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New data on amphibians and reptiles of the Northern Areas of Pakistan: distribution, genetic variability and conservation issues

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Abstract. The Northern Areas of Pakistan are at the boundary between the Paleartic and the Oriental biogeographical realm. This mountainous region is at the junction between major mountain ranges, has huge altitudinal and environmental gradients, and is the focus of efforts for biodiversity conservation. However, the amphibians and reptiles of this area remain poorly known. We used multiple approaches to describe species distribution and ecology, to perform a preliminary analysis of genetic diversity of toads, and to assess the occurrence of *Batrachochytrium dendrobatidis*, a pathogen largely responsible of global amphibian decline. We observed seven species of amphibians and reptiles: *Bufo pseudoraddei baturae*, *Bufo latastii*, *Scutiger* cf. *nyingchiensis*, *Altiphylax stoliczkai*, *Laudakia himalayana, L. pakistanica* and *Varanus bengalensis*; we describe the habitat and distribution of these species. In the case of *L. himalayana*, we observed a bimodal pattern of diurnal activity, and we report new data on the altitudinal range of this agamid. Sequences of a fragment of mitochondrial 16S rDNA from multiple populations of *B. pseudraddei baturae* show low genetic diversity among individual of this species. Conversely, we observed strong genetic substructuring among populations of *B. latastii* living in different valleys. A PCR-based analysis did not reveal the presence of *B. dendrobatidis* in any of the amphibians investigated. Distribution data show that amphibians and reptiles are limited to relatively low elevations; these areas suffer the strongest human impact, and might be overlooked by future conservation plans for the area, making the future of these organisms at risk.

Keywords: Agamidae; altitude; Amphibian conservation; Asia; Biodiversity; Chytridiomycosis; DNA barcoding; Himalaya; Karakoram.

Introduction

Pakistan hosts a highly diverse and unique herpetofauna, due to the huge environmental and altitudinal gradients, leading to complex zoogeographic patterns. This richness is particularly high for reptiles, with about 200 species described; furthermore, about 30% of reptiles and amphibians are endemic (Khan 2006, 2008). Despite this richness, knowledge of the herpetofauna of Pakistan is not complete (Minton 1966, Borkin 1999, Sindaco & Jeremčenko 2008, Xu et al. 2009). Only a few areas and species have been deeply investigated, and strong uncertainties persist with regards to the distribution and taxonomy of many amphibians and reptiles (Borkin 1999, Sindaco & Jeremčenko 2008). The Northern Areas of Pakistan represent an exceptional environment for both geographical and biogeographical reasons. This area is at the boundary between the Paleartic and the Oriental zoogeographical realms (Duellman 1999, Sindaco & Jeremčenko 2008), and it is the junction point of three of the world's greatest mountain ranges: Himalaya; Karakoram and Hindukush. Despite these unique features, the biodiversity of these mountain ranges remains poorly studied (Xu et al. 2009).

Here we combined multiple approaches to gather information on amphibians and reptiles of the Northern Areas of Pakistan. Specifically, the aims of this study were threefold. First, we performed intensive field surveys to evaluate the distribution of amphibians and reptiles in several valleys of the Northern Areas. Local authorities are establishing a new protected area in Central Karakoram (Central Karakoram National Park), and the knowledge on species distribution is the first, necessary step, to understand their response to environmental changes, and to identify conservation priorities in order to set up proper management plans (Xu et al. 2009).

Second, morphological identification might not be enough to undertake species inventories, causing vast underestimation of biodiversity (Vieites et al. 2009). In order to evaluate if cryptic entities were present within the recognised species of toads (*Bufo viridis* subgroup), we sequenced a fragment of the mitochondrial 16S rDNA gene, a molecular marker widely sequenced across vertebrates for molecular identification through DNA barcoding (Vences et al. 2005). In amphibians, strong molecular divergence is not always paralleled by morphological differentiation; therefore genetic information can provide key information on population structure, helping in the detection of cryptic taxa (Vieites et al. 2009). High intraspecific ranges of mitochondrial genetic distance are generally caused by deep phylogenetic divergences within and among species, rather than by a particularly fast evolutionary rate (Vences et al. 2005). Although the wide range of overlap between intra- and interspecific divergence may cause problems in molecular species identification (Vieites et al. 2009), genetic information can be a valuable help in the identification of conservation units (Marris 2007, Crottini et al. 2008, Gebremedhin et al. 2009a).

Finally, we evaluated whether amphibian populations were infected by the chytrid fungus, *Batrachochytrium dendrobatidis*. This fungus is considered to be the etiological agent of chytridiomycosis, an emerging disease largely implicated in amphibian decline in several areas of the world (Berger et al. 1998, Lips et al. 2008). *Batrachochytrium dendrobatidis* has been regularly identified in wild populations on all continents except Asia (Speare & Berger 2000, Bradley et al. 2002, Weldon et al. 2004, Lips et al. 2006, Lips et al. 2008), where few surveys for chytrid fungus occurrence have been carried out so far (Rowley et al. 2007, Kusrini et al. 2008, Une et al. 2008). The first report of amphibian chytridiomycosis in Asia was in 2006 (reported in Une et al. 2008, Goka et al. 2009), and until now, there have been very few data on the occurrence of *B. dendrobatidis* infection in wild Asian amphibians (Kusrini et al. 2008, Une et al. 2008). Knowledge of the distribution of *B. dendrobatidis* is extremely important to ascertain the conservation status of amphibian populations. However, to our knowledge, there is no information on the occurrence of *B. dendrobatidis* in Pakistan.

The combination of data on distributions, genetic diversity, and the analysis of *B. dendrobatidis* occurrence, provides new information on the unique herpetofauna of Northern Pakistan, and constitute the baseline data of future conservation plans.

Materials and methods

Study area

We investigated an area of approx. 150 × 200 km in the Northern Areas of Pakistan, included in the Giglit, Skardu and Ghanche districts. The climate is arid, with total annual precipitation < 250 mm in most of areas (Manzoom 2004). The study area is mountainous and it includes part of the Little Karakorum and of North-western Himalaya; several peaks are above 7000 m, while the bottom of the valleys are at 1500-2300 m. The northern part of the study area is dominated by large glacial valleys (e.g., Bagrot, Shigar, Hushey), with glacial streams flanked by steep mountains. In these valleys, the front of glaciers is at 2500-3500 m, but deglaciated areas are present also at higher altitudes. Deosai represents the southern part of the study area, this is a high elevation plateau (altitude: 3800-4500 m). At low altitudes (e.g., Gilgit, Skardu) average winter temperature is around 0°C, while the average summer temperature is around 25°C (Manzoom 2004, Hijmans et al. 2005). Natural vegetation was composed of Himalayan dry coniferous forests (Khan 2006), but deforestation and overgrazing reduced natural forests to small residual patches, mostly in remote areas. Currently, most of the study area is occupied by scrublands, croplands, poplar plantations and barren rocks.

Surveys were focused in seven major areas: Gilgit; Bagrot Valley; Skardu; Shigar Valley; Shyok Valley; Hushey Valley; Deosai Plateau (Fig.1). Geographic coordinates of the different areas are reported in Appendix 1. For most valleys, information on the distribution of amphibians and reptiles is nearly absent. Stöck and co-workers studied green toads from the Northern Areas of Pakistan, but did not sample most of lateral valleys of the Central Karakoram (Stöck et al. 1999, Stöck et al. 2001, Stöck & Grosse 2003, Stöck et al. 2006). Locality names follow Manzoom (2004).

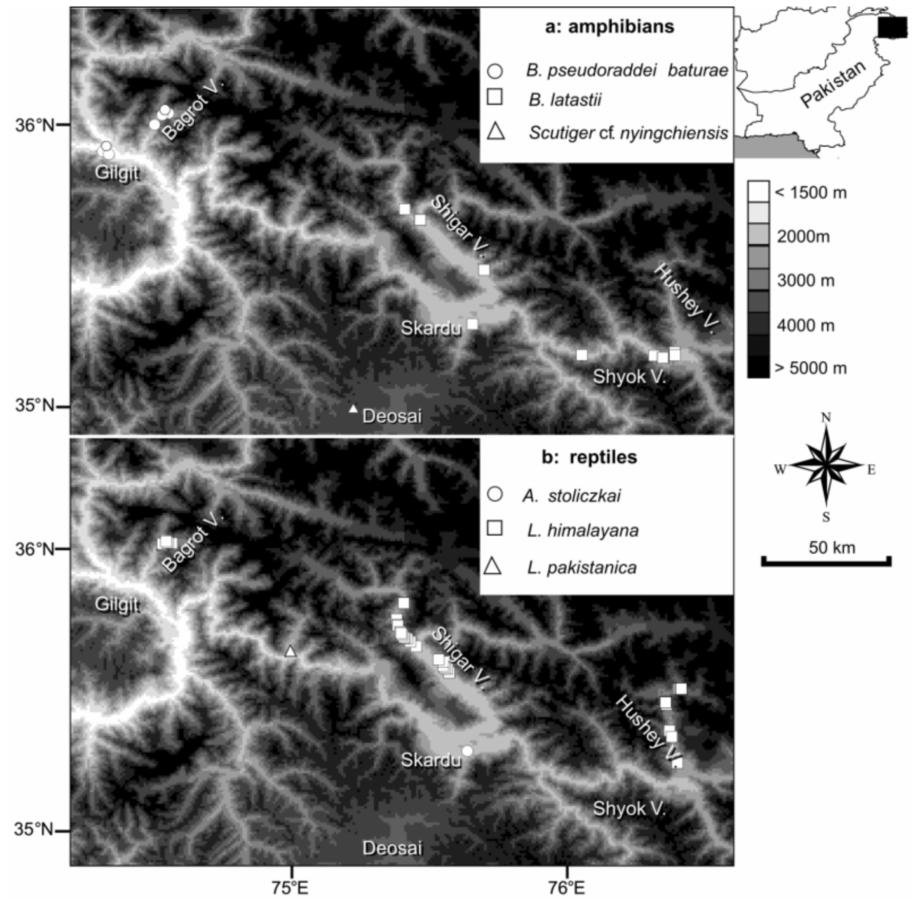

Figure 1. Study area, location of valleys sampled, and distribution of localities where we observed (a) amphibians and (b) reptiles. Darker shading represents higher elevation; altitude is obtained from a 30-arc seconds resolution digital terrain model (Hijmans et al. 2005).

Sampling

In June 2008 and late April-early May 2009, we used a combination of techniques to census amphibians and reptiles. In daytime, we performed random encounter surveys, mostly by slowly walking in natural and semi-natural vegetation; we also turned rocks and logs looking for hidden animals (Heyer et al. 1994). We performed nocturnal surveys and audio point counts in the wetlands potentially used for breeding by amphibians (Heyer et al. 1994). We also performed nocturnal surveys looking for nocturnal reptiles (e.g., Gekkonidae). We used a Garmin Extrex GPS to record geographic coordinates and altitude (accuracy: 3m). Where possible, adults were temporarily captured for morphological identification. Most surveys were performed at altitudes of 1500-4200 m. The sampling effort was approx. 150 man/days. Identification in the field was performed using the keys of Khan (2006, 2008). Morphological identification of toads was confirmed by M. Stöck, while morphological identification of reptiles was confirmed by R. Sindaco. Neo-metamorphosed amphibians and toe clips from a subset of adults were sampled for genetic analyses. Samples were stored in 100% ethanol; we sampled a maximum of one metamorph per breeding wetland.

Molecular analyses

Total genomic DNA of 10 samples (Table 1) was extracted using proteinase K digestion (10 mg/ml) followed by the protocol of Bandi et al. (1994). We used standardized cycling protocols and primers (Vences et al. 2000) to sequence a fragment of approximately 550bp of the mitochondrial 16S rRNA gene, which has proven to be suitable in anuran species identification (Vences et al. 2005). PCR products were loaded onto 1% agarose gels, stained with ethidium bromide, and visualised on a 'Gel Doc' system (PeqLab). If results were satisfactory, products were purified using QIAquick spin columns (Qiagen). The light strands were sequenced using an ABI3730XL by Macrogen Inc. Sequences were edited and aligned, and uncorrected pairwise distances (*p*-distances) were obtained using MEGA 4.1 (Kumar et al. 2008). DNA sequences were submitted to GenBank (accession numbers FJ861307-FJ861316).

The presence of *B. dendrobatidis* in amphibian skin was evaluated with a PCR-based approach, using ITS primers specific for *B. dendrobatidis* (Annis et al. 2004). Amplification conditions followed Federici et al. (2008). PCR products were loaded onto 1.5% agarose gel, stained with ethidium bromide, and visualised under ultraviolet light (365 nm). Samples were scored as positive or negative for *B. dendrobatidis* infection based on the presence of the approximately 300 bp band. Known samples infected by *B. dendrobatidis* were used as positive controls in all PCRs. Twelve individuals coming from eight localities were screened for the presence of *B. dendrobatidis*: 4 *Bufo pseudoraddei baturae*; 6 *B. latastii*; 2 *Scutiger* cf. *nyingchiensis* (Table 1).

Results and discussion

Species accounts

Amphibia: Anura: Bufonidae

Batura toad, *Bufo pseudoraddei baturae* Stöck, Schmid, Steinlein, and Grosse, 1999 (Fig. 2a). Prior to this study, most records of *B. pseudoraddei baturae* were limited to areas north or north-west of Gilgit (Stöck et al. 2001, Stöck & Grosse 2003, Stöck et al. 2006). The records in the Bagrot valley confirm the presence of this species in the valleys starting south-east of Gilgit (Fig. 1a) We found *B. pseudoraddei baturae* at the bottom of valleys, at altitudes of 1450-2600 m (Appendix 1). It breeds mostly in shallow, small temporary wetlands, with high sun exposition; at both high and low altitudes, we observed active individuals and mating during both day and night. Breeding wetlands are usually fed by rivers or glacial streams; we observed calling males also in artificial water-bodies.

Ladakh toad, *Bufo latastii* Boulenger, 1882 (Fig. 2b). This toad is common in the surroundings of Skardu (Stöck et al. 2001), but also in all the surveyed valleys North and East of Skardu, at altitudes ranging from 2200 to 2500 m (Fig. 1a; Appendix 1). The breeding habitat of *B. latastii* was similar to the one of *B. pseudoraddei baturae*: shallow, well exposed wetlands, mostly fed by streams, rivers or springs; we observed active individuals mostly at night (Dubois & Martens 1977). The analysis of mitochondrial 16S rDNA showed clear genetic differences among populations (see below for details). Khan (1997) described the species *B. siacheninsis* from the southern bank of the Shyok river. On the basis of morphological

Table 1. List of samples used for the molecular analyses, with reference to the locality, geographic coordinates and taxon.

Taxon	Locality	Latitude	Longitude
Bufo pseudoraddei baturae	Bagrot valley	36°00′	74°32′
Bufo pseudoraddei baturae	Bagrot valley	36°02′	74°34′
Bufo pseudoraddei baturae	Gilgit	35°53′	74°21′
Bufo pseudoraddei baturae	Gilgit	35°53′	74°21′
Bufo latastii MOTU 1	Shigar Valley	35°29′	75°42′
Bufo latastii MOTU 1	Shigar Valley	35°29′	75°42′
Bufo latastii MOTU 2	Shyok Valley	35°11′	76°19′
Bufo latastii MOTU 2	Shyok Valley	35°11′	76°23′
Bufo latastii MOTU 2	Skardu	35°17′	75°40′
Bufo latastii MOTU 2	Skardu	35°17′	75°40′
Scutiger cf. *nyingchiensis**	Deosai	34°59′	75°15′
Scutiger cf. *nyingchiensis**	Deosai	34°59′	75°15′

*: Analyzed only for the presence of *Batrachochytrium dendrobatidis*

features, Stöck et al. (1999) considered *B. siacheninis* as a synonym of *B. latastii*. Nevertheless, M.S. Khan suggested that further collections were required to confirm this synonymy (pers. comm. cited in Stöck et al. 2004). We analyzed mitochondrial 16S rDNA from specimens of the southern bank of the Shyok river, at approx. 20 km from the type locality of *B. siacheninsis*. Both morphological examination and mitochondrial 16S rDNA (see below) confirmed that all toads in this valley were indeed *B. latastii*. We never observed *B. latastii* and *B. pseudoraddei baturae* in the same valley (Fig. 1a).

Megophrydae

Alpine toad, *Scutiger* cf. *nyingchiensis* Fei, 1977 (Fig. 2c). Khan (2005) reported the presence of *Scutiger nyingchiensis* for the Deosai plateau. We confirm the presence of *Scutiger* in Deosai, at an altitude of 4150 m (Fig. 1a). Molecular genetic analyses are ongoing to ascertain the specific status of these specimens.

Reptilia: Squamata: Gekkonidae

Baltistan gecko, *Altiphylax stoliczkai* (Steindachner, 1867) (Fig. 2d). *Altiphylax stoliczkai* was very abundant on buildings in a single locality in Skardu (Fig. 1b); were a large numbers of both adults and juveniles was found. Other authors reported that this gecko does not live on human habitations (Auffenberg et al. 2004). Our observations suggest that human buildings can constitute a further habitat for *A. stoliczkai*, as for many other geckos.

Agamidae

Himalayan agama, *Laudakia himalayana* (Steindachner, 1869) (Fig. 2e). The Himalayan agama was the most abundant reptile of the study area; we observed *L. himalayana* mostly in the small valleys (Bagrot, Shigar and Hushey valley) (Fig. 1b; Appendix 1). *Laudakia himalayana* was associated to open shrublands dominated by *Juniperus*. In June 2008, we observed active individuals from 8.30 to 18.30, but most observations were in the late morning and afternoon. The activity pattern was bimodal, with two peaks around 11.00 and around 16.00, while activity was reduced during the hottest hours.

Khan (2006) reports that the altitudinal range of *L. himalayana* is 3000-3200 m. Baig

(1992) suggests a wider altitudinal range, but does not provide precise data. Our data confirm a broad range, as we observed *L. himalayana* at altitudes of 2300-3430 m. The altitudinal range might be wider, as we have unconfirmed reports at approx. 3600 m (Hushey Valley) and at approx. 3800 m (Bagrot Valley).

North-Pakistan agama, *Laudakia pakistanica* (Baig, 1989) (Fig. 2f). We observed *L. pakistanica* at very low density on the Northern bank of the Indus river. The locality of observation is not far from the easternmost locality of presence reported by Baig (1992).

Varanidae

Bengal monitor, *Varanus bengalensis* (Daudin, 1802). We observed the Bengal monitor in the upper Indus valley, near Sazin (Appendix 1). This locality is slightly outside the study area

Figure 2. Pictures of the study species. (a): *Bufo pseudoraddei baturae*, adult male (Bagrot Valley); (b): *B. latastii*, adult female (Shyok Valley); (c) *Scutiger* cf. *nyngchiensis*, juvenile (Deosai); (d): *Altiphylax stoliczkai* (Skardu); (e): *Laudakia himalayana*, juvenile (Hushey valley); (f) *L. pakistanica*, adult female, upper Indus valley.

depicted in Fig. 1; we report it because *V. bengalensis* was not recorded for this area of Pakistan (Khan 2006, Sindaco & Jeremčenko 2008).

Molecular analyses

Our molecular analyses, based on a fragment of the mitochondrial 16S rRNA gene, confirmed the presence of two distinct taxa, corresponding to *Bufo pseudoraddei baturae* and *Bufo latastii*. The uncorrected genetic divergence (*p*-distance, transformed into percent) within *Bufo pseudoraddei baturae* was very low (0.09%), although we analysed samples coming from two rather distant populations (Gilgit and Bagrot Valley: Fig. 2a) (Table 1, Table 2). These results are in agreement with the findings of Stöck et al. (2006), which found high homogeneity in the mitochondrial control region. The samples of *B. latastii* were less homogeneous, with an intraspecific uncorrected genetic divergence of 0.58% (Table 2). Indeed, within *B. latastii,* our data suggest the existence of two distinct Molecular Operational Taxonomic Units (MOTU, sensu Floyd et al. 2002): we assigned the samples from Shigar Valley to the MOTU 1, while we assigned the samples from Shyok Valley and Skardu to the MOTU 2. The genetic distance (uncorrected *p*-values) as a measure of the molecular divergence between these two MOTU of *Bufo latastii* is rather high (1.09%), indicating the occurrence of geographic substructure in *Bufo latastii* populations (Table 3). This genetic distance is similar to the divergence observed within highly polytypic species of anurans (e.g., Martìnez-Solano et al. 2004). The orography of the area, the complex landscape structure and the high environmental heterogeneity might explain the genetic substructuring within *B. latastii*. For example, the Skardu area is separated by the Shigar valley by an extremely arid, nearly desert region. This area is apparently unsuitable for *B. latastii* and might act as barrier. Nevertheless, analyses on a larger sample are required prior conclusions can be drawn. Finally, genetic distance (uncorrected *p*-values) between *Bufo pseudoraddei baturae* and the two MOTUs of *Bufo latastii* ranges from 5.86% and 6.04% (Table 3).

Table 2. Within group genetic divergence of the 16S rRNA gene fragment based on the pairwise distance calculation for the *Bufo pseudoraddei baturae*, *B. latastii*, and separately also for the two identified MOTUs of *B. latastii*.

Species	**divergence**
Bufo pseudoraddei baturae	0.09%
Bufo latastii	0.58%
Bufo latastii MOTU 1	0
Bufo latastii MOTU 2	0

Table 3. Among group genetic divergence of the 16S rRNA gene fragment based on the pairwise distance calculation for the *Bufo pseudoraddei baturae*, *B. latastii*, and separately also for the two identified MOTUs of *B. latastii*.

	B. pseudoraddei baturae	***B. latastii* MOTU 1**
B. pseudoraddei baturae	-	
B. latastii	5.98%	
B. latastii MOTU 1	5.86%	-
B. latastii MOTU 2	6.04%	1.09%

Chytridiomycosis

In several breeding wetlands, we observed the metamorphosis of a large numbers of *B. pseudoraddei baturae* and *B. latastii,* and we did not detect dead metamorphs or other episodes of mass mortality which are often associated to chytridiomycosis presence (Bosch et al. 2001). Indeed, none of the samples analyzed using PCR was positive to *B. dendrobatidis* occurrence.

In many areas of the word, chytridiomycosis has been spread into naïve ecosystems via biological invasions caused by alien invasive species, translocations of native species, and the amphibian trade (Cunningham et al. 2005, Fisher & Garner 2008). The Northern Areas of Pakistan is a remote region, where both pet trade and biological invasions are limited. Isolation of the area, lack of invasive amphibians, together with the unfavourable harsh climate (Rödder et al. 2009), probably explain the absence of *B. dendrobatidis*; nevertheless, human impact in this area is quickly increasing (Manzoom 2004), and we stress the importance to carry out surveys for *B. dendrobatidis* occurrence. Since *B. dendrobatidis* is a major issue of amphibian conservation, patchy distributed surveys can be extremely useful for the early detection of new infections hotspots across wide, poorly investigated areas.

Conclusions

Our surveys suggest that the richness of herpetofauna communities in the Northern Areas of Pakistan is quite low, particularly compared to the high richness of southern Pakistan. For example, we never found more than one species of amphibian in each surveyed area. The study area is one of the highest altitude regions of the world, in the Himalayas. In these high elevation regions, species richness decreases because of the unfavourable climatic conditions, but endemism is often very high (Xu et al. 2009). Indeed, for amphibians, different valleys often host distinct species or MOTUs (Fig. 1a) (Funk et al. 2005). The mountainous ranges, the patchy distribution of the favourable habitats, and the complex geological history of the region, dominated by some of the largest alpine glaciers of the world, probably explain the allopatric distribution and the limited geographic range of taxa.

Despite being adapted to severe environmental conditions, this unique herpetofauna is threatened by multiple factors. Human impact is quickly growing. For example, human population increased by 51% from 1981 to 1998 (Manzoom 2004). Increased human population results in the removal of natural vegetation, overgrazing and desertification of already arid landscapes; currently, human impact is low only at the highest altitude areas (above 4000m). Finally, high altitude Himalayan taxa have limited geographic range, being particularly susceptible to climate change (Salick et al. 2009). A large national park is going to be established in the study area. The target of this park is the conservation of the uninhabited areas at the highest altitude. However, amphibians and reptiles are associated to lower elevations (i.e., below 3500 m), therefore some low elevation areas should have high conservation priority. Further studies are needed for this area, possibly combining ecological and molecular analyses (see for example the work on *Walia ibex* by Gebremedhin et al. 2009b), to understand the factors determining the distribution of species and the pattern of their genetic diversity, to unravel the evolutionary adaptations of populations, and to better identify the actions for the conservation of poorly studied areas, such as the Norther Areas of Pakistan.

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Appendix 1. Localities of amphibians and reptiles observed in the Northern Areas of Pakistan.

Area	Latitude	Longitude	Altitude (m)
Bufo pseudoraddei baturae			
Gilgit	35°53′	74°21′	1486
	35°54′	74°22′	1450
	35°54	74°24	1407
Bagrot Valley	36°00′	74°32′	2221
	36°02′	74°34′	2400-2500
	36°02′	74°35′	2632
	36°03′	74°35′	2627
Bufo latastii			
Skardu	35°17′	75°40′	2204
Shigar Valley	35°29′	75°42′	2356
	35°42′	76°26′	2392
	35°40′	76°29′	2345
Shyok Valley	35°11′	76°03′	2350
	35°11′	76°19′	2486
	35°10′	76°21′	2480
	35°11′	76°23′	2500
Scutiger cf. *nyngchiensis*			
Deosai Plateau	34°59′	75°15′	4155
Altiphylax stoliczkai			
Skardu	35°18′	75°39′	2245
Laudakia himalayana			
Bagrot Valley	36°02′	74°34′	2470-2675
	36°02′	74°35′	2585-2880
	36°02′	74°36′	2840-2850
	36°03	74°35	2627
Shigar Valley	35°44′	75°24′	2475
	35°45′	75°24′	2479
	35°46′	75°24′	2460-2500
	35°42′	75°25′	2390-2460
	35°43′	75°25′	2420-2440
	35°49′	75°25′	2735-2745
	35°42′	75°26′	2400-2450
	35°41′	75°27′	2380
	35°40′	75°28′	2352
	35°37′	75°33′	2325
	35°35′	75°34′	2402
	35°36′	75°34′	2414
	35°37′	75°34′	2320
	35°34′	75°35′	2352

Appendix 1. (Continued)

Area	Latitude	Longitude	Altitude (m)
	35°35′	75°35′	2360
Hushey Valley	35°28′	76°21′	3175-3230
	35°21′	76°22′	2841
	35°22′	76°22′	2918
	35°15′	76°24′	2700
	35°31′	76°24′	3375-3430
Laudakia pakistanica			
Indus valley	35°38′	75°01′	1814
Varanus bengalensis			
Indus valley	35°31′	73°25′	960