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The puzzling taxonomic rank of *Pijnackeria hispanica*, a chimerical hybrid androgen (Insecta, Phasmida)

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1     **The puzzling taxonomic rank of *Pijnackeria hispanica*, a chimerical**  
2     **hybrid androgen (Insecta, Phasmida)**

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35

36 **Abstract**

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38 The stick-insect genus *Pijnackeria* includes four diploid bisexual and two polyploid (3n,  
39 4n) parthenogenetic species. Earlier analyses of the tetraploid parthenogen *P. hispanica*  
40 using mitochondrial markers allowed to trace its maternal ancestry to *Pijnackeria*  
41 *originis*, while no maternal nuclear contribution was found, thus suggesting an  
42 androgenetic and hybrid origin. The recently described *Pijnackeria recondita*—  
43 showing, among other features, a specific antennal structure linking it to the tetraploid  
44 parthenogen—prompted us to check whether the new species could be *P. hispanica*  
45 unknown paternal ancestor. In this work we use karyology and of molecular analysis of  
46 the mitochondrial gene cytochrome c oxidase subunit 2 (*cox2*), and the nuclear gene  
47 elongation factor 1 subunit  $\alpha$  (*efl- $\alpha$* ) to investigate the origin of such a complex  
48 tetraploid hybrid parthenogen.

49 The molecular analysis supported *P. recondita* as being a paternal ancestor of the *P.*  
50 *hispanica*, but also suggested that two more fathering species have to be taken into  
51 account: *P. barbarae* and the unknown paternal ancestor of the triploid hybrid *P.*  
52 *masettii*. Therefore, *P. hispanica* is apparently a polyphyletic chimeric androgen, which  
53 we propose to indicate as an *androgenetic complex*. Our data also revealed that *P.*  
54 *hispanica* is between 1.96 Myr and 3.31 Myr old, making it the oldest parthenogenetic  
55 taxon discovered among insects.

56

57 Keywords: androgenesis, hybridization, parthenogenesis, *Pijnackeria hispanica*,  
58 *Pijnackeria recondita*, reticulate evolution.

59

60

61 **Introduction**

62

63 The phasmid genus *Pijnackeria* Scali, 2009 is a Spanish monophyletic taxon  
64 embodying four diploid bisexual and two polyploid parthenogenetic species (3n, 4n), all  
65 sharing the same haploid chromosome set with  $n = 19$  (Brock 1991, 1993 for reviews;  
66 Bianchi, 1992, Bianchi and Meliado, 1998; Scali, 2009a; Scali et al. 2013).  
67 *P. hispanica* (Pantel, 1890) (Fig. 1), the nominal species of the genus, appears to be a  
68 very successful tetraploid hybrid parthenogen, ranging from the Sierra Nevada

69 (southern Spain) up to the Sistema Central Mountains, northwestwards and to the  
70 Serrania de Cuenca, northeastwards. Here, its distribution area overlaps that of the  
71 similarly successful triploid hybrid parthenogen *P. masettii* Scali et al., 2013 which is  
72 also distributed up to the southern French districts of Var, Herault and Basses Alps  
73 (Bianchi, 1992; Ghiselli et al. 2007, Scali, 2009; Scali et al. 2013) (Fig. 2). The  
74 remaining four taxa, namely *P. lucianae* (Scali et al. 2013), *P. barbarae* (Scali et al.  
75 2013), *P. lelongi* (Scali et al. 2013) and *P. originis* (Scali et al. 2013) are diploid and  
76 show much more limited distribution areas in the south-eastern Iberian Peninsula,  
77 clearly suggestive of relict distribution (Fig. 2).

78 Molecular genetic analyses, carried out using both the mitochondrial gene cytochrome c  
79 oxidase subunit 2 (*cox2*) and the nuclear gene elongation factor 1 subunit  $\alpha$  (*efl- $\alpha$* ) as  
80 markers, suggested the bisexual diploid *P. lelongi* Scali et al., 2013 as the maternal  
81 ancestor of *P. masettii*, and the diploid bisexual *P. originis* Scali et al., 2013 of Tiscar  
82 (Sierra de Cazorla) as the maternal ancestor of the tetraploid *P. hispanica* (Ghiselli et al.  
83 2007; Scali, 2009a). On the other hand, quite surprisingly, while *P. masettii* showed the  
84 *efl- $\alpha$*  allele of maternal derivation, as it could be expected, *P. hispanica* did not, so that  
85 its *efl- $\alpha$*  gene ought to be of only paternal derivation (Ghiselli et al. 2007). In order to  
86 explain such finding, three different hypotheses were considered: *i*) the effect of gene  
87 conversion; *ii*) the outcome of a non-equivalent gene silencing in the hybrid; *iii*) the  
88 clear-cut consequence of the maternal genome exclusion within an androgenesis  
89 scenario (Ghiselli et al. 2007; Milani et al. 2010, 2013). At any rate, the available data  
90 greatly stimulated us to trace the paternal ancestor(s) of *P. hispanica* and to try to shed  
91 light on its puzzling genetic structure, without forgetting that even the paternal ancestor  
92 of *P. masettii* was actually unknown.

93 Recently, a new *Pijnackeria* species, *P. recondita* Valero and Ortiz, 2015 was found in  
94 a very small area of the Sierra Nevada, right at the border of *P. hispanica* southernmost  
95 range (Fig. 2; Valero and Ortiz, 2015). Its general morphology differs from *P. hispanica*  
96 only in the lower amount of body granulation and chorionic egg sculpturing. All other  
97 morphological and morphometric characters are shared with the tetraploid hybrid,  
98 including a very similar structure and number of antennae articles (Scali et al. 2013). All  
99 these features point to the possibility to consider *P. recondita* as a candidate paternal  
100 ancestor of *P. hispanica*. We therefore decided to test the supposed paternal role of *P.*  
101 *recondita* by investigating its chromosome set—which obviously had to be congruent

102 with the tetraploid karyotype—and, more importantly, by comparing the *cox2* and the  
103 *efl- $\alpha$*  gene sequences of the newly described species with those of *P. hispanica*.

104

## 105 **Material and Methods**

106

107 On the second half of July 2016, 14 adult specimens (8 males and 6 females) were  
108 found in the tiny area of the Sierra Nevada, 2,000 meters a.s.l., as precisely indicated by  
109 Valero and Ortiz (2015) (Fig. 2). Owing to the very small size of the population, four  
110 males and two females were released on the spot to keep the population as steady as  
111 possible. At collection, few specimens were found on the common broom, *Cytisus*  
112 (*Sarothamnus*) *scoparius*, but most of them were caught resting on, or actually eating, a  
113 different leguminous plant, here tentatively referred to as *Cytisus sp.* (Fig. 3); no  
114 *Dorycnium pentaphyllum* was recorded in the collecting area, although it was easily  
115 accepted as lab feed, as stated by Valero and Ortiz (2015). The insects were therefore  
116 kept on their original food plants added with *D. pentaphyllum*, until their utilization for  
117 cytogenetic and molecular analyses.

118 During the same sampling campaign, two adult females of *P. hispanica*, also feeding on  
119 *Cytisus sp.*, were collected 15 kilometers away, along the route to El Purche, about 2  
120 Km from the A395 junction.

121 Chromosome plates of *P. recondita* were obtained from anaesthetized specimens by  
122 manual dissection of the gonads soaked in Ringer solution for insects. After a short  
123 hypotonic shock (5-10 min), testes or ovariole tips were put in an 1% sodium citrate  
124 solution, fixed for 30 min in a simplified Carnoy solution (3:1, absolute ethanol:acetic  
125 acid), and then gently pinched in drops of 45% acetic acid and dried on a hot plate (60°  
126 C). Finally, a post-fixation treatment with the same fixative was applied to the slides  
127 and then the Giemsa staining was performed. Dry stained slides were eventually  
128 mounted in some drops of Canadian balm. Later on, chromosome observations were  
129 carried out with a Zeiss photomicroscope, which also allowed picture recording on  
130 Ilford film or direct recording from the microscope camera.

131 Total genomic DNA was obtained according to the method described in Preiss *et al.*  
132 (1988). Total RNA was obtained with TRIzol reagent (ThermoFisher) according to  
133 manufacturer instructions, then the cDNA was reverse transcribed as indicated in  
134 Ghiselli *et al.* (2007). The partial sequences of mitochondrial gene *cox2* and of the  
135 nuclear gene *efl- $\alpha$*  were PCR-amplified as described in Ghiselli *et al.* (2007). Obtained

136 PCR product were purified using the Wizard PCR Preps DNA Purification System  
137 (Promega), and Sanger-sequenced at Macrogen Europe Lab.  
138 Sequence chromatograms checking and multiple sequence alignments with ClustalW  
139 algorithm were carried out using Mega v.7 (Kumar et al. 2016). New sequences were  
140 elaborated together with previously obtained ones (Fig. 4; Table 1 and Table 2;  
141 Supplementary material 1-3 for Genbank accession numbers and sequences) in order to  
142 get a more comprehensive analysis.  
143 Maximum Likelihood tree searches were performed with RAxML v. 8.2 (Stamatakis  
144 2014), using the GTR+G substitution model and 500 rapid bootstrap replicates for both  
145 genes. Bayesian inferences were conducted with Mr Bayes v3.2.6 (Ronquist et al.  
146 2012): two runs were launched, each with 1,000,000 generations, sampled every 500<sup>th</sup>  
147 generation, and using the GTR+G substitution model. Convergence was assessed  
148 through the variance of split frequencies (<0.01), PSRF (=1.00) and ESS (>200). Age  
149 estimates of cladogenetic event were calculated using a bayesian framework with  
150 BEAST v. 1.8 (Drummond and Rambaut, 2007) on the *cox2* dataset. Two independent  
151 searches were run, each 10,000,000 generations long, sampled every 1,000<sup>th</sup> generation,  
152 and using the GTR+G substitution model. Convergence was assessed through ESS  
153 values >200. Following Mantovani et al., 2000, time calibration was set to the split  
154 between *Bacillus rossius tripolitanus* and *B. rossius rossius/B. rossius redtenbacheri*:  
155 the separation of this two clades would date back to the end of Messinian salinity crisis,  
156 when the Mediterranean basin was filled up, separating North Africa, hosting *B. r.*  
157 *tripolitanus* only, and Southern Italy, where only *B. rossius rossius/B. rossius*  
158 *redtenbacheri* can be found. Calibration time was, therefore, set to  $5.33 \pm 0.5$  Myr ago  
159 and implemented with a normal distribution. Searches were run with an uncorrelated,  
160 log-normal relaxed molecular clock and the birth-death speciation process. Haplotype  
161 parsimony networks were calculated through TCS v. 1.21 (Clement et al. 2000).

## 162 163 **Results**

### 164 165 *Karyotype analysis*

166  
167 The chromosome set of *P. recondita* fully matched to expectations, for both number and  
168 structure, being  $2n=37,X0$  male /  $38,XX$  female (Fig. 5), and showing similarities to the  
169 *P. hispanica* quartets (Fig. 6). The main features of *P. recondita* karyotype are three

170 large pairs of metacentrics (1, 2, 4), and a smoothly decreasing series of acro- and  
171 subacrocentric pairs; the last four pairs, owing to their minute size, could also be  
172 envisaged as small metacentrics.  
173 The *P. recondita* karyotype shows an overall good correspondence with the *P.*  
174 *hispanica* chromosome set (Figs 5, 6), the main differences being the different  
175 centromere position in the 4<sup>th</sup> and 6<sup>th</sup> pairs of the former when compared to the  
176 corresponding quartets of the latter. It could also be noted that the first and 13<sup>th</sup> quartets  
177 have two chromosomes bearing small satellites, lacking in the corresponding positions  
178 of *P. recondita*, which, in turn, presents satellites on the 2<sup>nd</sup> and 4<sup>th</sup> pairs.

179 The peculiar features of *P. masettii* (3n = 57) can be summarized as follows: *P. masettii*  
180 is a triploid hybrid with one chromosome set derived from *P. lelongi* and the other two  
181 from an unknown heterospecific paternal ancestor, as both the structure of several  
182 chromosome triplets and the cytological satellite features clearly support (Fig. 6, triplets  
183 1-4, 6, 12, 17-19) (Ghiselli et al. 2007; Scali et al. 2013). Its link to *P. hispanica* will be  
184 commented in the Discussion section.

185

#### 186 *Molecular analysis*

187

188 Maximum Likelihood and Bayesian inference phylogenetic analyses carried out on the  
189 *cox2* mitochondrial gene are congruent (Fig. 7a) and cluster *Pijnackeria* and *Leptynia*  
190 species in a monophyletic clade (bootstrap = 64%; posterior probability = 0.99).  
191 However, relationships within this clade are not fully resolved. Overall, taxa are split in  
192 a polytomy where it is possible to recognize four well-supported clades: *i*) the *L.*  
193 *annaepaulae* clade, *ii*) a clade including *L. attenuata*, *L. caprai*, and *L. montana*, *iii*) the  
194 *P. recondita* clade, which embodies also the newly obtained sequences, and *iv*) a cluster  
195 comprising *P. lucianae* in sister relationship with the group of the remaining  
196 *Pijnackeria* species. In this latter group, *P. masettii* and *P. lelongi* cluster together with  
197 high support; on the other hand, the cluster including *P. hispanica* and *P. originis* is  
198 weakly supported (bootstrap = 71%; posterior probability < 0.9). The same analyses  
199 performed on the *ef-1 $\alpha$*  nuclear gene are congruent as well (Fig. 7b), and place the *P.*  
200 *recondita* clade in sister relationship with the remaining *Pijnackeria* species. *P.*  
201 *hispanica* and *P. originis* are not included in the same cluster, while three *P. masettii ef-*  
202 *1 $\alpha$*  sequences cluster with that of *P. lelongi*.



203 Parsimony network on *Pijnackeria cox2* sequences (Fig. 8) is consistent with the  
204 phylogenetic analyses. Three separate networks can be observed: i) one formed by *P.*  
205 *recondita* haplotypes, ii) one made by *P. lucianae* haplotypes, and iii) another one  
206 including sequences from *P. barbarae*, *P. lelongi*+*P. masettii*, and *P. hispanica*+*P.*  
207 *originis*.

208 The parsimony network of *ef-1α* (Fig. 9), though, shows a quite different pattern. The *P.*  
209 *hispanica* subnetwork is connected with two different subnetworks, one including *P.*  
210 *barbarae* and one including *P. masettii* and *P. lelongi*. *P. recondita*, and *P. originis* are  
211 included in two different networks. Interestingly, when the network connection limit is  
212 relaxed (< 90%), the three networks become connected and *P. recondita* appears more  
213 related to the *P. hispanica* sub-network. On the other hand, the *P. originis* sub-network  
214 results connected to that of *P. masettii* (Fig. 9).

215 The Bayesian time tree analysis (Supplementary Figure 1) produced a tree topology that  
216 is fully compatible with that obtained through Maximum Likelihood and Bayesian  
217 inference analyses. Age estimates of the main *Pijnackeria* clades are included between  
218 0.14 Mya (*P. barbarae*) and 3.25 Mya (*P. lucianae*) (Table 3; Supplementary Figure 1).  
219 The *P. hispanica*+*P. originis* clade resulted to be 1.96 Myr old and diverged from the  
220 sister clade (*P. masettii*+*P. lelongii*) 3.31 Mya (Supplementary Figure 1). The  
221 divergence of the *Leptynia-Pijnackeria* clade dates back to 29.73 Mya (Table 3;  
222 Supplementary Figure 1)

223

## 224 Discussion

225

### 226 *The origin of P. hispanica genome*

227

228 Our results suggest a quite complex scenario for the composition of *P. hispanica*  
229 genome and the possible role of *P. recondita* as a fathering species.

230 From a chromosome analysis standpoint, the karyotypes of *P. recondita* and *P.*  
231 *hispanica* are highly similar, especially the relative size and centromere positioning of  
232 most chromosomes. However, there are also differences such as the centromere position  
233 in the 4<sup>th</sup> and 6<sup>th</sup> pairs, and the position of cytological satellites. The karyotype of *P.*  
234 *recondita* shows the same basic haploid set of 19 elements consistently found in all  
235 other diploid species of the genus (Scali, 2009a; Scali et al. 2013), and also keeps the  
236 metacentric X chromosome as the largest. This finding clearly follows from the male

237 mitotic divisions which, being the male sex-chromosome formula X0, allow the  
238 identification of the unique X chromosome: therefore, we could reasonably assign the  
239 same role of sex-chromosomes to the first quartet in the karyotype of the  
240 parthenogenetic *P. hispanica*.  
241 The combined analysis of *cox2* mitochondrial sequences and of *efl-a* nuclear genes  
242 suggests a quite intriguing origin of *P. hispanica* hybrid genome. *P. originis* and *P.*  
243 *hispanica* *cox2* haplotypes always cluster together, indicating a clear genetic  
244 homogeneity. Therefore, in line with previous analyses (Ghiselli et al. 2007), the  
245 maternal ancestor would have been *P. originis*, as *P. hispanica* clearly incorporates its  
246 mitochondrial marker. On the other hand, the paternal contributions are less clear.  
247 Based on the *efl-a* phylogenetic and network analyses, *P. hispanica* is more related to  
248 *P. barbarae*, *P. masettii*, and *P. lelongi* than to *P. recondita* and *P. originis*. However,  
249 less stringent parameters for network construction allowed the connection of *P.*  
250 *recondita* and *P. hispanica* sub-networks. This suggests a common ancestry of the two  
251 nuclear sequences, but it also indicates a wide differentiation between *P. recondita* and  
252 *P. hispanica*. The time tree analysis clearly supports such distant relationship, dating the  
253 divergence of mitochondrial *cox2* sequences back to the Mid-Oligocene (29.73 Mya).  
254 Overall, the results are not in contrast with a possible contribution of *P. recondita* to the  
255 genome of *P. hispanica*, although the level of genetic divergence observed in both  
256 mitochondrial and nuclear markers would suggest caution. In summary, we can say that  
257 the data are compatible with the hypothesis of an ancestral contribution followed by  
258 diversification. It is worth noting that the *P. recondita* and the *P. hispanica* subnetworks  
259 are connected through the sample from El Purche (PUR), which has been collected very  
260 close to the distribution area of *P. recondita* (Sierra Nevada, SNE) (Fig. 8). Therefore, it  
261 is possible that widening the sampling in the area of sympatry would shed light on this  
262 issue.  
263 *P. hispanica* is a very unusual hybrid since it incorporates the mitochondrial marker of  
264 *P. originis* but it includes the nuclear sequences of different species. The tetraploid  
265 constitution of *P. hispanica* genome and the close similarity of chromosome sets shared  
266 by all *Pijnackeria* taxa suggest the possibility of polyploidization. On the other hand,  
267 some minute differences among specific chromosome complements indicate a  
268 heterogeneous structure, supporting a complex chromosome set with heterospecific  
269 genomes. Among these, at least one of *P. recondita* origin. However, further inspection  
270 of *P. hispanica* karyotype with heteromorphic quartets points to just a double *P.*

271 *recondita* chromosome set contribution—also in view of a diploid structure of the initial  
272 hybrid—with two paternal chromosome sets derived each from the unknown paternal  
273 ancestor of *P. masettii* and *P. barbarae*. Overall, *P. hispanica* genetic structure points to  
274 a complex, unusual derivation.

275 The occurrence of a mitochondrial genome from one species and the nuclear genome  
276 from a different species inherited by an individual of a third species was observed for  
277 the first time through allozyme analysis and cytological investigations of both field-  
278 collected and lab-reared specimens of the hybridogenetic *Bacillus rossius-grandii*  
279 strains (Mantovani and Scali, 1992; Tinti and Scali, 1996), thus providing clear  
280 evidence for androgenesis in stick insects. Afterwards, natural androgenesis was also  
281 discovered in several species of the freshwater clam *Corbicula* (Komaru et al. 1998;  
282 Byrne et al. 2000; Qiu et al. 2001) and in the cypress tree *Cupressus dupreziana* (Pichot  
283 et al. 2001). Our analysis with a mitochondrial gene and a single nuclear marker cannot  
284 be conclusive but, all considered, it is reasonable to suggest an androgenetic origin also  
285 for *P. hispanica*, which could date back to between 2 Mya (the estimated age of the  
286 clade) and 3.3 Mya (the estimate split age from the closest relative).

287 Because of the clonal structure of parthenogenetic taxa, with consequent loss of genetic  
288 variability, the causes and consequences of their longevity are debated (see Bell, 1982;  
289 Wrijenhoek and Lerman, 1982; Wrijenhoek, 1998; Normark et al. 2003). Data on stick  
290 insects obtained so far indicated that *Bacillus* hybrid taxa originated around 1 Mya  
291 (Mantovani et al. 2001), whereas parthenogenetic *Timema* lineages have evolved  
292 between 500,000 and 2 Mya (Schwander et al. 2011). In this view, *P. hispanica* may  
293 represent the oldest parthenogenetic taxon discovered so far among insects.

294

### 295 *The Pijnackeria karyotype and the evolution of egg maturation modes*

296

297 A great stability for chromosome number and structure within a cluster of related  
298 species is quite a rare finding among Phasmida: all species-rich genera investigated so  
299 far actually showed a marked karyotype plasticity for both number and structure of  
300 chromosomes, particularly when parthenogens are involved. Striking instances of this  
301 feature are the Australian *Didymuria* (Craddock, 1972, 1975) and *Sipyloidea* (John et al.  
302 1987), as well as the circum-Mediterranean *Bacillus* and *Clonopsis* (Scali, 2009b;  
303 Milani et al. 2010). Also the Iberian genus *Leptynia*—defined on both morphology and  
304 genetic basis, and encompassing only four species—revealed diploid chromosome sets

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305 ranging from 36 to 40, and a very significant structural variation, even entailing the sex-  
306 chromosome formula with a shift from the usual XX/X0, to the rarer XX/XY one (Scali,  
307 2009c; Scali et al. 2016). In addition, the karyotype stability of *Pijnackeria* species is  
308 mirrored by the number and localization of cytological NOR-bearing satellites (Ghiselli  
309 et al. 2007; Scali et al. 2016): taking into account the tens of specimens analyzed  
310 belonging to the seven *Pijnackeria* species, we were able to score 10 different satellite  
311 positions, but each species possesses a maximum of two different locations. This is a  
312 quite different scenario from that observed for NOR-bearing satellites of *Bacillus*,  
313 where a single species may encompass as much as 12 different locations for cytological  
314 satellites (Manaresi et al. 1991, 1992, 1993; Salvadori et al. 2018; Scali et al.  
315 submitted). Frequent changes in location and number of satellites are a common finding  
316 in stick insects, and it has been suggested that they could be an outcome of transposon  
317 activity (Meyne et al. 1990; Zhdanova et al. 2007; Ruiz Herrera et al. 2008; Ocalewicz,  
318 2013; Satovic et al. 2016). For example, R2 non-LTR transposons have been reported to  
319 be active in *Bacillus* species and even particularly prone to accumulate in  
320 parthenogenetic taxa (Bonandin et al. 2014, 2017; Scavariello et al. 2017).

321 The cytological satellite features are not just a trait of inter- and intra-specific  
322 variability, since in all investigated phasmatodean species—13 all together up to now—  
323 chromosomal satellites have been always found to be sites of highly enriched and co-  
324 localized rDNA/telomeric sequences (Scali et al. 2016; Liher et al. 2017; Salvadori et al.  
325 2018): therefore, this trait appears to have a biological and evolutionary bearing.

326 Actually, it has been already possible to observe that in the di-hybrid *Bacillus whitei*  
327 and the three-hybrid *B. lynceorum*, active NORs derive from all ancestors, although that  
328 of maternal *B. rossius* derivation appears as the most conserved (Manaresi et al.  
329 1991, 1992, 1993). However, owing to the androgenetic structure of *P. hispanica*, such a  
330 feature cannot be verified.

331 A careful karyotype analysis of *Pijnackeria* polyploids gives us some indirect clues  
332 about their egg maturation mechanisms, since a direct investigation has not been  
333 possible. In stick insects, egg meiosis is blocked at pachytene during the first instar  
334 larva, to be resumed in adults at laying. Consequently, eggs can be collected at precisely  
335 scheduled times and investigated. Unfortunately, in *Pijnackeria* such a direct analysis of  
336 oocyte maturation is not feasible, because the few laid eggs are firmly glued to the  
337 substratum and their chorionic capsule is too fragile to be handled and cut for fixation as  
338 it has been done in *Bacillus* and *Clonopsis* (Marescalchi et al. 1991; Scali et al. 2010).

339 Owing to such limitations, we can propose only conjectural hypotheses for *P. hispanica*,  
340 and considering its complex hybrid structure, an apomictic mechanism maintaining a  
341 steady karyotype structure appears the most likely. In this connection, we can observe  
342 that *P. masettii* is a triploid hybrid with one chromosome set derived from *P. lelongi*  
343 and the other two from an unknown heterospecific paternal ancestor, as both the  
344 structure of several chromosome triplets and the cytological satellite features clearly  
345 support (Fig. 6, triplets 1-4, 6, 12, 17-19; see also Ghiselli et al. 2007; Scali et al. 2013).  
346 At the time of its formation, such a hybrid certainly suffered from a diffuse imbalance in  
347 pairing and segregation of the trivalents during the first meiotic division, as actually  
348 observed in triploid specimens of *Bacillus atticus* (Marescalchi and Scali 1997, 2003);  
349 therefore, a modified meiosis escaping such constraints is likely to have been evolved  
350 by selecting an apomictic egg-maturation mechanism. A similar meiotic pathway could  
351 have been evolved in the tetraploid *P. hispanica* androgenetic parthenogen with a high  
352 (76) chromosome number.

353 Gathering all relevant observations on the issue, the most parsimonious series of  
354 gains/losses of whole chromosome sets leading to the extant structure of *P. hispanica*  
355 can be envisaged as follows: a seminal parthenogenetic *P. originis*/*P. recondita* hybrid  
356 with an apomictic reproduction owing to the marked genetic differentiation of the  
357 parental taxa was produced. Pre-mating isolating mechanisms were easily overcome,  
358 since in phasmids they are rather ineffective even between utterly differentiated species  
359 (Scali et al. 1995). Back-crosses to *P. recondita* males were still possible (see Tinti and  
360 Scali, 1996) and, thanks to the physiological egg-polyspermy (Scali, 1972), an all-  
361 paternal progeny was originated when syngamy with the hybrid egg nucleus failed and  
362 two spermatozoa fused to originate a 2n androgen, which only kept the mitochondrial  
363 DNA of the mother but continued an apomictic reproduction (Mantovani and Scali,  
364 1992). The 4n ploidy of *P. hispanica* could then be reached through a two-step  
365 acquisition of additional *Pijnackeria* genomes by the androgen. After the original  
366 hybridization of *P. recondita* with *P. originis* leading to an early diploid androgen, an  
367 additional fathering taxon, providing the third haploset, should have been different from  
368 *P. recondita* and likely similar to the unknown paternal ancestor of *P. masettii*. The last  
369 contribution of a fourth genome could have been provided by a *P. barbarae*-like  
370 paternal ancestor: the heterozygous structure of several quartets of *P. hispanica* (Fig. 6)  
371 and the high variability of its *efl-α* sequences (Fig. 9) are consistent with the above  
372 outlined assumptions. Being these correct, *P. hispanica* could be then envisaged as a

1 373 polyphyletic hybrid-complex endowed with a high colonizing potential. Moreover, it is  
2 374 tempting to speculate that such kind of multi-hybrid origin could be the reason behind  
3 375 the long evolutionary persistence (1.96-3.31 Myr) of this parthenogenetic taxon. The  
4 376 high variability produced by multiple hybridizations events might have compensated for  
5 377 the absence of sexual recombination (Ghiselli et al 2007 and references therein).  
6  
7 378 On the issue, we would like mentioning that androgenetic stick-insect strains of *Bacillus*  
8  
9 379 and *Clonopsis* contributed to the formation of a complex network among parental and  
10  
11 380 derived taxa, so that their reproductive and micro-evolutionary features were defined as  
12  
13 381 “reticulate evolution” (reviewed in Scali, 2009a), and, within it, androgenesis has been  
14  
15 382 proposed as a short-cut pathway for speciation (Ghiselli et al. 2007; Milani et al. 2010;  
16  
17 383 2015). To better envisage the cladogenetic potential of androgenesis, a simple model of  
18  
19 384 hybrid eggs maturation and genome transmission has been worked out for *Clonopsis*  
20  
21 385 hybrids, which would even explain the ascertained diploid structure of polyploid  
22  
23 386 karyotypes (Milani et al. 2009; 2010): the *Clonopsis* model also accommodates quite  
24  
25 387 easily the otherwise inexplicable chromosomal findings reported in the Australian  
26  
27 388 *Sipyloidea nelida* species complex by John et al. (1987).  
28  
29 389 On the whole, the targeted cytogenetic insight and transmission analysis of genomes,  
30  
31 390 although rather limited, appears an effective tool to reveal the exploitation of a wide  
32  
33 391 array of reproductive modes and evolutionary pathways in stick insects: these insights  
34  
35 392 seem to really add to the routinely accepted ideas about reproductive features,  
36  
37 393 evolutionary modes and phylogenetic relationships in animals.  
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39 394

#### 40 395 *Taxonomic implications*

41 396  
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43 397 Following the above described scenario of “reticulate” backcrosses, multiple tetraploid  
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45 398 populations arose in different areas of the *Pijnackeria* range, stepwise embodying  
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47 399 additional sets of fathering taxa, some of which can also be missing from the sampling.  
48  
49 400 These 4n populations are now spread and mixed in the region indicated in Figure 2.  
50  
51 401 According to this phylogeographic pattern, *P. hispanica* would then represent the  
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53 402 ensemble of many subpopulations of 4n parthenogenetic androgens in which the  
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55 403 multiple contributions from diverse diploid species can be appreciated in the *efl*-  
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57 404  $\alpha$  network (Fig. 9). Each *P. hispanica* specimen within the different subpopulations  
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59 405 appears to possess a chimerical genetic structure, even more strengthened by the  
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61 406 occurrence of the “foreign” mitochondrial DNA of the maternal ancestor. All this points  
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407 to a composite, polyphyletic structure of the tetraploid hybrid, which we propose to  
408 indicate as an *androgenetic complex*.  
409 Finally, we would like to point out that, if in *cox2* network the recently described Sierra  
410 Nevada (SNE) *P. recondita* taxon does not actually cluster together with the previously  
411 described *Pijnackeria* species (Fig. 7a,b), and Valero and Ortiz (2015) obtained the same  
412 tree topology from *cox1* and *cox 2* analyses. Although this topology does not fully resolve  
413 the relationships within the genus *Pijnackeria*, it clearly indicates a high degree of  
414 differentiation between *P. recondita* and the other conspecific species. Further molecular  
415 investigation may help to shed light on the evolution of this genus and its relationship  
416 with the closely related genus *Leptynia*.

417

418 **Data availability:** The datasets generated during and/or analyzed during the current  
419 study are available in the GenBank repository (see corresponding GenBank numbers in  
420 Supplementary Material 1). The sequences generated during this study are also available  
421 as Supplementary Material 2 and 3 in FASTA format.

422

423 **Conflict of Interest:** The authors declare that they have no conflict of interest.

424

425 **Ethical approval:** All applicable international, national, and/or institutional guidelines  
426 for the care and use of animals were followed.

427

## 428 **Figure Captions**

429

430 **Fig. 1** Specimen of *Pijnackeria hispanica*: the nominal species of the new genus  
431 (corresponding to the originally described *Leptynia hispanica* species by Pantel  
432 1890). Note the very short antennae and the pointed abdomen end peculiar to the taxon.  
433 Additional information and images of this tetraploid parthenogen and of all other  
434 congeneric species are to be found in Scali 2009, Scali et al. 2012, Scali et al.  
435 2013; Valero and Ortiz 2015.

436

437 **Fig. 2** Ranges of *Pijnackeria* taxa. **2n**: **A**, *P. lucianae*; **B**, *P. barbarae*; **C**, *P. lelongi*; **D**,  
438 *P. originis*; **3n**, *P. masettii*; **4n**, *P. hispanica*. Sample acronyms as in Ghiselli *et al*,  
439 2007. The area of *P. recondita* (Sierra Nevada) and *P. hispanica* (El Purche) samples is

440 represented by a single dot south-east of PRA (Puerto La Ragua). Acronyms as in Table  
441 1 and Table 2

442

443 **Fig. 3** *Pijnackeria recondita* food plants: *Cytisus scoparius* on the right and *Cytisus sp.*,  
444 on the left

445

446 **Fig. 4** Geographical distribution of the haplotypes obtained both by sampling and from  
447 literature contributing to the molecular analysis. A) *cox2*. B) *efl-α*. For the exact  
448 coordinates of the sampling sites, refer to Table 1 and Table 2

449

450 **Fig. 5** Karyotype of *Pijnackeria recondita*. The karyotype is very similar to those of all  
451 other *Pijnackeria* species. A) Female: 1st pair, heterochromosomes; the 2nd and  
452 4th pairs bear a heterozygous satellite; B) Male: its unique sex chromosome (X0) allows  
453 to indicate the first female pair as the heterochromosome pair in both *P. recondita* and,  
454 as a consequence, in *P. hispanica*

455

456 **Fig. 6** Karyotype of the triploid *Pijnackeria masettii* (on the top), and of the tetraploid  
457 *Pijnackeria hispanica* (on the bottom) modified from Scali et al. (2013). *P. hispanica*  
458 appears either a 2+2 structure, or, better, a 2+1+1 structure. *P. masettii* triplets 1-4, 6,  
459 12, 17-19 clearly support a 2+1 structure; 1<sup>st</sup>, 2<sup>nd</sup>, 13<sup>th</sup>, 15<sup>th</sup> and 19<sup>th</sup> quartets of *P.*  
460 *hispanica* seem to suggest either a 2+2 structure, or better a 2+1+1 structure

461

462 **Fig. 7** Schematic drawing of Maximum Likelihood/Bayesian Inference on *cox2* (A;  $-\ln L$   
463 = 3431.06/3503.44) and *efl-α* (B;  $-\ln L$  = 1958.30/20072.49) datasets. Number at nodes  
464 are bootstrap/posterior probabilities support values. Outgroup(s) have been omitted for  
465 graphical purposes

466

467 **Fig. 8** Parsimony network of the *cox2* gene sequences. Circles size is proportional to  
468 haplotype frequency; black dots represent missing/ideal haplotypes. Connections  
469 obtained with relaxed parameters are indicated with dashed, grey lines; grey numbers in  
470 parentheses are the number of missing/ideal haplotypes along the connection

471

472 **Fig. 9** Templeton network of the *efl-α* gene sequences. Circles size is proportional to  
473 haplotype frequency; black dots represent missing/ideal haplotypes. Connections



1 474 obtained with relaxed parameters are indicated with dashed, grey lines; grey numbers in  
2 475 parentheses are the number of missing/ideal haplotypes along the connection

3 476

4 477

5 478 **Legends to tables**

6 479

7 480 Table 1. Analyzed species for *cox2*, with collecting place with acronyms and geographic  
8 481 coordinates.

9 482

10 483 Table 2. Analyzed species for *efl- $\alpha$* , with collecting place with acronyms and  
11 484 geographic coordinates.

12 485

13 486 Table 3. Age estimates of the main *Pijnackeria* clades.

14 487

15 488

16 489 **Supplementary material**

17 490

18 491 Supplementary Figure 1. Time calibrated tree obtained on *cox2* gene sequence.

19 492 Numbers on branches represent the posterior probability nodal support; bars at nodes  
20 493 indicate the 95% high posterior density (HPD).

21 494

22 495 Supplementary Material 1. Genbank accession numbers of the analyzed sequences.

23 496

24 497 Supplementary Material 2. *cox2* sequences.

25 498

26 499 Supplementary Material 3. *efl- $\alpha$*  sequences.

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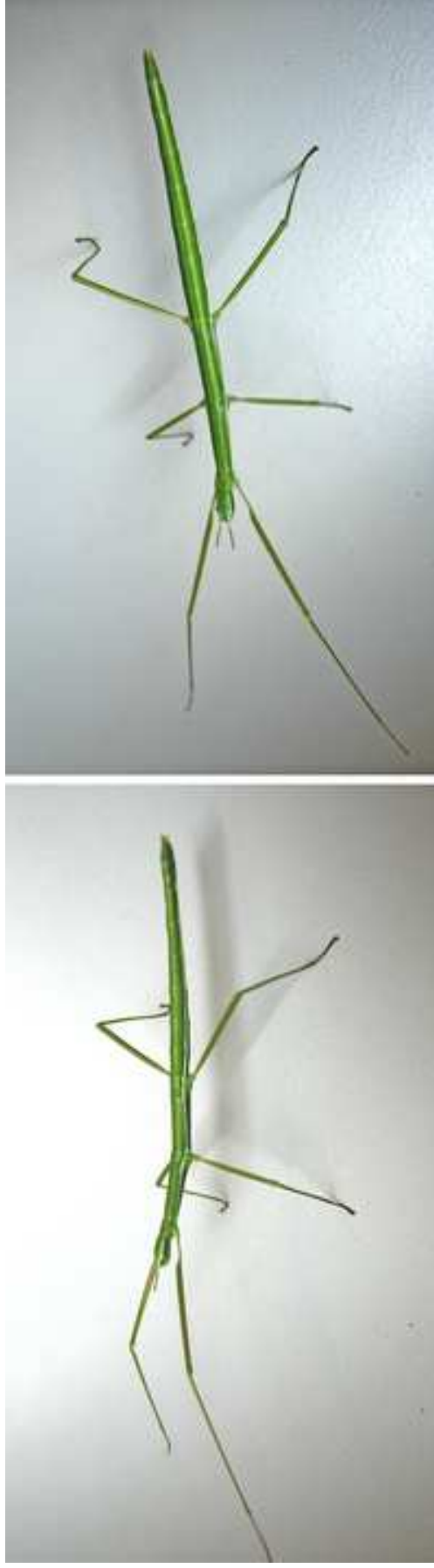
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FIGURE 1

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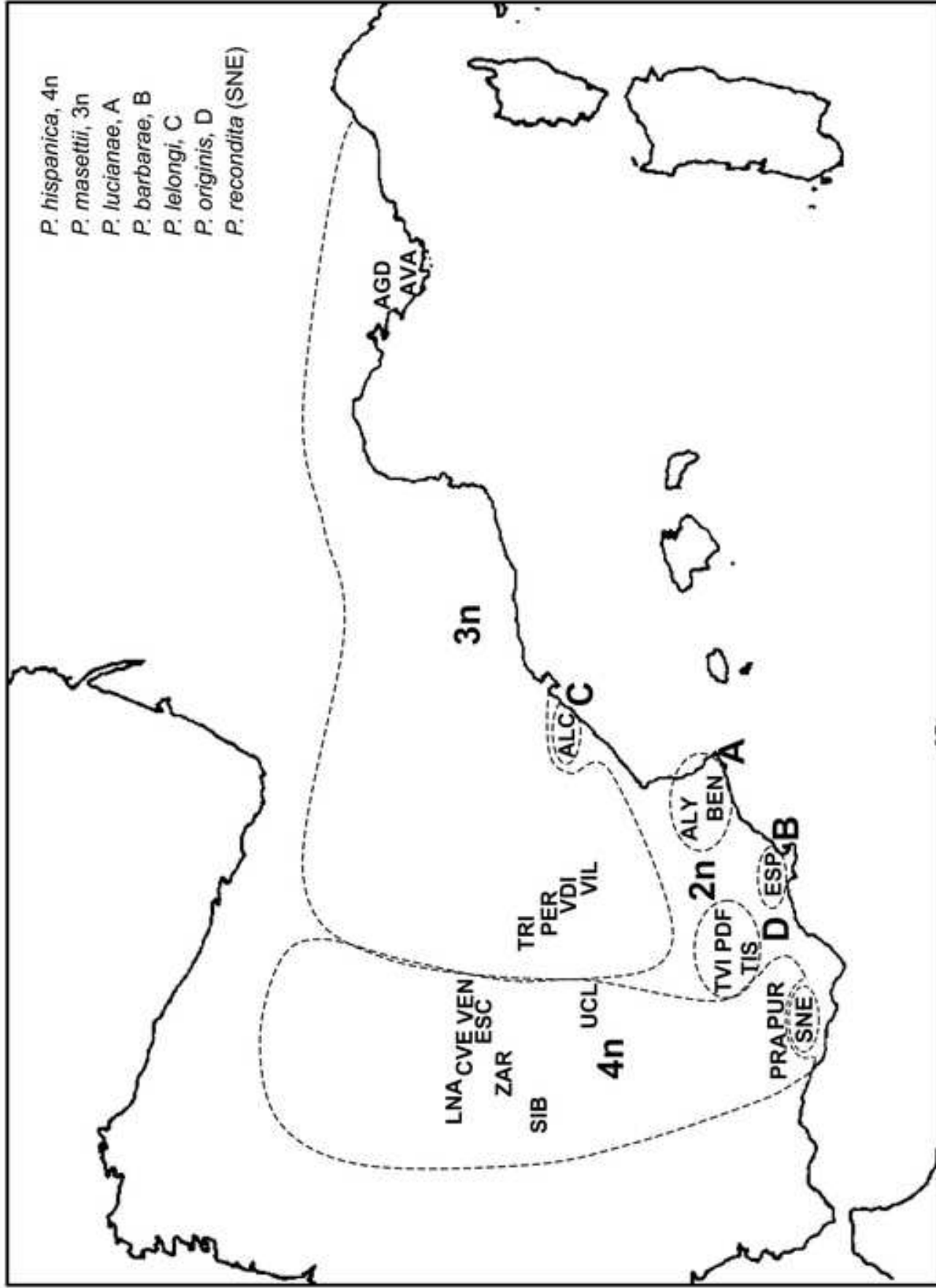


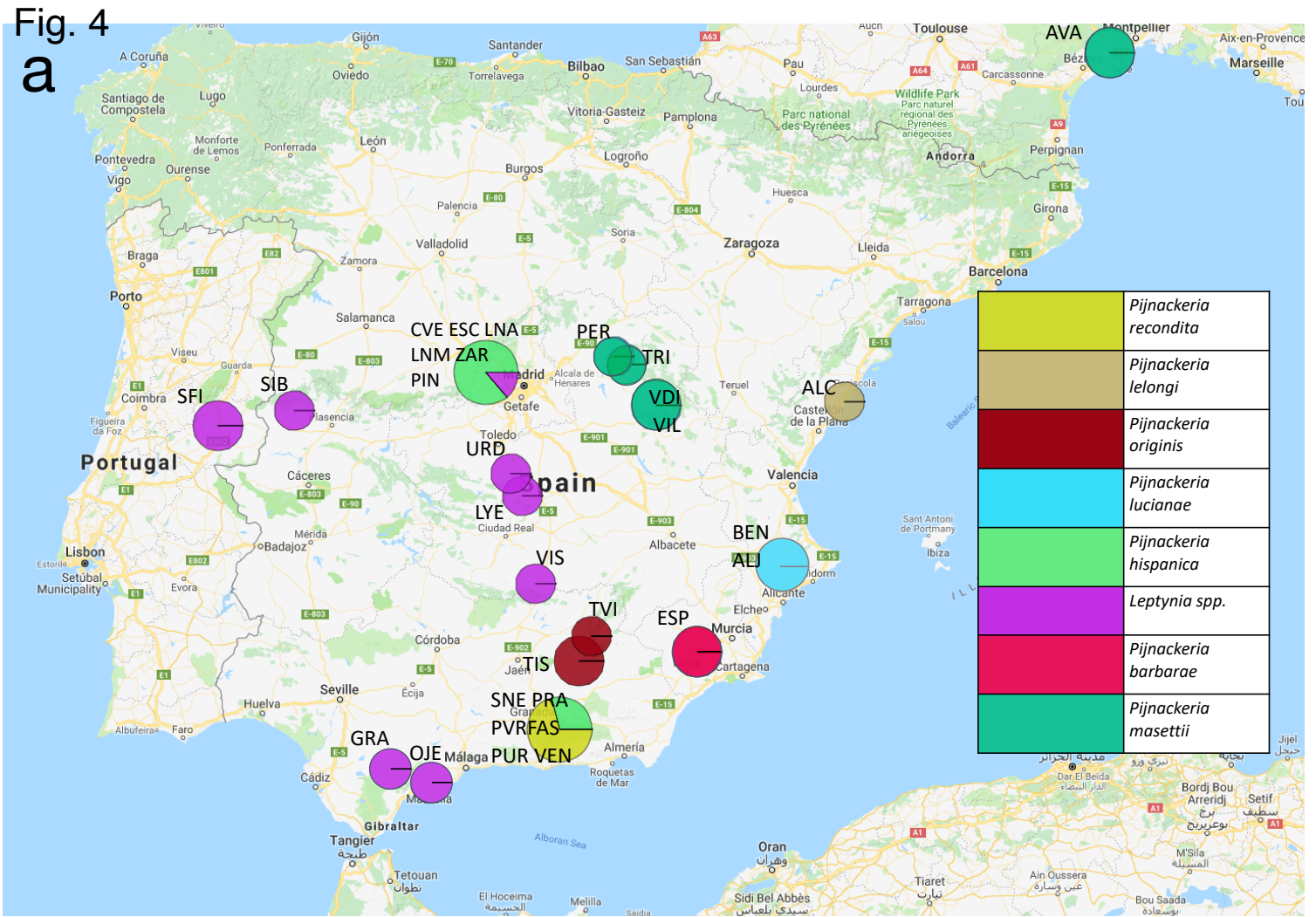
FIGURE 2

FIGURE 3

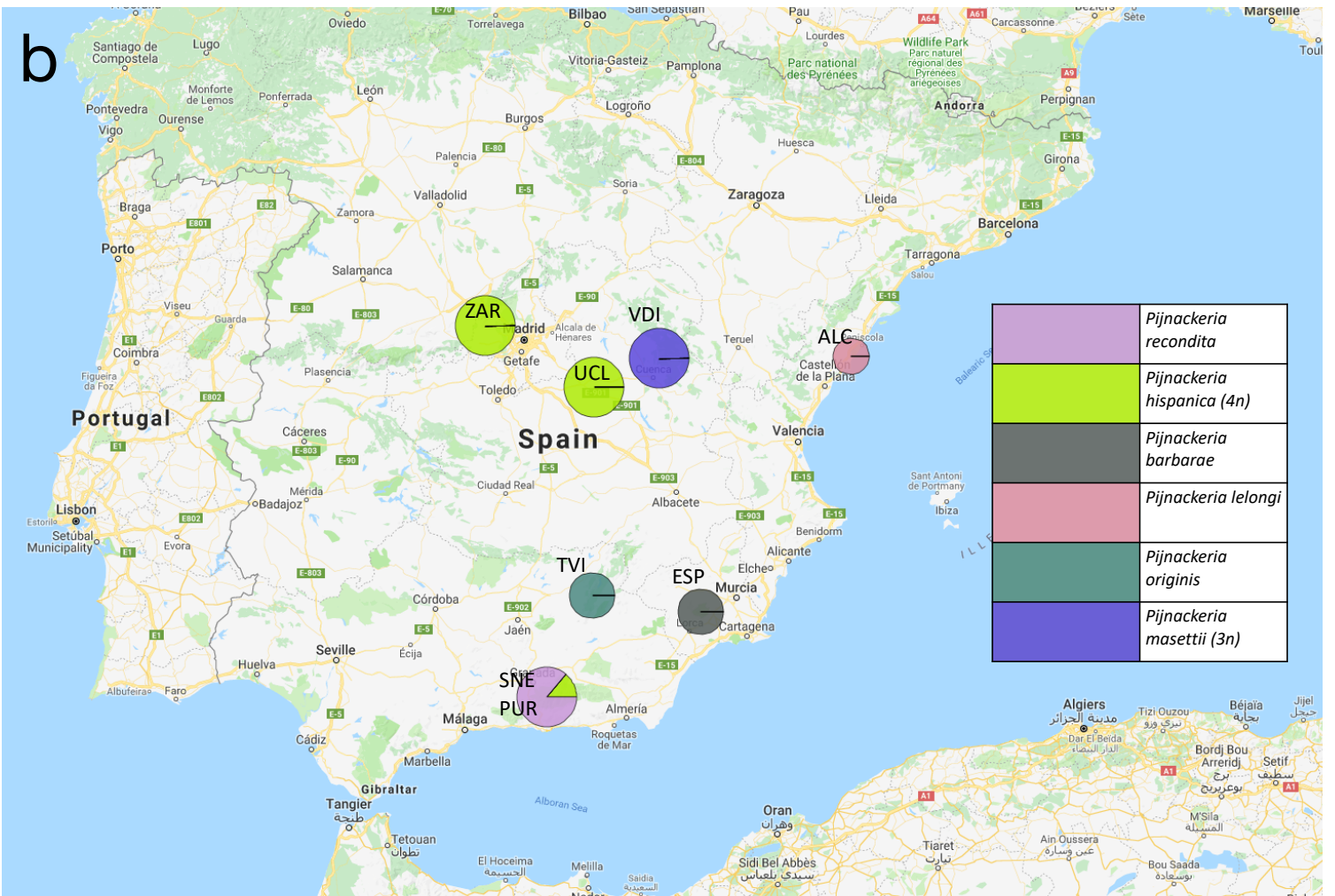
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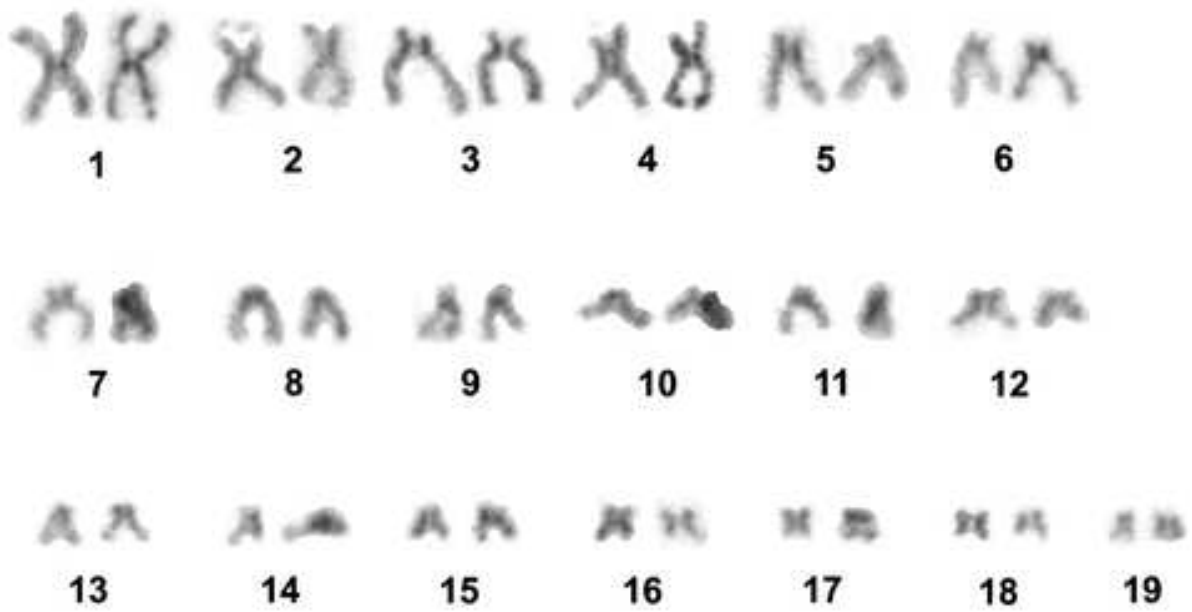
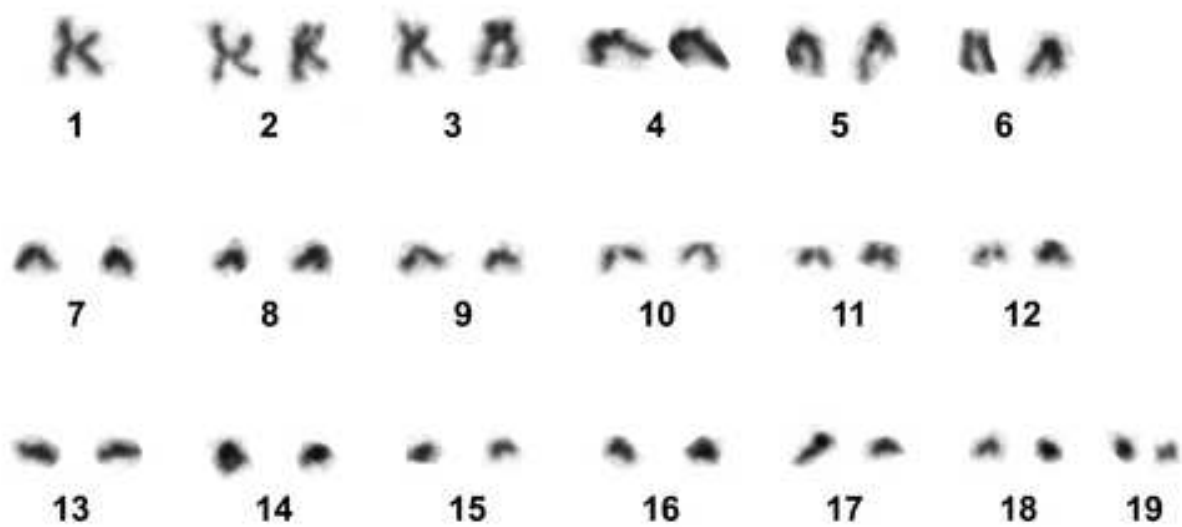


Fig. 4  
a

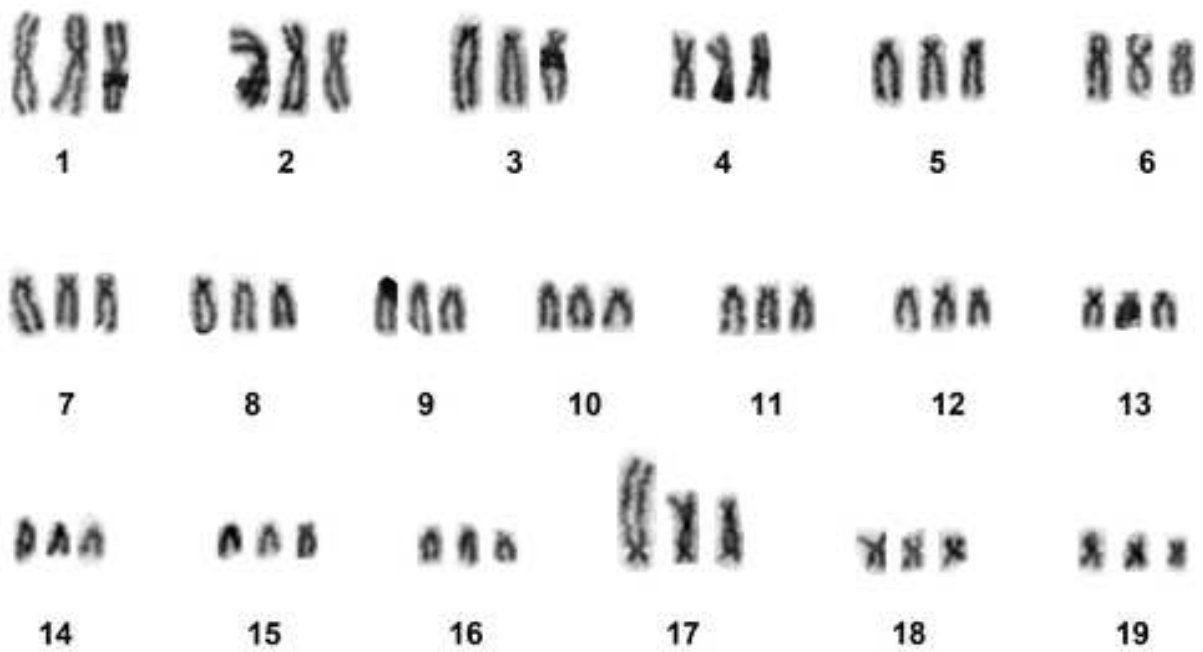


b

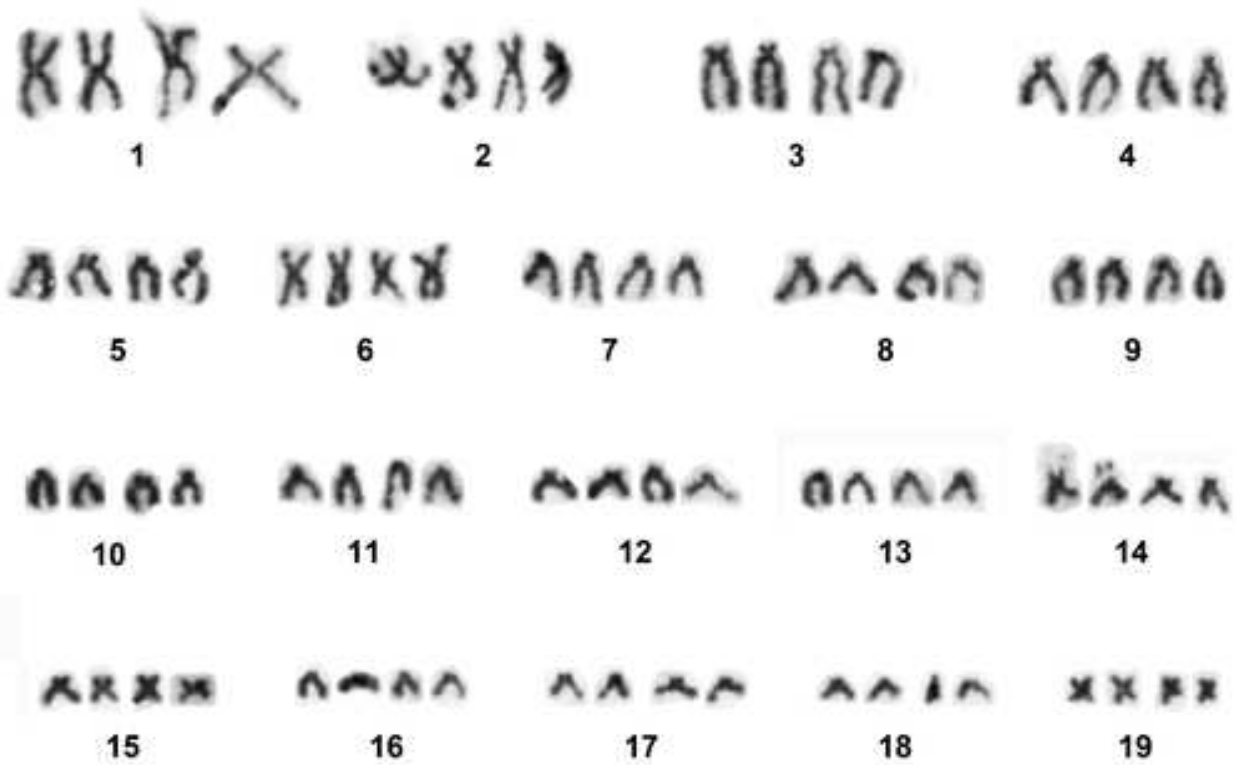


**A****B**

**A**

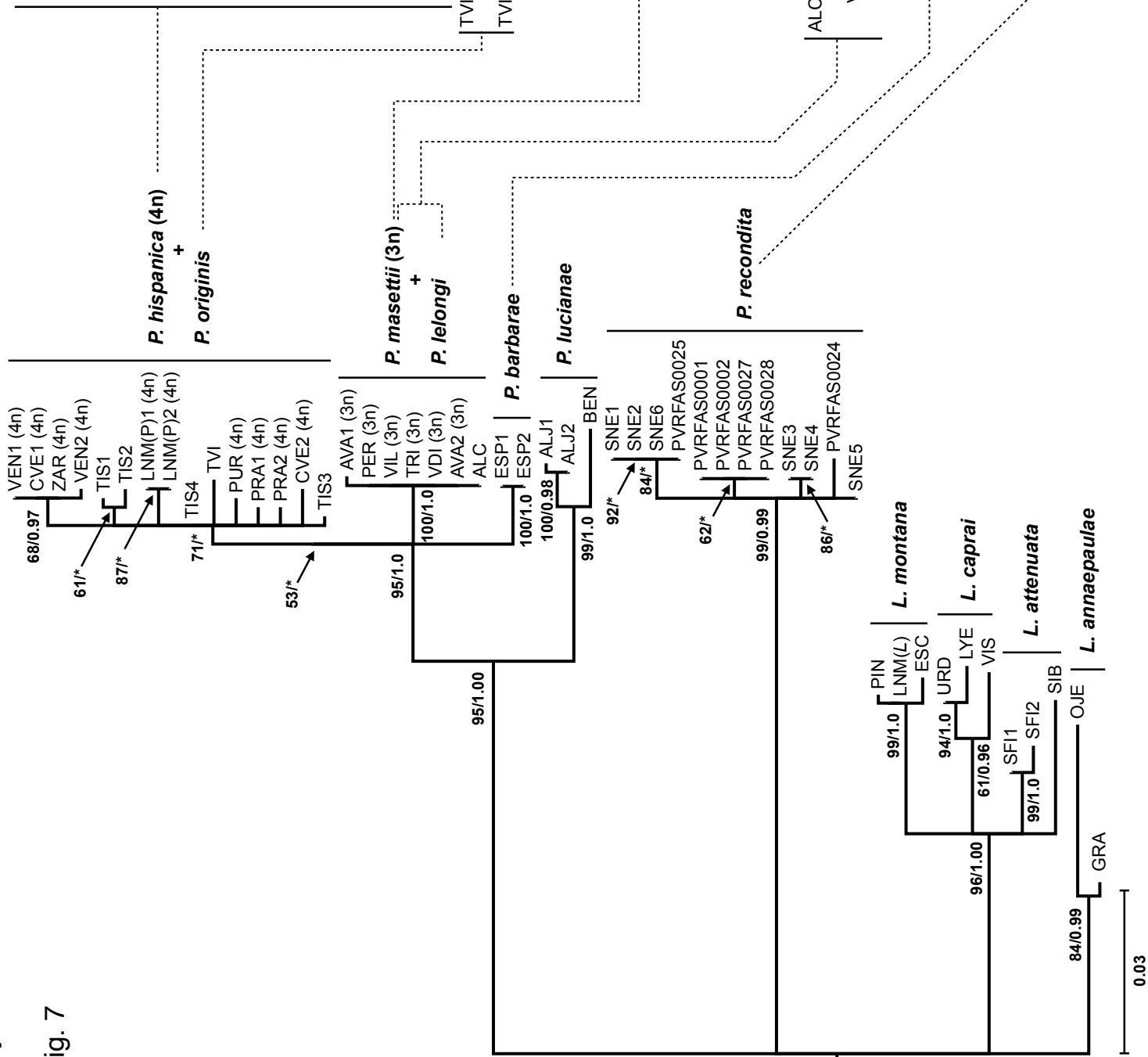


**B**



A)

Fig. 7



B)

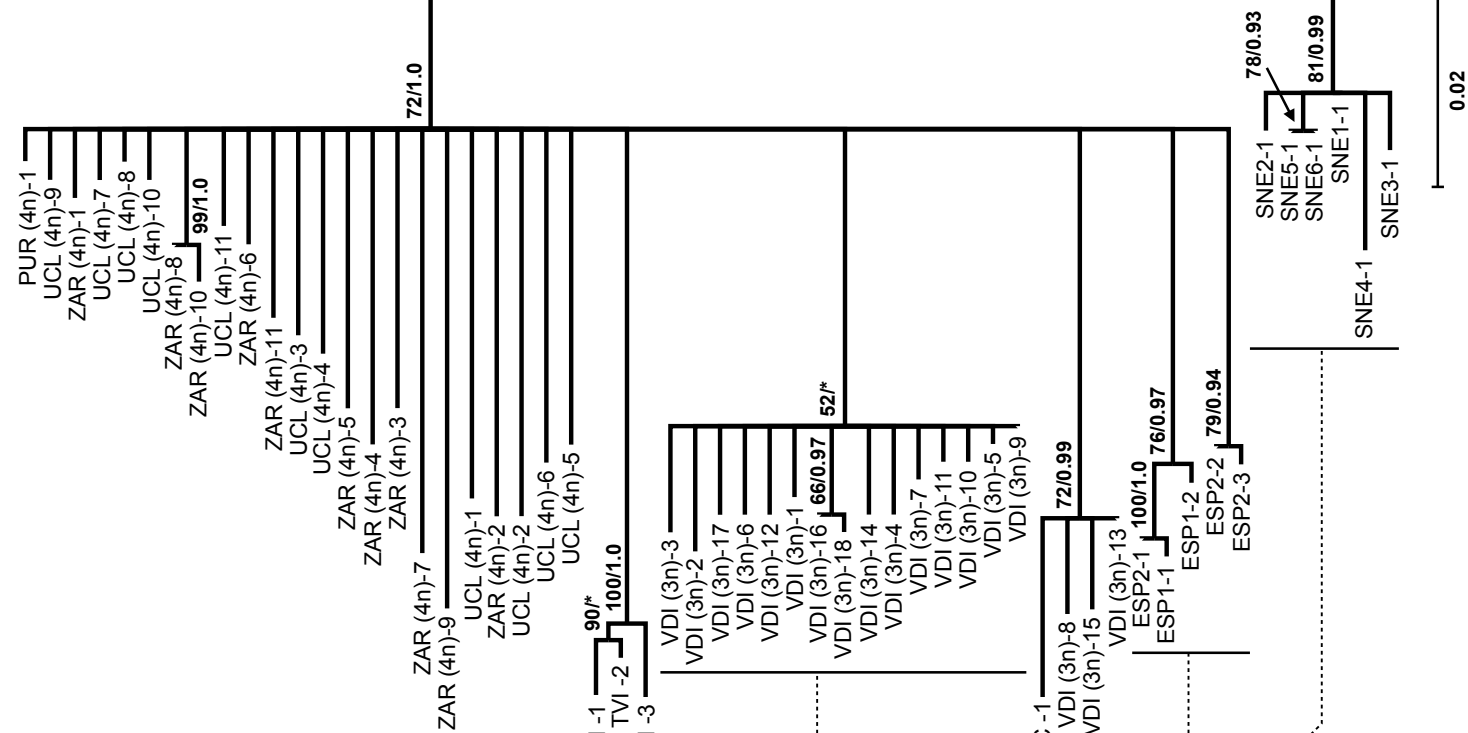


Fig. 8

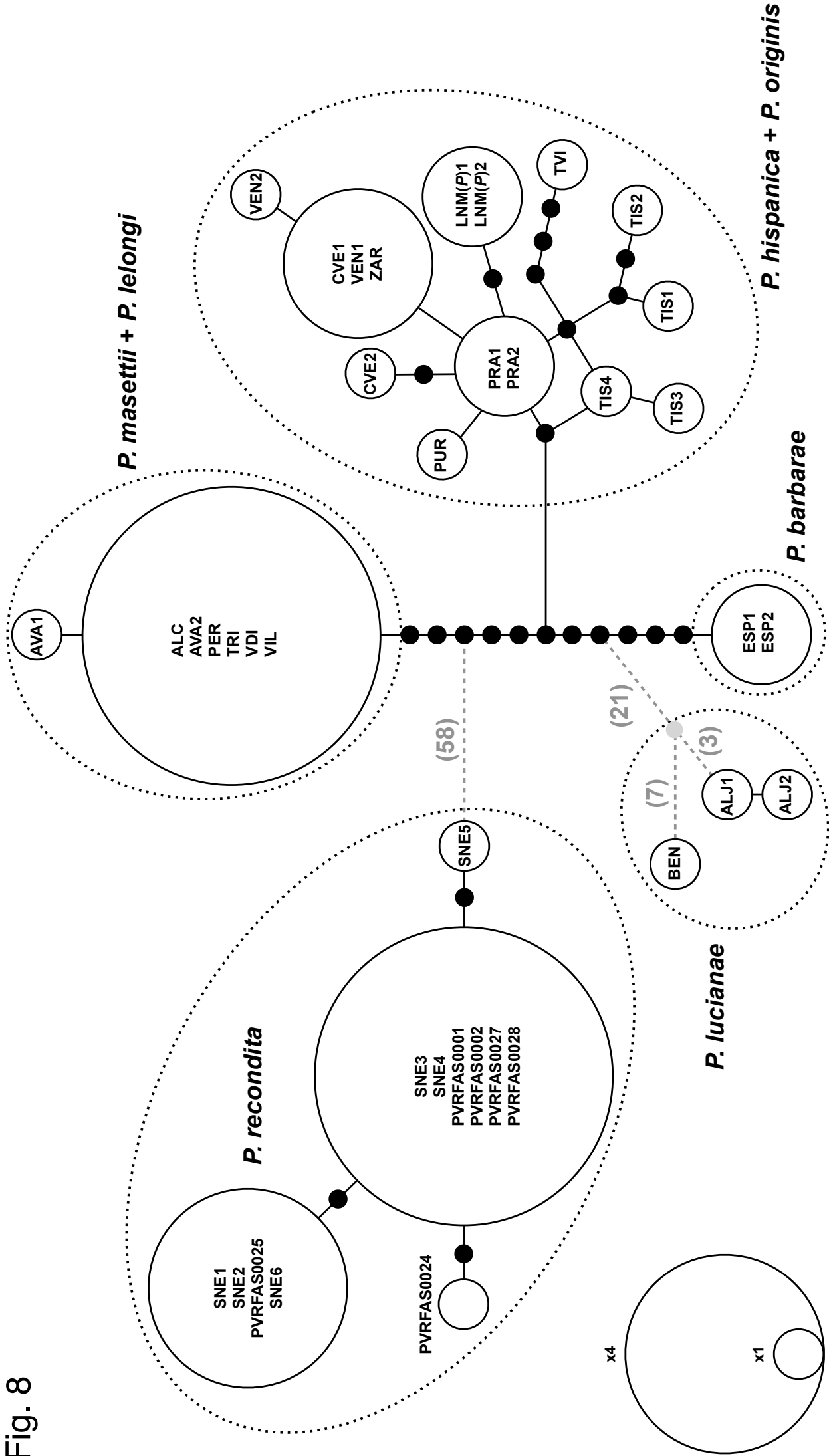


Fig. 9

