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The genus *Microhyla* (Anura: Microhylidae) in Pakistan: species status and origins

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The territory of Pakistan has been influenced by biota from different geographic directions, and is divided zoogeographically into the Palearctic and Oriental regions (Khan 2006; Masroor 2012). This makes Pakistan one of the important territories in Eurasia in the understanding of past biodiversity dynamics. Well-known examples of Oriental elements among its amphibian fauna are observed in all four families of toads and frogs currently known from Pakistan: Bufonidae, Microhylidae, Megophryidae, and Dicroglossidae. In this short contribution, we focused on the species status and the origins of the genus *Microhyla* (Microhylidae), known from the north-eastern part (Punjab, Islamabad, and Azad Jammu and Kashmir; Masroor 2012) of the country. However, Sarkar (1984), also reported *Microhyla* from Bhuj in Gujarat, India, very close to the southern Pakistani province of Sindh. This genus has not yet been reported from the Palearctic region of the country and all currently known localities are from the Oriental parts of Pakistan (i.e. eastward of the Indus River). The genus is represented in the country by *M. ornata* (Duméril & Bibron, 1841), originally reported as *Oxyglossus lima* (Khan 1968). However, in view of the overall distribution and diversity of the genus based on genetic data (Garg *et al*. 2018, 2019; Gorin *et al*. 2020), it appears that populations from Pakistan could possibly have a different evolutionary history and be different taxon (see the currently scattered range of the genus between northern and western India and Pakistan; Fig. 1). Therefore, we tested this assumption using mitochondrial (mt) and nuclear (n) DNA data.

Herein, we provide the first genetic data of the genus *Microhyla* from Pakistan. Following the methodology, primers, and PCR conditions of Garg *et al*. (2019) we used *16S* ribosomal RNA (*16S*/mtDNA/419bp) and brain-derived neurotrophic factor (*BDNF*/nDNA/662bp) genes to test the taxonomic status and origin of the genus in Pakistan. We tested DNA of four specimens from three localities with the following voucher numbers: Parera, Chakwal District, Punjab (DJ7882): 32.76°N, 73.15°E; Margalla Hills National Park, Islamabad (DJ7914, DJ7915): 33.73°N, 73.04°E; Rumli, Islamabad (DJ7926): 33.76°N, 73.13°E. For taxonomic affiliation of the species, the new sequences were checked via BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and then combined with the most similar and available sequences of relatives that comprise the *M. ornata* group (total of 79 sequences of *16S* and seven sequences of *BDNF*) from the following studies: Matsui *et al*. (2005), Howlader *et al*. (2015), Hasan *et al*. (2014), Garg *et al*. (2018, 2019) with accession sequence numbers mentioned in Figure 1. GenBank accession numbers for newly generated sequences are MT573521–MT573524 for *16S* and MT596699–MT596702 for *BDNF*. Both fragments were aligned using Clustal W algorithm (Thompson *et al*. 1994) as implemented in BioEdit (Hall 1999). The alignment was checked by eye and low-quality ends were trimmed. Ambiguously aligned regions/gaps were ignored for the analysis. To infer genealogical relationships within the examined sequences for each gene, we used a network approach (Posada & Crandall 2001) using the median-joining network implemented in the software PopArt (http://popart.otago.ac.nz). As sequences of nuclear *BDNF* were without the heterozygote positions, we did not perform a phased dataset. The DnaSP 5.10 (Librado & Rozas 2009) was used to estimate the number of haplotypes (h), nucleotide diversity (π), and uncorrected p distances.

All examined samples of *Microhyla* from Pakistan correspond in both genes with the species *M. nilphamariensis* Howlader, Nair, Gopalan, Merilä, 2015 (Fig. 1A,B) that originated from the type locality in "Koya Golahut, Saidpur, Nilphamari, Bangladesh" (*16S* GenBank sequences KP072787-93; Howlader *et al*. 2015). For *16S,* our sequences nested within three different haplotypes, two are unique for the Pakistani territory (samples DJ7882 and DJ7915+DJ7926) and one sequence (sample DJ7914) corresponds with widely distributed central network haplotype that includes populations

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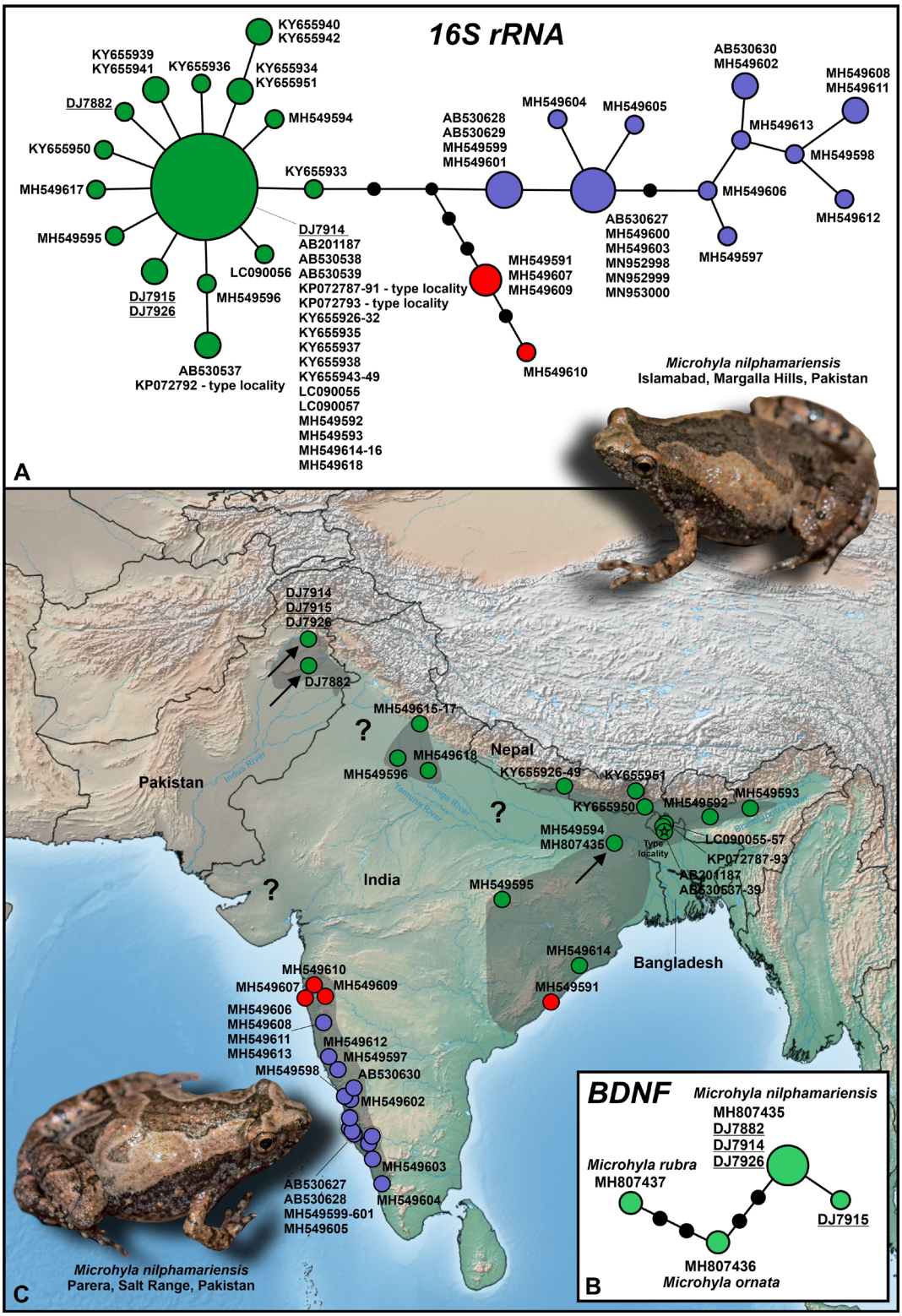

FIGURE 1. A: Median-joining haplotype network inferred from a *16S* rRNA showing the intraspecific relationships of *Microhyla nilphamariensis* with regards to Pakistani populations. B (inset): the haplotype network of *BDNF* gene representing relationships in *M. ornata* group according to Garg *et al*. (2019). C: map displaying the localities of genetically investigated (*16S* rRNA) population of the species with an expected range highlighted in grey (sensu Garg *et al*. 2018, 2019). GenBank accession numbers of analysed sequences are associated with a particular detected haplotype/locality. The type locality of *M. nilphamariensis* is marked with a star. The populations investigated using both genes are marked by arrows in the map. Question marks suggest unclear species presence. The pictured specimens originate from two investigated localities of Pakistan, Margalla Hills National Park, Islamabad (DJ7915) and Parera, Chakwal district, Punjab (DJ7882).

from Bangladesh (including the type locality of the species), India, and Nepal (Fig. 1A,C). A maximum of two mutation steps with nucleotide diversity π=0.28% were noted between all Pakistani *16S* sequences. For *BDNF*, sequences from Pakistan form two haplotypes, one that includes samples DJ7882, DJ7914, DJ7926 and published MH807435 (India: Bihar, Banka, Kaitha), and the second composed of only the sequence DJ7915 (Fig. 1B) with one mutation step between haplotypes and π=0.076%. The overall phylogeographic pattern detected in the *16S* network suggests the existence of three haplogroups in *M. nilphamariensis*. The Pakistani populations correspond with the most common haplogroup (shown in green colour) which included 15 haplotypes in total forming a star-like pattern (Fig. 1A) The distributions of this main haplogroup is centered mainly around the territories of the North Indian River Plain including the Brahmaputra, Yamuna, Ganga, and Indus rivers (Fig. 1C). The other defined haplogroups (red and violet) are more than three mutation steps in distance from the green haplogroup (uncorrected *p* distances 1.7% and 1.3%, respectively).

Our data revealed the species status of the genus in the country and showed the possible phylogeographic relationships of Pakistani population of the genus *Microhyla* to other populations in south Asia. As was expected from recent studies (Garg *et al*. 2018, 2019), *M. ornata* is not present in Pakistan and populations from the country are identified as *M. nilphamariensis*. Our results also report a significant range extension westward for *M. nilphamariensis*, that prior to this was only reported from northern Bangladesh, central and eastern Nepal, northwestern Uttar Pradesh, possibly northern Rajasthan, Kashmir, Assam, Western Ghats region of Maharashtra, Karnataka, and Kerala (Howlader *et al*. 2015; Khatiwada *et al*. 2017; Garg *et al*. 2018, 2019). Moreover, our data also suggest possible colonization scenarios for other amphibian genera that also have Oriental origins that have distributions in Pakistan (e.g. *Uperodon*, *Sphareoteca, Duttaphrynus*). Khan (2006) mentioned that several amphibian species used climatically stable conditions and a barrier-free environment of the northern part of the Indian subcontinent to colonize broad lowland areas from south-east Asia to the Indus and Kabul Rivers, where they reach the border with the Palearctic region. Similar colonization took place vice versa, although there are not too many species of amphibian and reptile in the Oriental region with Palearctic origins. Khan (1979) reported several morphological differences between populations of Pakistani *Microhyla* living in the plains and the uplands, but our genetic analysis clearly suggests a very shallow population divergence between the two studied sub-populations (localities in Islamabad vs. the Salt Range). Furthermore, our geographically broad comparison with more than 1,500 km between the Indian locality at Banka, Kaitha (Bihar; the only locality where we have published data from both studied markers of *M. nilphamariensis*) and the Pakistani populations showed genetic distance on the level of one to two mutation steps in the mtDNA. Along with the star-like pattern of the green haplogroup in which the Pakistani populations clustered, it suggests that the colonization from the east was recent and most possibly rapid (probably during the Pleistocene), which necessitates further research. There are a number of other mostly lowland amphibian species living in Pakistan that have Oriental origins, i.e. *Uperodon* (Microhylidae), *Minervarya*, *Hoplobatrachus*, or *Sphareoteca* (all Dicroglossidae), and/or endemic taxa, i.e. *Duttaphrynus melanostictus hazarensis* (Khan, 2001) (Bufonidae) or *Euphlyctis cyanophlyctis microspinulata* Khan, 1997 that would be of research interest as well.

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