An insight into molecular taxonomy of bufonids, microhylids, and dicroglossid frogs: First genetic records from Pakistan

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Abstract

The current study was focused on documentation of amphibian assemblage in North Punjab and Islamabad Capital Territory, Pakistan, by using mitochondrial gene sequences of 16S rRNA. Our study entailed 37% of the known amphibian species of the country. We provided a phylogenetic analysis based on 74 newly generated mitochondrial 16S rRNAs from nine species of genus Microlyla, Duttaphrynus, Allopaa, Nanorana, Sphaerotheca, Minervarya, Hoplobatrachus, and Euphlyctis. We employed the maximum-likelihood inference and Bayesian analysis to assess the taxonomic status of the samples obtained from Pakistan, with respect to other congeneric species from surrounding regions. Our findings confirmed the taxonomic status of South Asian anuran species Duttaphrynus stomaticus, Duttaphrynus melanostictus, Microhyla nilphamariensis, Allopaa hazarensis, Nanorana vicina, Sphaerotheca maskeyi (synonym: S. pashchima), Minervarya pierrei, Hoplobatrachus tigerinus, and Euphlyctis kalasgramensis in Pakistan. We have reported new country records of genus Minervarya (M. pierrei). Minervarya pierrei was previously misidentified as Fejervarya limnocharis, due to dearth of genetic information. We provided the first genetic records of our endemic species N. vicina. The results revealed the taxonomic placement of N. vicina with respect to its congeners and validated the taxonomic status of N. vicina from its type locality (Murree) for the first time. The findings of the present study also indicated the paraphyletic relationship of A.- hazarensis with Nanorana species. So, based on our phylogenetic inferences, morphological characters, and habitat preferences, validity of generic status of A. hazarensis is undecided. As our data were not enough to resolve this issue, we suggest sequencing of additional mitochondrial and nuclear genes in the future studies to get a better resolution. We recommend carrying out extensive surveys throughout the country for proper scientific documentation of amphibians of Pakistan. Many new species, some of them might be endemic to Pakistan, are expected to be discovered, and taxonomic status of other species would be resolved.

Keywords
endemism, Minervarya, Nanorana, new records, phylogeny
1 | INTRODUCTION

Identification of species by examining only morphological characters is difficult and may result in misidentifications (Stuart et al., 2006). Modern amphibian taxonomy relies heavily on molecular taxonomy and phylogeny (Frost et al., 2006). In the recent past, integrated taxonomic approaches have been applied successfully to resolve the complications associated with the species identification; see Phuge et al. (2020). In amphibian’s taxonomy, many new species are being described worldwide (Frost, 2019) and the discovery of new anuran species is an ongoing activity (Ohler et al., 2009). Despite these new findings and descriptions, the amphibian species in South and Southeast Asia remain underestimated generally due to the presence of homoplasies in morphology of amphibians (Stuart et al., 2006).

Boulenger (1890) provided a detailed description of amphibians of British India (Now Pakistan, India, Myanmar, and Sri Lanka). A total of 348 amphibians have been described so far from the eight countries of South Asia, with significant contribution from India and Sri Lanka (Molur, 2008; Pratihar et al., 2014). The territory of Pakistan, which is influenced by fauna from different geographic directions, is divided zoogeographically into the Palearctic and Oriental regions (Khan, 2006). Pakistan is one of the important territories in Eurasia in respect of past biodiversity dynamics (Jablonski et al., 2020). The amphibians of Pakistan are represented by a heterogeneous assemblage of 24 anuran species belonging to nine genera (Duttaphrynus, Scinax, Microhyla, Uperodon, Hoplobatrachus, Allopaa, Nanorana, Minervarya, and Sphaerotheca) distributed over four families Bufonidae, Microhylidae, and Dicroglossidae (Khan, 2006).

The contributions on diversity and ecology of amphibians of Pakistan include Khan (1976, 2001, 2006), Dubois and Khan (1979), Ohler and Dubois (2006), Ficetola et al. (2010), Yousaf et al. (2010), Tabassum et al. (2011), Rais et al. (2012, 2014), Pratihar et al. (2014), and Akram et al. (2015). Khan (2006) provided a checklist and identification key of anurans of Pakistan. The listing of species and their taxonomic status was based on morphological examination without any genetic confirmation and molecular taxonomy. As there is a vast sampling gap exists in the field of molecular taxonomy, strong uncertainties persist about the taxonomy of many anuran species of Pakistan. Molecular taxonomic studies are crucial to fill this gap. Few phylogenetic studies were recently conducted in Pakistan by Hussain et al. (2020) on genus Duttaphrynus, Jablonski et al. (2020) on genus Microhyla, Ali et al. (2020) on genus Euphlyctis, Jablonski et al. (2021) on genus Sphaerotheca, and Hofmann et al. (2021) on genus Allopaa. Despite these studies, there is scanty of information and anuran species from Pakistan are still underrepresented.

The detailed phylogenetic relationship of anurans within the family Bufonidae (genus Duttaphrynus), Microhylidae (genus Microhyla), and Dicroglossidae (Allopaa, Nanorana, Minervarya, Sphaerotheca, Hoplobatrachus, and Euphlyctis) is underrepresented in the samplings from Pakistan. The anuran fauna of this region also include endemic species such as Nanorana vicina, which is endemic to South Asia, and Allopaa hazarensis, which is endemic to Pakistan. We therefore in this study provided the molecular evidences and assemblage of reported anuran species of North Punjab (Rawalpindi District) and Islamabad Capital Territory, Pakistan.

2 | MATERIALS AND METHODS

2.1 Sampling area

The selected sampling area was Rawalpindi District (North Punjab) and Islamabad Capital Territory of Pakistan (Figures 1 and 2). The Rawalpindi District (33.4095°N, 72.9933°E) is located in the northwest of Punjab Province and covers an area of 5,286 km². The area is rocky and has mostly scrub vegetation. Administratively, the district has seven tehsils (Rawalpindi, Gujar Khan, Kallar Syedan, Kahuta, Murree, Kotli Sattian, and Taxila). The areas experience a humid subtropical climate with long and hot summers, a short monsoon period, and mild wet winters (Chaudhry & Rasul, 2004). The average temperature ranges from 2°C in January to 38.6°C in the June. Tehsil Murree have an elevation of 804–2,291 m, with mean annual precipitation of 1,789 mm. The area features mainly tropical Chir Pine (Pinus roxburghii) Forest (900–1,700 m elevation) and Himalayan Moist Temperate Forest. Other tehsils such as Gujar Khan, Taxila, Rawalpindi, Kotli Sattian, and Kallar Syedan of Rawalpindi District have predominantly Subtropical Broad-leaved Evergreen Forest or Scrub Forest with elevation <900 m (Sheikh & Hafeez, 2001). The district is drained by perennial and intermittent streams (Ahmed et al., 2020).

2.2 Field surveys

The specimens were collected from selected sites of study area (Figures 1 and 2) from February 2016 to October 2017. Field surveys were conducted in the morning (07:00–10:00) and evening (17:00–21:00). The length of field visits varied from a minimum of one day to a maximum of five days. All major habitats such as water, land, and vegetation were thoroughly searched, and only adult specimens were collected by hand or with dip net. The specimens were then brought to the Herpetology Laboratory, Department to Wildlife Management, Pir Mehr Ali Shah-Arid Agriculture University Rawalpindi, Pakistan.

2.3 Morphological identification

We collected nine anuran species, which included two toad species: Duttaphrynus stomaticus and Duttaphrynus melanostictus, and seven frog species: Microhyla nilphamariensis, Allopaa hazarensis, Nanorana vicina, Sphaerotheca maskeyi (synonym: S. pashchima), Minervarya
pierrei, Hoplobatrachus tigerinus, and Euphlyctis kalasgramensis (see Figure 3). The specimens were initially examined and identified based on morphological characters described in Khan (2006), Padhye et al. (2017), Howlader et al. (2015a, 2015b), and Howlader et al. (2016).

2.4 | Molecular analysis

The anuran specimens were euthanized by using chloroform, and toe clips were removed and stored in 95% ethanol in sample tubes for genetic analysis. The voucher specimens were fixed and later preserved in 10% formalin solution. The preserved specimens were then deposited in the museum of Herpetology Laboratory, Department to Wildlife Management, PMAS-AAUR. The general principles and guidelines of animal ethics were followed. The collected voucher specimens were not recognized as belonging to the threatened species and not listed in IUCN Red list or by CITES. Detailed lists of preserved voucher specimens are provided in Appendix 1.

2.4.1 | DNA extraction, amplification, and sequencing

We extracted DNA from stored tissue samples (toe clips) by using Promega Genomic DNA purification and extraction kit with the provided protocol for animal tissue. Quality of extracted DNA was assessed by Agarose Gel electrophoresis, and quantity of extracted DNA was calculated with Qubit 2.0 Fluorometer by using provided protocol of High Sensitivity Assay Kit. Two sets of primers (see Appendix 2) were used for the PCR amplification of 16S mitochondrial gene. For genus Duttaphrynus, Microhyla, Sphaerotheca,
Minervarya, Hoplobatrachus, and Euphlyctis, we used the primer pair 16SAR, 16SBR (Palumbi, 1996), and for genus Nanorana and Allopaa, we used the primer pair 16SC, 16SD (Cannatella et al., 1998). The PCR protocol of previous publication (Palumbi, 1996) was followed with few modifications for this study. Two mitochondrial fragments were amplified in 25 µl volume reaction. The recipe for the master mix is 2 µl of genomic DNA template, 2.5 µl of 10x PCR buffer, 0.5 µl of dNTP, 0.125 µl of Taq DNA polymerase, 0.5 µl of forward primer, 0.5 µl of reverse primer, and 18.875 µl of distilled water. The annealing temperature of the primers was 50°C. The thermocycler settings were as follows: initial denaturation step with 4 min at 94°C, 40 cycles of denaturation 30 s at 94°C, annealing for 30 s at 50°C, and extension for 90 s at 72°C. Final extension at 72°C was conducted for 7 min. DNA amplification was confirmed by agarose gel electrophoresis. The resulting PCR products were then sequenced using the Promega DNA purification kit (Wizard SV Gel and PCR Clean-Up System). The concentration of DNA was checked through NanoDrop Spectrophotometer (Invitrogen). The resulting PCR products were then sequenced in both directions using the same primers. Sanger sequencing was performed at the Institute for Cellular and Molecular Biology Core Facility, University of Texas Austin, Texas, USA. The sequences generated from the present study were deposited in the GenBank, and accession numbers are provided in Appendix 1.

2.4.2 | Data analysis

For reading, editing, and making consensus of forward and reverse sequences, the program Geneious (ver. R 7.1.9) (Kearse et al., 2012) was used. The new 16S sequences obtained were blasted on NCBI Nucleotide Blast Tool to identify and collect reference sequences of the same and closely related species. Sequences with more than 95% percentage similarity were retrieved and included in the analysis in order to find a good match. To analyze the taxonomic placements of our samples/species, we also included the representative samples of other species and genera of geographically linked species distributed throughout Pakistan, India, Bangladesh, Nepal, Sri Lanka, Iran, China, Japan, Taiwan, Uzbekistan, Greece, Turkey, Oman, Yemen, Indonesia, Malaysia, Vietnam, Thailand, Madagascar, and also from Himalayan range.

Alignments for each family, that is, Bufonidae, Microhylidae, and Dicroglossidae were prepared separately. A total of 37 samples including 8 newly sequenced and 29 from GenBank (two out-groups
Ansonia longidigita and Ingerophrynus divergens) were used for the phylogenetic analysis of Bufonidae. For the Microhylidae, 48 sequences were used including 4 newly sequenced samples and 44 sequences from GenBank (including two out-groups Uperodon systoma and Kaloula pulchra). For Dicroglossidae, a total of 260 sequences including 62 newly sequenced samples and 198 from GenBank (including out-groups Rana asiatica and Rana catesbeiana) were used in the analysis of Dicroglossidae. The details of sequences generated in the present study along with those recovered from GenBank are provided in Appendix 1.

Nucleotide sequences were aligned using MAFFT multiple sequence alignment program v. 7 with the option --auto that chooses the most appropriate algorithm for the data type (Katoh & Standley, 2013). The ambiguities, insertion, and deletion of single nucleotides from the sequences were manually edited using program Geneious (ver. R 7.1.9) (Kearse et al., 2012). Phylogenetic analysis of sequences was performed with maximum-likelihood analysis (Bootstrap value 100) on the CIPRES Science Gateway V. 3.3 (Miller et al., 2010) using the software IQ-TREE (Nguyen et al., 2015) with 1,000 ultrafast bootstraps approximation (Hoang et al., 2018). The model selection for each analysis tree was done as part of the run in IQ-TREE (Kalyaanamoorthy et al., 2017) using the option -TESTONLY. The best-fit model for Bufonidae family was TIM2+F+I+G4, for Dicroglossidae GTR+F+I+G4, and for Microhylidae TIM2e+F+I+G4. The consensus tree was calculated using SumTrees v.5.4.1 (Sukumaran & Holder, 2010). The tree with the highest maximum likelihood was selected, and the support from the bootstrap was mapped into that topology. The best value of maximum likelihood for Bufonidae was −3001.2908, for Dicroglossidae −9362.9580, and for Microhylidae −2110.5332.

To evaluate different strategy, an alignment that takes into account the secondary structure was made, with the option Q-INS-i of MAFFT online service: multiple sequence alignment, interactive sequence choice, and visualization (https://mafft.cbrc.jp/alignment/server/, Katoh et al., 2019). Based on secondary structure alignment, Bayesian phylogenetic inference (BI) (posterior probability 1) was performed in MrBayes ver. 3.2.6, (Ronquist et al., 2011). We ran the BI tree in CIPRES using MrBayes on XSEDE. We used the
reversible jump Markov chain Monte Carlo approach in order to calculate a model of DNA substitution, which allows us to examine among 203 substitutions model using AIC (Akaike, 1974) and BIC (Schwarz, 1978). We ran the BI analysis using a set parameters of two runs with eight chains with length of 40 million generations, sampling a tree every 1,000 generations. The convergence and effective sample sizes (ESSs) >200 of the runs were seen in TRACER v.1.6.0 (Rambaut et al., 2014). A burn-in was defined at 10%, by using SUMTREES v.4.2.0 (Sukumaran & Holder, 2010), and we discarded the burn-in and calculated a maximum clade credibility tree (MCCT).

The pairwise genetic distances between species groups were estimated. Within the group mean distance, between-group mean distance was calculated by using uncorrected p-distances in the software MEGA. 7.0 (Kumar et al., 2016).

3 | RESULTS

3.1 | Phylogenetic analysis of Bufonidae (genus Duttaphrynus)

We estimated phylogenies using the alternative alignments (primary and secondary structure), and found that the tree topologies are almost similar to each other. We conducted a maximum-likelihood and Bayesian analyses for taxonomic identification of bufonid toads (Genus Duttaphrynus). The data matrix was comprised of 37 samples related to 20 species, including two out-groups (Ansonia longidigita and Ingerophrynus divergens) and 18 in-groups (Adenomus kelaartii, Pedostibes tuberculosis, Xanthophryne koyayensis, Bufotes surdus, B. pezwowi, B. variabilis, B. viridis, Duttaphrynus melanostictus, D. stomaticus, D. brevirostris, D. atukoralei, D. dhufarensis, D. hololius, D. parietalis, D. scaber, D. stuarti, D. crocus, and D. himalayanus) (Figure 4; Appendix 3). All the newly sequenced samples of D. stomaticus and D. melanostictus recovered in both ML and BI trees with bootstrap value for D. stomaticus clade were 82% and for D. melanostictus 92%. The posterior probability value for D. stomaticus was 0.96, and D. melanostictus was 0.98 (Figure 4; Appendix 3). As there were two subclades of D. stomaticus observed in the phylogenetic inference, newly generated samples shared a same subclade with genetically identical sample of D. stomaticus (India), as uncorrected p-distance within the group is 0%. However, in the second subclade of D. stomaticus the uncorrected p-distance within the group was 1.7%. Moreover, between these two subclades of D. stomaticus was 3.9%, which reflects some genetic variation within species (Figure 4; Appendix 3; Tables S1 and S2).

The newly generated sequences of D. melanostictus are more closer/identical to the Indian samples (with uncorrected p-distance within group was 0.6%) as compared to D. melanostictus from China and Vietnam samples. The uncorrected p-distance between the two clades (China, Vietnam) and (Pakistan, India) was 2.7% (Figure 4; Appendix 3; Tables S1 and S2). The results showed the genetic confirmation of newly generated sequences of D. stomaticus and D. melanostictus from Pakistan.

3.2 | Phylogenetic analysis of Microhylidae (genus Microhyla)

The maximum-likelihood and Bayesian analyses of microhylid species were performed on data matrix comprised of 48 samples of 10 species including two out-groups (Uperodon systoma and Kaloula pulcha) and 8 ingroups of genus Microhyla (M. ornata, M. chakrapanii, M. fijissipes, M. mukhlesuri, M. mymensinghensis, M. nilphamariensis, M. rubra, and M. taraiensis). The tree topologies were observed similar, based on both maximum-likelihood and Bayesian analyses. The clade consisting of all sampled species of Genus Microhyla was highly supported in both ML and BI analyses (ML = 100 and PP = 1) (Figure 5; Appendix 4). However, the taxonomic status of our new samples of Microhyla nilphamariensis from Pakistan shared clade with the same species from India, Nepal, and Bangladesh which indicates its taxonomic status and wide distribution (BT = 37, PP = 0.83) (Figure 5; Appendix 4). The uncorrected p-distance within the clade of M. nilphamariensis was 0.3%, which clearly indicates the genetic confirmation of these species (Table S4).

3.3 | Phylogenetic analysis of family Dicroglossidae

The maximum-likelihood and Bayesian analyses were conducted on final alignments of 260 sequences of 8 genera (Allopaa, Nanorana, Quasipaa, Sphaerotheca, Fejervarya, Minervarya, Hoplobatrachus, and Euphlyctis), whereas two species of genus Rana (Rana catesbeiana and Rana asiatica) served as out-groups. We therefore inferred the phylogenetic analysis of our samples of N. vicina and A. hazienssis with 16S rRNA data of other species of genus Nanorana (N. karica, N. parkeri, N. ventripunctata, N. pleskei, N. yunnanensis, N. taihangnica, N. polunini, N. blanfordii, N. vicina, N. rostandi, N.ercepeae, N. liebigi, and unidentified samples of genus Nanorana from the Himalayan range). The phylogenetic trees infered from both maximum-likelihood and Bayesian analyses were similar, and the tree topologies were well resolved for the Quasipaa and Nanorana species (including Allopaa hazienssis samples) with BT = 99 and PP = 1 (Figure 6; Appendix 5). The N. vicina and A. hazienssis samples were nested within the clade of genus Nanorana with nodal support of BT = 96 and PP = 1. The species taxonomic placement of N. vicina and A. hazienssis was highly supported by ML (BT = 100) and BI (PP = 1) analyses (see Figures 6 and 7; Appendices 5 and 6). Newly sequenced samples of A. hazienssis appeared as paraphyletic with respect to Nanorana species. Allopaa hazienssis samples were recovered as nested within genus Nanorana, which may lead to the possibility of having same genus (Nanorana) (Figures 6 and 7; Appendices 5 and 6). Furthermore, our samples of N. vicina were identical (uncorrected p-distance of 0%) to the N. vicina from Northwest Himalayas (India: Himachal Pradesh), which depicts the existing distribution of N. vicina from Pakistan (type locality: Murree) to Himachal Pradesh (Figure 7; Tables S5 and S6). The uncorrected p-distance between N. vicina and A. hazienssis was 2.1% (Table S5).
The maximum likelihood and Bayesian inference trees recovered all sampled species of genera *Sphaerotheca*, *Fejervarya*, and *Minervarya* in their respective clades (Figure 8; Appendix 7). All the sampled species of genus *Sphaerotheca* from Pakistan appeared in the clade of *S. pashchima* (Figure 8; Appendix 7). The uncorrected p-distance within group of *S. pashchima* was 0%; however, between *S. pashchima* and *S. breviceps*, it was 6.4% (Tables S5 and S6).

Species of genus *Fejervarya* (*F. cancivora* and *F. limnocharis*) and *Minervarya* (*M. rufescens, M. greenei, M. kudremukhensis, M. sahyadris, M. caperata, M. asmati, M. granosa, M. syhadrensis*, and *M. pierrei*) appeared in their respective clade in both maximum likelihood and Bayesian inference (Figure 8; Appendix 7). The samples of *M. syhadrensis, M. granosa*, and *M. pierrei* were appeared in their respective subclades, under one main clade with maximum nodal support (BT = 100 and PP = 1). Newly generated sequences of *M. pierrei* are recovered as nested within *M. pierrei* clade. This species was previously misidentified in Pakistan as *F. limnocharis* (uncorrected p-distance between *F. limnocharis* and *M. pierrei* was 7.9%). The uncorrected p-distance between and within *M. pierrei* samples was observed as 0% (Tables S5 and S6). Therefore, we considered our samples as *M. pierrei* and reported first genetic record of this species from Pakistan.

For the genus *Hoplobatrachus*, we included species *H. rugulose* and *H. tigerinus* from its already established range of India and Bangladesh. Two haplotypes were observed in the *H. tigerinus*: One
is from Indian population and other is Bangladeshi population. Our samples of H. tigerinus from Pakistan showed a well-supported clade with Bangladesh samples of H. tigerinus (BT = 96 and PP = 1), which indicates validation of our samples as H. tigerinus (Figure 9; Appendix 8). The uncorrected p-distance between H. rugulose and H. tigerinus was 6.3% and between haplotypes of Bangladesh and India was 1.6% (Table S5).

The highly supported clade of genus Euphlyctis (BT = 97 and PP = 1) included species of E. hexadactylus, E. karaavali, E. aloisii, E. ehrenbergi, E. cyanophlyctis, E. mudigere, and E. kalasgramensis (Figure 9; Appendix 8). Both maximum-likelihood and Bayesian inference analyses of genus Euphlyctis strongly suggest that India, Pakistan, Bangladesh, and Iran populations of E. cyanophlyctis are split into four genetic lineages separated by nucleotide divergence (between group distance ranged from 0.9% to 3%) (Table S5). These lineages correspond to the clade from southern India, which include E. mudigere, the south Indian clade that we consider to be nominal E. cyanophlyctis because of its proximity to the type locality, the clade of E. kalasgramensis from specimens of Bangladesh, India (Assam), and Pakistan. The last clade was comprised of newly generated samples of Pakistan (Northern Punjab) and Iran (Figure 9; Appendix 8). Our samples of Euphlyctis appeared as a sister clade (BT=98; PP=1) of E. kalasgramensis clade constituting samples from Pakistan, India, and Bangladesh (Figure 9; Appendix 8).
However, the uncorrected p-distance between two sister clades of *E. kalasgramensis* was 2.1% (Table S5). Furthermore, two samples of Iran shared the same clade with our samples with 0% genetic divergence (Table S6).

4 | DISCUSSION

We conducted the first comprehensive molecular study on anurans of Pakistan, which entailed about 37% of the known anuran species of the country, including eight genera and nine species. The maximum-likelihood analysis based on standard alignment and Bayesian analysis based on secondary structure alignment validated the taxonomic status of *D. stomaticus*, *D. melanostictus*, *M. nilphamariensis*, *S. maskeyi* (synonym: *S. pashchima*), *H. tigerinus*, *E. kalasgramensis*, and *A. hazarensis* in Pakistan. We provided the genetic record of *N. vicina* (endemic to South Asia), for the first time from Pakistan and confirmed their species taxonomic ranks. We also reported first genetic record of genus *Minervarya* (*M. pierrei*) from Pakistan. This species were previously identified as *F. limnocharis* from the Rawalpindi District and Islamabad Capital Territory, Pakistan.

There are 12 true toad species of family Bufonidae, reported from Pakistan. Pakistani toad species can be represented in three groups *viridis*, *stomaticus*, and *melanostictus* (Khan, 1976). *Duttaphrynus stomaticus* and *D. melanostictus* toads can be morphologically identified by their parotid glands, rough skin with warts, and unwebbed toes. However, they can be distinguished from each other by distinct cranial crest present on head of *D. melanostictus* (Khan, 2006). *Duttaphrynus stomaticus* (Lütken, 1864) and *D. melanostictus* (Schneider, 1799) both include in a taxonomic framework of a monophyletic group of *Duttaphrynus* (Dubois & Ohler, 1999). Regardless of several studies on phylogenetic relationships in the family Bufonidae elsewhere, species from Indian subcontinent, especially from Pakistan, have not been studied in detail (Van Bocxlaer et al., 2009). In addition to many species with uncertain taxonomic affiliations, *D. stomaticus* and *D. melanostictus* (previously labeled as *Bufo*) require more detailed phylogenetic evidence (Van Bocxlaer et al., 2010). Van Bocxlaer et al. (2010) reported *D. stomaticus* being limited to the Western Ghats which is not true (Akram et al., 2015; Portik & Papenfuss, 2015). The previous sampling was limited to India leading to a sampling gap across Pakistan, a region that contains many widespread species in the genus *Duttaphrynus* and *Bufotes* (Portik & Papenfuss, 2015). Hussain et al. (2020) recently confirmed the taxonomic status of *D. stomaticus* and *D. melanostictus* from Punjab, but using one sample of each species, with limited dataset. We hereby presented an extensive dataset by incorporating 16S molecular data of 20 species, to confirm the taxonomic position of *D. stomaticus* and *D. melanostictus* with respect to other species of same genus. Our phylogenetic inferences yielded genetic confirmation of these two toads from Pakistan. The present study is largely in agreement with Portik and Papenfuss (2015) who recorded the species from Tharparkar (Sindh) and Lasbela (Baluchistan), and reported the possible distribution of *D. stomaticus* throughout Pakistan. We, however, in this
study provided genetic samples from northern Punjab, Pakistan and confirmed taxonomic status of *D. stomaticus*.

The species complex of *D. melanostictus* also entails taxonomic revision. It exhibits a wide geographical range (Wogan et al., 2016). The ancestral range for *D. melanostictus* is estimated to be the Myanmar–China border (Wogan et al., 2016). We validated the taxonomic status of *D. melanostictus* based on our maximum-likelihood and Bayesian analyses, which inferred that newly reported samples from the study area are closely related to the Indian samples with uncorrected p-distance within group of 0.6% (Table S2). The genetic divergence of 1.7% was observed between the two clades: (China, Vietnam) and (Pakistan, India) (Table S1). This genetic variation within species was previously reported by Khan (2001), who differentiated this species of *D. melanostictus* from South-East Asian congeners based on morphological parameters and ranked Pakistani samples as a subspecies named *D. melanostictus hazarensis*. Our genetic data are also in agreement with this existing variation across its range. Another study by Mulcahy et al. (2018) also indicated this genetic variation but referred all the samples as *D. melanostictus* until this species complex is revised.

Microhylid species are believed to be one of the most challenging taxonomical group of microhylid frogs due to their small size, conserved morphology, and widespread distribution of its members across Asia (Garg et al., 2018; Matsui et al., 2005, 2011). Molecular data have doubled the number of recognized *Microhyla* species found in South Asia (Hasan et al., 2014; Howlader et al., 2015a; Khatiwada et al., 2017; Seshadri et al., 2016; Vineeth et al., 2018; Wijayathilaka et al., 2016) and delineation of already known taxa (Garg et al., 2018; Hasan et al., 2012; Matsui, 2011; Matsui et al., 2005, 2011; Yuan et al., 2016), therefore elevating the importance of this region for *Microhyla* diversity. *Microhyla ornata* was assumed to be broadly distributed species throughout India, Bangladesh, Bhutan, Nepal, Pakistan, and Sri Lanka (Dutta et al., 2008), on the basis of century old range assumptions (Boulenger, 1882; Parker, 1934) and previous literature (Matsui et al., 2005, 2011). The checklists and records lacked vouchers or molecular information (Dinesh et al., 2009; Khan, 2006; Mathew & Sen, 2010). The genetic variations within populations of Microhylid species were first examined by Matsui et al. (2005), among three geographical regions of South Asia, South-East Asia, and East Asia. The study allocated and restricted the name *M. ornata* to the South Asian populations. Matsui et al. (2011) provided insights on phylogenetic relationships among *Microhyla* frogs across their known range; however, the South Asian members especially from Pakistan were not included. The South Asian *M. ornata* was recently described as a species complex (Hasan et al., 2012), followed by the description...
of four new and closely related species *M. mymensinghensis* (Hasan et al., 2014), *M. mukhlesuri* (Hasan et al., 2014), *M. nilphamariensis* (Howlader et al., 2015a), and *M. taraiensis* (Khatiwada et al., 2017). The recent reports of three new Microhylid species from India (*M. mukhlesuri, M. mymensinghensis,* and *M. nilphamariensis*) (Garg et al., 2018) lead to the urgency of the detailed sampling and genetic confirmation from its entire range. Based on extensive sampling by Wijayathilaka et al. (2016), *M. ornata* actually exhibits a narrow distribution limited to Peninsular India, more specifically in the states of Kerala, Karnataka, Tamil Nadu, Maharashtra, and Andhra Pradesh. Despite being a relatively newly identified microhylid, *M. nilphamariensis* is now genetically confirmed from the Western Ghats, Eastern Ghats up to Central India, East India, North India, Northeast India, Nepal, and Bangladesh (Garg et al., 2018).

The genus was represented in Pakistan by *M. ornata* (Duméril & Bibron, 1841); however, in view of the overall distribution and diversity of the genus based on genetic data (Garg et al., 2019; Gorin et al., 2020), populations from Pakistan are *M. nilphamariensis*. It was misidentified by previous studies such as Khan (2006), as previous research was only based on morphological examination. Our phylogenetic analysis inferred a resolved topology by showing an independent species taxonomic rank of each Microhylid species.
We confirmed existence of *M. nilphamariensis* in the study area, as our samples formed the same clade with *M. nilphamariensis* samples from India and Bangladesh. This evidence was supported by less support in ML analysis (<50%) but with high support in BI (0.83) (Figure 5; Appendix 4). As, *M. nilphamariensis* was misidentified as *M. ornata* from Pakistan, high genetic divergence (uncorrected p-distance 5%) was also observed between *M. nilphamariensis* and *M. ornata*, which leads to recognition of this species as *M. nilphamariensis* (Table S3). Our results are in agreement with Howlader et al. (2015a), who reported genetic divergence of *M. nilphamariensis* with its congeners by 5.7% to 13.2%. In a recent study, Jablonski et al. (2020) also revealed the species status of the genus from Pakistan (Islamabad and Northern Punjab) and reported the populations from the country as *M. nilphamariensis*. Based on recent studies of Garg et al. (2019) and Gorin et al. (2020), Jablonski et al. (2020) also reported the absence of *M. ornata* from Pakistan. *Microhyla nilphamariensis* species can also be morphologically characterized based on its dark brown diamond-shaped marking on dorsal side, dark streak from back of eyes to shoulders, lateral bands from tip of snout to the groin on either side of the body, blackish-brown mottling on throat, chest and margins of the belly, and indistinct inner metacarpal and metatarsal tubercles (Howlader et al., 2015a). We therefore in accordance with Jablonski et al. (2020) reported a significant range extension westward by confirmation of its presence in Pakistan 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 (Garg et al., 2018, 2019; Howlader et al., 2015a; Khatiwada et al., 2017).

We provided the first genetic records of *N. vicina* from Pakistan by sampling from its type locality (Murree). *Nanorana vicina* (Stoliczka, 1872) is a least studied anuran species from Pakistan. After its initial reports from Pakistan by Dubois (1976) from Azad Kashmir, Baig (2002) from Ayubia, Masroor (2011) from Margalla Hills National Park, and Rais et al. (2014) from Murree (type locality), the genetic validity of this species was still lacking. Previously this species was reported based on its morphological characters. The morphological diagnostic features included brownish smooth body dorsum with a few tubercles on flanks, dark bars on forearm, thighs and shank, distinct sooty stripes from snout to angle of jaws (see Figure 3; Khan, 2006; Rais et al., 2014). Hofmann et al. (2019) performed phylogeny of genus *Nanorana* from vast Himalayan range, but there was a sampling gap from Pakistan. They did not confirm the taxonomic status of several samples of the Himalayan range and left them as unidentified species. Latterly, Hofmann et al. (2021) referred *Nanorana* sp. samples from Himachal Pradesh as *N. vicina*, based on morphological (photographic) identifications. However, in the present study, we validated the taxonomic status of *N. vicina* genetically, as our samples of *N. vicina* appeared identical (uncorrected p-distance of 0%) to the samples of *Nanorana* (MNO12201, MNO12200, and MNO12199) collected from Northwest Himalayas (India: Himachal Pradesh) in the study of Hofmann et al. (2019). Moreover, our maximum-likelihood and Bayesian analyses showed the nesting of all species of genus *Nanorana* under one main clade with branch support of 96 (BT) and 1 (PP) (Figures 6 and 7; Appendices 5 and 6). So, we in accordance with Hofmann et al. (2021) referred these samples as *N. vicina*. The existing distribution of this South Asian endemic species from Pakistan (Type locality: Murree) to India (India: Himachal Pradesh) north-west Himalayas is now evident based on our phylogenetic analysis (Figures 6 and 7; Appendices 5 and 6). The geographical distribution of *N. vicina* in the west Himalayan range is also supported by several studies such as Sclater (1892), who reported its distribution range from Shimla, India. Litvinchuk et al. (2017) also reported its distribution in the west Himalayan range (Himachal Pradesh). As this species was understudied and had no previous genetic information, this study would be used as a reference for the future validation of conspecifics throughout its range.

*Allopaa hazarensis* (Dubois & Khan, 1979), which is endemic to Pakistan, was first placed in the supergroup of *Paa liebigi* (Dubois & Khan, 1979). Latterly, based on its unique combination of morphological characters, Ohler and Dubois (2006) proposed a separate genus for this species. In a recent study, Hofmann et al. (2021) conducted the first phylogenetic analysis based on genetic sampling of *A. hazarensis* from foothills of Kashmir Himalaya, which also includes its type locality (Dutta, District Mansehra). In this study, we included samples from same geographical range, that is, Murree (North Punjab), and our results showed genetic resemblance of *A. hazarensis* with all congeners of *Nanorana* and recovered as paraphyletic with respect to all sampled *Nanorana* species (Figures 6 and 7; Appendices 5 and 6), with genetic distance of 2.1% between *N. vicina* and *A. hazarensis* (Table S5). Hofmann et al. (2021) also reported this paraphyletic relationship of *Allopaa* with respect to genus *Nanorana*.

The separate genus of *Allopaa* which was described by Ohler and Dubois (2006) on morphological basis has appeared as nested within genus *Nanorana* by genetic analysis. *Allopaa hazarensis* also share its morphological characters with *N. vicina*, except having grayish dorsum with a superimposed network of dark olive green color, with horny spinules on dorsal and lateral sides and well-developed male secondary sex characters (nuptial spines) (Dubois & Khan, 1979) (see Figure 3). Both *A. hazarensis* and *N. vicina* share their habitat (freshwater streams) at higher elevation (>1,000 m) (Ahmed et al., 2020). So, based on our phylogenetic inferences, morphological characters, and habitat preferences, we doubt on the validity of generic status of *A. hazarensis*. As our data were not enough to resolve this taxonomic issue, we suggest sequencing of additional mitochondrial and nuclear genes in the future studies to get a better resolution. As a least studied genus, which is exclusive to Pakistan, with no other documented species till date, the genus is particularly important for Pakistan. However, to prevent taxonomic instability, we are hesitant to propose any taxonomic changes until further evidence is available.

Our results based on maximum-likelihood and Bayesian analyses agreed with previous studies by recovering three main subclades, corresponding to (a) genus *Sphaerotheca*, (b) South Asian clade (Minervarya), and (c) South-East Asian clade (Fejervarya) (Dinesh et al., 2015; Hasan et al., 2014; Howlader, 2011; Kotaki et al., 2008; Kuramoto et al., 2007; Pyron & Wiens, 2011; Sanchez et al., 2018). All the sampled species in our dataset of genus *Sphaerotheca* (*S. plumialis*, *S. dobsonii*, *S. magadha*, *S. rolandae*, *S. breviceps*, *S. pashchima*) recovered to have an independent taxonomic species rank albeit with low branch support (Figure 8; Appendix 7). *Sphaerotheca breviceps*, which is endemic to South Asia, was considered as a species complex (Dubois, 1983; Dutta, 1986), but Padhye et al. (2017) restricted its distribution range to the eastern coastal plains of India and described a new species, *S. pashchima* from western and northern India, which was previously misidentified as *S. breviceps*. *Sphaerotheca pashchima* is considered as a morphologically and genetically distinct species from western Maharasthra, Gujrat, and Karnataka, after its comparison with topotypic material of *S. breviceps*. *Sphaerotheca pashchima* differs from *S. breviceps* by minor differences such as rounded snout, second finger length equal to or less than fourth finger length; first finger length less than third finger (Padhye et al., 2017). Khatiwada et al. (2021) declared *S. pashchima* as a synonym of *S. maskeyi* (Schleich and Anders, 1998). Jablonski et al. (2021) also designated this species as *S. maskeyi*, from Himalayan foothills of Pakistan, based on two genetic samples collected from Khyber Pakhtunkhwa Province, Pakistan. In this study, *Sphaerotheca* sp. collected from north Punjab were clustered within *S. pashchima* clade, with uncorrected p-distance of 0%. However, genetic distance between *S. pashchima* and *S. breviceps* was 6.4% (Tables S5 and S6). Our results are in congruent with successive studies of Padhye et al. (2017), Jablonski et al. (2021), and Khatiwada et al. (2021), and we believe the presence of *S. maskeyi* (synonym:*S. pashchima*) in North Punjab and these molecular studies resolved.
the taxonomic status of this species complex. However, by extensive genetic sampling in future studies, we expect more species of *Sphaerotheca* to be discovered from the region.

The frogs from genus *Fejervarya* are morphologically similar to many new morphologically cryptic (but genetically distinct) species (Sanchez et al., 2018). Recent taxonomic rearrangement of *Fejervarya* treats South Asian and South-East Asian taxa as separate genera. The South-East Asian species *Rana limnocharis* Gravenhorst 1829 (the type species of *Fejervarya*) is the first-described genus named in the group (Frost, 2019). *Fejervarya Limnocharis* was reported by earlier workers (such as by Akram et al., 2015; Khan, 2006; Pratihar et al., 2014; Rais et al., 2012) from Pakistan, merely on basis of morphological characters. In the present study, we did first ever phylogenetic analysis based on genetic sampling from Pakistan. *Minervarya* species from the North Punjab which was misidentified previously as *F. limnocharis* is actually *M. pierrei* with 7.9% uncorrected p-distance between groups (Table S5). The second described clade (primarily South Asian taxa) contains the type species of both *Minervarya* and *Zakerana* (*Minervarya sahyadris*) Dubois et al. (2001), and *Rana limnocharis* syhadrensis Annandale (1919), from which *Minervarya* was described earlier and have taxonomic priority on *Zakerana*. *Minervarya* placement within the South Asian clade is also confirmed by our both maximum-likelihood and bayesian analyses, as our newly sequenced samples were nested within *Minervarya* genus with maximum branch support. The samples of *M. syhadrensis*, *M. granoasa*, and *M. pierrei* were appeared in their respective subclades, under one main clade with maximum nodal support (BT = 100 and PP = 1) (Figure 8; Appendix 7). Our results are in congruent with Köhler et al. (2019), who also grouped these species in one main clade by using Automated barcode recovery method.

Our sequences were placed in *M. pierrei* group, and the taxonomic status of our samples was also validated by having 0% uncorrected p-distance within *M. pierrei* samples (Tables S5 and S6). Phuge et al. (2020) examined two *M. syhadrensis*-like species, which they named as types A and B and comparison of these types with the type specimen of *M. syhadrensis* from Pune district (India) through morphological, call pattern, and phylogenetic analysis referred type A as *M. syhadrensis*, whereas the other type was referred as *M. pierrei* / *M. Agricola* complex. We also analyzed these samples having accession numbers AY882955, AY882953, and AY882948 originated from India (West Bengal). Therefore, we in accordance with Phuge et al. (2020) referred samples originated from Pakistan, Bangladesh, Nepal, and west Bengal (India) as *M. pierrei* clade (Figure 8; Appendix 7). This species can also be identified through morphological features of pointed snout, dorsum postulate, and vocal sacs, and markings on throat are laterally dark and medially pale, mid-dorsal line with constant width from snout to vent (see Figure 3) (Howlader et al., 2016). Furthermore, as type locality of *M. pierrei* is east Nepal, which is in closer proximity with Northern Punjab (Pakistan). Therefore, by considering geographical proximity, previous study of Phuge et al. (2020), our phylogenetic inferences (Figure 8; Appendix 7) and morphological characters, we identified our samples as *M. pierrei*. We believe that more extensive phylogenetic datasets are required in order to provide genetic evidences of *M. syhadrensis* and *M. granoasa* in Pakistan.

The phylogenetic analysis of genus *Hoplobatrachus* showed that the *Hoplobatrachus* samples from Pakistan (North Punjab) were similar to Bangladeshi samples of *H. tigerinus* with 0% genetic divergence; however, 1.6% genetic divergences were observed between Indian and Bangladeshi clades (Tables S5 and S6) (Figure 9; Appendix 8). Similarly, in a recent study of Khatiwada et al. (2021), Nepalese samples were appeared as closely related to Bangladesh samples as compared to Indian subclade, which indicates that the *H. tigerinus* present in geographical range of Pakistan (North Punjab), Bangladesh, and Nepal are genetically identical; however, there is some genetic variation exist between these and Indian lineage.

The genus *Euphlyctis* (Schneider, 1799) is one of the most widespread in southern Asia. It comprises of seven extant species: Description of new species has been reported in this genus from past few years, that is, *E. mudigere* (Joshi et al., 2009) from Southern India and Sri Lanka, *E. kalasgramensis* (Howlader et al., 2015b) from the Barisal district of Bangladesh, and *E. karavaali* (Priti et al., 2016) from West Coastal Plains of India. *E. cyanophlyctis*, the most common species, exhibits high degree of morphological similarity with other species of the genus (Joshi et al., 2009). Its distribution is known from Southeastern Iran, Southern Afghanistan, Pakistan, Nepal, Bhutan, India, Sri Lanka, Myanmar, Malaysia and Vietnam. Phylogenetic analyses of genus *Euphlyctis* strongly suggest that India, Pakistan, Bangladesh, and Iran populations of *E. cyanophlyctis* are split into four genetic lineages separated by nucleotide divergence (between groups uncorrected p-distance ranged from 0.9% to 3%) (Table S5). These lineages correspond to the separate clades, clade 1 corresponds to southern India, which include *E. mudigere*, and second clade is the south Indian clade that we consider to be nominal *E. cyanophlyctis* because of its proximity to the type locality. Third clade constitutes samples of *E. kalasgramensis* from Bangladesh, India (Assam), and Pakistan and the last clade with newly generated samples of Pakistan (Northern Punjab) and Iran (Figure 9; Appendix 8). Two samples (KF992800 and KF992815) from Iran in study of Khajeh et al. (2014) are genetically identical to our samples (0% uncorrected p-distance with our samples) (Table S6). Our results are in agreement with the study of Khajeh et al. (2014), which suggested that samples from Iran population of *E. cyanophlyctis* are more closely related to Bangladeshi population, as compared to south Indian population. This argument became strengthened when Howlader et al. (2015b) described a new species *E. kalasgramensis* from Bangladeshi population.

The existence of *E. kalasgramensis* from Pakistan was also reported by Ali et al. (2020) from Kasur (Punjab), Pakistan, based on molecular and morphological evidences, but with limited dataset. The identification features of *E. kalasgramensis* include the following: the body dorsum without longitudinal folds, plain whitish ventral coloration and limbs with incomplete dark bands (Howlader et al. (2015b) see Figure 3. In the present study, we analyzed extensive datasets by adding other congeneric species of this genus in our phylogenetic inferences. Our samples of *Euphlyctis* appeared as a sister clade of *E. kalasgramensis* comprised of samples originated
from Pakistan, India, and Bangladesh. The genetic divergence of 2.1% (uncorrected p-distance) was observed between these two sister clades (Table S5). However, we suggest detailed taxonomic study exclusively for this genus by including more mitochondrial and nuclear genes to clarify the presence of possible cryptic species under the taxonomic rank of Euphlyctis.

5 | CONCLUSIONS

This study was aimed to document the species taxonomic status based on 16S rRNA. The phylogenetic analysis of South Asian anuran species was performed, in which new genetic samples obtained from Pakistan and their respective congeners retrieved from GenBank were included. Our results based on maximum-likelihood and Bayesian analyses of 16S rRNA data validated the taxonomic status of nine anuran species which belong to eight genera from Pakistan. These species include Duttaphrynus stomaticus, Duttaphrynus melanostictus, Microhyla nilphamariensis, Allopaa hazarensis, Nanorana vicina, Spheothea maskeyi (synonym: S. paschchima), Minervarya pierrei, Hoplobatrachus tigerinus, and Euphlyctis kalahragensis. We reported the first genetic record of genus Minervarya (M. pierrei), This species was misidentified previously in Pakistan as Fejervarya limnocharis. Furthermore, we provided the first genetic records of N. vicina from its type locality (Murree), which will provide a baseline data for this understudied species.

5.1 | Recommendations

Systematic surveys have never been conducted in Pakistan to document amphibian diversity of the country. A deep review is still necessary to resolve the taxonomy morphologically undistinguishable putative species complexes in the future studies.

The known fauna was based on work carried by individuals within their own capacity without any institutional setup and coordination among the researchers. Either individuals working in academic and research institutions gather samples or their peers bring them the samples for species identification. Utilizing their full capacity and understanding, they try to identify the species. Amphibians in Pakistan have failed to find any place in policy and legislation. We suggest carrying out extensive surveys throughout the country for the collection of specimens and their genetic analysis in future studies. These analyses will confidently resolve the taxonomic issues caused due to morphologically undistinguishable species. This would result in proper scientific documentation of amphibians of Pakistan. Many new species, some of them might be exclusive to Pakistan, are expected to be discovered, and taxonomic status of other species would be resolved.

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CONFLICT OF INTEREST

The authors declare no conflict of Interest. The funding agencies had no role in design of study, in the collection, analysis, interpretation of data, in the writing of the manuscript, or in the decision to publish the results.

AUTHOR CONTRIBUTIONS

Ayesha Akram: Conceptualization (equal); Data curation (lead); Formal analysis (lead); Funding acquisition (equal); Investigation (lead); Methodology (lead); Project administration (equal); Resources (lead); Software (lead); Supervision (equal); Validation (lead); Visualization (lead); Writing-original draft (lead); Writing-review & editing (lead). Muhammad Rais: Conceptualization (lead); Funding acquisition (lead); Project administration (equal); Resources (equal). Karem Lopez-Hervas: Data curation (supporting); Formal analysis (equal); Methodology (equal); Software (equal); Supervision (supporting). Rebecca D. Tarvin: Conceptualization (supporting); Data curation (equal); Formal analysis (supporting); Methodology (supporting); Resources (supporting); Software (supporting); Writing-review & editing (supporting). Muhammad Saeed: Data curation (supporting); Project administration (supporting). Daniel I. Bolnick: Conceptualization (lead); Data curation (lead); Project administration (lead); Resources (lead); Supervision (lead); Writing-review & editing (equal). David C. Cannatella: Conceptualization (lead); Data curation (lead); Formal analysis (lead); Methodology (equal); Resources (supporting); Software (supporting); Supervision (lead); Validation (lead); Writing-review & editing (equal).

DATA AVAILABILITY STATEMENT

The sequence data generated in this study are readily available on GenBank with accession numbers MW885769 to MW885776, MW886319 to MW886322, and MW898152 to MW898213.

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### APPENDIX 1

Details of samples used in the phylogenetic analysis, as well as in genetic distances

| Genus         | Species               | Gene | Voucher No. | GenBank accession No. | Country/locality | Lat  | Long  | Publication          |
|---------------|-----------------------|------|-------------|-----------------------|------------------|------|-------|---------------------|
| Ansonia       | longidigita           | 16S  | VUB 0666    | FJ882796              | Malaysia: Borneo | –    | –     | Bocxlaer et al. (2009) |
| Ingerophrynus | divergens             | 16S  | VUB 0602    | FJ882802              | Malaysia: Borneo | –    | –     | Bocxlaer et al. (2009) |
| Adenomus      | kelaartii             | 16S  | VUB 0171    | FJ882780              | Sri Lanka        | –    | –     | Bocxlaer et al. (2009) |
| Pedostibes    | tuberculosus          | 16S  | SDB 4691    | FJ882793              | India: Western Ghats | – | – | Bocxlaer et al. (2009) |
| Bufo           | cf. surdus            | 16S  | ZMMSU A-4027| FJ882810              | Iran             | –    | –     | Bocxlaer et al. (2009) |
| Bufo           | cf. pewzowi           | 16S  | NP B-4-1    | FJ882811              | Uzbekistan       | –    | –     | Bocxlaer et al. (2009) |
| Bufo           | cf. variabilis        | 16S  | VUB 1813    | FJ882812              | Turkey           | –    | –     | Bocxlaer et al. (2009) |
| Bufo viridis   |                       | 16S  | NP B-2-1    | FJ882813              | Greece           | –    | –     | Bocxlaer et al. (2009) |
| Xanthophryne  | koyayensis            | 16S  | SDB 2004-012| FJ882782              | India: Western Ghats | – | – | Bocxlaer et al. (2009) |
| Duttaphrynus  | melanostictus         | 16S  | VUB 0052    | FJ882791              | India: Western Ghats | – | – | Bocxlaer et al. (2009) |
| Duttaphrynus  | sp.                   | 16S  | SDB 927     | FJ882792              | India: Western Ghats | – | – | Bocxlaer et al. (2009) |
| Duttaphrynus  | brevirostris          | 16S  | SDB 4714    | FJ882786              | India: Western Ghats | – | – | Bocxlaer et al. (2009) |
| Duttaphrynus  | atukoralei            | 16S  | VUB 0101    | FJ882835              | Sri Lanka        | –    | –     | Bocxlaer et al. (2009) |
| Duttaphrynus  | dhufarensis           | 16S  | CAS 227584  | FJ882837              | Oman             | –    | –     | Bocxlaer et al. (2009) |
| Duttaphrynus  | hololius              | 16S  | SDB 4240    | FJ882781              | India            | –    | –     | Bocxlaer et al. (2009) |
| Duttaphrynus  | parietalis            | 16S  | SDB 10100   | FJ882784              | India: Western Ghats | – | – | Bocxlaer et al. (2009) |
| Duttaphrynus  | scaber                | 16S  | SDB 532     | FJ882785              | India: Western Ghats | – | – | Bocxlaer et al. (2009) |
| Duttaphrynus  | stuarti               | 16S  | CAS 221485  | FJ882788              | Myanmar          | –    | –     | Bocxlaer et al. (2009) |
| Duttaphrynus  | crocus                | 16S  | CAS 220193  | FJ882789              | Myanmar          | –    | –     | Bocxlaer et al. (2009) |
| Duttaphrynus  | himalayanus           | 16S  | SDB4566     | FJ882790              | India            | –    | –     | Bocxlaer et al. (2009) |
| Duttaphrynus  | himalayanus           | 16S  | DH3         | KY000450              | India: Uttarakhand | 29.868236 | 79.310841 | Bahuguna et al. (2017) Unpublished |
| Duttaphrynus  | melanostictus         | 16S  | DM2         | KY000456              | India: Uttarakhand | 30.324483 | 78.102409 | Bahuguna et al. (2017) Unpublished |
| Duttaphrynus  | melanostictus         | 16S  | KIZ-95L001  | AF160790              | China: Yunnan Pingbian Co | 22.790772 | 103.90988 | Liu et al. (2000) |
| Duttaphrynus  | melanostictus         | 16S  | ROM 33162   | AF160791              | Vietnam: Gia Lia, Koh Rong | 11.190175 | 103.86354 | Liu et al. (2000) |

(Continues)
| Genus         | Species       | Gene | Voucher No. | GenBank accession No. | Country/locality                  | Lat     | Long     | Publication                       |
|---------------|---------------|------|-------------|-----------------------|-----------------------------------|---------|----------|-----------------------------------|
| Duttaphrynus  | melanostictus | 16S  | 1           | EU071740              | India: Pune, Maharashtra           | 18.576642 | 74.049723 | Shouche and Ghate (2007) Unpublished |
| Duttaphrynus  | sp.           | 16S  | SDB 4594    | FJ882839              | India                             | –       | –        | Bocxlaer et al. (2009)            |
| Duttaphrynus  | stomaticus    | 16S  | 27          | EU071742              | India                             | –       | –        | Shouche and Ghate (2007) Unpublished |
| Duttaphrynus  | stomaticus    | 16S  | CCMB D02_B247 | KT991344          | India                             | –       | –        | Chandramouli et al. (2016)        |
| Duttaphrynus  | stomaticus    | 16S  | SDB 4020    | FJ882787              | India: Western Ghats               | –       | –        | Bocxlaer et al. (2009)            |
| Duttaphrynus  | melanostictus | 16S  | WLM:DM296164 | MW885769              | Pakistan: Kotli Sattian, Nalari    | 33.81554079 | 73.47496593 | This study                        |
| Duttaphrynus  | melanostictus | 16S  | WLM:DM303174 | MW885770              | Pakistan: Islamabad, Bani Gala    | 33.719659 | 73.164594 | This study                        |
| Duttaphrynus  | stomaticus    | 16S  | WLM:DS48176 | MW885771              | Pakistan: Islamabad, Bani Gala    | 33.72553314 | 73.19205455 | This study                        |
| Duttaphrynus  | stomaticus    | 16S  | WLM:DS2361610 | MW885776          | Pakistan: Taxila: Bahtar road     | 33.75005567 | 72.71150025 | This study                        |
| Duttaphrynus  | stomaticus    | 16S  | WLM:DS46168 | MW885774              | Pakistan: Taxila: UET              | 33.75794448 | 72.82491694 | This study                        |
| Duttaphrynus  | stomaticus    | 16S  | WLM:DS236168 | MW885772              | Pakistan: Rawalpindi: Chakri       | 33.47833084 | 72.93900073 | This study                        |
| Duttaphrynus  | stomaticus    | 16S  | WLM:DS236169 | MW885773              | Pakistan: Kahuta: Barootheri       | 33.53938907 | 73.50488902 | This study                        |
| Duttaphrynus  | stomaticus    | 16S  | WLM:DS46169 | MW885775              | Pakistan: Gujor Khan: Manghot      | 33.2755836 | 73.12263851 | This study                        |
| Uperodon      | systoma       | 16S  | 1151UpeSys  | EF017960              | India                             | –       | –        | Bocxlaer et al. (2006)            |
| Kaloula       | pulchra       | 16S  | MBM-USNMFS36482 | MG935853         | Myanmar: Kandawgyi National Gardens | 21.9931 | 96.46713 | Mulchay et al. (2018)             |
| Microhyla     | fissipes      | 16S  | KUHE32943   | AB201185              | China: Anhui, Huangshan            | 30.034532 | 118.0078 | Matsui et al. (2005)              |
| Microhyla     | mukhlesuri    | 16S  | SDBDU 2010.1332 | MH549575          | India: Mizoram                     | 22.53 | 92.89 | Garg et al. (2018)                |
| Microhyla     | mukhlesuri    | 16S  | Not mentioned | JQ621935            | Thailand                          | 13.855145 | 100.76215 | Gao and Fan (2012) Unpublished    |
| Microhyla     | mukhlesuri    | 16S  | KIZHERP0138 | JX678905              | Vietnam                           | 19.991547 | 104.13737 | Li et al. (2012)                  |
| Microhyla     | mukhlesuri    | 16S  | TZ52        | AF205202              | Vietnam                           | 17.650995 | 106.01649 | Ziegler, T. (2000) Unpublished thesis |
| Microhyla     | mukhlesuri    | 16S  | 16SMicrohyla_ornata | AF215373       | Madagascar                        | -18.60814 | 46.61644 | Vences (2000), Unpublished        |

(Continues)
| Genus       | Species            | Gene | Voucher No. | GenBank accession No. | Country/locality          | Lat        | Long       | Publication                  |
|-------------|--------------------|------|-------------|-----------------------|---------------------------|------------|------------|-----------------------------|
| Microhyla   | chakrapanii        | 16S  | not preserved | MH807389               | India: Andaman Islands    | 12.565535  | 92.80566   | Garg et al. (2019)           |
| Microhyla   | mymensinghensis    | 16S  | SDBDU 2015.2904 | MH549590               | India: West Bengal        | 22.43      | 88.38      | Garg et al. (2018)           |
| Microhyla   | rubra              | 16S  | SDBDU 2014.2829 | MH807420               | India: Tamil Nadu, Meenakshipuram | 10.631822  | 76.86593   | Garg et al. (2019)           |
| Microhyla   | rubra              | 16S  | not preserved | AB201192               | India: Karnataka          | 15.587753  | 75.863205  | Matsui et al. (2005)         |
| Microhyla   | taraiensis         | 16S  | JRK201525    | KY655952               | Nepal: Mechi             | 27.152954  | 87.886133  | Khatiwada et al. (2017)      |
| Microhyla   | taraiensis         | 16S  | JRK201527    | KY655954               | East Nepal               | 29.10608   | 82.246342  | Khatiwada et al. (2017)      |
| Microhyla   | taraiensis         | 16S  | JRK201526    | KY655953               | East Nepal               | 29.326619  | 81.916752  | Khatiwada et al. (2017)      |
| Microhyla   | ornata             | 16S  | SDBDU 2008.1720 | MH549636               | India: Tamil Nadu, Coimbatore | 11.005298  | 76.942513  | Garg et al. (2018)           |
| Microhyla   | ornata             | 16S  | ZSIK-A9119   | AB201188               | India: Karnataka, Dharwad | 15.464739  | 74.98685   | Matsui et al. (2005)         |
| Microhyla   | ornata             | 16S  | RGCB15059    | KP072794               | India: Pulpally, Wayanad District, Kerala | 11.7902    | 76.181561  | Howlader et al. (2015a)      |
| Microhyla   | ornata             | 16S  | SDBDU 2015.2898 | MH549619               | India: Andhra Pradesh, Maredumili | 17.66      | 82.22      | Garg et al. (2018)           |
| Microhyla   | ornata             | 16S  | DZ 1432      | MH807404               | Sri Lanka: Kukalamalpotha | 7.412246   | 81.159342  | Garg et al. (2019)           |
| Microhyla   | ornata             | 16S  | DZ 1427      | MH807402               | Sri Lanka: Makandura      | 7.323117   | 79.977009  | Garg et al. (2019)           |
| Microhyla   | ornata             | 16S  | SDBDU 2008.1958 | MH549630               | India: Tamil Nadu, Keeriparai | 8.395153   | 77.412114  | Garg et al. (2018)           |
| Microhyla   | ornata             | 16S  | DZ 1104      | MH807400               | Sri Lanka: Puttalam       | 8.043706   | 79.835967  | Garg et al. (2019)           |
| Microhyla   | nilphamariensis    | 16S  | ABHU<JPN>:4213 | LC090056 | Bangladesh: Nilphamari, Barua, Berakhuti | 25.886874  | 88.945658  | Hasan et al. (2015)          |
| Microhyla   | nilphamariensis    | 16S  | Morn-Bd1     | ABS30537               | Bangladesh: Dinajpur, Parbatipur | 25.655386  | 88.912375  | Hasan et al. (2012)          |
| Microhyla   | nilphamariensis    | 16S  | Morn-Bd3     | ABS30539               | Bangladesh: Dinajpur, Parbatipur | 25.653732  | 88.918973  | Hasan et al. (2012)          |
| Microhyla   | nilphamariensis    | 16S  | Morn-Bd2     | ABS30538               | Bangladesh: Dinajpur, Parbatipur | 25.657774  | 88.916044  | Hasan et al. (2012)          |
| Microhyla   | nilphamariensis    | 16S  | ABHU<JPN>:4212 | LC090055 | Bangladesh: Nilphamari, Barua, Berakhuti | 25.887231  | 88.947224  | Hasan et al. (2015)          |
| Microhyla   | nilphamariensis    | 16S  | MHLB00206    | LC090057               | Bangladesh: Nilphamari, Barua, Berakhuti | 25.886102  | 88.944821  | Hasan et al. (2015)          |
| Genus     | Species              | Gene | Voucher No. | GenBank accession No. | Country/locality                          | Lat    | Long    | Publication                        |
|-----------|----------------------|------|-------------|-----------------------|-------------------------------------------|--------|---------|-----------------------------------|
| Microhyla | nilphamariensis      | 16S  | SDBDU 2015.2905 | MH549592              | India: Assam, Barpeta, Mandia             | 26.268615 | 90.960971 | Garg et al. (2018)               |
| Microhyla | nilphamariensis      | 16S  | MZH-2362    | KP072789              | Bangladesh: Saidpur                       | 25.781567 | 88.902439 | Howlader et al. (2015a)          |
| Microhyla | nilphamariensis      | 16S  | ADWII_DT1   | MH549616              | India: Uttarakhand, Tuntowala             | 30.279222 | 78.004864 | Garg et al. (2018)               |
| Microhyla | nilphamariensis      | 16S  | ADWII_M03   | MH549618              | Bangladesh: Saidpur                       | 25.79115 | 88.889736 | Howlader et al. (2015a)          |
| Microhyla | nilphamariensis      | 16S  | MZH-2363    | KP072790              | Bangladesh: Saidpur                       | 25.79115 | 88.889736 | Howlader et al. (2015a)          |
| Microhyla | nilphamariensis      | 16S  | M1_2_16SF-A09.ab1 | MN952999           | Laterite Plateau of Western India         | 23.868377 | 72.611802 | Mudke (2020), Unpublished         |
| Microhyla | nilphamariensis      | 16S  | M1_3_16SF-B09.ab1 | MN953000           | Laterite Plateau of Western India         | 24.056953 | 72.810493 | Mudke (2020), Unpublished         |
| Microhyla | nilphamariensis      | 16S  | M1_1_16SF-H08.ab1 | MN952998           | Laterite Plateau of Western India         | 19.559477 | 73.761439 | Mudke (2020), Unpublished         |
| Microhyla | nilphamariensis      | 16S  | SDBDU 2004.4507 | MH549606              | India: Maharashtra, Koyna                 | 19.636949 | 73.170211 | Garg et al. (2018)               |
| Microhyla | nilphamariensis      | 16S  | JRK201514   | KY655939              | Nepal: Jhuwani, district Chitwan, Narayani | 27.591617 | 84.525397 | Khatiwada et al. (2017)          |
| Microhyla | nilphamariensis      | 16S  | JRK201529   | KY655951              | Nepal: Hangdewa, Tapljung district, Mechi province | 27.378704 | 87.700172 | Khatiwada et al. (2017)          |
| Microhyla | nilphamariensis      | 16S  | JRK201501   | KY655926              | Nepal: Jhuwani, district Chitwan, Narayani province | 27.594508 | 84.522865 | Khatiwada et al. (2017)          |
| Microhyla | nilphamariensis      | 16S  | JRK201523   | KY655948              | Nepal: Jhuwani, district Chitwan, Narayani province | 27.588042 | 84.529431 | Khatiwada et al. (2017)          |
| Microhyla | nilphamariensis      | 16S  | JRK201518   | KY655943              | Nepal: Jhuwani, district Chitwan, Narayani province | 27.58595 | 84.526341 | Khatiwada et al. (2017)          |
| Microhyla | nilphamariensis      | 16S  | JRK201524   | KY655949              | Nepal: Jhuwani, district Chitwan, Narayani province | 27.584894 | 84.529398 | Khatiwada et al. (2017)          |
| Microhyla | nilphamariensis      | 16S  | WLM:MN236165 | MW886319              | Pakistan: Rawalpindi: Misrial            | 33.190561 | 72.808795 | This study                      |
| Microhyla | nilphamariensis      | 16S  | WLM:MN236163 | MW886320              | Pakistan: Rawalpindi, Chakri             | 33.5178509 | 72.9759799 | This study                      |
| Microhyla | nilphamariensis      | 16S  | WLM:MN46164 | MW886321              | Pakistan: Murree, Shangrila              | 33.856779 | 73.373913 | This study                      |
| Microhyla | nilphamariensis      | 16S  | WLM:MN236164 | MW886322              | Pakistan: Rawalpindi: Girja road         | 33.557083 | 72.995833 | This study                      |
| Rana      | catesbeiana          | 16S  | NIBRAM0000100407 | JQ815323           | Korea: Gyeongsangnam-do Jinsu-si Jeongchon-myeon | –       | –     | Jeong et al. (2013)              |
| Rana      | asiatica             | 16S  | Asia. R     | AB058884              | Russia: Kirghizia                        | –       | –     | Sumida et al. (2003)             |
| Genus         | Species       | Gene | Voucher No. | GenBank accession No. | Country/locality              | Lat  | Long  | Publication       |
|---------------|---------------|------|-------------|-----------------------|-------------------------------|------|-------|------------------|
| Quasipaa      | shini         | 16S  | XJW-LS-001  | KF199148              | China: Longsheng, Guangxi     | –    | –    | Zhang et al. (2018) |
| Quasipaa      | boulengeri    | 16S  | JFW-TS-002  | KF199152              | China: Tongshan, Hubei        | –    | –    | Zhang et al. (2018) |
| Quasipaa      | exilispinosa  | 16S  | XJW-WYS-001 | KF199151              | China: Wuyishan, Fujian       | –    | –    | Zhang et al. (2018) |
| Quasipaa      | jiulongensis  | 16S  | JLJW-WYS-002| KF199149              | China: Wuyishan, Fujian       | –    | –    | Zhang et al. (2018) |
| Nanorana      | cf. rarica    | 16S  | A1961/13_NME| M012202               | Central Himalaya              | 29.51| 82.09| Hofmann et al. (2019) |
| Nanorana      | cf. rarica    | 16S  | A1970/13_NME| M012203               | Central Himalaya              | 29.51| 82.09| Hofmann et al. (2019) |
| Nanorana      | cf. rarica    | 16S  | A2015/13_NME| M012204               | Central Himalaya              | 29.51| 82.09| Hofmann et al. (2019) |
| Nanorana      | cf. rarica    | 16S  | A1960/13_NME| M012207               | Central Himalaya              | 29.36| 82.2 | Hofmann et al. (2019) |
| Nanorana      | cf. rarica    | 16S  | A2019/13_NME| M012205               | Central Himalaya              | 29.51| 82.09| Hofmann et al. (2019) |
| Nanorana      | cf. rarica    | 16S  | A1965/13_NME| M012206               | Central Himalaya              | 29.513| 82.092| Hofmann et al. (2019) |
| Nanorana      | sp.           | 16S  | R5_12_NME   | M012169               | Central Himalaya              | 28.513| 83.033| Hofmann et al. (2019) |
| Nanorana      | sp.           | 16S  | SH070556_NME| M012172               | Central Himalaya              | 28.622| 83.662| Hofmann et al. (2019) |
| Nanorana      | sp.           | 16S  | Ne13_13_NME | M012194               | Central Himalaya              | 27.576| 86.514| Hofmann et al. (2019) |
| Nanorana      | sp.           | 16S  | Ne1_13_NME  | M012195               | Central Himalaya              | 27.689| 86.731| Hofmann et al. (2019) |
| Nanorana      | sp.           | 16S  | Ne2_13_NME  | M012196               | Central Himalaya              | 27.689| 86.731| Hofmann et al. (2019) |
| Nanorana      | sp.           | 16S  | Ne9_13_NME  | M012197               | Central Himalaya              | 27.671| 86.765| Hofmann et al. (2019) |
| Nanorana      | sp.           | 16S  | SH080545_NME| M012187               | Central Himalaya              | 27.703| 86.337| Hofmann et al. (2019) |
| Nanorana      | sp.           | 16S  | SH080593_NME| M012180               | Central Himalaya              | 27.686| 86.252| Hofmann et al. (2019) |
| Nanorana      | sp.           | 16S  | R1_09_13_NME| M012174               | Central Himalaya              | 28.38 | 84.065| Hofmann et al. (2019) |
| Nanorana      | sp.           | 16S  | R2_09_13_NME| M012175               | Central Himalaya              | 28.38 | 84.065| Hofmann et al. (2019) |
| Nanorana      | sp.           | 16S  | SH070510_NME| M012177               | Central Himalaya              | 28.074| 85.302| Hofmann et al. (2019) |
| Nanorana      | sp.           | 16S  | SH080551_NME| M012190               | Central Himalaya              | 27.703| 86.337| Hofmann et al. (2019) |
| Nanorana      | sp.           | 16S  | SH080592_NME| M012179               | Central Himalaya              | 27.686| 86.252| Hofmann et al. (2019) |
| Nanorana      | sp.           | 16S  | SH080594_NME| M012181               | Central Himalaya              | 27.686| 86.252| Hofmann et al. (2019) |
| Nanorana      | sp.           | 16S  | SH080570_NME| M012182               | Central Himalaya              | 27.697| 86.275| Hofmann et al. (2019) |
| Genus     | Species | Gene | Voucher No.                | GenBank accession No. | Country/locality                      | Lat    | Long    | Publication                  |
|-----------|---------|------|---------------------------|-----------------------|---------------------------------------|--------|---------|------------------------------|
| *Nanorana* | sp.     | 16S  | SH080571_NME              | MN012183              | Central Himalaya                     | 27.697 | 86.275 | Hofmann et al. (2019)        |
| *Nanorana* | sp.     | 16S  | SH080572_NME              | MN012184              | Central Himalaya                     | 27.697 | 86.275 | Hofmann et al. (2019)        |
| *Nanorana* | sp.     | 16S  | SH080553_NME              | MN012185              | Central Himalaya                     | 27.718 | 86.311 | Hofmann et al. (2019)        |
| *Nanorana* | sp.     | 16S  | SH080555_NME              | MN012186              | Central Himalaya                     | 27.718 | 86.311 | Hofmann et al. (2019)        |
| *Nanorana* | sp.     | 16S  | SH080546_NME              | MN012188              | Central Himalaya                     | 27.703 | 86.337 | Hofmann et al. (2019)        |
| *Nanorana* | sp.     | 16S  | SH080548_NME              | MN012189              | Central Himalaya                     | 27.703 | 86.337 | Hofmann et al. (2019)        |
| *Nanorana* | sp.     | 16S  | SH080552_NME              | MN012191              | Central Himalaya                     | 27.703 | 86.337 | Hofmann et al. (2019)        |
| *Nanorana* | sp.     | 16S  | SH080512_NME              | MN012192              | Central Himalaya                     | 27.595 | 86.34  | Hofmann et al. (2019)        |
| *Nanorana* | sp.     | 16S  | SH080591_NME              | MN012178              | Central Himalaya                     | 27.686 | 86.252 | Hofmann et al. (2019)        |
| *Nanorana* | sp.     | 16S  | SH080523_NME              | MN012193              | Central Himalaya                     | 27.694 | 86.351 | Hofmann et al. (2019)        |
| *Nanorana* | parkeri | 16S  | SYNU-1706031              | MH315959              | China                                 | –      | –       | Qi et al. (2019). Unpublished |
| *Nanorana* | parkeri | 16S  | KizYP205                  | DQ118498              | China                                 | –      | –       | Hu et al. (2006). Unpublished |
| *Nanorana* | parkeri | 16S  | TP3_06_NME                | MN012136              | Transhimalaya and adjacent parts of the Tibetan Plateau | 29.578 | 90.435 | Hofmann et al. (2019)        |
| *Nanorana* | parkeri | 16S  | TP7_06_NME                | MN012155              | Transhimalaya and adjacent parts of the Tibetan Plateau | 31.166 | 92.061 | Hofmann et al. (2019)        |
| *Nanorana* | parkeri | 16S  | N7_06_NME                 | MN012126              | Transhimalaya and adjacent parts of the Tibetan Plateau | 29.589 | 90.214 | Hofmann et al. (2019)        |
| *Nanorana* | parkeri | 16S  | TP5_06_NME                | MN012153              | Transhimalaya and adjacent parts of the Tibetan Plateau | 31.166 | 92.061 | Hofmann et al. (2019)        |
| *Nanorana* | parkeri | 16S  | CAS805L                   | MN012141              | Transhimalaya and adjacent parts of the Tibetan Plateau | 30.09  | 90.48  | Hofmann et al. (2019)        |
| *Nanorana* | ventripunctata | 16S | KizYP200                  | DQ118502              | China                                 | –      | –       | Hu et al. (2006). Unpublished |
| *Nanorana* | ventripunctata | 16S | KizYP201                  | DQ118503              | China                                 | –      | –       | Hu et al. (2006). Unpublished |
| *Nanorana* | ventripunctata | 16S | SH050538_NME              | MN012208              | (sub) alpine parts of the eastern margin of the Tibetan Plateau | 27.788 | 99.855 | Hofmann et al. (2019)        |

(Continues)
| Genus     | Species          | Gene | Voucher No.        | GenBank accession No. | Country/locality                                                                 | Lat    | Long     | Publication                  |
|-----------|------------------|------|-------------------|-----------------------|--------------------------------------------------------------------------------|--------|----------|------------------------------|
| Nanorana  | ventripunctata   | 16S  | SH050539_NME      | MN012209              | (sub) alpine parts of the eastern margin of the Tibetan Plateau                  | 27.788 | 99.855   | Hofmann et al. (2019)        |
| Nanorana  | pleskei          | 16S  | KQ20_14_NME       | MN012165              | (sub) alpine parts of the eastern margin of the Tibetan Plateau                  | 30.377 | 101.675  | Hofmann et al. (2019)        |
| Nanorana  | pleskei          | 16S  | KQ9_14_NME        | MN012166              | (sub) alpine parts of the eastern margin of the Tibetan Plateau                  | 30.377 | 101.675  | Hofmann et al. (2019)        |
| Nanorana  | pleskei          | 16S  | KQ17_14_NME       | MN012162              | (sub) alpine parts of the eastern margin of the Tibetan Plateau                  | 30.377 | 101.675  | Hofmann et al. (2019)        |
| Nanorana  | pleskei          | 16S  | CAS201             | MN012167              | (sub) alpine parts of the eastern margin of the Tibetan Plateau                  | 33.467 | 102.75   | Hofmann et al. (2019)        |
| Nanorana  | pleskei          | 16S  | CAS202             | MN012168              | (sub) alpine parts of the eastern margin of the Tibetan Plateau                  | 33.467 | 102.75   | Hofmann et al. (2019)        |
| Nanorana  | pleskei          | 16S  | KizYP203           | DQ118504              | China                                                                            | –      | –        | Hu et al. (2006). Unpublished |
| Nanorana  | pleskei          | 16S  | KizYP204           | DQ118505              | China                                                                            | –      | –        | Hu et al. (2006). Unpublished |
| Nanorana  | yunnanensis      | 16S  | -                  | KF199150              | China                                                                            | –      | –        | Zhang and Yu (2017). Unpublished |
| Nanorana  | taihangnica      | 16S  | -                  | KJ569109              | China                                                                            | –      | –        | Chen et al. (2015)           |
| Allopaa   | hazarensis       | 16S  | WLM:AH265163      | MW898152              | Pakistan: Murree: Parhana                                                        | 33.84322266 | 73.46941712 | This study                  |
| Allopaa   | hazarensis       | 16S  | WLM:AH461611      | MW898153              | Pakistan: Murree: Bastal Mor (Shangrila Park)                                   | 33.8608605 | 73.37946601 | This study                  |
| Allopaa   | hazarensis       | 16S  | WLM:AH305164      | MW898154              | Pakistan: Murree: Patriata road                                                  | 33.86869227 | 73.4672    | This study                  |
| Allopaa   | hazarensis       | 16S  | WLM:AH305168      | MW898155              | Pakistan: Murree: Patriata road                                                  | 33.86869227 | 73.4672    | This study                  |
| Allopaa   | hazarensis       | 16S  | WLM:AH265161      | MW898156              | Pakistan: Murree: Bell Garan                                                    | 33.88477419 | 73.50041681 | This study                  |
| Allopaa   | hazarensis       | 16S  | AH2651611         | MW898157              | Pakistan: Murree: Bell Garan                                                    | 33.88477419 | 73.50041681 | This study                  |
| Allopaa   | hazarensis       | 16S  | WLM:AH299171      | MW898158              | Pakistan: Murree: Mureer expressway                                              | 33.84833345 | 73.42944468 | This study                  |
| Allopaa   | hazarensis       | 16S  | WLM:AH299174      | MW898159              | Pakistan: Murree: Parhana                                                        | 33.84324964 | 73.46955605 | This study                  |
| Allopaa   | hazarensis       | 16S  | WLM:AH305167      | MW898160              | Pakistan: Murree: Aliyot                                                         | 33.90124184 | 73.43383471 | This study                  |

(Continues)
### APPENDIX 1 (Continued)

| Genus       | Species                  | Gene | Voucher No. | GenBank accession No. | Country/locality                  | Lat       | Long       | Publication                  |
|-------------|--------------------------|------|-------------|-----------------------|-----------------------------------|-----------|------------|------------------------------|
| Allopaa     | hazarensis               | 16S  | WLM:AH296163| MW898161              | Pakistan: Murree: Mall road        | 33.9877782| 73.4927785| This study                   |
| Allopaa     | hazarensis               | 16S  | WLM:AH305162| MW898162              | Pakistan: Murree: Jhika Gali      | 33.91422267| 73.4167499| This study                   |
| Allopaa     | hazarensis               | 16S  | WLM:AH285161| MW898163              | Pakistan: Murree: Army dog center | 33.91430535| 73.39388838| This study                   |
| Allopaa     | hazarensis               | 16S  | WLM:AH305165| MW898164              | Pakistan: Murree: Jhika Gali      | 33.91422267| 73.39388838| This study                   |
| Allopaa     | hazarensis               | 16S  | WLM:AH299175| MW898165              | Pakistan: Kotli Sattian: Kyonian  | 33.75205517| 73.49188943| This study                   |
| Allopaa     | hazarensis               | 16S  | WLM:AH289174| MW898166              | Pakistan: Kotli Sattian: New Koreana | 33.82288846| 73.52886145| This study                   |
| Allopaa     | hazarensis               | 16S  | WLM:AH299173| MW898167              | Pakistan: Kotli Sattian: Kyonian  | 33.75205517| 73.49188943| This study                   |
| Allopaa     | hazarensis               | 16S  | WLM:AH289173| MW898168              | Pakistan: Kotli Sattian: Nalari   | 33.81372258| 73.50108327| This study                   |
| Allopaa     | hazarensis               | 16S  | WLM:AH289175| MW898169              | Pakistan: Kotli Sattian: Nalari   | 33.81372258| 73.50108327| This study                   |
| Allopaa     | hazarensis               | 16S  | WLM:AH252171| MW898170              | Pakistan: Murree: Chaka Begwal    | 33.797    | 73.37674988| This study                   |
| Allopaa     | hazarensis               | 16S  | WLM:AH296161| MW898171              | Pakistan: Murree: Angoori         | 33.81458356| 73.36883365| This study                   |
| Allopaa     | hazarensis               | 16S  | WLM:AH296162| MW898172              | Pakistan: Murree: Angoori police station | 33.79941651| 73.35383292| This study                   |
| Nanorana    | cf. polunini             | 16S  | R3_09_13_NME | MN012087              | Central Himalaya                 | 28.38     | 84.065     | Hofmann et al. (2019)        |
| Nanorana    | cf. blanfordii           | 16S  | JS040534_NME | MN012071              | Central Himalaya                 | 27.617    | 87.233     | Hofmann et al. (2019)        |
| Nanorana    | cf. blanfordii           | 16S  | JS060520_NME | MN012073              | Central Himalaya                 | 27.173    | 87.421     | Hofmann et al. (2019)        |
| Nanorana    | cf. blanfordii           | 16S  | JS060515_NME | MN012074              | Central Himalaya                 | 27.214    | 87.463     | Hofmann et al. (2019)        |
| Nanorana    | cf. blanfordii           | 16S  | JS060508_NME | MN012075              | Central Himalaya                 | 27.413    | 87.734     | Hofmann et al. (2019)        |
| Nanorana    | cf. blanfordii           | 16S  | JS040529_NME | MN012067              | Central Himalaya                 | 27.617    | 87.233     | Hofmann et al. (2019)        |
| Nanorana    | cf. blanfordii           | 16S  | JS040532_NME | MN012069              | Central Himalaya                 | 27.617    | 87.233     | Hofmann et al. (2019)        |
| Nanorana    | cf. blanfordii           | 16S  | JS040531_NME | MN012068              | Central Himalaya                 | 27.617    | 87.233     | Hofmann et al. (2019)        |
| Nanorana    | cf. blanfordii           | 16S  | JS040535_NME | MN012072              | Central Himalaya                 | 27.617    | 87.233     | Hofmann et al. (2019)        |
| Nanorana    | cf. blanfordii           | 16S  | JS040533_NME | MN012070              | Central Himalaya                 | 27.617    | 87.233     | Hofmann et al. (2019)        |
| Nanorana    | cf. polunini             | 16S  | R15_12_NME | MN012082              | Central Himalaya                 | 28.502    | 83.129     | Hofmann et al. (2019)        |
| Nanorana    | sp.                      | 16S  | 2Pul_RAS    | MN012200              | NW Himalaya, (Himachal Pradesh)   | 31.996    | 77.448     | Hofmann et al. (2019)        |

(Continues)
## APPENDIX 1 (Continued)

| Genus       | Species        | Gene | Voucher No. | GenBank accession No. | Country/locality                  | Lat    | Long    | Publication               |
|-------------|----------------|------|-------------|-----------------------|-----------------------------------|--------|---------|---------------------------|
| Nanorana    | sp.            | 16S  | 782_RAS     | MN012201              | NW Himalaya, (Himachal Pradesh)   | 31.261 | 77.45   | Hofmann et al. (2019)     |
| Nanorana    | sp.            | 16S  | 2Bhan_RAS   | MN012199              | NW Himalaya, (Himachal Pradesh)   | 32.873 | 75.858  | Hofmann et al. (2019)     |
| Nanorana    | vicina         | 16S  | WLM:NV28917 | MW898173              | Pakistan: Murree: Sursi           | 33.86869227 | 73.46719617 | This study               |
| Nanorana    | vicina         | 16S  | WLM:NV289171| MW898174              | Pakistan: Murree: Bell Garan      | 33.84777419 | 73.50041681 | This study               |
| Nanorana    | vicina         | 16S  | WLM:NV299172| MW898175              | Pakistan: Murree: expressway       | 33.84833345 | 73.42944468 | This study               |
| Nanorana    | vicina         | 16S  | WLM:NV265166| MW898176              | Pakistan: Murree: Mall road        | 33.9877782 | 73.49277785 | This study               |
| Nanorana    | vicina         | 16S  | WLM:NV289172| MW898177              | Pakistan: Murree: Parhara          | 33.84324964 | 73.46955605 | This study               |
| Nanorana    | vicina         | 16S  | WLM:NV131017| MW898178              | Pakistan: Murree: Jhika Gali       | 33.91422267 | 73.4167499  | This study               |
| Nanorana    | vicina         | 16S  | WLM:NV265163| MW898179              | Pakistan: Kotli: Sattian: New Koreana | 33.82288846 | 73.52886145 | This study               |
| Nanorana    | vicina         | 16S  | WLM:NV265164| MW898180              | Pakistan: Kotli: Sattian: Ratta Kas | 33.72438924 | 73.45886097 | This study               |
| Nanorana    | vicina         | 16S  | WLM:NV265167| MW898181              | Pakistan: Murree: Angoori (Police station) | 33.79941651 | 73.35383292 | This study               |
| Nanorana    | cf. ercepeae   | 16S  | A2017/13_NME| MN012076              | Central Himalaya                  | 29.374 | 81.137  | Hofmann et al. (2019)     |
| Nanorana    | cf. ercepeae   | 16S  | A5_12_NME   | MN012080              | Central Himalaya                  | 28.857 | 82.976  | Hofmann et al. (2019)     |
| Nanorana    | cf. rostandi   | 16S  | R17_12_NME  | MN012101              | Central Himalaya                  | 28.519 | 83.264  | Hofmann et al. (2019)     |
| Nanorana    | cf. rostandi   | 16S  | SH070550_NME| MN012102              | Central Himalaya                  | 28.683 | 83.591  | Hofmann et al. (2019)     |
| Nanorana    | sp.            | 16S  | A1966/13_NME| MN012198              | Central Himalaya (Chainpur Himal)  | 29.374 | 81.137  | Hofmann et al. (2019)     |
| Nanorana    | cf. polunini   | 16S  | SH070507_NME| MN012084              | Central Himalaya                  | 28.06  | 85.294  | Hofmann et al. (2019)     |
| Nanorana    | cf. polunini   | 16S  | SH070509_NME| MN012085              | Central Himalaya                  | 28.08  | 85.295  | Hofmann et al. (2019)     |
| Nanorana    | cf. polunini   | 16S  | SH070531_NME| MN012086              | Central Himalaya                  | 27.965 | 85.472  | Hofmann et al. (2019)     |
| Nanorana    | liebigii       | 16S  | A17_12_NME  | MN012104              | Central Himalaya                  | 28.519 | 83.264  | Hofmann et al. (2019)     |
| Nanorana    | liebigii       | 16S  | SH070515_NME| MN012106              | Central Himalaya                  | 28.099 | 85.317  | Hofmann et al. (2019)     |
| Nanorana    | liebigii       | 16S  | SH080506_NME| MN012108              | Central Himalaya                  | 27.609 | 86.295  | Hofmann et al. (2019)     |
| Nanorana    | liebigii       | 16S  | Ne16_13_NME | MN012115              | Central Himalaya                  | 27.584 | 86.411  | Hofmann et al. (2019)     |
| Nanorana    | liebigii       | 16S  | JS040512_NME| MN012119              | Central Himalaya                  | 27.631 | 87.224  | Hofmann et al. (2019)     |

(Continues)
| Genus         | Species       | Gene | Voucher No. | GenBank accession No. | Country/locality     | Lat  | Long  | Publication                      |
|---------------|---------------|------|-------------|-----------------------|----------------------|------|-------|----------------------------------|
| Nanorana      | liebigii      | 16S  | JS060503_NME | MN012124              | Central Himalaya     | 27.407 | 87.752 | Hofmann et al. (2019)            |
| Nanorana      | liebigii      | 16S  | JS060511_NME | MN012122              | Central Himalaya     | 27.296 | 87.535 | Hofmann et al. (2019)            |
| Nanorana      | liebigii      | 16S  | KIZ-RDXZL1   | DQ118499              | Central Himalaya     | 27.485 | 88.907 | Hofmann et al. (2019)            |
| Nanorana      | liebigii      | 16S  | 2003.308     | KR827956              | Nepal: Pangum        | –     | –     | Grosjean et al. (2015)           |
| Nanorana      | liebigii      | 16S  | SH0805109_NME| MN012107              | Central Himalaya     | 27.673 | 86.24 | Hofmann et al. (2019)            |
| Nanorana      | liebigii      | 16S  | Ne12_13_NME  | MN012117              | Central Himalaya     | 27.584 | 86.594 | Hofmann et al. (2019)            |
| Nanorana      | liebigii      | 16S  | Ne10_13_NME  | MN012118              | Central Himalaya     | 27.586 | 86.635 | Hofmann et al. (2019)            |
| Sphaerotheca  | pluvialis     | 16S  | -            | AF249042              | Sri Lanka            | –     | –     | Bossuyt and Milinkovitch (2000)  |
| Sphaerotheca  | dobsonii      | 16S  | Sdob-In      | AB530608              | India: Bajipe, Mangalore | 12.9804 | 74.883618 | Hasan et al. (2014)               |
| Sphaerotheca  | dobsonii      | 16S  | 16S-dob      | AB277305              | India: Bajipe        | 12.992151 | 74.878932 | Kotaki et al. (2008)             |
| Sphaerotheca  | dobsonii      | 16S  | INHER Amphibia-86 | KY215970              | India: Tamhini, Pune, Maharashtra | 18.477 | 73.427 | Padhye et al. (2017)             |
| Sphaerotheca  | dobsonii      | 16S  | WILD-16-AMP-651 | KY215971              | India: Devi Hasool, Maharashtra | 16.742 | 73.432 | Padhye et al. (2017)             |
| Sphaerotheca  | breviceps     | 16S  | BNHS 6005    | KY215977              | India: Tranquebar (Tharangambadi), Tamil Nadu | 11.062 | 79.813 | Padhye et al. (2017)             |
| Sphaerotheca  | breviceps     | 16S  | WILD-16-AMP-645 | KY215978              | India: Tranquebar (Tharangambadi), Tamil Nadu | 11.062 | 79.813 | Padhye et al. (2017)             |
| Sphaerotheca  | magadha       | 16S  | ZSI/WRC/2179 | MK694738              | India: Nawadih village, Koderma, Jharkhand | 24.417985 | 85.468 | Prasad et al. (2019)             |
| Sphaerotheca  | breviceps     | 16S  | WILD-16-AMP-647 | KY215976              | India: Maithon, Jharkhand | 23.776 | 86.809 | Padhye et al. (2017)             |
| Sphaerotheca  | breviceps     | 16S  | BNHS 6006    | KY215975              | India: Maithon, Jharkhand | 23.776 | 86.809 | Padhye et al. (2017)             |
| Sphaerotheca  | rolandae      | 16S  | Sptr         | GU191122              | India: Rajasthan     | –     | –     | Sharma et al. (2009), Unpublished |
| Sphaerotheca  | pashchima     | 16S  | WILD-16-AMP-644 | KY215994              | India: Maharashtra, Pune, Tamhini | 18.477 | 73.427 | Padhye et al. (2017)             |
| Sphaerotheca  | pashchima     | 16S  | WILD-16-AMP-642 | KY215991              | India: Maharashtra, Saswad-Waghapur Road, Ambodi village | 18.308 | 74.083 | Padhye et al. (2017)             |
| Sphaerotheca  | pashchima     | 16S  | ZSI-WRC A/1549 | KY215980              | India: Karnataka, Yellapur-Haliyal Road | 15.16 | 74.759 | Padhye et al. (2017)             |
### APPENDIX 1 (Continued)

| Genus       | Species      | Gene | Voucher No. | GenBank accession No. | Country/locality                           | Lat      | Long     | Publication                  |
|-------------|--------------|------|-------------|-----------------------|---------------------------------------------|----------|----------|------------------------------|
| *Sphaerotheca* | *pashchima* | 16S  | BNHS 6013   | KY215983              | India: Maharashtra, Raigad District, Kolad    | 18.404   | 73.321   | Padhye et al. (2017)         |
| *Sphaerotheca* | *pashchima* | 16S  | BNHS 6018   | KY215988              | India: Gujarat, Dang District, Waghai-Ahwa road | 20.709   | 73.709   | Padhye et al. (2017)         |
| *Sphaerotheca* | *pashchima* | 16S  | WILD-16-AMP-641 | KY215990 | India: Maharashtra, Ahmednagar District, Karjat, near Rehekuri WLS | 18.598 | 74.974 | Padhye et al. (2017) |
| *Sphaerotheca* | *pashchima* | 16S  | BNHS 6017   | KY215987              | India: Maharashtra, Chinchli to Salher fort road | 20.747   | 73.973   | Padhye et al. (2017)         |
| *Sphaerotheca* | *pashchima* | 16S  | WILD-16-AMP-643 | KY215993 | India: Gujarat, Veghai Road, Ahwa-Dang | 20.764   | 73.676   | Padhye et al. (2017)         |
| *Sphaerotheca* | *pashchima* | 16S  | BNHS 6012   | KY215981              | India: Karnataka, Near Yellapur               | 14.98    | 74.731   | Padhye et al. (2017)         |
| *Sphaerotheca* | *pashchima* | 16S  | ZSI-WRC A/1550 | KY215982 | India: Karnataka, Near Yellapur | 14.98    | 74.731 | Padhye et al. (2017) |
| *Sphaerotheca* | *pashchima* | 16S  | SbHR25HNBGU | KK815437              | India                                        | 29.91889 | 78.12618 | Chowdhary et al. (2017). Unpublished |
| *Sphaerotheca* | *pashchima* | 16S  | Sb31HNBGU   | KK815435              | India                                        | 30.28476 | 77.97365 | Chowdhary et al. (2017). Unpublished |
| *Sphaerotheca* | *pashchima* | 16S  | SbSG17HNBGU | KK815440              | India                                        | 30.22177 | 78.78434 | Chowdhary et al. (2017). Unpublished |
| *Sphaerotheca* | *pashchima* | 16S  | WLM:SP46163 | MW898191              | Pakistan: Rawalpindi: Chakri                  | 33.55389 | 73.015766 | This study |
| *Sphaerotheca* | *pashchima* | 16S  | WLM:SP236166 | MW898192 | Pakistan: Islamabad: River Korang (Way to Angoori) | 33.78342224 | 73.26690085 | This study |
| *Sphaerotheca* | *pashchima* | 16S  | WLM:SP177161 | MW898193              | Pakistan: Kahuta: Bandhya                      | 33.62661116 | 73.47552765 | This study |
| *Sphaerotheca* | *pashchima* | 16S  | WLM:SP177162 | MW898194              | Pakistan: Rawalpindi: Adiala Road              | 33.50751564 | 73.03706598 | This study |
| *Sphaerotheca* | *pashchima* | 16S  | SbJM1HNBGU  | KK815439              | India                                        | 30.55444 | 79.5635 | Chowdhary et al. (2017). Unpublished |
| *Fejervarya*   | *cancrivora* | 16S  | - | AF346810 | Indonesia: Java | - | - | Veith et al. (2001) |
| *Fejervarya*   | *cancrivora* | 16S  | FC-3 | AB570273 | Indonesia: Malang, East Java | - | - | Kurniawan et al. (2014) |
### APPENDIX 1 (Continued)

| Genus   | Species    | Gene | Voucher No. | GenBank accession No. | Country/locality        | Lat     | Long    | Publication                  |
|---------|------------|------|-------------|-----------------------|-------------------------|---------|---------|------------------------------|
| Fejervarya | limnocharis | 16S   | TZ25        | AF285211              | North Vietnam: Vietnam  | –       | –       | Ziegler (2000). Unpublished thesis |
| Fejervarya | limnocharis | 16S   | MVZ226347   | EU979847              | Vietnam: Tam Dao, Vinh Phu Prov. | 21.406484 | 105.6417 | Che et al. (2009)                |
| Fejervarya | limnocharis | 16S   | FL-1        | AB570262              | Indonesia: Padang, West Sumatra | –0.943817 | 100.41878 | Kurniawan et al. (2014)          |
| Fejervarya | limnocharis | 16S   | FL-4        | AB570265              | Indonesia: Rokan Hilir, Riau, Sumatra | 1.763345 | 100.75021 | Kurniawan et al. (2014)          |
| Fejervarya | limnocharis | 16S   | -           | JQ621940              | China: Yunnan (south west china) | –       | –       | Gao and Fan (2012). Unpublished |
| Fejervarya | limnocharis | 16S   | 1999.5721   | KR827740              | Vietnam: Lao Cal, Sapa   | 22.335302 | 103.84612 | Grosjean et al. (2015)          |
| Fejervarya | limnocharis | 16S   | Okin(2)     | AB070734              | Japan: Okinawa           | 26.388602 | 127.78929 | Sumida et al. (2002)            |
| Fejervarya | limnocharis | 16S   | 16SF15      | KU840567              | China: Long Quan, Sichuan Prov. | −0.716667 | 112.44245 | Goutte et al. (2016)            |
| Fejervarya | limnocharis | 16S   | H001        | HQ226055              | Taiwan                   | –       | –       | Chang and Liu (2010). Unpublished |
| Fejervarya | limnocharis | 16S   | H006        | HQ226060              | Taiwan                   | –       | –       | Chang and Liu (2010). Unpublished |
| Minervarya | rufescens  | 16S   | SDBDU 2015.2882 | KY447323            | India: Pozhuthana, Kerala | 11.590328 | 76.02067 | Garg and Biju (2017)            |
| Minervarya | greenel    | 16S   | -           | AB488891              | Sri Lanka: Hakgala       | 6.918789 | 80.83113 | Kotaki et al. (2010)            |
| Minervarya | kudremukhensis | 16S | -          | AB488898 | India: Kudremukh (Karnataka) | 13.222713 | 75.250694 | Kotaki et al. (2010)            |
| Minervarya | sahyadris  | 16S   | Fsah-In2    | AB530605              | India: Aralam (Kerala)   | 11.970321 | 75.800625 | Hasan et al. (2014)             |
| Minervarya | caperata   | 16S   | -           | AB488894              | India: Mudigere (Karnataka) | 13.132451 | 75.640472 | Kotaki et al. (2010)            |
| Minervarya | asmati     | 16S   | FaCSE_17    | KP849815              | Bangladesh: Dhaka        | 23.742528 | 90.453325 | Howlader et al. (2016)          |
| Minervarya | asmati     | 16S   | HJZG-04     | MG010390              | Bangladesh               | –       | –       | Jahan et al. (2018). Unpublished |
| Minervarya | granosa    | 16S   | BNHS 4652   | AB355839              | India: Western Ghats, Madikeri | 10.246409 | 76.827714 | Kuramoto et al. (2007)          |
| Minervarya | granosa    | 16S   | -           | AB488895              | India: Mudigere (Karnataka) | 13.131135 | 75.64207 | Kotaki et al. (2010)            |
| Minervarya | syhadrensis | 16S | -          | AB488892              | Sri Lanka: Hakgala       | 6.918789 | 80.831063 | Kotaki et al. (2010)            |
| Minervarya | syhadrensis | 16S   | WHT2665     | AY141843              | Sri Lanka               | –       | –       | Meegaskumbura et al. (2002)     |
| Minervarya | pierrei    | 16S   | FSP 04      | MK635483              | Bangladesh               | –       | –       | Jahan et al. (2019). Unpublished |
| Genus       | Species | Gene | Voucher No. | GenBank accession No. | Country/locality            | Lat     | Long     | Publication                        |
|-------------|---------|------|-------------|------------------------|-----------------------------|---------|----------|-----------------------------------|
| Minervarya  | pierrei | 16S  | F1DSE_14    | KP849816               | Bangladesh                  | -       | -        | Howlader et al. (2016)            |
| Minervarya  | pierrei | 16S  | Fsp.S-Bd1   | ABS30509               | Bangladesh: Mymensingh, Char Nilokhia | 24.08494 | 90.8911  | Hasan et al. (2012)               |
| Minervarya  | pierrei | 16S  | FSP 02      | MK635481               | Bangladesh                  | -       | -        | Jahan et al. (2019), Unpublished  |
| Minervarya  | pierrei | 16S  | -           | AB488888               | Nepal: Chinw                        | 27.533841 | 84.436593 | Kotaki et al. (2010)             |
| Minervarya  | syhadrensis | 16S | IN023       | AY882955               | India: Kolkata: West Bengal    | 22.571807 | 88.427181 | Tandon et al. (2005), Unpublished |
| Minervarya  | pierrei | 16S  | Fsp.S-Bd2   | ABS30510               | Bangladesh: Cox’ s Bazar, Laboni point | 21.438741 | 92.016288 | Hasan et al. (2012)               |
| Minervarya  | pierrei | 16S  | WLM:MP48171 | MW898182               | Pakistan: Taxila: Margalla Hills National Park | 33.731587 | 72.94793  | This study                        |
| Minervarya  | pierrei | 16S  | WLM:MP48173 | MW898183               | Pakistan: Gujor Khan: Susral    | 33.15186109 | 73.24997274 | This study                        |
| Minervarya  | pierrei | 16S  | WLM:MP48174 | MW898184               | Pakistan: Gujor Khan: Morha Phool | 33.16852748 | 73.08461077 | This study                        |
| Minervarya  | pierrei | 16S  | WLM:MP48172 | MW898185               | Pakistan: Islamabad: Korang river | 33.719977 | 73.147451  | This study                        |
| Minervarya  | pierrei | 16S  | WLM:MP48175 | MW898186               | Pakistan: Islamabad: Korang river | 33.719977 | 73.147451  | This study                        |
| Minervarya  | pierrei | 16S  | WLM:MP236167 | MW898187              | Pakistan: Rawalpindi: Kalyal Sharef | 33.53288389 | 73.05085836 | This study                        |
| Minervarya  | syhadrensis | 16S | IN021       | AY882953               | India: Kolkata: West Bengal    | 22.630128 | 88.418941 | Tandon et al. (2005), Unpublished |
| Minervarya  | syhadrensis | 16S | IN016       | AY882948               | India: Kolkata: West Bengal    | 22.573076 | 88.442974 | Tandon et al. (2005), Unpublished |
| Hoplobatrachus | rugulose | 16S | Chin-Chaco-3663-16S | AB636617 | Thailand: Chachoengsao | 13.696414 | 101.64166 | Alam et al. (2012)                |
| Hoplobatrachus | rugulose | 16S | Chin-Chaco-3911-16S | AB636615 | Thailand: Chachoengsao | 13.746443 | 101.54347 | Alam et al. (2012)                |
| Hoplobatrachus | tigerinus | 16S | 31          | AB167944               | India: Mangalore, Padil         | 12.872854 | 74.888555 | Kurabayashi et al. (2005)         |
| Hoplobatrachus | tigerinus | 16S | 30          | AB167943               | India: Mangalore, Padil         | 12.869214 | 74.882375 | Kurabayashi et al. (2005)         |
| Hoplobatrachus | tigerinus | 16S | Htig-In     | AB530600               | India: Padil                   | 12.867122 | 74.882085 | Hasan et al. (2014)               |
| Hoplobatrachus | tigerinus | 16S | RGCB 5794   | KU179090               | India: Kerala                  | 9.797816  | 76.895336 | Anoop and George (2016), Unpublished |

(Continues)
| Genus            | Species       | Gene | Voucher No. | GenBank accession No. | Country/locality             | Lat       | Long        | Publication                          |
|------------------|---------------|------|-------------|-----------------------|------------------------------|-----------|-------------|-------------------------------------|
| Hoplobatrachus   | tigerinus     | 16S  | RGCB 5060   | KU179088              | India: Kerala                | 9.803033  | 76.912811   | Anoop and George (2016). Unpublished |
| Hoplobatrachus   | tigerinus     | 16S  | RGCB 5731   | KU179089              | India: Kerala                | 9.797383  | 76.915643   | Anoop and George (2016). Unpublished |
| Hoplobatrachus   | tigerinus     | 16S  | tig-baji-A  | AB290412              | India: Bajipe                 | 12.962778 | 74.890833   | Alam et al. (2008)                  |
| Hoplobatrachus   | tigerinus     | 16S  | tig-padi-A  | AB272594              | India: Padile                 | 12.869167 | 74.8825     | Alam et al. (2008)                  |
| Hoplobatrachus   | tigerinus     | 16S  | WLM:HT46162 | MW898188              | Pakistan: Islamabad: Naval anchorage | 33.568652 | 73.183754   | This study                          |
| Hoplobatrachus   | tigerinus     | 16S  | WLM:HT236161| MW898189              | Pakistan: Kallar Syedan: Bhayakrial | 33.483528 | 73.35569476 | This study                          |
| Hoplobatrachus   | tigerinus     | 16S  | WLM:HT48177 | MW898190              | Pakistan: Rawalpindi: Adiala Road | 33.482533 | 73.005784   | This study                          |
| Hoplobatrachus   | tigerinus     | 16S  | IABHU<JPN>:4001 | AB671183           | Bangladesh: Mymensingh          | 24.745336 | 90.404392   | Hasan et al. (2012)                 |
| Hoplobatrachus   | tigerinus     | 16S  | Tig-Control-3920-165 | AB636619           | Bangladesh: Mymensingh, BAU Campus | 24.724225 | 90.428289   | Alam et al. (2012)                 |
| Hoplobatrachus   | tigerinus     | 16S  | Httig-Bd1   | AB530500             | Bangladesh: Mymensingh, BAU Campus | 24.45     | 90.24       | Hasan et al. (2012)                 |
| Hoplobatrachus   | tigerinus     | 16S  | IABHU<JPN>:4000 | AB671182           | Bangladesh: Mymensingh          | 24.745024 | 90.404563   | Hasan et al. (2012)                 |
| Hoplobatrachus   | tigerinus     | 16S  | tig-jaga-B  | AB272590             | Bangladesh: Jagannathganj      | 24.75     | 89.816667   | Alam et al. (2008)                  |
| Hoplobatrachus   | tigerinus     | 16S  | tig-sylh-A  | AB272588             | Bangladesh: Sylhet              | 24.92     | 92          | Alam et al. (2008)                  |
| Hoplobatrachus   | tigerinus     | 16S  | tig-BAUC-B  | AB272584             | Bangladesh: Mymensingh, BAU Campus | 24.747222 | 90.406667   | Alam et al. (2008)                  |
| Hoplobatrachus   | tigerinus     | 16S  | tig-BAUC-A  | AB272583             | Bangladesh: Mymensingh, BAU Campus | 24.738321 | 90.401817   | Alam et al. (2008)                  |
| Euphlyctis       | aloysii       | 16S  | BNHS5995    | KU870382             | India: Karnataka               | 13.3728   | 74.8016     | Priti et al. (2016)                 |
| Euphlyctis       | hexadactylus  | 16S  | hex-mudi-A  | AB272608             | India: Mudigere, Karnataka     | 12.87     | 74.92       | Alam et al. (2008)                  |
| Euphlyctis       | hexadactylus  | 16S  | hex-adya-B  | AB272607             | India: Adyar, Karnataka         | 13.134444 | 75.641111   | Alam et al. (2008)                  |
| Euphlyctis       | karaavali     | 16S  | BNHS5986    | KU870373             | India: Uttara Kannada, Karnataka | 14.5512   | 74.3378     | Priti et al. (2016)                 |
| Euphlyctis       | ehrenbergi    | 16S  | MNHN 2000.649 | AY014367           | Yemen                          | –         | –           | Kosuch et al. (2001)                |
| Euphlyctis       | mudigere      | 16S  | Emud-In     | AB530599             | India: Mudigere                | 13.142503 | 75.646705   | Hasan et al. (2014)                 |
| Euphlyctis       | cyanophlyctis | 16S  | RGCB 79     | KU179080             | India: Kerala                  | 10.046289 | 77.003533   | Anoop and George (2016). Unpublished |

APPENDIX 1 (Continued)
| Genus        | Species          | Gene | Voucher No. | GenBank accession No. | Country/locality | Lat    | Long    | Publication                          |
|--------------|------------------|------|-------------|-----------------------|------------------|--------|---------|--------------------------------------|
| *Euphlyctis* | cyanophlyctis    | 16S  | -           | EU523739              | India: Kerala    | 10.026056 | 77.07216 | Liya et al. (2008). Unpublished      |
| *Euphlyctis* | cyanophlyctis    | 16S  | RGCB 1018   | KU179079              | India: Nagapattinam, Tamil Nadu | 10.780117 | 79.83786 | Anoop and George (2016). Unpublished |
| *Euphlyctis* | mudigere         | 16S  | -           | AF249053              | India: Mudigere  | 12.904912 | 74.85309 | Bossuyt and Milinkovitch (2000)     |
| *Euphlyctis* | cyanophlyctis    | 16S  | MNHN 2000.650 | AY014366              | India: Cochin    | –      | –       | Kosuch et al. (2001)                |
| *Euphlyctis* | cyanophlyctis    | 16S  | cya-padi-B  | AB272604              | India: Padil     | 12.869167 | 74.8825  | Alam et al. (2008)                  |
| *Euphlyctis* | cyanophlyctis    | 16S  | 030523-03   | AB167938              | India: Western Ghats, Madikeri | 12.427069 | 75.731093 | Kurabayashi et al. (2005)           |
| *Euphlyctis* | cyanophlyctis    | 16S  | -           | AB488901              | India: Mangalore | 12.904912 | 74.85309 | Kotaki et al. (2010)                |
| *Euphlyctis* | cyanophlyctis    | 16S  | Ecya-In     | AB530596              | India: Madikeri  | 12.429919 | 75.73281 | Hasan et al. (2014)                 |
| *Euphlyctis* | kalasgramensis   | 16S  | ZMUVAS5     | MK920114              | Pakistan: Pattoki, Kasur | 31.044  | 73.87492 | Ali et al. (2020)                  |
| *Euphlyctis* | kalasgramensis   | 16S  | ZMUVAS1     | MK881165              | Pakistan: Pattoki, Kasur | 31.044  | 73.87492 | Ali et al. (2020)                  |
| *Euphlyctis* | kalasgramensis   | 16S  | cya-assa-A  | AB290420              | India: Assam     | 26.165556 | 92.841389 | Alam et al. (2008)                 |
| *Euphlyctis* | kalasgramensis   | 16S  | cya-BAUC-A  | AB272601              | Bangladesh: Mymensingh, BAU Campus | 24.747222 | 90.406667 | Alam et al. (2008)                 |
| *Euphlyctis* | kalasgramensis   | 16S  | Ecya-Bd3    | AB530496              | Bangladesh: Cox's Bazar, Laboni point | 21.41  | 91.98  | Hasan et al. (2012)                |
| *Euphlyctis* | kalasgramensis   | 16S  | Ecya-Bd4    | AB530497              | Bangladesh: Cox's Bazar, Laboni point | 21.41  | 91.98  | Hasan et al. (2012)                |
| *Euphlyctis* | kalasgramensis   | 16S  | Ecya-Bd2    | AB530495              | Bangladesh: Mymensingh, Char Nilokhia | 21.45  | 90.24  | Hasan et al. (2012)                |
| *Euphlyctis* | kalasgramensis   | 16S  | ESP 02      | MK635488              | Bangladesh: Kalashram | 22.760392 | 90.319291 | Jahan et al. (2019). Unpublished    |
| *Euphlyctis* | kalasgramensis   | 16S  | HJZG-03     | MG010388              | Bangladesh: Barisal | 22.696171 | 90.355864 | Jahan et al. (2019). Unpublished    |
| *Euphlyctis* | kalasgramensis   | 16S  | MZH-3381    | KP091868              | Bangladesh: Kalashram | 22.760392 | 90.317961 | Howlader et al. (2015b)            |
| *Euphlyctis* | kalasgramensis   | 16S  | PUCZM/IX/SL64 | MH087080              | India: Mizoram   | 23.759  | 92.799  | Lalonungna et al. (2019) Unpublished |
| *Euphlyctis* | kalasgramensis   | 16S  | PUCZM/IX/SL613 | MH087081              | India: Mizoram   | 23.712  | 92.662  | Lalonungna et al. (2019) Unpublished |

(Continues)
| Genus       | Species                  | Gene | Voucher No. | GenBank accession No. | Country/locality       | Lat    | Long    | Publication                  |
|-------------|--------------------------|------|-------------|-----------------------|------------------------|--------|---------|------------------------------|
| Euphlyctis  | *kalasgramensis*         | 16S  | PUCZM/IX/SL62 | MH087078              | India: Mizoram         | 23.759 | 92.799  | Lalronunga et al. (2019)     |
|             |                          |      |             |                       |                        |        |         | Unpublished                 |
| Euphlyctis  | *kalasgramensis*         | 16S  | PUCZM/IX/SL63 | MH087079              | India: Mizoram         | 23.759 | 92.799  | Lalronunga et al. (2019)     |
|             |                          |      |             |                       |                        |        |         | Unpublished                 |
| Euphlyctis  | *kalasgramensis*         | 16S  | MZH-3383     | KP091856              | Bangladesh: Barisal     | 22.694206 | 90.351395 | Howlade et al. (2015b)       |
| Euphlyctis  | *cyanophlyctis*          | 16S  | F1           | KF992815              | Iran: Tiran             | 32.701346 | 51.153867 | Khajeh et al. (2014)         |
| Euphlyctis  | *cyanophlyctis*          | 16S  | A1           | KF992800              | Iran: Apatan            | 27.350647 | 62.098627 | Khajeh et al. (2014)         |
| Euphlyctis  | *kalasgramensis*         | 16S  | WLM:EK303173 | MW898195              | Pakistan: Taxila: Village Khurram | 33.7345002 | 72.86511099 | This study                  |
| Euphlyctis  | *kalasgramensis*         | 16S  | WLM:EK48179  | MW898196              | Pakistan: Islamabad: Shahdara | 33.78513863 | 73.17800038 | This study                  |
| Euphlyctis  | *kalasgramensis*         | 16S  | WLM:EK48178  | MW898197              | Pakistan: Islamabad: Shahdara | 33.78513863 | 73.17800038 | This study                  |
| Euphlyctis  | *kalasgramensis*         | 16S  | WLM:EK481710 | MW898198              | Pakistan: Islamabad: Shahdara | 33.78513863 | 73.17800038 | This study                  |
| Euphlyctis  | *kalasgramensis*         | 16S  | WLM:EK236162 | MW898199              | Pakistan: Gujrat Khan: Doltana | 33.19633336 | 73.26858302 | This study                  |
| Euphlyctis  | *kalasgramensis*         | 16S  | WLM:EK10917  | MW898200              | Pakistan: Murree: Manga | 33.798224 | 73.294917 | This study                  |
| Euphlyctis  | *kalasgramensis*         | 16S  | WLM:EK265166 | MW898201              | Pakistan: Islamabad: Talhar | 33.770179 | 73.049072 | This study                  |
| Euphlyctis  | *kalasgramensis*         | 16S  | WLM:EK265168 | MW898202              | Pakistan: Islamabad: Talhar | 33.770179 | 73.049072 | This study                  |
| Euphlyctis  | *kalasgramensis*         | 16S  | WLM:EK2010175| MW898203              | Pakistan: Kahuta: Panjpeer | 33.65313917 | 73.5337495 | This study                  |
| Euphlyctis  | *kalasgramensis*         | 16S  | WLM:EK2010173| MW898204              | Pakistan: Kahuta: Panjpeer | 33.65313917 | 73.5337495 | This study                  |
| Euphlyctis  | *kalasgramensis*         | 16S  | WLM:EK2010174| MW898205              | Pakistan: Kahuta: Panjpeer | 33.65313917 | 73.5337495 | This study                  |
| Euphlyctis  | *kalasgramensis*         | 16S  | WLM:EK2010176| MW898206              | Pakistan: Kahuta: Panjpeer | 33.645182 | 73.460291 | This study                  |
| Euphlyctis  | *kalasgramensis*         | 16S  | WLM:EK2010172| MW898207              | Pakistan: Kahuta: Panjpeer | 33.645182 | 73.460292 | This study                  |
| Euphlyctis  | *kalasgramensis*         | 16S  | WLM:EK46161  | MW898208              | Pakistan: Kotli Sattian: Near Kahuta Checkpost | 33.75554159 | 73.44597954 | This study                  |
| Euphlyctis  | *kalasgramensis*         | 16S  | WLM:EK461612 | MW898209              | Pakistan: Rawalpindi: Soan river | 33.436292 | 72.99796 | This study                  |
| Euphlyctis  | *kalasgramensis*         | 16S  | WLM:EK252173 | MW898210              | Pakistan: Islamabad: Bani Gala | 33.711083 | 73.161416 | This study                  |

(Continues)
| Genus        | Species          | Gene | Voucher No. | GenBank accession No. | Country/locality                  | Lat   | Long  | Publication          |
|--------------|------------------|------|-------------|-----------------------|----------------------------------|-------|-------|----------------------|
| Euphlyctis   | kalasgramensis   | 16S  | WLM:EK252172| MW898211              | Pakistan: Islamabad: Bani Gala   | 33.711083 | 73.161416 | This study           |
| Euphlyctis   | kalasgramensis   | 16S  | WLM:EK305161| MW898212              | Pakistan: Rawalpindi: Choanthera | 33.352238 | 72.775779 | This study           |
| Euphlyctis   | kalasgramensis   | 16S  | WLM:EK31317t| MW898213              | Pakistan: Rawalpindi: Udhuaj      | 33.312832 | 72.912557 | This study           |

**APPENDIX 2**

Primer used in the present study for PCR amplification

| Gene | Primer | Sequence 5′-3′ | bp  | Reference               |
|------|--------|----------------|-----|-------------------------|
| 16S  | 16SAR  | 5′-CGCCTGTAYCAAAAACAT-3′ | 550 | Palumbi (1996)           |
|      | 16SBR  | 5′-CGGTYTGAACGTACAGATCAYGT-3′ |     |                         |
| 16S  | 16SC   | 5′-GTRGGCTAAAGCAGCCAC-3′ | 950 | Cannatella et al. (1998) |
|      | 16SD   | 5′-CTCCGGTCTGAACGTACAGATCAGTAG-3′ |     |                         |
APPENDIX 3
Bayesian inference analysis based on the 16S rRNA, of genus Duttaphrynus, Family Bufonidae. Posterior probability values are indicated near each node. Sequences generated in the present study are given in red.
APPENDIX 4
Bayesian inference analysis based on the 16S rRNA, of genus Microhyla, Family Microhylidae. Posterior probability values are indicated near each node. Sequences generated in the present study are given in red.
APPENDIX 5
Bayesian inference analysis based on the 16S rRNA, of genus *Nanorana* and *Allopaa*. Posterior probability values are indicated near each node. Sequences generated in the present study are given in red.
APPENDIX 6
Bayesian inference analysis based on the 16S rRNA, of genus Nanorana. Posterior probability values are indicated near each node. Sequences generated in the present study are given in red.
APPENDIX 7
Bayesian inference analysis based on the 16S rRNA, of genus Sphaerotheca, Fejervarya, and Minervarya. Posterior probability values are indicated near each node. Sequences generated in the present study are given in red.
APPENDIX 8
Bayesian inference analysis based on the 16S rRNA, of genus *Hoplobatrachus* and *Euphlyctis*. Posterior probability values are indicated near each node. Sequences generated in the present study are given in red.