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Funding: This work was supported by the Canziani bequest.

Acknowledgements: We would like to thank Luciana Eliopoli for the Sierra Nevada sample collection.

Abstract

The stick-insect genus *Pijnackeria* includes four diploid bisexual and two polyploid (3n, 4n) parthenogenetic species. Earlier analyses of the tetraploid parthenogen *P. hispanica* using mitochondrial markers allowed to trace its maternal ancestry to *Pijnackeria originis*, while no maternal nuclear contribution was found, thus suggesting an androgenetic and hybrid origin. The recently described *Pijnackeria recondita*—showing, among other features, a specific antennal structure linking it to the tetraploid parthenogen—prompted us to check whether the new species could be *P. hispanica* unknown paternal ancestor. In this work we use karyology and of molecular analysis of the mitochondrial gene cytochrome c oxidase subunit 2 (*cox2*), and the nuclear gene elongation factor 1 subunit α (*ef1-α*) to investigate the origin of such a complex tetraploid hybrid parthenogen.

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

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

Introduction

The phasmid genus *Pijnackeria* Scali, 2009 is a Spanish monophyletic taxon embodying four diploid bisexual and two polyploid parthenogenetic species (3n, 4n), all sharing the same haploid chromosome set with n = 19 (Brock 1991, 1993 for reviews; Bianchi, 1992¸ Bianchi and Meliado, 1998; Scali, 2009a; Scali et al. 2013).

P. hispanica (Pantel, 1890) (Fig. 1), the nominal species of the genus, appears to be a very successful tetraploid hybrid parthenogen, ranging from the Sierra Nevada

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

Molecular genetic analyses, carried out using both the mitochondrial gene cytochrome c oxidase subunit 2 (*cox2*) and the nuclear gene elongation factor 1 subunit α (*ef1-α*) as markers, suggested the bisexual diploid *P. lelongi* Scali et al., 2013 as the maternal ancestor of *P. masettii*, and the diploid bisexual *P. originis* Scali et al., 2013 of Tiscar (Sierra de Cazorla) as the maternal ancestor of the tetraploid *P. hispanica* (Ghiselli et al. 2007; Scali, 2009a). On the other hand, quite surprisingly, while *P. masettii* showed the *ef1-α* allele of maternal derivation, as it could be expected, *P. hispanica* did not, so that its *ef1-α* gene ought to be of only paternal derivation (Ghiselli et al. 2007). In order to explain such finding, three different hypotheses were considered: *i)* the effect of gene conversion; *ii)* the outcome of a non-equivalent gene silencing in the hybrid; *iii)* the clear-cut consequence of the maternal genome exclusion within an androgenesis scenario (Ghiselli et al. 2007; Milani et al. 2010, 2013). At any rate, the available data greatly stimulated us to trace the paternal ancestor(s) of *P. hispanica* and to try to shed light on its puzzling genetic structure, without forgetting that even the paternal ancestor of *P. masettii* was actually unknown.

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

with the tetraploid karyotype—and, more importantly, by comparing the *cox2* and the *ef1-α* gene sequences of the newly described species with those of *P. hispanica.*

Material and Methods

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

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

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

Total genomic DNA was obtained according to the method described in Preiss *et al*. (1988). Total RNA was obtained with TRIzol reagent (ThermoFisher) according to manufacturer instructions, then the cDNA was reverse transcribed as indicated in Ghiselli et al. (2007). The partial sequences of mitochondrial gene *cox2* and of the nuclear gene *ef1-α* were PCR-amplified as described in Ghiselli et al. (2007). Obtained

PCR product were purified using the Wizard PCR Preps DNA Purification System (Promega), and Sanger-sequenced at Macrogen Europe Lab.

Sequence chromatograms checking and multiple sequence alignments with ClustalW algorithm were carried out using Mega v.7 (Kumar et al. 2016). New sequences were elaborated together with previously obtained ones (Fig. 4; Table 1 and Table 2; Supplementary material 1-3 for Genbank accession numbers and sequences) in order to get a more comprehensive analysis.

Maximum Likelihood tree searches were performed with RAxML v. 8.2 (Stamatakis 2014), using the GTR+G substitution model and 500 rapid bootstrap replicates for both genes. Bayesian inferences were conducted with Mr Bayes v3.2.6 (Ronquist et al. 2012): two runs were launched, each with 1,000,000 generations, sampled every 500th generation, and using the GTR+G substitution model. Convergence was assessed through the variance of split frequencies (<0.01), PSRF (=1.00) and ESS (>200). Age estimates of cladogenetic event were calculated using a bayesian framework with BEAST v. 1.8 (Drummond and Rambaut, 2007) on the *cox2* dataset. Two independent searches were run, each 10,000,000 generations long, sampled every 1,000th generation, and using the GTR+G substitution model. Convergence was assessed through ESS values >200. Following Mantovani et al., 2000, time calibration was set to the split between *Bacillus rossius tripolitanus* and *B. rossius rossius*/*B. rossius redtenbacheri*: the separation of this two clades would date back to the end of Messinian salinity crisis, when the Mediterranean basin was filled up, separating North Africa, hosting *B. r. tripolitanus* only, and Southern Italy, where only *B. rossius rossius*/*B. rossius redtenbacheri* can be found. Calibration time was, therefore, set to 5.33 ± 0.5 Myr ago and implemented with a normal distribution. Searches were run with an uncorrelated, log-normal relaxed molecular clock and the birth-death speciation process. Haplotype parsimony networks were calculated through TCS v. 1.21 (Clement et al. 2000).

Results

Karyotype analysis

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

large pairs of metacentrics (1, 2, 4), and a smoothly decreasing series of acro- and subacrocentric pairs; the last four pairs, owing to their minute size, could also be envisaged as small metacentrics.

The *P. recondita* karyotype shows an overall good correspondence with the *P. hispanica* chromosome set (Figs 5, 6), the main differences being the different centromere position in the 4th and 6th pairs of the former when compared to the corresponding quartets of the latter. It could also be noted that the first and 13th quartets have two chromosomes bearing small satellites, lacking in the corresponding positions of *P. recondita*, which, in turn, presents satellites on the 2nd and 4th pairs.

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

Molecular analysis

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

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

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

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

Discussion

The origin of P. hispanica *genome*

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

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

mitotic divisions which, being the male sex-chromosome formula X0, allow the identification of the unique X chromosome: therefore, we could reasonably assign the same role of sex-chromosomes to the first quartet in the karyotype of the parthenogenetic *P. hispanica*.

The combined analysis of *cox2* mitochondrial sequences and of *ef1-α* nuclear genes suggests a quite intriguing origin of *P. hispanica* hybrid genome. *P. originis* and *P. hispanica cox2* haplotypes always cluster together, indicating a clear genetic homogeneity. Therefore, in line with previous analyses (Ghiselli et al. 2007), the maternal ancestor would have been *P. originis,* as *P. hispanica* clearly incorporates its mitochondrial marker. On the other hand, the paternal contributions are less clear. Based on the *ef1-α* phylogenetic and network analyses, *P. hispanica* is more related to *P. barbarae*, *P. masettii,* and *P. lelongi* than to *P. recondita* and *P. originis*. However, less stringent parameters for network construction allowed the connection of *P. recondita* and *P. hispanica* sub-networks. This suggests a common ancestry of the two nuclear sequences, but it also indicates a wide differentiation between *P. recondita* and *P. hispanica*. The time tree analysis clearly supports such distant relationship, dating the divergence of mitochondrial *cox2* sequences back to the Mid-Oligocene (29.73 Mya). Overall, the results are not in contrast with a possible contribution of *P. recondita* to the genome of *P. hispanica*, although the level of genetic divergence observed in both mitochondrial and nuclear markers would suggest caution. In summary, we can say that the data are compatible with the hypothesis of an ancestral contribution followed by diversification. It is worth noting that the *P. recondita* and the *P. hispanica* subnetworks are connected through the sample from El Purche (PUR), which has been collected very close to the distribution area of *P. recondita* (Sierra Nevada, SNE) (Fig. 8). Therefore, it is possible that widening the sampling in the area of sympatry would shed light on this issue.

P. hispanica is a very unusual hybrid since it incorporates the mitochondrial marker of *P. originis* but it includes the nuclear sequences of different species. The tetraploid constitution of *P. hispanica* genome and the close similarity of chromosome sets shared by all *Pijnackeria* taxa suggest the possibility of polyploidization. On the other hand, some minute differences among specific chromosome complements indicate a heterogeneous structure, supporting a complex chromosome set with heterospecific genomes. Among these, at least one of *P. recondita* origin. However, further inspection of *P. hispanica* karyotype with heteromorphic quartets points to just a double *P.*

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

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

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

The Pijnackeria karyotype and the evolution of egg maturation modes

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

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

The cytological satellite features are not just a trait of inter- and intra-specific variability, since in all investigated phasmatodean species—13 all together up to now—chromosomal satellites have been always found to be sites of highly enriched and co-localized rDNA/telomeric sequences (Scali et al. 2016; Liher et al. 2017; Salvadori et al. 2018): therefore, this trait appears to have a biological and evolutionary bearing. Actually, it has been already possible to observe that in the di-hybrid *Bacillus whitei* and the three-hybrid *B. lynceorum*, active NORs derive from all ancestors, although that of maternal *B. rossius* derivation appears as the most conserved (Manaresi et al. 1991,1992, 1993). However, owing to the androgenetic structure of *P. hispanica*, such a feature cannot be verified.

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

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

Gathering all relevant observations on the issue, the most parsimonious series of gains/losses of whole chromosome sets leading to the extant structure of *P. hispanica* can be envisaged as follows: a seminal parthenogenetic *P. originis/P. recondita* hybrid with an apomictic reproduction owing to the marked genetic differentiation of the parental taxa was produced. Pre-mating isolating mechanisms were easily overcome, since in phasmids they are rather ineffective even between utterly differentiated species (Scali et al. 1995). Back-crosses to *P. recondita* males were still possible (see Tinti and Scali, 1996) and, thanks to the physiological egg-polyspermy (Scali, 1972), an all-paternal progeny was originated when syngamy with the hybrid egg nucleus failed and two spermatozoa fused to originate a 2n androgen, which only kept the mitochondrial DNA of the mother but continued an apomictic reproduction (Mantovani and Scali, 1992). The 4n ploidy of *P. hispanica* could then be reached through a two-step acquisition of additional *Pijnackeria* genomes by the androgen. After the original hybridization of *P. recondita* with *P. originis* leading to an early diploid androgen, an additional fathering taxon, providing the third haploset, should have been different from *P. recondita* and likely similar to the unknown paternal ancestor of *P. masettii*. The last contribution of a fourth genome could have been provided by a *P. barbarae*-like paternal ancestor: the heterozygous structure of several quartets of *P. hispanica* (Fig. 6) and the high variability of its *ef1-α* sequences (Fig. 9) are consistent with the above outlined assumptions. Being these correct, *P. hispanica* could be then envisaged as a

polyphyletic hybrid-complex endowed with a high colonizing potential. Moreover, it is tempting to speculate that such kind of multi-hybrid origin could be the reason behind the long evolutionary persistence (1.96-3.31 Myr) of this parthenogenetic taxon. The high variability produced by multiple hybridizations events might have compensated for the absence of sexual recombination (Ghiselli et al 2007 and references therein).

On the issue, we would like mentioning that androgenetic stick-insect strains of *Bacillus* and *Clonopsis* contributed to the formation of a complex network among parental and derived taxa, so that their reproductive and micro-evolutionary features were defined as "reticulate evolution" (reviewed in Scali, 2009a), and, within it, androgenesis has been proposed as a short-cut pathway for speciation (Ghiselli et al. 2007; Milani et al. 2010; 2015). To better envisage the cladogenetic potential of androgenesis, a simple model of hybrid eggs maturation and genome transmission has been worked out for *Clonopsis* hybrids, which would even explain the ascertained diploid structure of polyploid karyotypes (Milani et al. 2009; 2010): the *Clonopsis* model also accommodates quite easily the otherwise inexplicable chromosomal findings reported in the Australian *Sipyloidea nelida* species complex by John et al. (1987).

On the whole, the targeted cytogenetic insight and transmission analysis of genomes, although rather limited, appears an effective tool to reveal the exploitation of a wide array of reproductive modes and evolutionary pathways in stick insects: these insights seem to really add to the routinely accepted ideas about reproductive features, evolutionary modes and phylogenetic relationships in animals.

Taxonomic implications

Following the above described scenario of "reticulate" backcrosses, multiple tetraploid populations arose in different areas of the *Pijnackeria* range, stepwise embodying additional sets of fathering taxa, some of which can also be missing from the sampling. These 4n populations are now spread and mixed in the region indicated in Figure 2. According to this phylogeographic pattern, *P. hispanica* would then represent the ensemble of many subpopulations of 4n parthenogenetic androgens in which the multiple contributions from diverse diploid species can be appreciated in the *ef1-α* network (Fig. 9). Each *P. hispanica* specimen within the different subpopulations appears to possess a chimerical genetic structure, even more strengthened by the occurrence of the "foreign" mitochondrial DNA of the maternal ancestor. All this points

to a composite, polyphyletic structure of the tetraploid hybrid, which we propose to indicate as an *androgenetic complex*.

Finally, we would like to point out that, if in *cox2* network the recently described Sierra Nevada (SNE) *P. recondita* taxon does not actually cluster together with the previously described *Pijnackeria* species (Fig. 7a,b), and Valero and Ortiz (2015) obtained the same tree topology from *cox1* and *cox 2* analyses. Although this topology does not fully resolve the relationships within the genus *Pijnackeria*, it clearly indicates a high degree of differentiation between *P. recondita* and the other conspecific species. Further molecular investigation may help to shed light on the evolution of this genus and its relationship with the closely related genus *Leptynia*.

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

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

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

Figure Captions

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

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

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

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

Fig. 4 Geographical distribution of the haplotypes obtained both by sampling and from literature contributing to the molecular analysis. A) *cox2.* B) *ef1-α.* For the exact coordinates of the sampling sites, refer to Table 1 and Table 2

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

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

Fig. 7 Schematic drawing of Maximum Likelihood/Bayesian Inference on *cox2* (A; *-lnL* = 3431.06/3503.44) and *ef1-α* (B; *-lnL*= 1958.30/20072.49) datasets. Number at nodes are bootstrap/posterior probabilities support values. Outgroup(s) have been omitted for graphical purposes

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

Fig. 9 Templeton network of the *ef1-α* gene sequences. Circles size is proportional to haplotype frequency; black dots represent missing/ideal haplotypes. Connections

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

Legends to tables

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

Table 2. Analyzed species for *ef1-α*, with collecting place with acronyms and geographic coordinates.

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

Supplementary material

Supplementary Figure 1. Time calibrated tree obtained on *cox2* gene sequence. Numbers on branches represent the posterior probability nodal support; bars at nodes indicate the 95% high posterior density (HPD).

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

Supplementary Material 2. *cox2* sequences.

Supplementary Material 3. *ef1-α* sequences.

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FIGURE 1

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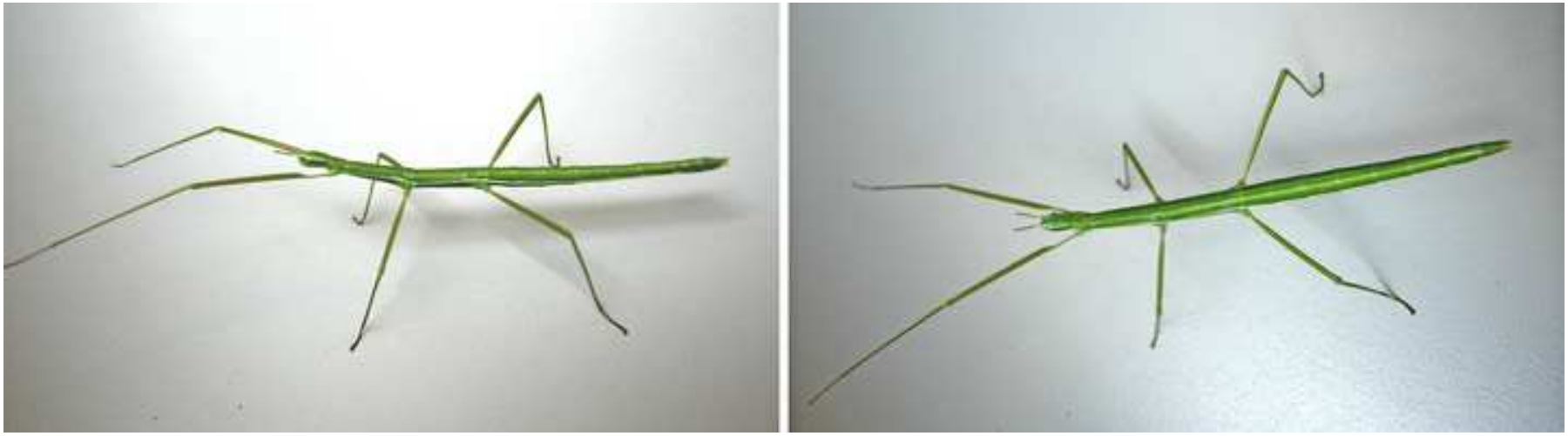

FIGURE 2

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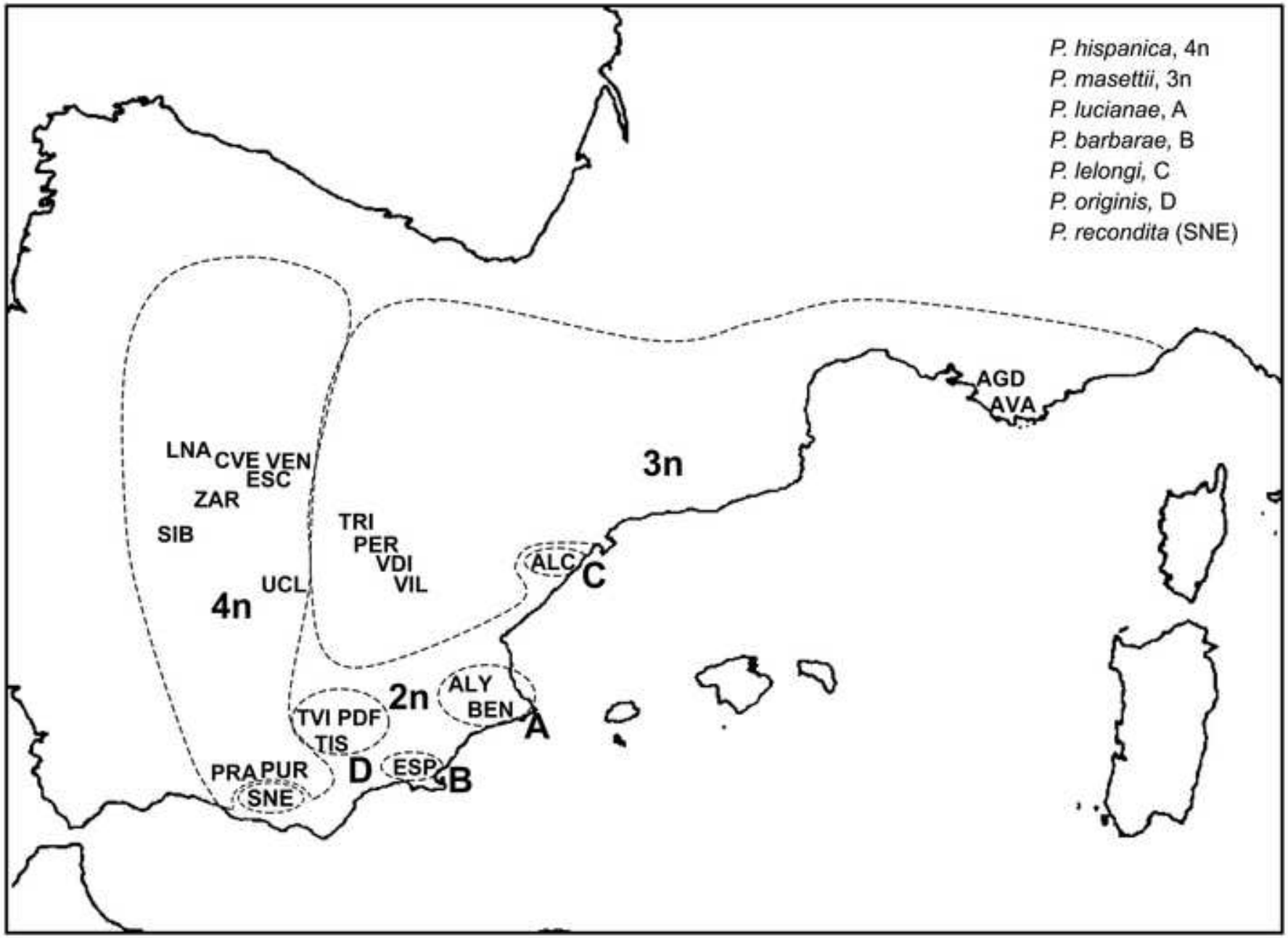

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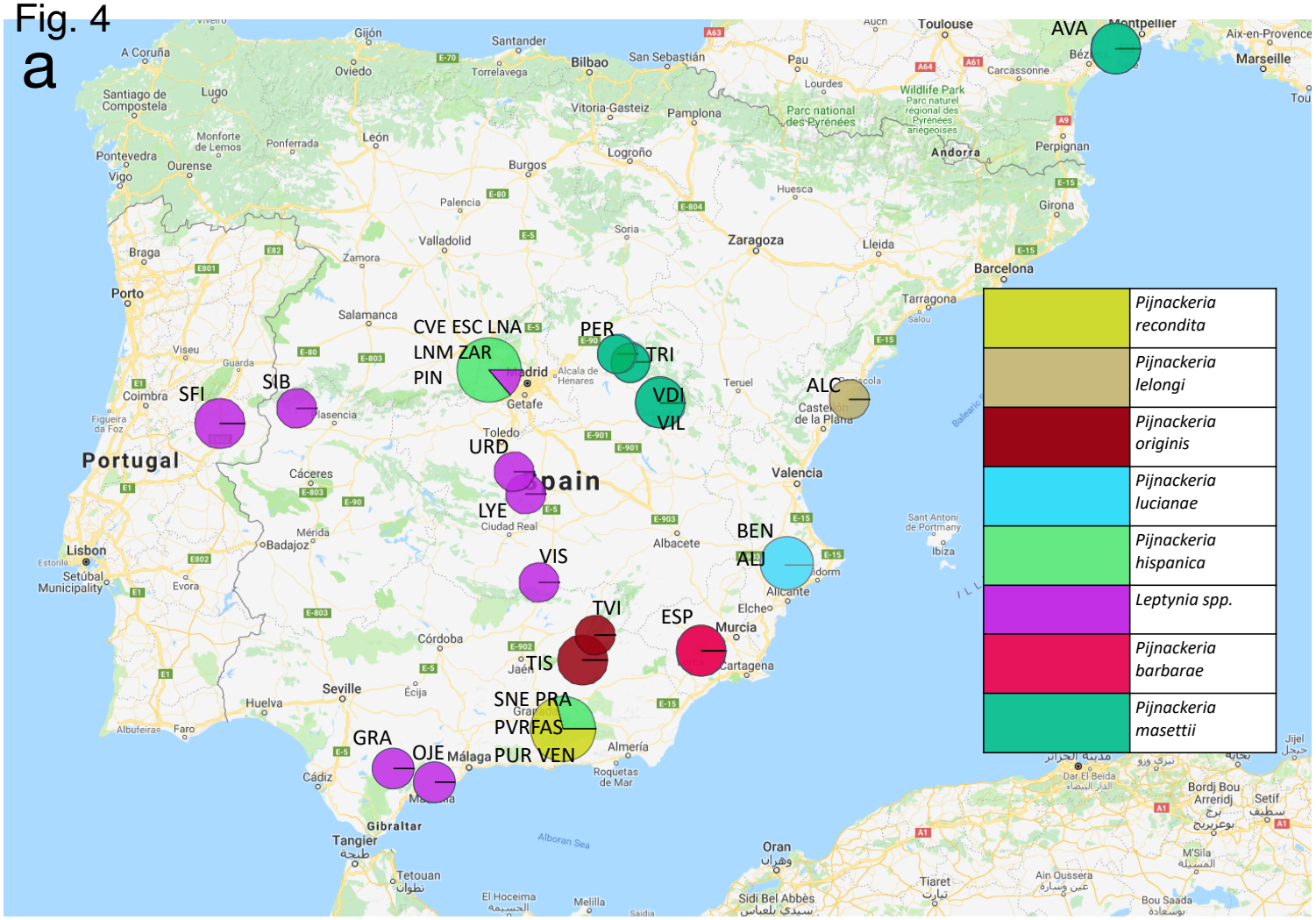
Fig. 4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Pijnackeria recondita
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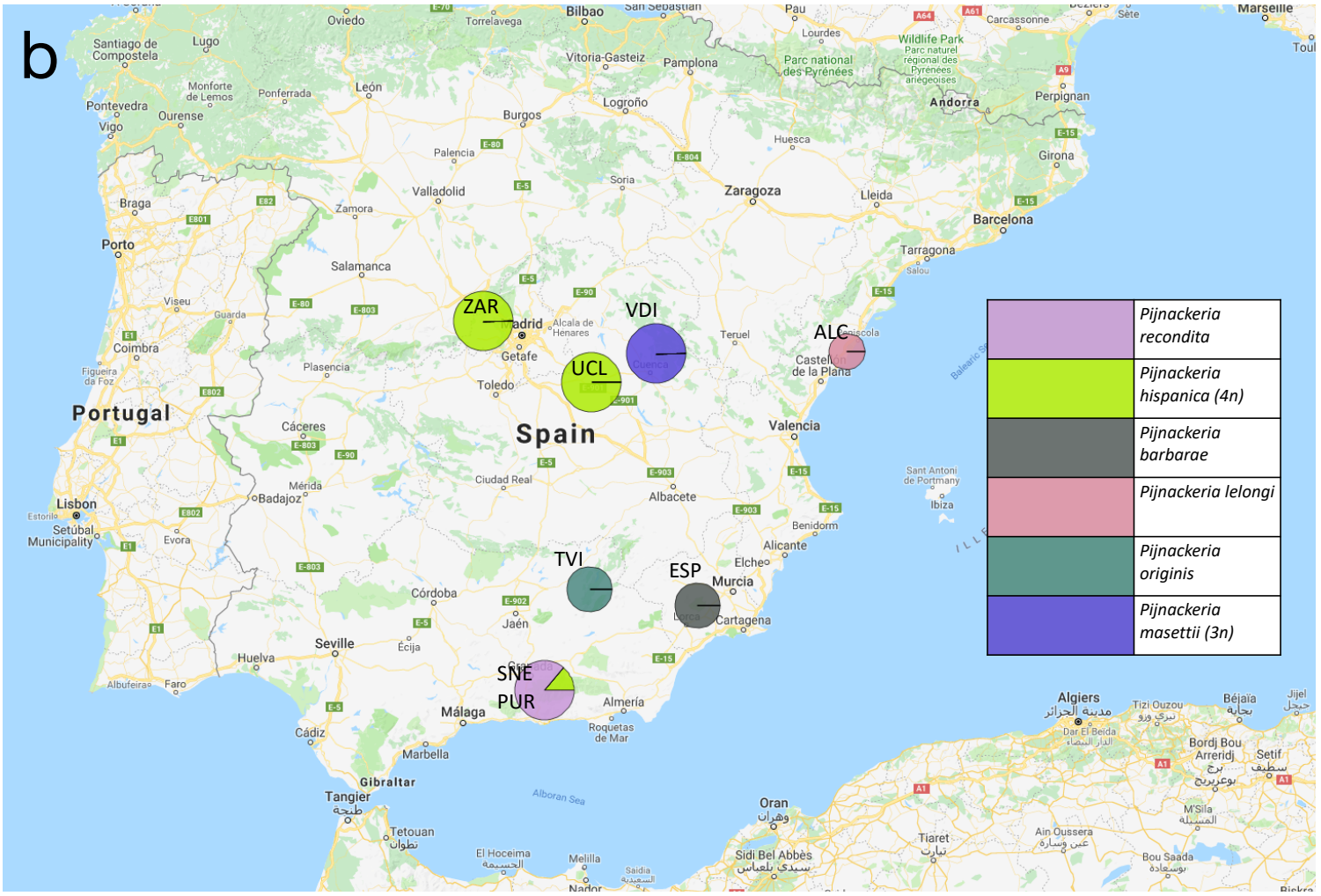
Pijnackeria hispanica
Leptynia spp.
Pijnackeria barbarae
Pijnackeria masettii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Pijnackeria hispanica (4n)
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Pijnackeria lelongi
Pijnackeria originis
Pijnackeria masettii (3n)

FIGURE 5

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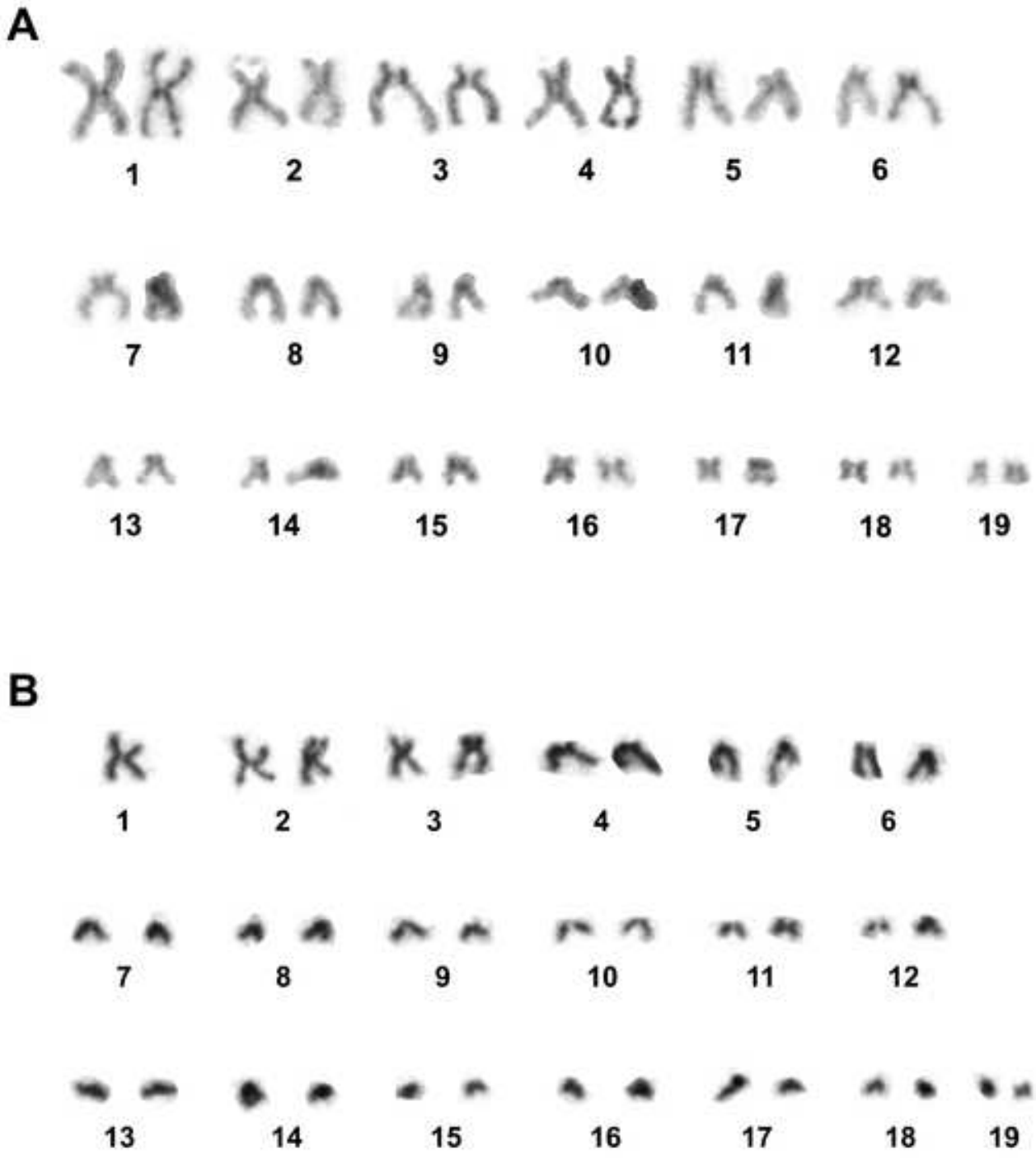

FIGURE 6

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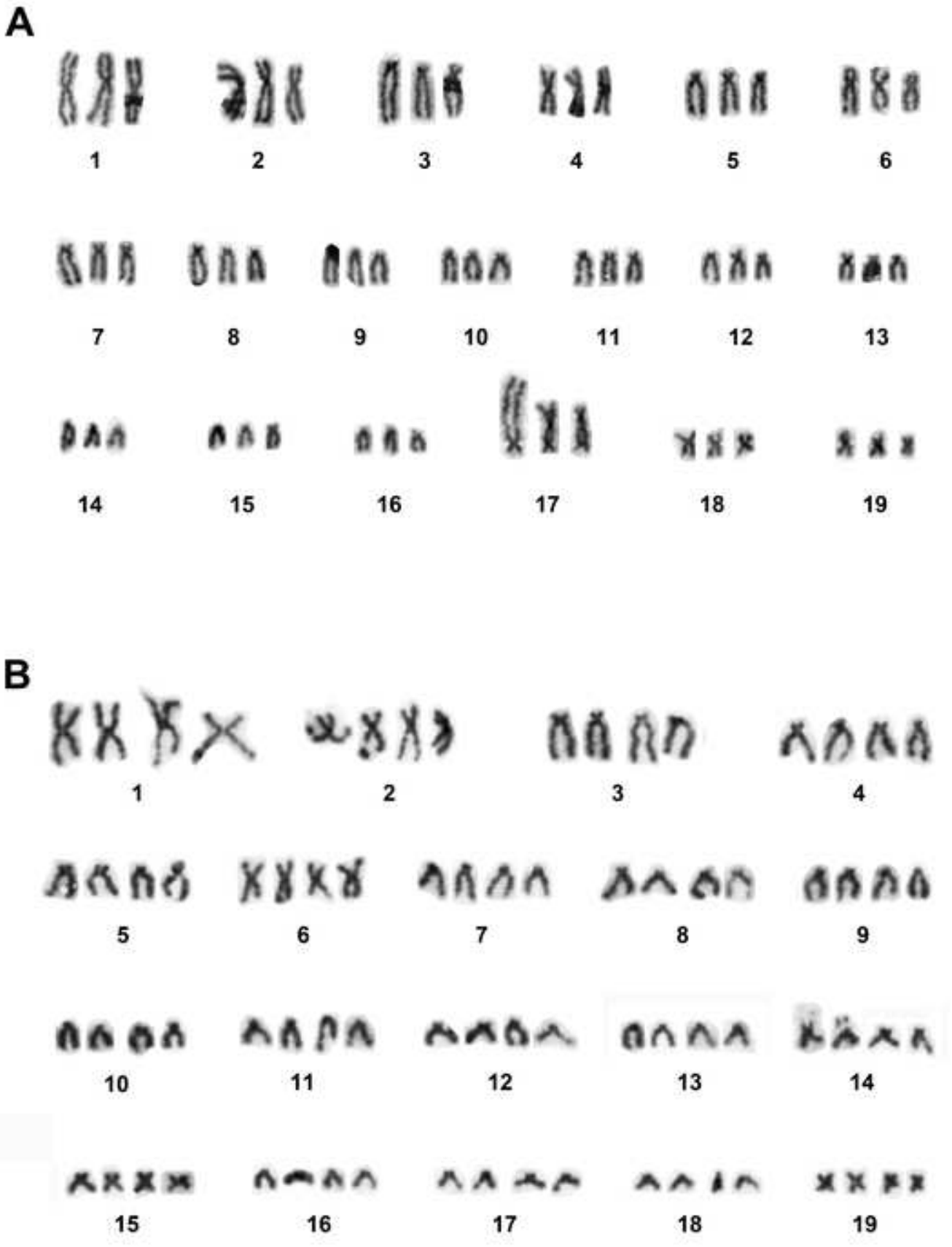
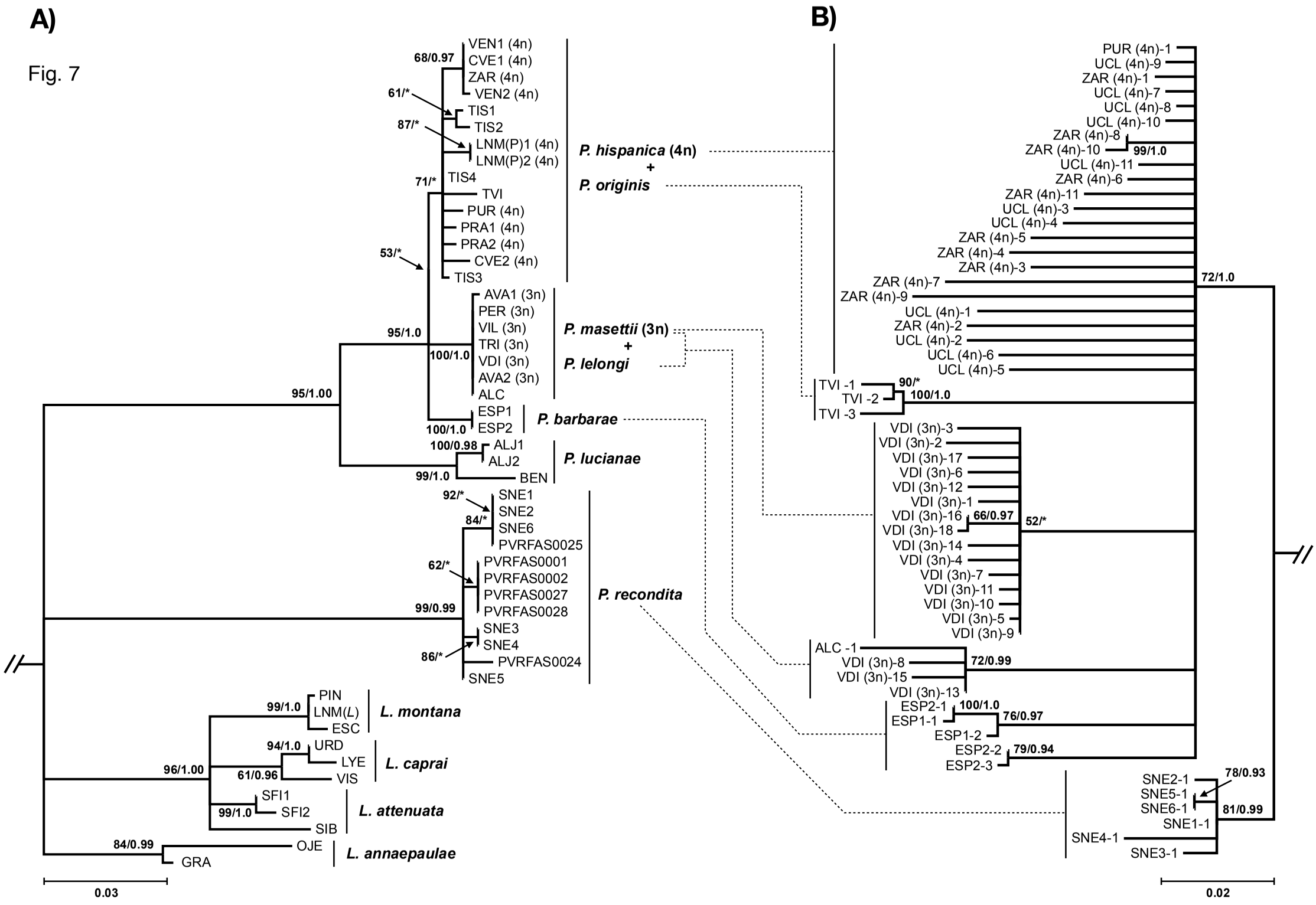


Fig. 7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P. masettii (3n)
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P. lelongi
ESP1
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P. barbarae
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P. lucianae
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PUR (4n)-1
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VDI (3n)-11
VDI (3n)-10
VDI (3n)-5
VDI (3n)-9
ALC -1
VDI (3n)-8
VDI (3n)-15
VDI (3n)-13
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ESP1-1
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ESP2-2
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100/1.0
76/0.97
79/0.94
SNE2-1
SNE5-1
SNE6-1
SNE1-1
SNE4-1
SNE3-1
78/0.93
81/0.99
0.02

Fig. 8

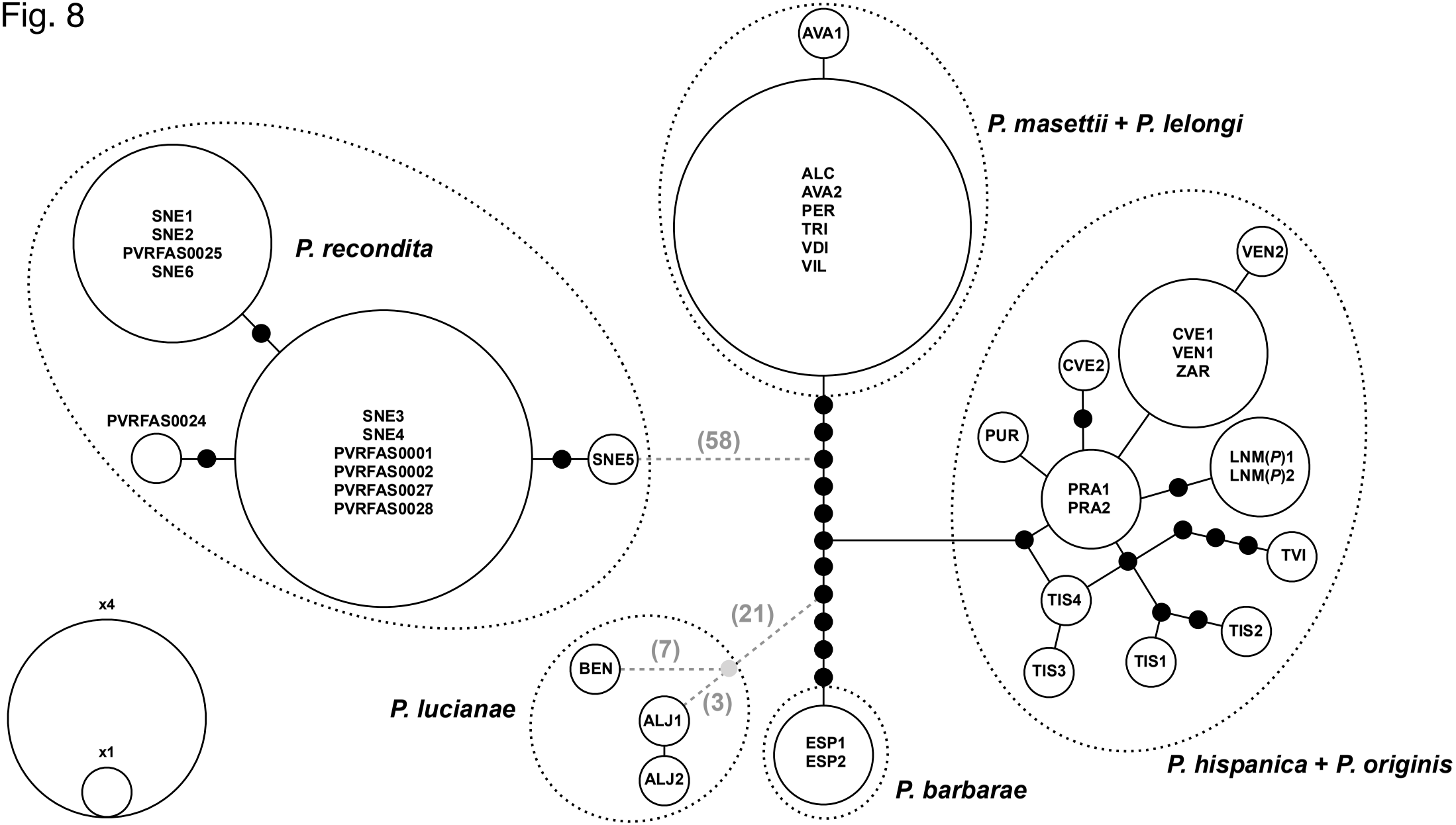
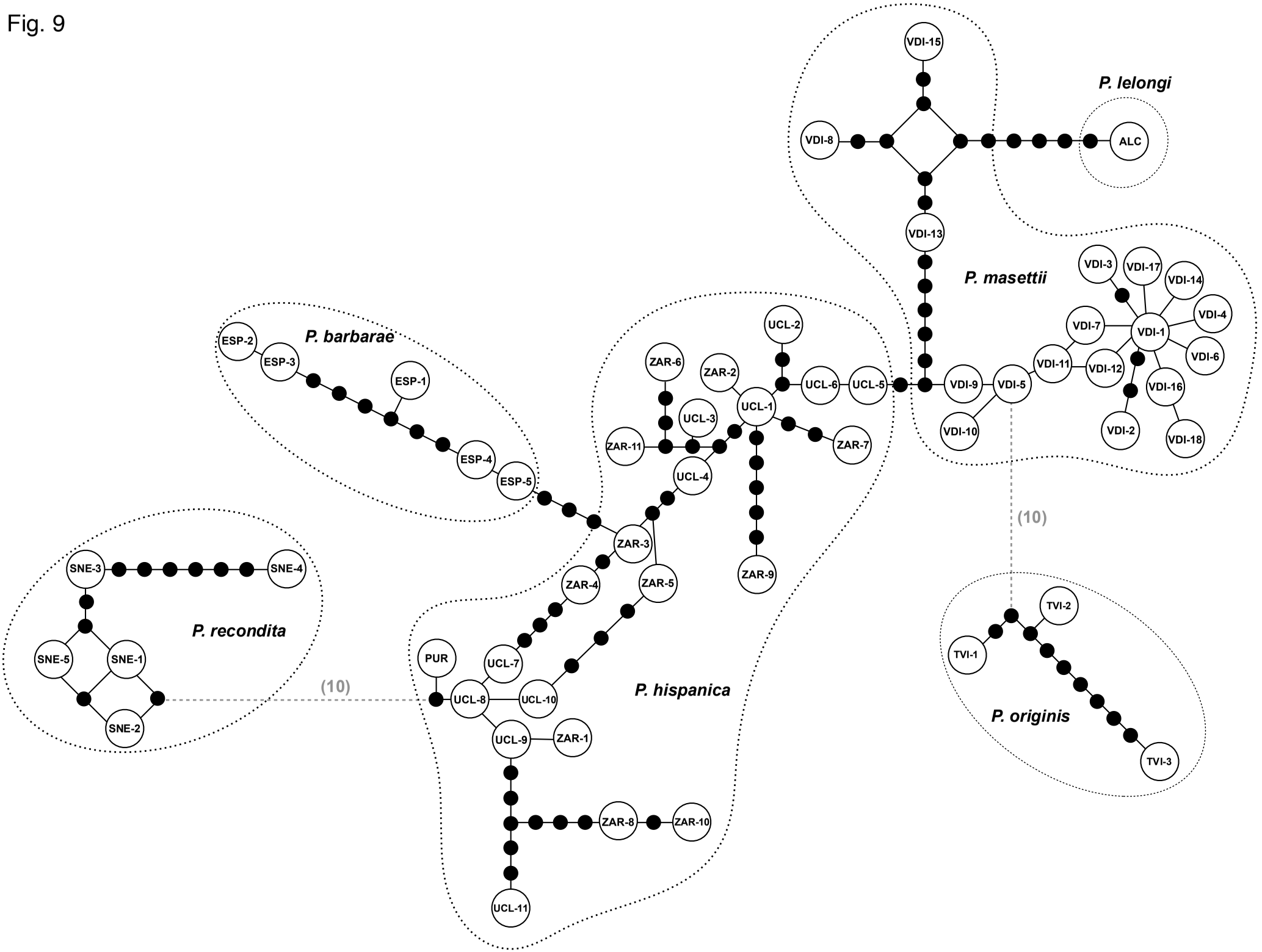

AVA1
ALC
AVA2
PER
TRI
VDI
VIL
P. masettii + P. lelongi
SNE1
SNE2
PVRFAS0025
SNE6
P. recondita
PVRFAS0024
SNE3
SNE4
PVRFAS0001
PVRFAS0002
PVRFAS0027
PVRFAS0028
SNE5
(58)
VEN2
CVE1
VEN1
ZAR
CVE2
PUR
PRA1
PRA2
LNM(P)1
LNM(P)2
TVI
TIS4
TIS2
TIS3
TIS1
(21)
(7)
BEN
(3)
ALJ1
ALJ2
P. lucianae
ESP1
ESP2
P. barbarae
P. hispanica + P. originis
x4
x1

Fig. 9


P. lelongi
VDI-15
VDI-8
ALC
VDI-13
P. masettii
VDI-3
VDI-17
VDI-14
VDI-4
VDI-7
VDI-1
VDI-6
VDI-11
VDI-12
VDI-16
VDI-2
VDI-18
VDI-9
VDI-5
VDI-10
P. barbarae
ESP-2
ESP-3
ESP-1
ESP-4
ESP-5
UCL-2
ZAR-6
ZAR-2
UCL-6
UCL-5
UCL-3
UCL-1
ZAR-11
ZAR-7
UCL-4
ZAR-3
ZAR-9
ZAR-4
ZAR-5
(10)
P. recondita
SNE-3
SNE-4
SNE-5
SNE-1
SNE-2
PUR
UCL-7
UCL-8
UCL-10
P. hispanica
UCL-9
ZAR-1
ZAR-8
ZAR-10
UCL-11
(10)
TVI-2
TVI-1
P. originis
TVI-3