What can genetics tell us about the history of a human-mediated introduction of the Golden-striped salamander south of its native range

Sequeira F[1], Aguilar FF[1,4], Madeira FM[4], Teixeira J[3], Crespo E[2], Ferrand N[1,5,6], Rebelo R[4]

[1]CIBIO, Centro de Investigação em Biodiversidade e Recursos Genéticos, InBIO Laboratório Associado, BIOPOLIS Program in Genomics, Biodiversity and Land Planning, Campus de Vairão, Universidade do Porto, 4485-661 Vairão, Portugal

[2]Departamento de Biologia Animal, Faculdade de Ciências da Universidade de Lisboa, Campo Grande, 1749-016 Lisboa, Portugal

[3]CIIMAR - Interdisciplinary Centre of Marine and Environmental Research, U. Porto – University of Porto, Terminal de Cruzeiros do Porto de Leixões, Av. General Norton de Matos s/n, 4450-208 Matosinhos, Portugal

[4]cE3c centre for Ecology, Evolution and Environmental Changes & CHANGE - Global Change and Sustainability Institute, Faculdade de Ciências da Universidade de Lisboa, Campo Grande, 1749-016 Lisboa, Portugal

[5]Departamento de Biologia, Faculdade de Ciências, Universidade do Porto, Rua do Campo Alegre s/n, Porto, 4169-007, Portugal

[6]Department of Zoology, University of Johannesburg, PO Box 524, Johannesburg, Auckland Park, 2006, South Africa

*Corresponding author – Fernando Sequeira, CIBIO, Centro de Investigação em Biodiversidade e

Recursos Genéticos, InBIO Laboratório Associado, Campus de Vairão, Universidade do Porto, 4485-661

Vairão, Portugal. Telephone: 00351 252660400; E-mail: fsequeira@cibio.up.pt

Running title: Human-mediated introduction of an Iberian salamander

Abstract

28 The golden-striped salamander is a streamside species endemic to the northwestern corner of the Iberian

29 Peninsula. In the first half of the twentieth century, an undisclosed number of individuals of this species

30 were reportedly captured in Buçaco, central Portugal, and deliberately introduced in Serra de Sintra, 170

31 km south of its native distribution range. The discovery of a breeding population of this salamander in

32 Sintra during 2015 prompted this work: we used neutral genetic markers, the mitochondrial DNA

33 Cytochrome b (cyt b) and seven microsatellite loci to elucidate on the relic/human-introduced nature of

34 Sintra population, identify the potential source population and infer the severity of founder effect. Our

35 results support a human-mediated introduction. First, sequencing analysis of cyt b showed the presence of

36 a unique haplotype (h31) in Sintra, which was detected only in Buçaco and in two additional populations

37 located close to Mondego river. Second, microsatellite analysis showed that Sintra is more closely related

38 with populations in between Douro and Mondego rivers (Central Portugal), instead of its geographically

39 closest populations (southernmost), as would be expected if Sintra were a relic population isolated in an

40 interglacial refuge. Third, Sintra presents both reduced levels of genetic variability and effective population

41 size when compared to native populations, particularly to those of Central Portugal. Consistent with an

42 isolated population funded by a small number of individuals (inferred herein to be ca. 10 salamanders),

43 Sintra forms a geographically coherent genetic unit that is significantly differentiated from the extant native

44 *C. lusitanica* populations. Although changes in the genetic makeup of Sintra do not allow to track

45 unequivocally the origin of the introduced individuals, genetic signs from both nuclear and mtDNA data

46 provide supporting evidence for Buçaco as the most likely source population, which coincides with the

47 documented history of the introduced population in Sintra.

48 **Introduction**

49 Elucidating the history of species introductions requires a multidisciplinary endeavour in that molecular

50 approaches appear as powerful tools. For instance, crossing historical records with patterns of genetic

51 variation has contributed to identify introduced populations that were previously considered native ones

52 (Gippoliti and Amori, 2006; Clavero et al., 2016; Tiberti and Splendiani, 2019; Kehlmaier et al., 2020).

53 Since introduced populations are expected to have a genetic makeup that reflects their source population

54 (Sakai et al., 2001; Antzen et al., 2010), genetic analyses can also give important insights on the

55 reconstruction of the history of the introduction, including the identification of the source population as

56 well as the number of founders that formed the initial introduced population ("propagule size"), the severity

57 of the founder effect and bottleneck, and the number of generations over which the population has been

58 isolated from the source population (e.g. Nei et al., 1975; Frankham, 1995; Ficetola et al., 2008; Simberloff,

59 2009).

60 Amphibians are currently the most endangered tetrapod group (IUCN, 2022). This is often explained by

61 their exquisite requirements, such as of humid or unpolluted freshwater microhabitats, that makes them

62 vulnerable to even slight changes in their quality or microclimate (Button and Borzée, 2021). Dependence

63 on microclimate has led several species to cling on relic, small extent habitats, increasing their vulnerability

64 to extinction (Ceballos et al. 2020). As a response to range reductions or to a threatened status, a possible

65 conservation tool is population translocations and/or reintroductions to climatically suitable areas, but this

66 has rarely been attempted with amphibians (Kraaijeveld-Smit et al. 2006).

67 The golden-striped salamander, *Chioglossa lusitanica* (Bocage 1864), is a monotypic endemic caudate of

68 Northwestern Iberian Peninsula, where it inhabits the banks of swift running streams with dense

69 surrounding vegetation in fairly mountainous areas, characterized by high rainfall, high topographical relief

70 and mild summer and winter temperatures (Arntzen 1981; Sequeira and Alexandrino 2008). This

71 salamander has unique morpho-physiological traits that make displacements outside moist habitats severely

72 restricted (Arntzen 1995). In an undisclosed date before 1943, a Portuguese zoologist (Anthero Seabra)

73 ordered that "a few specimens" (unknown number) of the golden-striped salamander should be collected at

74 Mata do Buçaco (a 400-ha State Forest in Buçaco Mountains, Coimbra, central Portugal; Figure 1) and

75 released in the Sintra Mountains (located 20 km NW of Lisbon and about 170 km of the currently southern

76 distribution limit of the species; Figure 1) (Seabra 1943). At the time, the association of this salamander

77 with the mountain streams in the North of the country was already clear to Seabra, as well as its probable

78 absence south of the region of Coimbra-Buçaco (Seabra 1943). Seabra mentions that environmental

79 conditions of Sintra and other mountains in the south of the country were similar to those of the northern

80 mountains where *C. lusitanica* was common, and that the species should thrive there if it could somehow

81 cross the inhospitably hot and dry habitats in between (Seabra 1943). However, the species presence had

82 been cited for the Sintra Mountains at the end the XIX century by Vieira (1886) and, based on a distribution

83 ecological modelling approach, this area was indicated as a potentially suitable area for *C. lusitanica*.

84 (Teixeira et al. 2001), leading to the possibility that the species already existed in the region in the past or

85 some relict rear edge population may still subsisted till the present (Arntzen and Teixeira 2006). The success

86 of Seabra's "re-stocking essay" (as he referred to it) was never monitored, and in fact Seabra mentions it

87 only in a footnote, and specifically "so that in the future, if by chance the species is found at Sintra, its

88 origin will be explained" (Seabra 1943). Indeed, only 60 years after its putative introduction, one single

89 individual of the species was observed by the naturalist Gaston-Denis Guex in Sintra (Arntzen 1999). The

90 occurrence of a reproducing population of *C. lusitanica* in Sintra was only confirmed in 2015 (Aguilar et

91 al. 2018). Through a 2 years monitoring program, these authors found a relatively small population

92 (estimated at *ca.* 340 individuals) confined to a small area along ca.110 m stretch of a single stream (Aguilar

93 et al. 2018).

94 Here we use neutral genetic markers, mitochondrial DNA cytochrome b (mtDNA cyt b) sequences and

95 seven microsatellite loci, to address whether the genetic composition of Sintra population is compatible

96 with the introduction history described by Seabra (1943) or the possibility of a relict population. By

97 comparing genetic variation between Sintra population with that reported for populations across the entire

98 species' native range based on the same genetic markers (Alexandrino et al. 2000, 2002; Sequeira et al.

99 2008), we aimed to identify the history of this population, the possible occurrence and source of introduced

100 individuals and gain insights about the severity of founder effects by determining the reduction in levels of

101 genetic diversity and the potential number of founder individuals of the introduced population.

102 **Materials and methods**

103 *Sampling and data collection*

104 A total of 97 salamander tail-tip tissue samples were collected from three localities: Sintra (47), two

105 additional sites close to Mondego river: Misarela (25) and Riba de Cima (25); and 5 individuals from

106 Buçaco (Figure 1, Table 1). Tissue samples were preserved in 70% ethanol. Whole genomic DNA was

107 extracted using QIA Quick DNEasy columns (Qiagen, Inc., Valencia, CA, USA) following standard DNA

108 extraction protocols. Seven microsatellite polymorphic loci (*CL5, CL6*, *CL17*, *CL19*, *CL39*, *CL136*, and

109 *CL145*) described by Sequeira et al. (2005) and sequences of the mitochondrial cytochrome b (cyt b), 700-

110 bp long fragment (Alexandrino et al. 2002), were chosen for analysis.

111 PCR amplification of cyt b and sequencing protocols are as in Alexandrino et al (2000). PCR products were

112 sequenced in both directions by using the PCR primers with the BigDye Terminator v3.1 Cycle sequencing

113 protocol (Applied Biosystems), an ABI Prism 3130XL Genetic Analyzer automated sequencer (Applied

114 Biosystems). Sequences were edited and aligned using BioEdit, version 7.2 (Hall, 1999). DNA samples

115 were amplified at seven microsatellite markers, accomplished with fluorescently labelled primers, using

116 multiplexed PCR and published protocols optimized by Sequeira et al (2005, 2008) with slight

117 modifications. PCR products were separated by capillary electrophoresis on an automatic sequencer

118 ABI3130xl Genetic Analyzer (AB Applied Biosystems). Fragments were scored against the GeneScan-500

119 LIZ Size Standard using the GENEMAPPER 4.1 (Applied Biosystems). To ensure no bias in allele sizing,

120 several samples previously analysed by Sequeira et al. (2008) were genotyped as control.

121 *Genetic diversity and population assignment*

122 For the mtDNA cyt b gene dataset, we used DnaSP 6.0 software (Rozas et al. 2017) to estimate diversity

123 parameters, including nucleotide diversity and haplotype diversity (h). For microsatellites, MICRO-

124 CHECKER 2.2.1 (van Oosterhout et al. 2004) was used to check amplified microsatellite genotypes for

125 large allele dropout, scoring errors due to stuttering and the presence of null alleles. Measures of genetic

126 diversity, including the mean number of alleles, the expected hererozygosity (He) and *f* estimator of FIS

127 per population, was estimated using GENETIX v.4.05 (Belkhir et al. 2000). Allelic Richness was estimated

128   using a rarefaction procedure implemented in HP-RARE 1.0 (Kalinowski 2005). The minimum number of

129   genes in analysed populations (Table 1, but see details below for total dataset and Sequeira et al. 2008) was

130   32 (16 genotypes), so this was used as a basis for rarefaction. To test for linkage disequilibrium (LD) and

131   departures from Hardy-Weinberg equilibrium (HWE) among all pairs of loci in each population, we used

132   GENEPOP 3.3 (Raymond and Rousset 1995). All probability tests were based on MCMC simulations (Guo

133   and Thompson 1992; Raymond and Rousset 1995) using default values, with significance levels adjusted

134   for multiple tests using sequential Bonferroni corrections to minimize type I errors ($\alpha = 0.05$; Rice 1989).

135   BOTTLENECK 1.2.02 (Piry et al. 1999) was used to investigate signatures of a recent reduction in effective

136   population size of the (introduced) Sintra population by using the mode-shift test and one-tailed Wilcoxon

137   signed-rank test (Cornuet and Luikart 1996), under a two-phase model (TPM; Di Rienzo et al. 1994) with

138   90% stepwise mutation and 10000 iterations.

139   To accomplish the main goals of this study (elucidate the origin and genetic diversity of the introduced

140   population – Sintra), for downstream analyses we used mtDNA (Alexandrino et al. 2002) and microsatellite

141   data (Sequeira et al. 2008) that had been analysed previously (Figure 1; Table 1). Microsatellite data

142   consisted of allele frequencies (total of 96 alleles) at the same seven polymorphic loci scored for 286 (16

143   to 27 individuals/location) salamanders (Sequeira et al. 2008). MtDNA data consisted of 120 sequences (2

144   to 12 individuals/location) of a cyt b fragment (30 distinct haplotypes; GenBank accession numbers:

145   AF329285-AF329314), sampled from the same 13 locations as used for microsatellite analyses by

146   Alexandrino et al. (2002). Altogether, a total dataset composed of 140 cyt b sequences and 375 individuals

147   genotyped at seven microsatellite loci, from 16 populations distributed across the entire species' range,

148   were analysed in this study (Table 1; Supplementary Table S1). Intraspecific relationships of the cyt b

149   haplotypes were inferred by a median-joining network using the NETWORK software v. 5.0.0.1 (Bandelt

150   et al. 1999). Pairwise genetic differentiation between populations based on microsatellite data was evaluated

151   using Weir and Cockerham's unbiased F-statistics (F_{ST}) (Weir and Cockerham 1984) and Shared Allele

152   Distance (DAS; Chakraborty and Jin 1993) using Populations 1.2.31 (Langella 1999). To infer relationship

153   among individuals and populations, a phylogenetic tree was reconstructed by the Neighbor-Joining method

154   using the Populations software. The Tree was based on the DAS with 1000 bootstraps, and was viewed and

155   edited in Mega 11.0 (Kumar et al. 2018). We further assigned individuals to genetic groups using the

156   clustering-based approach STRUCTURE v.2.3.4 (Pritchard et al. 2000). We ran STRUCTURE with 5

157   replicates for each K value ranging from K=1 to K=10, with a burnin period of 100,000 and 500,000 steps

158   under the admixture model and uncorrelated allele frequencies. The optimal number of ancestral

159   populations was determined using Evanno's ΔK method (Evanno et al. 2005) as implemented in

160   STRUCTURE HARVESTER (Earl and von Holdt 2012), and to account for label switching between results

161   of different runs with same K, replicate runs were merged using the CLUMPAK (Kopelman et al. 2015) on

162   the webserver http://clumpak.tau.ac.il. Additionally, a Factorial Correspondence Analysis on the allelic

163   frequencies, as implemented in GENETIX 4.05, was used as a model-independent approach (i.e., free of

164   assumptions on the underlying population genetics model) to identify and describe clusters of genetically

165   related individuals.

166   *Effective population size and minimum number of founders*

167   We estimated contemporary effective population size (Ne) with a single temporal sample through the

168   linkage disequilibrium (LD) method with Jacknifing implemented in NeEstimator 2.1 (Do et al. 2014). We

169   also estimated Ne based on theoretical predictions of the relationship between genetic drift and population

170   size (see Hendrick 2000 and references therein). Based on the assumption that heterozygosity decreases

171   approximately at a rate of $1/(2Ne)$ per generation, the effective size of a population over time can be

172   calculated using the equation (Hedrick 2000): $Ne = 1/ [2 * (1 - H^{1/t})]$, where H is the ratio ($H_T/H_E$) of the

173   expected heterozygosity found in Sintra (H_T) to that expected (H_E) in the source population, and t is the

174   number of generations. For calculations we used a generation time estimate of four years (Lima et al. 2001),

175   and thus, a T of 18 generations, assuming that population was founded 72 years ago (Seabra 1943). For the

176   calculations we used Buçaco as the most likely source population, but because populations around Buçaco

177   and Mondego river (SA, BU, VA, MI and RC) group together, we also used H_E averaged across those

178   populations (see Results).

179   To obtain a minimum estimate of the founder population, we used an approach based on Rasner et

180   al. (2004), which requires empirical information from microsatellite alleles observed in the present

181   population. We simulated in R 4.0.2 (R Core Team 2020), via a custom written script (see Fisher et al.

182   2015), genetic profiles of the founder individuals by randomly sampling alleles (10,000 replicates)

183   independently for each locus, and without replacement from the genetic profiles. The smallest number of

184   founders consistent with the observed data (i.e. smallest number of individuals containing all the alleles

185   identified at the seven microsatellites), was taken to be the number that gave a p-value > 0.05 of capturing

186   the observed alleles.

187   **Results**

188   Sequencing of the mtDNA cyt b fragment (700 bp long) performed on 25 samples from the three newly

189   sampled populations and additional five individuals from Buçaco, uncovered a total of four haplotypes.

190   Considering the published data (Alexandrino et al. 2002), three haplotypes (h31-33; XXX-XXX GenBank

191   accession no. upon acceptance) were newly described (Fig. 2; Supplementary Table S3). Samples from

192   Sintra were fixed for the newly described haplotype h31. This haplotype was only detected among

193   populations close to Mondego river (RC and MI) and Buçaco (Fig. 2; Supplementary Table S3). The

194   haplotype network showed that the newly described haplotypes, including the one observed in Sintra,

195   belong to *C. lusitanica longipes,* the subspecies that occurs in southern part of *C. lusitanica* range, south of

196   Buçaco population (Fig. 2).

197   We successfully genotyped 85 individuals at all seven microsatellite markers. No evidence of

198   scoring errors due to stuttering or large allele dropout was found. For the analysed populations and across

199   all loci, there were no significant deviations from Hardy-Weinberg or linkage equilibrium. STRUCTURE

200   analysis revealed that the most likely number of genetically distinct clusters of *C. lusitanica* is four ($K = 4$;

201   Fig. 3 and Supplementary Fig. S1), albeit a second clear peak at $K = 5$ was also apparent (Supplementary

202   Fig. S1). At $K = 3$, Sintra population clustered with populations located in between Douro and Mondego

203   rivers, while populations south of Mondego River and the ones north of Douro river represent the other two

204 clusters. At $K \geq 4$, Sintra formed a separate population cluster (Supplementary Fig. S1). At $K = 4$-5, BU is

205 the population with higher average levels of proportion of assignment to Sintra cluster (12.4 -14.0%,

206 respectively; Supplementary Fig. S1 and Table S4). In addition, individuals partly admixed (membership

207 proportion $\geq$ 30%) with the Sintra cluster were only found in SA (2) and BU (5) populations. The FCA

208 analysis is in line with the STRUCTURE results, supporting the population allocation of four spatial groups

209 (Fig. 3). According to FCA, Sintra appears clearly separated from the extant groups, being closely related

210 with populations of Central Portugal between Douro and Mondego rivers (in between rivers), while

211 populations north of Douro and south of Mondego river, form independent groups. The microsatellite-based

212 phylogenetic tree showed similar results to those obtained with FCA and STRUCTURE. However, the

213 separation of Sintra from the other populations of Central Portugal (in between rivers) is not statistically

214 supported (Fig. 3). Pairwise F_{ST} and DAS genetic distance are in agreement with the genetic differentiation

215 of the introduced population (Sintra). Sintra is strongly differentiated ($F_{ST} >0.25$; DAS>0.4) from most

216 populations, except those close to Mondego river ($F_{ST} = 0.19$-0.21; DAS = 0.29-0.31) and, in particular

217 with Buçaco ($F_{ST} = 0.14$ and DAS=0.20), from which Sintra appears as only moderately differentiated

218 (Supplementary Table S5). A summary of all pairwise F_{ST}s and DASs values is provided in Supplementary

219 Table S5.

220 For the Sintra population, the seven microsatellite loci yielded a total of 19 alleles, with a N_a of 2.7,

221 an A_R of 2.5, and H_E of 0.452 (Table 1, Supplementary Table S2). With exception of the four populations

222 north of Douro river, genetic diversity statistics in Sintra are consistently lower than those of the extant

223 populations. When compared (One sample t-test) to populations from Central Portugal (populations

224 phylogenetically most closely allied with Sintra, and therefore harboring the potential source population

225 from which founder individuals have been taken), all measures of genetic diversity in Sintra are

226 significantly lower (N_a: t=17.269, df=6, p<0.001; A_R: t= 16.352, df=6, p<0.001; H_E: t=12.205, df=6,

227 p<0.001).

228 Bottleneck tests indicated (using Wilcoxon tests of significance, and mode-shift of allele frequency

229 distributions) that Sintra population has undergone a significant size reduction (TPM model, p = 0.023;

230   shifted mode distribution of allele frequencies). Results from NeEstimator returned several infinite

231   estimates, both for point estimates and upper confidence limits (Supplementary Table S6). Despite of this,

232   our results showed that estimates for Sintra were relatively lower ($\approx$ 24; 95% CI 10.5-77.8) than most of

233   the other populations from its native range, especially those close to Mondego river ($Ne \approx$ 182-997, 95%

234   CI 25.3-∞). Based on the average decline of the expected heterozygosity in Sintra relative to that in Buçaco

235   after 18 generations ($\approx$ 18%), we estimated a current $Ne \approx$ 25, which is similar to that returned by LD

236   method implemented in NeEstimator. Using the decline of heterozygosity relative to the averaged value

237   (15%) of populations from Central Portugal around Buçaco and Mondego river, our estimate was slightly

238   higher ($Ne \approx$ 34), albeit within the confidence interval returned by NeEstimator (Table S6). Based on

239   resampling technique of empirical data set, the effective number of founders was estimated to be of around

240   10-11 individuals (Supplementary Fig. S3).

241   **Discussion**

242   According to scarce historical available information, there was an old and dubious citation of the species

243   presence in the area at the end of the XIX century (Vieira 1886) and the report of relatively few individuals

244   of *C. lusitanica* collected at Buçaco mountains being deliberated introduced in Sintra at mid XX century

245   (Seabra, 1943). Our genetic analyses provide several evidences that support this documented human-

246   mediated introduction as the source of the present population. First, individuals from Sintra form a

247   geographically coherent genetic unit that are differentiated from the extant *C. lusitanica* populations.

248   Second, Sintra appears more closely related with populations in between Douro and Mondego rivers

249   (Central Portugal), instead of its geographically closest populations (southernmost), as would be expected

250   if Sintra were a natural population that has been kept isolated in an interglacial refuge (see example of the

251   Iberian north-western lacertid, *Lacerta schreiberi*, in Brito et al. 1986). Third, in line with theoretical

252   predictions and empirical studies of introduced populations (e.g. Nei et al. 1975; Allendorf and Lundquist

253   2003), Sintra presents reduced levels of genetic diversity when compared to native populations, particularly

254   to those of Central Portugal, which is consistent with the significant signs of population reduction returned

255   by the TPM model, and shifted mode distribution of allele frequencies in the bottleneck analysis. Finally,

256   in agreement with the hypothesis that Sintra population experienced a bottleneck at the founding event, the

257   reduction of genetic diversity is much more pronounced in number of alleles than in levels of heterozygosity

258   (Nei et al. 1975; Luikart et al. 1998; Dlugosch and Parker 2008). Sintra has 60% less of the allelic richness

259   and 15-18% less of heterozygosity when compared to its closely related populations (Central Portugal),

260   which agrees with the proportional losses of allelic richness *versus* heterozygosity (on average 5.1% higher

261   for allelic richness) reported by several studies of introduced populations (see review in Dlugosch and

262   Parker 2008). Besides, when compared to the northernmost populations (GE, PO, SAL), which correspond

263   to a recent range expansion and postglacial colonization of northern Iberia (Alexandrino et al., 2000;

264   Sequeira et al., 2008), Sintra has similar number of alleles but higher levels of heterozygosity. The rationale

265   is that allelic richness does not consider the frequency of the alleles but only their presence, being thus

266   particularly sensitive to the loss of rare alleles (as expected in founder events) that contribute little to

267   heterozygosity (Allendorf 1986; Spencer 2000, Leberg 2002; Greenbaum et al. 2014). This is particularly

268   evident when using microsatellite markers because a higher proportion of their alleles are normally at low

269   frequency (Dlugosch and Parker 2008).

270   Amongst the genetic cluster composed by the populations in between Douro and Mondego rivers,

271   which is the most closely related with Sintra, Buçaco is the population that presents the lower levels of

272   genetic differentiation (F_{ST} and DAS) and higher levels of admixture proportion with Sintra cluster (12.4 -

273   14.0%, as revealed by Structure analyses at $K = 4$-5, respectively; Supplementary Table S4). Despite these

274   signatures from nuclear data together with the presence of the mtDNA haplotype h31 (that is fixed in Sintra)

275   provide supporting evidence for Buçaco as the source population, overall we cannot definitively exclude

276   other neighboring closely related populations as alternative source. Indeed, the single mtDNA haplotype

277   observed in Sintra (h31) also occurs in two populations close to Mondego river (MI and RC; Fig. 1) and at

278   higher frequency (0.8-0.6, respectively) than observed in Buçaco (0.07). Furthermore, some individuals

279   from SA appeared partly assigned and clustered to Sintra according to STRUCTURE and FCA analyses,

280   respectively (Fig. 3). Finally, SA and other populations close to Mondego river (VA and MI) are also only

281   moderately differentiated from Sintra (F_{ST}= 0.19-0.21). It is worth mentioning that substantial loss of

282   genetic diversity and shifts in allele/haplotype frequencies are expected in small isolated populations

283   through the process of random genetic drift (Chakraborty and Nei 1977; Keller et al. 2012). The effects of

284   this process could even be more exacerbated in the case of mtDNA because its effective population size is

285   one-fourth of the one calculated from nuclear markers (e.g. Ballard and Whitlock 2004). In any case, the

286   expected changes in the genetic makeup of an introduced population after several generations of isolation

287   make difficult the identification of its source population, especially if founder individuals represented only

288   a fraction of the total amount of genetic variation (Keller et al. 2012). A visual inspection of alleles profile

289   among loci showed that Sintra has an overall lower number of alleles and a substantial shift in their

290   frequency. For instances, many alleles, independently of its frequency in between rivers cluster (putative

291   source populations), were not found in Sintra, and some low-frequency alleles present on that cluster

292   reached relatively high frequencies in Sintra (e.g. allele 119; locus Cl5), as expected under the effect of

293   random drift acting on a small founding population (e.g. Nei et al. 1975; Bartlett 1985). Other factor that

294   may affect the ability of identifying the source population is the level of genetic differentiation within

295   species' native populations (source area). Although significant, the level of differentiation between Buçaco

296   and its closely related neighboring populations, especially among SA, VA and MIS is relatively low (F_{ST}=

297   0.014-0.034). In spite of the limitations aforementioned, further work increasing the number of loci

298   involved and the intensity of sampling in the potential source area, especially in Buçaco population could

299   improve the accuracy in tracking the origin of Sintra.

300   The high proportion of heterozygosity retained in Sintra (∼82%) is likely to reflect this population's

301   relatively recent isolation (Furlan et al. 2012), supposedly around 72 years ago (Seabra, 1943). Based on

302   the assumption that heterozygosity decreases approximately at a rate of $1/(2Ne)$ per generation (Hedrick

303   2000), a decline in heterozygosity of 18% after 18 generations (72 years) of genetic drift would correspond

304   to a current effective population size (Ne) of ≈ 25, which is similar to the estimates returned by LD method

305   implemented in NeEstimator (24.3; 95% CI 8.5-125.7). Besides, using our Ne estimate together with the

306   previous estimate of population size (N ≈ 340) from a capture-mark-recapture study in Sintra (Aguilar et

307   al. 2018), we found a ratio of $Ne/N \approx 0.1$, which is within estimates found for other amphibians (Frankham

308   1995; Jehle et al. 2001; Álvarez et al. 2015).

309   Depending on the life-history traits of the organism and on other biotic and abiotic factors, patterns

310   of genetic variation may be strongly affected by different selective regimes and/or demographic

311   stochasticity (e.g. Roderick and Navajas 2003). So, estimating the number of founders could be difficult

312   when information on demographic history of the population is missing. For example, when introduced to

313   new areas, some species' populations often grow to a large population size after only few generations or

314   remains small for several generations before sudden, rapid growth. In other cases, the introduced population

315   size may be continuously small and stable (Monnet et al. 1993; Crooks and Soulé 1999; Ficetola et al. 2008;

316   Sendell-Price et al. 2020). Although the number of individuals of *C. lusitanica* that were introduced in

317   Sintra is unknown, based on the Seabra's writings (Seabra 1943) and the pronounced reduction of genetic

318   diversity observed in the present study, there are reasons to expect that the founding population was

319   relatively small. Actually, our estimate based on genotypes simulated by resampling all alleles across the

320   entire microsatellite dataset, indicated that Sintra population may has resulted from the introduction of about

321   10-11 individuals (Supplementary Fig. S2). This estimate, however, should be treated as a proxy of the

322   effective number of founders, which may be lower than the size of the founding population.

323   Estimates of current population size ($N \approx 340$) and density (3.2 individuals per m of brook) of the

324   introduced population by 2015/2016 (Aguilar et al. 2018) are lower than estimates (size $\approx$1250-2200 and

325   density 11-17 per m of brook) for local populations among the native range (Arntzen 1981; Arntzen et al.

326   2015; Teixeira et al. 1998; Lima 1995), suggesting that present-day Sintra population is facing ecological

327   limitations. According to Aguilar et al. (2018), salamanders were restricted to a very small site, being

328   confined to a stretch of $\approx$ 100 m along the margins of one small stream and its tiny tributaries. Although

329   the exact location of the introduction was not referred by Seabra (1943), it is possible that the current

330   population is limited to the vicinity of the release site and may have reached carrying capacity. Despite

331   documented movements over distances of 700 m along the wet banks of streams (Arntzen 1981, 1984) and

332   the species' propensity for dispersal by larval drift (Arntzen 1995; Thiesmeier 1994), displacements of this

13

333 species outside moist habitats are severely restricted (Arntzen 1995). According to Aguilar et al. (2018),

334 considering both microclimate and vegetation cover, the habitat surrounding the stream occupied by this

335 population is not suitable for *C. lusitanica*. This, together with the fact that *C. lusitanica* presents relatively

336 "slow" life history traits (*sensu* Allen et al. 2017), including low fecundity (average clutch size = 18;

337 Sequeira et al., 2003) and long reproductive lifespan (age of sexual maturity = 4 years; generation time = 4

338 years; and, longevity = 10 years; Lima et al., 2001), may hamper the species to expand its range into other

339 waterways and thus increase the total population size. Together with possible scenarios of climate change

340 or stochastic local extinctions due to e.g. increasing forest fires, the long-term survival or expansion

341 prospects of this introduced rear edge population of an ecologically demanding species may be in risk;

342 however, its maintenance for more than 70 years without any human-assisted management highlights the

343 potential of amphibian reintroductions or assisted migrations as effective conservation tools.

Data availability

351 Data generated and analysed in this study are included in the Supplementary Information file and
352 mitochondrial cytochrome b sequences are available in the GenBank repository (Accession Nos.
353 XXXXX– XXXXX).

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Table 1 - Genetic variation at seven microsatellite loci and mitochondrial Cyt b in 16 *Chioglossa lusitanica* populations. Total number of analyzed samples (N), number of alleles per locus (N_a), allelic richness (A_R), expected heterozygosity (H_E), fixation index (F_{IS}), number of mitochondrial Cyt b haplotypes (N_H), haplotype diversity (h) and nucleotide diversity (π) for each sampled population. Population codes are as in Fig.1.

Population ID/code	Lat.	Long.	Microsatellites					Mitochondrial cytb				Reference
			N	H_E	N_a	A_R	F_{IS}	N	N_H	h	π	
Salas (SAL)	43.394	-6.256	24	0.286	2.9	2.7	-0.039	6	1	0	0.0000	1, 2
Pontevedra (PO	42.502	-8.482	16	0.273	3.6	3.6	-0.048	9	2	0.286	0.0004	1, 2
Gerês (GE)	41.757	-8.146	20	0.286	2.9	2.8	0.073	10	2	0.222	0.0003	1, 2
Valongo (VAL)	41.179	-8.49	22	0.474	4.7	4.5	0.051	9	3	0.556	0.0020	1, 2
Montemuro (MO)	41.043	-8.066	20	0.553	5.6	5.2	-0.033	10	4	0.533	0.0324	1, 2
Covelo (CO)	40.777	-8.213	25	0.569	6.6	5.9	0.015	12	4	0.561	0.0012	1, 2
Saide (SA)	40.446	-8.324	21	0.578	7.3	6.6	0.1	4	2	0.5	0.0007	1, 2
Buçaco (BU)	40.377	-8.367	22	0.634	6.9	6.5	0.053	14	5	0.758	0.0088	1, 2, this study
Várzea (VA)	40.248	-8.375	22	0.594	7.0	6.5	0.028	10	5	0.756	0.0026	1, 2
Misarela (MI)	40.218	-8.358	26	0.619	7.6	7.0	0.028	5	2	0.4	0.0011	this study
Riba de Cima (RC)	40.259	-8.236	24	0.587	6.9	6.9	-0.055	5	3	0.7	0.0017	this study
Lousã (LO)	40.114	-8.224	26	0.756	8.4	7.7	0.043	11	3	0.655	0.0027	1,2
Castanheira Pêra (CP)	40.091	-8.201	22	0.712	7.4	6.9	0.012	2	1	0	0.0000	1,2
Açor (AC)	40.221	-7.919	18	0.640	6.9	6.6	-0.053	10	5	0.756	0.0023	1,2
Muradal (MU)	40.007	-7.697	21	0.579	5.1	4.8	-0.068	10	3	0.378	0.0006	1,2
Sintra (SI)	38.796	-9.424	47	0.452	2.7	2.5	0.049	10	1	0	0.0000	this study

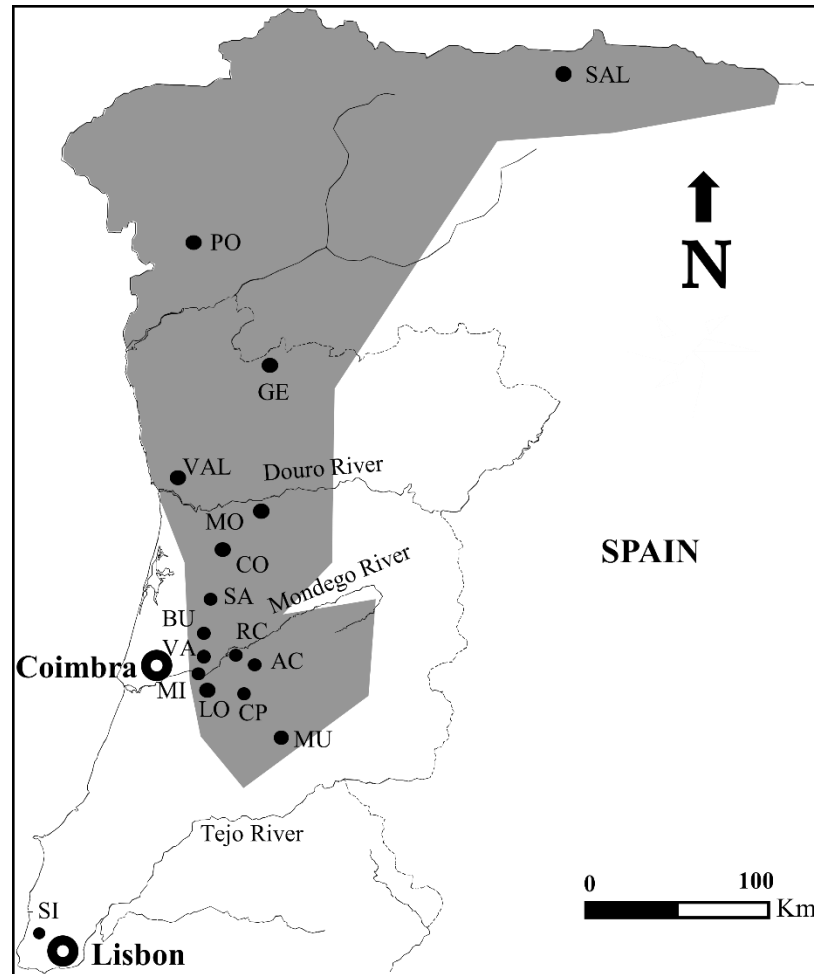1 – Alexandrino et al. (2002)

2 – Sequeira et al. (2008)

Figure 1. Map showing the distribution of *Chioglossa lusitanica* in the Iberian Peninsula (grey shading; Arntzen 1999) and sampling localities as identified in Table 1.
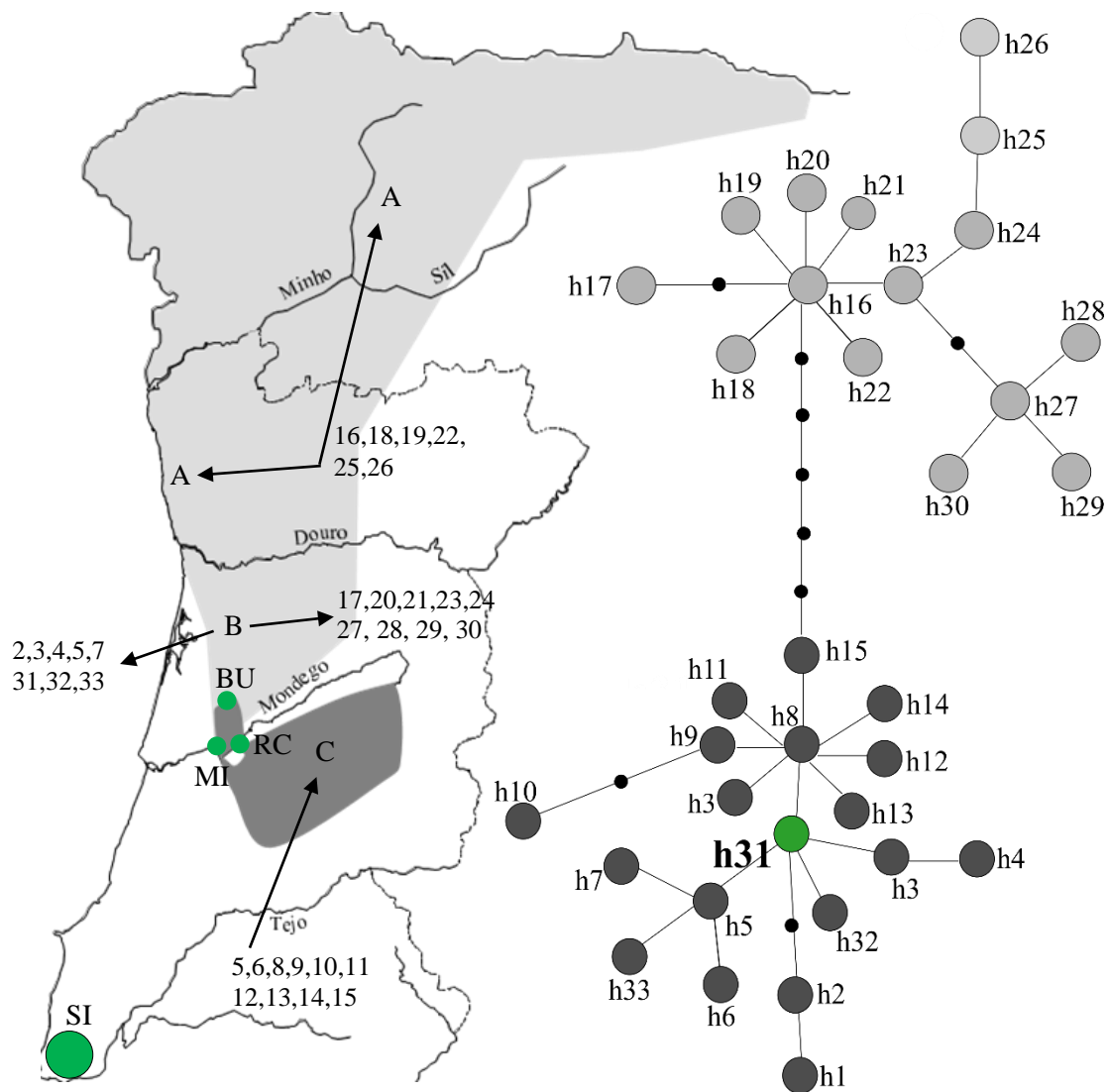
Figure 2. Median-joining network and geographic distribution of cytochrome b haplotypes observed in *Chioglossa lusitanica*. Each circle represents a specific haplotype: h1-h30, described by Alexandrino et al. (2002); and, haplotypes h31-h33, newly described in the present study. Black dots represent hypothetical undetected haplotypes, and each line represents one nucleotide substitution. The light gray haplotypes correspond to the subspecies *C. lusitanica longipes*, and the dark gray haplotypes are from *C. l. lusitanica* (Alexandrino et al., 2002; Arntzen et al. (2007). The green circles in the map correspond to populations that share the haplotype h31 (the haplotype found in Sintra). Numbers in the map correspond to the geographic distribution of haplotype as shown in the network. Letters across *C. lusitanica* range distribution, corresponds to: A, north of Douro river; B, between Douro and Mondego rivers; and; C, south of Mondego river (see Supplementary Table S3 for detailed information on haplotype frequency across populations and identification of haplotype numbers).
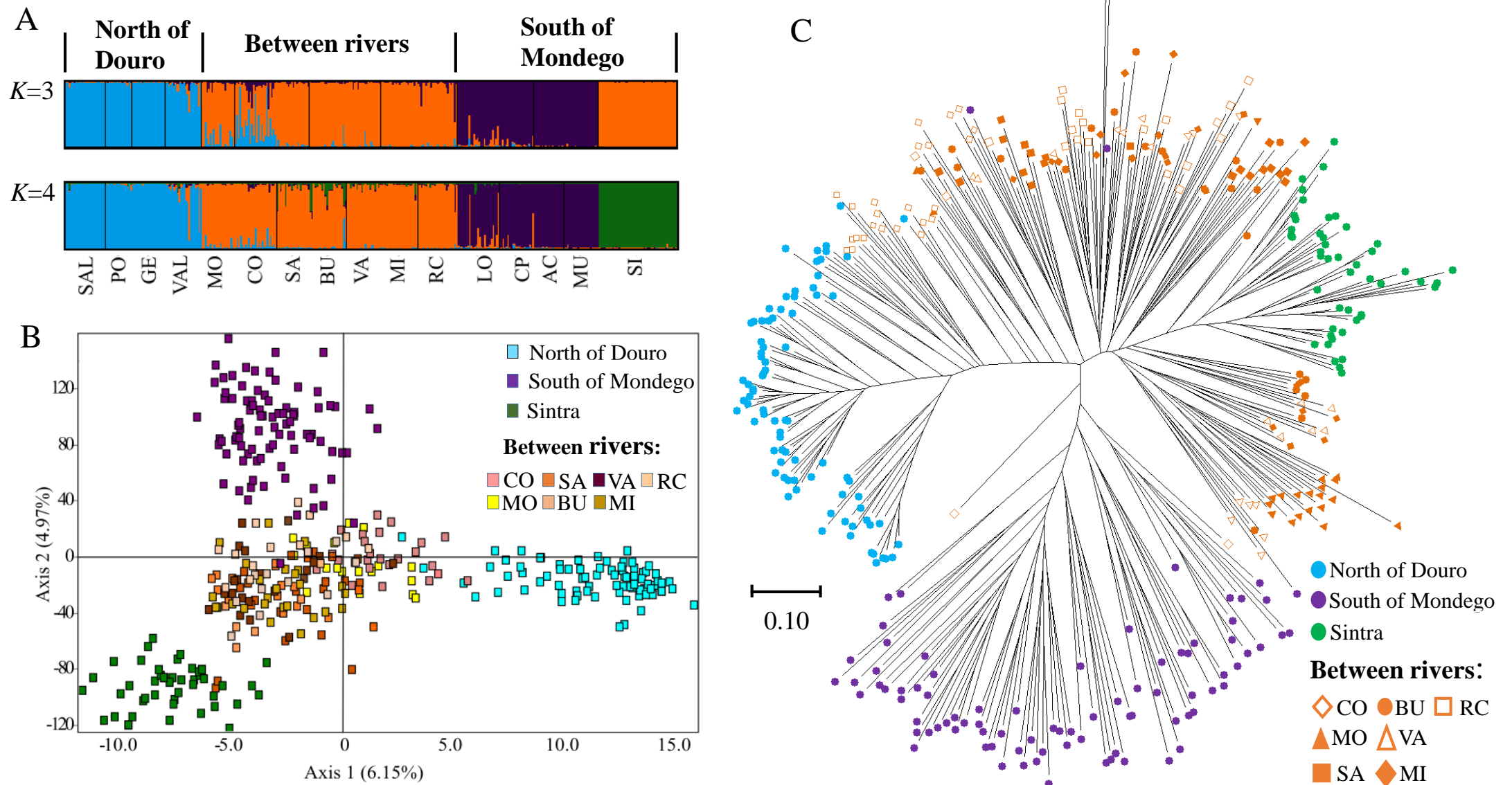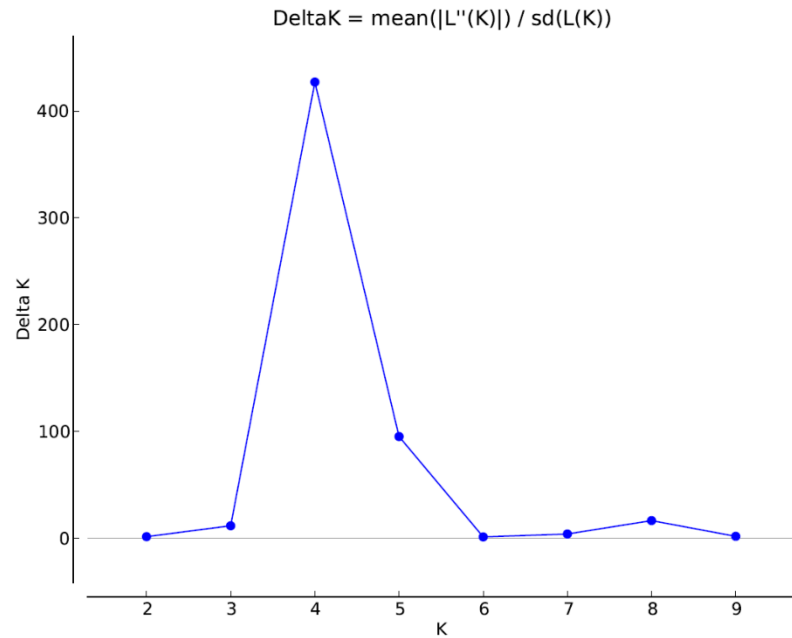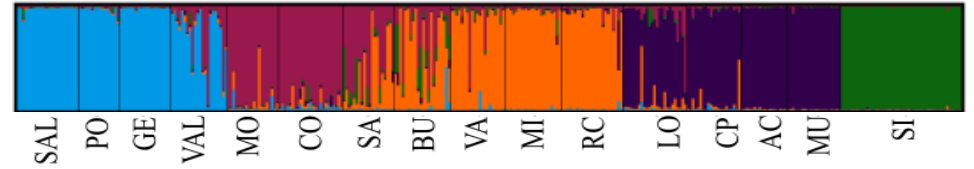
Figure 3. Analyses based on microsatellite genotypes at seven loci for 286 *Chioglossa lusitanica* individuals: A) Bayesian clustering results of STRUCTURE. Each vertical bar represents one individual and its assignment proportion into one of the three (*K=3*) or four (*K=4*) clusters. A black line separates individuals of different populations. These are labelled below the figure (as identified in the Table 1) and are sorted from north to south (from the left to the right of the figure). Population localisation according to geographical region is indicated on the top of the figure. B) Factorial Correspondence Analysis (FCA) of population multilocus scores computed using GENETIX 4.0543. Multilocus scores are computed in the bivariate space defined by the first two factorial components.. C) Neighbor-Joining tree based on allele-sharing distance (DAS) using Populations 1.2.31 software.
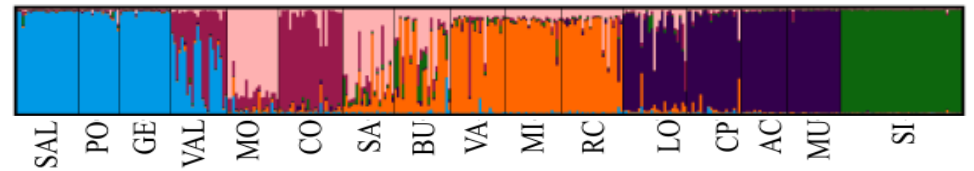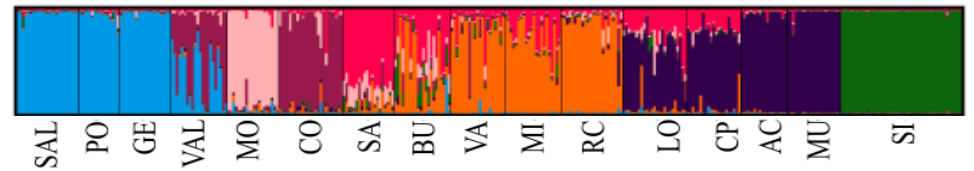
Figure S1. Analyses based on microsatellite genotypes at seven loci for 375 *Chioglossa lusitanica* individuals: A) Delta K values estimated according to Evanno et al. 2005 Method. B) Bayesian clustering results of STRUCTURE for (*K=5-7*). Each vertical bar represents one individual and its assignment proportion into one of the clusters. A black line separates individuals of different populations. These are labelled below the figure (as identified in the Table 1) and are sorted from north to south (from the left to the right of the figure).
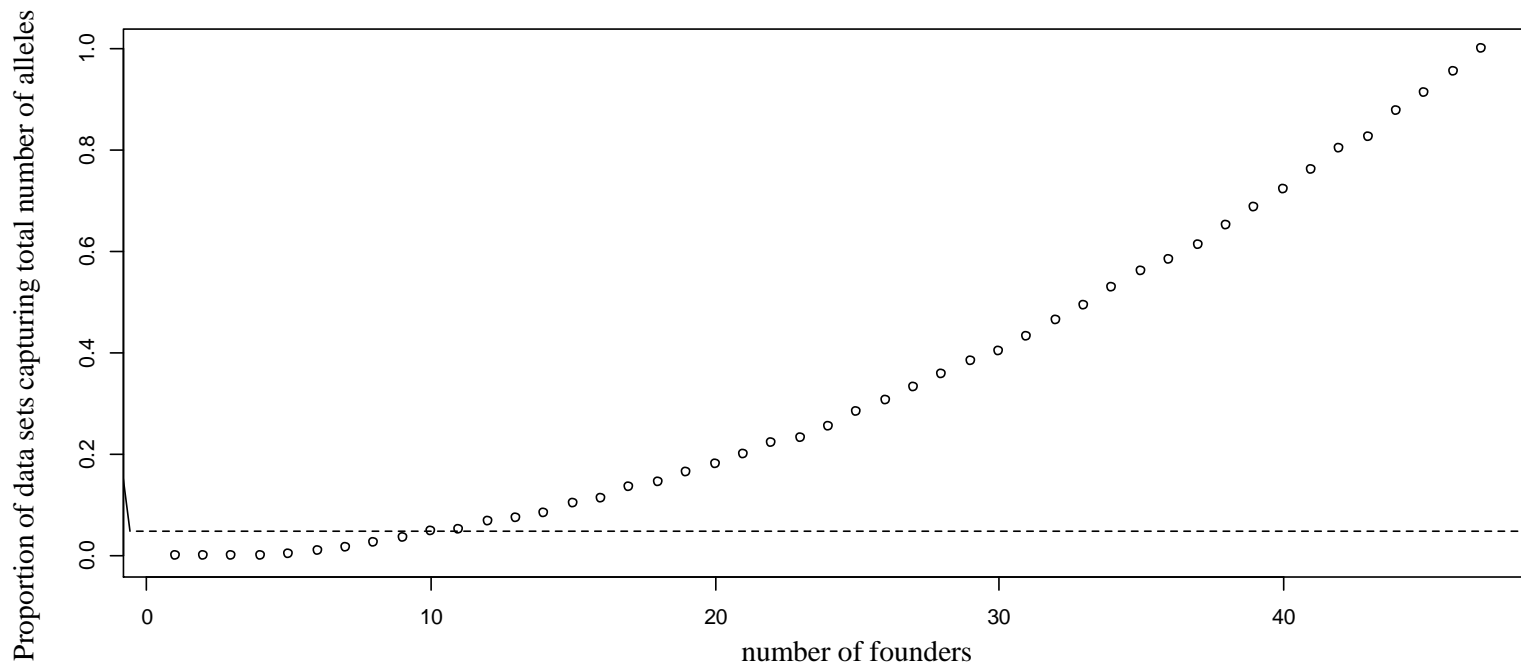
Figure S2. Minimum number of founders consistent with the observed data (smallest number of individuals containing all the alleles identified at the seven microsatellites), taken to be the number that gave a probability > 0.05 of capturing the observed alleles (dotted line).
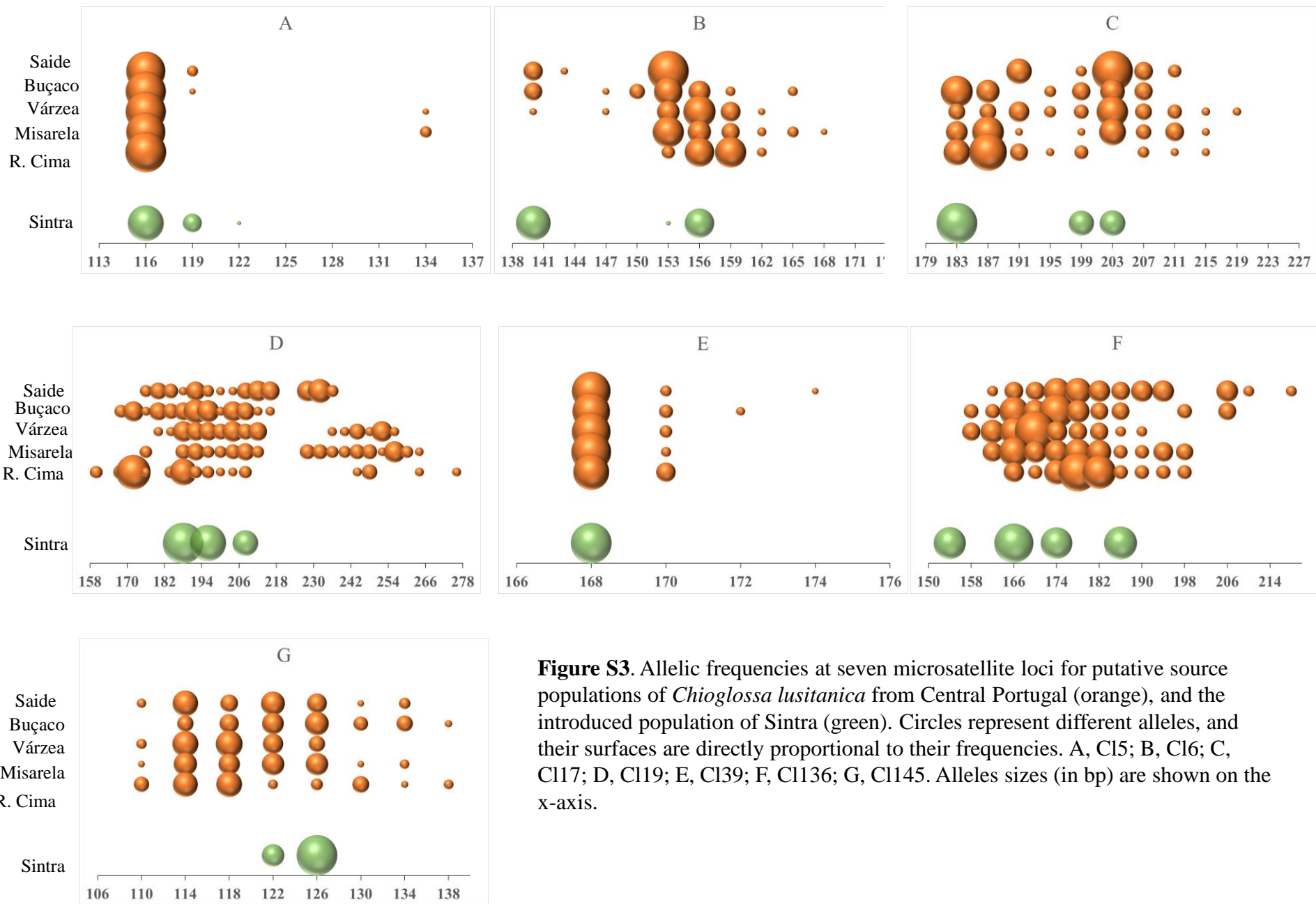
Figure S3. Allelic frequencies at seven microsatellite loci for putative source populations of *Chioglossa lusitanica* from Central Portugal (orange), and the introduced population of Sintra (green). Circles represent different alleles, and their surfaces are directly proportional to their frequencies. A, Cl5; B, Cl6; C, Cl17; D, Cl19; E, Cl39; F, Cl136; G, Cl145. Alleles sizes (in bp) are shown on the x-axis.