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Resolving Recent Plant Radiations: Power and Robustness of Genotyping-by-Sequencing

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Abstract.—Disentangling species boundaries and phylogenetic relationships within recent evolutionary radiations is a challenge due to the poor morphological differentiation and low genetic divergence between species, frequently accompanied by phenotypic convergence, inter-specific gene flow and incomplete lineage sorting. Here we employed a genotyping-by-sequencing (GBS) approach, in combination with morphometric analyses, to investigate a small western Mediterranean clade in the flowering plant genus *Linaria* that radiated in the Quaternary. After confirming the morphological and genetic distinctness of eight species, we evaluated the relative performances of concatenation and coalescent methods to resolve phylogenetic relationships. Specifically, we focused on assessing the robustness of both approaches to variations in the parameter used to estimate sequence orthology (clustering threshold). Concatenation analyses suffered from strong systematic bias, as revealed by the high statistical support for multiple alternative topologies depending on clustering threshold values. By contrast, topologies produced by two coalescent-based methods (NJ$_{st}$, SVDquartets) were robust to variations in the clustering threshold. Reticulate evolution may partly explain incongruences between NJ$_{st}$, SVDquartets and concatenated trees. Integration of morphometric and coalescent-based phylogenetic results revealed (1) extensive morphological divergence associated with recent splits between geographically close or sympatric sister species, and (2) morphological convergence in geographically disjunct species. These patterns are particularly true for floral traits related to pollinator specialisation, including nectar spur length, tube width and corolla colour, suggesting pollinator-driven diversification. Given its relatively simple and inexpensive implementation, GBS is a promising technique for the phylogenetic and systematic study of recent radiations, but care must be taken to evaluate the robustness of results to variation of data assembly parameters. [Genotyping-by-sequencing; RAD-Seq; radiation; phylogeny; coalescence; concatenation; speciation; *Linaria*.]

47   Recent evolutionary radiations constitute ideal systems to investigate evolution,

48   speciation and adaptation (Schluter 2000; Hughes and Eastwood 2006; Seehausen 2006;

49   Valente et al. 2010; Farrington et al. 2014). To understand the causes and direction of

50   evolutionary change, robust systematic treatments and well-resolved phylogenies are

51   required. However, disentangling species boundaries and phylogenetic relationships within

52   recent evolutionary radiations is a challenge due to the low genetic divergence between

53   species, frequently accompanied by inter-specific gene flow and incomplete lineage sorting

54   (ILS) (Shaw 2002; Shaffer and Thomson 2007). This leads to poor resolution, low support

55   values and short branch lengths in phylogenetic trees, interpreted as signatures of rapid

56   diversification (Valente et al. 2010; Meiklejohn et al. 2016). Although morphological

57   diversity may be considerable, the high frequency of phenotypic convergence further

58   confounds systematics and phylogeny, yet it has high evolutionary interest because it is

59   potentially informative about recurrent adaptive traits and their molecular basis (e.g. Whittall

60   et al. 2006; Muschick et al. 2012). Some authors cast doubt on the possibility of resolving

61   recent radiations in the tree of life because of these difficulties (Vargas and Zardoya 2014),

62   and even state-of-the-art approaches have methodological challenges to overcome (Harvey et

63   al. 2016).

64   In plants, classical approaches to phylogenetic analysis are based on a small number

65   of loci, frequently nuclear ribosomal DNA (nrDNA), plastid DNA (ptDNA) and low-copy

66   number genes (Sang 2002; Álvarez and Wendel 2003; Shaw et al. 2007). This strategy has

67   been proven effective to resolve some recent radiations, and is frequently able to recover

68   major clades and evolutionary patterns (Givnish et al. 2009; Guzmán et al. 2009). However,

69   incongruence between loci that may result from hybridisation and ILS, amongst other causes,

70   often brings about uncertainty regarding species boundaries and species-level relationships

71   (Blanco-Pastor et al. 2012). Under these conditions, the sequencing of a large number of

72   genome-wide genetic markers, accompanied by thorough intra-specific sampling of

73   individuals, is a requirement to resolve systematic and phylogenetic questions. To achieve

74   this, several approaches based on next generation sequencing (NGS) have been proposed in

75   recent years, including sequence capture, transcriptomics and restriction digest-based

76   methods (Lemmon and Lemmon 2013; McCormack et al. 2013; Harvey et al. 2016).

77   Restriction digest-based NGS methods comprise a number of procedures, including those

78   known as restriction-site associated DNA sequencing (RAD-Seq) and genotyping-by-

79   sequencing (GBS) (van Orsouw et al. 2007; Baird et al. 2008; Davey et al. 2011; Elshire et al.

80   2011; Peterson et al. 2012; Poland et al. 2012). Despite the plethora of protocols that have

81   been published, a NGS-based method that is universally and routinely applicable by

82   systematic biologists is yet to be established as a replacement to conventional phylogenetic

83   approaches. Such a method should not only be capable of providing genome-wide

84   phylogenetic information, but should also be cost- and time-effective, involve relatively

85   simple laboratory protocols, and should not require a sequenced reference genome or

86   previous genomic information (Lemmon and Lemmon 2013; McCormack et al. 2013).

87   Ideally, it should also be successful with fragmented DNA available from biological

88   collections of various ages.

89   In addition to laboratory protocols, uncertainties also remain concerning the

90   bioinformatic analysis of large numbers of genomic loci with potentially conflicting

91   phylogenetic signals. The concatenated approach has frequently been favoured because of its

92   analytical speed (Wagner et al. 2013; Hipp et al. 2014). However, simulation studies show

93   that, even when analysing thousands of loci, concatenated analysis can produce inconsistent

94   results in the presence of ILS (Kubatko and Degnan 2007). Species tree methods based on the

95   multi-species coalescent approach therefore should be preferred to deal with this problem but

96   they may be computationally intensive (reviewed by Liu et al. 2015). In addition, as for

97 concatenation methods, species tree methods may be inconsistent in the presence of

98 hybridisation (Solís-Lemus et al. 2016). Coalescent-based phylogenetic network methods,

99 capable of dealing with gene flow, are even more computationally intensive, but more

100 efficient implementations of these are currently in development (Solís-Lemus and Ané 2016).

101   Here we have analysed species boundaries, phylogenetic relationships and patterns of

102 morphological evolution during speciation in a small radiation of flowering plants, the

103 Iberian clade of *Linaria* subsect. *Versicolores* (Fernández-Mazuecos et al. 2013a). According

104 to current taxonomic treatments, this clade comprises eight taxa endemic or sub-endemic to

105 the Iberian Peninsula, in the western Mediterranean biodiversity hotspot (Sáez and Bernal

106 2009; Vigalondo et al. 2015; Blanca et al. 2017). This clade constitutes an excellent study

107 system for the understanding of pollinator-driven floral evolution and speciation because it

108 has high phenotypic diversity in floral traits promoting pollinator specialisation, including

109 corolla tube width, corolla colour and nectar spur length (Fernández-Mazuecos et al. 2013a).

110 Nectar spurs in particular have been considered a key evolutionary innovation, promoting

111 speciation and morphological diversity (Kay et al. 2006; Fernández-Mazuecos and Glover

112 2017). However, the Iberian clade also displays a number of features that make it a

113 challenging study case from both a systematic and phylogenetic standpoint (Fernández-

114 Mazuecos et al. 2013a; Fernández-Mazuecos and Vargas 2015; Vigalondo et al. 2015): (i)

115 recent and rapid radiation, dated back to the Quaternary; (ii) potential instances of phenotypic

116 convergence; (iii) incongruence between ptDNA and nrDNA (internal transcribed spacer,

117 ITS) phylogenies; (iv) inconsistent results between concatenation- and coalescent-based

118 phylogenies of combined ptDNA+ITS data; and (v) lack of monophyly of intra-specific

119 samples and extensive sharing of ptDNA haplotypes across species. As a result, previous

120 attempts to resolve relationships with standard phylogenetic markers were unable to recover a

121 reliable species-level phylogeny for this clade.

122   To generate genome-wide phylogenetic markers we selected the genotyping-by-

123   sequencing approach first described by Elshire et al. (2011) as it does not require previous

124   genomic information and is relatively simple and inexpensive compared to related restriction

125   digest-based methods. This makes it a good candidate to become a universally applicable

126   NGS replacement for conventional phylogenetic approaches. This method has recently been

127   successfully applied to species phylogenetics and species delimitation (Escudero et al. 2014;

128   Nicotra et al. 2016), but questions remain concerning the analytical approach to GBS data,

129   such as the treatment of missing data (whether loci with large proportions of missing

130   sequences should be excluded or not; Roure et al. 2013; Huang and Knowles 2014; Eaton et

131   al. 2016) or the relative merits of concatenated and coalescent-based approaches to infer

132   species phylogenies (DaCosta and Sorenson 2016).

133   Our objective was to use both morphometric data and genome-wide genetic markers

134   generated by GBS in the Iberian clade of *Linaria* subsect. *Versicolores* to: (1) test species

135   limits, thus achieving a robust systematic treatment; (2) infer a well-supported species

136   phylogeny comparing the relative performances of concatenation and coalescent-based

137   methods; (3) analyse the patterns of morphological evolution during speciation, particularly

138   those potentially linked to pollinator specialisation. The ultimate goal was to establish a

139   unified approach that allows the resolution of the elusive species limits and phylogenetic

140   relationships in a recent radiation.

141

142   Materials and Methods

143   *Study System*

144   The eight taxa currently recognised in the Iberian clade of *Linaria* subsect.

145   *Versicolores* and acronyms used hereafter in this paper are listed in Table 1 (Sáez and Bernal

146   2009; Vigalondo et al. 2015; Blanca et al. 2017; see Online Appendix 1). One of these taxa

147   has been treated as either a species or a subspecies (*L. salzmannii* or *L. viscosa* subsp.

148   *spicata*), while the other seven have been consistently treated as species. An additional taxon

149   has been described, *L. viscosa* subsp. *crassifolia* (CRA) (Sutton 1988), but its taxonomic

150   status is uncertain (Sáez and Bernal 2009). These taxa inhabit grasslands and open shrubby

151   formations on soils developed from diverse lithologies. They are endemic to the Iberian

152   Peninsula, except for SPA, which is also present in southern France. They are diploid species

153   with chromosome number 2n=12 (Sutton 1988; Sáez and Bernal 2009) and genome sizes

154   around 2C=1.1 pg (Castro et al. 2012), and they all seem to be predominantly allogamous

155   (Valdés 1970; Fernández-Mazuecos M., unpublished data).

156   The Iberian clade has been strongly supported as monophyletic by both concatenated

157   and coalescent-based analyses of combined ITS and ptDNA sequences, and it is sister to a

158   mostly North African clade of c. 20 taxa (Fig. S1a; Supplementary Material can be found in

159   the Dryad data repository at http://datadryad.org/review?doi=doi:10.5061/dryad.mp818)

160   (Fernández-Mazuecos et al. 2013a; Vigalondo et al. 2015). Both the Iberian and North

161   African clades constitute *Linaria* subsect. *Versicolores*, which is in turn sister to *Linaria*

162   subsect. *Elegantes* (two species). Both subsections make up *Linaria* sect. *Versicolores*, one of

163   the major clades of the toadflax genus (Fernández-Mazuecos et al. 2013b). Divergence

164   between the Iberian and North African clades has been dated back to the Pliocene or early

165   Quaternary, and speciation of the Iberian clade is estimated to have happened in the late

166   Quaternary, during the last million years, with high diversification rates (Fig. S1a;

167   Fernández-Mazuecos and Vargas 2011; Fernández-Mazuecos et al. 2013a; Fernández-

168   Mazuecos and Vargas 2015). Two major geographically-structured ptDNA lineages have

169   been detected in the Iberian clade (Fernández-Mazuecos and Vargas 2015).

170

171   *Morphometric Analysis*

172   A morphometric analysis of all taxa included in the Iberian clade was performed

173   following the methods of our previous study of INC and ONU (Vigalondo et al. 2015). A full

174   account of methods is found in Online Appendix 2. Specimens were obtained from herbaria

175   (Online Appendix 1) and from the authors' collections (vouchers in BCB and MA). The

176   selected characters included 24 quantitative and three qualitative variables (Table S1). Ten to

177   thirty-three specimens per taxon were analysed and one measure per character per specimen

178   was taken. Specimens of two different flower colour morphs of SAL (purple and yellow)

179   were included, as well as three specimens collected in the *locus classicus* of CRA. The final

180   morphometric dataset included 166 samples.

181   To assess the morphological affinities between specimens and taxa, principal

182   coordinate analysis (PCoA) was conducted using Gower's coefficient of similarity for mixed

183   data (Gower 1971). A discriminant function analysis (DFA) was then performed to reveal the

184   main characters contributing to the morphological differentiation of taxa and to test the

185   assignment of specimens to taxa using a cross-validation approach. The R package *rgl* (Adler

186   and Murdoch 2016) was used to represent the first three axes of both the PCoA and DFA in

187   3D scatter plots with 95% confidence ellipses of concentration for the eight taxa.

188   Distributions of quantitative characters were summarised in the form of beanplots using the R

189   package *beanplot* (Kampstra 2008). To evaluate the impact of sample size on the results, we

190   repeated the PCoA and DFA analyses replacing the missing data with mean values, without

191   obtaining significantly different results. We used the basic packages of R v3.2.5 (R

192   Development Core Team 2016), *ape* (Paradis et al. 2004) and *vegan* (Oksanen et al. 2011) to

193   construct the PCoA and *MASS* (Venables and Ripley 2002) for the DFA.

194

195   *Phylogenomic Analysis*

196   *Specimen sampling and DNA extraction.*—We sampled a total of 89 individuals of

197   *Linaria*. Of these, 76 individuals represented the distribution and morphological variation of

198   all species and subspecies of the Iberian clade of *Linaria* subsect. *Versicolores*, including

199   specimens of the two flower colour morphs of SAL and one individual of CRA (Tables 1, S2;

200   Fig. S1b). Thirteen individuals of additional species of *Linaria* sect. *Versicolores* were

201   sampled to be used as the outgroup, including the two species of *Linaria* subsect. *Elegantes*

202   and six species of the North African clade of *Linaria* subsect. *Versicolores* (Tables 1, S2).

203   Seventy-five individuals were sampled in the field and dried in silica gel between 2006 and

204   2015, while 14 individuals were obtained from generally older (1970-2006) herbarium

205   specimens (MA, RNG). For each sampled individual, total genomic DNA was extracted from

206   c. 20 mg of leaf tissue using a modified CTAB protocol (Doyle and Doyle 1987; Cullings

207   1992). DNA concentrations were quantified using a Qubit 2.0 fluorometer (Invitrogen, CA,

208   USA) with the dsDNA Broad-Range Assay Kit.

209

210   *Genotyping-by-sequencing library preparation.*—A GBS library was prepared using

211   all DNA samples with the *Pst*I-HF restriction enzyme and following previously published

212   procedures (Elshire et al. 2011; Escudero et al. 2014; Grabowski et al. 2014) with some

213   modifications. A full description of laboratory protocols is found in Online Appendix 2, and a

214   summary is provided here. For each sample, 500 ng (where available) of genomic DNA were

215   combined with 0.6 pmol of a sample specific barcode adapter and 0.6 pmol of common

216   adapter (Tables S2, S3). Each sample was digested with 4 units of *Pst*I-HF (NEB, MA, USA)

217   at 37ºC overnight. Adapters were ligated using 400 units of T4 DNA Ligase (NEB, MA,

218   USA) at room temperature for 4 h. 50 ng of each sample were combined, and the pool was

219   then purified with Agencourt AMPure XP (Beckman Coulter, CA, USA). DNA fragments

220   were amplified for 15 PCR cycles starting from 35 ng of DNA and using NEB 2x Taq

221   MasterMix (NEB, MA, USA). The PCR product was purified using different volume ratios of

222   AMPure XP beads to sample, and fragment size distributions were assessed in a 2100

223   Bioanalyzer (Agilent Technologies, CA, USA; Fig. S2). The library with the optimal profile

224   and concentration (AMPure to sample ratio 0.8:1, concentration 1.87 ng/μl, average size 595

225   bp) was submitted to BGI-Europe (Copenhagen, Denmark) for 100 bp HiSeq 2000 Illumina

226   sequencing. Quality control of sequencing results was conducted in FastQC 0.11.3 (Andrews

227   2010).

228

229   *Data assembly and genetic structure.*—Assembly of GBS loci was performed using

230   the pyRAD 3.0.61 pipeline (Eaton 2014). The pyRAD procedure consisted of seven

231   sequential steps, with parameters based on those recommended for single-end GBS data in

232   the pyRAD documentation (http://dereneaton.com/software/pyrad/; see Online Appendix 2

233   for details). Since phylogenetic results are known to be sensitive to the similarity threshold

234   employed for within-sample and across-sample sequence clustering (c) (Takahashi et al.

235   2014; Mastretta-Yanes et al. 2015; Shafer et al. 2016), 16 assemblies of GBS loci were

236   generated using a range of clustering thresholds from c=0.80 to c=0.95. Statistical base

237   calling was conducted following Li et al. (2008), with a minimum depth of coverage of 6 and

238   a maximum of 5 Ns in consensus sequences. Filtering of putative paralogs was performed by

239   setting a maximum of two alleles (corresponding to diploid organisms) and 8 heterozygous

240   sites per consensus sequence. Loci with a minimum taxon coverage (m, minimum number of

241   samples with data) of 4 and a maximum of 3 individuals with shared polymorphic sites (as a

242   further filter of putative paralogs) were retained. Additional assemblies were generated for

243   particular downstream analyses by excluding specific individuals and varying the minimum

244   taxon coverage (m=10, m=20; see below). Henceforth, assemblies are denoted as cXmY,

245   with X being the clustering threshold and Y being the minimum taxon coverage (Table 2).

10

246   Before starting phylogenetic analyses, we tested the correspondence between

247   morphologically defined taxa and genetic clusters by analysing unlinked single nucleotide

248   polymorphism (SNP) matrices using three approaches: principal component analysis (PCA;

249   Waterhouse et al. 2009), Neighbour-Net phylogenetic network estimation (Bryant and

250   Moulton 2004) and Bayesian clustering (Pritchard et al. 2000; Corander et al. 2004) (see

251   Online Appendix 2 for details). As a result of these analyses, eight individuals of uncertain

252   genetic identity, either because of their putative recent hybrid origin or low-quality

253   sequencing results (>99% missing data in the final datasets) were excluded from downstream

254   phylogenetic analyses.

255

256   *Concatenation-based phylogenies.*—Maximum likelihood (ML) analyses of the 16

257   full-sequence concatenated datasets obtained under 16 clustering threshold values were

258   implemented in RAxML 8.2.8 (Stamatakis 2014) using the GTRCAT substitution model

259   (Stamatakis 2006) during tree search, followed by evaluation and optimisation of the final

260   tree under GTRGAMMA. The four *Linaria* subsect. *Elegantes* samples were set as the

261   outgroup.

262   Four alternative species-level topologies were found among the 16 ML trees (see

263   Results). We selected four datasets, each yielding a distinct topology with high bootstrap

264   support (BS) values, for further analysis and testing: c84m4, c85m4, c87m4 and c92m4. For

265   these datasets, we additionally explored the performance of ML analyses based on

266   concatenated SNP matrices (i.e. excluding invariant sites) using two acquisition bias

267   corrections (Leaché et al. 2015a) implemented in RAxML (see Online Appendix 2 for

268   details). The four full-sequence matrices were also analysed using Bayesian inference (BI), as

269   implemented in ExaBayes 1.4.1 (Aberer et al. 2014), with the GTRGAMMA substitution

270   model. To assess the sensitivity of the results to the minimum taxon coverage, assemblies

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271   generated with m=10 and m=20 and the same clustering threshold values (see Table 2) were

272   analyzed by ML in RAxML using the same methods described above.

273

274   *Coalescent-based phylogenies.*—It is known that the concatenated approach may

275   produce spurious phylogenetic results, particularly under high ILS, expected in rapid

276   radiations. Amongst available gene-tree-based methods accounting for ILS, we selected NJ_{st}

277   (Liu and Yu 2011) because of the following features: it is able to infer the species tree from

278   unrooted gene trees (outgroup samples would be absent from many gene trees in our dataset,

279   impeding the rooting of gene trees); it can accommodate missing data; and it can handle

280   allele data from multiple individuals per species. The four selected datasets yielding

281   contrasting topologies in concatenated analyses (c84m4, c85m4, c87m4 and c92m4) were

282   separately analysed as follows. For all loci showing variability, gene trees were estimated

283   using RAxML with the GTR+GAMMA substitution model and 100 bootstrap replicates.

284   Fifty multilocus bootstrap replicates (Seo 2008; Mallo 2015) were generated, thus resampling

285   nucleotides within loci, as well as loci within the dataset. The NJ_{st} method was implemented

286   on the fifty bootstrapped matrices using the R script NJstM (Mallo 2016), which relies on the

287   *phybase* package (Liu and Yu 2010). A 50% majority-rule (MR) consensus tree was then

288   built from the 50 bootstrap replicates in PAUP* 4 (Swofford 2002). Additionally, assemblies

289   generated with higher values of m (m=10 and m=20) and the same clustering threshold

290   values (see Table 2) were analysed using the same methods described above, to assess the

291   sensitivity of the results to the minimum taxon coverage.

292   For the same datasets analysed in NJ_{st}, we additionally implemented the quartet-based

293   method SVDquartets (Chifman and Kubatko 2014), also accounting for ILS. This method can

294   handle both unlinked SNP and multi-locus full-sequence datasets. Therefore, we analysed

295   both types of matrices generated under the same four clustering threshold values and three

296   minimum taxon coverage values. Analyses were run in PAUP* 4 under the multispecies

297   coalescent, evaluating all possible quartets. For each matrix, one hundred bootstrap replicates

298   were conducted, and results were summarised in a 50% MR consensus tree.

299

300   *Tests of alternative topologies.*—To examine if alternative tree topologies could be

301   statistically rejected by each of the four selected concatenated datasets (c84m4, c85m4,

302   c87m4 and c92m4), topology tests were conducted. First, for each of the four sequence

303   matrices, constrained ML analyses were conducted in RAxML using the four alternative

304   species-level topologies encountered in unconstrained analyses (topologies 1-4), the

305   topologies obtained from coalescent-based NJ_{st} (topology 5) and SVDquartets (topology 6)

306   analyses and two additional topologies recovered from previous concatenated (topology 7;

307   Vigalondo et al. 2015) and coalescent-based (topology 8; Fernández-Mazuecos et al. 2013a)

308   analyses of ITS+ptDNA sequences. For each matrix, per-site log likelihoods using all

309   constrained ML topologies were computed in RAxML under the GTR+GAMMA model, re-

310   estimating model parameters for each tree. Then we used CONSEL v0.20 (Shimodaira and

311   Hasegawa 2001) to calculate the *p*-values of topology tests, including the approximately

312   unbiased (AU) test (Shimodaira 2002), the Shimodaira-Hasegawa (SH) test (Shimodaira and

313   Hasegawa 1999) and the weighted Shimodaira-Hasegawa (WSH) test (Buckley et al. 2001).

314   To visualise differences in the numbers of loci supporting alternative topologies, we

315   used the "partitioned RAD" approach (Hipp et al. 2014) implemented in the R package

316   *RADami* (Hipp 2014). The numbers of loci supporting and disfavouring each of the eight

317   constrained topologies generated for topology tests (see above) were compared for each of

318   the four selected datasets. A set of unique trees was generated for each locus (by pruning the

319   original eight trees to those tips present in each locus), and the log likelihood of each unique

320   tree for each locus was calculated in RAxML under the GTR+GAMMA model. For each

321   locus, trees were then ranked as supported if they were within 2 log likelihood units of the

322   best supported tree for that locus, or disfavoured if they were within 2 log likelihood units of

323   the least supported tree for that locus. Finally, for each dataset, the number of loci supporting

324   or disfavouring each of the eight alternative trees was plotted against the tree log likelihood

325   calculated using the concatenated matrix.

326

327   *Hybridisation analyses.*—Coalescent-based species tree analyses provided two

328   alternative topologies (see Results). We explored ancestral hybridisation as a potential source

329   of these conflicting results using two approaches: D-statistic tests (Durand et al. 2011; Eaton

330   et al. 2015) and the pseudolikelihood-based phylogenetic network method SNaQ (Species

331   Networks applying Quartets), which incorporates both incomplete lineage sorting and

332   hybridisation, and employs unrooted gene trees (Solís-Lemus and Ané 2016).

333   Four-taxon D-statistic tests (ABBA-BABA tests) were conducted in pyRAD,

334   including heterozygous sites (Durand et al. 2011; Eaton et al. 2015). Analyses were

335   performed on the c84m4, c85m4, c87m4 and c92m4 datasets using a set of selected

336   individuals with good-quality sequencing, and assuming either the NJ_{st} or the SVDquartets

337   phylogeny as the species tree (representing the major vertical inheritance pattern, MVIP). For

338   each dataset, three sets of tests were conducted exploring the potential role of hybridisation

339   on the alternative positions of CLE and SPA in the NJ_{st}, SVDquartets and concatenation-

340   based trees (see Online Appendix 2 for details).

341   The SNaQ method (Solís-Lemus and Ané 2016) was implemented using a small set of

342   27 individuals to reduce computational demand. For each of the c84m4, c85m4, c87m4 and

343   c92m4 assemblies, ML gene trees were generated in RAxML and used as input for SNaQ

344   analyses in PhyloNetworks 0.5.0. Since preliminary searches for an optimal network

345   produced highly inconsistent results in independent runs, we focused on evaluating each of

14

346   the NJ_{st} and SVDquartets species trees as fixed candidate topologies representing the MVIP,

347   both without reticulation and with reticulation events (hybrid edges) accounting for

348   incongruences with the alternative species tree topology. After optimisation, the fit of trees

349   and networks to the data was evaluated based on pseudo-deviance values, and estimated

350   inheritance probabilities (i.e. the proportion of genes contributed by each parental population

351   to a hybrid taxon) were visualised.

352

353   *Patterns of Morphological Evolution*

354   Morphometric and phylogenomic data were integrated to investigate patterns of

355   morphological evolution during the radiation of the study clade. A phylomorphospace

356   approach, implemented in the R package *phytools* (Revell 2012), was used to map the history

357   of morphological diversification and explore the magnitude and direction of morphological

358   changes (Sidlauskas 2008). The coalescent-based MR phylogenies obtained in NJ_{st} and

359   SVDquartets, which provided the most robust phylogenetic hypotheses (see below), were

360   used. After estimating branch lengths by ML (see Online Appendix 2 for details), both trees

361   were projected onto the multivariate morphospace defined by the first three canonical

362   discriminant functions of our DFA of vegetative and reproductive traits, with each taxon

363   represented by the mean values of the functions for all examined individuals. The same

364   approach was used to project the phylogeny into the two-dimensional flower morphospace

365   defined by the geometric morphometric study of Fernández-Mazuecos et al. (2013a), based

366   on canonical variate (CV) analysis of landmark data. For comparison with geographic

367   patterns of speciation, the phylogeny was also mapped onto the geographic distribution of the

368   eight taxa in the Iberian Peninsula.

369   In addition, we investigated shifts in particular morphological characters and

370   instances of phenotypic convergence by conducting ancestral state reconstructions (ASRs) in

371 Mesquite 3.04 (Maddison and Maddison 2011) using the coalescent-based species trees (NJ_{st}

372 and SVDquartets). Maximum parsimony (MP) and ML methods were applied. Four key traits

373 determining morphological diversity in the study clade were analysed: habit (annual vs.

374 perennial), inflorescence density (lax vs. dense), dominant corolla colour (purple vs. yellow)

375 and corolla shape. For corolla shape, three types based on geometric morphometric analyses

376 were defined (Fernández-Mazuecos et al. 2013a): type I (broad corolla tube and long nectar

377 spur), type II (broad corolla tube and short nectar spur) and type III (narrow corolla tube and

378 long nectar spur).

379

380   RESULTS

381   *Morphometric Analysis*

382   Morphometric analyses revealed the eight taxa in the Iberian clade of *Linaria* subsect.

383 *Versicolores* as morphologically distinct units (Figs. 1a, b, S3; Table S4). Beanplots (Fig. S3)

384 depicted differences between taxa in particular morphological traits, especially between CLE

385 and other taxa for stem length, flower pedicel length, spur width, and corolla/spur length

386 ratio. The first three coordinates of the PCoA (explaining 54.8% of variance) depicted a

387 complex morphospace (Fig. 1a), with morphological affinities between individuals and taxa

388 consistent with taxonomic expectations, and different degrees of morphological variation

389 within taxa. VIS, SPA and SAL displayed the highest overall morphological variation, while

390 BEC, CLE and ALG displayed the smallest variation. Individuals of different species did not

391 generally intermingle in the morphospace, despite some overlap of 95% confidence ellipses

392 of concentration. CRA specimens were found to fall within the variation of SPA and were

393 considered as SPA for the DFA analysis.

394   The first three canonical discriminant functions of the DFA (explaining 79.5% of

395 variation among groups) clearly discriminated CLE from two other clusters of species, one

396   formed by INC and ONU, and the other formed by ALG, BEC, SPA, SAL and VIS (Fig. 1b;

397   Table S5). The isolation of CLE was mainly the result of significant differences in the ratio

398   corolla/spur length (Table S5; Fig. S3). In the DFA, 100% of cases were correctly classified

399   in the original species grouping, and 96.98% in the cross-validated cases, with ALG, BEC

400   and CLE showing 100% of correct classification (Table S6).

401

402   *Phylogenomic Analysis*

403   *Data assembly and genetic structure.*—Illumina sequencing provided c. 155 million

404   raw reads, with a GC content of 43.05% and bases with quality >Q20 at 96.36%. Assessment

405   in FastQC showed high quality throughout the full 100 bp length of the first reads and little

406   signs of contamination (based on per sequence GC content). Assembly in pyRAD with a

407   minimum taxon coverage of 4 and the full set of individuals resulted in a total number of loci

408   between 21,283 and 36,435 and a concatenated length of 1.97-3.30 Mbp depending on the

409   clustering threshold. Percentages of missing data were high (86.41 to 88.85%). The number

410   of loci, concatenated length and percentage of missing data increased with the clustering

411   threshold, while the number of parsimony-informative characters (PICs) decreased (Table 2).

412   The steep increase in number of loci for the highest values of clustering threshold suggested

413   "over-splitting", where orthologous sequences are divided into separate loci (Harvey et al.

414   2015). The average number of loci per species of the study clade ranged between 2,630 (VIS)

415   and 4,464 (BEC), and the number of sequenced loci per individual decreased quickly with

416   sample age (Fig. S4), with four herbarium specimens of VIS older than 25 years producing

417   low-quality results. After excluding problematic individuals, similar numbers of loci and

418   percentages of missing data were found when using a minimum taxon coverage of 4. When

419   increasing the minimum taxon coverage, the percentage of missing data improved, but the

420   number of loci, concatenated length and number of PICs decreased dramatically (Table 2).

421   SNP-based genetic structure analyses produced qualitatively similar results across

422   datasets. Genetic clusters broadly corresponding with morphologically defined taxa were

423   recovered by PCA (Fig. 1c), Neighbour-Net (Fig. S5a) and Bayesian clustering (Fig. S5b)

424   analyses. The CRA individual was consistently included in the SPA cluster. Individuals of

425   the two colour morphs of SAL were consistently included in the same genetic cluster. Eight

426   individuals with uncertain genetic affinities were excluded from further analyses, including

427   six individuals of VIS, one of SPA and one of ONU (see Online Appendix 2 for details).

428

429   *Concatenation-based phylogenies.*—ML phylogenetic analyses of the 16 datasets

430   supported the monophyly of the Iberian clade of *Linaria* subsect. *Versicolores* and its sister

431   relationship to the North African clade (Figs. 2a-d, S6). The eight taxa were recovered as

432   monophyletic groups with 100% BS, and four alternative species-level topologies were

433   obtained (Figs. 2a-d, 3). Three pairs of sister species were consistently recovered from all

434   datasets: (BEC,SAL), (VIS,ONU) and (SPA,INC). In contrast, two species displayed two

435   alternative phylogenetic placements each with variable BS: CLE was recovered as either

436   sister to a clade formed by the remaining 7 species (8 datasets, e.g. Fig. 2c, d) or sister to the

437   (BEC,SAL) clade (8 datasets, e.g. Fig. 2a, b); and ALG was recovered as either sister to a

438   ((VIS,ONU),(SPA,INC)) clade (10 datasets, e.g. Fig. 2a, d) or sister to the (SPA,INC) clade

439   (6 datasets, e.g. Fig. 2b, c). The c84m4, c85m4, c87m4 and c92m4 datasets were selected as

440   representatives of the four alternative topologies (topologies 1-4) for further analysis because

441   of their high average BS for species-level divergences. Topologies produced by ML analyses

442   of concatenated SNP matrices fell within the range of topologies produced by full-sequence

443   analyses (Fig. S7; see Online Appendix 2 for details).

444   BI phylogenetic analyses of the c84m4, c85m4, c87m4 and c92m4 datasets recovered

445   MR trees topologically identical to the ML trees obtained from the same datasets, and

446   displaying high posterior probabilities (PP≈1) for all species divergences (Fig. 2a-d). When

447   increasing the minimum taxon coverage parameter, ML analyses (Fig. S6) displayed varied

448   topologies with a general decrease in statistical support of clades (particularly for m=20).

449

450   *Coalescent-based phylogenies*.—Unlike the contrasting topologies obtained from

451   concatenated analyses, coalescent-based NJ_{st} analyses of the c84m4, c85m4, c87m4 and

452   c92m4 datasets produced identical MR topologies, although with variable BS values (Figs.

453   2e, S8). The monophyly of the Iberian clade of *Linaria* subsect. *Versicolores* (98-100% BS)

454   and its sister relationship to the North African clade were strongly supported. Two major,

455   geographically structured subclades were recovered within the Iberian clade (Figs. 2e, 4a, 5):

456   a clade formed by SAL, BEC and CLE, endemic to the south-eastern Iberian Peninsula (74-

457   94% BS); and a clade formed by VIS, ONU, ALG, SPA and INC, mostly distributed in the

458   western Iberian Peninsula (100% BS in all analyses). Within the south-eastern Iberian clade,

459   SAL was recovered as sister to a (BEC,CLE) clade. This phylogenetic position of CLE as

460   sister to BEC (94-100% BS) was not recovered from concatenated analyses. Within the

461   western Iberian clade, two sister species pairs found in the NJ_{st} tree were also consistently

462   recovered from concatenated analyses: (VIS,ONU) (100% BS in all analyses) and (SPA,INC)

463   (88-100% BS). ALG was recovered as sister to the (SPA,INC) pair, one of the two alternative

464   positions identified in concatenated analyses. When increasing the minimum taxon coverage

465   parameter to m=10, we obtained the same MR topology for all datasets, although with lower

466   BS values. For m=20, there was a general loss of resolution, including basal polytomies and

467   poor BS values (Fig. S8).

468   SVDquartets analyses produced another topology (Figs. 2f, S8) that was highly robust

469   to variations in clustering threshold and minimum taxon coverage. The monophyly of the

470   Iberian clade (98-100% BS for analyses with m=4) and its sister relationship to the North

471   African clade were again strongly supported, but there were two differences with the NJ_{st}

472   tree: CLE was consistently recovered as sister to a clade formed by the remaining seven

473   species (100% BS), and SPA was found to form a clade (69-97% BS) with the (VIS,ONU)

474   pair (75-88% BS). The same topology was obtained for all analyses with m=10 and three out

475   of four analyses with m=20 (Fig. S8).

476

477   *Tests of alternative topologies.*—The two topologies obtained in previous analyses of

478   ITS+ptDNA sequences (topologies 7, 8) were strongly rejected by all tests implemented for

479   all analysed GBS datasets (Table 3). The SVDquartets tree (topology 6) was rejected by all

480   tests for the c85m4, c87m4 and c92m4 datasets. None of the remaining five topologies (four

481   concatenated and NJ_{st}) were consistently rejected. SH tests could not reject any of these five

482   topologies for any of the datasets, while WSH tests only rejected the NJ_{st} topology (topology

483   5) for the c92m4 dataset. AU tests rejected the NJ_{st} topology for the c87m4 and c92m4

484   datasets and one of the concatenation-based topologies (topology 2) for the c92m4 dataset.

485   All remaining *p*-values were non-significant (Table 3).

486   Topologies 7 and 8 were also consistently supported by the smallest numbers of loci

487   and disfavoured by the highest numbers of loci (Fig. 3). The ML tree for each concatenated

488   dataset was also supported by the highest number and disfavoured by the smallest number of

489   loci. The remaining coalescent- and concatenation-based topologies were supported and

490   disfavoured by intermediate numbers of loci, with the NJ_{st} tree being consistently supported

491   by more loci than the SVDquartets tree (Fig. 3).

492

493   *Hybridisation analyses.*—When assuming the NJ_{st} species tree as MVIP in *D*-statistic

494   tests, we found clear evidence for an excess of shared derived alleles between BEC and SAL

495   in three of the datasets (24-30% of significant tests depending on the dataset) but weaker

496 evidence for an excess of shared derived alleles between SPA and VIS/ONU (8-13% of

497 significant tests) (Table 4). When assuming the SVDquartets species tree as MVIP, the tests

498 (Table 4) found evidence for an excess of shared derived alleles between SPA and INC (24-

499 41% of significant tests), and between BEC and CLE (18-35% of significant tests).

500 Evaluation of the NJ$_{st}$ and SVDquartets trees without reticulations in SNaQ (Table 5,

501 Fig. 2g, h) consistently supported the NJ$_{st}$ tree for all analysed datasets. When including

502 reticulations accounting for incongruences between species tree topologies, three datasets

503 (c85m4, c87m4, c92m4) supported the NJ$_{st}$ topology as MVIP, while the c84m4 dataset

504 supported the SVDquartets topology (although in this case with a small difference in pseudo-

505 deviance values). For the best supported network (with the NJ$_{st}$ tree as MVIP), CLE was

506 estimated to contain a 81-85% genomic contribution from a lineage sister to BEC and 15-

507 19% from an ancestral lineage sister to the other seven species of the clade. Similarly, SPA

508 was estimated to contain a 81-87% contribution from a lineage sister to INC and a 13-19%

509 contribution from a lineage sister to the (VIS,ONU) clade.

510

511

512 *Patterns of Morphological Evolution*

513 The phylomorphospace based on vegetative and reproductive traits and the NJ$_{st}$

514 species tree (Fig. 4b) suggested that SAL has retained morphological traits close to those of

515 the common ancestor of the Iberian clade, while other species have experienced extensive

516 morphological change in multiple directions. Changes of great magnitude in opposite

517 directions of the morphospace are inferred during the divergence of pairs of sister species,

518 including BEC/CLE, VIS/ONU and, to a lesser extent, SPA/INC. At the same time,

519 convergence is suggested by the proximity in the morphospace of non-closely related species

520 pairs, such as INC/ONU, BEC/ALG and SAL/SPA. When focusing on flower shape (Fig.

521   4c), morphological disparity between sister species was even more striking. The ancestral

522   flower for the clade seems to have had a corolla with a broad tube and relatively long nectar

523   spur (type I), a basic shape that has been retained by five species in both subclades (SAL,

524   BEC, SPA, VIS and ALG). Speciation in the sister species pairs VIS/ONU and SPA/INC

525   involved extensive change in flower shape along CV1, which independently gave rise to

526   narrow-tubed, long-spurred flowers (Type III) in ONU and INC. By contrast, speciation of

527   the BEC/CLE pair involved extensive change along CV2, producing the short-spurred, broad-

528   tubed flowers (type II) of CLE. The longest-spurred type I flowers (with the lowest values of

529   CV2) have evolved independently in BEC (sister to CLE) and ALG. Mapping of the

530   phylogeny onto geographic ranges showed that pairs of morphologically divergent sister

531   species display geographically close or even overlapping distributions (Fig. 5): BEC and CLE

532   are both narrow endemics to the same small region in south-eastern Spain; VIS and ONU

533   occur in south-western Iberia; and the distributions of SPA and INC largely overlap in the

534   central-western Peninsula. MP reconstructions based on the NJ_{st} tree (Fig. S9a)

535   unambiguously inferred a single shift from annual to perennial habit (in CLE), and multiple

536   shifts in inflorescence habit, dominant corolla colour and corolla shape. ML reconstructions

537   (Fig. S9b) produced similar results.

538   Broadly similar patterns of morphological evolution were inferred from the

539   SVDquartets tree (Fig. S9c-f), including extensive morphological change associated with

540   speciation events, as well as phenotypic convergence. In phylomorphospace analyses, the

541   main differences resulted from the early-diverging position of CLE in this tree. As a

542   consequence, the extensive morphological change leading to CLE was associated with the

543   first speciation event in the Iberian clade, instead of a more recent divergence with BEC.

544

545   DISCUSSION

546   *GBS, an Effective Method to Resolve Recent Radiations*

547   Our genotyping-by-sequencing approach proved successful in generating highly

548   informative genome-wide genetic markers, not only across the recent (Quaternary) radiation

549   of our 8-species study clade, but also across the whole *Linaria* sect. *Versicolores*, whose most

550   recent common ancestor has been dated back to the late Miocene-Pliocene (Fernández-

551   Mazuecos and Vargas 2011; Fernández-Mazuecos et al. 2013a). Regardless of the method

552   used for phylogenetic inference, inferred basal relationships (i.e. the monophyly and sister

553   relationship of the Iberian and North African clades of subsect. *Versicolores*; Figs. 2, 4a)

554   were identical to those obtained using conventional phylogenetic markers. The two major

555   subclades within the Iberian clade supported by coalescent-based analyses of GBS data in

556   NJ$_{st}$ (Fig. 4a) broadly corresponded to those already obtained (with varying support) in a

557   coalescent-based analysis of ITS and ptDNA sequences and in a concatenated analysis of

558   ptDNA sequences (but they were inconsistent with those obtained using ITS sequences alone)

559   (Fernández-Mazuecos et al. 2013a; Fernández-Mazuecos and Vargas 2015). However, apart

560   from major subclades, previous species-level topologies found little support in our genome-

561   wide data, and comparatively more reliable phylogenetic hypotheses were inferred herein. In

562   addition, genetic structure analyses recovered unprecedented resolution of species

563   boundaries, as shown by the congruence between inferred genetic clusters and

564   morphologically-defined species (Figs. 1, S5).

565   Therefore, despite the uncertainties remaining (possibly explained by hybridisation;

566   see below), our results show that GBS can provide unprecedented systematic and

567   phylogenetic resolution in recent radiations where conventional markers failed, and without

568   the need for previous genomic information (Escudero et al. 2014). Given their relatively

569   simple and inexpensive implementation, GBS and similar methods of the GBS/RAD-Seq

570   family may be universally and routinely applicable by systematic biologists as a NGS-based

571   replacement to conventional phylogenetic approaches (Ree and Hipp 2015; de la Harpe et al.

572   2017; Hamon et al. 2017). Indeed, after DNA extraction, GBS library preparation can be

573   carried out in 2-3 days using equipment available in most molecular systematic laboratories,

574   and successful results may be obtained by multiplexing c.100 individuals in a single HiSeq

575   Illumina lane (although the allowed level of multiplexing would depend on genome sizes and

576   GC content as well as number of reads per lane). Interestingly, even though recently collected

577   tissue is clearly preferable due to a decline in sequencing success with sample age as a result

578   of DNA degradation, we still obtained >1000 sequenced loci with herbarium samples as old

579   as 20-30 years (Fig. S4; but see an improved method for highly degraded DNA samples in

580   Suchan et al. 2016). Compared to the RAD-Seq procedure (Baird et al. 2008), which has been

581   more widely used in phylogenetic and population genetic studies in recent years, the GBS

582   protocol of Elshire et al. (2011) has the advantage of a much simpler protocol for library

583   preparation, including fewer purification steps (thus increasing the probability of success with

584   low starting amounts) and of not requiring accurate size selection of DNA fragments

585   (therefore involving less specialised equipment).

586   The main drawback of GBS, particularly when applied to phylogenetics, is the high

587   proportion of missing data in the assembled datasets (Table 2). This problem is shared by

588   RAD-Seq, although to a lesser extent thanks to size selection. Datasets with lower

589   percentages of missing data may be obtained by increasing the minimum taxon coverage,

590   therefore restricting the final dataset to those loci that were sequenced in a high percentage of

591   individuals. Still, simulations show that accurate phylogenies can be obtained from datasets

592   including vast amounts of missing data (Rubin et al. 2012). Furthermore, increasing the

593   minimum taxon coverage may result in the loss of valuable phylogenetic information due to

594   reduced dataset size and a biased representation of the mutation spectrum across sequenced

595   loci (Huang and Knowles 2014). This is consistent with our empirical results, in which the

596   numbers of loci dramatically decreased when increasing the minimum taxon coverage (Table

597   2), resulting in a loss of phylogenetic resolution in both concatenated and coalescent-based

598   analyses (Fig. S6, S8). Nevertheless, more complete GBS datasets may be obtained, at the

599   same time preventing information loss, by multiplexing smaller numbers of samples per

600   Illumina lane (thus increasing sequencing coverage) and optimising the quality of starting

601   DNA.

602

603   *Concatenation vs. Coalescent-based Approaches to GBS Phylogenetics*

604   A concatenation approach has been widely used for the analysis of restriction digest-

605   based phylogenomic datasets (e.g. Nadeau et al. 2013; Wagner et al. 2013; Escudero et al.

606   2014; Hipp et al. 2014; Cavender-Bares et al. 2015). In particular, ML in RAxML is the

607   current standard for concatenated analysis thanks to its high efficiency with large data

608   matrices. However, there are several well-known drawbacks of concatenated analysis

609   (Wagner et al. 2013). Concatenation assumes a shared phylogenetic history for all sequenced

610   genes, and therefore it is inconsistent in the presence of gene tree discordance caused by ILS

611   and hybridisation (Kubatko and Degnan 2007; Solís-Lemus et al. 2016). Accuracy of species

612   phylogenies inferred by concatenation relies on the most frequent gene tree topology being

613   the same as the species tree topology, but "anomaly zones" exist under certain conditions

614   (involving short branch lengths) in which the most likely gene tree topology differs from the

615   species tree (Degnan and Rosenberg 2006). Additionally, as phylogenomic datasets grow

616   larger, concatenation is more likely to produce anomalously high statistical support for

617   incorrect topologies as a result of systematic biases (Gadagkar et al. 2005; Kumar et al.

618   2011). Bias may result from the specification of a single substitution model, which assumes

619   substitution rate homogeneity across the whole dataset. Partitioned analysis may prevent this

620   problem, but it may be computationally problematic with high numbers of loci. Issues with

25

621 orthology determination and alignment can also result in biases (Kumar et al. 2011), which

622 may be alleviated if a sequenced genome is available as reference for data assembly. Finally,

623 another well-known cause of bias is long-branch attraction, which may be mitigated by a

624 complete taxonomic sampling and by avoiding those methods that are more sensitive to it,

625 such as parsimony (Bergsten 2005).

626   Analyses of our GBS datasets highlight such shortcomings of concatenation. The high

627 statistical support, as measured by both ML bootstrap and Bayesian posterior probabilities,

628 for alternative topologies (Fig. 2a-d) is a sign of systematic bias (Kumar et al. 2011) resulting

629 from the different clustering thresholds. This is true for both concatenated full-sequence and

630 SNP matrices (Figs. S6, S7). Indeed, changes in the clustering threshold produce contrasting

631 optimal topologies (with alternative phylogenetic positions for two species) as a result of

632 significant changes in the numbers of loci supporting and disfavouring alternative trees (Fig.

633 3). Interestingly, in contrast to the misleading confidence suggested by BS and PP values,

634 topology tests revealed no such certainty, as they were generally unable to reject topologies

635 produced by alternative GBS datasets (they did reject previously-published topologies based

636 on only two loci; Table 3). This bias potentially introduced during data assembly at the

637 crucial steps of orthology determination casts doubts on any concatenation analysis based on

638 a fixed clustering threshold. While methods have been proposed to optimise clustering

639 parameters (Mastretta-Yanes et al. 2015), the significant topological changes that we found

640 associated with small changes in the clustering threshold (as small as 1%; Fig. 2a-d) indicate

641 that multiple analyses based on a range of values (Takahashi et al. 2014; Leaché et al.

642 2015b), combined with topology tests, are still necessary to evaluate if high clade support

643 values provide a realistic measurement of confidence.

644 In contrast to concatenation, gene-tree-based coalescent methods have been less frequently

645 used for the inference of species trees from restriction digest-based NGS data. The short

646   length of sequenced loci when analysing single-end data (c. 100-200 bp) may result in poorly

647   informative gene trees based on individual loci, which may be a problem for species tree

648   inference (Salichos and Rokas 2013; Mirarab et al. 2016), and full probabilistic methods co-

649   estimating gene trees and species trees (Liu 2008; Heled and Drummond 2010) are generally

650   unable to handle large NGS datasets (Wagner et al. 2013). Unlike full probabilistic methods,

651   summary methods that operate by combining previously estimated gene trees (e.g. Liu and

652   Yu 2011; Mirarab et al. 2014) are computationally efficient with large datasets. Even with

653   short loci, one of these methods (NJ_{st}) allowed us to infer a resolved species tree (Figs. 2e,

654   4a), showing that the implementation of summary methods is computationally feasible with

655   GBS and RAD-Seq data (see also Ebel et al. 2015; DaCosta and Sorenson 2016). Moreover,

656   the same species tree was produced by this coalescent method from datasets assembled using

657   different clustering thresholds that produced contrasting topologies in concatenation analyses

658   and, furthermore, the NJ_{st} topology could not be rejected by a majority of topology tests using

659   the concatenated datasets (Table 3). The NJ_{st} topology was similar to the concatenation-based

660   topologies, with one of the problematic species, ALG, consistently recovered at one of the

661   two alternative positions of the concatenated analyses (although with varying support). By

662   contrast, the other problematic species, CLE, was recovered as sister to BEC, a relationship

663   not found in concatenated analyses but biogeographically reasonable, given the close

664   proximity of the two species' narrow distributions (Fig. 5). All other species remained at the

665   phylogenetic placements found in concatenation analyses.

666   As an alternative to gene-tree-based methods, coalescent-based approaches that

667   bypass the inference of gene trees have been proposed (Bryant et al. 2012; Chifman and

668   Kubatko 2014) and applied to RAD-Seq datasets (DaCosta and Sorenson 2016; Manthey et

669   al. 2016). We tested the performance of the SVDquartets method, which infers relationships

670   among quartets of taxa using algebraic statistics (Chifman and Kubatko 2014). While poor

671 resolution and support values were recovered when analysing unlinked SNP matrices (Fig.

672 S8), analyses of full-sequence matrices produced a well-supported topology that was highly

673 robust to variations in assembly parameters (Figs. 2f, S8), but different to the NJ_{st} topology.

674 In the SVDquartets tree, CLE was sister to the remaining seven species, a position

675 encountered in some concatenated analyses. SPA was related to VIS and ONU, a position

676 only obtained for one concatenated dataset (c84m20; Fig. S6), but consistent with the close

677 proximity of these tree species in the SNP-based PCA (Fig. 1c).

678   Robustness of phylogenetic patterns across methods and data is considered crucial in

679 phylogenomics (Kumar et al. 2011). The constancy of the NJ_{st} and SVDquartets species trees

680 across datasets indicates that coalescent analyses may be more robust than concatenation to

681 systematic bias introduced during dataset assembly. This result adds to the list of advantages

682 of coalescent methods over concatenation, including their accuracy in the presence of gene

683 tree discordance caused by ILS (Mirarab et al. 2016) and their more realistic measures of

684 clade statistical support (Giarla and Esselstyn 2015).

685   The question remains as to the robustness of our results to the violation of the main

686 assumption of most coalescent methods, i.e. the absence of inter-specific gene flow. Indeed,

687 historical hybridisation may be behind the contrasting topologies of the NJ_{st} and SVDquartets

688 trees. We minimized the effect of recent hybridisation by excluding individuals suggested as

689 hybrids by genetic structure analyses. However, *D*-statistic tests (Table 4) still supported

690 ancestral hybridisation events during diversification of the clade, as indicated by significant

691 asymmetries in the sharing of derived alleles between non-sister species when using either

692 tree as the major vertical inheritance pattern. These results show that a fully bifurcating

693 species tree is an oversimplified representation of the diversification of young clades.

694 Simulations indicate that coalescent methods, including NJ_{st}, are able to infer the correct

695 dominant species tree in the presence of low levels of gene flow, given a sufficiently large

696   number of genes (Solís-Lemus et al. 2016). With high levels of gene flow, coalescent

697   methods may not be able to recover the true dominant topology, but they are still more

698   accurate than concatenation (Solís-Lemus et al. 2016). We propose that both coalescent-

699   based topologies (and possibly the concatenation-based topologies to an extent) obtained

700   from our GBS data represent different aspects of a reticulate history of diversification (Fig.

701   4a). At the same time, we favour the NJ$_{st}$ tree over the SVDquartets tree as the current best

702   estimate of the major pattern of speciation based on the following lines of evidence: (i) the

703   NJ$_{st}$ tree generally fitted the data better in SNaQ analyses, both with and without

704   hybridisation (Table 5); (ii) the SVDquartets tree was more frequently rejected in topology

705   tests (Table 3); and (iii) the NJ$_{st}$ tree was supported by more gene trees (Fig. 3). In any case,

706   we cannot minimise the potential role of homoploid hybrid speciation, already suggested for

707   other *Linaria* clades (Blanco-Pastor et al. 2012; see also Nieto Feliner et al. 2017). The origin

708   of *L. clementei* is particularly intriguing, as it may have involved hybridisation between an

709   early-diverging lineage and a recently-derived one (probably with a higher contribution of the

710   latter according to SNaQ analyses; Table 5; Fig. 2g).

711

712   *Phenotypic Divergence and Convergence during Speciation*

713   Our combination of multivariate morphometric, genetic structure and coalescent-

714   based phylogenetic analyses for the first time provide a well-resolved picture of systematics

715   and patterns of morphological evolution in a Mediterranean plant lineage, the Iberian clade of

716   *Linaria* subsect. *Versicolores*. The data support a systematic hypothesis with eight

717   morphologically and genetically distinct species. Taxonomic notes are provided in Online

718   Appendix 1. Our results are essentially consistent with previous taxonomic revisions (Sutton

719   1988; Sáez and Bernal 2009), except that *L. salzmannii* had been generally considered a

720   subspecies of *L. viscosa* (*L. viscosa* subsp. *spicata*) but is now supported as a distinct species

721 as a result of both taxa being independently derived in all phylogenetic analyses (Figs. 2, 4a;

722 see also Blanca et al. 2017). A flower colour polymorphism within *L. salzmannii* (Boissier

723 1841; Sáez and Bernal 2009) is confirmed, as shown by the morphological and genetic

724 similarity of the yellow- and purple-flowered morphs, and *L. becerrae* is well supported as

725 distinct from *L. salzmannii* (Blanca et al. 2017). The morphologically similar (particularly in

726 flower traits) *L. spartea* and *L. viscosa* are established as genetically distinct and non-sister

727 species (Figs. S5, 2, 4a), although further population genetic sampling would be required to

728 determine the exact limits of their geographic ranges. Finally, plants collected in the *locus*

729 *classicus* of *L. viscosa* subsp. *crassifolia* are found to fall within the morphological and

730 genetic variation of *L. spartea* (Figs. 1, S5, S3).

731   Mapping of morphological traits and geography onto the NJ$_{st}$ phylogeny (which we

732 consider a good estimate of major speciation events) shows a pattern in which closely related

733 species have geographically close or overlapping distributions, but they are strikingly

734 divergent in phenotypic traits, and potentially ecological interactions (Figs. 4, 5, S9a, b). At

735 the same time, unrelated species have evolved convergent phenotypes in different geographic

736 regions. This pattern has been found in large scale replicated island radiations (Mahler et al.

737 2013), but is documented here for a smaller scale radiation in a continental setting (see

738 Hughes and Eastwood 2006; Mittelbach and Schemske 2015). The deeper splits show a

739 geographic pattern, reflecting the progressive colonization of the Iberian Peninsula during the

740 Quaternary (Fernández-Mazuecos et al. 2013a; Fernández-Mazuecos and Vargas 2015). The

741 major phylogenetic split between south-eastern and western species coincides with a

742 phylogeographic discontinuity found for ptDNA markers (Fernández-Mazuecos and Vargas

743 2015). There is no clear pattern of morphological differentiation linked to this split, as

744 indicated by the phylomorphospace based on both vegetative and reproductive characters

745 (Fig. 4b). In contrast, extensive morphological divergence is associated with recent splits

746   between sister species (Figs. 4b, c, S9a, b). This is particularly true for floral traits related to

747   pollinator specialisation (Fenster et al. 2004), including corolla colour, corolla shape, nectar

748   spur length and tube width. Three recent splits between sister species are revealed by the

749   phylogeny. One of them, in the south-eastern subclade, has led to the divergent flower

750   morphologies of *L. clementei* and *L. becerrae*, respectively displaying the shortest and one of

751   the two longest nectar spurs in the clade (Fig. 4). In addition, *L. clementei* is the only

752   perennial species in the clade, which adds to the singularity of this species (see also DFA

753   results; Fig. 1b) and its phenotypic divergence with the annual *L. becerrae*. The two other

754   recent speciation events, in the western subclade, *L. viscosa – L. onubensis* in southwestern

755   Iberia and *L. spartea – L. incarnata* in central Iberia, also represent high degrees of

756   phenotypic divergence. Interestingly, they have both followed nearly identical pathways of

757   floral change in different geographic regions, including divergence in corolla colour (yellow

758   in *L. viscosa* and *L. spartea*, and purple-dominated in their sisters *L. onubensis* and *L.*

759   *incarnata*), corolla tube width (broad in *L. viscosa* and *L. spartea*, narrow in *L. onubensis* and

760   *L. incarnata*), and overall flower shape (with parallel divergence along the first axis of the

761   geometric morphometric analysis) (Fig. 4, S9a, b). The most likely floral ancestral

762   morphology for the western subclade, and for the two pairs of sister species, corresponds to a

763   purple corolla with broad tube and type I shape. *L. algarviana* is the only species in the

764   subclade that has retained this ancestral morphology (except for its elongated nectar spur),

765   while convergent changes have happened in the two other pairs of sister species: a yellow

766   corolla has independently evolved in *L. spartea* and *L. viscosa* from purple-flowered

767   ancestors, and a narrow-tubed, type III corolla has independently evolved in *L. onubensis* and

768   *L. incarnata* from broad-tubed, type I ancestors (Figs. 4, S9a, b). Broad- and narrow-tubed

769   flowers have been suggested as distinct evolutionary optima representing divergent strategies

770   of pollen placement on nectar-feeding insects (Fernández-Mazuecos et al. 2013a). Other

771 instances of phenotypic convergence in geographically disjunct species include the additional

772 shift to a yellow from a purple-dominated corolla in most of the range of *L. salzmannii*, the

773 independent evolution of dense inflorescences from lax inflorescences in the south-eastern

774 subclade and *L. viscosa*, and the independent evolution of the longest nectar spurs in *L.*

775 *becerrae* and *L. algarviana* (Figs. 4, S9a, b).

776 General patterns of morphological evolution are similar when analysing the

777 SVDquartets tree (Fig. S9c-f). Even though some of the details are different, morphological

778 divergence between close relatives and convergence between non-close relatives is still

779 detected, indicating that our main evolutionary inferences are robust to the uncertainty in

780 topology and to hypothesised reticulation events. Similar results have been obtained,

781 although with lower resolution, in the North African clade of *Linaria* subsect. *Versicolores*

782 (Fernández-Mazuecos et al. 2013a) and in *Linaria* sect. *Supinae* (Blanco-Pastor et al. 2015),

783 pointing to a general pattern in the genus.

784

785 In summary, our results indicate a higher robustness of coalescent approaches, as

786 compared to concatenation, for GBS-based species phylogenetics. In our study clade,

787 combined roles of geographical and ecological isolation in speciation are suggested, together

788 with a likely role of hybridisation. Recent speciation events involve extensive divergence in

789 key traits linked to pollinator interactions, such as the length of the nectar spur, considered a

790 key innovation and a model system for eco-evo-devo studies of plant speciation (Fernández-

791 Mazuecos and Glover 2017). These patterns suggest adaptive, ecological diversification,

792 including cases of "parallel speciation" likely associated with similar isolating barriers and

793 selective pressures in different regions.

794

795 FUNDING

32

813   REFERENCES

814   Aberer A.J., Kobert K., Stamatakis A. 2014. ExaBayes: massively parallel Bayesian tree

815   inference for the whole-genome era. Mol. Biol. Evol. 31:2553-2556.

816   Adler D., Murdoch D. 2016. RGL - 3D real-time visualization device system for R. Available

817   in http://rgl.neoscientists.org/.

818   Álvarez I., Wendel J.F. 2003. Ribosomal ITS sequences and plant phylogenetic inference.

819   Mol. Phylogenet. Evol. 29:417-434.

820   Andrews S. 2010. FastQC: a quality control tool for high throughput sequence data.

821   Available in http://www.bioinformatics.babraham.ac.uk/projects/fastqc/.

822   Baird N.A., Etter P.D., Atwood T.S., Currey M.C., Shiver A.L., Lewis Z.A., Selker E.U.,

823   Cresko W.A., Johnson E.A. 2008. Rapid SNP discovery and genetic mapping using

824   sequenced RAD markers. PLOS ONE 3:e3376.

825   Bergsten J. 2005. A review of long-branch attraction. Cladistics 21:163-193.

826   Blanca G., Cueto M., Fuentes J. 2017. *Linaria becerrae* (Plantaginaceae), a new endemic

827   species from the southern Spain, and remarks on what *Linaria salzmannii* is and is

828   not. Phytotaxa 298:261-268.

829   Blanco-Pastor J.L., Ornosa C., Romero D., Liberal I.M., Gómez J.M., Vargas P. 2015. Bees

830   explain floral variation in a recent radiation of *Linaria*. J. Evolution. Biol. 28:851-

831   863.

832   Blanco-Pastor J.L., Vargas P., Pfeil B.E. 2012. Coalescent simulations reveal hybridization

833   and incomplete lineage sorting in Mediterranean *Linaria*. PLOS ONE 7:e39089.

834   Boissier P.E. 1841. Voyage botanique dans le midi de l'Espagne. Tome II. Paris: Gide et Cie.

835   Bryant D., Bouckaert R., Felsenstein J., Rosenberg N.A., RoyChoudhury A. 2012. Inferring

836   species trees directly from biallelic genetic markers: bypassing gene trees in a full

837   coalescent analysis. Mol. Biol. Evol. 29:1917-1932.

838   Bryant D., Moulton V. 2004. Neighbor-net: an agglomerative method for the construction of

839   phylogenetic networks. Mol. Biol. Evol. 21:255-265.

840   Buckley T.R., Simon C., Shimodaira H., Chambers G.K. 2001. Evaluating hypotheses on the

841   origin and evolution of the New Zealand alpine cicadas (Maoricicada) using multiple-

842   comparison tests of tree topology. Mol. Biol. Evol. 18:223-234.

843   Castro M., Castro S., Loureiro J. 2012. Genome size variation and incidence of polyploidy in

844   Scrophulariaceae sensu lato from the Iberian Peninsula. AoB Plants 2012:pls037.

845 Cavender-Bares J., González-Rodríguez A., Eaton D.A.R., Hipp A.A.L., Beulke A., Manos

846   P.S. 2015. Phylogeny and biogeography of the American live oaks (*Quercus*

847   subsection *Virentes*): A genomic and population genetics approach. Mol. Ecol.

848   24:3668-3687.

849 Chifman J., Kubatko L. 2014. Quartet inference from SNP data under the coalescent model.

850   Bioinformatics 30:3317-3324.

851 Corander J., Waldmann P., Marttinen P., Sillanpää M.J. 2004. BAPS 2: enhanced

852   possibilities for the analysis of genetic population structure. Bioinformatics 20:2363-

853   2369.

854 Cullings K.W. 1992. Design and testing of a plant-specific PCR primer for ecological and

855   evolutionary studies. Mol. Ecol. 1:233-240.

856 DaCosta J.M., Sorenson M.D. 2016. ddRAD-seq phylogenetics based on nucleotide, indel,

857   and presence-absence polymorphisms: analyses of two avian genera with contrasting

858   histories. Mol. Phylogenet. Evol. 94:122-135.

859 Davey J.W., Hohenlohe P.A., Etter P.D., Boone J.Q., Catchen J.M., Blaxter M.L. 2011.

860   Genome-wide genetic marker discovery and genotyping using next-generation

861   sequencing. Nat. Rev. Genet. 12:499-510.

862 de la Harpe M., Paris M., Karger D., Rolland J., Kessler M., Salamin N., Lexer C. 2017.

863   Molecular ecology studies of species radiations: current research gaps, opportunities,

864   and challenges. Mol. Ecol. 26:2608-2622.

865 Degnan J.H., Rosenberg N.A. 2006. Discordance of species trees with their most likely gene

866   trees. PLOS Genet. 2:e68.

867 Doyle J.J., Doyle J.L. 1987. A rapid DNA isolation procedure for small quantities of fresh

868   leaf tissue. Phytochem. Bull. 19:11-15.

869   Durand E.Y., Patterson N., Reich D., Slatkin M. 2011. Testing for ancient admixture between

870   closely related populations. Mol. Biol. Evol. 28:2239-2252.

871   Eaton D.A.R. 2014. PyRAD: assembly of de novo RADseq loci for phylogenetic analyses.

872   Bioinformatics 30:1844-1849.

873   Eaton D.A.R., Hipp A.L., González-Rodríguez A., Cavender-Bares J. 2015. Historical

874   introgression among the American live oaks and the comparative nature of tests for

875   introgression. Evolution 69:2587-2601.

876   Eaton D.A.R., Spriggs E.L., Park B., Donoghue M.J. 2016. Misconceptions on missing data

877   in RAD-seq phylogenetics with a deep-scale example from flowering plants. Syst.

878   Biol. 66:399-412.

879   Ebel E.R., DaCosta J.M., Sorenson M.D., Hill R.I., Briscoe A.D., Willmott K.R., Mullen S.P.

880   2015. Rapid diversification associated with ecological specialization in Neotropical

881   *Adelpha* butterflies. Mol. Ecol. 24:2392-2405.

882   Elshire R.J., Glaubitz J.C., Sun Q., Poland J.A., Kawamoto K., Buckler E.S., Mitchell S.E.

883   2011. A robust, simple genotyping-by-sequencing (GBS) approach for high diversity

884   species. PLOS ONE 6:e19379.

885   Escudero M., Eaton D.A.R., Hahn M., Hipp A.L. 2014. Genotyping-by-sequencing as a tool

886   to infer phylogeny and ancestral hybridization: A case study in *Carex* (Cyperaceae).

887   Mol. Phylogenet. Evol. 79:359-367.

888   Farrington H.L., Lawson L.P., Clark C.M., Petren K. 2014. The evolutionary history of

889   Darwin's finches: speciation, gene flow, and introgression in a fragmented landscape.

890   Evolution 68:2932-2944.

891   Fenster C.B., Armbruster W.S., Wilson P., Dudash M.R., Thomson J.D. 2004. Pollination

892   syndromes and floral specialization. Annu. Rev. Ecol. Evol. Systemat. 35:375-403.

893   Fernández-Mazuecos M., Blanco-Pastor J.L., Gómez J.M., Vargas P. 2013a. Corolla

894   morphology influences diversification rates in bifid toadflaxes (*Linaria* sect.

895   *Versicolores*). Ann. Bot. 112:1705-1722.

896   Fernández-Mazuecos M., Blanco-Pastor J.L., Vargas P. 2013b. A phylogeny of toadflaxes

897   (*Linaria* Mill.) based on nuclear internal transcribed spacer sequences: systematic and

898   evolutionary consequences. Int. J. Plant Sci. 174:234-249.

899   Fernández-Mazuecos M., Glover B.J. 2017. The evo-devo of plant speciation. Nat. Ecol.

900   Evol. 1:0110.

901   Fernández-Mazuecos M., Vargas P. 2011. Historical isolation *versus* recent long-distance

902   connections between Europe and Africa in bifid toadflaxes (*Linaria* sect.

903   *Versicolores*). PLOS ONE 6:e22234.

904   Fernández-Mazuecos M., Vargas P. 2015. Quaternary radiation of bifid toadflaxes (*Linaria*

905   sect. *Versicolores*) in the Iberian Peninsula: low taxonomic signal but high geographic

906   structure of plastid DNA lineages. Plant Syst. Evol. 301:1411-1423.

907   Gadagkar S.R., Rosenberg M.S., Kumar S. 2005. Inferring species phylogenies from multiple

908   genes: concatenated sequence tree versus consensus gene tree. J. Exp. Zool. B Mol.

909   Dev. Evol. 304:64-74.

910   Giarla T.C., Esselstyn J.A. 2015. The challenges of resolving a rapid, recent radiation:

911   empirical and simulated phylogenomics of Philippine shrews. Syst. Biol. 64:727-740.

912   Givnish T.J., Millam K.C., Mast A.R., Paterson T.B., Theim T.J., Hipp A.L., Henss J.M.,

913   Smith J.F., Wood K.R., Sytsma K.J. 2009. Origin, adaptive radiation and

914   diversification of the Hawaiian lobeliads (Asterales: Campanulaceae). Proc. Roy. Soc.

915   Lond. B Biol. Sci. 276:407-416.

916   Gower J.C. 1971. A general coefficient of similarity and some of its properties. Biometrics

917   27:857-874.

918   Grabowski P.P., Morris G.P., Casler M.D., Borevitz J.O. 2014. Population genomic variation

919   reveals roles of history, adaptation and ploidy in switchgrass. Mol. Ecol. 23:4059-

920   4073.

921   Guzmán B., Lledó M.D., Vargas P. 2009. Adaptive radiation in Mediterranean *Cistus*

922   (Cistaceae). PLOS ONE 4:e6362.

923   Hamon P., Grover C.E., Davis A.P., Rakotomalala J.J., Raharimalala N.E., Albert V.A.,

924   Sreenath H.L., Stoffelen P., Mitchell S.E., Couturon E., Hamon S., de Kochko A.,

925   Crouzillat D., Rigoreau M., Sumirat U., Akaffou S., Guyot R. 2017. Genotyping-by-

926   sequencing provides the first well-resolved phylogeny for coffee (*Coffea*) and insights

927   into the evolution of caffeine content in its species: GBS coffee phylogeny and the

928   evolution of caffeine content. Mol. Phylogenet. Evol. 109:351-361.

929   Harvey M.G., Judy C.D., Seeholzer G.F., Maley J.M., Graves G.R., Brumfield R.T. 2015.

930   Similarity thresholds used in DNA sequence assembly from short reads can reduce the

931   comparability of population histories across species. PeerJ 3:e895.

932   Harvey M.G., Smith B.T., Glenn T.C., Faircloth B.C., Brumfield R.T. 2016. Sequence

933   capture versus restriction site associated DNA sequencing for shallow systematics.

934   Syst. Biol. 65:910-924.

935   Heled J., Drummond A.J. 2010. Bayesian inference of species trees from multilocus data.

936   Mol. Biol. Evol. 27:570-580.

937   Hipp A.L. 2014. RADami: R package for phylogenetic analysis of RADseq data. Available in

938   https://cran.r-project.org/web/packages/RADami/index.html.

939   Hipp A.L., Eaton D.A.R., Cavender-Bares J., Fitzek E., Nipper R., Manos P.S. 2014. A

940   framework phylogeny of the American oak clade based on sequenced RAD data.

941   PLOS ONE 9:e93975.

942 Huang H., Knowles L.L. 2014. Unforeseen consequences of excluding missing data from

943   next-generation sequences: simulation study of RAD sequences. Syst. Biol. 65:357-

944   365.

945 Hughes C., Eastwood R. 2006. Island radiation on a continental scale: exceptional rates of

946   plant diversification after uplift of the Andes. P. Natl. Acad. Sci. USA 103:10334-

947   10339.

948 Kampstra P. 2008. Beanplot: A boxplot alternative for visual comparison of distributions. J.

949   Stat. Softw. 28:1-9.

950 Kay K.M., Voelckel C., Yang J.Y., Hufford K.M., Kaska D.D., Hodges S.A. 2006. Floral

951   characters and species diversification. In: Harder L.D., Barrett S.C.H., editors.

952   Ecology and evolution of flowers, Oxford: Oxford University Press. pp. 311-325.

953 Kubatko L.S., Degnan J.H. 2007. Inconsistency of phylogenetic estimates from concatenated

954   data under coalescence. Syst. Biol. 56:17-24.

955 Kumar S., Filipski A.J., Battistuzzi F.U., Pond S.L.K., Tamura K. 2011. Statistics and truth in

956   phylogenomics. Mol. Biol. Evol. 29:457-472.

957 Leaché A.D., Banbury B.L., Felsenstein J., de Oca A.N.-M., Stamatakis A. 2015a. Short tree,

958   long tree, right tree, wrong tree: new acquisition bias corrections for inferring SNP

959   phylogenies. Syst. Biol. 64:1032-1047.

960 Leaché A.D., Chavez A.S., Jones L.N., Grummer J.A., Gottscho A.D., Linkem C.W. 2015b.

961   Phylogenomics of phrynosomatid lizards: conflicting signals from sequence capture

962   versus restriction site associated DNA sequencing. Genome Biol. Evol. 7:706-719.

963 Lemmon E.M., Lemmon A.R. 2013. High-throughput genomic data in systematics and

964   phylogenetics. Annu. Rev. Ecol. Evol. Systemat. 44:99-121.

965 Li H., Ruan J., Durbin R. 2008. Mapping short DNA sequencing reads and calling variants

966   using mapping quality scores. Genome Res. 18:1851-1858.

967   Liu L. 2008. BEST: Bayesian estimation of species trees under the coalescent model.

968   Bioinformatics 24:2542-2543.

969   Liu L., Wu S., Yu L. 2015. Coalescent methods for estimating species trees from

970   phylogenomic data. J. Syst. Evol. 53:380-390.

971   Liu L., Yu L. 2010. Phybase: an R package for species tree analysis. Bioinformatics 26:962-

972   963.

973   Liu L., Yu L. 2011. Estimating species trees from unrooted gene trees. Syst. Biol. 60:661-

974   667.

975   Maddison W.P., Maddison D.R. 2011. Mesquite: a modular system for evolutionary analysis.

976   Available in http://mesquiteproject.org.

977   Mahler D.L., Ingram T., Revell L.J., Losos J.B. 2013. Exceptional convergence on the

978   macroevolutionary landscape in island lizard radiations. Science 341:292-295.

979   Mallo D. 2015. Multi-locus bootstrapping script. Available in

980   https://github.com/adamallo/multi-locus-bootstrapping.

981   Mallo D. 2016. NJstM. Available in https://github.com/adamallo/NJstM.

982   Manthey J.D., Campillo L.C., Burns K.J., Moyle R.G. 2016. Comparison of target-capture

983   and restriction-site associated DNA sequencing for phylogenomics: a test in

984   cardinalid tanagers (Aves, Genus: *Piranga*). Syst. Biol. 65:640-650.

985   Mastretta-Yanes A., Arrigo N., Alvarez N., Jorgensen T.H., Piñero D., Emerson B.C. 2015.

986   Restriction site-associated DNA sequencing, genotyping error estimation and de novo

987   assembly optimization for population genetic inference. Mol. Ecol. Resour. 15:28-41.

988   McCormack J.E., Hird S.M., Zellmer A.J., Carstens B.C., Brumfield R.T. 2013. Applications

989   of next-generation sequencing to phylogeography and phylogenetics. Mol.

990   Phylogenet. Evol. 66:526-538.

991 Meiklejohn K.A., Faircloth B.C., Glenn T.C., Kimball R.T., Braun E.L. 2016. Analysis of a
992   rapid evolutionary radiation using ultraconserved elements: evidence for a bias in
993   some multispecies coalescent methods. Syst. Biol. 65:612-627.

994 Mirarab S., Bayzid M.S., Warnow T. 2016. Evaluating summary methods for multilocus
995   species tree estimation in the presence of incomplete lineage sorting. Syst. Biol.
996   65:366-380.

997 Mirarab S., Reaz R., Bayzid M.S., Zimmermann T., Swenson M.S., Warnow T. 2014.
998   ASTRAL: genome-scale coalescent-based species tree estimation. Bioinformatics
999   30:i541-i548.

1000 Mittelbach G.G., Schemske D.W. 2015. Ecological and evolutionary perspectives on
1001   community assembly. Trends Ecol. Evol. 30:241-247.

1002 Muschick M., Indermaur A., Salzburger W. 2012. Convergent evolution within an adaptive
1003   radiation of cichlid fishes. Curr. Biol. 22:2362-2368.

1004 Nadeau N.J., Martin S.H., Kozak K.M., Salazar C., Dasmahapatra K.K., Davey J.W., Baxter
1005   S.W., Blaxter M.L., Mallet J., Jiggins C.D. 2013. Genome-wide patterns of
1006   divergence and gene flow across a butterfly radiation. Mol. Ecol. 22:814-826.

1007 Nicotra A.B., Chong C., Bragg J.G., Ong C.R., Aitken N.C., Chuah A., Lepschi B., Borevitz
1008   J.O. 2016. Population and phylogenomic decomposition via genotyping-by-
1009   sequencing in Australian *Pelargonium*. Mol. Ecol. 25:2000-2014.

1010 Nieto Feliner G., Álvarez I., Fuertes-Aguilar J., Heuertz M., Marques I., Moharrek F., Piñeiro
1011   R., Riina R., Rosselló J.A., Soltis P.S., Villa-Machío I. 2017. Is homoploid hybrid
1012   speciation that rare? An empiricist's view. Heredity 118:513-516.

1013 Oksanen J., Blanchet F.G., Kindt R., Legendre P., O'Hara R.B., Simpson G.L., Solymos P.,
1014   M.H.H. S., Wagner H. 2011. *vegan*: Community Ecology Package. R package version
1015   1.17-9. Available in http://CRAN.R-project.org/package=vegan.

1016 Paradis E., Claude J., Strimmer K. 2004. APE: analyses of phylogenetics and evolution in R

1017   language. Bioinformatics 20:289-290.

1018 Peterson B.K., Weber J.N., Kay E.H., Fisher H.S., Hoekstra H.E. 2012. Double digest

1019   RADseq: an inexpensive method for de novo SNP discovery and genotyping in model

1020   and non-model species. PLOS ONE 7:e37135.

1021 Poland J.A., Brown P.J., Sorrells M.E., Jannink J.L. 2012. Development of high-density

1022   genetic maps for barley and wheat using a novel two-enzyme genotyping-by-

1023   sequencing approach. PLOS ONE 7:e32253.

1024 Pritchard J.K., Stephens M., Donnelly P. 2000. Inference of population structure using

1025   multilocus genotype data. Genetics 155:945-959.

1026 R Development Core Team. 2016. R: A language and environment for statistical computing.

1027   Vienna: R Foundation for Statistical Computing.

1028 Ree R.H., Hipp A.L. 2015. Inferring phylogenetic history from restriction site associated

1029   DNA (RADseq). In: Hörandl E., Appelhans M.S., editors. Next-generation

1030   sequencing in plant systematics, Königstein: Koeltz Scientific Books. pp. 181-204.

1031 Revell L.J. 2012. phytools: an R package for phylogenetic comparative biology (and other

1032   things). Methods Ecol. Evol. 3:217-223.

1033 Roure B., Baurain D., Philippe H. 2013. Impact of missing data on phylogenies inferred from

1034   empirical phylogenomic data sets. Mol. Biol. Evol. 30:197-214.

1035 Rubin B.E.R., Ree R.H., Moreau C.S. 2012. Inferring phylogenies from RAD sequence data.

1036   PLOS ONE 7:e33394.

1037 Sáez L., Bernal M. 2009. *Linaria* Mill. In: Castroviejo S., Herrero A., Benedí C., Rico E.,

1038   Güemes J., editors. Flora iberica XIII (Plantaginaceae – Scrophulariaceae), Madrid:

1039   CSIC. pp. 232-324.

1040   Salichos L., Rokas A. 2013. Inferring ancient divergences requires genes with strong

1041   phylogenetic signals. Nature 497:327-331.

1042   Sang T. 2002. Utility of low-copy nuclear gene sequences in plant phylogenetics. Crit. Rev.

1043   Biochem. Mol. 37:121-147.

1044   Schluter D. 2000. The ecology of adaptive radiation. Oxford: Oxford University Press.

1045   Seehausen O. 2006. African cichlid fish: a model system in adaptive radiation research. Proc.

1046   Roy. Soc. Lond. B Biol. Sci. 273:1987-1998.

1047   Seo T.K. 2008. Calculating bootstrap probabilities of phylogeny using multilocus sequence

1048   data. Mol. Biol. Evol. 25:960-971.

1049   Shafer A., Peart C.R., Tusso S., Maayan I., Brelsford A., Wheat C.W., Wolf J.B.W. 2016.

1050   Bioinformatic processing of RAD-seq data dramatically impacts downstream

1051   population genetic inference. Methods Ecol. Evol. doi 10.1111/2041-210X.12700.

1052   Shaffer H.B., Thomson R.C. 2007. Delimiting species in recent radiations. Syst. Biol.

1053   56:896-906.

1054   Shaw J., Lickey E.B., Schilling E.E., Small R.L. 2007. Comparison of whole chloroplast

1055   genome sequences to choose noncoding regions for phylogenetic studies in

1056   angiosperms: the tortoise and the hare III. Am. J. Bot. 94:275-288.

1057   Shaw K.L. 2002. Conflict between nuclear and mitochondrial DNA phylogenies of a recent

1058   species radiation: what mtDNA reveals and conceals about modes of speciation in

1059   Hawaiian crickets. P. Natl. Acad. Sci. USA 99:16122-16127.

1060   Shimodaira H. 2002. An approximately unbiased test of phylogenetic tree selection. Syst.

1061   Biol. 51:492-508.

1062   Shimodaira H., Hasegawa M. 1999. Multiple comparisons of log-likelihoods with

1063   applications to phylogenetic inference. Mol. Biol. Evol. 16:1114-1116.

1064 Shimodaira H., Hasegawa M. 2001. CONSEL: for assessing the confidence of phylogenetic

1065   tree selection. Bioinformatics 17:1246-1247.

1066 Sidlauskas B. 2008. Continuous and arrested morphological diversification in sister clades of

1067   characiform fishes: a phylomorphospace approach. Evolution 62:3135-3156.

1068 Solís-Lemus C., Ané C. 2016. Inferring phylogenetic networks with maximum

1069   pseudolikelihood under incomplete lineage sorting. PLOS Genet. 12:e1005896.

1070 Solís-Lemus C., Yang M., Ané C. 2016. Inconsistency of species-tree methods under gene

1071   flow. Syst. Biol. 65:843-851.

1072 Stamatakis A. 2006. Phylogenetic models of rate heterogeneity: a high performance

1073   computing perspective. Proceedings of the IPDPS2006, Rhodos, Greece: IEEE.

1074 Stamatakis A. 2014. RAxML version 8: a tool for phylogenetic analysis and post-analysis of

1075   large phylogenies. Bioinformatics 30:1312-1313.

1076 Suchan T., Pitteloud C., Gerasimova N.S., Kostikova A., Schmid S., Arrigo N., Pajkovic M.,

1077   Ronikier M., Alvarez N. 2016. Hybridization capture using RAD probes (hyRAD), a

1078   new tool for performing genomic analyses on collection specimens. PLOS ONE

1079   11:e0151651.

1080 Sutton D.A. 1988. A revision of the tribe Antirrhineae. Oxford: Oxford University Press.

1081 Swofford D. 2002. PAUP*. Phylogenetic analysis using parsimony (*and other methods).

1082   Version 4. Sunderland, Massachusetts: Sinauer.

1083 Takahashi T., Nagata N., Sota T. 2014. Application of RAD-based phylogenetics to complex

1084   relationships among variously related taxa in a species flock. Mol. Phylogenet. Evol.

1085   80:137-144.

1086 Valdés B. 1970. Taxonomía experimental del género *Linaria* IV. Reproducción sexual:

1087   autogamia y polinización intraespecífica. Bol. R. Soc. Esp. Hist. Nat. Secc. Biol.

1088   68:79-89.

1089   Valente L.M., Savolainen V., Vargas P. 2010. Unparalleled rates of species diversification in

1090   Europe. Proc. Roy. Soc. Lond. B Biol. Sci. 277:1489-1496.

1091   van Orsouw N.J., Hogers R.C.J., Janssen A., Yalcin F., Snoeijers S., Verstege E., Schneiders

1092   H., van der Poel H., Van Oeveren J., Verstegen H. 2007. Complexity reduction of

1093   polymorphic sequences (CRoPS™): a novel approach for large-scale polymorphism

1094   discovery in complex genomes. PLOS ONE 2:e1172.

1095   Vargas P., Zardoya R. 2014. The tree of life: evolution and classification of living organisms.

1096   Sunderland, Massachusetts: Sinauer Associates Inc.

1097   Venables W.N., Ripley B.D. 2002. Modern applied statistics with S. 4th edition. New York:

1098   Springer.

1099   Vigalondo B., Fernández-Mazuecos M., Vargas P., Sáez L. 2015. Unmasking cryptic species:

1100   morphometric and phylogenetic analyses of the Ibero-North African *Linaria*

1101   *incarnata* complex. Bot. J. Linn. Soc. 177:395-417.

1102   Wagner C.E., Keller I., Wittwer S., Selz O.M., Mwaiko S., Greuter L., Sivasundar A.,

1103   Seehausen O. 2013. Genome-wide RAD sequence data provide unprecedented

1104   resolution of species boundaries and relationships in the Lake Victoria cichlid

1105   adaptive radiation. Mol. Ecol. 22:787-798.

1106   Waterhouse A.M., Procter J.B., Martin D.M.A., Clamp M., Barton G.J. 2009. Jalview

1107   Version 2—a multiple sequence alignment editor and analysis workbench.

1108   Bioinformatics 25:1189-1191.

1109   Whittall J.B., Voelckel C., Kliebenstein D.J., Hodges S.A. 2006. Convergence, constraint and

1110   the role of gene expression during adaptive radiation: floral anthocyanins in

1111   *Aquilegia*. Mol. Ecol. 15:4645-4657.

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FIGURE CAPTIONS

FIGURE 1. Results of morphometric and genetic structure analyses of the eight taxa

included in the Iberian clade of *Linaria* subsect. *Versicolores* (see Table 1 for the meaning of

acronyms). (a) Morphospace based on principal coordinate analysis of all morphometric data

(24 quantitative and 3 qualitative variables; 166 individuals); values of the first three

coordinates, explaining 54.8% of variance, are represented. (b) Morphospace based on

discriminant function analysis of quantitative variables; values of the first three canonical

discriminant functions, explaining 79.5% of variation among groups, are represented. (c)

Principal component analysis based on the unlinked single nucleotide polymorphism (SNP)

matrix obtained from the c85m4 dataset (5265 SNPs; 76 individuals); values of coordinates 1,

2 and 4, explaining 72.1% of variance, are represented. In all panels, 95% confidence ellipses

of concentration for each of the eight species are shown, and arrows indicate CRA

individuals (ultimately assigned to SPA; see text).

FIGURE 2. Results from concatenation- and coalescent-based phylogenetic analyses of

genotyping-by-sequencing datasets of the Iberian clade of *Linaria* subsect. *Versicolores*. (a-d)

Four alternative maximum-likelihood (ML) phylogenetic trees obtained in RAxML from

concatenated analyses of datasets assembled using different clustering thresholds: c84m4 (a),

c85m4 (b), c87m4 (c), c92m4 (d) (see Table 2). Numbers above branches are percentage

bootstrap values from ML analyses. Numbers below branches are posterior probabilities from

Bayesian analyses in ExaBayes. (e) Coalescent-based species tree obtained using the NJ$_{st}$

method, after estimation of unrooted gene trees by ML in RAxML with 100 bootstrap

replicates. The 50% majority-rule consensus of 50 multilocus bootstrap replicates is shown.

The same majority-rule topology was obtained from four genotyping-by-sequencing datasets

1140   assembled using different clustering thresholds (c84m4, c85m4, c87m4, c92m4; see Table 2).

1141   Numbers above branches are multilocus bootstrap support values (in percentage) from the

1142   c84m4 and c85m4 datasets. Numbers below branches are multilocus bootstrap support values

1143   from the c87m4 and c92m4 datasets. (f) Coalescent-based species tree obtained using the

1144   SVDquartets method with 100 bootstrap replicates. The 50% majority-rule consensus

1145   obtained from the same four datasets is shown. Numbers above branches are bootstrap

1146   support values from the c84m4 and c85m4 datasets. Numbers below branches are bootstrap

1147   support values from the c87m4 and c92m4 datasets. (g-h) Fixed network topologies evaluated

1148   in a coalescent with hybridisation framework using the SNaQ method: a network with the

1149 NJ_{st} tree as major vertical inheritance pattern (MVIP) and reticulations representing

1150   incongruences with the SVDquartets tree (g), and a network with the SVDquartets tree as

1151   MVIP and reticulations representing incongruences with the NJ_{st} tree (h). The four values

1152   attached to hybrid edges represent inheritance probabilities (i.e. the proportions of genes

1153   contributed by each parental population to a hybrid taxon) estimated from the c84m4, c85m4,

1154   c87m4 and c92m4 datasets respectively.

1155

1156   FIGURE 3. Numbers of loci supporting and disfavouring constrained concatenated

1157   trees with eight alternative topologies (1-8, left) in four genotyping-by-sequencing datasets

1158   assembled using different clustering thresholds (c84m4, c85m4, c87m4, c92m4; right; see

1159   Table 2). Topologies 1-4 are the four alternative optimal topologies respectively produced by

1160   concatenated analyses of the same four datasets (see Fig. 3a-d). Topology 5 is the one

1161   produced by coalescent-based analyses of all four datasets in NJ_{st} (see Fig. 3e). Topology 6 is

1162   the one produced by coalescent-based analyses of all four datasets in SVDquartets (see Fig.

1163   3f). Topologies 7 and 8 were produced by previously published concatenated (topology 7)

1164   and coalescent-based (topology 8) analyses of combined ITS and ptDNA sequences

(Fernández-Mazuecos et al. 2013a; Vigalondo et al. 2015). For each dataset, numbers of loci significantly supporting and disfavouring each tree topology are plotted against the log-likelihood of the tree when using the full concatenated matrix. Black dots represent the maximum likelihood tree for each dataset. White dots represent constrained trees that were significantly rejected by Shimodaira–Hasegawa tests, and grey dots represent constrained trees that were not significantly rejected (see Table 3).

FIGURE 4. Patterns of morphological evolution in the Iberian clade of *Linaria* subsect. *Versicolores* based on coalescent-based phylogenetic analysis of genotyping-by-sequencing data. (a) Coalescent-based species tree obtained using the NJ_{st} method, with dotted lines representing the alternative position of two species in the SVDquartets tree. Flowers of the eight species of the Iberian clade of *Linaria* subsect. *Versicolores* are shown (photos by J. Ramírez and M. Fernández-Mazuecos), and mean values of spur length and tube width according to Fernández-Mazuecos et al. (2013a) are plotted. (b) Phylomorphospace defined by the NJ_{st} phylogeny and the first three canonical discriminant functions of the morphometric analysis based on vegetative and reproductive traits. Each species is represented by the mean values of the coordinates. (c) Phylomorphospace defined by the NJ_{st} phylogeny and the first two canonical variates of a previously published geometric morphometric analysis of flowers (Fernández-Mazuecos et al. 2013a).

FIGURE 5. Biogeographic patterns in the Iberian clade of *Linaria* subsect. *Versicolores* based on coalescent-based phylogenetic analysis of genotyping-by-sequencing data. The NJ_{st} species tree is projected onto the geographic distribution of the eight species in the Iberian Peninsula. Dashed lines at the limit between *L. spartea* and *L. viscosa* indicate uncertainty on distribution ranges.

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1192   TABLE 1. Study taxa and summary of plant materials included in morphometric and

1193   genotyping-by-sequencing (GBS) analyses.

Taxon[a]	Acronym	No. sampled individuals, morphometry	No. sampled individuals, GBS[b]	No. loci (mean ± SD)[c]
Linaria subsect. *Elegantes* (2/2)	OUT	-	4 / 4	2130 ± 296
Linaria subsect. *Versicolores*, North African clade (6/20)	NAF	-	9 / 9	2524 ± 618
Linaria subsect. *Versicolores*, Iberian clade (8/8)	-	-	76 / 68	3586 ± 1048
L. algarviana Chav.	ALG	17	10 / 10	3219 ± 808
L. becerrae Blanca, Cueto & J.Fuentes	BEC	11	8 / 8	4464 ± 706
L. clementei Haens.	CLE	10	9 / 9	4229 ± 845
L. incarnata (Vent.) Spreng.	INC	33	10 / 10	3526 ± 670
L. onubensis Pau	ONU	32	9 / 8	3805 ± 668
L. salzmannii Boiss. (=L. viscosa subsp. spicata (Kunze) D.A.Sutton)	SAL	21	10 / 10	3801 ± 875
L. spartea (L.) Chaz.	SPA	27	10 / 8	3213 ± 1218
L. viscosa (L.) Chaz.	VIS	22	10 / 5	2630 ± 1412

1194   *Notes:* The final taxonomic treatment is followed, with a previous treatment in brackets for *L. salzmannii*. The

1195   specimens sampled in the *locus classicus* of *L. viscosa* subsp. *crassifolia* (CRA) are included in *L. spartea* (see

1196   text).

1197   [a]Sampled/total numbers of species and subspecies for the three major clades are shown in brackets.

1198   [b]For each taxon, total number of sampled individuals / number of individuals included in phylogenetic analyses

1199   is shown.

1200   [c]For each taxon, the mean (±SD) number of loci recovered across individuals and pyRAD assemblies (from

1201   clustering threshold 0.80 to 0.95, with minimum taxon coverage of 4) is shown. Figures calculated after

1202   taxonomic reassignment of some individuals resulting from genetic structure analyses.

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1207   TABLE 2. Characteristics of assembled genotyping-by-sequencing datasets generated

1208   in pyRAD and used in phylogenetic analyses (81 individuals).

Dataset	Clustering threshold	Minimum taxon coverage	No. loci	Concatenated length (bp)	% Missing data	No. PICs
c84m4	0.84	4	22106	2039028	85.50	81001
c84m10	0.84	10	9090	850257	74.72	57815
c84m20	0.84	20	3888	367013	62.18	32559
c85m4	0.85	4	22586	2079729	85.62	80502
c85m10	0.85	10	9203	858302	74.81	57033
c85m20	0.85	20	3894	366417	62.24	31842
c87m4	0.87	4	23593	2166830	85.91	78488
c87m10	0.87	10	9475	880128	75.28	54553
c87m20	0.87	20	3881	363324	62.65	29259
c92m4	0.92	4	29037	2647482	86.90	72514
c92m10	0.92	10	10738	986997	76.42	47175
c92m20	0.92	20	3899	359814	63.09	22620

1209   *Notes:* Assembly parameters, dataset sizes, completeness and numbers of parsimony-informative characters

1210   (PICs) are indicated.

1211

TABLE 3. Results from topology tests in CONSEL.

Dataset	Rank	Topology	Obs.	AU	SH	WSH
c84m4	1	1	-32.5	0.841	0.98	0.985
	2	4	32.5	0.466	0.825	0.758
	3	2	59.2	0.262	0.752	0.511
	4	3	76.7	0.244	0.663	0.57
	5	5	164.2	0.102	0.398	0.251
	6	6	239.1	0.061	0.207	0.195
	7	8	978.7	1.00E-07*	0*	0*
	8	7	6270.7	2.00E-08*	0*	0*
c85m4	1	2	-29.9	0.823	0.986	0.991
	2	5	29.9	0.365	0.791	0.694
	3	3	46.1	0.335	0.746	0.647
	4	1	85.1	0.12	0.615	0.294
	5	4	137	0.063	0.411	0.247
	6	6	456.7	0.001*	0.004*	0.001*
	7	8	1055	1.00E-04*	0*	0*
	8	7	6398.8	4.00E-05*	0*	0*
c87m4	1	3	-61.4	0.897	0.991	0.997
	2	4	61.4	0.205	0.69	0.489
	3	2	71.4	0.228	0.655	0.477
	4	1	115.5	0.137	0.491	0.356
	5	5	182.4	0.015*	0.287	0.099
	6	6	442.4	1.00E-04*	0.005*	2.00E-04*
	7	8	1323.9	1.00E-06*	0*	0*
	8	7	6279	2.00E-06*	0*	0*
c92m4	1	4	-75.7	0.89	0.993	0.996
	2	1	75.7	0.198	0.64	0.496
	3	3	77	0.173	0.639	0.435
	4	2	155.7	0.033*	0.346	0.174
	5	5	268.1	0.005*	0.136	0.027*
	6	6	488.9	4.00E-11*	0.004*	0*
	7	8	1282.5	3.00E-24*	0*	0*
	8	7	5344.1	5.00E-08*	0*	0*

Notes: Four genotyping-by-sequencing datasets were used to test eight alternative topologies in a maximum likelihood framework with concatenated matrices. Tested topologies (summarized in Fig. 3) included the four alternative optimal topologies produced by concatenated analyses of the same four datasets (topologies 1-4; Fig. 2a-d), the topologies produced by coalescent-based NJ_{st} (topology 5; Fig. 2e) and SVDquartets (topology 6; Fig. 2f) analyses of all four datasets and two topologies produced by previously-published analyses of internal transcribed spacer and plastid DNA sequences (topologies 7, 8). For each dataset, topologies are ranked by the test statistics (Obs.), and *p*-values of the Approximately Unbiased (AU), Shimodaira–Hasegawa (SH) and Weighted Shimodaira–Hasegawa (WSH) tests are shown. Asterisks indicate significant results ($p<0.05$).

TABLE 4. *D*-statistic tests conducted on four genotyping-by-sequencing datasets given a four-taxon tree (((P1,P2),P3),O).

Dataset	Set	Assumed species tree	P1	P2	P3	O	N	% Sig. (ABBA>BABA)	% Sig. (BABA>ABBA)
c84m4	1	NJ_{st}	ALG, INC	SPA	VIS, ONU	BEC, SAL	648	9.26	3.55
	2	NJ_{st}	CLE	BEC	SAL	VIS, ONU, ALG, SPA, INC	405	23.95	0
	3	SVDquartets	VIS, ONU	SPA	INC	BEC, SAL	324	25.00	0.31
	4	SVDquartets	ALG, INC, SPA, VIS, ONU	BEC	CLE	NAF	540	22.59	0.00
c85m4	1	NJ_{st}	ALG, INC	SPA	VIS, ONU	BEC, SAL	648	8.33	2.31
	2	NJ_{st}	CLE	BEC	SAL	VIS, ONU, ALG, SPA, INC	405	27.16	0
	3	SVDquartets	VIS, ONU	SPA	INC	BEC, SAL	324	24.07	0.62
	4	SVDquartets	ALG, INC, SPA, VIS, ONU	BEC	CLE	NAF	540	34.63	0.00
c87m4	1	NJ_{st}	ALG, INC	SPA	VIS, ONU	BEC, SAL	648	11.11	5.40
	2	NJ_{st}	CLE	BEC	SAL	VIS, ONU, ALG, SPA, INC	405	29.88	0
	3	SVDquartets	VIS, ONU	SPA	INC	BEC, SAL	324	38.27	0.31
	4	SVDquartets	ALG, INC, SPA, VIS, ONU	BEC	CLE	NAF	540	17.96	0.74
c92m4	1	NJ_{st}	ALG, INC	SPA	VIS, ONU	BEC, SAL	648	12.65	2.01
	2	NJ_{st}	CLE	BEC	SAL	VIS, ONU, ALG, SPA, INC	405	5.68	1.73
	3	SVDquartets	VIS, ONU	SPA	INC	BEC, SAL	324	41.36	0.31
	4	SVDquartets	ALG, INC, SPA, VIS, ONU	BEC	CLE	NAF	540	18.89	0.93

Notes: Three individuals per species of the Iberian clade and four individuals from different species of the North African clade were selected, and introgression hypotheses potentially explaining topological differences between the two species trees obtained in coalescent-based analyses (Fig. 2e, f) were tested. For each set of tests, we show the number of tests conducted using different combinations of individuals (N) and the percentage of significant tests ($p<0.05$) for ABBA>BABA (potential introgression between P2 and P3) and BABA>ABBA (potential introgression between P1 and P3).

TABLE 5. Results from tree and network evaluation using the SNaQ method.

	NJ$_{st}$ tree without reticulations	SVDquartets tree without reticulations	NJ$_{st}$ tree with reticulations	SVDquartets tree with reticulations
c84m4	734.72	1084.15	736.67	732.19
c85m4	758.05	1120.34	761.38	801.18
c87m4	817.74	1201.44	829.88	896.67
c92m4	1547.23	1648.7	1166.42	1406.25

Notes: Four reduced datasets with 27 individuals were used to evaluate four fixed topologies, including the NJ$_{st}$ and SVDquartets species tree topologies without reticulations (Figs. 2e, f), the NJ$_{st}$ tree as major vertical inheritance pattern (MVIP) with reticulations representing incongruences with the SVDquartets tree (Fig. 2g), and the SVDquartets tree as MVIP with hybrid edges representing incongruences with the NJ$_{st}$ tree (Fig. 2h). Pseudo-deviance values are shown, with lower values indicating a better fit.

a) Morphology (PCoA)

VIS   SAL   CLE   INC   BEC   ONU   ALG   SPA

b) Morphology (DFA)

CLE   ONU   INC   SAL   SPA   ALG   VIS   BEC

c) SNPs (PCA)

BEC   CLE   ALG   SAL   VIS   INC   SPA   ONU
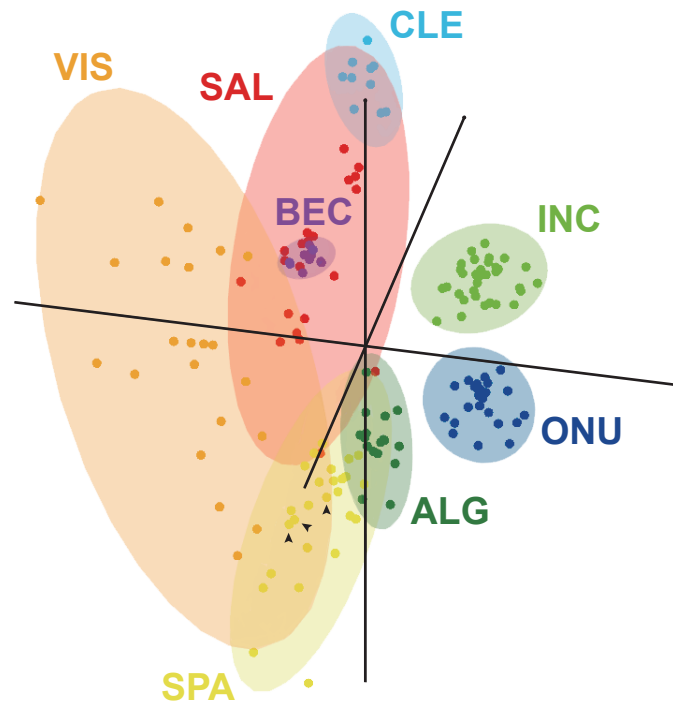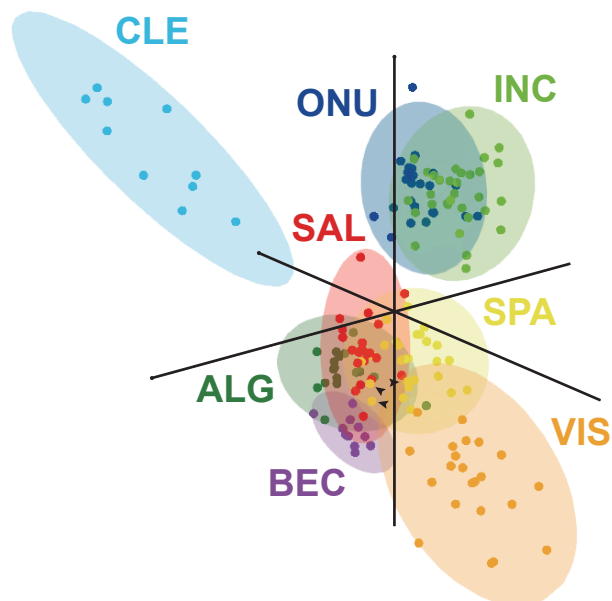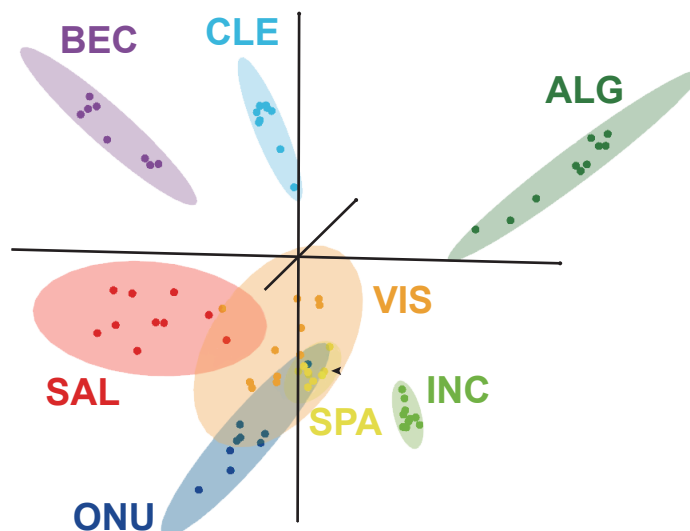
a) Concatenated, RAxML/ExaBayes
TOPOLOGY 1 (c84m4)
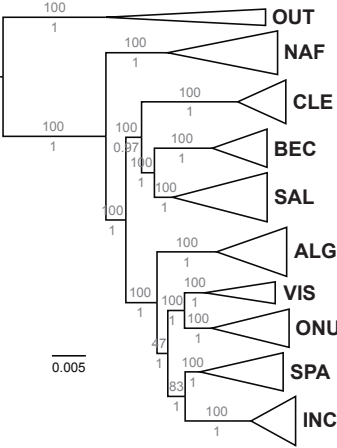
b) Concatenated, RAxML/ExaBayes
TOPOLOGY 2 (c85m4)
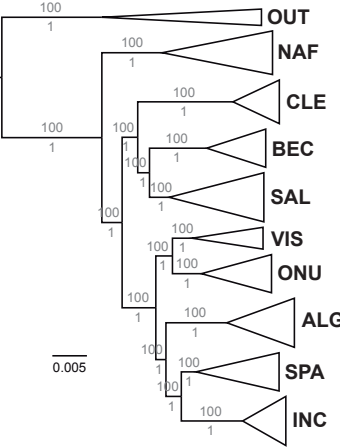
c) Concatenated, RAxML/ExaBayes
TOPOLOGY 3 (c87m4)
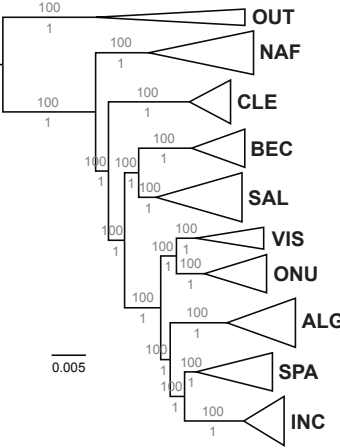
d) Concatenated, RAxML/ExaBayes
TOPOLOGY 4 (c92m4)
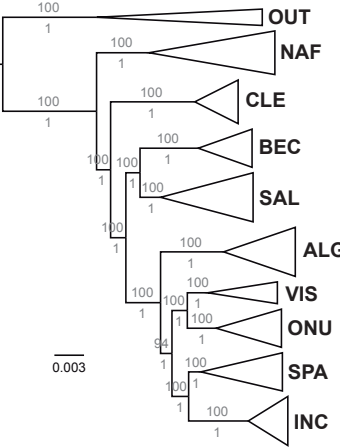
e) Coalescent, NJ$_{st}$
TOPOLOGY 5 (4 datasets)
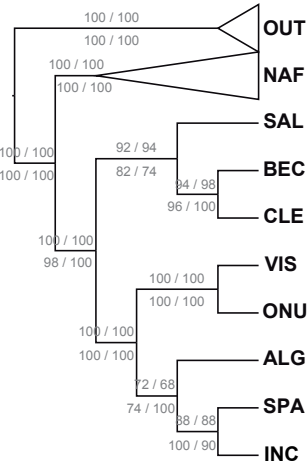
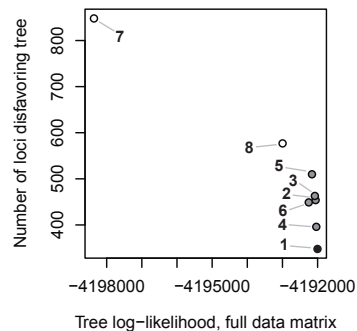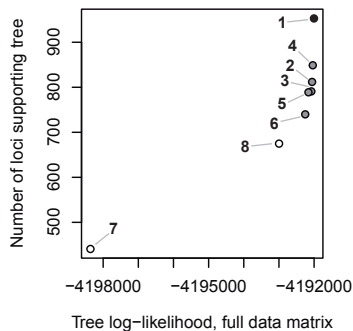**f) Coalescent, SVDquartets**
TOPOLOGY 6 (4 datasets)
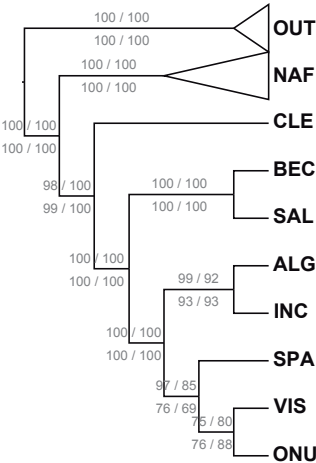
g) Coalescent with hybridisation, SNaQ
TOPOLOGY 5 fixed as MVIP (4 datasets)
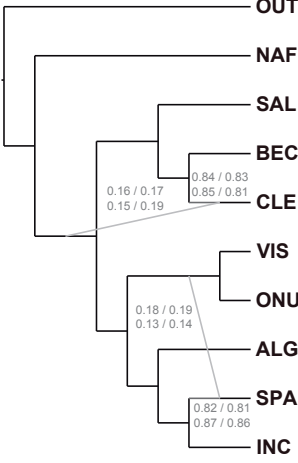
h) Coalescent with hybridisation, SNaQ
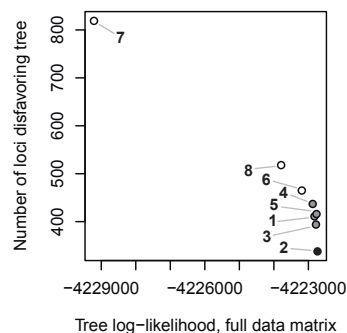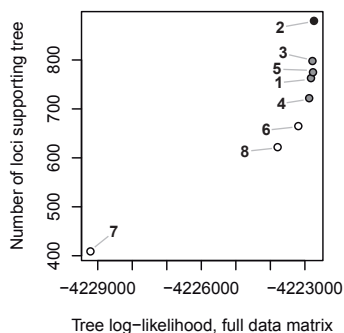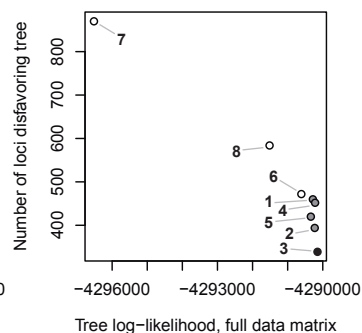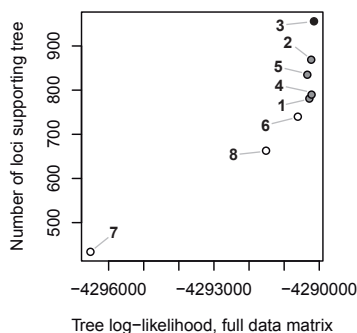TOPOLOGY 6 fixed as MVIP (4 datasets)

c84m4

c85m4

c87m4

c92m4

a)

Spur length (mm)

Tube width (mm)

Subsect. *Elegantes*

North African clade

Subsect. *Versicolores*

Iberian clade

L. salzmannii

L. becerrae

L. clementei

L. viscosa

L. onubensis

L. algarviana

L. spartea

L. incarnata

b)

CAN2

CAN3

CAN1

CLE

ONU

INC

root

SAL

SPA

ALG

BEC

VIS

c)

CV2

CV1

CLE

Type II

Type III

root

SAL

VIS

ONU

INC

SPA

Type I

BEC

ALG