Basic and Applied Herpetology 35 (2021) 77-83

Further molecular assessment of the distribution of Spanish sand racers (Lacertidae; *Psammodromus*)

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Received: 10 November 2020; returned for review: 14 December 2020; accepted 21 January 2021.

Three related and morphologically similar small *Psammodromus* species (*P. hispanicus* complex) occur in the Iberian Peninsula and Mediterranean France, *Psammodromus hispanicus*, *Psammodromus occidentalis*, and *Psammodromus edwarsianus*. Despite recent works, overall ranges of the species remain partially uncertain, in particular for Westernmost part of Iberia and South-western France. Here we include data from 15 additional specimens that were sequenced for part of the mitochondrial gene Cytochrome *b* to allow comparison with published data. We provide genetic confirmation that *P. edwarsianus* is the species of the complex occurring in France, and that *P. occidentalis* occurs on coastal and inland Portugal. Within *P. occidentalis* notable diversity occurs for this marker, which warrants further investigation. Some areas, particularly southern Portugal, still need assessment to clarify the distribution of these mostly cryptic species across the region.

Key words: cytochrome *b; Psammodromus hispanicus; Psammodromus occidentalis; Psammodromus edwarsianus*.

The genus *Psammodromus* is currently considered to consist of six species, and together with *Gallotia* forms the subfamily Gallotiinae, the sister taxa to remaining Lacertidae (ARNOLD *et al.*, 2007; GARCIA-PORTA *et al.*, 2019). Two related species, *P. microdactylus* and *P. blanci*, are endemic to North Africa, while *P. algirus* occurs across the Iberian Peninsula and the Maghreb (MENDES *et al.*, 2017). The remaining three species form a monophyletic group; *P. occidentalis* from Western Iberia, *P. hispanicus* from Central Iberia, and *P. edwarsianus* from Eastern Iberian into Mediterranean France. Until recently these three were considered as a single species, with *P. edwarsianus* as a subspecies that could be readily identified by the presence of a supralabial scale below the subocular scale (BOULENGER, 1920; SAN-JOSE *et al.*, 2012). Genetic data led to the recognition of these three species, but distinguishing *P. hispanicus* and *P. occidentalis* in the field is complex, with substantial morphological overlap and limited differences concerning more pointed snout and higher numbers of femoral pores and of ocelli in *P. hispanicus* (FITZE *et al.*, 2012). Due to this overall morphological similarity, there are many areas where it is not clear which species of

DOI: http://dx.doi.org/10.11160/bah.205

Table 1: Locality data of all newly sequenced samples included in this work. The exact coordinates for the samples from Montpellier (France) are unknown.

Sample	Species	Country	Locality	Latitude	Longitude
DB19	*P. occidentalis*	Spain	Valencia del Ventoso, Badajoz	38.227030	-6.483000
DB29	*P. edwarsianus*	Spain	Boniche, Cuenca	39.985380	-1.628710
DB1297	*P. hispanicus*	Spain	Cañada del Provencio, Albacete	38.521757	-2.346722
DB1303	*P. edwarsianus*	Spain	Corredor de Almansa, Albacete	38.764807	-0.982542
DB1723	*P. hispanicus*	Spain	Siles, Jaén	38.359322	-2.509787
DB1784	*P. hispanicus*	Spain	Orcera, Jaén	38.337277	-2.607107
DB1850	*P. hispanicus*	Spain	Orcera, Jaén	38.293301	-2.591243
DB1851	*P. hispanicus*	Spain	Orcera, Jaén	38.293301	-2.591243
DB1856	*P. hispanicus*	Spain	Bogarra, Albacete	38.536241	-2.416859
DB4219	*P. hispanicus*	Spain	Cazorla, Jaén, Andalusia	37.91337	-3.00320
DB4222	*P. edwarsianus*	France	Montpellier, Hérault	n/a	n/a
DB4223	*P. edwarsianus*	France	Montpellier, Hérault	n/a	n/a
DB4225	*P. occidentalis*	Portugal	Setúbal, Alto Alentejo	38.47177	-9.17084
DB4226	*P. occidentalis*	Portugal	Setúbal, Alto Alentejo	38.47177	-9.17084
DB22065	*P. occidentalis*	Spain	Huelva, Huelva, Andalusia	37.047798	-6.567283

Psammodromus is present – in particular that has been almost no sampling in Portugal to confirm that the "western" lineage does indeed spread all the way to the Atlantic. This is needed, as many genetic studies of Iberian herpetofauna have identified distinct lineages in Southern Portugal (Martínez-Solano *et al.*, 2006; Kaliontzopoulou *et al.*, 2011; Martínez-Sampaio *et al.*, 2015; Martínez-Freiría *et al.*, 2020). Furthermore, samples from France, where the type locality of *P. edwarsianus* is located (following Crochet, 2015), have not been included in molecular analyses. Here we build on the molecular assessments of Fitze *et al.*, 2011 and Molina *et al.*, 2020 to better determine distributions of these species, in particular including samples from the Atlantic coast of Portugal and from France.

A total of 15 individuals were sequenced (Fig 1 and Table 1). Samples consisted of tail-tip muscle, stored in 96% ethanol. A standard high-salt method was employed for DNA extraction (Sambrook *et al.*, 1989). A fragment of the mitochondrial Cytochrome b gene (approximately 300bp) was amplified with universal primers (Kocher *et al.* 1989). Purification and sequencing were carried out using a commercial service (Genewiz, Germany). New sequences were deposited on GenBank (Accession numbers MW56224 - MW56238), and aligned against the data from Molina *et al.* (2020) using MAFFT in Geneious® v4.8.5 (Biomatters). We included only data from the three target Iberian species since it is clear they form a monophyletic group (Mendes *et al.*, 2017; Molina *et al.*, 2020) with *P. algirus* as an outgroup. Translated sequences were checked for the unexpected presence of stop codons, and no gaps were needed for alignment.

Bayesian inference (BI) was used to estimate a phylogeny. The most appropriate model of molecular evolution was defined with the AIC, using PartitionFinder2 (Lanfear *et al.*, 2016) and the phylogenetic

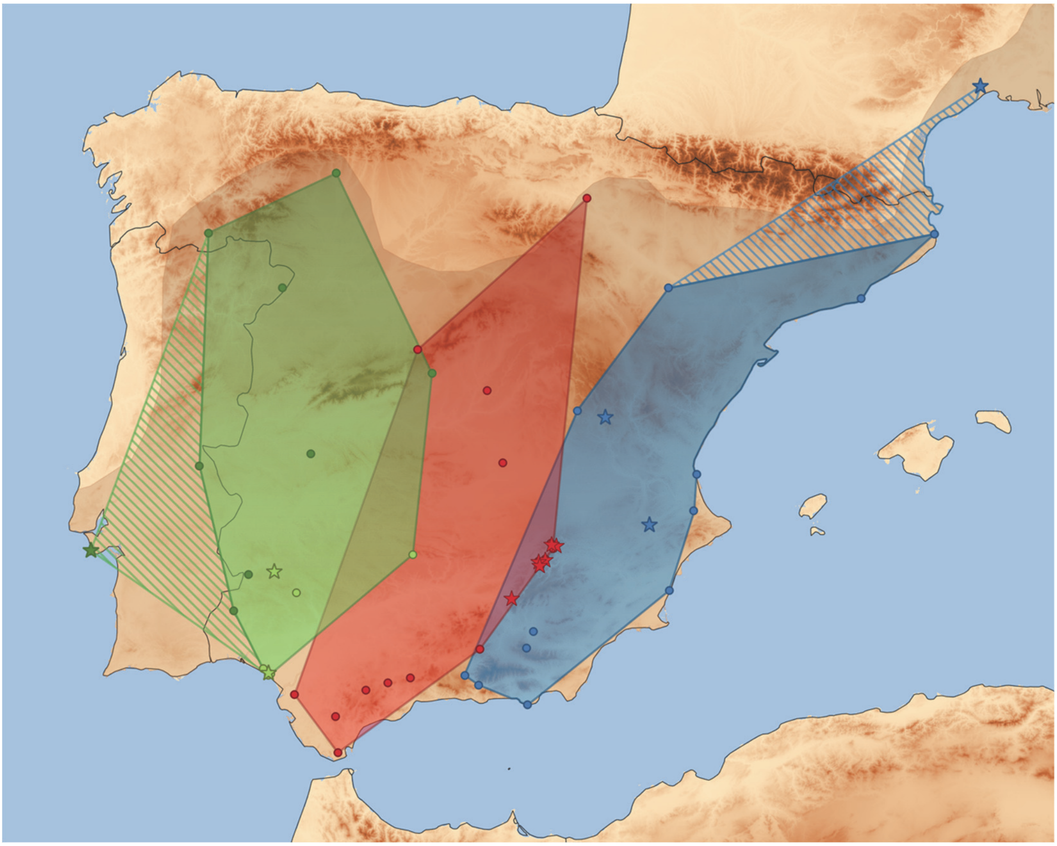

Figure 1: Map showing distribution of the three species (*P. occidentalis* in green, *P. hispanicus* in red, and *P. edwarsianus* in blue) across the Iberian Peninsula. Minimum convex polygons are modified from MOLINA *et al.* (2020), with hashed areas showing the additional areas incorporated based on the new sequences from this study. Previously published data are marked with circles, new data with stars. The two lineages within *P. occidentalis* are indicated in light and dark green. For a correct interpretation of the figure, the reader is referred to the online, coloured version of the article.

analyses implemented with BEAST2 v2.6.3 (BOUCKAERT *et al.*, 2019). BI was performed with the HKY+G model, using random starting trees, run for 10×10^6 generations, and sampled every 5000 generations. A 10% burn-in was applied to the obtained trees. Remaining data was used to estimate posterior nodal probabilities and produce a summary phylogeny. Three separate replicates were performed and compared to check for local optima (HUELSENBECK & BOLLBACK, 2001).

As expected, we recovered three major groups corresponding to the three described species (Fig. 2). The same estimate of relationships was also recovered, with *P. edwarsianus* sister taxa to *P. hispanicus*. Our new sequences all fell within these clades, regarding both genetic diversity and geographical distribution. Samples

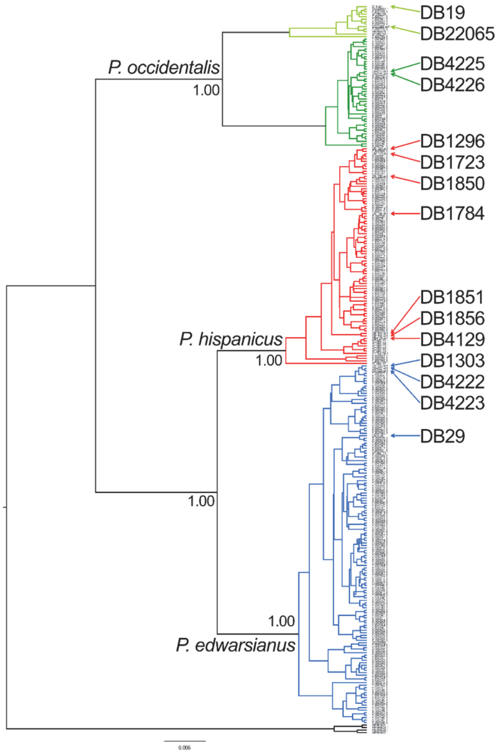

Figure 2: Obtained estimate of relationships between the three Iberian species. Bayesian Posterior Probabilities are indicated by relevant nodes. Species are distinguished by colour, with the two lineages within *P. occidentalis* indicated in light and dark green. The tree was rooted with *Gallotia caesaris.*

from France were as expected identified as *P. edwarsianus*, and those from Portugal, either coastal or inland, as *P. occidentalis*. This allows us to extend the polygons proposed by Molina *et al.* (2020) to cover most of the known ranges (Fig. 2). Our estimate of relationships also indicates a relatively deep divergence within *P. occidentalis*, with five localities from the southeastern part of the range notably distinct from remaining populations (indicated as light green versus dark green in Fig. 2). It is clear from Fig. 2 that this divergence is notable, almost as great as the divergence between *P. edwarsianus* and *P. hispanicus*. Indeed, based on the number of base substitutions per site from averaging over all sequence pairs between groups, estimated in MEGA X v10.0.5 (Kumar *at al.*, 2018), divergence between *P. hispanicus* and *P. edwarsianus* is 0.059, while divergence between the two lineages of *P. occidentalis* is 0.071.

As expected, the three species are easily identifiable using this genetic marker, and show a strong geographic pattern with *P. occidentalis* in the west, *P. hispanicus* in central Iberia and *P. edwarsianus* in Eastern Iberia and Mediterranean France. Our additional samples provide genetic confirmation of the presence of *P. edwarsianus* in France, and of *P. occidentalis* in Atlantic Portugal. In this way the proposed minimum area polygons of Molina *et al.* (2020) can be notably extended, so that occurrence across the majority of the range can be predicted. Certainly, this is only a first approximation since better geographic representation and stronger predictive power could be achieved using ecological models (e.g. Kaliontzopoulou *et al.*, 2008). This is really needed since current knowledge on ecological niche separation between all three species relies solely on Spanish records (Carretero *et al.* 2018). One of the few areas that still needs to be confirmed remains southern Portugal, across Alentejo and the Algarve. All of the new samples included in this study from the potential area of overlap between *P. hispanicus* and *P. edwarsianus*, as proposed by Molina *et al.* (2020), all belong to *P. hispanicus*. Thus, even with additional sampling in these areas, no signs of sympatry have been detected.

Again, the two previously identified deep sublineages within *P. occidentalis* (FITZE *et al.*, 2011; MENDES *et al.*, 2017; MOLINA *et al.*, 2020) have been recovered, with an estimated diversity of around 7%, close to the value obtained by MOLINA *et al.* (2020). Two more samples have been added for the southeastern lineage, with one expanding its distribution further to the east in southern Badajoz, in Valencia del Ventoso. This puts the closest records of individuals from the two lineages at just over 30 km. This intra-specific diversity, while slightly lower than that typically identified between species of lacertids for this mitochondrial marker, clearly warrants further assessment.

Additional sampling has helped to determine the distribution of *P. occidentalis*, *P. hispanicus* and *P. edwarsianus* within the Iberian Peninsula and Mediterranean France. This is relevant for Portugal and France, where until now environmental authorities were determining the species present in their respective countries mostly based on distribution and on morphological identification based on a single scalation trait rather than any genetic evidence. Remarkably, there continues to be no known areas of sympatry. Samples from southern Portugal are still needed to confirm that this area is occupied by *P. occidentalis*. Within *P. occidentalis*, two deep mitochondrial lineages occur. Further work is needed to evaluate whether these coincide with variation at nuclear loci, or with morphological variation.

Acknowledgements

DJH was funded through an FCT contract (IF/01257/2012). We thank our colleagues from CIBIO for assistance during the fieldwork, and two reviewers for useful comments on an earlier version.

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