1	KP670307
B. terrestris	F	5	Poland	Katowice	50°15053 N	19°01025 E	01/06/2007	Moreira *et al.* (2015)	x	HB / H1	KP670307
B. terrestris	F	5	Denmark	Bilund	56°15050 N	9°30006 E	01/08/2007	Moreira *et al.* (2015)	x	HB / H1	KP670307
B. terrestris	F	5	Latvia	Jelgava	56°56047 N	24°06017 E	07/05/2008	Moreira *et al.* (2015)	x	HB / H1	KP670307
B. t. sassaricus	M	7	Sardinia	--	40°47'27"N	08°03'12"E	--	Coppée (2010)	x	H11	JQ769086
B. t. terrestris	M	4	France (Southeast)	--	43°25'32"N	06°25'23"E	--	Coppée (2010)	x	H1	JQ769069
B. t. terrestris	M	4	France (North)	--	48°50'37"N	02°21'35"E	--	Coppée (2010)	x	H1	JQ769080
B. t. terrestris	M	4	France (Southwest)	--	42°28'38"N	01°55'04"E	--	Coppée (2010)	x	H1	JQ769088
B. t. terrestris	M	4	Belgium	--	50°50'22"N	04°23'44"E	--	Coppée (2010)	x	H1	JQ769070
B. t. terrestris	M	3	Germany	--	51°56'27"N	07°33'03"E	--	Coppée (2010)	x	H13	JQ769081
B. t. terrestris	M	3	Denmark	--	54°59'01"N	12°00'32"E	--	Coppée (2010)	x	H1	JQ769082
B. t. terrestris	M	5	Sweden	--	59°51'43"N	17°38'00"E	--	Coppée (2010)	x	H1	JQ769083
B. t. xanthopus	M	5	Corsica	--	42°17'25"N	08°52'40"E	--	Coppée (2010)	x	H15	JQ769071
B. t. dalmatinus	M	5	Greece (Crete)	--	35°23'28"N	23°34'30"E	--	Coppée (2010)	x	H1	JQ769072
B. t. dalmatinus	M	4	Turkey	--	39°55'54"N	32°51'48"E	--	Coppée (2010)	x	H1	JQ769078
B. t. dalmatinus	M	4	France (Southeast)	--	43°25'32"N	06°25'23"E	--	Coppée (2010)	x	H1	JQ769079
B. t. lusitanicus	M	3	Madeira	--	32°48'01"N	16°50'43"W	--	Coppée (2010)	x	H1	JQ769073
B. t. lusitanicus	M	3	France (Southwest)	--	42°28'51"N	01°55'25"E	--	Coppée (2010)	x	H1	JQ769087
B. t. lusitanicus	M	5	Spain	--	36°42'59"N	04°25'03"W	--	Coppée (2010)	x	H1	JQ769076

Species	Sex		Country	Locality	Latitude	Longitude	Date	Reference		Haplotype	Accession
B. t. africanus	M	5	Morocco	--	35°03'40"N	05°09'60"W	--	Coppée (2010)	x	H16	JQ769074
B. t. calabricus	M	5	Italy	--	35°59'35"N	13°52'49"E	--	Coppée (2010)	x	H1	JQ769075
B. t. audax	M	5	Ireland	--	52°57'03,3"N	9°04'41,8"W	--	Coppée (2010)	x	H2	JQ769077
B. t. audax	M	3	Great Britain (North)	--	51°05'43,0"N	0°38'33,2"E	--	Coppée (2010)	x	H8	JQ769084
B. t. audax	M	5	Great Britain (Southwest)	--	50°21'33"N	03°50'15"W	--	Coppée (2010)	x	H8	JQ769085
B. terrestris	M	1	Great Britain	--	--	--	--	Williams *et al.* (2012b)	x	H8	JQ843647
B. terrestris	M	1	Great Britain	--	--	--	--	Williams *et al.* (2012b)	x	H8	JQ843646
B. terrestris	F	1	Turkey	Sinop	--	--	2010	Williams *et al.* (2012b)	x	H1	JQ843645
B. terrestris	F	1	Turkey	--	--	--	--	Williams *et al.* (2012b)	x	H1	JQ843658
B. terrestris	F	1	Turkey	--	--	--	--	Williams *et al.* (2012b)	x	H1	JQ843669
B. terrestris	F	1	Turkey	--	--	--	--	Williams *et al.* (2012b)	x	H12	JQ843663
B. terrestris	F	1	Turkey	--	--	--	--	Williams *et al.* (2012b)	x	H1	JQ843643
B. terrestris	F	1	Turkey	--	--	--	--	Williams *et al.* (2012b)	x	H1	JQ843664
B. terrestris	F	1	Turkey	--	--	--	--	Williams *et al.* (2012b)	x	H1	JQ843644
B. terrestris	F	1	Turkey	--	--	--	--	Williams *et al.* (2012b)	x	H1	JQ843611
B. terrestris	F	1	Turkey	--	--	--	--	Williams *et al.* (2012b)	x	H1	JQ843612
B. terrestris	F	1	Turkey	--	--	--	--	Williams *et al.* (2012b)	x	H1	JQ843613
B. terrestris	F	1	Turkey	--	--	--	--	Williams *et al.* (2012b)	x	H1	JQ843614
B. terrestris	F	1	Algeria	Bouira	--	--	2005	Williams *et al.* (2012b)	x	H14	JQ843667
B. terrestris	F	1	Italy	Siena	--	--	1995	Williams *et al.* (2012b)	x	H14	JQ843662
B. terrestris	F	1	Spain	Canary Islands	--	--	2008	Williams *et al.* (2012b)	x	H17	JQ843661
B. terrestris	F	1	Spain	Canary Islands	--	--	2008	Williams *et al.* (2012b)	x	H17	JQ843660
B. terrestris	F	1	Spain	Canary Islands	--	--	2008	Williams *et al.* (2012b)	x	H17	JQ843659
B. terrestris	F	1	France	Perpignan	--	--	2008	Williams *et al.* (2012b)	x	H1	JQ843670
B. terrestris	F	1	Latvia	--	--	--	--	Williams *et al.* (2012b)	x	H1	JQ843609
B. terrestris	F	1	Latvia	--	--	--	--	Williams *et al.* (2012b)	x	H1	JQ843610
B. terrestris	F	1	Great Britain	--	--	--	--	Williams *et al.* (2012b)	x	H8	JQ843628
B. terrestris	F	1	Great Britain	--	--	--	--	Williams *et al.* (2012b)	x	H8	JQ843631
B. terrestris	F	1	Great Britain	Bromley, close to London	--	--	2011	Williams *et al.* (2012b)	x	H8	JQ843632
B. terrestris	F	1	Germany	--	--	--	--	Williams *et al.* (2012b)	x	H1	JQ843633
B. terrestris	F	1	Great Britain	--	--	--	--	Williams *et al.* (2012b)	x	H8	JQ843634
B. terrestris	F	1	Greece	--	--	--	--	Williams *et al.* (2012b)	x	H1	JQ843635
B. terrestris	F	1	Sweden	Uppsala	--	--	2011	Williams *et al.* (2012a)	x	H1	JQ692970
B. terrestris	F	1	Sweden	--	--	--	--	Williams *et al.* (2012b)	x	H1	JQ843636
B. terrestris	F	1	Sweden	--	--	--	--	Williams *et al.* (2012b)	x	H1	JQ843637
B. terrestris	F	1	Sweden	--	--	--	--	Williams *et al.* (2012b)	x	H1	JQ843638
B. terrestris	F	1	Switzerland	--	--	--	--	Williams *et al.* (2012b)	x	H1	JQ843639
B. terrestris	F	1	Switzerland	--	--	--	--	Williams *et al.* (2012b)	x	H1	JQ843640
B. terrestris	F	1	Switzerland	--	--	--	--	Williams *et al.* (2012b)	x	H1	JQ843641
B. terrestris	F	1	Switzerland	--	--	--	--	Williams *et al.* (2012b)	x	H12	JQ843642
B. terrestris	F	1	Switzerland	--	--	--	--	Williams *et al.* (2012b)	x	H1	JQ843629
B. terrestris	--	1	Germany	Brandenburg, Mallnow	52.56 N	14.48E	28/03/2012	Schmidt *et al.* (2015)	x	H12	KJ838396
B. terrestris	--	1	Germany	Brandenburg, Mallnow	52.56 N	14.48E	28/03/2012	Schmidt *et al.* (2015)	x	H1	KJ838178
B. terrestris	--	1	Germany	Baden-Wuerttemberg, Muell	47.82 N	7.62 E	07/04/2012	Schmidt *et al.* (2015)	x	H1	KJ838879

Table S2. The sample size and diversity indices across *B. t. lusitanicus* samples by Iberian region considering mitochondrial DNA COI data and RADseq dataset BT_BTL. Note that six individuals were excluded from RADseq dataset. *n*, number of individuals analysed; *h*, average haplotype diversity; π, average nucleotide diversity; H_O, observed heterozygosity; H_E, expected heterozygosity.

Geographic region	COI			RADseq			
	n (total = 198)	*h*	π	*n* (total = 31)	H_O	H_E	π
IP-NW	24 (2 hap.)	0.08330	0.00014	2	--	--	--
IP-WE	56 (2 hap.)	0.07010	0.00012	11	0.2696	0.2919	0.2706
IP-SW	20 (2 hap.)	0.10000	0.00034	3	--	--	--
IP-NO	28 (2 hap.)	0.07140	0.00012	2	--	--	--
IP-CE	32 (1 hap.)	0	0	5	0.3148	0.3600	0.2702
IP-SE	38 (5 hap.)	0.19700	0.00044	8	0.2537	0.3128	0.2706

STACKS parameter testing

Methods

Preliminary tests of parameter values for each STACKS module were carried out in order to identify the optimal parameter values for use in the final STACKS analysis. The three datasets (BT_OUT, BT_SSP and BT_BTL) were tested separately. For the three first modules – *pstacks*, *cstacks* and *sstacks* – tests were performed using three sets of three randomly chosen individuals, each set corresponding to each one of the three datasets. For the last module – *populations* – all the samples were used because the number of SNPs obtained vary with population size and number.

Pstacks extract stacks that have been aligned to a reference genome and identify SNPs in each individual (Catchen *et al.*, 2013). In this module, the parameter -m (minimum depth of coverage to report a stack) was tested. Several tests were performed by increasing the 'm' value in increments of 2, from 2 to 10, while non-test parameters were kept as default. For each test, the number of created loci and respective mean coverage was evaluated. The input for *cstacks* module was created by running *pstacks* with the selected "optimal" parameters. *Cstacks* merges loci from multiple individuals, creating a set of consensus loci or catalog (Catchen et al., 2013). For the module, the parameter -n (maximum number of mismatches allowed between homologous loci among individuals when building the catalog) was also tested by increasing the value in increments of 2 from 2 to 10, while keeping non-test parameters as default. The number of loci added to the catalog for each 'n' value, as well as the number of verified matches against the catalog was then evaluated.

Populations is used to compute population-level summary statistics and to output SNPs according to some parameters (Catchen *et al.*, 2013). In this module two main parameters were tested: -p, which represents the minimum number of populations that a locus must be present in for it to be retained in the final dataset; -r, which represents the minimum percentage of individuals in a population that a locus must be present in for it to be retained in the final dataset (Catchen *et al.*, 2013). Several combinations using these two parameters were tested as follows: 'p' was set at values between 3 and 6 for dataset BT_BTL, at values between 5 and 9 for dataset BT_SSP and at values between 7 and 14 for dataset BT_OUT, which corresponds to 50% of taxa or above, for each dataset; 'r' was set to 50% and 100% for each 'p' setting. Only these two values of 'r' were tested because some taxa are only represented by two or less individuals. After testing each combination of parameters (-r; -p), we evaluated the number of retained SNP loci in each final dataset, the average coverage per SNP per sample and the average frequency of missing data per sample, using VCFTOOLS version 0.1.14.

Results

Figure S1 shows the results of parameter tests for *pstacks*. In *pstacks*, an 'm' parameter value of two (minimum of two reads to report a stack) resulted in a very large number of loci (min: 405 555, máx: 1 700 054), and with a low coverage, as expected (min: 7.26, máx 10.57). Increasing the minimum stack depth parameter to four prevented reads with possible sequencing errors from forming stacks and the number of

loci decreases considerably, as mean coverage increases. After m=6 (number of loci: min: 117 490, máx: 624 304; mean coverage: min: 17.73, máx: 27.95), further increases in 'm' resulted in slowed changes in number of loci, while coverage increases considerably, probably because the rate at which stacks absent from the reference were removed slowed. After this value, true stacks began to be dismantled, resulting in a lower number of loci, while the stacks that are maintained have very high coverage (Catchen *et al.*, 2013). The results from m=6 seem to be the ones which result in the best combination between loci number and coverage. In this way, the value of m set to 6 was chosen to perform the final STACKS analysis. The output results obtained from the parameter test using m=6 were used as input for the *cstacks* parameter tests.

Figure S2 shows the results of parameter tests for *cstacks* and *sstacks*. Increments in the maximum number of mismatches allowed between homologous loci from multiple individuals when building the catalog, seem to have a very small impact in the number of loci added to the catalog and in the number of verified matches of an individual against the catalog. This value varies with the evolutionary distance of the members being examined, and the small variation observed is probably the result of the small divergence among individuals (Catchen *et al.*, 2011). The value n=2 was chosen for final STACKS analysis and for running the input for *populations* parameters tests.

Figure S3 shows the results of parameter tests for *populations* module. Results for r=50% typically showed higher numbers of SNP loci with lower coverage but also with lower amount of missing data. This means that few SNPs are present in all individuals of each population. Higher values of 'p' typically yielded lower numbers of loci but with higher coverage and also lower amount of missing data. This means that, as the stringency of 'p' increased, less SNPs are available, but they have a relatively good coverage across all samples, and as a consequence, less missing data. Among the different tests, the number of retained loci ranged from 40 045 (average coverage per SNP per sample: 50.70; average frequency of missing data per sample: 0.10) to 14 231 for dataset BT_BTL (average coverage per SNP per sample: 73.25; average frequency of missing data per sample: 0), from 41 849 (average coverage per SNP per sample: 49.7; average frequency of missing data per sample: 0.1) to 14 580 (average coverage per SNP per sample: 72.6; average frequency of missing data per sample: 0) for dataset BT_SSP and from 45 184 (average coverage per SNP per sample: 44.69; average frequency of missing data per sample: 0.20) to 3 772 (average coverage per SNP per sample: 75.18; average frequency of missing data per sample: 0) for dataset BT_OUT. Based on these results, outputs from runs using the parameters p5r50 (dataset BT_BTL), p8r50 (dataset BT_SSP) and p13r50 (dataset BT_OUT) retain the better combination of number of loci, average coverage per SNP per sample and amount of missing data and these parameters were chosen for final STACKS analysis.
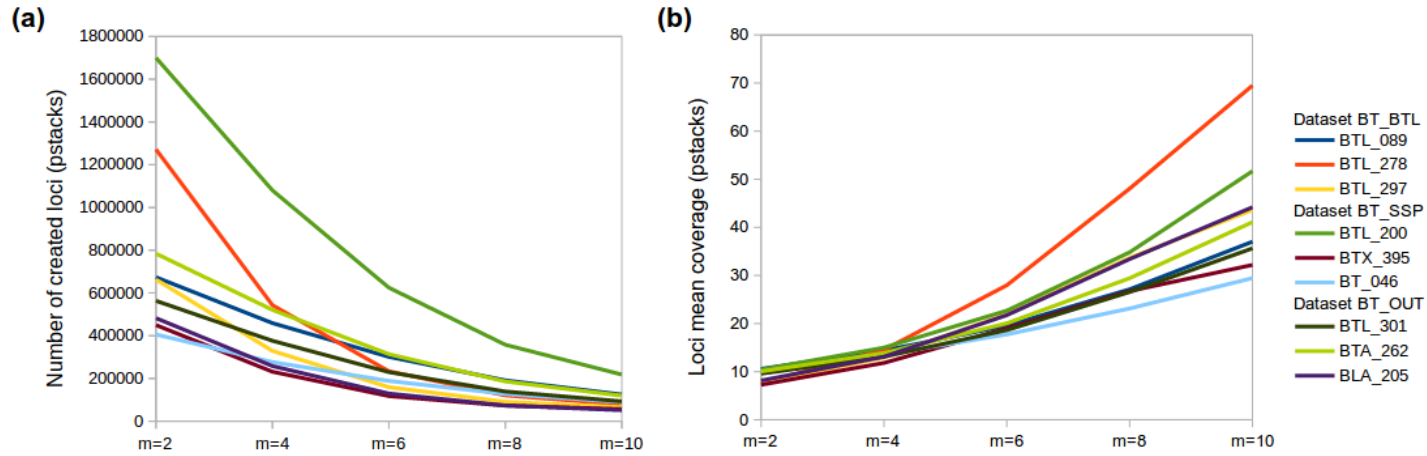
Figure S1. Results of parameter tests for the STACKS module *pstacks*. (a) Changes in number of created loci (*pstacks*) and (b) loci mean coverage obtained (*pstacks*) for each value of 'm' tested for three randomly selected individuals from each dataset.
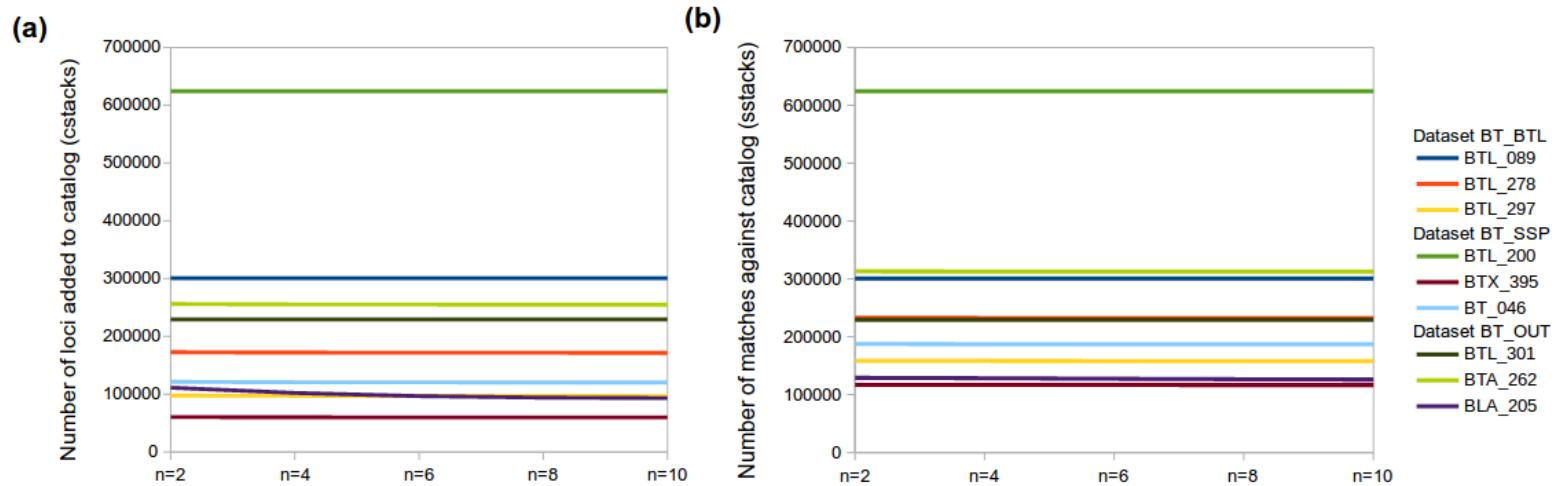

Figure S2. Results of parameter tests for the STACKS modules *ctsacks* and *sstacks*. (a) Changes in number of loci added to the catalog (*cstacks*) and (b) number of matches against the catalog (*sstacks*) for each value of 'n' tested for three randomly selected individuals from each dataset.
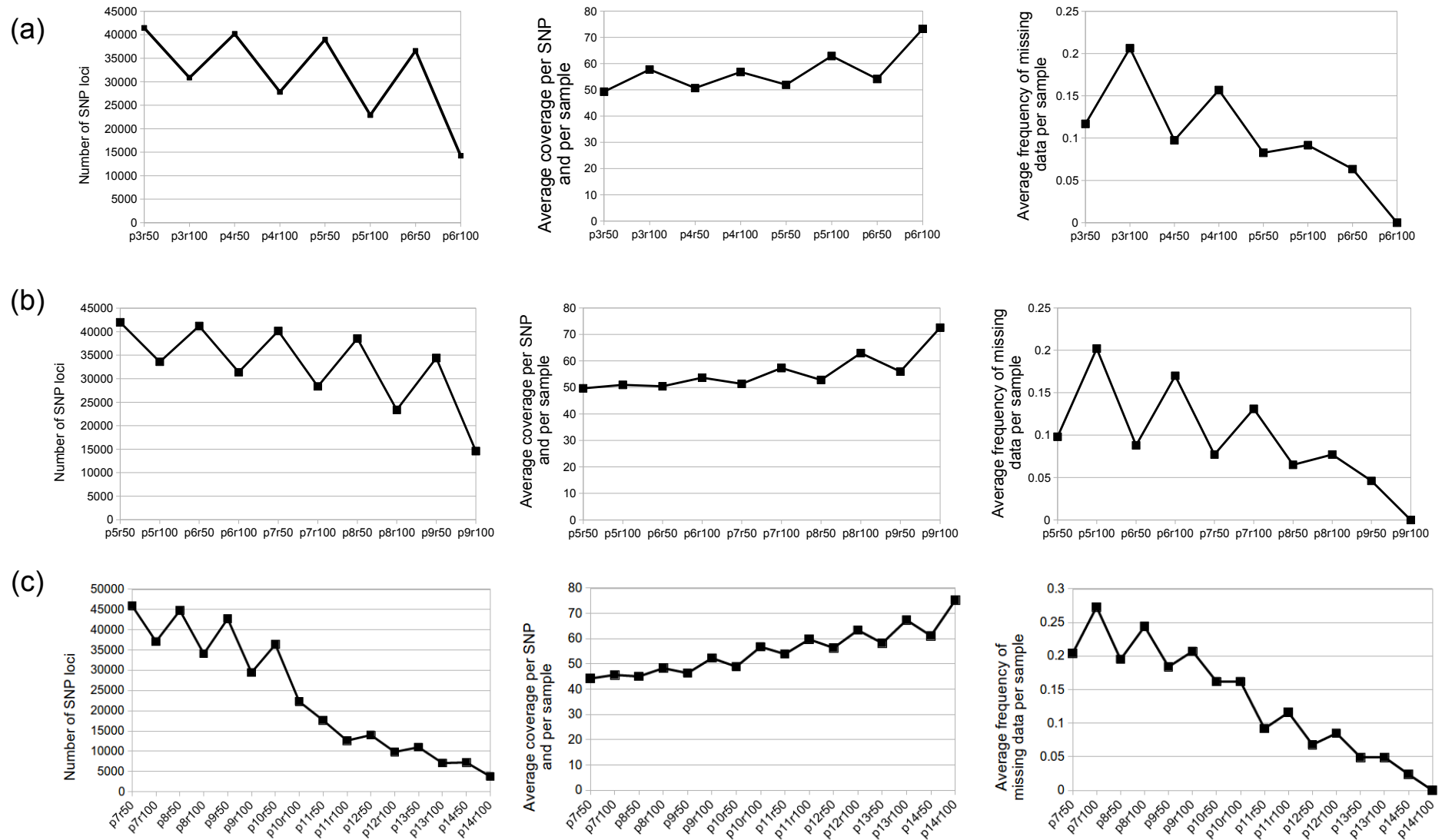
Figure S3. Results of parameter tests for the STACKS module *populations*. Variation in number of SNP loci in the final dataset, in average coverage per SNP and per sample and in average frequency of missing data per sample obtained for each combination of parameters (-r; -p) tested in *populations* module, using (a) dataset BT_BTL, (b) dataset BT_SSP and (c) dataset BT_OUT.

```
###################################################################
# List of command line commands used for RADseq dataset analyses ##
###################################################################
# in the commands below
# input_files_path = input file path
# output_files_path = output path to write results
# output_directory_path = output path to directory where to write the results
# barcodes_file_path = path to a file containing barcodes
# log_files_path = path to write logfile
# list_of_samples = list of the files corresponding to each sample
# populations_file_path = path to a file containing the list of populations


### Build index using BOWTIE2 version 2.1.0
bowtie2-build -f input_files_path Bt_all


### Prepare reads (cleaning and demultiplexing) with STACKS version 1.29 - pipeline
process_radtags
process_radtags -1 input_files_path -2 input_files_path -o output_files_path -b
barcodes_file_path -c -q -r -e pstI -E phred33 -i gzfastq -D


### Convert barcodes to sample name
#!/bin/bash
mv input_files_path/sample_ATTAG.1.fq.gz output_files_path/BTL_236.1.fq.gz
...


### Align reads with reference genome using BOWTIE2 version 2.1.0 #
#! /bin/bash

samples="
list_of_samples
"

for sample in $samples
do

bowtie2 --trim3 3 --sensitive -p 6 -x input_files_path/Bt_all -1 input_files_path/$
{sample}.1.fq.gz -2 input_files_path/${sample}.2.fq.gz -S input_files_path/$
{sample}_Bt_all.sam

done
```

```
### Conver SAM files to BAM using SAMTOOLS version 0.1.19
#!/bin/bash

for samfile in $(ls input_files_path/*.sam); do samtools view -Sb ${samfile} > $
{samfile}.bam; done



### Sort and filter BAM files using SAMTOOLS version 0.1.19
#! /bin/bash

samples="
list_of_samples
"

for sample in $samples
do
samtools sort input_files_path/${sample}_Bt_all.sam.bam output_files_path/$
{sample}_Bt_all.sam.bam.sorted
samtools index input_files_path/${sample}_Bt_all.sam.bam.sorted.bam
samtools view -q 20 -F 0x0004 -b input_files_path/$
{sample}_Bt_all.sam.bam.sorted.bam > output_files_path/$
{sample}_Bt_all.sorted.quality.bam
samtools flagstat input_files_path/${sample}_Bt_all.sam.bam.sorted.bam >
output_files_path/${sample}_Bt_all.sorted_flagstat
samtools flagstat input_files_path/${sample}_Bt_all.sorted.quality.bam >
output_files_path/${sample}_Bt_all.sorted.quality_flagstat

done



### Build loci from ref using STACKS version 1.45 - pipeline pstacks
# !/bin/bash
samples="
list_of_samples
"

i=1
for sample in $samples
do
        pstacks -t bam -i $i -m 6 -p 4 \
                -f input_files_path/$sample \
                -o output_files_path &>> log_files_path/Log_pstacks
        let "i+=1";
done
```

```bash
### Create catalog and match samples to catalog using STACKS version 1.45 -
pipeline cstacks and sstacks
#!/bin/bash

samples="
list_of_samples
"

samp=""
for sample in $samples
do
 samp+="-s input_files_path/$sample ";
done

cstacks -p 4 -b 1 -n 2 -o output_files_path $samp &>> log_files_path/Log_cstacks

for sample in $samples
do
        sstacks -p 4 -b 1 -c input_files_path/batch_1 \
        -s input_files_path/${sample} \
        -o output_files_path &>> log_files_path/Log_sstacks
done


### Filter and export SNPs to VCF file format using STACKS version 1.45 - pipeline
populations, using dataset BT_OUT
#!/bin/bash

populations -b 1 -P input_files_path -M input_files_path -t 4 -p 13 -r 50 --vcf --
write_random_snp


### Filter and export SNPs to VCF file format using STACKS version 1.45 - pipeline
populations, using dataset BT_SSP
#!/bin/bash

populations -b 1 -P input_files_path -M input_files_path -t 4 -p 8 -r 50 --vcf --
write_random_snp


### Filter and export SNPs to VCF file format using STACKS version 1.45 - pipeline
populations, using dataset BT_BTL
#!/bin/bash
```

```
populations -b 1 -P input_files_path -M input_files_path -t 4 -p 5 -r 50 --vcf --
write_random_snp
```

Determine missing data per individual and filter VCF files using VCFTOOLS version 0.1.15
```
vcftools --vcf input_file_path --missing-indv
vcftools --vcf input_file_path --max-missing 0.75 --maf 0.05 --recode --out
output_file_path
```

Determine per-SNP nucleotide diversity, observed and expected heterozygosities
```
vcftools –-vcf input_file_path –-site-pi
vcftools –-vcf input_file_path –-het
```

Principal component analysis of the three datasets using the R script snp_pca_static.R (https://github.com/CoBiG2/RAD_Tools) as of commit 134b11d
```
snp_pca_static.R input_files_path output_files_path populations_file_path
```

Run BAYESCAN version 2.1 using both datasets BT_SSP and BT_BTL
```
./bayescan_2.1 input_file_path -snp -threads 14 -od output_directory_path
```

Run SELESTIM version 1.1.7 using both datasets BT_SSP and BT_BTL
```
./selestim -threads 12 -file input_files_path -outputs output_directory path -thin
20 -npilot 50 -lpilot 1000 -burnin 100000 -length 1000000 -calibration
```

Run Maverick version 1.0.4 with Structure_threader version 1.2.4, using dataset BT_SSP
```
~/.local/bin/structure_threader run -K 5 -i input_file_path -o output_file_path -t
10 -mv ~/.local/bin/MavericK --params parameters.txt --log=1
```

Run Maverick version 1.0.4 with Structure_threader version 1.2.4, using dataset BT_BTL
```
~/.local/bin/structure_threader run -K 4 -i input_file_path -o output_file_path -t
10 -mv ~/.local/bin/MavericK --params parameters.txt --log=1
```

Final set of parameters (parameters.txt) used for run Maverick, using dataset BT_SSP

Data properties

\# headerRow_on   t

\# popCol_on   f

\# ploidyCol_on   f

\# ploidy   2

\# missingData -9

Model parameters

\# Kmin 1

\# Kmax 5

\# admix_on   t

\# fixAlpha_on f

\# alpha   1.0,0.845,0.448,0.414,0.32

\# alphaPropSD 1.0,0.2125,0.0975,0.065,0.063

Simulation parameters

\# exhaustive_on   f

\# mainRepeats 10

\# mainBurnin 5000

\# mainSamples 50000

\# thermodynamic_on t

\# thermodynamicRungs   50

\# thermodynamicBurnin   5000

\# thermodynamicSamples   50000

\# EMalgorithm_on   f

\# EMrepeats   100

\# EMiterations   100

Basic output properties

\# outputLog_on   t

\# outputLikelihood_on   t

\# outputQmatrix_ind_on   t

\# outputQmatrix_pop_on   f

\# outputQmatrixError_ind_on   t

\# outputQmatrixError_pop_on   f

\# outputEvidence_on t

\# outputEvidenceNormalised_on   t

\# outputEvidenceDetails_on t

Final set of parameters (parameters.txt) used for run Maverick, using dataset BT_BTL

Data properties

\# headerRow_on   t

\# popCol_on   f

\# ploidyCol_on   f

```
# ploidy      2
# missingData -9
#### Model parameters
# Kmin 1
# Kmax 4
# admix_on    t
# fixAlpha_on f
# alpha       1.0, 3.94, 1.58, 1.05
# alphaPropSD 1.0, 0.51, 0.18, 0.12
#### Simulation parameters
# exhaustive_on      f
# mainRepeats 10
# mainBurnin  5000
# mainSamples 50000
# thermodynamic_on   t
# thermodynamicRungs        50
# thermodynamicBurnin       5000
# thermodynamicSamples      50000
# EMalgorithm_on     f
# EMrepeats   100
# EMiterations       100
#### Basic output properties
# outputLog_on        t
# outputLikelihood_on       t
# outputQmatrix_ind_on      t
# outputQmatrix_pop_on      f
# outputQmatrixError_ind_on        t
# outputQmatrixError_pop_on        f
# outputEvidence_on t
# outputEvidenceNormalised_on      t
# outputEvidenceDetails_on t
```