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6 Phylogeography of *Psammodromus algirus* (Lacertidae) revisited:
7 systematic implications
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26

27 **Abstract**

28

29 Relationships among *Psammodromus algirus* populations from the Iberian Peninsula
30 and North Africa, including recently described *P. jeanneae* and *P. manuelae,* were
31 estimated from mitochondrial DNA gene sequences. This enlarged data set confirmed
32 the presence of two divergent eastern and western mitochondrial DNA lineages on the
33 Iberian Peninsula, the distributions for which are separated by a narrow zone of contact
34 across the centre of the Peninsula. Paratypes of *P. jeanneae* and topotypes of *P.*
35 *manuelae* represent southern and northern clades of the western lineage, respectively,
36 making *P. algirus* paraphyletic. This, together with the low level of allozymic and
37 mitochondrial DNA substructuring within western populations, is not sufficient to retain
38 *P. jeanneae* and *P. manuelae* as valid species, and we relegate them to the status of
39 junior synonyms of *P. algirus*.

40

41 *Key words*: Iberian Peninsula, lacertid lizards, mitochondrial DNA, *Psammodromus*
42 *algirus, Psammodromus jeanneae*, *Psammodromus manuelae*.

43

44 Since its original designation as a species in 1758, *Psammodromus algirus* has been
45 considered representative of a single, invariant, species throughout the Iberian
46 Peninsula. Recently, however, Carranza et al. (2006) demonstrated that *P. algirus* in
47 Iberia was actually comprised of eastern and western mtDNA clades. Working
48 independently of Carranza et al., Busack and Lawson (2006) noted mtDNA
49 differentiation and allozyme differentiation between northern and southern Iberian
50 populations (2006: Figs. 2 & 3, respectively), and later described *P. manuelae* from
51 Manzanares el Real (Madrid province) and *P. jeanneae* from 25.6 km NE of Facinas

52 (Cádiz province) following a morphological analysis (Busack, Salvador and Lawson,
53 2006). In this analysis we utilize DNA sequences from seven mitochondrial genes and
54 take advantage of a larger, more robust, specimen sample including the holotype and
55 paratypes of *P. jeanneae* and topotypes of *P. manuelae* to revisit the phylogeography of
56 Iberian *P. algirus*. This expanded data set allows us to infer genetic relationship among
57 populations of *P. algirus, P. manuelae* and *P. jeanneae* throughout much of its current
58 geographical range on Iberia and to revise the taxonomy of this group with special
59 reference to the status of *P. manuelae* and *P. jeanneae*.

60 A total of 104 individuals from 68 populations, including most samples used by Carranza et al.
61 (2006) and Busack and Lawson (2006), the holotype and one paratype of *Psammodromus jeanneae*
62 (E232055 and 232056, respectively), topotypes of *P. manuelae* (E232060 and 232062) and 32 specimens
63 from 16 previously unsampled populations (Fig. 1), were sequenced. *Psammodromus h. hispanicus* from
64 Encinasola (Huelva province) and *P. h. edwarsianus* from Sierra de Baza (Granada province) served as
65 outgroups (following Carranza et al., 2006).

66 Total genomic DNA was extracted from ~40 mg of tissue using the Qiagen BioSprint 15 DNA
67 Kit® following the manufacturer's protocol. Resulting DNA was visually inspected after migration on
68 agarose gels and quantified with a NanoDrop spectophotometer. Amplifications were performed in 50 µl
69 of 1x reaction buffer, 2 mM $MgCl_2$, 0.4 µM each primer, 0.2 mM each dNTP, 1.25 U of GoTaq Flexi
70 DNA Polymerase (Promega), and 3 µl of previously extracted DNA (50-100 ng). A 709 bp fragment of
71 the fourth subunit of the NADH dehydrogenase mitochondrial gene (ND4) and adjacent $tRNA^{His}$ (68 bp)
72 $tRNA^{Ser}$ (67 bp) and $tRNA^{Leu}$ (48 bp) were amplified using primers ND4 and Leu (Arévalo, Davis and
73 Sites, 1994). PCR consisted of 3-min pre-denaturing step at 94ºC, followed by 35 cycles of denaturing for
74 30 sec at 94ºC, primer annealing for 30 sec at 54ºC and elongation for 40 sec at 72ºC with a final 4-min
75 elongation step at 72ºC. Fragments of the mitochondrial cytochrome *b* gene (CytB, 300 bp), 12S rRNA
76 (363 bp) and 16S rRNA (410 bp) were amplified using primers cytb1 and cytb2 (Palumbi, 1996), 12Sa
77 and 12Sb (Kocher et al., 1989), and 16Sa and 16Sb (Palumbi, 1996), respectively. PCR consisted of a 5-
78 min pre-denaturing step at 94ºC, followed by 35 cycles of denaturing for 30 sec at 94ºC, annealing
79 primers for 45 sec at 48ºC and elongation for one min at 72ºC with a final 5-min elongation step at 72ºC.
80 PCR effectiveness was visually quantified after migration of PCR products on agarose gels. PCR

81 products were purified by an ammonium acetate/ethanol cleaning process and sequenced using the ABI
82 Prism Big Dye Terminator Cycle sequencing protocol in an ABI Prism 310 automated sequencer
83 (Applied Biosystems).

84 All sequence chromatograms were edited with Sequencer (v. 4.2.2, Gene Codes). Once
85 corrected, sequences were aligned independently for each gene with CLUSTALX (Thompson et al.,
86 1997) under program default parameters (opening gap = 10; gap extension = 0.2) and visually inspected
87 with Bioedit v.7.0.5 (Hall, 2005). Topological incongruence among partitions was tested using the
88 incongruence length difference (ILD) test (Michkevich and Farris, 1981; Farris et al., 1994). In this test,
89 10,000 heuristic searches were carried out after removing all invariable characters from the data set
90 (Cunningham, 1997). To test for incongruence among data sets, we also used a reciprocal 70% bootstrap
91 proportion or a 95% posterior probability threshold (Mason-Gamer and Kellogg, 1996). Topological
92 conflicts were considered significant if two different relationships for the same set of taxa were each
93 supported. Results of all tests indicated that independent data sets were not incongruent (data not shown)
94 and therefore a combined analysis involving 7 mitochondrial genes (ND4 - $tRNA^{His}$ - $tRNA^{Ser}$ - $tRNA^{Leu}$ -
95 CytB - 12S - 16S) was carried out. For many samples, however, it was not possible to amplify 12S and
96 16S genes and a smaller data set including only 5 genes (ND4 - $tRNA^{His}$ - $tRNA^{Ser}$ - $tRNA^{Leu}$ - CytB) was
97 also elaborated.

98 Phylogenetic trees were inferred using Maximum-Likelihood (ML; Felsenstein, 1981) and
99 Bayesian methods. The most appropriate model of sequence evolution was determined with jModelTest
100 v.0.1.1 (Posada, 2008) using the Akaike information criterion. In the Bayesian analyses each partition had
101 its evolutionary model and these were: the HKY for $tRNA^{His}$ and $tRNA^{Leu}$, HKY + G for $tRNA^{Ser}$ and
102 16S, GTR + G for ND4 and 12S, and GTR + I + G for CytB. ML analyses were performed using PhyML
103 version 2.4.3 (Guindon and Gascuel, 2003), with model parameters fitted to the data by likelihood
104 maximization. In this case a single model of sequence evolution was selected for each concatenated data
105 set (5 genes and 7 genes). In both cases the best model was the GTR+I+G. Reliability of the ML trees was
106 assessed by bootstrap analysis with 1,000 replications (Felsenstein, 1985). Bayesian analyses were
107 performed with MrBayes version 3.0b4 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck,
108 2003) for 2.5×10^6 generations, with a sampling frequency of 100 generations. After verifying that
109 stationarity had been reached in terms of likelihood scores and parameter estimation, the first 5,000 trees
110 for the two data sets were discarded from both runs and independent majority-rule consensus trees were

111 generated from the remaining (post burn-in) trees. The frequency of any particular clade of the consensus
112 tree represents the posterior probability of that node (Huelsenbeck and Ronquist, 2001); only values equal
113 to or greater than 95% were considered to indicate that nodes were significantly supported (Wilcox et al.,
114 2002).

115 The ILD test indicated that the 7 gene partitions (ND4 - $tRNA^{His}$ - $tRNA^{Ser}$ -
116 $tRNA^{Leu}$ - CytB - 12S - 16S) were not incongruent (P = 0.23); analyses of independent
117 partitions confirmed there were no topological conflicts (Mason-Gamer and Kellogg,
118 1996) and two independent data sets of mitochondrial fragments were combined for
119 further analysis. The first data set included 1192 bp for ND4-$tRNA^{His}$ - $tRNA^{Ser}$-
120 $tRNA^{Leu}$-CytB from 104 individuals in which 318 bp were variable and 236 bp were
121 parsimony-informative, and the second was comprised of 1965 bp for ND4-$tRNA^{His}$-
122 $tRNA^{Ser}$-$tRNA^{Leu}$-CytB-12S-16S from 46 individuals in which 459 bp were variable
123 and 319 bp were parsimony-informative. All sequence data not currently available in
124 GenBank will be added upon publication (see Figs. 2 and 3 for specimen identification
125 information).

126 Results of Maximum-Likelihood (ML) and Bayesian analyses are illustrated in
127 Fig. 2 (ND4-$tRNA^{His}$-$tRNA^{Ser}$-$tRNA^{Leu}$-CytB) and Fig. 3 (ND4-$tRNA^{His}$-$tRNA^{Ser}$-
128 $tRNA^{Leu}$-CytB-12S-16S). Each method and dataset produced trees with very similar
129 topologies. Log-likelihood values of the trees obtained by ML for ND4-$tRNA^{His}$-
130 $tRNA^{Ser}$-$tRNA^{Leu}$-CytB and ND4-$tRNA^{His}$-$tRNA^{Ser}$-$tRNA^{Leu}$-CytB-12S-16S
131 combinations were -5824.03 and -6584.01, respectively.

132 These data confirm that *Psammodromus algirus* consists of two well-supported,
133 reciprocally monophyletic, mitochondrial lineages (Carranza et al., 2006); an eastern
134 lineage confined to Iberia, and a western lineage present in both Iberia and North Africa
135 (Figs 1-3). This analysis, which includes a larger and more geographically-
136 representative sample of individuals and a more robust mitochondrial sampling than

137 Carranza et al. (2006), strongly supports the African clade as being sister to the western
138 lineage on Iberia. Iberian representatives of the western lineage are partitioned into
139 three groups: a basal clade restricted to three samples from southern localities, and two
140 main northwestern and southwestern clades (Figs. 2-3). Colonization of the African
141 continent occurred after differentiation between the western and eastern lineages on
142 Iberia, but before the split between southwestern and northwestern clades within the
143 western lineage.

144 Phylogenetic analyses clearly place the type specimens of *Psammodromus*
145 *jeanneae* in the southwestern clade of the western lineage and specimens of *P.*
146 *manuelae* from Manzanares el Real (Madrid province, the type locality) in the
147 northwestern clade (Figs. 2 and 3). If *P. jeanneae* and *P. manuelae* were, in fact, well-
148 differentiated species, *P. algirus* would be a paraphyletic unit. Within the western clade,
149 however, whose African populations are associated with *P. algirus*, the level of genetic
150 substructuring is much lower than that between western and eastern clades (Carranza et
151 al., 2006; this study) and our molecular data (Figs. 2 and 3) do not support *P. jeanneae*
152 and *P. manuelae* as well-differentiated species. As a result, we hereby relegate these
153 names to the status of junior synonyms of *P. algirus*.

154 Our greater geographic coverage, relative to that initially reported by Carranza et
155 al. (2006) and Busack and Lawson (2006), allows us to reject the hypothesis that
156 variation in mtDNA haplotypes is gradual. Currently available data do, however,
157 suggest that highly divergent colour patterns in *Psammodromus algirus* are found in
158 central Spain and in three northeastern populations (Carretero, 2002). If such (or other)
159 phenotipic differences were consistent on a broader geographical scale, the eastern
160 lineage of *P. algirus* might represent a separate species. Combined, this information
161 suggests that ecological or behavioural mechanisms may be currently acting to maintain

162 differentiation, resulting in a relatively narrow contact zone between eastern and
163 western lizards across the centre of the Iberian Peninsula (Fig. 1). Additional work,
164 which should include studies of nuclear markers (Godinho et al., 2008) and proper
165 morphometric analyses of these clades, is needed to fully understand genetic and
166 phenotypic variation between and within these lineages, especially at contact zones.

167

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169

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174 Mancha, Extremadura, and Murcia.

175

176 **References**

177

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231

232 Figure legends

233

234 **Figure 1**. Localities sampled; solid symbols represent the western lineage, open
235 symbols represent the eastern lineage and half-solid symbols indicate localities
236 representing both lineages (type localities for *P. manuelae* [8] and *P. jeanneae* [38]
237 highlighted with asterisk). Locality Key: **SPAIN**: TARRAGONA: (1) Tarragona. LERIDA: (2)
238 Tartareu. HUESCA: (3) Ainsa. ZARAGOZA: (4) El Frasno. ZAMORA: (5) Cabañas de Tera.
239 SEGOVIA: (6) Sotos de Sepúlveda. MADRID: (7) Navacerrada, (8) Manzanares el Real, (9) El Pardo,
240 (10) Aranjuez. GUADALAJARA: (11) Pioz. CUENCA: (12) Torrejoncillo del Rey, (13) Hoces del
241 Cabriel. CACERES: (14) Santiago del Campo, (15) Villuercas. TOLEDO: (16) Espinoso del Rey.
242 CIUDAD REAL: (17) Valdepeñas, (18) Solana del Pino. JAEN: (19) Despeñaperros. ALBACETE: (20)
243 Embalse Fuensanta, (21) Embalse de Bayco. MURCIA: (22) Morata, (23) Águilas. ALMERIA: (24)
244 Cabo de Gata, (25) Sierra Gador, (26) Abrucena. GRANADA: (27) La Calahorra, (28) Cortijo del Ciprés,
245 (29) Dehesa de los Montes. MALAGA: (30) Málaga, (31) Marbella, (32) Sierra Bermeja, (33)
246 Genaguacil, (34) La Sauceda, (35) Río Hozgarganta. CADIZ: (36) Castellar de la Frontera, (37) Getares,
247 (38) Facinas, (39) Barbate, (40) Caños de Meca, (41) Medina Sidonia. SEVILLA: (42) Lebrija, (47)
248 Cañada de los Pájaros, (48) Gelves, (49) Gerena. HUELVA: (43) Matalascañas, (44) Bodegones, (45) El
249 Portil, (46) Ayamonte, (50) Berrocal, (51) Linares de la Sierra. BADAJOZ: (52) Oliva de la Frontera,
250 (53) Tentudia, (54) Pallarés. CORDOBA: (55) Doña Rama, (56) Virgen de la Cabeza. **MOROCCO**: (57)
251 Tangier, (58) Ued Lau, (59) Chefchaouen, (60) Jebala, (61) Bab-Berret, (62) Beni-Mellal, (63)
252 Boulemane, (64) Middle Atlas, (65) Berkana. **ALGERIA**: (66) Tlemcen, (67) Sidi Feredj. **TUNISIA**:
253 (68) Ain Draham.

254

255 **Figure 2**. Phylogenetic relationship of *Psammodromus algirus*, *P. jeanneae* and *P.*
256 *manuelae* derived from ML and Bayesian analyses using ND4, $tRNA^{His}$, $tRNA^{Ser}$,
257 $tRNA^{Leu}$ and CytB (see text for details). Numbers above and below nodes represent
258 bootstrap support (> 70%) from ML analysis and Posterior Probabilities (> 0.95) for
259 Bayesian analysis (not shown in polytomous nodes), respectively. Dashed lines indicate

260 basal branch lengths not proportional to total number of changes. Plain text letter and
261 number allocation identifies individuals, boldface number refers to locality on Fig. 1.
262
263 **Figure 3**. Estimate of relationships of *Psammodromus algirus*, *P. jeanneae* and *P.*
264 *manuelae* derived from ML and Bayesian analyses using ND4, $tRNA^{His}$, $tRNA^{Ser}$,
265 $tRNA^{Leu}$, CytB, 12S and 16S (see text for details). Numbers above and below nodes
266 represent bootstrap support (> 70%) for ML analysis and Posterior Probabilities (> 0.95)
267 for Bayesian analyses (not shown in polytomous nodes), respectively. Dashed lines
268 indicate basal branch lengths not proportional to total number of changes. Numbers
269 after specimens refer to the geographic locations shown in Fig. 1. Plain text letter and
270 number allocation identifies individuals, boldface number refers to locality on Fig. 1.

Fig 1

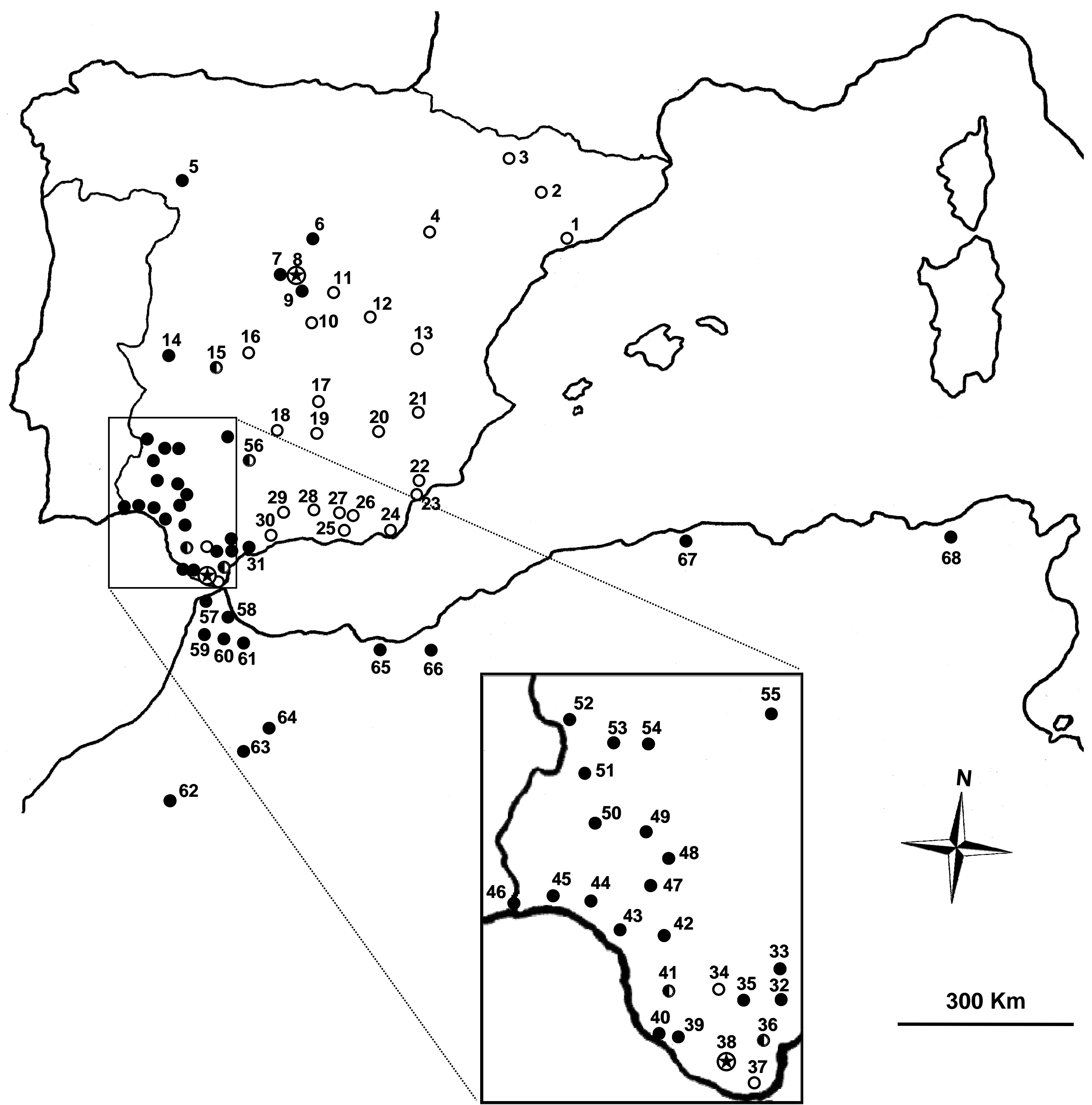

Fig 2

E281145 - *P.h.edwarsianus*
E281143 - *P.h.hispanicus*
E281125 - ***24***
E1071 - ***27***
ABRUM1 - ***26***
E1074 - ***23***
MORAH2 - ***22***
E281118 - ***30***
E3119 - ***29***
E10718 - ***18***
VIM2 - ***15***
VH2 - ***17***
VM1 - ***17***
E10711 - ***56***
VH5 - ***17***
VIM1 - ***15***
ER1 - ***16***
DH2 - ***19***
E232051 - ***41***
E281133 - ***37***
E10712 - ***34***
E232054 - ***36***
ABRUH2 - ***26***
E3118 - ***28***
E10714 - ***25***
E20396 - ***1***
E140118 - ***2***
E20398 - ***2***
E3117 - ***3***
FRASH1 - ***4***
AM1 - ***10***
PISA05 - ***11***
PIM04 - ***11***
PIH02 - ***11***
HCM3 - ***13***
FRASH2 - ***4***
E1079 - ***21***
AH1 - ***10***
AH3 - ***10***
E281129 - ***20***
CH3 - ***12***
CH6 - ***12***
E3112 - ***66***
E28119 - ***58***
E28116 - ***65***
E28113 - ***61***
E281135 - ***68***
E281134 - ***68***
E281138 - ***68***
E281137 - ***68***
E281136 - ***68***
E3111 - ***67***
E20392 - ***64***
E3113 - ***62***
E3114 - ***63***
E28112 - ***61***
E28114 - ***65***
E3115 - ***57***
E28117 - ***57***
E281110 - ***57***
E140115 - ***59***
E28115 - ***60***
E10713 - ***31***
E10717 - ***32***
E10710 - ***33***
E20397 - ***14***
PH3 - ***9***
PH6 - ***9***
PM3 - ***9***
E281126 - ***52***
NM1 - ***7***
CABAH2 - ***5***
SH1 - ***6***
SM3 - ***6***
SM1 - ***6***
CABAM1 - ***5***
E232062 - ***8***
E232060 - ***8***
P. manuelae
E1075 - ***46***
E10719 - ***53***
E31110 - ***45***
E281120 - ***49***
E281119 - ***51***
E281116 - ***49***
VIH5 - ***15***
PALM1 - ***54***
E1072 - ***55***
E281114 - ***56***
E281128 - ***43***
E281117 - ***56***
E281115 - ***56***
E1076 - ***50***
E1077 - ***47***
E1078 - ***40***
E281127 - ***44***
E232053 - ***36***
E232055 - ***38***
E232056 - ***38***
P. jeanneae
E3116 - ***39***
E140117 - ***40***
E232050 - ***41***
E20399 - ***42***
E232059 - ***35***
E140116 - ***48***
E232057 - ***38***
E232052 - ***41***

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Eastern lineage

African clade

Iberian northwestern clade

Iberian southwestern clade

Western lineage

Fig 3