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New and highest record of the rare Szechwan ratsnake, *Euprepiophis perlaceus* (Serpentes: Colubridae) from Muge Co, Kangding, Garzê, China

Dedicated to Klaus-Dieter Schulz (†2019)

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Abstract

Little is known about the distribution, biology, and ecology of the rare, endemic Szechwan ratsnake. Here, molecular data of the species are provided from a habitat at 3370 m a.s.l., the so far highest record of this snake. Genetic analysis (mtDNA and nDNA) confirmed the identity of the specimen as *E. perlaceus* and its close relationship to *E. mandarinus*. P-distances are in the range known for Elaphids and *Euprepiophis* as interspecific.

Key words: *Euprepiophis*, high elevation, Muge Co lake basin, southeast margin of Tibet, species identification

Introduction

The Szechwan ratsnake, *Euprepiophis perlaceus* (Stejneger, 1929), had been originally described as *Elaphe perlacea* based on a single male specimen from Yachow (Ya'an Prefecture), Sichuan Province, China (holotype USNM 76257), Fig. 1. Schulz (1989) suggested assigning this species as a subspecies of *Elaphe mandarinus* due to the morphological similarity and overlapping distribution range of the two taxa. Morphological data of three additional *Elaphe perlacea* specimens were presented by Zhao (1990). More recently, the phylogenetic relationships within the genus *Elaphe* were revised, placing *Elaphe mandarina* and *Elaphe conspicillata* in the genus *Euprepiophis*, as well as *Elaphe perlacea* because of its close morphological relationship with these two species (Utiger et al. 2002). However, the taxonomic status of the Szechwan ratsnake remained controversial until a phylogenetic study which included three specimens of that species from unknown localities (Chen et al. 2014). Based on genetic data and coalescent models, this study determined *Euprepiophis perlaceus* as distinct species. Supporting morphological data for the species validity were recently provided, based on 20 specimens collected in Shimian County, Chongzhou City, and Kangding County (Zhou et al. 2019).

Very little is known about the ecology, biology, and distribution of *Euprepiophis perlaceus*. So far, this species has been recorded from mountainous regions between elevations of 1600 and 2400 m a.s.l., within a narrow range in western Sichuan, and Shaanxi Province, China (Fig. 1; supplementary Tab. S1) where it is found in natural, humid broad-

leaf forests and shrubby grassland (Ding et al. 2017; Hu et al. 2002; Tang et al. 2021). Their life history characteristics and ecology probably resemble those of *E. mandarinus* (Schulz 1996).

In the present study, molecular data, and a new record of *Euprepiophis perlaceus* from Sichuan are provided. Mitochondrial and nuclear DNA sequence data were used to validate the identity of the specimen by assigning them to existing *E. perlaceus* sequences. These recent data may support future research on this taxon in the mountains of Western Sichuan and potentially suitable adjacent regions.

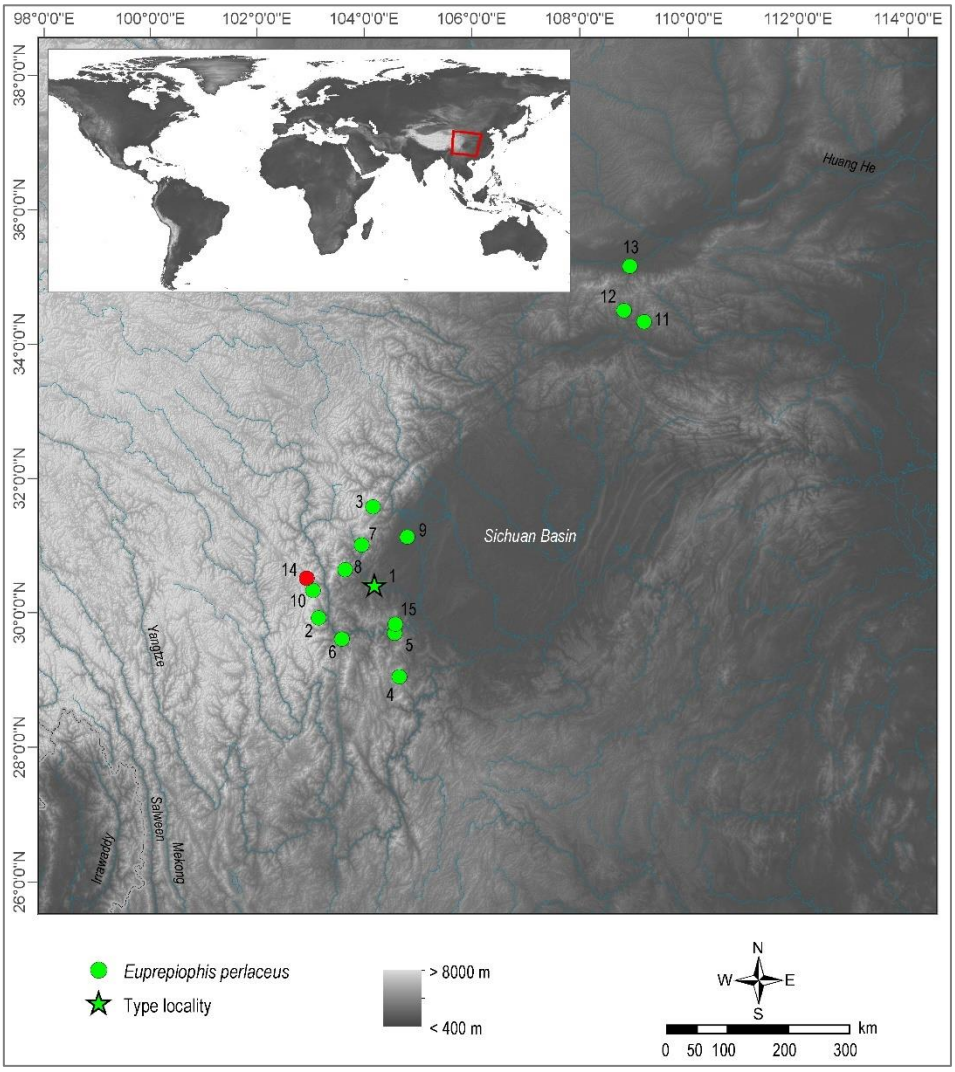

Figure 1. Map showing the locality of the *Euprepiophis perlaceus* specimen reported herein (red dot; locality number 14) and further known records (for details, see supplementary Tab. S1). The type locality of *E. perlaceus* is indicated by a star.

Methods

Tissue sample, DNA sequencing and analysis

Two adult individuals (one dead, one alive) were found in Muge Co area of Gongge mountains, Kangding County, Garzê, China (30.19°N, 101.87°E, 3370 m; Fig. 1, supplementary Tab. S1). A scale sample was taken from the dead specimen, transferred into absolute ethanol, and stored at −20 °C. Genomic DNA was extracted from tissue using the DNeasy Blood & Tissue Kit (Qiagen, Venlo, Netherlands) following the manufacturer's protocol. Approximately 351 bp of the 12S and 446 bp of the 16S ribosomal RNA (rRNA), 666 bp of the COI, 662 bp of the ND2, 678 bp of the ND4, 781 bp of the cytb, and a fragment

of 567 bp of the nuclear c-mos gene were amplified via the polymerase chain reaction (PCR) using primers and PCR conditions as previously described (12S: Utiger et al. 2002; 16S: Hedges 1994; COI: Nagy et al. 2012; ND2: L4437b/tRNA-trpR, Macey et al. 1997, Ashton and de Queiroz 2001; ND4: Forstner et al. 1995; cytb: H15547/L14724, Edwards et al. 1991, Irwin et al. 1991; c-mos: Lawson et al. 2005). PCR products were purified using the mi-PCR Purification Kit (Metabion, Planegg, Germany) and sequenced in both directions by Macrogen (Amsterdam, Netherlands; http://www.macrogen.com).

We aligned the new sequences (accession numbers 12S: xxx, 16S: xxx, COI: xxx, ND2: xxx, ND4: xxx; cytb: xxx; c-mos: xxx) to data of the three *Euprepiophis* species, *Elaphe taeniura*, *Elaphe carinata*, and *Ptyas korros* (outgroup) available from GenBank (supplementary Tab. S1) by eye. Alignment of protein coding regions based on nucleotides and amino acids produced similar results, since no ambiguities, such as deletions, insertions, or stop codons, were found. The final concatenated rRNA + mtDNA + nDNA sequence dataset consisted of 11 taxa and contained 4151 alignment positions of which 590 were phylogenetically informative. We generated a Bayesian-inference (BI) tree using MrBayes v.3.2.6 (Ronquist et al. 2012). The dataset was partitioned a priori by gene and codon fragments, and PartitionFinder 1.1.1 (Lanfear et al. 2012) was applied to optimize partitions using linked branch lengths, the corrected Aikaike Information Criterion, the greedy search algorithm, and the substitution models implemented in MrBayes. Analysis was run for 10 million generations, with four parallel Markov chain Monte Carlo simulations and four chains (one cold and three heated), sampling trees every 1000th generation. Inspection of the standard deviation of split frequencies after the final run as well as the effective sample size value of the traces using Tracer v. 1.7.1 (Rambaut et al. 2018) indicated convergence of Markov chains. The first 25% of the samples of each run were dropped as burn-in.

Pairwise uncorrected p-distances were assessed separately for c-mos and ND2 sequence data using Mega-X v.11.0 (Kumar et al. 2018) with the pairwise deletion option, 1000 bootstrap replications, and by considering both transitions and transversions.

Results and Discussion

The first Szechwan ratsnake was spotted near a road surrounded by deciduous broad-leaved forest, moving fast under rocks. The second snake was found dead beside the road, not far from the first individual (Figs. 2).

Figure 2. Dead specimen of *Euprepiophis perlaceus* (left panel) found in a mountainous habitat (right panel) at an elevation of 3370 m a.s.l. in Muge Co area, Kangding County, Garzê, China.

The identity of the dead snake specimen was confirmed as *Euprepiophis perlaceus*. Our sequences nested in the clade of *E. perlaceus*; the placement within this clade was highly supported (Fig. 3). Pairwise p-distances between mitochondrial ND2 sequences of different *Euprepiophis* species ranged between 11.9 (*E. conspicillatus*/*E. perlaceus*) and 14.2 % (*E. conspicillatus*/*E. mandarinus*); for c-mos p-distances were 0.53 (*E. conspicillatus*/*E. mandarinus*; *E. mandarinus*/ *E. perlaceus*) and 0.71 % (*E. conspicillatus*/*E. perlaceus*), respectively (Tab. 1, 2). These genetic distances are moderately lower than the evolutionary distances between *Elaphe* species but in the range of interspecific distances.

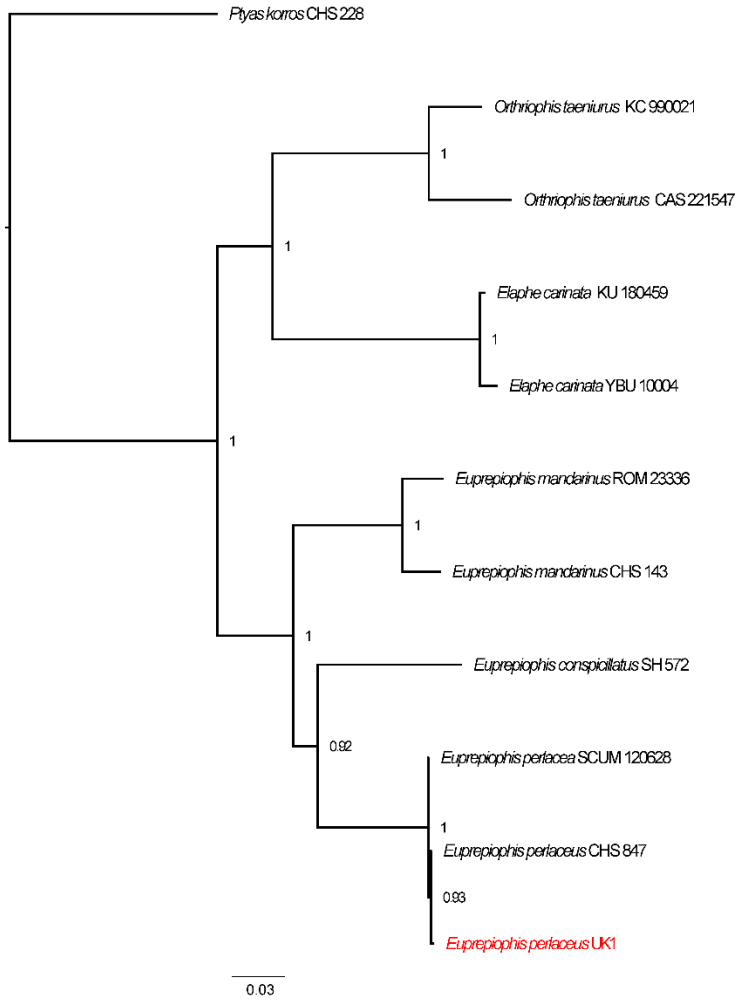

Figure 3. Bayesian inference tree based on concatenated rRNA, mtDNA and nDNA sequence data inferred with MrBayes. Species name is followed by the sample or voucher ID. Numbers on branch nodes represent posterior probability values. ID of the sample sequenced herein is indicated red.

Table 1. Pairwise distance matrix (lower: uncorrected p-distances, %; upper: standard error) for ND2 sequences of samples. Cells with intraspecific distances are indicated grey.

	carinata	*carinata*	*taeniurus*	*taeniura*	*conspicillatus*	*mandarinus*	*mandarinus*	*perlaceus*	*perlaceus*	UK1
E. carinata KU180459		0.40	2.22	2.39	2.46	2.52	2.47	2.26	2.26	2.34
E. carinata YBU10004	1.06		2.24	2.42	2.52	2.60	2.55	2.33	2.33	2.40
E. taeniura AH015912	18.00	18.40		1.19	2.54	2.46	2.45	2.30	2.30	2.29
E. taeniurus KC990021	19.75	20.16	7.05		2.58	2.44	2.42	2.25	2.25	2.35

E. conspicillatus DQ902213	19.77	20.38	20.37	20.38		1.87	1.90	1.61	1.61	1.66
E. mandarinus CHS143	19.68	20.29	19.60	19.48	14.12		1.00	1.76	1.76	1.87
E. mandarinus ROM23336	19.95	20.57	19.86	19.27	14.24	5.22		1.73	1.73	1.82
E. perlaceus CHS847	18.10	18.70	17.59	17.52	11.85	12.55	12.21		0.00	0.00
E. perlaceus SCUM120628	18.10	18.70	17.59	17.52	11.85	12.55	12.21	0.00		0.00
UK1 (this study)	18.35	18.78	17.01	17.63	11.71	13.05	12.49	0.00	0.00	

Table 1. Pairwise distance matrix (lower: uncorrected p-distances, %; upper: standard error) for c-mos sequences of samples.

	carinata	*conspicillatus*	*mandarinus*	UK1	*taeniura*
E. carinata YBU10004		0.47	0.46	0.54	0.35
E. conspicillatus DQ902065	1.28		0.30	0.36	0.38
E. mandarinus ROM23336	1.28	0.53		0.29	0.42
UK1 (this study)	1.65	0.71	0.53		0.46
E. taeniura CAS221547	0.73	0.89	1.07	1.25	

The new record matches the preferred habitat conditions of *E. perlaceaus*, which include deciduous broadleaf forest and mixed evergreen deciduous broad-leaved forest in temperate mountains (Ding et al. 2017; Gan et al. 2017; Tang et al. 2021). However, given the elevation of the new observation, the vertical distribution of the snake seems to be wider than previously reported. Thus, potential habitats of the species might be available in large parts of the Hengduan Shan and mountains around the Sichuan Basin, e.g., the Daba Shan, Qinling Shan and even the Wu Shan. Indeed, a recent study reported on records of *E. perlacea* from Qinling Mountains, Foping County, Shaanxi Province and suspected a continuous distribution range of the snake in the neighboring provinces Sichuan and Gansu (Tang et al. 2021).

According to the on IUCN Red List assessments of Asian snake species (DECISION 16.104), major threats for the Szechwan ratsnake are habitat loss, and overharvesting for food and skins. Another threat is the increasing, unregulated trade in this snake for pet (Tang et al. 2021), especially in the European and American markets, highlighting the need for detailed *in situ* research (e.g., biology, distribution).

Conclusions

Here a new and so far, highest record of *Euprepiophis perlaceus* from Kangding County is reported. Species validation is based on multi-loci DNA sequence data of *E. perlaceus* and congeners. The study provides the first high-altitude record of the Szechwan ratsnake, supporting the assumption of a wider distribution of that species than previously believed.

Acknowledgements

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