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Research Article

First evidence of the genetic structure of *Neurergus strauchii munzurensis*

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Abstract

Until 2016, two subspecies of *Neurergus strauchii* were identified; at this time, one more subspecies was described from Tunceli as *N. s. munzurensis*. However, this study mainly focused on morphological characters. We aim to test of *Neurergus* species, including *N. s. munzurensis* taxa by phylogenetic analysis, using partial base sequences of 12S rRNA and D-loop genes. For this purpose, we reconstructed Bayesian Inference and Maximum Likelihood trees with other *Neurergus* samples. According to the results, phylogenetic tree topologies demonstrate that *N. crocatus* was separated from the *N. strauchii-N. barani* lineage and the *N. strauchii* group have shown monophyly. Also, *N. s. munzurensis* showed higher genetic distances with *N. barani* (1.1%) and *N. s. strauchii* (0.8%) while genetic distances were only determined as 0.5% within *N. barani* and *N. s. strauchii*. *N. s. munzurensis* was found to be a genetically distinct taxon, following its previous morphological description. This study obtained the first evidence on better explaining the taxonomic status of *N. s. munzurensis*.

Keywords: Anatolian newt, Phylogeny, Salamandridae, Urodela

Introduction

Since Anatolian biogeography is the intersection of 3 different biodiversity hotspots, it has wealthy biodiversity in terms of amphibians and reptiles (Ficetola et al., 2018; Kurnaz, 2020). This location offers an important feature to Anatolia in terms of endemism (Kurnaz, 2020). In addition, its 180 amphibian and reptile species correspond to almost 60% of continental Europe (Kurnaz, 2020). This situation shows that Anatolia alone can have the potential of continental characteristics. A total of 22 salamander species belonging to 7 genera (*Lissotriton*, *Lyciasalamandra*, *Mertensiella*, *Neurergus*, *Ommatotriton*, *Salamandra*, and *Triturus*) are found in Anatolia. Ten of these species are endemic to Anatolia (Kurnaz, 2020). Therefore, the endemism rate of Anatolia (45%) is quite high in terms of the salamanders it hosts.

Genus *Neurergus* is represented by 5 species described in the Middle East (Iran and Iraq) and Türkiye (Rancilhac et al., 2019). While three of these species (*N. barani*, *N. crocatus*, and *N. strauchii*) are distributed in Anatolia, *N. barani* and *N. strauchii* are endemic species to Anatolia (Kurnaz, 2020). In 2016, a new subspecies of *N. strauchii* was morphologically described in Tunceli and named *N. s. munzurensis* (Olgun et al., 2016). Although various studies have been carried out to date on the species within the Genus *Neurergus*, the most comprehensive study is performed by Rancilhac et al. (2019). In this study, researchers examined the phylogeny of all species and subspecies in the *Neurergus* genus (except *N. s. munzurensis*) using the ddRADseq (double-digest Restriction Site Associated DNA sequencing) method. The research results elevated the *N. s. barani* taxa, previously known as the subspecies of *N. strauchii*, to the species level. Researchers have reported a profound genetic difference between *N. strauchii* and *N. barani*. This situation was confirmed in a different study examining the ecological niches of the two taxa, and it was reported that these two species are not only genetically different but also require different requirements in terms of ecological niches (Kurnaz & Şahin, 2021).

Rancilhac et al. (2019) study, the only missing *N. strauchii* is the other subspecies, *N. s. munzurensis* was not included in the study. This situation revealed a missing aspect of this comprehensive study. Based on this, the present study aimed to conduct a new phylogenetic study with all *Neurergus* species to clarify their taxonomic status, including the *N. s. munzurensis* taxa, using partial base sequences of 12S rRNA and D-loop genes.

Material and methods

Tissue samples were obtained from the newt specimens (1 male, 2 females, 1 subadult male, 1

subadult female, Figure 1) gathered in the Munzur Valley, Tunceli Province, Eastern Anatolia. The collection site is located at coordinates Lat: 39.195°N–Long: 39.678°E and an elevation of 1531 meters above sea level (Figure 2). The collected specimens were conserved by submerging the tissue samples in 96% ethanol at a temperature of -20°C. Afterward, the tissues were broken down into little pieces to facilitate the DNA separation process using the CTAB approach (Doyle & Doyle, 1990). A pair of samples was employed to amplify a segment of the mitochondrial 12S rRNA and D-loop genes. The amplification was performed utilizing the SThr15300L forward primer and the 12S600H reverse primer, as described by Zang et al. (2008). The amplification of 12S rRNA and D-loop genes commenced with an initial incubation phase at a temperature of 96 °C for 2 minutes. Subsequently, a total of 35 denaturation cycles were performed at a temperature of 94 °C for 30 seconds. This was followed by annealing at a temperature ranging between 45 and 55 °C for 60 seconds. Finally, the extension was carried out at a temperature of 72 °C for 5 minutes. Lastly, there was a concluding elongation phase at a temperature of 72 °C for 10 minutes. The PCR amplifications for the 12S rRNA and D-loop genes were conducted using the methodology described by Zang et al. (2008). The amplified DNA segments were purified and sequenced at BM Labosis in Ankara, Türkiye.

Figure 1. General view of the *N. s. munzurensis* specimen

Figure 2. Type locality of the *N. s. munzurensis*

The phylogenetic analyses were performed using the sequences of 12S rRNA and D-loop genes derived from the specimens collected in Türkiye, together with additional sequences of *Neurergus* species retrieved from GenBank. The optimal substitution model was determined using

JModelTest v.2.1.8 (Darriba et al., 2012), and the model with the lowest Akaike's information criteria (AIC) value was chosen (Akaike, 1974). To reconstruct the phylogenetic tree, we utilized Bayesian Inference (BI) analysis with MrBayes v.3.2.6 (Ronquist et al., 2012) and Maximum Likelihood (ML) analysis with MEGA X (Kumar et al., 2018). The BI analysis included the following parameters: ten million iterations of Markov Chain Monte Carlo (MCMC); a sampling frequency of 100; and a burn-in duration of 25%. The ML studies were conducted using a heuristic search approach called 10,000 random addition replicates, tree-bisection-reconnection branch switching. In addition, bootstrap analyses were performed using 1000 replications of the ML method (Felsenstein, 1985). Both transitions and transversions were accorded equal significance, and gaps were treated as missing data. The ML trees were evaluated using bootstrap analyses comprising 1000 iterations. The statistical validation of the generated BI trees was determined using Bayesian posterior probability (BPP). Nodes with a BPP equal to or greater than 95% were considered significant, as mentioned by Leaché and Reeder (2002). The BPP was used to infer the BI tree topology. The unaltered disparities in genetic sequences across pairs of organisms were ascertained for the 12S rRNA and D-loop genes utilizing MEGA X (Kumar et al., 2018).

Results

A 719-base pair (bp) fragment of the 12S rRNA and D-loop genes was sequenced from two newly collected *N. s. munzurensis* specimens from Türkiye, revealing important insights into their genetic structure. In analyzing this fragment, we identified 137 variable positions across the sequences. Notably, the 407th position within these 719 bp was deleted in both *N. s. munzurensis* specimens, a consistent genetic marker for this subspecies. To determine the best-fit model for our data, we conducted a model test, which indicated that the HKY+G substitution model provided the most accurate fit, allowing us to build an ML tree for these gene fragments. This ML tree, as illustrated in Figure 2, provides a visual representation of the phylogenetic relationships among the taxa, organized into two strongly supported clades with bootstrap values of 100.

The phylogenetic analysis divides the ML tree into two distinct, well-supported clades. The first clade includes *N. kaiseri* and *N. derjugini*, highlighting a close relationship between these species. The second clade encompasses four taxa: *N. crocatus*, *N. barani*, *N. s. strauchii*, and *N. s. munzurensis*. Within this second clade, the topology indicates that *N. crocatus* has diverged significantly from the *N. strauchii-N. barani* lineage (Figure 3). This branching pattern supports the monophyletic status of the *N. strauchii* group. Despite this grouping, *N. barani* was previously

identified as a separate species in literature, suggesting either a recent divergence or ongoing gene flow within these taxa.

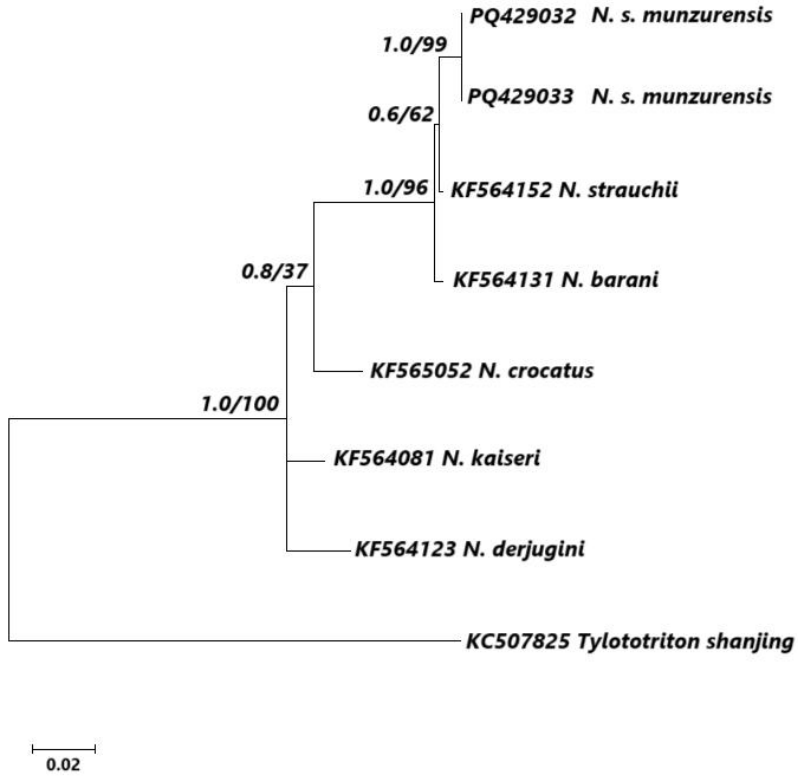

Figure 3. BI and ML tree presenting the phylogenetic relationship between the specimens of the genus *Neurergus*. The numbers left of separatrix's are BI posterior probabilities, and the numbers right of separatrix's are ML bootstrap values.

The genetic distances between these taxa, as shown in Table 1, further elucidate these relationships. Overall, the 12S rRNA and D-loop genes demonstrate low genetic distances, ranging from 0.8% to 6.2% across all taxa studied. The genetic distance between *N. barani* and *N. s. strauchii* is 0.5%, while that between *N. s. strauchii* and *N. s. munzurensis* is 0.8%. Furthermore, the distance between *N. barani* and *N. s. munzurensis* reaches 1.1%, underscoring a slightly greater divergence between these taxa.

Table 1. Genetic distances between *Neurergus* taxa, in terms of 12S rRNA and D-loop regions with best model HKY+G

	1	2	3	4	5	6
N. s. munzurensis						
N. s. munzurensis	0,000					
N. strauchii	0,008	0,008				
N. barani	0,011	0,011	0,006			
N. crocatus	0,054	0,054	0,051	0,051		
N. kaiseri	0,059	0,059	0,056	0,056	0,030	
N. derjugini	0,062	0,062	0,057	0,059	0,041	0,032

Discussion

Pasmans et al. (2006) studied only the mitochondrial 16s and 12s regions and stated that *N. s. strauchii* and *N. s. barani* are independent lineages that diverge from each other. Özdemir et al. (2009) reported that when they investigated the 12s and 16s mitochondrial gene regions, there was a nucleotide difference between *N. s. strauchii* and *N. s. barani* of 0.48% and 1.2%, but these two taxa should still be evaluated at the subspecies level. In our study, the genetic distance between *N. barani* and *N. strauchii* was found to be 0.5% and these rates are consistent with previous studies. However, *N. s. munzurensis* has a greater genetic distance to these two taxa.

Hendrix et al. (2014) studied mitochondrial 12s and nuclear gene regions (KIAA and SACS) in the genus *Neurergus* and their results showed that mitochondrial segregation was at the population level while nuclear segregation remained at the species level. They also reported that *N. s. strauchii* and *N. s. barani* were genetically separated at the population level, but not at the species level. According to the RADseq results obtained by Rancilhac et al. (2019), it would be correct to consider the two subspecies of *N. strauchii*, which are morphologically and geographically separated, as separate species due to the deep genetic differences they have. In terms of genetic distance, two different species, *N. strauchii* and *N. barani* taxa, seem to be closer to each other in terms of this gene. However, it is known that there is a deep genetic distance between these two taxa. For this reason, using this gene region for the definitive separation of taxa may not be appropriate. However, the results can be useful as a guide for further studies. According to used

gene regions, *N. s. munzurensis* was found to be a genetically distinct taxon, in accordance with its previous morphological description, such as the number and diameter of spots on head, dorsum, tail, and limbs (Olgun et al., 2016)

Kurnaz and Şahin (2021) stated that there are significant statistical differences between the ecological niches of *N. barani* and *N. strauchii*, and that this distinction may be due to the Euphrates River, which acts as a barrier during the distribution of the two species. Vaissi (2022) stated that the ecological niche between *N. strauchii* and *N. barani* is equivalent. Here, the Murat River, which also acts as a barrier between *N. s. strauchii* and *N. s. munzurensis*, is also located and *N. s. munzurensis* is located further north than *N. s. strauchii* and *N. barani*. In this case, ecological niche differentiation and genetic differentiation due to the barriers may have occurred and this taxon may have differentiated from the others.

As a result of our study, using a single gene region and only the mitochondrial region is limiting. Therefore, we recommend that more gene regions be studied by including mitogenome studies or nuclear gene regions in future studies.

Conclusion

The genetic analysis of *N. s. munzurensis* based on the 12S rRNA and D-loop gene sequences provides valuable insights into its phylogenetic position and genetic divergence. Phylogenetic reconstruction using the HKY+G model revealed two well-supported clades, reinforcing the close evolutionary relationship between *N. kaiseri* and *N. derjugini* in one group, while *N. s. munzurensis* clustered within the *N. strauchii* group along with *N. crocatus* and *N. barani*. Notably, the low genetic distances (0.5%–1.1%) observed among *N. barani*, *N. s. strauchii*, and *N. s. munzurensis* suggest a close genetic affinity, yet the greater divergence of *N. s. munzurensis* highlights its potential differentiation from the other subspecies. Given these findings, future studies should incorporate a broader set of genetic markers, including nuclear genes or complete mitogenome analyses, to further clarify the evolutionary history and taxonomic status of N. *s. munzurensis* within the *Neurergus* genus.

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