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Molecular phylogenetic and historical biogeographical relationships of *Laudakia* (Squamata: Agamidae) and intraspecific differentiation of *L. stoliczkana* inferred from mitochondrial DNA sequences

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Abstract

The rock lizard genus *Laudakia* is representative agamid species from the arid zone, and its genus division has not been resolved yet. *Laudakia stoliczkana*, which occurs in both Xinjiang, China, and the Gobi Altai, Mongolia, is divided into two subspecies, *Laudakia stoliczkana stoliczkana* and *Laudakia stoliczkana altaica,* based on morphological differences, but little is known about the molecular genetic differences between the two subspecies. This study reconstructs the phylogenetic tree of *Laudakia* and analyses molecular differences between two subspecies of *L. stoliczkana* by DNA barcoding (COI and 16S). Our results show that: (1) *Laudakia* is monophyletic and the phylogenetic tree is broadly divided into three main branches, namely branch A (*L. caucasia* and *L. stoliczkana*), which occurs mainly in Central Asia and the Gobi Altai region to the north, branch B (*L. stellio*), which occurs in the Middle East, and branch C (*L. tuberculata*, *L. papenfussi*, *L. himalayana*, *L. wui*, *L. stellio*), which occurs mainly near the Himalayas; (2) The biogeographic analysis of *Laudakia* suggests that the genus probably originated at 43.72 Ma (95% confidence interval HPD: 23.53–66.12Ma) and is associated with the uplift of the Tibetan Plateau and the aridification of Central Asias subsequently; (3) Molecular genetic distances and morphological differences support the delimitation of the two subspecies of *L. stoliczkana*, with divergence between the two subspecies estimated to have occurred at 3.27 Ma (95% confidence interval HPD: 1.58–5.87Ma), in associated with the recent uplift of the Tian Shan Mountains. The results highlight the importance of the uplift of the Central Asian mountains and the Tibetan Plateau for the divergence of *Laudakia*.

Key words: Phylogeny, DNA barcoding, Subspecies, Divergence time

Introduction

The species of *Laudakia* (Squamata, Agamidae) are primarily found in the Middle East and Central Asia, from Greece and the Nile Delta in the West to Mongolia in the East; from Central Asia in the North to the Himalayas in the South. They typically live in rocky habitats in arid regions (Wang *et al.* 2014). The morphological investigation conducted by Moody in 1980 led to the present classification of *Laudakia* (Gray, 1845). The revision of *Laudakia* has been presented in recent years by Baig *et al.* (2012), who divided *Laudakia* into three genus—*Stellagama*, *Laudakia*, and *Paralaudakia*, based on morphological distinctions. However, this new generic division based on morphological differences was not supported by the results of phylogenetic that followed. The molecular analysis indicated that the genus *Laudakia* is monophyletic (Pyron *et al.* 2013; Zou *et al.* 2016; Speybroeck *et al.* 2020).

There are six *Laudakia* species distributed in China (Wang *et al.* 2020): *Laudakia himalayana*, *Laudakia papenfussi*, *Laudakia sacra*, *Laudakia stoliczkana*, *Laudakia tuberculata*, and *Laudakia wui*. *Laudakia* is domestically distributed in the Tibetan and Xinjiang, and only one species of *L. stoliczkana* is found in the Xinjiang.

L. stoliczkana inhabits the rocky zone of arid regions and can be found both domestically and abroad in the Tarim Basin, the Beita Mountains, the eastern Altai Mountains, and the northwestern Mahaizan Mountains (Li & Zhao 2015), as well as in the Mongolian Altai and Gobi Altai. *L. stoliczkana* is currently poorly studied, only in

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terms of skeleton and resistance to erosion (Smirina & Ananjeva 2007; Liang *et al.* 2019) and the mitochondrial whole sequence of this species has been sequenced to satisfy basic biological (or phylogenetic) studies (Lin *et al.* 2019). *L. stoliczkana* is divided into two subspecies, *Laudakia stoliczkana stoliczkana* (with a 3-ringed segment at the tail and only the basal 2 to 3 segments with a 4-ringed segment) and *Laudakia stoliczkana altaica* (with a 4-ringed segment at the tail), based on morphological differences(Shi & Zhao 2008). *L. s. stoliczkana* occurs mainly on the margins of the Tarim Basin and in the eastern part of Xinjiang; *L. s. altaica* is found in the northern Tashan and eastern Altai Mountains to the Mongolian Gobi (Shi & Zhao 2008). Morphological differences have been found and no molecular phylogenetic studies are available for the time being.

The aim of our study is to explore species relationships within *Laudakia* based on DNA barcoding (COI and 16S), as well as to analyse the molecular phylogeny of species in the genus *Laudakia* and the relationships of subspecies *L. stoliczkana*, and to provide molecular data to support the classification of subspecies of *L. stoliczkana*.

Materials and methods

2.1 Samples

Ten tissues samples of *L. stoliczkana* preserved in 95% ethanol from four different locations (Appendix 1) were used for DNA extraction experiments.

At the same time, available sequences or mitochondrial full sequence information for other species of the genus *Laudakia* were collected and downloaded from BOLD (Barcode of Life Database) and GenBank at NCBI (National Center for Biotechnology Information) as reference sequences (Appendix 2).

2.2 DNA extraction and PCR amplification

Total DNA from the tissue samples was extracted using the Foregene Animal Tissue Genomic DNA Extraction Kit, according to the kit instructions. The mitochondrial cytochrome oxidase subunit I gene (COI) and 16S rRNA gene (16S) were used as DNA barcoding and amplified using polymerase chain reaction (PCR) with published primers and cycling parameters (Table 1).

TABLE 1. The sequence of primer.

Gene	Primer	Sequences	References
COI	RepCOI-F	TNTTMTCAACNAACCACAAAGA	Nagy *et al.* (2012)
	RepCOI-R	ACTTCTGGRTGKCCAAARAATCA	
16S	LE2190(F)	GTAGGCCTCAAAGCAGCCAC	Pavlicev *et al.* (2009)
	HO3056(R)	CCGGTCTGAACTCAGATCACG	

The PCR reaction system volume was 25μl, containing 1μl of template DNA, 1μl each of upstream and downstream primers, 12.5μl of 2x Taq PCR Mix and 9.5μl ddH2O. The PCR conditions for the COI gene were as follows: pre-denaturation, 94°C, 4min; denaturation, 94°C, 30s; annealing, 52°C, 30s; extension, 72°C, 50s; Cycle number 35; sequential extension, 72°C, 10min. The PCR conditions for the 16S gene were as follows: pre-denaturation, 94°C, 2min; denaturation, 95°C, 30s; annealing, 59°C, 30s; extension, 72°C, 50s; cycle number 35; sequential extension, 72°C, 7min. Both genes were finally kept at 10°C after completion of PCR. After completion of the PCR reactions, the amplified samples were tested for successful amplification by gel electrophoresis with 0.5% TBE solution and agarose. The successfully amplified PCR stock solution was then sent to Sangon Biotechnology company for purification and sequencing.

2.3 Sequence alignment and phylogenetic tree construction

All sequences were aligned and manually edited by SeqMan in DNASTAR (Burland 1999). The forward and

reverse sequences were spliced together in SeqMan and the 5` and 3` end ambiguous bases were removed, keeping the middle stable part. We finally obtained a 16S sequence of about 680 bp and a COI sequence of 590–625 bp, as well as a tandem sequence of 1189 bp. We aligned the tandem sequences on MEGA 7.0.26 (Kumar *et al.* 2016) and converted the sequences into amino acid sequences by protein coding to check the stop codons. We used PAUP4W and MrModeltest2.3 to calculate models for COI and 16S sequences, respectively. We used the Akaike information criterion (AIC) and ran the Hierarchical Likelihood Ratio Tests (hLRT) for all four levels to determine the best model. Also, we used partitionfinder-2.1.1 (Lanfear *et al.* 2017) to calculate the best model for tandem sequences (Appendix 3).

Phylogenetic analysis of the two datasets and the tandem sequences was performed by using Bayesian Inference (BI) and Maximum Likelihood (ML). The outgroups were set as follows (Appendix 2): *Acanthosaura crucigera* as outgroups of phylogenetic tree, as the same time we complements other species sequences. BI ran in MrBayes 3.2.2 (Ronquist *et al.* 2012), using the Markov chain Monte Carlo (MCMC) approach, and the program was run for a total of 5,000,000, with one sample drawn every 1,000, and 4 chains were run for each analysis, removing the top 25% of the data, with the other parameters being the default ones. The constructed phylogenetic tree was checked for branching and Bayesian posterior probabilities (BPP) using FigTree v1.4.3, and we checked the effective sample size (ESS) using Tracer 1.7.2 (Rambaut *et al.* 2018); all parameter values for ESS values were >200. ML was analysed in IQ-TREE in PhyloSuite (Zhang *et al.* 2020) with model K80 (K2P) and default parameters. Finally, the phylogenetic tree was drawn using ITOLV6 (Letunic & Bork 2021). We consider a posteriori probability (BPP) or maximum likelihood bootstrap value (BS) >95% as strong support for monophyly. Finally, genetic distances (*p*-distance) were calculated for 16S and COI sequences by MEGA7.0.

2.4 Divergence time estimates

We used the mtDNA dataset (COI+16S) to estimate species divergence times within *Laudakia* using BEAST 2.6.6 (Bouckaert *et al.* 2014) based on the lognormal relaxed clock assumption. We used three calibration points (Melville *et al.* 2009): (1) the Middle Jurassic (154–180 Myr ago), a fossil calibration point for the common ancestor of all iguanas; (2) the early Miocene (22.8 Myr ago), the divergent nodes of *Sceloporus*, *Uma*, *Holbrookia*, *Phrynosoma*, *Callisaurus*; (3) the late Miocene (10 Myr ago), the timing of the uplift of the Pamir-Tian Shan. *Draco* species were selected as the outgroup (Appendix 2) and sampled every 10,000 iterations by using the GTR model and the Yule model, with 25% of the initial sample discarded as an aged sample. Tracer 1.7.2 was used to evaluate the estimated ESS for all parameters.

2.5 Morphological measurements

Based on the morphological descriptions of two subspecies of *L. stoliczkana* by Wang *et al.* (1986) and Shi & Zhao (2008), we measured and described the number of caudal links and some morphological indices of the main identifying features of adult *L. stoliczkana* stored in the zoological herbarium of Xinjiang Agricultural University. These include: snout-vent length (SVL), tail length (TL), supra-labials, infra-labials, Scales Around No5 annulus of Tail (SAT). Morphometric data were also collected from the specimens in the published papers (Shi & Zhao 2008). We then used SPSS software to perform covariance analysis and T-test on the morphological indices of the specimens by male and female. The seventy-five percent rule for subspecies definition (Mayr *et al.* 1953; Amadon 1949; Wang & Zhao 2006; Liu *et al.* 2014) means that two populations are considered to be distinct if all individuals in one (A) differ from those in the other (B) by 75% of the number of individuals. The formula for the Coefficient of Difference (C.D.): the difference between the means (M) of individuals in two samples (A and B), divided by the sum of the standard deviations (S.D.). According to the 75% rule, a C.D. > 1.28 means that 90% of the individuals in the population A are different from 90% of the individuals in population B, which means that the two populations are subspecies differentiated. Based on the above formula, three morphological indicators, SVL, TL and SAT, were selected for calculation in this paper.

The formula of S.D.:

$$S.D.=\sqrt{\frac{\sum x^2-(\sum x)^2/n}{n}} \quad n\geq 15 \quad \text{Or} \quad S.D.=\sqrt{\frac{\sum x^2-(\sum x)^2/n}{n-1}} \quad n<15$$

Where 'X' means the measured value; 'n' means the number of samples

The formula of C.D.:

$$C.D.=\frac{M_A-M_B}{S.D._A+S.D._B}$$

Results

3.1 Phylogenetic tree

Bayesian (BI) and Maximum Likelihood (ML) phylogenetic trees were constructed using 16S and COI and tandem sequences of both, respectively, and the phylogenetic trees of all three show consistent topology, all showing *Laudakia* as monophyletic (Figs. 1, 2, 3): 16S: 1.00/100 (BPP/BS); COI: 1.00/100 (BPP/BS); and tandem dataset: 1.00/100 (BPP/BS). In the phylogenetic tree of 16S sequences (Fig. 1), the samples from this study clustered into one clade (BPP/BS: 1.00/98) with the reference sequence of *L. stoliczkana*. *L. tuberculata* and *L. papenfussi* are closely related (BPP/BS: 0.99/95); *L. himalayana*, *L. wui* and *L. sacra* are clustered into one branch (BPP/BS: 1.00/98).

In the phylogenetic tree of the COI sequences (Fig. 2), the tree structure can be found to be presumably consistent with the phylogenetic tree of 16S, with a branching support of 1.00/100 for the samples and *L. stoliczkana* in this study; *L. lehmanni*, *L. caucasia* and *L. stoliczkana* are strongly related (BPP/BS; 1.00/99). The two phylogenetic trees are distinguished in the branching position of *L. stellio* and *L. nupta*.

In the phylogenetic trees of COI and 16S tandem sequences (Fig. 3). There are three branches within *Laudakia*. Among them, branch A includes *L. caucasia* and *L. stoliczkana*; branch B includes *L. stellio*; branch C includes six species of *L. tuberculata*, *L. papenfussi*, *L. himalayana*, *L. wui*, *L. sacra*, and *L. nupta*. In branch C, C1 is *L. nupta*; C2 includes *L. tuberculata* and *L. papenfussi*; and C3 includes *L. himalayana*, *L. wui*, and *L. sacra*.

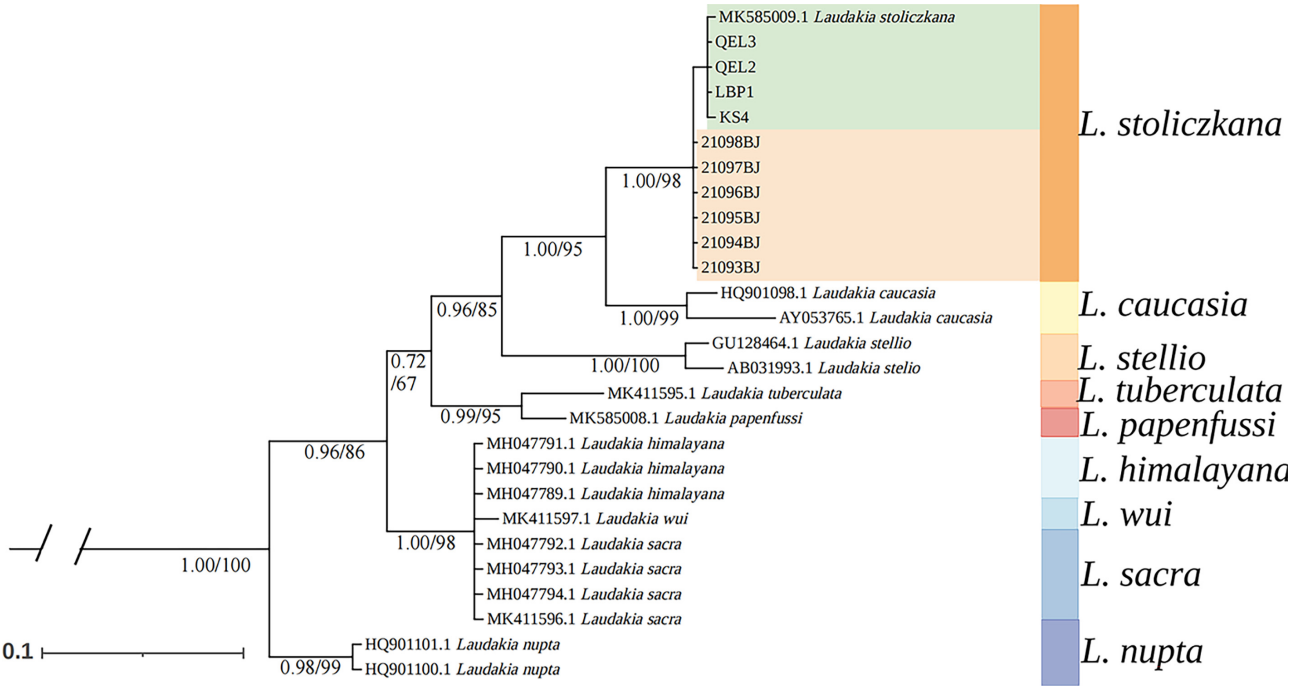

FIGURE 1. Bayesian phylogenetic trees of *Laudakia* on the sequenced of 16S. (note: the values of nodes near is BPP/BS).

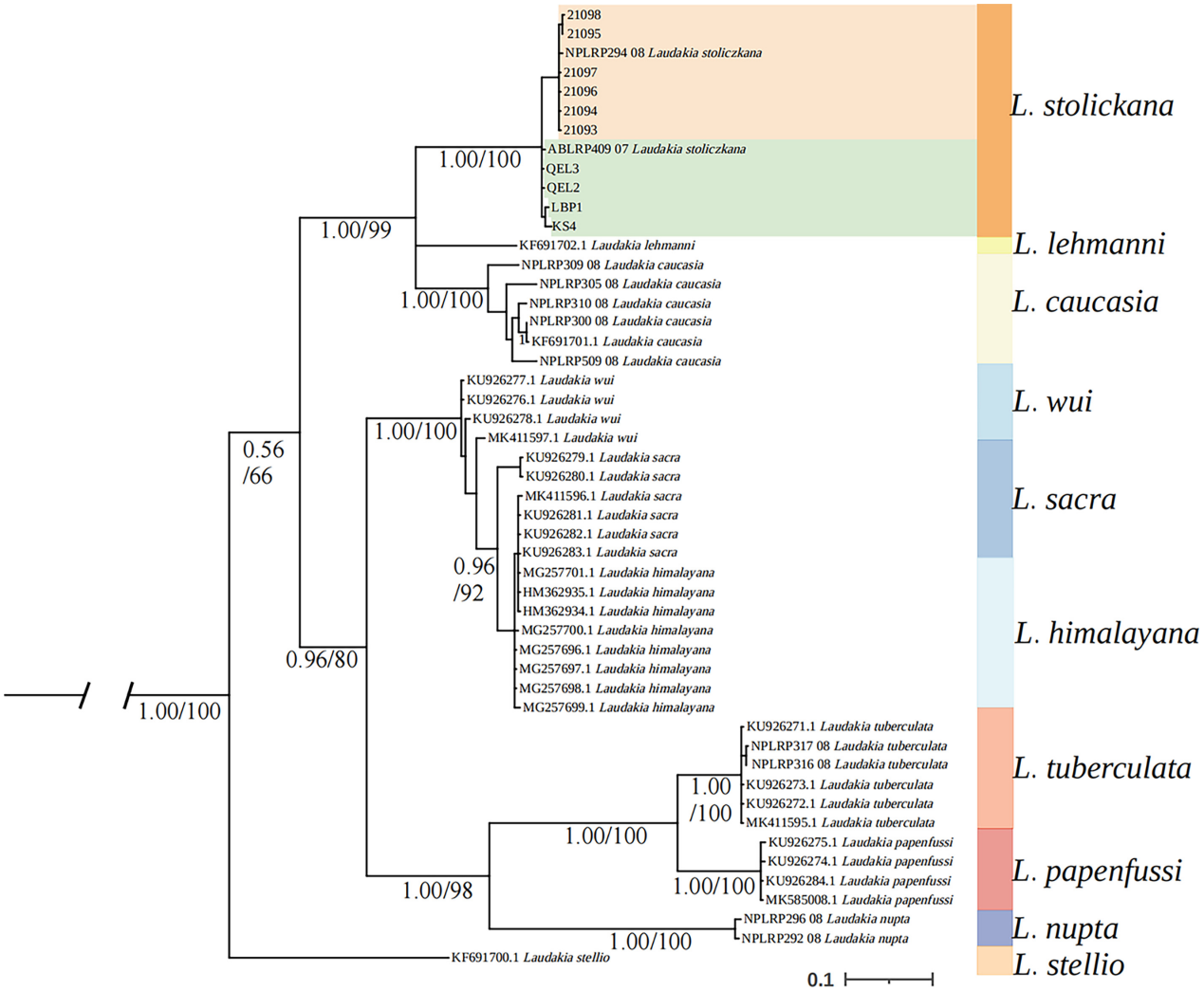

FIGURE 2. Bayesian phylogenetic trees of *Laudakia* on the sequenced of COI. (note: the values of nodes near is BPP/BS).

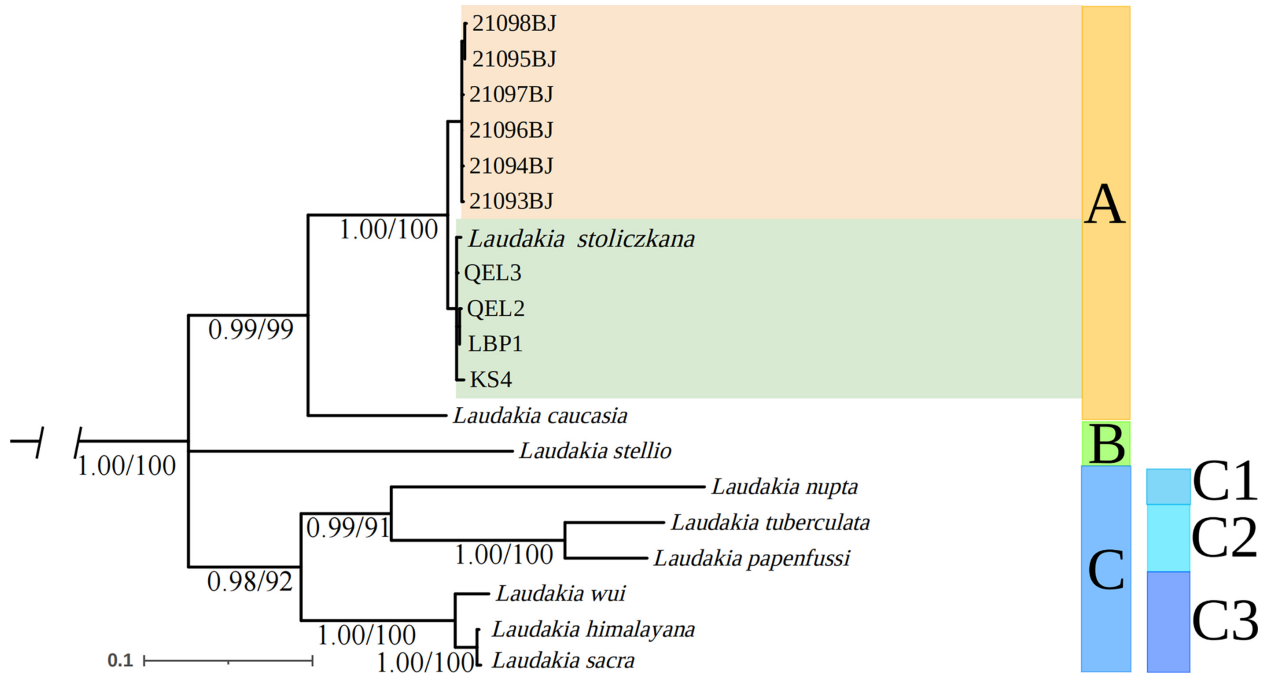

FIGURE 3. Bayesian phylogenetic trees of *Laudakia* species on tandem sequences (CO1 and 16S). (note: the values of nodes near is BPP/BS).

3.2 Genetic distance

The 10 molecular samples of this study were collected from Korla, Ruoqiang, Tashkurgan and Qinghe, which can be roughly divided into two groups according to their geographical location, based on the boundaries of the Tian Shan Mountains, one group is the southern part of Xinjiang (Group 2), including Korla, Ruoqiang and Tashkurgan (QEL2, QEL3, LBP1, KS4); and one group is the northern part of Xinjiang (Group 1), namely Qinghe (21093, 21094, 21095, 21096, 21097, 21098).

The results of the genetic distance(*p*-distance) calculated from the two sequences show in Table 2. The *p*-distance (uncorrected) based on COI sequences of 0.0% (Group 1, Group 2 and *L. stoliczkana*) to 22.0% (*L. stellio* and *L. tuberculata*, *L. nupta*, *L. papenfussi*); The *p*-distance (uncorrected) based on 16S sequences ranged from 0.0% (Group 2 and *L. stoliczkana*) to 11.5% (*L. stellio* and *L. nupta*). The results for two genetic distances (*p*-distance) were consistent with the structure of the relationship in the phylogenetic tree, two groups of samples had small values of *p*-distance with *L. stoliczkana* (0.0% to 1.5%), and the remaining genetic distances between species were consistent with the branching results of the phylogenetic tree for tandem sequences.

TABLE 2. Uncorrected *p*-distances (%) between species of *Laudakia* based on COI(below the diagonal) and 16S(above the diagonal).

	Group 1	Group 2	*L. stoliczkana*	*L. caucasia*	*L. nupta*	*L. papenfussi*	*L. sacra*	*L. wui*	*L. tuberculata*	*L. stellio*	*L. himalayana*
Group 1	\	1.5	1.5	2.3	7.7	6.2	10.8	10.8	3.8	8.5	10.8
Group 2	1.2	\	0.0	1.5	7.7	7.7	10.0	10.0	4.2	8.5	10.0
L. stoliczkana	0.0	0.0	\	1.5	7.7	7.7	10.0	10.0	4.2	8.5	10.0
L. caucasia	11.2	10.4	10.0	\	6.9	7.7	8.8	8.8	2.7	7.7	8.8
L. nupta	20.4	20.6	19.7	18.0	\	4.6	8.5	8.5	3.8	11.5	8.5
L. papenfussi	20.2	20.5	19.7	17.8	17.1	\	5.4	5.4	0.8	7.7	5.4
L. sacra	17.5	17.2	16.6	15.6	18.4	19.0	\	0.0	4.2	11.5	0.0
L. wui	17.2	16.8	16.2	15.1	18.0	18.7	2.3	\	4.2	11.5	0.0
L. tuberculata	20.7	20.1	19.7	18.4	18.6	8.8	17.8	17.6	\	2.7	4.2
L. stellio	18.7	18.1	17.7	16.9	22.0	22.0	17.2	16.3	22.0	\	11.5
L. himalayana	18.3	17.9	17.3	16.2	19.2	19.7	0.2	2.9	18.6	18.1	\

3.3 Divergence date estimation

Divergence date estimates (Fig. 4) suggest that the divergence of *Laudakia* occurred at 43.72 Ma (95% confidence interval HPD: 23.53–66.12 Ma), in the middle Eocene. The differentiation of subspecies *L. stoliczkana* occurred at 3.27 Ma (95% confidence interval HPD: 1.58–5.87 Ma). Branch A (*L. stoliczkana* and *L. caucasia*) diverged at 10.60 Ma (95% confidence interval HPD: 8.56–12.34 Ma); while branch A and *L. stellio* diverged at 30.59 Ma (95% confidence interval HPD: 15.62–51.09 Ma). Branch C (*L. tuberculata*, *L. papenfussi*, *L. himalayana*, *L. wui*, *L. sacra* and *L. nupta*) diverged at 35.72 Ma (95% confidence interval HPD: 19.16–55.16 Ma).

3.4 Morphological comparison on two subspecies of *L. stoliczkana*

Morphological indications are detailed in Appendix 4. Among the adult specimens corresponding to the above molecular results, the number of caudal links in the samples from Qinghe County is consistent with the identifying characters of *L. s. altaica*, with three rings of one segment in the tail, while the samples from the southern Xinjiang region (Korla, Ruoqiang and Tashkurgan) have the same four rings of one segment in the tail as *L. s. stoliczkana* (Fig. 5). Identification of morphology identified the samples in this study as two subspecies of *L. stoliczkana*.

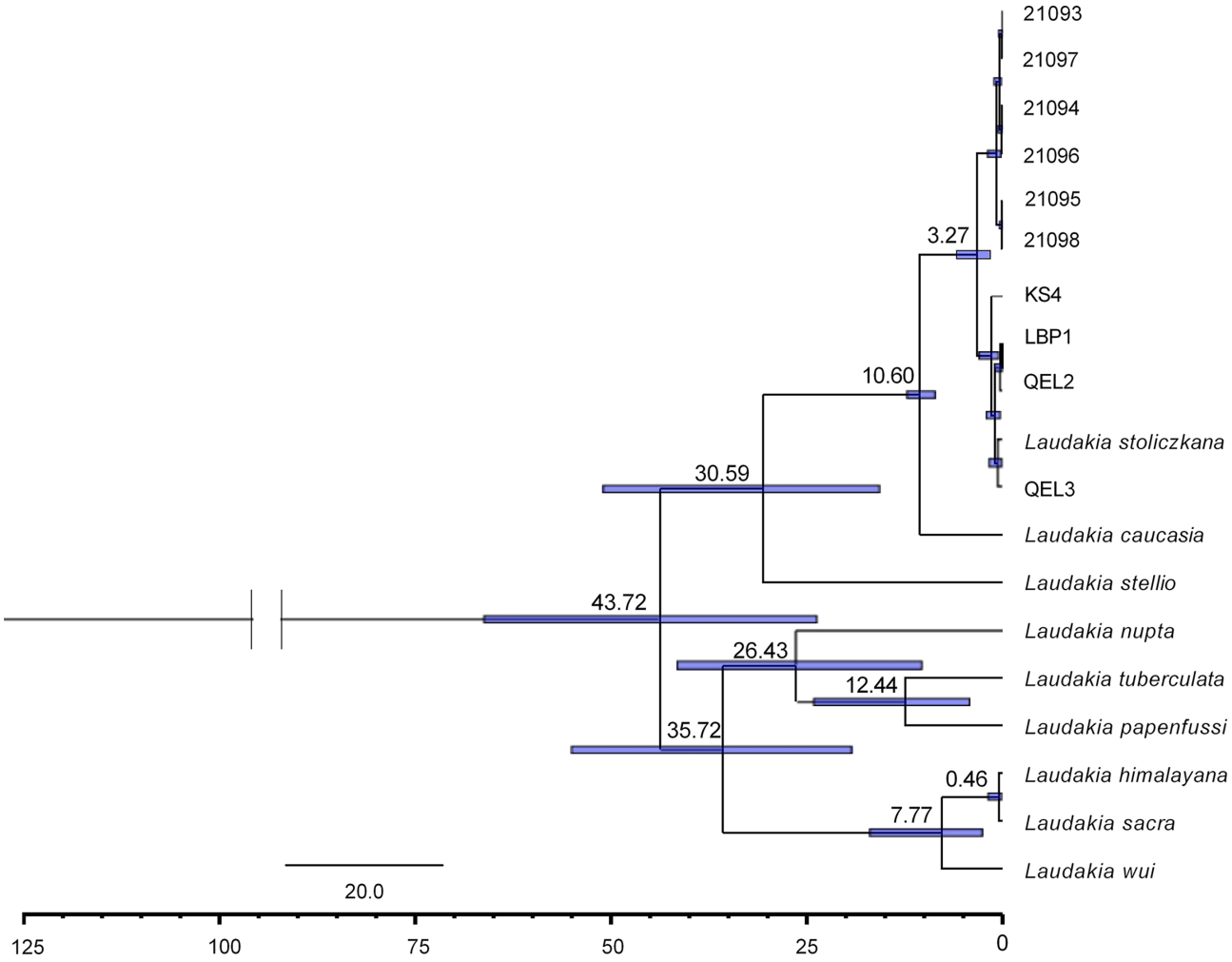

FIGURE 4. The estimation of divergence time for *Laudakia.* (Note: Values are estimated divergence times, and the blue bars are 95% confidence intervals HPD)

There was no significant difference in SVL between males from different locations (P = 0.740); secondly, using SVL as a covariate, analysis of covariance revealed that males of *L. s. altaica* had significantly shorter TL (157.96 ± 23.95 mm) than males of *L. s. stoliczkana* (184 ± 36.09 mm) (P = 0.010); males of *L. s. altaica* had highly significant less SAT (35.05 ± 3 scales) than males of *L. s. stoliczkana* (44.3 ± 2.99 scales) (P = 0.000) (Figure 6) . SVL significantly differed between females from different locations (P = 0.049), with females of *L. s. altaica* having a significantly shorter SVL (117.04 ± 7.4 mm) than females of *L. s. stoliczkana* (124.19 ± 9.85 mm); secondly, using SVL as a covariate, analysis of covariance showed that females of *L. s. altaica* had a very significantly shorter TL (137.16 ± 30 mm) than females of *L. s. stoliczkana* (183.12 ± 31.07 mm) (P = 0.001); and the SAT of females of *L. s. altaica* (38.1 ± 3.51 scales) was significantly less than that of females of *L. s. stoliczkana* (42.22 ± 3.42 scales) (P = 0.002)(Figure 6).

The calculation of C.D. according to the 75% rule for subspecies definition (Mayr *et al.* 1953) shows (Appendix 5) that: SVL (C.D. = 0.530) and TL (C.D. = 0.434) failed to reach subspecific distinction levels for males from different locations (C.D. > 1.28), while SAT (C.D. = 1.544) reached subspecific distinction levels; all three morphological indicators for females from different locations failed to reach subspecific distinction levels (SVL C.D. = 0.415; TL C D. = 0.753; SAT C.D. = 0.594).

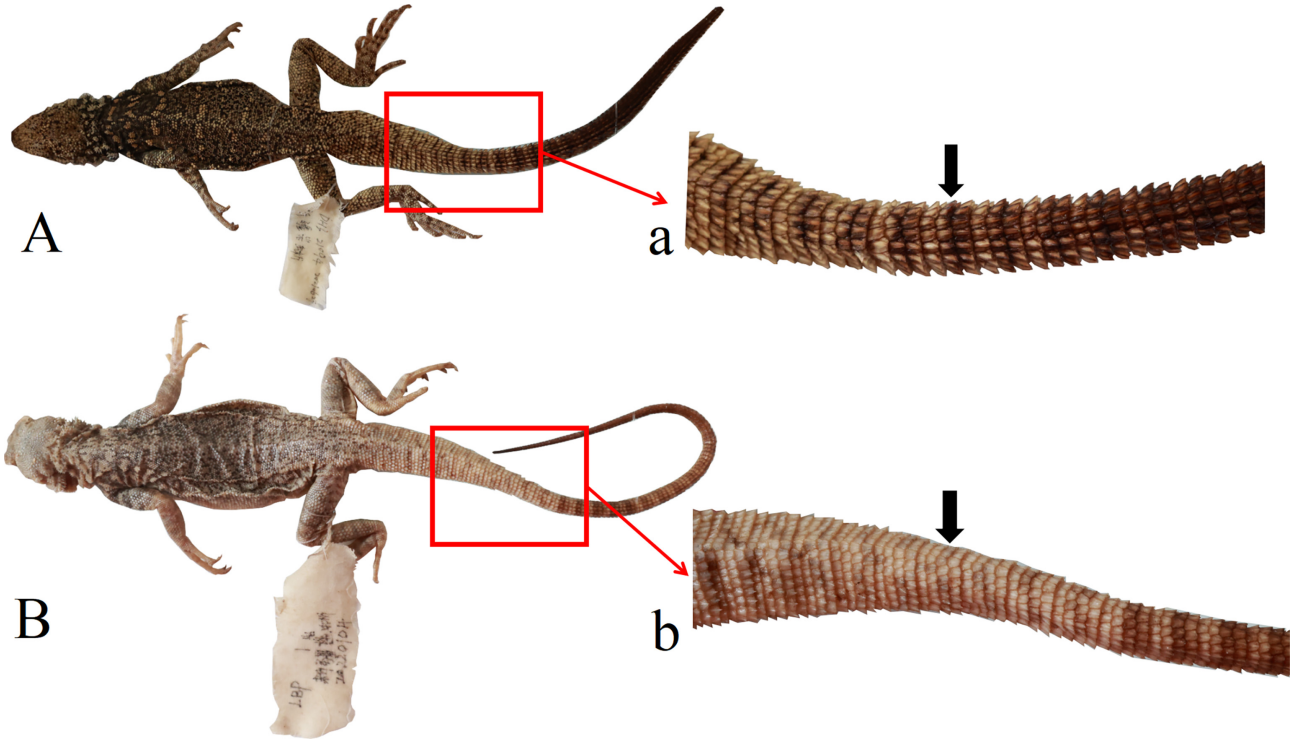

FIGURE 5. The morphology and tail of *Laudakia stoliczkana* subspecies. (A & a: *L. s. altaica*; B & b: *L. s. stoliczkana*)

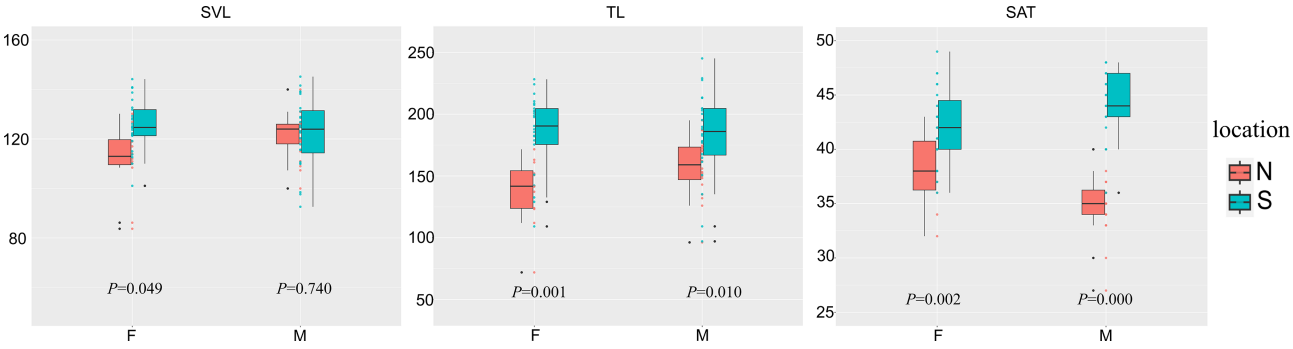

FIGURE 6. T-test results for morphological indicators. (F = females, M = males, N = northern Xinjiang, S = south Xinjiang)

Discussion

4.1 Phylogeny of *Laudakia*

The phylogenetic results of this study indicate that *Laudakia* is monophyletic, consistent with the earlier molecular results (Pyron *et al.* 2013; Zou *et al.* 2016; Speybroeck *et al.* 2020), and do not support the three genus division of Baig *et al.* (2012). *L. himalayana* was assigned to the newly established genus *Paralaudakia* by Baig *et al.* (2012). However, the molecular results show that it clusters with *L. wui* and *L. sacra*, and has a high level of support for their close relationship. Although the taxonomic revision of *Laudakia* by Baig *et al.* (2012) was based on 54 morphological analyses, the results of this revision do not appear to be supported by molecular data.

In our study, the phylogenetic tree of species of *Laudakia* was reconstructed by COI and 16S and tandem sequences, and the results shown the same topology and interspecific relationships. Our results are similar to the phylogenetic tree results of Zou *et al.* (2016), and the branches of the tandem sequence phylogenetic tree delineated here are also similar to the results of Zou *et al.* (2016), with all three phylogenetic trees showing high support, but with different branch positions for *L. stellio*. We found that *L. himalayana*, *L. wui* and *L. sacra* clustered into one branch with a strong support. Both *L. wui* and *L. sacra* were formerly subspecies of *L. himalayana* and were later

elevated to species status (Ananjeva *et al.* 1990; Zhao *et al.* 1999), so the close phylogenetic relationship between the three species is understandable. The phylogenetic relationships of other species, e.g. *L. tuberculata* and *L. papenfussi*, *L. stoliczkana* and *L. caucasia*, are likely to be related to the geographical location of distribution as well as evolutionary history (Zhao *et al.* 1999; Melville *et al.* 2012; Zou *et al.* 2016; Uetz *et al.* 2018).

4.2 Biogeography of *Laudakia*

The three branches (A, B and C) shown by the results of the phylogenetic tree of the tandem sequences in this study correspond to the geographical pattern of distribution of the species. *L. caucasia* and *L. stoliczkana* of branch A are mainly distributed in Central Asia and the Gobi Altai region. *L. caucasia* is widely distributed, from Iraq in the Middle East to the Caucasus region of Central Asia, Tajikistan, etc (Baig *et al.* 2012; Khisroon *et al.* 2012; Mazanaeva & Ananjeva 2016). *L. stoliczkana* is widely distributed from Xinjiang, China to Mongolia, and is the most northerly species in the genus (Shi & Zhao 2008; Baig *et al.* 2012). The B branch of *L. stellio* is found mainly in the eastern Mediterranean and the Middle East (western Greece, Turkey and northwestern Iraq to northern Egypt, etc.) (Brammah *et al.* 2010; Baig *et al.* 2012; Kadry *et al.* 2020; Karameta *et al.* 2022). The six species of branch C, *L. tuberculata*, *L. papenfussi*, *L. himalayana*, *L. wui*, *L. sacra* and *L. nupta*, occur mainly in the Himalayas and near Tibet. *L. nupta* is mainly distributed in the southwestern Himalayas (Iraq to Pakistan) (Cheatsazan *et al.* 2008; Baig *et al.* 2012). *L. tuberculata* and *L. papenfussi* are mainly distributed in the southern Himalayas and Tibet, with *L. tuberculata* extending from eastern Afghanistan and northwestern Pakistan and finally to southwestern Nepal and Tibet (Baig *et al.* 2012; Uetz *et al.* 2018; Vishwakarma *et al.* 2019), while *L. papenfussi* occurs in western Tibet and is endemic to Tibet in China (Zhao *et al.* 1999). *L. himalayana*, *L. wui* and *L. sacra* are distributed in the northern slopes of the Himalayas, the Yarlung Tsangpo River basin and other nearby areas, with *L. himalayana* widely distributed from the Southern Pamir Plateau and the Ladakh Karakorum Mountains in Central Asia to the Xinjiang region of Tibet in the Himalayas (Ananjeva *et al.* 2011; Baig *et al.* 2012). *L. wui* occurs in the alpine valley region of eastern Tibet in the lower Yarlung Tsangpo River, while *L. sacra* occurs in the upper Yarlung Tsangpo River near the southern Tibetan valley, both of which are endemic to Tibet in China (Zhao *et al.* 1999; Baig *et al.* 2012; Zou *et al.* 2016).

Based on the geographical distribution, it can be inferred that the three branches of the phylogenetic tree of *Laudakia* in this study showed a trend of spreading to the north (branch A), west (branch B) and south (branch C), with the Pamir Plateau in Central Asia as the midpoint. This inference is consistent with the hypothesis of Baig *et al.* (1992), who suggested that the ancestral origin and evolution of *Laudakia* occurred around the Pamir knot, with various dispersals over time along the alpine systems of southern Asia and Central Asia, and expansion into the northeast (e.g. *L. stoliczkana*), southeast (e.g. *L. tuberculata*), southwest (e.g. *L. nupta*) and western mountains (e.g. *L. stellio*). Rastegar-Pouyani & Nilson (2002) also suggested that the centre of origin of *Laudakia* was the high mountain ranges of northeastern Afghanistan and southeastern Tajikistan (Pamir Knot), and that subsequent Tertiary and Quaternary orogeny and climatic fluctuations led to the present distribution pattern of *Laudakia*. The species formation of *Laudakia* is intimately associated with the Hindu Kush, Pamir and Himalayan ranges (Ananjeva *et al.* 1990; Ananjeva & Tuniev 1994; Baig *et al.* 1992; Rastegar-Pouyani & Nilson 2002). Macey *et al.* (2006) suggested that species divergence in *Laudakia* may have resulted from the uplift of the Pamir-Tian Shan (10 Myr ago).

The results of this study suggested that the divergence of *Laudakia* began at 43.72 Ma (95% confidence interval HPD: 23.53–66.12 Ma), which is similar to the age of divergence of 40.6 Mya (95% confidence interval HPD: 34.1–55.9 Ma) estimated by Melville *et al.* (2009). The relatively recent topographic changes in the Tian Shan inferred from Abdrakhmatov *et al.* (1996) suggested that the Tian Shan developed in the last 10 Ma, and the divergence time estimates for branch A (*L. caucasia* and *L. stoliczkana*) in this study are consistent with this. The six species of branch C are distributed near the Tibetan Plateau, and the uplift of the plateau and its surrounding mountains has played an important role in the formation of the species distributed there. The collision of the Indian subcontinent with Eurasia occurred in the Eocene at around 50–55 Ma, while the Tibetan Plateau-Himalayan complex began to uplift at around 40 Ma, with uplift increasing between 25–20 Ma (Guo *et al.* 2002), followed by mountain building on the Tibetan Plateau between 10–25 Ma (Sun 1997). The timing of the divergence of the major branches of *Laudakia* in this study is estimated to coincide with the occurrence of important geographical events. The period of

aridification in Central Asia occurred during the late Oligocene to early Miocene (Guo *et al.* 2002), and the effects of aridification, contributed even more to the formation of species diversity in *Laudakia*.

4.3 Subspecies differentiation of *L. stoliczkana*

The formation of mountains and rivers affects the divergence of species, producing geographic isolation that contributes significantly to subspecies formation (Macey *et al.* 2000; Tamar *et al.* 2016; Shahamat *et al*. 2020; Qi *et al.* 2023; Jablonski *et al.* 2023). The Tian Shan Mountains are located in the hinterland of the Eurasian continent, the Tarim Basin in the south and the Junggar Basin in the north, so they play a very important role in the species formation and differentiation (Zhang & Zhang 2012; Wang *et al.* 2018; Li *et al.* 2020; Hou *et al.* 2022).

Laudakia stoliczkana has a relatively wide distribution in China, in southern Xinjiang on the periphery of the Tarim Basin and in the eastern Xinjiang region, in northern Xinjiang in the Beita Mountains, the eastern Altai Mountains and the northwestern Mazong Mountains, and abroad in Mongolia (Zhao *et al.* 1999). *L. s. stoliczkana* is mainly distributed around the Tarim Basin south of the Tian Shan Mountains (Zhao *et al.* 1999), while *L. s. altaica* distributed in Xinjiang was determined to be mainly distributed near the Altai Mountains north of the Tian Shan Mountains based on the morphological description of specimens collected in the Qinghe county by Shi & Zhao (2008).

The results of both the phylogenetic tree and the genetic distance support this geographical division. In the phylogenetic tree of COI sequences, the Group 1 clusters with a sequence of *L. stoliczkana* from Mongolia (NPLRP294-08), while the Group 2 clusters with a sequence from Ruoqiang, Xinjiang (ABLRP409-07). The phylogenetic tree of 16S and tandem sequences showed the same results, with Group 2 clustered with a sequence from Turpan, Xinjiang (MK585009). The results for genetic distances showed *p*-distances ≤1.5% between both Group 1 and Group 2 (COI/16S/tandem sequences: 1.2%/1.5%/1.1%), consistent with published studies of molecular differences in subspecies of species of *Laudakia*, e.g. genetic distances between subspecies of *L. microlepis* < 7.8% (Sanchooli *et al.* 2015); and < 2.9% between *L. stellio* subspecies (Kadry *et al.* 2020).

The recent uplift of the Altai and Tian Shan topography, which undulates over 3500 m above sea level, developed during the Neoproterozoic (5.33 Ma) (Yuan *et al.* 2006), and the present study found that the divergence of *L. stoliczkana* subspecies occurred at 3.27 Ma (95% confidence interval HPD: 1.58–5.87 Ma), after the recent uplift of Tian Shan. This suggests that the uplift of Tian Shan Mountains has influenced the differentiation of *L. stoliczkana* subspecies.

Also, according to the results of the subspecies morphological comparison, there were significant differences in tail length and the number of scales in the 5th ring of the caudal base between the two localities, both for females and males, and the tail length of *L. s. altaica* (Northern) was significantly shorter than that of *L. s. stoliczkana* (Southern) for both males and females, in agreement with previous results (Peters 1971; Shi & Zhao 2008).

The habitats of *L. stoliczkana* subspecies are distinctly different, with *L. s. altaica* mainly in rocky mountainous areas and *L. s. stoliczkana* mainly in loess and sandy desert areas (Peters 1971; Wang *et al.* 1986; Shi & Zhao 2008). *L. s. stoliczkana* is mainly found on river terraces, within *Populus euphratica* forests, on bank walls and in the crevices of abandoned earthen walls (Guoying *et al.* 1986). Zhao *et al.* (1999) described the vertical distribution of *L. stoliczkana* as 100m (Turpan City)–1760m (Hami City), while according to Peters (1971), who examined the capture sites in the study, the altitude distribution of *L. s. altaica* was found to be 1000m–2000m, showing that the vertical distribution of the two subspecies does not differ significantly. Whether the intraspecific genetic differentiation of *L. stoliczkana* correlates with the altitude of its distribution needs further study.

Conclusion

The phylogenetic tree reconstruction of *Laudakia* in this study shows that the genus is monophyletic and does not support the division of the three genus by Baig *et al.* (2012)—*Stellagama*, *Laudakia* and *Paralaudakia*. The phylogeny of *Laudakia* can be divided into three main branches, the relationships of which are related to the geographical distribution of the species. The uplift and development of the Pamir-Tian Shan and Qinghai-Tibet Plateau-Himalayan ranges have had an important influence on and contributed to the divergence of species in this

genus. This study explores the differences between subspecies of *L. stoliczkana* from a molecular genetic perspective, and the results show that there are significant genetic differences between the two subspecies, consistent with subspecific differentiation. Our study estimates that the two subspecies diverged at about 3.27 Ma (95% confidence interval HPD: 1.58–5.87 Ma), which is consistent with the recent uplift in the Tian Shan. The results highlight the importance of the uplift of the Central Asian mountains and the Tibetan Plateau for the divergence of *Laudakia*.

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Conflict of interest statement

The authors declare no competing interests.

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APPENDIX 1. Molecular sample source.

Voucher No.	Number	Locality	Longitude and Latitude
XJA0010021	QEL2	Korla, Xinjiang	86º8’ E, 41º37’ N
XJA0010022	QEL3	Korla, Xinjiang	
XJA0010023	LBP1	Ruoqiang, Xinjiang	90º5’ E, 40º48’ N
XJA0010024	KS4	Tashkurgan, Xinjiang	76º12’ E, 37º47’ N
XJA0010013	21093	Qinghe, Xinjiang	90º26’ E, 45º28’ N
XJA0010014	21094		
XJA0010015	21095		
XJA0010016	21096		
XJA0010017	21097		
XJA0010018	21098		

APPENDIX 2. Reference sequence downloaded on GenBank or BOLD.

Species	GenBank or BOLD Voucher	Location
Laudakia stoliczkana	ABLRP409-07 (BOLD)	Ruoqiang, Xinjiang
	NPLRP294-08 (BOLD)	Mongolia
	MK585009.1	Mayao Village, Turpan, Xinjiang
Laudakia nupta	HQ901100.1	Iran
	HQ901101.1	
	NPLRP292-08 (BOLD)	
	NPLRP296-08 (BOLD)	
Laudakia papenfussi	MK585008.1	Zanda, Tibet
	KU926284.1	Qinghai-Tibetan Plateau
	KU926275.1	
	KU926274.1	
Laudakia sacra	MH047794.1	\
	MH047793.1	
	MH047792.1	Nyemo, Tibet
	MK411596.1	
	KU926279.1	Mainling, Tibet
	KU926280.1	
	KU926281.1	
	KU926282.1	
	KU926283.1	
Laudakia wui	MK411597.1	Pome, Tibet
	KU926278.1	Qinghai-Tibetan Plateau
	KU926277.1	
	KU926276.1	
Laudakia tuberculata	MK411595.1	\
	KU926273.1	Qinghai-Tibetan Plateau
	KU926272.1	
	KU926271.1	
	NPLRP316-08 (BOLD)	Nepal
	NPLRP317-08 (BOLD)	

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APPENDIX 2. (Continued)

Species	GenBank or BOLD Voucher	Location
Laudakia stellio	AB031993.1	West Asia
	GU128464.1	Turkey
	KF691700.1	\
	HQ901098.1	Europe and Asia
	AY053765.1	Iran
Laudakia caucasia	KF691701.1	Azerbaijan
	NPLRP300-08 (BOLD)	Azerbaijan
	NPLRP305-08 (BOLD)	\
	NPLRP309-08 (BOLD)	Turkmenistan
	NPLRP310-08 (BOLD)	Russia
	NPLRP509-08 (BOLD)	Turkey
Laudakia lehmanni	KF691702.1	\
Laudakia himalayana	MG257699.1	\
	MG257698.1	
	MG257697.1	
	MG257696.1	
	MG257700.1	
	MH047791.1	
	MH047790.1	
	MH047789.1	
Draco volans	KU986311.1	\
	AB023768.1	\
Draco maculatus	AB023759.1	\
	MT609388.1	\
Draco taeniopterus	MT608780.1	\
	MT609389.1	\
Acanthosaura crucigera	MT608654.1	\
	MG935416.1	\

APPENDIX 3. Best models for the three sequences.

Sequence	Best model	Partition name
COI	GTR+I+G	\
16S	GTR+G	\
Tandem sequence	GTR+G	16S pos1, 16S pos2, 16S pos2
	TIMEF+I+G	COI pos1
	GTR+I+G	COI pos2
	HKY+I+G	COI pos3

APPENDIX 4. Morphometric data of *Laudakia stoliczkana* subspecies.

Voucher No.	Sex	SVL (mm)	TL (mm)	Supra-labials	Infra-labials	SAT	Source
XJA0010010	m	131.00	170.00 +	13/13	10/11	36	
XJA0002923	m	119.00	177.00	13/13	12/11	38	
XJA0002924	m	119.00	187.00	13/13	11/11	34	
XJA0002925	m	125.00	162.00 +	12/12	10/10	35	
XJA0002926	m	123.00	132.00 +	13/13	11/11	34	
XJA0002927	m	120.00	170.00	12/12	11/11	33	
XJA0002928	m	125.00	172.00 +	13/13	11/11	34	
XJA0002929	m	115.00	195.00	13/13	11/11	36	
XJA0010011	m	123.00	135.00 +	13/13	11/11	35	
XJA0002930	m	100.00	150.00	13/13	11/11	37	
XJA0002931	m	111.00	173.00	13/13	10/10	35	Shi and Zhao, 2008
XJA0010012	m	125.00	152.00 +	13/13	11/11	36	
XJA0002932	f	122.00	72.00 +	13/13	11/11	43	
XJA0002933	f	119.00	124.00	13/13	11/11	42	
XJA0002934	f	110.00	112.00 +	13/13	11/11	41	
XJA0002935	f	114.00	142.00 +	13/13	11/11	36	
XJA0002936	f	112.00	161.00	13/13	11/11	38	
XJA0002937	m	109.00	126.00 +	13/13	11/11	40	
XJA0002938	m	140.00	187.00	12/12	10/10	36	
XJA0002939	m	131.00	156.00 +	13/13	11/11	40	
XJA0002940	m	125.00	143.00 +	13/13	11/11	37	
XJA0002941	f	117.00	152.00 +	13/13	11/11	40	
XJA0010013	m	126.37	148.28	11/11	12/12	33	
XJA0010014	f	126.84	171.60	11/11	11/10	34	
XJA0010015	f	130.18	123.26	11/11	10/11	32	
XJA0010016	f	108.41	163.07	11/13	10/9	38	This study
XJA0010017	m	125.85	153.11	11/10	13/11	35	
XJA0010018	f	110.94	150.71	12/11	12/11	37	
XJA0010019	m	126.71	96.28 +	10/11	13/13	30	
XJA0010020	m	107.33	174.50	10/9	12/12	27	
Range		140.00–100.00	195.00–72.00			27–43	*L. s. altaica*
XJA0010021	f	109.92	204.60	15/14	13/14	43	
XJA0010022	m	131.12	227.63	15/15	13/12	43	
XJA0010023	f	124.46	202.48	13/12	12/13	40	
XJA0010024	f(juvenile)	67.81	140.46	11/10	12/12	42	
XJA0006605	f	140.90	224.19	11/11	13/12	38	
XJA0006606	f	119.17	210.44	12/13	12/12	36	
XJA0006608	f	140.68	204.53	12/12	12/12	37	
XJA0010025	f	110.02	182.59	11/12	12/12	40	
XJA0010026	m	125.41	183.87	11/11	11/11	43	
XJA0010027	m	145.20	188.09	12/12	11/12	40	This study
XJA0010028	m	120.32	195.27	12/12	14/13	44	
XJA0007327	f	135.84	197.66	13/13	13/12	42	
XJA0007274	f	121.47	141.96	11/11	11/13	43	
XJA0007322	f	125.15	208.61	12/13	14/13	47	
XJA0007324	m	132.45	164.71	14/13	14/14	44	
XJA0007325	f	128.37	207.51	14/13	12/13	45	
XJA0007336	m	138.88	245.12	11/11	12/11	43	
XJA0007334	f	138.76	129.07	13/14	11/13	40	
XJA0007332	m	139.20	161.47	12/12	13/13	36	
XJA0007333	f	121.31	132.64	13/15	14/14	40	
XJA0007335	f	129.30	175.45	12/13	14/14	38	
XJA0007277	f	101.06	190.42	12/12	×	43	

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APPENDIX 4. (Continued)

Voucher No.	Sex	SVL (mm)	TL (mm)	Supra-labials	Infra-labials	SAT	Source
XJA0006618	m	123.13	189.76	13/13	12/12	47	This study
XJA0006639	m	97.67	182.63	10/12	14/15	48	
XJA0006635	m	122.65	213.12	13/13	14/14	44	
XJA0006619	f	122.31	200.74	11/12	12/12	46	
XJA0006625	f	131.85	178.80	11/12	12/13	43	
XJA0006630	m	141.60	203.04	12/12	14/13	47	
XJA0006614	f	126.14	142.18	12/13	12/12	46	
XJA0006632	m	126.83	178.00	12/12	12/13	45	
XJA0006628	f	127.92	216.52	14/14	13/13	49	
XJA0006620	m	138.23	229.06	13/13	12/11	40	
XJA0006636	f	123.71	198.84	12/12	12/12	44	
XJA0006629	f	113.72	185.50	12/14	13/12	43	
XJA0006641	m	124.79	150.72	13/11	13/13	47	
XJA0006627	f	121.85	202.50	12/13	11/12	47	
XJA0006623	m	117.83	200.22	15/14	14/13	43	
XJA0006617	m	92.61	176.90	14/13	12/13	48	
XJA0006638	m	128.92	135.25	11/12	13/12	48	
XJA0006615	m	128.60	204.69	13/13	11/12	47	
XJA0006613	m	110.06	213.39	12/12	12/12	46	
XJA0006624	m	110.64	97.11	11/12	12/12	42	
XJA0006640	f	112.66	109.19	10/12	11/12	41	
XJA0006616	m	115.68	109.23	12/12	12/12	46	
XJA0007284	f	124.68	182.15	12/12	12/12	41	
XJA0007272	f	115.00	187.74	11/12	12/12	42	
XJA0007281	m	98.49	178.19	13/13	12/11	45	
Range		145.2–92.61	245.12–97.11			36–49	*L. s. stoliczkana*

Note: f = females; m = males; + = Broken tail; × = Specimen damage cannot be counted.

APPENDIX 5. T-test results for morphological indicators.

Sex	location	number	SVL (mm)			TL (mm)			SAT		
			Mean ± SD	P	C.D.	Mean ± SD	P*	C.D.	Mean ± SD	P*	C.D.
males	N	20	121.36 ± 9.27	0.740	0.530	157.96 ± 23.95	0.010	0.434	35.05 ± 3	0.000	1.544
	S	23	122.62 ± 14.42			184 ± 36.09			44.3 ± 2.99		
females	N	10	117.04 ± 7.4	0.049	0.415	137.16 ± 30	0.001	0.753	38.1 ± 3.51	0.002	0.594
	S	23	124.19 ± 9.85			183.12 ± 31.07			42.22 ± 3.42		

Note: N is North of Xinjiang; S is South of Xinjiang; * Significant values are the results of analysis of covariance; $P < 0.05$ is a significant difference, $P < 0.01$ is a highly significant difference; C.D. > 1.28 indicates differences at the subspecies level.