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26 **The mitogenome of *Elaphe bimaculata* (Reptilia: Colubridae) has never been published:**  
27 **a case with the complete mitochondrial genome of *E. dione***

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48 Abstract. The steppes ratsnake, *Elaphe dione* (Pallas, 1773), is widely distributed across Eurasia, but the  
49 systematics and phylogeography of this species remain poorly studied. Sequencing of the full  
50 mitochondrial genome of this species provides a reference for its further study. Here, we report the full  
51 mitochondrial genome of an *E. dione* specimen from Krasnoyarsk Krai (East Siberia, Russia). We  
52 found that it is highly similar to the previously reported mitochondrial genome of the sister species, *E.*  
53 *bimaculata*. Both species misidentification by the authors of *E. bimaculata* mitogenome and the  
54 introgressive hybridization between these taxa can possibly explain this observation.

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56 Keywords. Colubridae, *Elaphe*, mitogenome, phylogeny, Siberia

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59 Ratsnakes of the genus *Elaphe* make up a widely distributed colubrid group of 15 species  
60 (The Reptile Database: Uetz et al., 2018), which inhabits a range from Western Europe to the  
61 Russian Far East and China. Some closely related genera (often also referred to as “ratsnakes”  
62 or *Elaphe* sensu lato) such as *Pantherophis*, *Zamenis*, *Gonyosoma*, etc. inhabit zones with  
63 temperate, subtropical and tropical climate almost all over Eurasia and North America.  
64 Relatively few mitochondrial genomes of ratsnakes have been sequenced so far, excluding *E.*  
65 *anomala* (Liu and Zhao, 2015a), *E. bimaculata* (Yan et al., 2014), *E. carinata* (Ding et al.,  
66 2016), *E. davidi* (Xu et al., 2015), and *E. schrenckii* (Liu and Zhao, 2015b).

67 The steppes ratsnake, *Elaphe dione* (Pallas, 1773), is the most widespread species of the  
68 genus. It is present from Ukraine in the west to the shores of the Pacific Ocean in the east, and  
69 from the 56th degree of latitude in Russia in the north to Iran in the south (Schulz, 2013).  
70 Type locality of the species is “Gratscheffskoi outpost, near Semijarsk, upper Irtysh area,  
71 Semipalatinsk district”, Kazakhstan [currently Grachi village, Beskaragay district of East  
72 Kazakhstan Region] (restricted by Mertens and Mueller, 1928). The systematics of this  
73 species remains controversial: so far, several subspecies have been described (such as *E. d.*  
74 *tenebrosa* Sobolevsky, 1929 and *E. d. czerskii* Vedmederya et al., 2009), but none of them  
75 have been widely accepted. While the mitogenome of the steppes ratsnake has never been  
76 sequenced, it would provide an important resource for further studies in systematics and  
77 phylogeography of this widespread species. Therefore, we sequenced and annotated the  
78 complete mitochondrial genome of *E. dione* specimen and reconstructed the mitogenome  
79 phylogeny with other related species of the genus.

80 DNA was sampled via non-lethal buccal swabs from *E. dione* collected in Krasnoyarsk  
81 Krai, Russia (53.59°N 91.64°E) in June 2016, and extracted using standard proteinase K and  
82 phenol-chloroform methods (Sambrook et al., 1989). DNA quality and concentration were  
83 examined by electrophoresis in 1.5% agarose gel and Qubit fluorimeter, respectively (Thermo

84 Fisher Scientific, USA). The DNA was fragmented using an ultrasonic Bioruptor Sonication  
85 System (Diagenode), and paired-end libraries were prepared using the TruSeq DNA LT  
86 Sample Prep Kit (Illumina) according to the TruSeq DNA Sample Preparation Guide. The  
87 quality control of the prepared library was carried out on the electrophoretic system  
88 Bioanalyzer 2100 (Agilent Technologies) using Agilent DNA 1000 Reagents (Agilent  
89 Technologies). The fragment size was approximately 400 bp (with insert size 260-280 bp).  
90 The library was sequenced on the MiSeq Illumina platform using the MiSeq Reagent Kit v3  
91 (300-cycle, 2x150 bp) Illumina kit at the Laboratory of Forest Genomics, Siberian Federal  
92 University.

93 Read quality was assessed with FastQC 0.11.7 (Andrews, 2010). Adapter and quality  
94 trimming was performed using CLC Genomics Workbench (CLC bio, Aarhus, Denmark). To  
95 assemble the mitochondrial genome, reads were mapped to previously published  
96 mitogenomes of congeneric species: *E. bimaculata* (KM065513.1) and *E. schrenckii*  
97 (KP888955.1). Successfully mapped reads were merged into single consensus sequence  
98 representing mtDNA of *E. dione*. All aforementioned steps were also done with CLC  
99 Genomics Workbench.

100 The *E. dione* mitochondrial genome was annotated in the MITOS2 web server  
101 (<http://mitos2.bioinf.uni-leipzig.de/index.py>), manually checked and corrected for errors.  
102 Mitochondrial genomes of *E. anomala* (KP900218.1), *E. bimaculata* (KM065513.1), *E.*  
103 *carinata* (KU180459.1), *E. davidi* (KM401547.1), and *E. schrenckii* (KP888955.1) were  
104 obtained from GenBank to examine phylogenetic relationships between *E. dione* and related  
105 taxa basing on complete mtDNA sequences. Some members of closely related genera were  
106 used as outgroup: *Oocatochus rufodorsatus* (KC990020.1), *Orthriophis taeniurus*  
107 (KC990021.1), *Oreocryptophis porphyraceus* (GQ181130.1), *Pantherophis slowinskii*  
108 (DQ523162.1), and *Pituophis catenifer* (KU833245.1). A multiple sequence alignment was

109 produced by Clustal Omega (Sievers et al., 2011) and trimmed with Gblocks (Talavera and  
110 Castresana, 2007); 95% (16,631) of the original 17,330 bp alignment remained after  
111 trimming. Maximum likelihood (ML) phylogenetic tree was inferred with IQ-TREE 1.6.1  
112 (Nguyen et al., 2015) using the TIM2+F+I+G4 substitution model (selected within 286 tested  
113 models by ModelFinder; Kalyaanamoorthy et al., 2017) and 1,000 ultrafast bootstrap  
114 replicates (Hoang et al., 2018). The uncorrected genetic distances between species were  
115 calculated in MEGA7 (Kumar et al., 2016) with pairwise deletion of gaps/missing data.

116 In total, 2,132,080 Illumina paired-end reads were generated. We successfully  
117 retrieved 16,994 bp of sequence data of the *E. dione* mitochondrial genome with an average  
118 coverage of 15x (0.09% of all reads were mapped to mtDNA). No differences were found  
119 between the mtDNA sequences generated by mapping to *E. bimaculata* or *E. schrenckii*  
120 reference mitogenomes. The very small portions of the ND5 gene and the second D-loop  
121 region were not covered by the obtained reads. We estimated the length of the non-covered  
122 region was 178 bp. Thus, the full length of the *E. dione* mitochondrial DNA was around  
123 17,172 bp with only ~1% not covered. The newly generated mitogenome is available under  
124 NCBI GenBank accession number MH460961.

125 The phylogenetic tree based on full mitochondrial genomes agreed with previous  
126 studies, placing members of genus *Elaphe* into a distinct monophyletic group (Utiger et al.,  
127 2002; Chen et al., 2010). The uncorrected genetic distance (*p*-distance) between the  
128 mitogenome of *E. dione* and the previously published mitogenome of *E. bimaculata*  
129 (KM065513.1) was 0.89% (for 16,989 aligned sites), while the mean distance between other  
130 *Elaphe* species is 10.1% (Table 1). Thus, the mitogenome of *E. dione* from Krasnoyarsk Krai  
131 was highly similar to the recently sequenced genome of its sister species *E. bimaculata*. The  
132 observed distance between the two genomes was too low even for closely related species and  
133 is rather at the intra-specific level.

134 The same result was reported by Hofman et al. (2016), when they compared 12S, ND4,  
135 Cyt b, and COI sequences with this *E. bimaculata* mitogenome. However, the pronounced  
136 genetic difference between the two considered species has been shown previously (Utiger et  
137 al., 2002) and confirmed by Hofman et al. (2016). To further clarify this situation, we  
138 extracted partial sequences of the 12S rRNA mitochondrial gene from mitogenomes of both  
139 species and compared them to the 12S sequences of *E. bimaculata* and *E. dione* available in  
140 GenBank. On the 12S gene tree (Fig. 1B), some *E. bimaculata* sequences form a separate  
141 clade, distinct from the *E. dione* lineage. Two other *E. bimaculata* sequences (including the  
142 one extracted from the mitogenome) clearly fall into the *E. dione* cluster. The *E. dione* sample  
143 used in our work belongs to the *E. dione* clade. It is evident that the mitogenome of “*E.*  
144 *bimaculata*” sequenced by Yan et al. (2016) belongs to *E. dione*. The two species are very  
145 similar phenotypically, and their ranges overlap in China (Schulz, 2013; Wallach et al.,  
146 2014). Thus, species misidentification by the authors of *E. bimaculata* mitogenome could be a  
147 possible explanation. An alternative explanation is introgressive hybridization between the  
148 species. This phenomenon is well documented in animals (including reptiles) and results in  
149 bidirectional or unidirectional introgression of mtDNA (e.g. Plötner et al., 2008; Machado et  
150 al., 2014; Ermakov et al., 2015; Johnson et al., 2015). If hybridization between these two  
151 species indeed occurs in the area of their sympatry, an *E. bimaculata* specimen with  
152 introgressed mtDNA could be accidentally used for the mitogenome sequencing.  
153 Unfortunately, Yan et al. (2016) did not provide any information about geographical origin or  
154 other details of the specimen they used for mtDNA sequencing.

155 By this communication, we would like to not only provide a reliable mitogenome of *E.*  
156 *dione*, but also highlight the need for careful selection and documentation of specimens  
157 intended for full mitogenome/genome sequencing to avoid further confusion.

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234 **Table 1.** Uncorrected genetic distances (%) between mitochondrial genomes of some species  
 235 of the genera *Elaphe*, *Orthriophis*, *Oocatochus*, *Pituophis*, *Pantherophis*, and  
 236 *Oreocryptophis*.

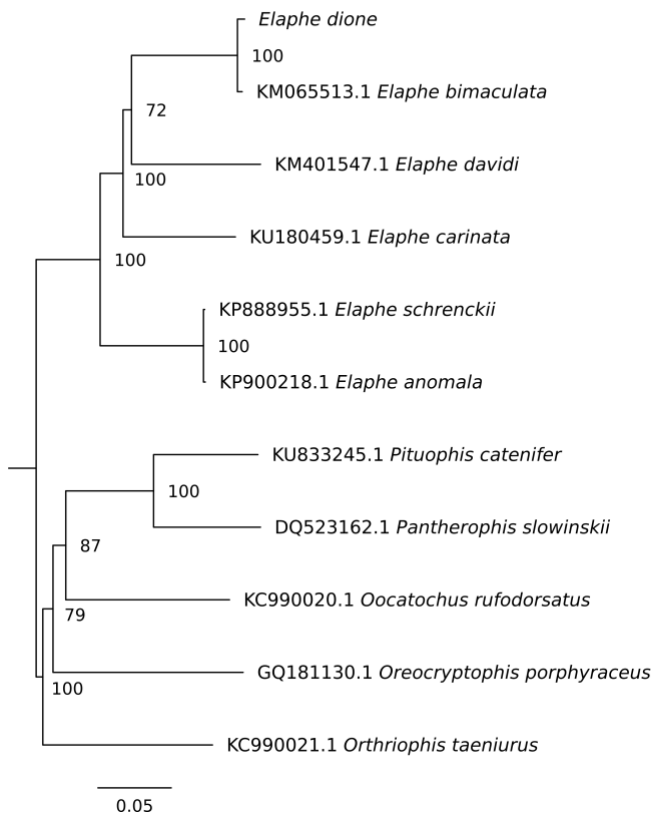
Species	1	2	3	4	5	6	7	8	9	10
1. <i>Elaphe dione</i>	-									
2. <i>Elaphe bimaculata</i>	<b>0.9</b>	-								
3. <i>Elaphe schrenckii</i>	<b>10.9</b>	<b>10.8</b>	-							
4. <i>Elaphe carinata</i>	<b>10.7</b>	<b>10.7</b>	<b>10.6</b>	-						
5. <i>Elaphe anomala</i>	<b>10.9</b>	<b>10.9</b>	<b>0.2</b>	<b>10.6</b>	-					
6. <i>Elaphe davidi</i>	<b>11.0</b>	<b>10.8</b>	<b>11.2</b>	<b>10.7</b>	<b>11.2</b>	-				
7. <i>Orthriophis taeniurus</i>	13.7	13.6	13.1	13.7	13.1	14.1	-			
8. <i>Oocatochus rufodorsatus</i>	13.7	13.6	13.6	14.1	13.6	14.5	13.6	-		
9. <i>Pituophis catenifer</i>	14.3	14.2	13.8	14.2	13.8	14.9	14.0	13.5	-	
10. <i>Pantherophis slowinskii</i>	14.3	14.3	14.0	14.3	14.0	14.8	14.0	13.4	9.9	-
11. <i>Oreocryptophis porphyraceus</i>	14.5	14.4	14.1	14.7	14.2	15.0	13.7	13.3	14.6	14.3

237 The distances between species of the genus *Elaphe* are highlighted in bold.

238 **Fig. 1.** (A) Maximum likelihood phylogenetic tree of the *Elaphe* sensu lato group based on  
239 full mitochondrial genomes; (B) maximum likelihood gene tree of *E. dione* and *E. bimaculata*  
240 based on 12S rRNA sequences. Bootstrap values above 50 are indicated.

241

A



B

