

Unexpectedly low mitochondrial DNA variation within the ladder snake *Rhinechis scalaris*

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Abstract. The Iberian Peninsula, as an important glacial refugia for much fauna and flora, is a well known biodiversity hotspot. Of the studied herpetofauna almost all species that are not recent colonizers of the area show extensive intraspecific genetic variation, often at a level that has implied cryptic speciation. In this study variation within the ladder snake, *Rhinechis scalaris*, essentially an Iberian endemic, was examined using cytochrome b mitochondrial DNA sequences. Surprisingly no variation across the species range was found. This is an extremely unusual situation, that deserves further investigation into the causes of the low variation.

Key words: *Rhinechis scalaris*, cytochrome b, mitochondrial DNA, phylogeography, Iberian Peninsula

The ladder snake, *Rhinechis scalaris*, is a large colubrid inhabiting the Iberian Peninsula except parts of the north, and Southeast France (Arnold 2002). Although historically included with the other European rat snakes in the genus *Elaphe*, it is genetically very differentiated from these, which has led to the revalidation of the monotypic genus *Rhinechis* Michaelis, 1833 (Lenk et al. 2001, Utiger et al. 2002). Using molecular dating techniques, Nagy et al. (2004) indicate *R. scalaris* may have split from its closest relatives, a clade comprising *Elaphe quatuorlineata* and *Coronella*, approximately 20 million years ago. This would suggest that

there has been considerable time for intraspecific variation to have evolved. This is particularly relevant, since molecular phylogeographic assessments have illuminated a number of cryptic species in other Iberian reptiles including *Podarcis* (Pinho et al. 2006), *Blanus* (Vasconcelos et al. 2006) and *Iberolacerta* (Carranza et al. 2004a). Diversity has also been considerable in most widespread reptile groups studied in this region, including *Lacerta schreiberi* (Godinho et al. 2006), *Timon lepidus* (Paulo 2001), and *Psammodromus algirus* (Carranza et al. 2006a). It is well known that Southern European areas such as the Balkans, Italy and the

Iberian Peninsula acted as refugia for many species during the climatic fluctuations of the Pleistocene (Hewitt 2000). Further, the findings of highly divergent groups within species from the Iberian Peninsula have led to the description of “refugia within refugia” (Gomez & Lunt 2007), with distinct parts of the Iberian Peninsula acting as separate refuges and allowing extensive genetic differentiation to develop. In this context, the aim of this study was to assess levels of diversity within *R. scalaris* using Cytochrome *b* mitochondrial DNA sequence data to assess if this species showed similarly high levels of genetic diversity.

The number and geographic locations of the specimens used in this study are given in Table 1 and Figure 1. Most samples were comprised of roadkills. Live individuals were released after tail tips were collected. Tissue samples were stored in 100% ethanol. Total genomic DNA was extracted from tissue samples following the Sambrook *et al.* (1989) proto-

cols. Polymerase Chain Reaction primers used in both amplification and sequencing were GluDG and Cytb2 from Palumbi *et al.* (1991) and Kocher *et al.* (1989) respectively. Amplification conditions were the same as described by Harris *et al.* (1998). Amplified fragments were sequenced on a 310 Applied Biosystem DNA Sequencing Apparatus. DNA sequences were aligned by eye. Three specimens from GenBank (Lenk *et al.* 2001; Nagy *et al.* 2004) were also analysed.

In total 333 base pairs of mitochondrial DNA were analysed for 12 new specimens and three specimens from GenBank, although two of these (Lenk *et al.* 2001) were 16 base pairs shorter. All the sequences for this region were identical, except for one C-T transition in one sample from Nagy *et al.* (2004), from Spain. The single haplotype recovered from the specimens sequenced in this study has been submitted to GenBank (EU497634).

Table 1. Localities of samples used, and their position in Figure 1.

Locality:	Country:	Coordinates:
Vila Cova, Barcelos, Braga district	Portugal	41° 32' N, 8° 42' W
Castelo Branco, Castelo Branco district	Portugal	39° 52' N, 7° 07' W
Resende, Viseu district	Portugal	41° 06' N, 7° 58' W
Sintra, Lisboa district	Portugal	38° 47' N, 9° 23' W
Motilla del Palancar, Cuenca province	Portugal	39° 34' N, 1° 55' W
Touro, Vila Nova de Paiva, Viseu district	Portugal	40° 53' N, 7° 45' W
Laroya, Almeria province	Spain	37° 17' N, 2° 19' W
Fermoselle, Zamora province	Spain	41° 18' N, 6° 24' W
Ribeira de Pena, Vila Real district	Portugal	41° 31' N, 7° 48' W
Vale de Algosos, Vimioso, Bragança district	Portugal	41° 29' N, 6° 32' W
Fornillos, Zamora province	Spain	41° 22' N, 6° 19' W
Monte La Algaida, Sevilla province	Spain	36° 50' N, 6° 18' W



Figure 1. Map showing the sampling locations of *R. scalaris* sequenced for this study. Codes are given in Table 1.

Such low diversity within a widespread snake is unexpected, especially given the very high levels of intraspecific variation typically seen in herpetofauna from the Iberian Peninsula. Although sampling was limited, they do cover a wide area and so this is unlikely to be the reason that diversity is so low. In the viperine snake, *Natrix maura*, there is high differentiation between samples in Southern Iberia and the rest of the Peninsula (Guiking et al. 2006; Barata et al. in press). This southern region shows no differentiation in *R. scalaris*. Diversity within the Iberian Peninsula

is also high in the relatively closely related *Coronella austriaca* partially using the same gene region (Santos et al. 2008). Diversity is low in the Iberian Peninsula in the snakes *Malpolon monspessulanus*, *Hemorrhois hippocrepis* (Carranza et al. 2006b) and *Macroprotodon brevis* (Carranza et al. 2004b; Vasconcelos & Harris 2006), but these species all show extensive variation in North Africa, and seem to be relatively recent colonizers of the Iberian Peninsula. The low diversity within *R. scalaris* would then require that it too was found in North Africa but has either gone unnoticed or went extinct if

a similar hypothesis for this species was made. A different hypothesis would be that *R. scalaris* suffered an extreme range decline during the last glacial periods, and lost most genetic diversity. Current populations would then reflect a recent expansion from a single refugium. A similar situation occurs in the Wall lizard *Podarcis bocagei*, but this species is found only in the Northwest Iberian Peninsula – other *Podarcis* species in the south of the Iberian Peninsula still contain extensive genetic variation (Pinho *et al.* 2007). In comparison with the other common Iberian large snakes *Malpolon monspessulanus* and *Hemorrhois hippocrepis*, *R. scalaris* displays early breeding due to summer spermatogenesis (Pleguezuelos & Feriche *in press*); a stenophagous diet restricted to endotherms and in particular mammals, all actively hunted (Pleguezuelos *et al.* 2007); and high optimal temperatures despite partial nocturnal activity (Blázquez 1995). Such life history traits may have allowed *R. scalaris* to face the rigorous conditions during the glaciations in better conditions than the other two species, both late breeders, with more reptile consumption and diurnal (Moreno-Rueda & Pleguezuelos, 2007), which went extinct in Iberia and then recolonized the area from North Africa (Carranza *et al.* 2006b). Nevertheless, the ladder snake was probably the largest reptile surviving in Iberia during this period in a worse situation than small lizards and it apparently became restricted to a small, single refugial area. A very different explana-

tion for the low diversity could be the phenomenon of a “selective sweep”, where direct or indirect selection leads to a near fixation of a mitochondrial DNA lineage within a species (reviewed in Ballard & Whitlock, 2004). It is now more clearly understood how the history of the mitochondria may not always reflect the history of the species, and assessment of nuclear markers is clearly needed to fully understand the situation in *R. scalaris*. The species does however seem to stand as the exception to the rule of very high intraspecific mitochondrial variation in Iberian herpetofauna.

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