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Molecular phylogeny of *Eremias* spp. from Pakistan contributes to a better understanding of the diversity of racerunners

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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. aporosceles*, *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. aporosceles* and *E. acutirostris* (both clade A), no unambiguous genetic support was found for these two taxa. Therefore, we regard *E. aporosceles* 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.

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KEYWORDS
Central Asia, genetic diversity, lizards, South Asia, taxonomy

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 *Eremias* 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 & Pavlicev, 2007; Sindaco & Jeremčenko, 2008) and are typically confined to temperate grasslands of Eurasia and desert regions of Central Asia (Guo, Chen, Wan, & Wang, 2010; Guo et al., 2011). The species diversity and taxonomy of *Eremias* remain unresolved, despite almost two centuries of research (Bedriaga, 1912; Boulenger, 1918, 1921; Lantz, 1928; Nikolsky, 1915; Strauch, 1876; Szczerbak, 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*), *Eremias* Fitzinger, 1834 (type species: *E. arguta*), *Pareremias* Szczerbak, 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 *Eremias* sensu stricto, while the species currently assigned to the *Eremias* subgenus were previously known as members of the subgenus *Ommateremias* Lantz, 1928 (Barabanov, 2009; Orlova et al., 2017; Sindaco & Jeremčenko, 2008; Szczerbak, 1974).

About twenty species of the genus *Eremias* 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; Dujsebayeva, Chirikova, & Belyalov, 2009; Masroor, Khisroon, Khan, & Jablonski, 2020; Mozaffari, Ahmadzadeh, & Parham, 2011; Orlova, 2008; Orlova et al., 2017; Orlova & Terbish, 1997; Rastegar-Pouyani et al., 2016; Sindaco & Jeremčenko, 2008; Wagner et al., 2016; Zhao & Adler, 1993; Zhao, Zhao, & 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, Dujsebayeva, Liu, & Guo, 2016). Over the past three decades, several attempts were made to revise *Eremias*, mostly based on external morphology, hemipenial traits, and ecology (Anderson, 1999; Arnold, 1986; Barabanov, 2009; Szczerbak, 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 *Eremias* 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* (Boulenger, 1887), *E. aporosceles* Alcock and Finn, 1897, *E. cholistanica* Baig & Masroor, 2006, *E. fasciata* Blanford, 1874, *E. kakari* Masroor et al., 2020, *E. persica* Blanford, 1874, and *E. scripta* Strauch, 1867. *Eremias aporosceles* was regarded for a long time as a synonym of *E. acutirostris* (Szczerbak, 1974). However, some authors regarded it later as a valid species (Anderson, 1999; Das, 1992; Khan, 2006). Except for apparently lost specimens reported by Boulenger (1921),

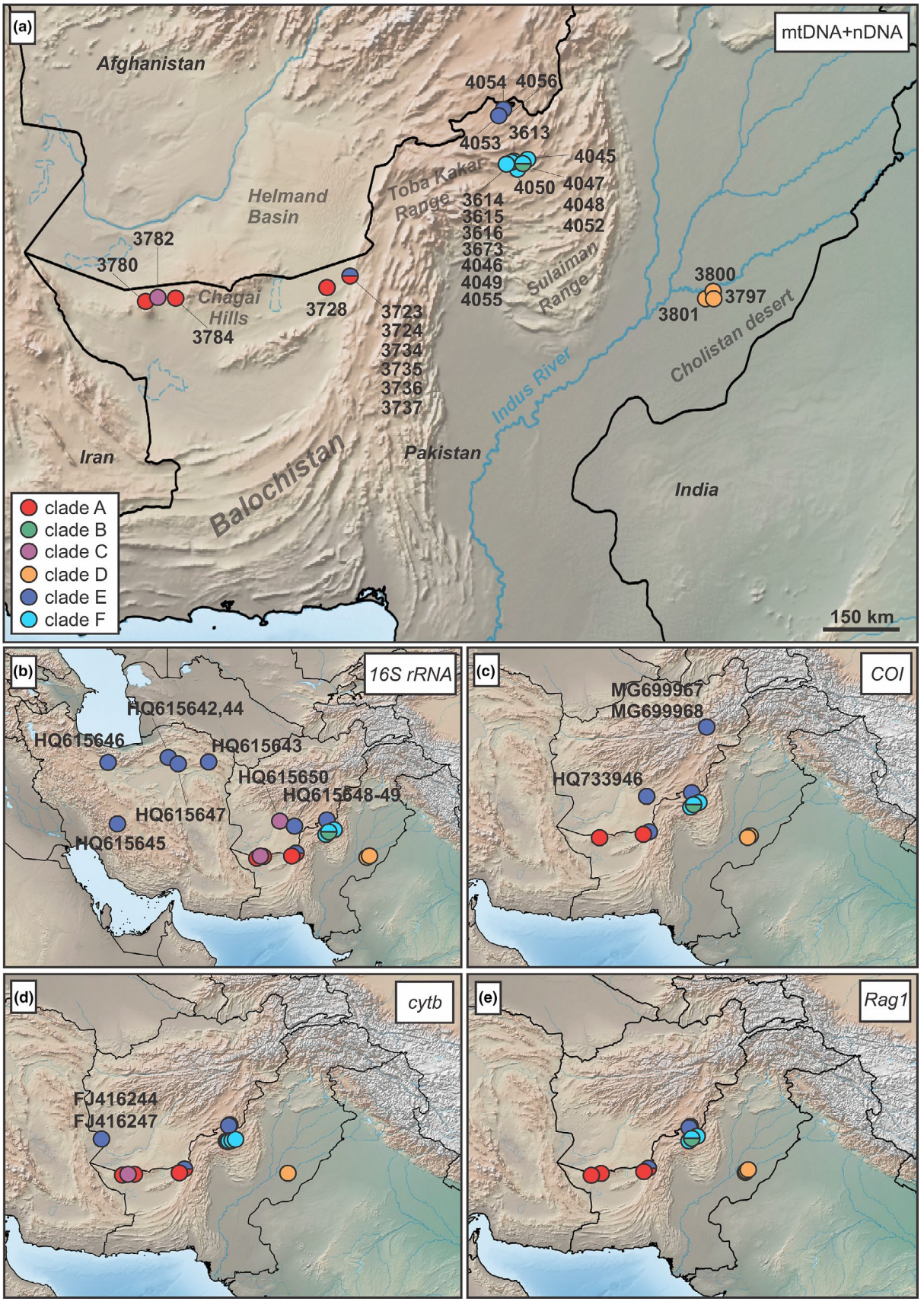

FIGURE 1 (a) Geographic origin of *Eremias* samples used in the present study. Clades are defined according to results of the concatenated data set (Figure 2). Numbers correspond to vouchers in the Pakistan Museum of Natural History (PMNH), Islamabad. (b-e) Records for our data and previously published 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. cholistanica*, *E. fasciata*, *E. kakari*, *E. scripta*) and *Scapteira* (*E. acutirostris*, *E. aporosceles*; Khan, 2006; Sindaco & Jeremčenko, 2008; Szczerbak, 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 racerunners 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. aporosceles*, *E. cholistanica*, *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 $MgCl_2$ 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 $MgCl_2$ 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 amplicons 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 *Acanthodactylus*, *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,

TABLE 1 Material from Pakistan used in molecular phylogenetic analyses. Taxonomy follows the present study

					ENA/GenBank accession numbers				
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, Mizal Darband	29.2456	62.7994	MT524478	n/a	MT554468	MT554491	A
19354	3784	Balochistan: Nok Kundi, Mizal 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, Mizal Darband	29.2946	62.9843	MT524477	n/a	MT554462	n/a	C
Eremias cholistanica									
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)

TABLE 1 (Continued)

MTD	PMNH	Provenance	N	E	ENA/GenBank accession numbers				Clade
					16S	*COI*	*cytb*	*Rag1*	
19482	4052	Balochistan: Torghar Mts., Tanishpa valley	30.9553	68.6912	MT524484	MT524260	MT554459	MT554497	F/F1
19340	4050	Balochistan: Torghar Mts., Tanishpa valley	30.9319	68.6472	MT524468	MT524247	MT554473	MT554483	F/F1
19334	4046	Balochistan: Torghar Mts., Kundar valley	30.9764	68.5047	MT524463	MT524244	MT554453	MT554479	F/F2
19339	3615	Balochistan: Torghar Mts., Kundar valley	30.9755	68.4465	MT524467	n/a	MT554456	MT554482	F/F2
19345	3673	Balochistan, Torghar Mts., Kundar valley	30.9680	68.4166	MT524473	MT524252	n/a	n/a	F/F2
19336	4055	Balochistan: Torghar Mts., Kundar valley	30.9764	68.5047	MT524465	MT524246	MT554455	MT554481	F/F2
19338	3616	Balochistan: Torghar Mts., Kundar valley	30.9643	68.4879	MT524466	n/a	n/a	n/a	F/F2
19333	3614	Balochistan: Torghar Mts., Kundar valley	30.9764	68.5047	MT524462	MT524243	MT554466	MT554478	F/F2
19356	4049	Balochistan: Torghar Mts., Kundar valley	30.9764	68.5047	MT524481	MT524257	MT554458	MT554494	F/F2

Abbreviations: MTD: Museum für Tierkunde (Tissue Collection), Senckenberg Dresden, Germany; PMNH: Pakistan Museum of Natural History; n/a: not available.

among them 911 ENA/GenBank sequences (Tables 1 and S1; Alignment S4); and

5. a data set of the nuclear *Rag1* sequences (2,414 bp length) contained 44 sequences of 12 currently recognized *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 RAXML 8.0.0 (Stamatakis, 2014). As a consequence, seven 676- to 690-bp-long *cytb* sequences (*E. isfahanica*, *E. papenfussi*; KP317957-KP317963) from Rastegar-Pouyani et al. (2016) were removed from the alignment. These sequences are highly variable and the RAXML analysis placed them outside *Eremias*, although both species should represent the subgenus *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 *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 PARTITIONFINDER 2 (Lanfear, Frandsen, Wright, Senfeld, & Guindon, 2017) and the implemented Bayesian Information Criterion (Table S3). Maximum Likelihood analyses were run with 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 MRBAYES 3.2.6 (Ronquist et al., 2012) with two parallel runs (each with four chains) and default parameters. Calculation parameters were analyzed using TRACER 1.7.1 (Rambaut, Drummond, Xie, Baele, & Suchard, 2018). With the exception of the *cytb* analysis, the chains ran on the Mac Pro for 20 million generations with every 1,000th generation sampled. For *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 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).

3 | RESULTS

3.1 | Phylogenetic analyses of Pakistani *Eremias* using multilocus data

Using our concatenated mitochondrial and nuclear DNA sequences from Pakistani racerunners, similar tree topologies were returned by

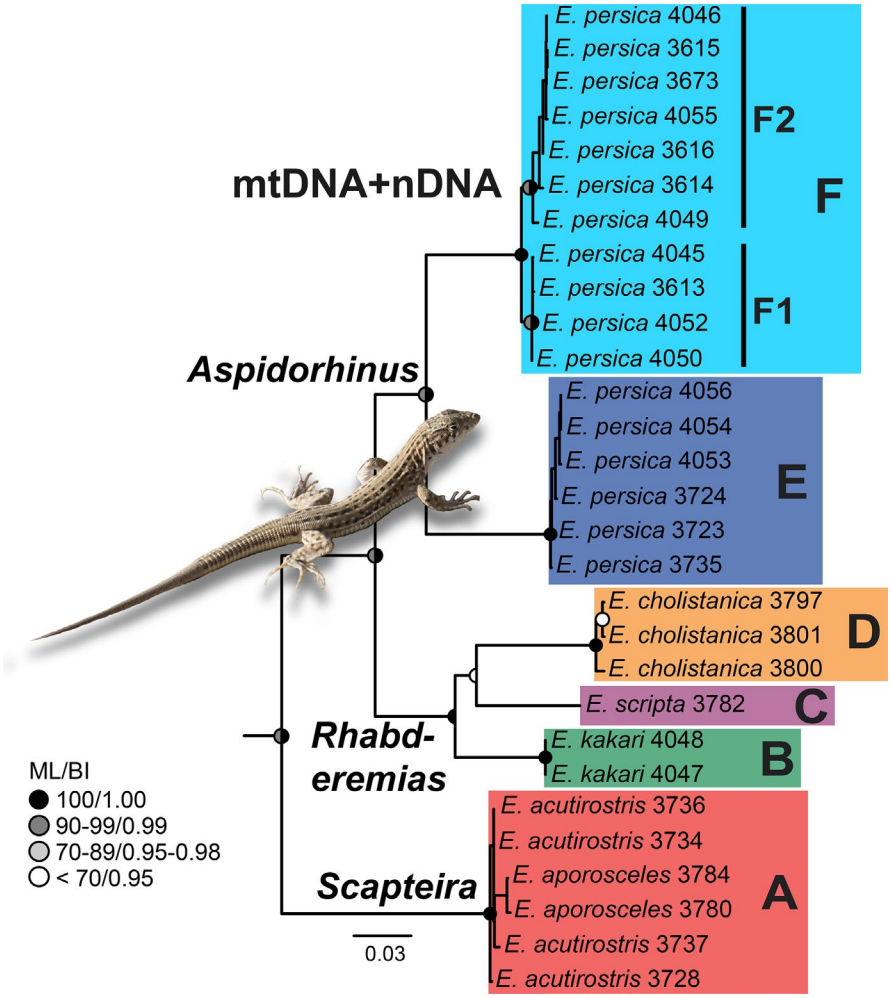

FIGURE 2 Maximum Likelihood tree for concatenated *16S*, *COI*, *cytb*, and *Rag1* sequences (3,528 bp) of 29 Pakistani *Eremias* samples (outgroups removed for clarity). Sample codes correspond to vouchers in the Pakistan Museum of Natural History (PMNH), Islamabad. Circles at nodes codify bootstrap values and posterior probabilities; half circles indicate that the node was not found under BI. Inset: *Eremias persica* from Kundar, Torghar, Killa Saifullah District, Balochistan, Pakistan. Photograph: R. Masroor

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 *Rag1* locus (Table 2). Clade A, maximally supported under both tree-building approaches, contained sequences of *E. acutirostris* and *E. aporosceles* and represents the *Scapteira* subgenus. The two species from north-western Balochistan (Figure 1a) were not reciprocally monophyletic, with *E. acutirostris* being paraphyletic with respect to *E. aporosceles* (Figure 2). Genetic divergences within clade A were shallow (approx. 1% in mtDNA and 0.3% in the nuclear *Rag1* gene; Table 2). Clade A was sister to the remaining clades (Figure 2).

The sequences of three *Rhabderemias* species, *E. cholistanica*, *E. kakari* (both endemic to Pakistan), and *E. 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. kakari* from the Toba Kakar Range (Torghar Mountains of north-eastern Balochistan), and clade C corresponded to the sequence of an *E. scripta* from the Pakistani part of the Helmand Basin. Clade D consisted of three *E. cholistanica* from the Cholistan Desert. The branching pattern within this *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. 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 individual *Rhabderemias* clades were high (mtDNA: 7.5%–16.6%, *Rag1*: 1.2%; Table 2).

The monophyly of the *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. 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	12.2/–/23.6/–	8.4/–/–/–	**0/–/0/–**								
D	12.7/17.6/22.8/3.3	7.5/15.3/–/1.2	8.6/–/16.6/–	**0.43/0/0/0.54**							
E	12.6/17.8/22.0/2.6	10.8/19.4/–/0.4	11.6/–/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/–	11.2/17.6/21.6/1.5	8.0/14.7/17.6/1.0	**0.92/2.48/2.97/0.44**					
F1	12.7/18.1/23.5/–	10.9/16.3/–/–	11.5/–/21.1/–	10.9/16.9/20.9/–	8.3/14.1/17.0/–	1.0/2.1/2.6/–	**0/0.08/0.10/–**				
F2	13.1/18.7/23.8/–	11.1/16.4/–/–	11.8/–/21.8/–	11.5/18.0/22.2/–	8.1/15.0/18.0/–	0.7/2.2/2.7/–	1.6/3.9/4.6/–	**0.28/1.10/1.51/–**			
Acantho-dactylus	16.3/20.2/26.3/5.0	16.3/21.5/–/4.7	16.1/–/25.8/–	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	17.0/17.8/26.1/5.6	17.2/21.3/–/4.6	17.6/–/25.7/–	17.9/17.6/23.8/6.5	18.5/20.3/25.9/5.3	17.6/18.9/26.4/4.8	16.9/18.2/25.8/–	17.5/19.5/26.8/–	20.5/21.1/20.0/6.2	–/–/–/–	
Mesalina	17.2/20.5/23.8/5.8	17.0/21.8/–/5.6	17.0/–/23.3/–	17.3/20.2/24.2/8.3	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/–	17.1/21.9/21.5/6.2	19.0/19.2/24.9/7.6	–/–/–/–

two subclades differed from one another by uncorrected *p* distances ranging from 1.6% (*16S*) to 4.6% (*cytb*) in mtDNA. *Rag1* did not distinguish these two subclades. In contrast, within the maximally supported clade E divergence values ranged only from 0.3% (*16S*) to 1.4% (*cytb*) in mtDNA; the value for the *Rag1* gene was 0.1% (Table 2). The average *p* distances between clades E and F varied from 8.0% (*16S*) to 17.6% (*cytb*) in mtDNA; for the nuclear *Rag1* gene, the divergence was 1.0% (Table 2).

3.2 | Phylogenetic analyses of Pakistani *Eremias* using single-locus data sets

Our four single-locus data sets using previously published sequences from ENA/GenBank and BOLD supported the clades revealed by our multilocus phylogeny for *Eremias* taxa from Pakistan. Due to missing data, clade B was not included in the *cytb* analysis, and clade C was not included in the analyses of *COI* and *Rag1*. Using *Rag1*, the monophyly of clades B (66/0.56) and F (28/0.78) was not supported. All other clades were moderately to well supported, with bootstrap and posterior probability values ranging from 75–100 and 0.99–1.00, respectively (Figures 2-6).

Using the *16S* data set comprising 154 sequences of 19 taxa, our Pakistani *Eremias* sequences clustered again into six clades, with two subclades in clade F (Figure 3). All six clades for Pakistani *Eremias* were well supported, and four of the six clades were previously unknown. One clade that has been identified before corresponds to our clade E (92/1.00). Our sequences of clade E (*E. persica*) clustered with high support (92/1.00) together with previously published sequences of *E. persica* and *E. "acutirostris"* (compare Guo et al., 2011 and our Figure 3) from Iran (HQ615642-47) and Afghanistan (HQ615648, HQ615649). Thus, this clade has a broader distribution in the Middle East and Central Asia (Figure 1b). Our clade C, corresponding to a sequence of *E. scripta*, clustered with a deeply divergent sequence (HQ615650) of the same species from Afghanistan (79/0.99; Figure 3). However, another sequence of *E. scripta* (MT509440) from Uzbekistan was even more divergent from ours. Under ML and BI it was, with poor support, sister to our clade F. The two clades E and F, corresponding to racerunners morphologically identified as *E. persica*, were confirmed and well supported by the *16S* data set but occurred in an unresolved polytomy.

According to the tree topology (Figure 3), the subgenera *Aspidorhinus*, *Rhabderemias*, and *Scapteira* were not monophyletic. However, while terminal clades in the *16S* analyses were typically well supported, many deeper branching patterns received only weak support. In any case, the type species of *Scapteira* (*E. grammica*) was placed in both analyses among taxa of the *Rhabderemias* subgenus (*E. pleskei*, *E. lineolata*, and clades B-D from Pakistan). Another *Rhabderemias* species, *E. vermiculata*, was deeply divergent and placed outside this group, albeit with weak statistical support (Figure 3).

With respect to our data set of 242 *COI* sequences of 20 taxa, only five clades were revealed for our Pakistani material (Figure 4)

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*, *Rhabderemias*, 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 *cytb* 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. aporosceles* 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 *cytb* data set, it is noteworthy that *Rhabderemias* was not found monophyletic. Our Pakistani clades C and D, corresponding to the *Rhabderemias* species *E. scripta* and *E. cholistanica*, respectively, were well-supported sister taxa (95/1.00). The third representative of *Rhabderemias*, *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. aporosceles* (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. aporosceles* 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*, *cytb*) 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. aporosceles*, *E. cholistanica*, and *E. kakari*) were never before included in molecular genetic investigations. Guo et al. (2011) reported to have studied

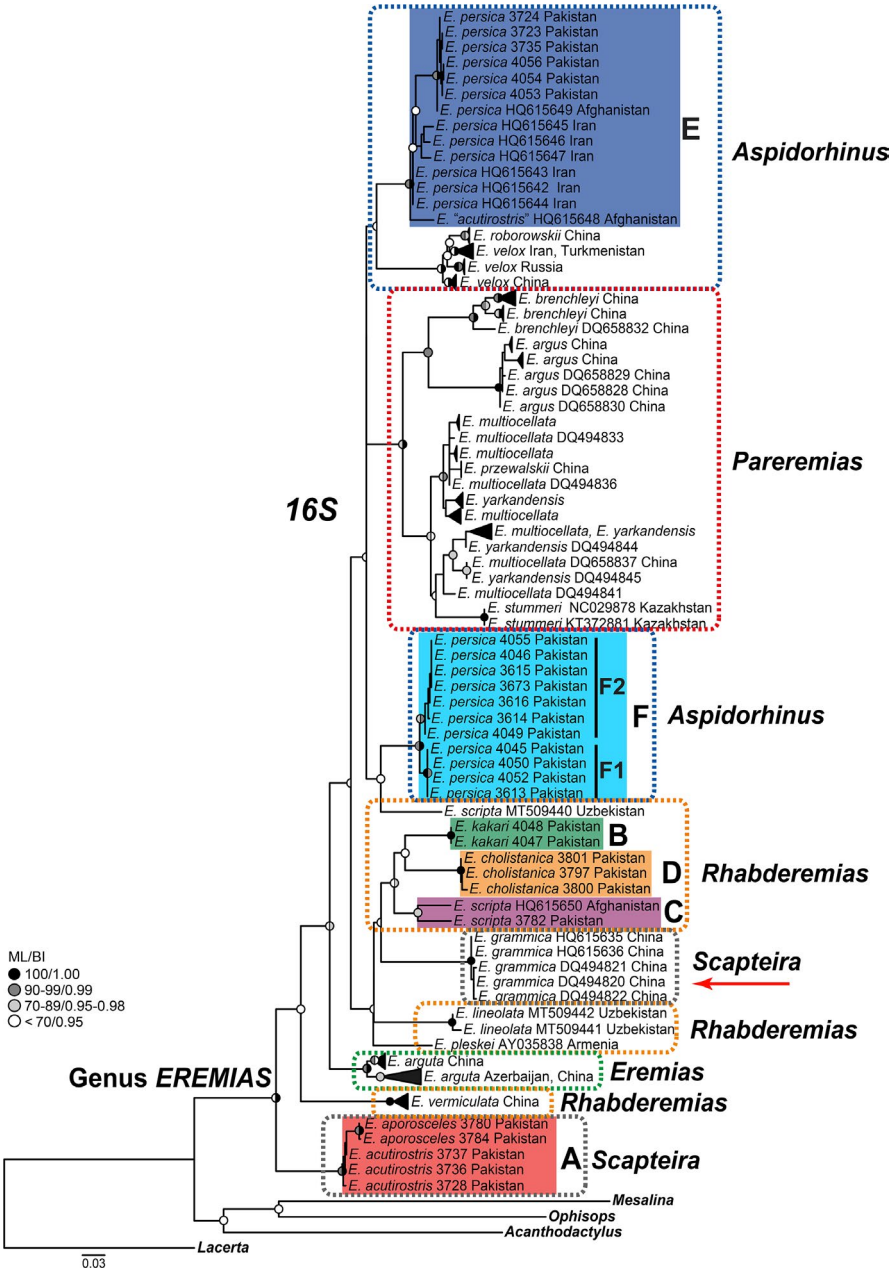

FIGURE 3 Maximum Likelihood tree based on *16S* sequences of 154 *Eremias* specimens (data from previous studies plus new material from Pakistan, $n = 28$). Sample codes correspond to vouchers in the Pakistan Museum of Natural History (PMNH), Islamabad, or to ENA/GenBank accession numbers. Circles at nodes codify bootstrap values and posterior probabilities; half circles indicate that the node was not found under BI. Clades containing Pakistani sequences shown in the same colors as in Figures 1 and 2. Some clades represented by ENA/GenBank sequences were collapsed. The red arrow highlights *E. grammica*, the type species of the *Scapteira* subgenus

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 Huocheng, China; *COI*: HQ733951 Xinjiang Uygur Autonomous Region, China; Wan, Sun, Jin, Yan, & Liu, 2007; Guo et al., 2011) of *E. grammica*, the type species of *Scapteira*, 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*

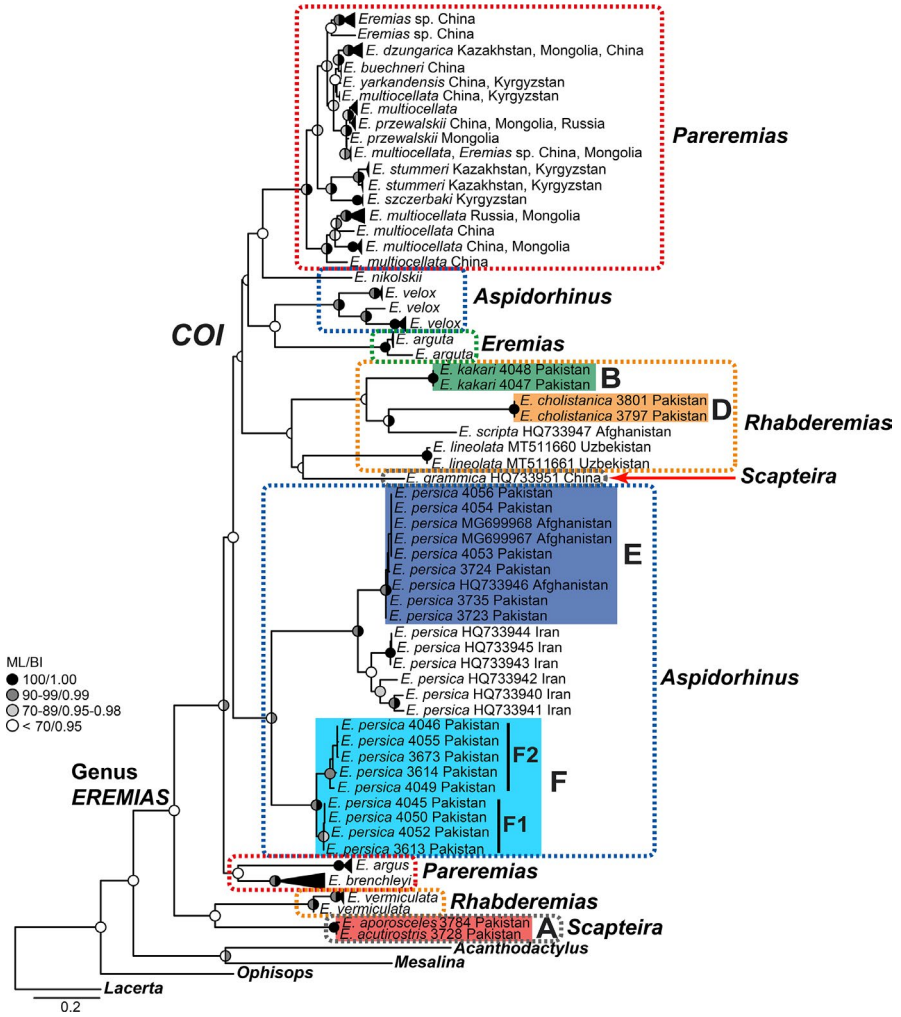

FIGURE 4 Maximum Likelihood tree based on *COI* sequences of 242 *Eremias* specimens (data from previous studies plus new material from Pakistan, $n = 21$). Sample codes correspond to vouchers in the Pakistan Museum of Natural History (PMNH), Islamabad, or to ENA/GenBank accession numbers. Circles at nodes codify bootstrap values and posterior probabilities; half circles indicate that the node was not found under BI. Clades containing Pakistani sequences shown in the same colors as in Figures 1 and 2. Some clades represented only by ENA/GenBank and BOLD sequences were collapsed. The red arrow highlights *E. grammica*, the type species of the *Scapteira* subgenus

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,

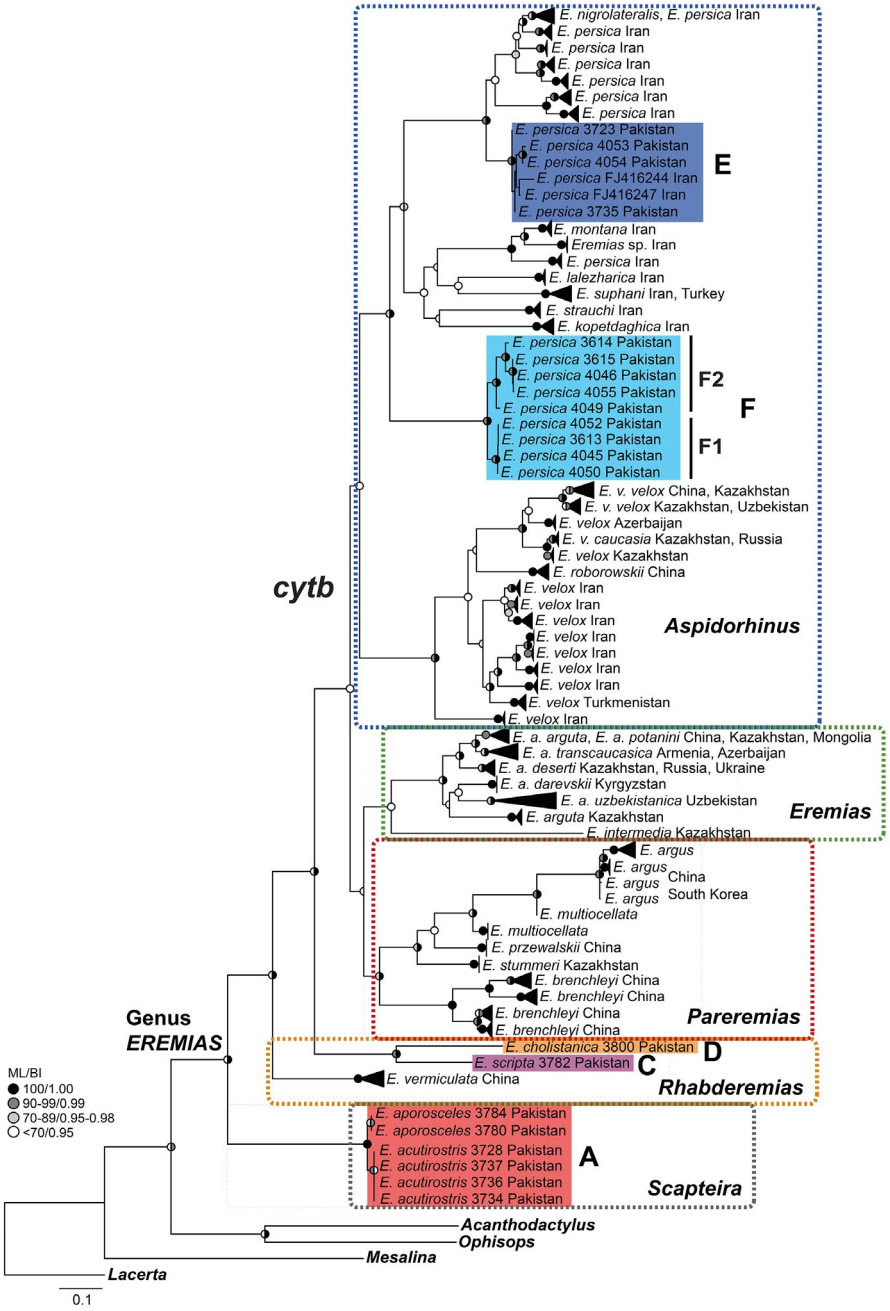

FIGURE 5 Maximum Likelihood tree based on *cytb* sequences of 932 *Eremias* specimens (data from previous studies plus new material from Pakistan, $n = 21$). Sample codes correspond to vouchers in the Pakistan Museum of Natural History (PMNH), Islamabad, or to ENA/GenBank accession numbers. Circles at nodes codify bootstrap values and posterior probabilities; half circles indicate that the node was not found under BI. Clades containing Pakistani sequences shown in the same colors as in Figures 1 and 2. Some clades represented only by ENA/GenBank sequences were collapsed

Chirikova, Milto, & Guo, 2014; Liu et al., 2019; Orlova et al., 2017; Rastegar-Pouyani et al., 2010, 2012). 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

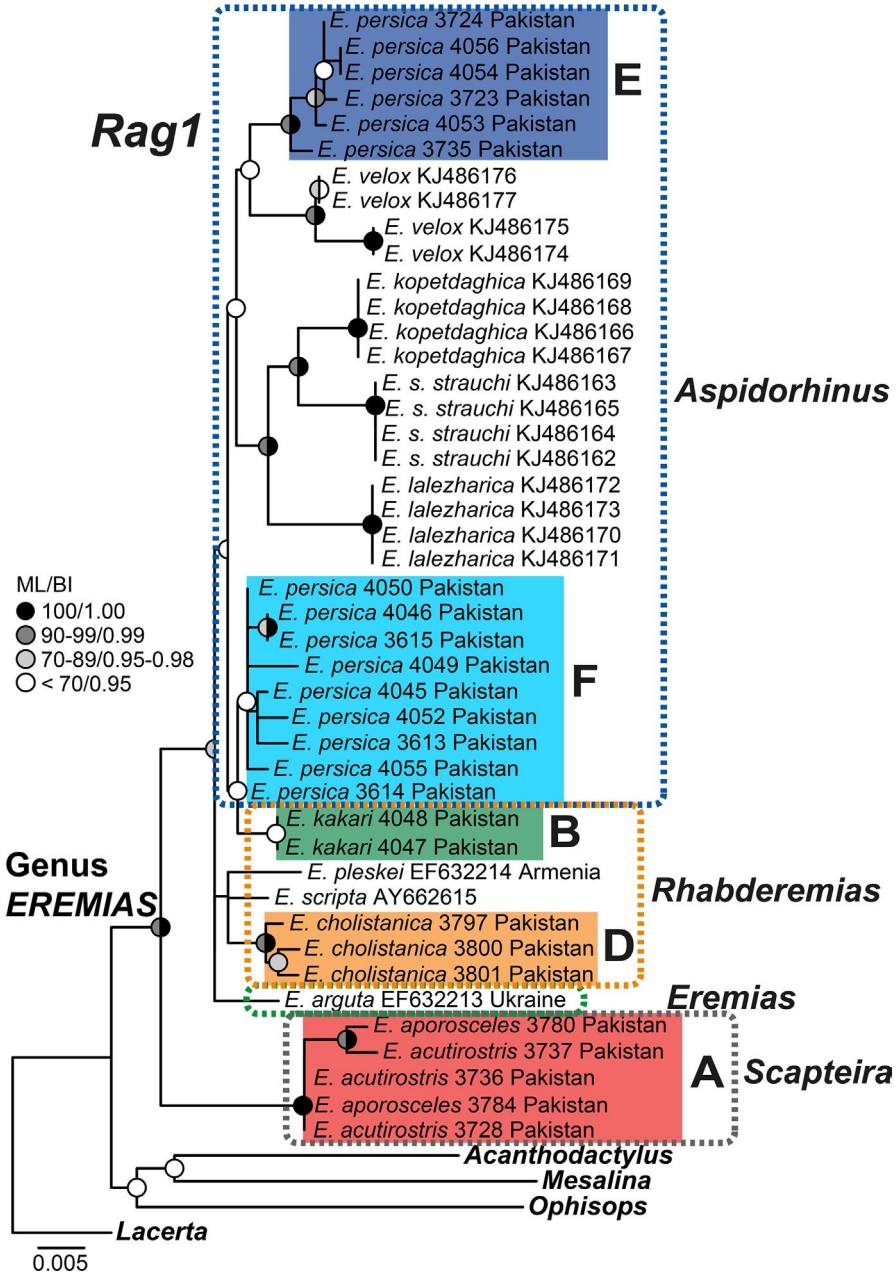

FIGURE 6 Maximum Likelihood tree based on *Rag1* sequences of 44 *Eremias* specimens (data from previous studies plus new material from Pakistan, $n = 25$). Sample codes correspond to vouchers in the Pakistan Museum of Natural History (PMNH), Islamabad, or to ENA/GenBank accession numbers. Circles at nodes codify bootstrap values and posterior probabilities; half circles indicate that the node was not found under BI. Clades containing Pakistani sequences shown in the same colors as in Figures 1 and 2

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)

	Previous phylogenies		This study	
Taxon	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 kokshaaliensis* 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 quadrifrons* (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 Szczerbak, 1978	—	—	—	—
30—*Eremias cholistanica* 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, Hosseinian Yousefkhani, 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 Pavlicev (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; Szczerbak & 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 & Szczerbak, 1991. Samples of this species from central and south-eastern Afghanistan (Böhme & Szczerbak, 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 Eremiadini 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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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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