Molecular phylogeny of Eremias spp. from Pakistan contributes to a better understanding of the diversity of racers runners

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Abstract

Eremias Fitzinger, 1834 is a speciose Eurasian genus of true lizards with approximately 40 species. Eremias species occurring in Pakistan have never been examined before using molecular genetics. In the present study, six out of seven morphologically defined taxa distributed in Pakistan (E. acutirostris, E. aperosceles, E. cholistanica, E. kakari, E. persica, and E. scripta) were studied using mitochondrial (16S rRNA, cytochrome c oxidase subunit I, cytochrome b) and nuclear (Rag1) genes. Data of 29 individuals were included in phylogenies using ENA/GenBank sequences. With a maximum of 20 species per analyzed data set, this study represents the most complete phylogeny of the genus to date. Maximum Likelihood and Bayesian analyses were run for concatenated (3,528 bp) and single-locus data sets and supported by uncorrected p distance calculations to evaluate the phylogenetic placement and divergence of Pakistani taxa. Among the Pakistani taxa, we detected six mostly well-supported and deeply divergent clades (A-F) differing by uncorrected p distances of up to 23.8% for mtDNA (cytb) and 3.3% for the nuclear Rag1 locus. Despite morphological differences between E. aperosceles and E. acutirostris (both clade A), no unambiguous genetic support was found for these two taxa. Therefore, we regard E. aperosceles as a synonym of E. acutirostris. On the other hand, E. persica was found to represent a species complex with deeply diverged clades (E and F) in Pakistan. Eremias cholistanica (clade D) and E. kakari (clade B), two morphologically defined endemic taxa of Pakistan, were revealed as phylogenetically clearly distinct. Eremias scripta (clade C) from Pakistan was found to be genetically deeply divergent compared to sequences of this taxon from Afghanistan and Uzbekistan. Our study provides evidence that the current taxonomy of Eremias does neither reflect the genetic diversity nor the evolutionary history of the genus, necessitating a comprehensive integrative taxonomic revision of the whole genus.
1 | INTRODUCTION

The area between the Iranian Plateau and the Himalayas, where Pakistan lies, is zoogeographically of outstanding interest. This area is a crossroads of two major zoogeographic regions, the Palearctic and the Oriental Regions, and three to four of their biogeographic subdivisions (Iranian, Western Central Asian Mountains, and Palearctic-Oriental Transition Zone) with four main biomes (desert and xeric shrublands, tropical and subtropical dry broadleaf forests, temperate broadleaf and mixed forests, and montane grasslands; Sindaco & Jeremčenko, 2008). Such a biogeographic variation is exceptional and suggestive of high biodiversity. Therefore, it is not a coincidence that Pakistan harbors more than 200 extant species of reptiles, and this number is probably vastly underestimated.

The understanding of reptile diversity in Pakistan increased much during the past two decades (Baig & Masroor, 2006; Khan, 2006; Masroor, 2012), and most of the current knowledge was obtained only during the 20th century (Mertens, 1969, 1970, 1971, 1974; Minton, 1966). Nevertheless, the knowledge of the Pakistani herpetofauna is still poor, and most studies are only morphology-based. The species diversity, their distribution patterns, phylogenetic relationships, and natural history remain largely unexplored (Khan, 2006; Masroor, 2012). One prominent example is the family Lacertidae. Only four genera with around 12 species are currently known from Pakistan (Masroor, 2012). The known diversity in neighboring countries is higher. Iran harbors nine lacertid genera with 49 species, China has four genera with 26 species, Afghanistan has four genera with 16 species, and India has four genera with 13 species (Uetz & Hošek, 2019; Wagner, Bauer, Leviton, Wilms, & Böhme, 2016). It seems likely that the putatively low diversity of Pakistan reflects rather a deficit in research, in particular using molecular approaches, than country size or other causes.

Racerunners (genus Ereemias Fitzinger, 1834) are small lizards closely related to the lacertid genera Acanthodactylus, Mesalina, and Ophisops, all belonging to the Saharo-Eurasian clade of the family Lacertidae. Their radiation is expected to have occurred in Southwestern Asia 40 Mya (Arnold, 1989; Arnold, Arribas, & Carranza, 2007; Fu, 1998, 2000; Harris, Arnold, & Thomas, 1998; Tamar et al., 2016). Lacertids occur mostly in the Western Palearctic (Mayer & Pavlíček, 2007; Sindaco & Jeremčenko, 2008) and are typically confined to temperate grasslands of Eurasia and desert regions of Central Asia (Guo, Chen, Wang, & Wang, 2010; Guo et al., 2011). The species diversity and taxonomy of Ereemias remain unresolved, despite almost two centuries of research (Bedriaga, 1912; Bouleneger, 1918, 1921; Lantz, 1928; Nikolsky, 1915; Strauch, 1876; Szczerek, 1971, 1974). The genus comprises around 40 species (Guo et al., 2011; Orlova et al., 2017; Rastegar-Pouyani, Kazemi-Noureini, Rastegar-Pouyani, Joger, & Wink, 2012; Rastegar-Pouyani, Rastegar-Pouyani, Kazemi-Noureini, Joger, & Wink, 2010). Five subgenera are currently distinguished (Orlova et al., 2017): Aspidorhinus Eichwald, 1841 (type species: E. velox), Ereemias Fitzinger, 1834 (type species: E. arguta), Pareremias Szczerek, 1973 (type species: E. multiocellata), Rhabderemias Lantz, 1928 (type species: E. scripta), and Scapteira Wiegmann, 1834 (type species: E. grammica). The Aspidorhinus subgenus was previously known under the name Dimorphea Eremchenko, 1999 or as Ereemias sensu stricto, while the species currently assigned to the Ereemias subgenus were previously known as members of the subgenus Ommeremias Lantz, 1928 (Barabanov, 2009; Orlova et al., 2017; Sindaco & Jeremčenko, 2008; Szczerek, 1974).

About twenty species of the genus Ereemias occur mainly in Southwestern Asia (Iranian Plateau); the remaining taxa are distributed in Southern, Central, and Eastern Asia (Barabanov, 2009; Guo et al., 2011; Orlova et al., 2017). Several endemic species are known from Afghanistan, China, Iran, Kyrgyzstan, Pakistan, and Turkmenistan (Anderson & Leviton, 1967; Baig & Masroor, 2006; Duisbayeva, Chirikova, & Belyakov, 2009; Masroor, Khisroon, Khan, & Jablonski, 2020; Mozaffari, Ahmadzadeh, & Parham, 2011; Orlova, 2008; Orlova et al., 2017; Orlova & Terbisch, 1997; Rastegar-Pouyani et al., 2016; Sindaco & Jeremčenko, 2008; Wagner et al., 2016; Zhao & Adler, 1993; Zhao, Zhou, & Zhou, 1999; Zhao, Liu, Luo, & Ji, 2011). For some species, morphologically defined subspecies are recognized, whose validity has been only rarely examined using molecular approaches (Guo et al., 2011; Liu et al., 2019; Orlova et al., 2017; Poyarkov, Orlova, & Chirikova, 2014; Zhou, Li, Duisabayeva, Liu, & Guo, 2016). Over the past three decades, several attempts were made to revise Ereemias, mostly based on external morphology, hemipenial traits, and ecology (Anderson, 1999; Arnold, 1986; Barabanov, 2009; Szczerek, 1974). Recently, the validity of some racerunner species was supported by molecular studies and several new species were described (Orlova et al., 2017; Rastegar-Pouyani et al., 2016). However, the phylogenetic relationships among many species remain unstudied (Guo et al., 2010, 2011; Orlova et al., 2017).

For Pakistan, the knowledge of Ereemias species is largely anecdotal, with big sampling and knowledge gaps. Five to seven taxa are currently recognized (Baig & Masroor, 2006; Khan, 2004, 2006; Masroor et al., 2020; Mertens, 1969; Smith, 1935): E. acutirostris of Eichwald, 1841, E. aporosceles Alcock and Finn, 1897, E. choli- tanica Baig & Masroor, 2006, E. fasciata Blanford, 1874, E. kakari Masroor et al., 2020, E. persica Blanford, 1874, and E. scripta Strauch, 1867. Ereemias aporosceles was regarded for a long time as a synonym of E. acutirostris (Szczerek, 1974). However, some authors regarded it later as a valid species (Anderson, 1999; Das, 1992; Khan, 2006). Except for apparently lost specimens reported by Bouleneger (1921),
FIGURE 1  (a) Geographic origin of Eremias samples used in the present study. Clades are defined according to results of the concatenated data set (Figure 2). Numbers correspond to vouchers in the Pakistan Museum of Natural History (PMNH), Islamabad. (b-e) Records for our data and previously published sequences (codes are ENA/GenBank accession numbers). For details, see Tables 1 and S1
E. fasciata has never been subsequently recorded from Pakistan (Lantz, 1928; Masroor et al., 2020; Mertens, 1969; Minton, 1966; Smith, 1935).

Pakistani species of Eremias are of outstanding interest because they are thought to represent the poorly known subgenera Rhabderemias (E. choliastica, E. fasciata, E. kakari, E. scripta) and Scapeotea (E. acutirostris, E. aprofosces; Khan, 2006; Sindaco & Jeremčenko, 2008; Szczerebak, 1971, 1974). However, their phylogenetic relationships were never tested using molecular genetic approaches.

In the present paper, we (i) analyze the phylogenetic relationships of Pakistani racers using newly generated mitochondrial and nuclear DNA sequences, (ii) combine our data set with sequences of Eremias from previous studies, and (iii) infer the phylogenetic placement and taxonomy of the Pakistani species in the context of the whole genus.

2 | MATERIALS AND METHODS

2.1 | Sampling and wet laboratory approaches

Tissue samples of 29 specimens representing different populations and six of the currently recognized Eremias taxa from Pakistan (E. acutirostris, E. aprofosces, E. choliastica, E. kakari, E. persica, and E. scripta) were collected in 2017 and 2018 (Figure 1). A piece of muscle tissue of each specimen was preserved in 96% ethanol and kept at −20°C until processing. All specimens were deposited in the herpetological collection of the Pakistan Museum of Natural History (PMNH), Islamabad, Pakistan. Tissues and extracted DNA are stored in the Museum of Zoology, Senckenberg Dresden, Germany (MTD; Tables 1 and S1).

Total genomic DNA was extracted from the tissues using the innuPREP DNA Mini Kit (Analytik Jena AG, Jena, Germany). As mitochondrial markers, the 16S rRNA (16S), cytochrome c oxidase subunit I (COI), and the cytochrome b (cytb) genes were used; as a nuclear marker, the recombination activating gene 1 (Rag1) was used. PCRs were performed using 20 ng of DNA in a 25 µl volume containing 1.25 units Taq polymerase (BIORON GmbH, Ludwigshafen, Germany), and the recommended reaction buffer (complete 10x) containing MgCl₂ at 25 mM, 0.5 µl of dNTP mix at 10 mM (Roth, Karlsruhe, Germany), 0.25 µl bovine serum albumin at 20 µg/µl (BSA; Thermo Fisher Scientific, Waltham, MA, USA), and 1 µl of each primer at 10 µM (Table S2). For challenging samples with fragmented DNA, the loci cytb and Rag1 were amplified in two smaller, overlapping fragments, and an additional 0.25 µl MgCl₂ at 100 mM was added to the reaction mix. PCR conditions for 16S were as follows: initial denaturation at 94°C for 2 min, 35 cycles with denaturation at 95°C for 30 s, annealing for 30 s at 59°C, and extension at 72°C for 50 s, followed by a final extension step at 72°C for 7 min. PCR conditions remained the same for cytb and Rag1, except for the annealing temperature, which was 53°C and 50°C, respectively. PCR products were purified using the ExoSAP-IT PCR Product Cleanup Reagent (Applied Biosystems, Foster City, CA, USA; 1:20 dilution; modified protocol: 30 min at 37°C, 15 min at 80°C). Before the primers were removed, the individual amplics had the following lengths: 864–874 bp (16S, length variation due to indels), 710 bp (COI), 1,213 bp (cytb—complete), 800 bp (cytb part a), 538 bp (cytb part b, overlap without primers: 84 bp), 1,053 bp (Rag1—complete), 625 bp (Rag1 part a), and 674 bp (Rag1 part b, overlap without primers: 206 bp). For cycle sequencing, the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and the respective PCR primers were used (Table S2). After an initial denaturation at 96°C for 1 min, 25 cycles were run with denaturation for 10 s, annealing at 50°C for 5 s, and elongation at 60°C for 75 s. Products from cycle sequencing were purified using Performa DTR V3 96-Well Short Plates (Edge Biosystems, Gaithersburg, MD, USA); each well was filled with 400 µl Sephadex (GE Healthcare, Munich, Germany; 1:20 solution). Sequences were resolved on an ABI 3730 Genetic Analyzer (Applied Biosystems).

2.2 | Alignment and phylogenetic analyses

Forward and reverse sequences were checked visually, and a consensus sequence was compiled with biOEDIT 7.0.5.2 (Hall, 1999). Sequences were submitted to a BLAST search in ENA/GenBank to confirm that the targeted loci were amplified; protein-coding sequences were translated into amino acids to confirm the absence of stop codons. All protein-coding mtDNA sequences translated perfectly into amino acids. Therefore, we conclude to have sequenced authentic mtDNA and not nuclear mitochondrial insertions (numts). Sequences were aligned in biOEDIT. After primer trimming and excluding ambiguous bases at the 5’ and 3’ ends, the obtained 16S sequences had a length of 805 bp; the COI sequences, 658 bp; the cytb sequences, 1,143 bp; and the nuclear Rag1 locus, 922 bp. Details on used outgroup sequences of the genera Acantodactylus, Ophisops, Mesalina, and Lacerta are presented in Table S1.

Due to inconsistent lengths and availability of published sequences, we compiled five data sets:

1. a data set of 3,528 bp length contained as ingroup only the Pakistani sequences generated in this study, consisting of 29 concatenated mitochondrial and nuclear genes (16S: 1–805, COI: 806–1,463, cytb: 1,464–2,606, Rag1: 2,607–3,528; Tables 1 and S1; Alignment S1);
2. a data set of 16S sequences (854 bp length) contained as ingroup 154 sequences of 19 currently recognized Eremias taxa, among them 123 ENA/GenBank and BOLD sequences (Tables 1 and S1; Alignment S2);
3. a data set of COI sequences (658 bp length) contained as ingroup 242 sequences of 20 currently recognized Eremias taxa, among them 219 ENA/GenBank and BOLD sequences (Tables 1 and S1; Alignment S3);
4. a data set of cytb sequences (1,143 bp length) contained as ingroup 939 sequences of 22 currently recognized Eremias taxa,
| MTD   | PMNH  | Provenance                                              | N     | E    | 16S  | COI  | cytb | Rag1 | Clade |
|-------|-------|---------------------------------------------------------|-------|------|------|------|------|------|-------|
| Eremias acutirostris |       |                                                         |       |      |      |      |      |      |       |
| 19328 | 3734  | Balochistan: Nushki, Khar                               | 29.5528 | 66.0110 | n/a | n/a | MT554463 | n/a | A     |
| 19329 | 3736  | Balochistan: Nushki, Khar                               | 29.5537 | 66.0102 | MT524458 | n/a | MT554464 | MT554474 | A     |
| 19330 | 3737  | Balochistan: Nushki, Khar                               | 29.5527 | 66.0097 | MT524459 | n/a | MT554465 | MT554475 | A     |
| 19435 | 3728  | Balochistan: Nushki, Zangi Nawar                        | 29.4413 | 65.7309 | MT524482 | MT524258 | MT554471 | MT554495 | A     |
| Eremias aporosceles |       |                                                         |       |      |      |      |      |      |       |
| 19353 | 3780  | Balochistan: Nok Kundi, Mizada Darband                  | 29.2456 | 62.7994 | MT524478 | n/a | MT554468 | MT554491 | A     |
| 19354 | 3784  | Balochistan: Nok Kundi, Mizada Darband                  | 29.2795 | 63.2668 | MT524479 | MT524255 | MT554469 | MT554492 | A     |
| Eremias kakari |       |                                                         |       |      |      |      |      |      |       |
| 19341 | 4047  | Balochistan: Torghar Mts., Tanishpa valley              | 30.9553 | 68.6912 | MT524469 | MT524248 | n/a | MT554484 | B     |
| 19355 | 4048  | Balochistan: Torghar Mts., Tanishpa valley              | 30.9555 | 68.6917 | MT524480 | MT524256 | n/a | MT554493 | B     |
| Eremias scripta |       |                                                         |       |      |      |      |      |      |       |
| 19351 | 3782  | Balochistan: Nok Kundi, Mizada Darband                  | 29.2946 | 62.9843 | MT524477 | n/a | MT554462 | n/a | C     |
| Eremias chalistanica |       |                                                         |       |      |      |      |      |      |       |
| 19347 | 3797  | Punjab: Bahawalpur: Cholistan Institute of Desert Studies, Baghdad-ul-Jadeed Campus | 29.2943 | 71.6356 | MT524474 | MT524253 | n/a | MT554488 | D     |
| 19349 | 3800  | Punjab: Bahawalpur: Cholistan Institute of Desert Studies, Baghdad-ul-Jadeed Campus | 29.3263 | 71.6444 | MT524475 | n/a | MT554472 | MT554489 | D     |
| 19350 | 3801  | Punjab: Bahawalpur: Cholistan Institute of Desert Studies, Baghdad-ul-Jadeed Campus | 29.3004 | 71.5999 | MT524476 | MT524254 | n/a | MT554490 | D     |
| Eremias persica |       |                                                         |       |      |      |      |      |      |       |
| 19344 | 4056  | Balochistan: Qamar-ud-Din Karez, Ashiwat                | 31.6871 | 68.4456 | MT524472 | MT524251 | n/a | MT554487 | E     |
| 19335 | 4054  | Balochistan: Qamar-ud-Din Karez, Ashiwat                | 31.6859 | 68.4370 | MT524464 | MT524245 | MT554454 | MT554480 | E     |
| 19342 | 4053  | Balochistan: Qamar-ud-Din Karez, Ashiwat                | 31.7087 | 68.4515 | MT524470 | MT524249 | MT554457 | MT554485 | E     |
| 19331 | 3724  | Balochistan: Nushki, Khar                               | 29.5530 | 66.0087 | MT524460 | MT524241 | n/a | MT554476 | E     |
| 19474 | 3723  | Balochistan: Nushki, Khar                               | 29.5528 | 66.0097 | MT524483 | MT524259 | MT554470 | MT554496 | E     |
| 19332 | 3735  | Balochistan: Nushki, Khar                               | 29.5535 | 66.0092 | MT524461 | MT524242 | MT554461 | MT554477 | E     |
| 19343 | 4045  | Balochistan: Torghar Mts., Tanishpa valley              | 30.9741 | 68.7308 | MT524471 | MT524250 | MT554467 | MT554486 | F/F1  |
| 19484 | 3613  | Balochistan: Torghar Mts., Tanishpa valley              | 30.9556 | 68.6917 | MT524485 | MT524261 | MT554460 | MT554498 | F/F1  |

(Continues)
among them 911 ENA/GenBank sequences (Tables 1 and S1; Alignment S4); and
5. a data set of the nuclear \textit{Rag1} sequences (2,414 bp length) contained 44 sequences of 12 currently recognized \textit{Eremias} species, among them 19 ENA/GenBank sequences (Tables 1 and S1; Alignment S5).

Each ENA/GenBank and BOLD sequence was quality-controlled by visual inspection and translation into amino acids. In addition, exploratory phylogenetic trees were built using \textit{raxml} 8.0.0 (Stamatakis, 2014). As a consequence, seven 676- to 690-bp-long \textit{cytb} sequences (\textit{E. isfahanica}, \textit{E. papenfusi}; KP317957-KP317963) from Rastegar-Pouyani et al. (2016) were removed from the alignment. These sequences are highly variable and the \textit{raxml} analysis placed them outside \textit{Eremias}, although both species should represent the subgenus \textit{Aspidorhinus} (Mozaffari et al., 2011; Rastegar-Pouyani et al., 2016). This suggests that these sequences are compromised and might represent nuclear insertions of mtDNA (numts). For the remaining \textit{cytb} data set of 932 sequences and 20 species and the other abovementioned data sets, evolutionary relationships were inferred using Maximum Likelihood (ML) and Bayesian approaches. The best partitioning scheme and the best model of sequence evolution were determined using \textit{partitionfinder} 2 (Lanfear, Frandsen, Wright, Senfeld, & Guindon, 2017) and the implemented Bayesian Information Criterion (Table S3). Maximum Likelihood analyses were run with \textit{raxml} 8.0.0 on a Mac Pro (3 GHz 8 Core, 64 GB RAM). Five independent searches were performed using different starting conditions and the fast bootstrap algorithm to explore the robustness of the results by comparing the best trees. Then, 1,000 non-parametric thorough bootstrap replicates were calculated and plotted against the best tree. Bayesian Inference (BI) of phylogeny was performed using \textit{MrBayes} 3.2.6 (Ronquist et al., 2012) with two parallel runs (each with four chains) and default parameters. Calculation parameters were analyzed using \textit{tracer} 1.7.1 (Rambaut, Drummond, Xie, Baele, & Suchard, 2018). With the exception of the \textit{cytb} analysis, the chains ran on the Mac Pro for 20 million generations with every 1,000th generation sampled. For \textit{cytb}, 50 million generations were necessary, and the analysis was conducted on the CIPRES Science Gateway (Miller, Pfeiffer, & Schwartz, 2010) because of computation power. CIPRES is part of the Extreme Science and Engineering Discovery Environment (XSEDE) running on the Comet supercomputer, located in San Diego, California. The analysis ran there for 152.3 hr on 8 XSEDE cores.

In addition, uncorrected p distances for particular genes (16S: 854 bp, COI: 658 bp, cytb: 1,143 bp, Rag1: 2,414 bp) and clades were calculated in \textit{dnasp} 6 (Rozas et al., 2017), with alignment gaps and missing data excluded.

The final alignments used for all calculations are provided in the Supporting Information (Alignments S1–S5).

### RESULTS

#### 3.1 Phylogenetic analyses of Pakistani \textit{Eremias} using multilocus data

Using our concatenated mitochondrial and nuclear DNA sequences from Pakistani racers, similar tree topologies were returned by
ML and Bayesian analyses. The ingroup sequences clustered into six, mostly well-supported and deeply divergent clades (A-F; Figure 2) with uncorrected \( p \) distances of up to 23.8% for mtDNA (cytb) and 3.3% for the nuclear \( \text{Rag1} \) locus (Table 2). Clade A, maximally supported under both tree-building approaches, contained sequences of \( E. \text{acutirostris} \) and \( E. \text{aporosceles} \) and represents the \textit{Scapteira} subgenus. The two species from north-western Balochistan (Figure 1a) were not reciprocally monophyletic, with \( E. \text{acutirostris} \) being paraphyletic with respect to \( E. \text{aporosceles} \) (Figure 2). Genetic divergences within clade A were shallow (approx. 1% in mtDNA and 0.3% in the nuclear \( \text{Rag1} \) gene; Table 2). Clade A was sister to the remaining clades (Figure 2).

The sequences of three \textit{Rhabderemias} species, \( E. \text{cholistanica} \), \( E. \text{kakari} \) (both endemic to Pakistan), and \( E. \text{scripta} \) occurred in three clades (B-D) that formed together a maximally supported clade under ML. However, this more inclusive clade was not supported under BI. Clade B contained two \( E. \text{kakari} \) from the Toba Kakar Range (Torghar Mountains of north-eastern Balochistan), and clade C corresponded to the sequence of an \( E. \text{scripta} \) from the Pakistani part of the Helmand Basin. Clade D consisted of three \( E. \text{cholistanica} \) from the Cholistan Desert. The branching pattern within this \textit{Rhabderemias} clade was not well resolved, with a poorly supported clade containing \( C + D \) under ML, whereas BI placed the three clades B, C, and D in a polytomy. However, \( E. \text{cholistanica} \) (clade D) represented always a maximally supported clade, showing a within-clade divergence of less than 1% in the used markers. In contrast, the average uncorrected \( p \) distances between the three \textit{Rhabderemias} clades were high (mtDNA: 7.5%–16.6%, \( \text{Rag1} \): 1.2%; Table 2).

The monophyly of the \textit{Rhabderemias} subgenus (clades B-D) and its sister group, formed by the two deeply divergent clades E and F (Figure 2), was well supported (99/1.00). Clades E and F corresponded to sequences of lizards from northern Balochistan that were morphologically identified as \( E. \text{persica} \) (Aspidorhinus subgenus; Figures 1 and 2). This Aspidorhinus clade (E + F) received high support (96/1.00), and clades E and F were both maximally supported. Clade F consisted of two well-supported reciprocally monophyletic subclades (F1 and F2, each with support of 98/1.00; Figure 2). The
TABLE 2  Average uncorrected p distances (percentages) between the *Eremias* clades from Pakistan and phylogenetically close genera using 16S/COI/cytb/Rag1 sequences (854 bp/658 bp/1,143 bp/2,414 bp). Within-clade values are shown in bold on the diagonal.

| Clades | A     | B     | C     | D     | E     | F     | F1    | F2     | Acanthodactylus | Ophisops | Mesalina |
|--------|-------|-------|-------|-------|-------|-------|-------|-------|----------------|----------|----------|
| A      | 0.94/1.31/0.90/0.27 |       |       |       |       |       |       |       |                 |          |          |
| B      | 13.7/20.0/–/2.2 | 0/0/0– |       |       |       |       |       |       |                 |          |          |
| C      | 12.7/12.3/–/0.4 |       | 8.4/6.4/0/0 |       |       |       |       |       |                 |          |          |
| D      | 12.7/17.6/22.8/3.3 | 7.5/15.3/–/1.2 | 8.6/6.1/16.6/– |       |       |       |       |       |                 |          |          |
| E      | 12.6/17.8/22.8/2.6 | 10.8/19.4/–/0.4 | 11.6/6.1/18.4/– | 11.2/19.1/20.1/2.3 | 0.26/0.50/1.37/0.09 |       |       |       |                 |          |          |
| F      | 12.8/18.3/23.7/2.5 | 11.0/16.4/–/0.4 | 11.6/21.5/–/1.5 | 8.0/14.7/17.6/1.0 | 0.92/2.48/2.97/0.44 |       |       |       |                 |          |          |
| F1     | 12.7/18.1/23.9/– | 10.9/16.3/–/1.5 | 11.5/21.1/–/1.5 | 8.3/14.1/17.0/– | 1.0/2.1/2.6/– |       |       |       |                 |          |          |
| F2     | 13.1/18.7/23.9/– | 11.1/16.4/–/1.5 | 11.8/21.8/–/2.2 | 8.1/15.0/18.0/– | 0.7/2.2/2.7/– |       |       |       |                 |          |          |
| Acanthodactylus | 16.3/20.2/26.3/5.0 | 16.3/21.5/–/4.7 | 16.1/25.8/–/6.8 | 16.9/20.3/20.0/6.8 | 15.7/19.3/23.2/6.8 | 17.1/21.3/21.1/4.4 | 16.8/20.7/15.8/– | 17.1/21.7/25.3/– | –/–/–/– |          |
| Ophisops | 170/17.8/26.1/5.6 | 172/21.3/–/4.6 | 17.6/25.7/–/1.5 | 179/17.6/23.8/6.5 | 18.5/20.3/25.9/5.3 | 176/18.9/26.4/4.8 | 16.9/18.2/25.8/– | 17.5/19.5/26.8/– | 20.5/21.1/20.0/6.2 | –/–/–/– |
| Mesalina | 172/20.5/23.8/5.8 | 170/21.8/–/5.6 | 17.0/23.3/–/1.5 | 173/20.2/24.2/8.3 | 15.6/21.0/25.5/5.1 | 16.6/20.8/24.4/5.5 | 16.0/21.5/24.0/– | 16.6/20.4/24.7/– | 17.1/21.9/25.1/6.2 | 19.0/19.2/24.9/7.6 | –/–/–/– |
because no sequences were available for clade C (E. scripta). These five clades were all well supported (97-100/1.00). Four Pakistani clades (A, B, D, and F) were not represented by any previously published sequences. Within the well-supported clade F (97/1.00), two subclades occurred again (F1: 98/0.97; F2: 95/0.99). Our clade E (97/1.00) contained ENA/GenBank sequences from Afghanistan, representing the E. persica complex (HQ733946, MG699967, MG699968; Figure 1c). This clade was sister to another clade comprised of sequences of E. persica from Iran (95/1.00; HQ733940-45), and these two clades together were sister to clade F (E. persica) from Pakistan.

Many deeper nodes were only weakly supported. Yet, the subgenera Aspidorhinus, Pareremias, Rhaberderemias, and Scapteira were not monophyletic, and for Aspidorhinus and Pareremias, the polyphyletic topologies received some statistical support. For Aspidorhinus, there was one well-supported clade (99/1.00) containing three deeply divergent clades corresponding to E. velox. However, excluded from this more inclusive clade was another major clade (69/0.99) including all sequences of E. persica (clades E and F and additional sequences from Iran). The placement of a deeply divergent sequence of another Aspidorhinus species (E. nikolskii) was not resolved, but it did not cluster with any of the two more inclusive clades containing sequences of E. velox or E. persica. Several species from China, Kazakhstan, Kyrgyzstan, Mongolia, and Russia identified with the Pareremias subgenus constituted a well-supported clade (85/1.00). However, this clade did not include two other species of the Pareremias subgenus (E. argus, E. brenchleyi), even though the placement of these two species was not resolved.

Also, in the most diverse data set including 932 cyt b sequences of 20 species, all clades and subclades of our multilocus analysis of Pakistani material were confirmed, except for clade B, for which data were missing. The Pakistani clades with more than one representative were well supported, with bootstrap and posterior probability values between 98/1.00 and 100/1.00 (Figure 5). Our maximally supported clade A (100/1.00) was sister to all other clades; in clade A, E. acutirostris and E. aropolisceles were reciprocally monophyletic. Yet, the sister group relationship of clade A, and most other basal nodes, were not supported under ML (in contrast to BI). Except for clade E, all Pakistani clades were novel, with no previously published Eremias sequences clustering with these clades. Again, sequences morphologically identified with E. persica corresponded to two deeply divergent clades that were not sister groups. Our well-supported clade E (99/1.00; E. persica from northern Balochistan) was sister to a weakly resolved diverse clade (48/0.70) comprised of seven well-supported terminal clades corresponding to E. persica and its synonym E. nigrolateralis, both from Iran (Figures 1d and 5). In addition to our Pakistani sequences, clade E contained also two ENA/GenBank sequences of E. persica from Iran (FJ416244, FJ416247). Our clade F (98/1.00; E. persica from Pakistan) was sister to other E. persica from Iran and Pakistan and all other species of the E. persica complex. Even though the deeper branching patterns were generally not resolved by the cyt b data set, it is noteworthy that Rhaberderemias was not found monophyletic. Our Pakistani clades C and D, corresponding to the Rhaberderemias species E. scripta and E. cholistanica, respectively, were well-supported sister taxa (95/1.00). The third representative of Rhaberderemias, E. vermiculata, was not their sister taxon but, with maximum support under BI, sister to all other species of Eremias sensu lato, except E. acutirostris and E. aropolisceles (clade A). However, under ML this branching pattern received only a bootstrap value of 34.

The tree derived from the Rag1 data set is largely in line with the abovementioned mtDNA results (Figure 6). The Rag1 data set included 44 sequences of 12 currently recognized Eremias species. All clades retrieved by the multilocus trees were confirmed, except for clade C, for which data were missing. Within clade F, the two subclades were not resolved. Clade A (subgenus Scapteira) was maximally supported and with high support (96/1.00) sister to all other Eremias taxa. Within clade A, E. acutirostris and E. aropolisceles were not reciprocally monophyletic. The monophyly of the Pakistani clades B (66/0.56) and F (25/0.78) was weakly supported, whereas clades D (94/1.00) and E (99/1.00) were well supported. Many deeper nodes within the Eremias genus were weakly supported, preventing any considerations about the monophyly of the subgenera.

4 | DISCUSSION

The herpetofauna of the arid and mountainous regions of the Middle East and Central Asia is insufficiently explored, although these regions lie partly or totally within biodiversity hotspots (Sindaco & Jeremčenko, 2008). Thus, an exact knowledge of the biodiversity of these regions is of paramount interest for biogeography and conservation. Until today, there exist only a handful of herpetological studies using molecular genetic approaches (Asadi et al., 2019; Dufresnes et al., 2019; Macey, Wang, Ananjeva, Larson, & Papenfuss, 1998; Poyarkov et al., 2014; Shahamat, Rastegar-Pouyani, Rastegar-Pouyani, Hosseinian Yousefkhani, & Wink, 2020; Solovyeva et al., 2018). This is explained by the cultural and ethnic diversity and complicated political circumstances preventing broadscale research. Thus, our present study contributes to a better understanding of the herpetological diversity of Pakistan and Central Asia.

Using previously published data merged with our new sequences, we present the most comprehensive phylogenetic assessment of Eremias to date. We include 12 (Rag1), 19 (16S), and 20 (COI, cyt b) of the approximately 40 species of the genus (Figures 2-6; Table 3). Furthermore, with our multilocus data set, we present the first molecular-based assessment of Eremias from Pakistan and provide a sound basis for further biogeographic and taxonomic research.

Despite our geographically limited sampling, we obtained some significant new insights. Four of our six clades of Pakistani Eremias were for the first time identified by our study (A, B, D, and F). Four clades (A, D, E, and F) show variability and call for further research and additional sampling in the whole region.

Four morphologically defined taxa (E. acutirostris, E. aropolisceles, E. chollistanica, and E. kakari) were never before included in molecular genetic investigations. Guo et al. (2011) reported to have studied
one specimen of *E. acutirostris* (HQ615648, Takhteh Pol, Kandahar, Afghanistan). However, this 16S sequence belongs to the *E. persica* complex (Figure 3) and cannot represent *E. acutirostris*. This misidentification led Guo et al. (2011) to conclude that the subgenus *Scapteira* is not monophyletic. The two Pakistani taxa representing *Scapteira*, *E. acutirostris* and *E. aporosceles* (Khan, 2006) constitute a well-supported monophylum (clade A) in our analyses of all data sets. Clade A is sister to all remaining *Eremias* taxa (Figures 2-6). Yet, ENA/GenBank sequences (16S: DQ494820-DQ494822, HQ615635, HQ615636) of *E. grammica*, the type species of the *Scapteira* subgenus, surprisingly were excluded from the well-supported clade containing the two Pakistani *Scapteira* taxa (*E. acutirostris*, *E. aporosceles*). The phylogenetic placement of *E. grammica* was not resolved and it clustered, albeit without statistical support, with species currently placed in *Rhabderemias* (Figures 3 and 4; see also below under "Taxonomic Conclusions"). Also, *Aspidorhinus*, *Pareremias*, and *Rhabderemias* were not monophyletic in our analyses, and the polyphyly of *Aspidorhinus*
and Pareremias was relatively well supported using our COI data set (Figure 4).

With respect to 16S sequences, our analyses found *E. scripta* from Pakistan, Afghanistan, and Uzbekistan deeply divergent (Figure 3), suggestive of taxonomic differentiation. *Eremias scripta* is the type species of the subgenus *Rhabderemias* (Lantz, 1928). Therefore, the proper identification and delineation of the taxon on which the subgenus was founded is of taxonomic relevance. This situation calls for further research and extended sampling.

*Eremias cholistanica* and *E. kakari*, two species endemic to Pakistan, were confirmed by our analyses as clearly distinct, representing previously unsampled and deeply divergent clades of *Eremias*.

Our Pakistani sequences of *E. persica* clustered in the two distinct clades E and F that were not sister taxa in our single-gene analyses using previously published sequences (Figures 3-6). Based on these expanded data sets, a broader distribution of clade E can be inferred that spans from western Iran to eastern Afghanistan including the border region with Pakistan (Figure 1b-d). In contrast, clade F, discovered in the present study, is currently only known from the northeast of Balochistan, Pakistan. This clade comprises two subclades (F1 and F2) suggestive of phylogeographic variation, despite the close proximity of the collection sites in the Toba Kakar Range (Torghar Mountains). This differentiation could have been caused by the isolation of the populations in the Tanishpa and Kundar valleys, which also correspond to distinct habitat types. The Tanishpa valley is composed of mountainous rocky terrain, while the Kundar valley is an open alluvial plain with the dominance of gravel and some sandy patches.

### 5 | TAXONOMIC CONCLUSIONS

The phylogenetic relationships within *Eremias* are poorly understood (Figures 2-6) and plagued by a high level of cryptic diversity coupled with taxonomic misidentifications (Guo et al., 2011; Liu, Ananjeva,
Our results underline that neither the phylogeny nor the taxonomy of this morphologically challenging group can be resolved without molecular genetic studies and that integrative taxonomic approaches are needed for understanding its diversity.

Compared to other Central Asian regions, the diversity of *Eremias* is not high in Pakistan (five to seven species; Khan, 2006; Masroor et al., 2020). However, our results suggest that this is an underestimation as the morphologically identified samples of *E. persica* turned out to represent the two deeply divergent clades E and F, the latter showing further substructure (subclades F1/F2). When it is considered that we studied DNA data of only 29 specimens from Pakistan, it seems likely that a broadscale investigation will reveal further diversity. However, comprehensive sampling is still challenging, not only in Pakistan, due to the inaccessibility of some regions in Central and Southern Asia, the low density of lizards, and demanding dry and hot climate. Some species are only known from a few specimens or localities and were
never examined with molecular approaches (e.g., *E. afghanistanica*, *E. andersoni*, *E. aria*, *E. fasciata*, *E. kavirensis*). Even though our analyses using three mitochondrial genes and one nuclear locus are the most comprehensive assessment of the systematic phylogeny of *Eremias* to date (cf. Guo et al., 2011; Liu et al., 2019; Orlova et al., 2017; Poyarkov et al., 2014; Rastegar-Pouyani et al., 2010), we included only approximately two thirds of the currently known taxa. Nevertheless, our analyses allow for some important new taxonomic insights.

We found *E. acutirostris* and *E. aporosceles* reciprocally monophyletic in our phylogenetic analysis of the cytb gene (Figure 3). However, neither the combined data set (Figure 2) nor the analyses of the 16S and Rag1 loci supported the monophyly of the two taxa. Their low genetic divergence (Table 2) casts further doubts on their species status. Based on morphology, in particular the absence of femoral pores and the greater snout–vent length in *E. aporosceles*, the two taxa were considered as distinct species (Khan, 2004, 2006). However, Mertens (1969) suggested that *E. aporosceles* could be only a divergent population of *E. acutirostris*. Fifty years after Mertens' suggestion, our results support his hypothesis and are in accordance with Szczerbak (1974), who proposed *E. aporosceles* as a synonym of *E. acutirostris*. We therefore concur with Szczerbak (1974) and regard *E. aporosceles* Alcock
TABLE 3  Overview of currently recognized species of *Eremias*, with a summary of previous results and those of the present study. Subgenera follow Guo et al. (2011), Orlova et al. (2017), and Masroor et al. (2020)

| Taxon | Previous phylogenies | This study |
|-------|---------------------|------------|
|       | Marker | Clade/species | Marker | Clade/species |
| Subgenus *Aspidorhinus* Eichwald, 1841 |
| 1—*Eremias afghanistanica* Böhme & Szczerbak, 1991 | — | — | — | — |
| 2—*Eremias isfahanica* Rastegar-Pouyani et al., 2016 | 12S, cytb (1) | Monophyletic | cytb | Compromised sequences, not evaluated |
| 3—*Eremias kopetdaghica* Szczerbak, 1972 | 12S, cytb, Rag1 (2) | Monophyletic | cytb, Rag1 | Monophyletic |
| 4—*Eremias lalezharica* Moravec, 1994 | 12S, cytb, Rag1 (2) | Monophyletic | cytb, Rag1 | Monophyletic |
| 5—*Eremias montana* Rastegar-Pouyani and Rastegar-Pouyani, 2001 | 12S, cytb (3) | Monophyletic | cytb | Monophyletic |
| 6—*Eremias nikolskii* Bedriaga in Nikolsky, 1905 | COI (4) | Monophyletic | COI | Unsupported topology |
| 7—*Eremias papenfussi* Mozaffari et al., 2011 | 12S, cytb (1) | Monophyletic | cytb | Compromised sequences, not evaluated |
| 8—*Eremias persica* Blanford, 1874 | 12S, cytb (3) | Species complex | 16S, COI, cytb, Rag1 | Species complex |
| 9—*Eremias regeli* Bedriaga in Nikolsky, 1905 | — | — | — | — |
| 10—*Eremias roborowskii* (Bedriaga, 1912) | 12S, cytb (7); 16S (8) | Monophyletic | 16S, cytb | Monophyletic |
| 11—*Eremias strauchi* Kessler, 1878 | 12S, cytb (2) | Monophyletic | cytb | Monophyletic |
| 12—*Eremias suphani* Başoğlu and Hellmich, 1968 | 12S, cytb (1) | Monophyletic | cytb | Monophyletic |
| 13—*Eremias velox* (Pallas, 1771) | 12S, cytb (3,5–7); 16S (8) | Species complex | 16S, COI, cytb, Rag1 | Species complex |
| Subgenus *Eremias* Fitzinger, 1834 |
| 14—*Eremias arguta* (Pallas, 1773) | 16S (8); cytb (9) | Species complex | 16S, COI, cytb, Rag1 | Species complex |
| 15—*Eremias aria* Anderson & Leviton, 1967 | — | — | — | — |
| 16—*Eremias intermedia* (Strauch, 1876) | cytb (9) | Monophyletic | cytb | Monophyletic |
| 17—*Eremias nigrocellata* Nikolsky, 1896 | — | — | — | — |
| Subgenus *Pareremias* Szczerbak, 1973 |
| 18—*Eremias argus* Peters, 1869 | 16S (8); cytb (10) | Monophyletic | 16S, COI, cytb | Monophyletic |
| 19—*Eremias brenchleyi* Günther, 1872 | 16S (8), cytb (10) | Species complex | 16S, COI, cytb | Species complex |
| 20—*Eremias buechneri* Bedriaga, 1907 | COI (4) | Monophyletic | COI | Monophyletic |
| 21—*Eremias dzungarica* Orlova et al., 2017 | COI (4) | Monophyletic | COI | Monophyletic |
| 22—*Eremias kokshaoliensis* Eremchenko and Panfilov, 1999 | — | — | — | — |
| 23—*Eremias multiocellata* Günther, 1872 | 16S (8); COI (4) | Paraphyletic species complex | 16S, COI, cytb | Paraphyletic species complex |

(Continues)
TABLE 3 (Continued)

| Taxon | Previous phylogenies | This study |
|-------|----------------------|------------|
|       | Marker | Clade/species | Marker | Clade/species |
| 24—Eremias przewalskii (Strauch, 1876) | 16S (8); COI (4) | Distinct lineage within E. multiocellata complex (16S, COI) | 16S, COI, cytb | Monophyletic (cytb), distinct lineage within E. multiocellata complex (16S, COI) |
| 25—Eremias quadriprongs (Strauch, 1876) | — | — | — | — |
| 26—Eremias stummeri Wettstein, 1940 | COI (4) | Monophyletic | 16S, COI, cytb | Monophyletic |
| 27—Eremias szczerbaki Eremchenko, Panfilov and Zarinenko, 1992 | COI (4) | Monophyletic | COI | Monophyletic |
| 28—Eremias yarkandensis Blanford, 1875 | 16S (8,12); COI (4) | Distinct lineage within E. multiocellata complex; monophyletic | 16S, COI | Distinct lineage within E. multiocellata complex |
| Subgenus Rhabderemias Lantz, 1928 | | | | |
| 29—Eremias andersoni Darevsky and Szczerek, 1978 | — | — | — | — |
| 30—Eremias choliostanica Baig & Masroor, 2006 | — | — | 16S, COI, cytb, Rag1 | Monophyletic |
| 31—Eremias fasciata Blanford, 1874 | — | — | — | — |
| 32—Eremias kakari Masroor, Khisroon, Khan, & Jablonski, 2020 | — | — | 16S, COI, Rag1 | Monophyletic |
| 33—Eremias lineolata (Nikolsky, 1896) | — | — | 16S, COI | Monophyletic |
| 34—Eremias pleskei Bedriaga, 1905 | 16S (8), Rag1 (11) | Monophyletic (unsupported, 16S) | 16S, Rag1 | Monophyletic (unsupported) |
| 35—Eremias scripta (Strauch, 1867) | 16S (8) | Monophyletic | 16S, COI, cytb, Rag1 | Possible species complex |
| 36—Eremias vermiculata Blanford, 1875 | 16S (8,12), COI (4) | Monophyletic | 16S, COI, cytb | Monophyletic |
| Subgenus Scapteira Wiegmann, 1834 | | | | |
| 37—Eremias acutirostris (Boulenger, 1887) | 16S (8) | Erroneously identified as E. acutirostris; sequence represents actually E. persica complex | 16S, COI, cytb, Rag1 | Monophyletic |
| 38—Eremias aporosceles Alcock and Finn, 1897 | — | — | 16S, COI, cytb, Rag1 | Synonym of E. acutirostris |
| 39—Eremias grammica (Lichtenstein, 1823) | 16S (8,12) | Monophyletic | 16S, COI, cytb | Monophyletic |
| 40—Eremias kavirensis Mozaffari and Parham, 2007 | — | — | — | — |

Note: Sources: (1) Rastegar-Pouyani et al. (2016); (2) Rastegar-Pouyani et al. (2015); (3) Rastegar-Pouyani et al. (2010); (4) Orlova et al. (2017); (5) Rastegar-Pouyani, Hosseini Yousekhani, and Wink (2012); (6) Liu et al. (2014); (7) Liu et al. (2019); (8) Guo et al. (2011); (9) Poyarkov et al. (2014); (10) Zhao et al. (2011); (11) Mayer and Pavlichev (2007); (12) Wan et al. (2007).

& Finn, 1897 (type locality "near Nushki, northern Baluchistan, Pakistan") as a synonym of E. acutirostris (Boulenger, 1887) with a type locality of "between Nushki and Helmand in the Afghan–Pakistani border area (N. Baluchistan)." However, it is noteworthy that the two morphotypes have never been collected at the same locality; our samples originally identified as E. acutirostris and E. aporosceles originated from two regions having an aerial distance of approximately 400 km (Nushki and Mizal Darband, Nuk Kundi).
Our analyses revealed that some morphologically defined taxa contain genetically deeply divergent lineages that most likely correspond to distinct species. *Eremias scripta* surely represents a species complex, as indicated by the deep divergences and the phylogenetic placement of our single Pakistani sample compared to material from Afghanistan and Uzbekistan. Without additional broad sampling from Central Asia (especially from the type localities of the currently recognized subspecies; Sindaco & Jeremčenko, 2008; Szczerek & Vashetko, 1973), we can only speculate about the taxonomic identity of the involved lineages.

Our Pakistani racerunner samples morphologically identified with *E. persica* corresponded to two deeply divergent clades (E and F). Clade F was identified for the first time in the present study. The comparison with previously published sequences supports that *E. persica* is a complex of several distinct species, as suggested by Rastegar-Pouyani et al. (2010, 2016). Our clade E has been recorded before from Iran ("clade 3" or "Zabol clade" of Rastegar-Pouyani et al., 2010). Our 16S and COI data sets (Figures 1, 3, 4) indicate that it also occurs in Afghanistan and that a previously published 16S sequence (HQ615648) labeled as *E. acutirostris* (Afghanistan) falls into this clade. Rastegar-Pouyani et al. (2010) suggested that their "Zabol clade" (i.e., our clade E) could represent *E. afghanistanica* Böhme & Szczerek, 1991. Samples of this species from central and south-eastern Afghanistan (Böhme & Szczerek, 1991) have never been analyzed by molecular means (Table 3). Thus, if Rastegar-Pouyani et al. (2010) should be right, it is possible that the range of *E. afghanistanica* extends into Pakistan. However, *E. afghanistanica* seems to be morphologically distinct from our specimens (M. A. Khan, R. Masroor, unpubl. observ.).

Our study did not support the monophyly of the currently recognized subgenera of *Eremias*. However, our single-gene analyses could not resolve the phylogeny in most cases. Yet, the *Aspidorhinus*, *Pareremias*, and *Rhabderemias* subgenera were found polyphyletic with some statistical support using the COI and cytb data sets (Figures 4, 5). This underlines that broader sampling and additional genetic information is needed for a better understanding of the subgeneric differentiation of *Eremias*.

In this context, we wish to mention that the sequence divergences between some clades within *Eremias* exceed 20% in mtDNA and 2.5% in nDNA. These values resemble those between distinct genera of the Eremiadiini tribe (*Acanthodactylus*, *Mesalina*, *Ophisops*; Table 2) or some European lacertids (e.g., *Podarcis* and *Hellenolacerta*; Kapli et al., 2013), implying that *Eremias* could be split in future in distinct genera.

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**NOTE ADDED IN PROOF**

Recently, another racerunner was described from Iran, *Eremias fahimii*. An exploratory phylogenetic assessment of the cytb data of the original description (Mozaffari, Ahmadzadeh, & Saberi-Pirooz, 2020) revealed that *E. fahimii* corresponds to one of the seven Iranian clades (labeled "E. persica") that are together sister to our Pakistani clade E (Figure 5).

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**REFERENCES**

Anderson, S. C. (1999). *The lizards of Iran*. Oxford, Ohio: Society for the Study of Amphibians and Reptiles.

Anderson, S. C., & Leviton, A. E. (1967). A new species of *Eremias* (Reptilia: Lacertidae) from Afghanistan. *Occasional Papers of the California Academy of Sciences*, 64, 1–4.

Arnold, E. N. (1986). The hemipenis of lacertid lizards (Reptilia: Lacertidae): Structure, variation and systematic implications. *Journal of Natural History*, 20, 1221–1257. https://doi.org/10.1080/00222938600707811

Arnold, E. N. (1989). Towards a phylogeny and biogeography of the Lacertidae: Relationships within an Old-World family of lizards derived from morphology. *Bulletin of the British Museum (Natural History)*, 55, 209–257.

Arnold, E. N., Arribas, O., & Carranza, S. (2007). Systematics of the Palaeartic and Oriental lizard tribe Lacertini (Squamata: Lacertidae), with descriptions of eight new genera. *Zootaxa*, 1430, 1–86. https://doi.org/10.11646/zootaxa.1430.1.1

Asadi, A., Montgelard, C., Nazarizadeh, M., Mohaddasi, A., Fatemizadeh, F., Simonov, E., ... Kaboli, M. (2019). Evolutionary history and post-glacial colonization of an Asian pit viper (*Gloydius halys caucasicus*) into Transcaucasia revealed by phylogenetic and phylogeographic analyses. *Scientific Reports*, 9, 1224. https://doi.org/10.1038/s41598-018-37558-8

Baig, K. J., & Masroor, R. (2006). A new species of *Eremias* (Sauria: Lacertidae) from Cholistan Desert, Pakistan. *Russian Journal of Herpetology*, 13, 167–174.

Barabanov, A. V. (2009). *Aspidorhinus* Eichwald, 1841 as a valid subgeneric name for *Eremias* velox species group (Sauria, Lacertidae). *Current Studies in Herpetology*, 9, 59–61 [in Russian, with English summary].

Böhme, W., & Szczerek, N. N. (1991). Ein neuer Wüstenrenner aus dem Hochland Afghanistans, *Eremias* (*Eremias*) *afghanistanica* sp. n. (Reptilia: Sauria: Lacertidae). *Bonner Zoologische Beiträge*, 42, 137–141.

Boulenger, G. A. (1918). A synopsis of the lizards of the genus *Eremias*. *Journal of Zoological Research*, 3, 1–12.
A molecular phylogenetic perspective and an area cladogram for Central Asia. *Molecular Phylogenetics and Evolution*, 12, 320–332. https://doi.org/10.1006/mpev.1999.0641

Masroor, R. (2012). A contribution to the herpetology of northern Pakistan. Oxford, Ohio: Society for the Study of Amphibians and Reptiles.

Masroor, R., Khisroon, M., Khan, M. A., & Jablonski, D. (2020). A new species of *Eremias* (Squamata: Lacertidae) from the arid mountains of Pakistan. *Zootaxa*, 4786, 101–121. https://doi.org/10.11646/zootaxa.4786.1.8

Mayer, W., & Pavlicev, M. (2007). The phylogeny of the family Lacertidae (Reptilia) based on nuclear DNA sequences: Convergent adaptations to arid habitats within the subfamily Eremiainae. *Molecular Phylogenetics and Evolution*, 44, 1155–1163. https://doi.org/10.11646/zootaxa.4786.1.8
KHAN ET AL.

Fitzinger, 1834 (Squamata: Lacertidae) from Central Iran, supported by mtDNA sequences and morphological data. Zootaxa, 4132, 207–220. https://doi.org/10.11646/zootaxa.4132.2.2

Rastegar-Pouyani, E., Hosseinian Yousefkhani, S. S., & Wink, M. (2015). Taxonomic reevaluation of Eremias strauchi strauchi Kessler, 1878 and Eremias strauchi kopetdaghica Szczerek, 1972, based on nuclear and mitochondrial DNA sequences (Reptilia: Lacertidae). Zoology in the Middle East, 61, 118–124. https://doi.org/10.1080/0939140.2015.1020615

Rastegar-Pouyani, E., Kazemi-Noureini, S., Rastegar-Pouyani, N., Joger, U., & Wink, M. (2012). Molecular phylogeny and intraspecific differentiation of the Eremias velox complex of the Iranian Plateau and Central Asia (Sauria, Lacertidae). Journal of Zoological Systematics and Evolutionary Research, 50, 220–229. https://doi.org/10.1111/j.1439-0469.2012.00662.x

Rastegar-Pouyani, E., Rastegar-Pouyani, N., Kazemi-Noureini, S., Joger, U., & Wink, M. (2010). Molecular phylogeny of the Eremias persica complex of the Iranian plateau (Reptilia: Lacertidae), based on mtDNA sequences. Zoological Journal of the Linnean Society, 158, 641–660. https://doi.org/10.1111/j.1096-3642.2009.00553.x

Ronquist, F., Teslenko, M., van der Mark, P., Ayres, D. L., Darling, A., Höhna, S., … Hueslenbeck, J. P. (2012). MrBayes 3.2: Efficient Bayesian phylogenetic inference and model choice across a large model space. Systematic Biology, 61, 539–542. https://doi.org/10.1093/sysbio/sys029

Rozas, J., Ferrer-Mata, A., Sánchez-DelBarrio, J. C., Guirao-Rico, S., Librado, P., Ramos-Onsins, S. E., & Sánchez-Gracia, A. (2017). Dnasp 6: DNA sequence polymorphism analysis of large data sets. Molecular Biology and Evolution, 34, 3299–3302. https://doi.org/10.1093/molbev/msx248.

Shahamat, A.-A., Rastegar-Pouyani, E., Rastegar-Pouyani, N., Hosseinian Yousefkhani, S. S., & Wink, M. (2020). Molecular phylogeny and intraspecific differentiation of the Trapelus agilis species complex in Iran (Squamata: Agamidae) inferred from mitochondrial DNA sequences. PeerJ, 8, e8295. https://doi.org/10.7717/peerj.8295.

Sindaco, R., & Jeremčenko, V. K. (2008). The reptiles of the Western Palearctic. Volume 1. Latina, Italy: Edizioni Belvedere.

Smith, M. A. (1935). The fauna of British India, including Ceylon and Burma. Volume II. London, UK: Taylor & Francis.

Solovyeva, E. N., Lebedev, V. S., Dunayev, E. A., Nazarov, R. A., Bannikova, A. A., Che, J., … Poyarkov, N. A. (2018). Cenozoic aridization in Central Eurasia shaped diversification of toad-headed agamas (Phrynocephalus; Agamidae, Reptilia). PeerJ, 6, e4543. https://doi.org/10.7717/peerj.4543.

Stamatakis, A. (2014). Raxml version 8: A tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics, 30, 1312–1313. https://doi.org/10.1093/bioinformatics/btu033.

Strauch, A. A. (1876). Reptiles and amphibians. In N. M. Przewalski (Ed.), Mongolia and the land of Tanguts. Three-year journey in eastern mountainous Asia. Volume 2, Section 2 (pp. 1–55). St. Petersburg: Imperial Academy of Sciences [in Russian].

Szczerek, N. N. (1971). Taxonomy of the genus Eremias (Sauria, Reptilia) in connection with the focuses of the desert-steppe fauna development in Palearctic. Vestnik Zoolozii, 1971, 47–55 [in Russian].

Szczerek, N. N. (1974). Racerunners of the Palearctic. Kiev, Ukraine: Naukova dumka [in Russian].

Szczerek, N. N., & Vashetko, E. V. (1973). New subspecies – Eremias scripta pherganensis ssp. n. (Reptilia, Sauria) from Uzbekistan. Vestnik Zoolozii, 1973, 84–86 [in Russian].

Tamar, K., Carranza, S., Sindaco, R., Moravec, J., Trape, J. F., & Meiri, S. (2016). Out of Africa: Phylogeny and biogeography of the widespread genus Acanthodactylus (Reptilia: Lacertidae). Molecular Phylogenetics and Evolution, 103, 6–18. https://doi.org/10.1016/j.ympev.2016.07.003.

Uetz, P., & Hošek, J. (2019). The reptile database. Available from: http://www.reptile-database.org.

van Bedriaga, J. (1912). Wissenschaftliche Resultate der von N. M. Przewalski nach Central-Asien unternommenen Reisen, Zoologischer Theil. Band III, Abtheilung 1. Amphibien und Reptilien. St. Petersburg, Russia: Kaiserliche Akademie der Wissenschaften [in Russian and German].

Wagner, P., Bauer, A. M., Leviton, A. E., Wilms, T. M., & Böhme, W. (2016). A checklist of the amphibians and reptiles of Afghanistan. Exploring herpetodiversity using biodiversity archives. Proceedings of the California Academy of Sciences, 63, 457–565.

Wan, L., Sun, S., Jin, Y., Yan, Y., & Liu, N. (2019). Molecular phylogeny and intraspecific differentiation of the Eremias velox complex of the Iranian plateau (Reptilia: Lacertidae), based on 16S rRNA mitochondrial DNA sequences. Amphibia-Reptilia, 28, 33–41. https://doi.org/10.1163/156853807797998956.

Zhao, E. M., & Adler, K. (1993). Herpetology of China. Oxford, Ohio: Society for the Study of Amphibians and Reptiles.

Zhao, E. M., Zhao, K. T., & Zhou, K. Y. (1999). Fauna Sinica: Reptilia. Squamata, Lacertilia. Beijing, China: Science Press.

Zhao, Q., Liu, H. X., Luo, L. G., & Ji, X. (2011). Comparative population genetics and phylogeography of two lacertid lizards (Eremias argus and E. brenchleyi) from China. Molecular Phylogenetics and Evolution, 58, 478–491. https://doi.org/10.1016/j.ympev.2010.12.017.

Zhou, T., Li, D., Dujsebayeva, T. N., Liu, J., & Guo, X. (2016). Complete mitochondrial genome of Stummer’s racerunner (Eremias stummeri) from Kazakhstan. Mitochondrial DNA Part A, 27, 4340–4341. https://doi.org/10.3109/19401736.2015.1089491.

**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.

Table S1. Samples, ENA/GenBank sequences and their accession numbers.

Table S2. Primers used for PCR amplification and sequencing.

Table S3. Partition schemes and substitution models used in RaxML and MrBayes analyses.

Alignment S1. Concatenated data set for Pakistani Eremias (16S, COI, cytB, Rag1).

Alignment S2. Data set for 16S sequences.

Alignment S3. Data set for COI sequences.

Alignment S4. Data set for cytB sequences.

Alignment S5. Data set for Rag1 sequences.

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