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Diversity, Phylogenetic Relationships and Distribution of Marsh Frogs (the *Pelophylax ridibundus* complex) from Kazakhstan and Northwest China

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Abstract: In order to study the diversity, phylogenetic relationships and distribution of marsh frogs of the *Pelophylax ridibundus* complex in Kazakhstan and northwest China, we conducted phylogeographic analyses of 125 samples from 53 localities using the mtDNA ND2 and COI genes and the SAI nuclear gene sequences. Phylogenetic inference of mtDNA revealed three main lineages—sister lineages Balkhash and Syrdarya (as the Central Asian *P.* sp. *novum*), and the Anatolian *P.* cf. *bedriagae*, while from nDNA data, we additionally detected the western form, *P. ridibundus*. According to mtDNA data, the mean genetic distances between *P.* sp. *novum* and two other forms of marsh frogs was more than 5%. Genetic homogeneity within populations of the Syrdarya lineage and *P.* cf. *bedriagae* is characterized by low nucleotide diversity and high haplotype diversity. Demographic analyses of the lineages showed past population expansions of the Balkhash and the Syrdarya forms. Divergence from the most recent ancestor had occurred in the Early Pleistocene period (2.46 Mya) for the Balkhash and the Syrdarya lineages, and 1.27 Mya for the *P.* cf. *bedriagae*. Our findings provide a first investigation of the lineage diversification and population dynamics of the Central Asian marsh frogs and will be useful for further taxonomic implications and conservational actions.

Keywords: phylogeography; marsh frogs; Ranidae; Central Asia; mtDNA; nDNA

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Citation: Ualiyeva, D.; Ermakov, O.A.; Litvinchuk, S.N.; Guo, X.; Ivanov, A.Y.; Xu, R.; Li, J.; Xu, F.; Arifulova, I.I.; Kaptyonkina, A.G.; et al. Diversity, Phylogenetic Relationships and Distribution of Marsh Frogs (the *Pelophylax ridibundus* complex) from Kazakhstan and Northwest China. *Diversity* **2022**, *14*, 869. https://doi.org/10.3390/d14100869

Academic Editor: Alessandro Catenazzi

Received: 1 September 2022
Accepted: 12 October 2022
Published: 14 October 2022

1. Introduction

The Eurasian water frogs (*Pelophylax*) consist of at least 20 species and several hybridogenous forms [1]. The marsh frogs of the *P. ridibundus* complex inhabit huge territories of Eurasia from Western Europe to Central Asia and northwestern China [2–5]. About a dozen lineages have been observed within the complex [6–9]. Some of them (*P. bedriagae*, *P. caralitanus*, *P. cerigensis*, *P. cypriensis*, *P. kurtmuelleri* and *P. terentievi*) are currently considered as separate species. Others remain undescribed and are usually noted as *P. ridibundus*, *P.* cf. *bedriagae* or *P.* sp. *novum* [10–12]. The situation in the complex is also complicated by the fact that hybridization between various marsh frog species, which is very common,

and asymmetric introgression of mitochondrial (mt) genomes often results in the capture of alien mtDNA [13–15]. An additional nomenclature problem is related to the undefined type territory of *P. ridibundus*, since specimens of this type are absent and the species was described from a vast region (lower parts of the Volga and Ural rivers) where both the European and Anatolian species and their hybrids co-occur [16,17]. This problem led to a discussion about which of the scientific names should be used for each of these two species. For example, depending on which lectotype will be chosen, the European species should be named as *P. ridibundus* or *P. fortis* [18]. Thus, the taxonomic structure of the *P. ridibundus* complex remains uncertain.

In contrast to the European part of range of the *P. ridibundus* complex, Kazakhstan, even Central Asia, is practically unexplored. For example, the eastern border of native range of marsh frogs on the map of "The IUCN Red List of Threatened Species" [19] runs through western Kazakhstan from the north of the Caspian Sea to the Ural Mountains. The remaining major part of Kazakhstan and the adjacent territories of western China remain a "blank spot".

Mezhzherin [20,21] was the pioneer who studied taxonomic status of marsh frogs in Kazakhstan. He compared two samples from eastern Kazakhstan with marsh frogs from Ukraine (*P. ridibundus*), Turkmenistan, Uzbekistan (the Fergana Valley) and south Tajikistan (*P. terentievi*). Based on results of allozyme analysis, he found that the Eastern Kazakhstan populations are not conspecific with *P. terentievi*.

Additionally, the marsh frogs of the genus *Pelophylax* from northwest China were reportedly studied [22–24]. Several findings of marsh frogs from Xinjiang province were recorded as *P. ridibundus* [25–30]. Considering that western Xinjiang of China is adjacent to Tajikistan, and the morphological characteristics of the specimens are basically the same as those of *R. terentievi*, Fei et al. [31] revised the specimens originally called as *P. ridibunda* in western Xinjiang to the Central Asian Pond Frog *P. terentievi* (the original *Rana terentievi*), in light of the above scholars' research. Since then, this view of *P. terentievi* occurring in Xinjiang has been adopted by books on herpetofauna and atlases in China [32–34]. So far, however, the identity of "marsh frogs" in China has not been verified using molecular-genetic evidence, that is, based on phylogenetic systematics, to test whether this viewpoint is reliable. Confusingly, Che et al. [35] considered two specimens of marsh frog in Yili (=Ily) (China) as *P. ridibunda*, with COI sequences (JN700829, JN700830) deposited in GenBank. Later, Ye et al. [36] analyzed the genetic diversity and phylogeny of frogs in Xinjiang based on 12S rRNA, and tentatively recognized five specimens from Wujiaqu city as *P. terentievi*.

Akin et al. [8] conducted the mitochondrial phylogeography of West-Palearctic water frogs and revealed that the mtDNA of marsh frogs from western Kazakhstan (Atyrau) are very close to those of the Anatolian lineage (*P.* cf. *bedriagae*). On the other hand, samples from southeastern Kazakhstan (Almaty) contained haplotypes of another Central Asian lineage ("Central Asia 2" or *P.* sp. *novum*). After that, Mazepa [14] attempted to study the evolution of water frogs *Pelophylax* in Central Asia and determined two native forms and their contact zone: *P.* sp., which populates the Syrdarya drainage, and *P. terentievi, which* inhabits the Amudarya drainage. Recently, molecular typing of mitochondrial lineages of the marsh frogs from Kazakhstan was conducted by PCR-RFLP method based on ND2 markers, which revealed the three lineages inhabiting the country—two lineages Syrdarya and Balkhash, and the Anatolian *P.* cf. *bedriagae* [37].

The study of a nuclear (n) DNA marker, the serum albumin intron-1 (SAI-1), revealed that the marsh frogs from Atyrau are heterozygous for harboring a European *P. ridibundus* and an Anatolian *P.* cf. *bedriagae* specific allele [16]. The variability of the nuclear and some mtDNA markers in the marsh frogs from several localities from Kazakhstan was previously observed in doctoral theses [38,39] and our preliminary publication [40]. These authors found alleles and haplotypes in Kazakhstan of three lineages of marsh frogs (*P. ridibundus*, *P.* cf. *bedriagae* and *P.* sp. *novum*). However, these fragmentary data do not allow an assessment of the peculiarities of distribution of these species in Kazakhstan and adjacent China.

It is important to note that the marsh frogs are among the most invasive amphibian species in Eurasia [41,42]. For example, the Anatolian marsh frog (*P.* cf. *bedriagae*) was widely introduced to Italy, Belgium, France, Switzerland, Germany and some regions of Russia [43–53]. Introgression of mt-nuclear DNA genes of this species was found in eastern European populations of *P. ridibundus* and *P. esculentus* [54–56], while the native range of the species is located in western Iran, Turkey, southern Bulgaria, eastern Greece, the Caucasus and Crimea [8,9,17,57–61].

The native distributional range of marsh frogs in Kazakhstan consists of two isolated segments located in the Caspian Sea drainage in western Kazakhstan and the Syrdarya River drainage in southeastern Kazakhstan [42,62]. Starting from the beginning of the twentieth century, with the help of humans, marsh frogs began to actively populate the entire territory of Kazakhstan and penetrate into western China. Now they can be found almost throughout this huge territory. However, the sources of introductions are usually unknown. Therefore, the study of genetic variability can help to reveal the means of these invasions, as well.

The species composition, phylogeny and distribution of the marsh frogs in Kazakhstan and adjacent areas of China have not been investigated in detail. Therefore, the purpose of this study was to examine the genetic variability and phylogeography of marsh frogs using both nuclear and mitochondrial DNA markers.

2. Materials and Methods

2.1. Sampling

We collected 125 samples of *P. ridibundus* from 53 localities across the distribution range in Kazakhstan and adjacent Northwest China (Xinjiang Uyghur Autonomous Region) (Figure 1, Table S1) during the fieldwork of 2015–2021. Fresh muscle tissue from the samples' toes or liver tissue was dissected and stored at −20 °C before DNA extraction. Voucher specimens of populations were deposited in the herpetological collections of Institute of Zoology SC MES RK (Almaty, Kazakhstan), Chengdu Institute of Biology CAS (China), College of Life Science and Technology, Xinjiang University (China), and State Key Laboratory of Desert and Oasis Ecology, Xinjiang Institute of Ecology and Geography, Chinese Academy of Sciences (China).

2.2. Laboratory Protocol

Genomic DNA was extracted from tissue samples preserved in 95% ethanol by using a standard salt protocol [63]. Fragments of nuclear serum albumin intron 1 (SAI, 837 bp) and two mitochondrial genes (NADH dehydrogenase subunit 2, ND2, 1038 bp; cytochrome c oxidase subunit 1, COI, 647 bp) were determined by PCR and Sanger sequencing.

The ND2 gene sequence was amplified with use of the universal primer ND2L1 5′-AAG CTT TTG GGC CCA TAC CCC-3′ [64] and the specific primer ND2H1 5′-GCA AGT CCT ACA GAA ACT GAA G-3′ [50]. The following amplification conditions were used: initial denaturation for 1 min at 95 °C followed by 32 cycles of 95 °C for 30 s, 60 °C for 30 s, 72 °C for 60 s, and final extension for 5 min at 72 °C. The COI gene fragment was amplified using the primer pair UTF 5′-TGT AAA ACG ACG GCC AGT TCT CAA CCA AYC AYA ARG AYA TYG G-3′ and UTR 5′-CAG GAA ACA GCT ATG ACT ARA CTT CTG GRT GKC CRA ARA AYC A-3′ [65]. The following amplification conditions were used: initial denaturation for 1 min at 95 °C followed by 30 cycles of 95 °C for 30 s, 50 °C for 30 s, 72 °C for 40 s. The SAI fragment was amplified using the primer pair SA1F-Pu 5′- CCA TAC AAA TGT GCT AAG TAG GTT-3′ [66] and SA1R 5′-GAC GGT AAG GGG ACA TAA TTC A-3′. The following amplification conditions were used: initial denaturation for 1 min at 95 °C followed by 32 cycles of 95 °C for 30 s, 60 °C for 30 s, 72 °C for 50 s. The PCR mixture (25 µL) contained 50–100 ng of DNA, 0.5 µM of each primer, 0.2 mM dNTPs, 1.5 mM MgCl2, 2.5 µL 10 × PCR buffer (10 mM Tris–HCl, pH 8.3, 50 mM KCl), and 2 units of Taq polymerase (Thermo Scientific). Sequencing was performed on an ABI 3500 automatic sequencer (Applied Biosystems) using the BigDye®Terminator 3.1 (Applied Biosystems) kit and the

same primers that were used for amplification. The sequences obtained were deposited in GenBank (COI: ON796820-ON796944; ND2: ON809573-ON809697; SAI: ON856262-ON856335; Table S1).

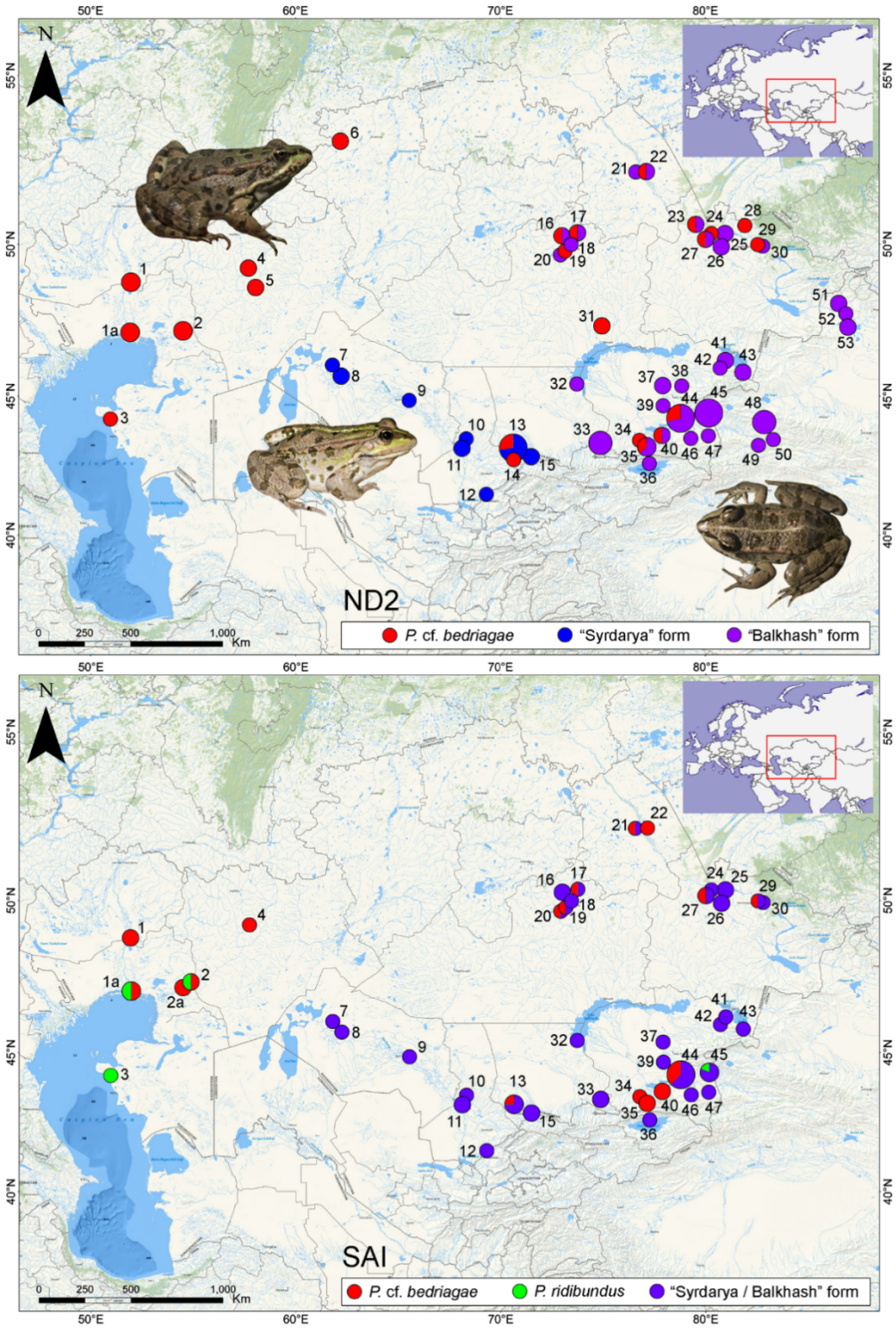

Figure 1. Distribution range of haplotypes of marsh frog of the complex *Pelophylax ridibundus* on its eastern peripheral (Kazakhstan and adjacent Northwest China) based on ND2 and SAI markers. Geographic position of the localities are given in Tables S1 and S2. Photographs of frogs (*P.* cf. *bedriagae*; Balkhash lineage) by T.N. Dujsebayeva represent individuals from sampling sites 28 and 43 respectively. Picture of Syrdarya form "Reproduced with permission from Amirekul K".

2.3. Phylogenetic Reconstruction

All obtained nucleotide sequences were checked and assembled using SeqMan II program included in LASERGENE 7.0 software package (DNAStR Inc., Madision, WI, USA). An alignment was performed by Clustal X v.1.83 [67]. Multiple sequence alignments were checked manually, and sequences of protein coding genes were translated into amino acids to check for erroneous stop codons. Mitochondrial haplotypes were generated using DnaSP v.6.0 [68]. Concatenated dataset of ND2 and COI gene sequences (1685 bp) was used to infer phylogenetic relationships with Bayesian Inference (BI) using MrBayes 3.2 [69]. Additionally, 76 sequences of previously published ND2 gene [8,70], 40 sequences of COI gene, and 3 outgroup sequences (*P. cretensis*, *P. epeiroticus*, *P. nigromaculatus*) were retrieved from GenBank and included in our dataset. Nine main haplogroups [MHG 1–9] of marsh frogs were included and designated according to the classification of Akın et al. [8].

Phylogenetic analysis on SAI gene dataset was conducted separately by using the Maximum Likelihood method in MEGA v.X [71]. Sequences from previous studies [8,9,16] of SAI were retrieved from GenBank. The SAI data (without sequences) from Kazakhstan presented in Akın [39] were also used in the analysis of the distribution of species. *Pelophylax perezi* and *P. saharicus* were chosen as outgroups.

The software PartitionFinder v.1.1.0 [72] was used to test for the best partitioning scheme for each dataset. The input configuration file contained 6 partitions, corresponding to individual codon positions for the two mtDNA genes. The "all" algorithm [73] (heuristic search) was used with branch lengths estimated as "linked" to search for the best scheme. A total of 24 a priori schemes with varying degrees of complexity were statistically compared in PartitionFinder using the BIC (Bayesian Information Criterion) [74]. Bayesian analysis was performed sampling two runs and four chains per run for 1×10^7 generations (started on random trees) and four heated Markov chains (using default heating values) and sampling every 1000th tree. After assessing the distribution of log-likelihoods and parameter values using Tracer v.1.6 [75] the burn-in threshold was set to 25%. The resulting phylogram and posterior probabilities were visualized in FigTree 1.3.1 [76].

To reveal evolutionary relationships and current mutational variations among mitochondrial haplotypes and nuclear alleles the median-joining network [77] was constructed using the program PopART, under the default parameters [78]. MtDNA dataset were defined on trait blocks regarding the attribution of the localities to the hydrology (main water drainages): Caspian Sea, Syrdarya River, Irtysh River and Balkhash–Alakol lakes. Whereas nDNA dataset was defined regarding to mitochondrial haplotypes with 95% connection limit using the same software.

2.4. Bayesian Molecular Dating

Divergence time for concatenated mtDNA dataset was calculated in the program BEAST v.1.7.5 [79]. The input file (xml format) was created using BEAUti v.1.7.5. The taxon set was including Western and Eastern Palearctic marsh frogs MHG1–9. *Pelophylax nigromaculatus* was chosen as an outgroup. We used HKY site substitution model with two unlinked partitions, one for mtDNA (unlinked site and clock models and linked trees), under the relaxed uncorrelated lognormal clock model. The calibration point was taken as a reference from the study [7], which correspond to the palaeogeological event—the isolation of the Crete Island from the Peloponnese occurred about 5.2 million years ago (Mya) [80,81] and corresponded to the divergence of *P. cretensis*. It should be noted that while this dating has been disputed [9], it was recently supported by Yang et al. [82]. The calibration was implemented as a normal prior with standard deviation equal to 1.0; the Yule model was chosen as a tree prior. The MCMC analysis was run for 1×10^7 generations, with random starting tree sampling every 1000th tree. The first saved 1000 trees were discarded as burn-in using LogCombiner and maximum clade credibility (MCC) was identified using TreeAnnotator [79]. The obtained molecular clocks (in Mya) were referenced with the geochronological scale of epochs' periods https://stratigraphy.org/timescale/ (Accessed on 10 June 2022) [83].

2.5. Population Structure and Demographic History

The genetic diversity indices for each population (number of haplotypes [H], haplotype diversity [h] and nucleotide diversity [π]) were determined using DnaSP v.6.0 [68]. Uncorrected pairwise sequence divergences (*p*-distances) between phyloclades were calculated using MEGA v.X. Mismatch distribution analysis under the constant population size model and neutrality tests [84,85] were used to assess demographic history of the major matrilines in DnaSP v.6.0. Raggedness statistic [86] was used to quantify the smoothness of the observed pairwise differences distribution. Additionally, we employed the R_2 statistics [87], which can capably detect population expansion.

3. Results

3.1. Data Analysis

3.1.1. Sequence Characteristics

We obtained 125 sequences of two mtDNA markers (COI and ND2 genes), as well as 75 sequences of nDNA marker SAI. The length of the COI dataset after the alignment and edition was 647 bp, in which the presence of 39 polymorphic sites and 15 haplotypes were revealed. The ND2 dataset length was 1038 bp. It contained 73 polymorphic sites with 17 haplotypes, respectively. No stop codons were observed. The total length of the aligned concatenated data set of ND2 + COI gene fragments was 1685 bp, in which, the total number of variable sites was 112 and 103 parsimony-informative sites. In total, 25 haplotypes were identified, among which, 14 haplotypes were unique (Table S3).

Among the 62 sequences of the SAI gene (only homozygous alleles), 835 sites were revealed (47 sites informative and 48 sites variable). The average nucleotide frequencies of T(U) = 30%, C = 16.4%, A = 32% and G = 21.7%, respectively. The haplotype diversity and nucleotide diversity for the whole data Hd = 0.824 $\pm$ 0.035 and π = 0.0183 $\pm$ 0.0026, respectively. The mean number of nucleotide differences k = 15.25.

3.1.2. Combined Mitochondrial DNA Phylogeny

Concatenated mtDNA data with previously published sequences generated 104 haplotypes. The best fitting evolutionary model was K80 + G for the first subset, HKY + I for the second and GTR + G for the third position. The best-partitioned scheme topology resulted ln *L*: −6347.97, and BIC: 14,367.59, respectively.

The monophyletic clade of Central Asian marsh frogs (*P.* sp. *novum* sensu Akin et al. [8]) consisted of the lineages of the Balkhash, Syrdarya, MHG8 Central Asian 2 andsouthwestern Iran, and were closely related to the marsh frog complex of the Cilician clade (Figure 2). The subclades were highly supported, except for the lineage of "Middle East" (MHG9) from Iran (PP < 0.50), which can be explained by the lack of an adequate sampling from this region (MHG9—single specimen haplogroup).

Anatolian marsh frog *P.* cf. *bedriagae* comprised two clades *P.* cf. *bedriagae* sensu lato that are widely distributed in Kazakhstan and Eastern Palearctic, and *P.* cf. *bedriagae* sensu stricto, inhabiting only the Anatolian mainland itself. All mentioned clades yielded a high posterior support (>0.9). Based on mtDNA markers, the European *P. ridibundus* was not observed among Kazakhstan populations.

The haplotype networks supported the division of the marsh frogs of Kazakhstan into three lineages according to both mtDNA markers (ND2 and COI) (Figure 3) and low level of variability in the Balkhash lineage. The topology of the ND2 gene network yielded a star-like structure in the Syrdarya lineage, suggested recent population expansion. Moreover, haplotype networks illustrated distribution of the Syrdarya lineage in the Syrdarya River drainage only, whereas the Balkhash lineage is distributed in two adjacent drainages. Haplotypes of the widely distributed Anatolian lineage were observed in all four drainages of Kazakhstan.

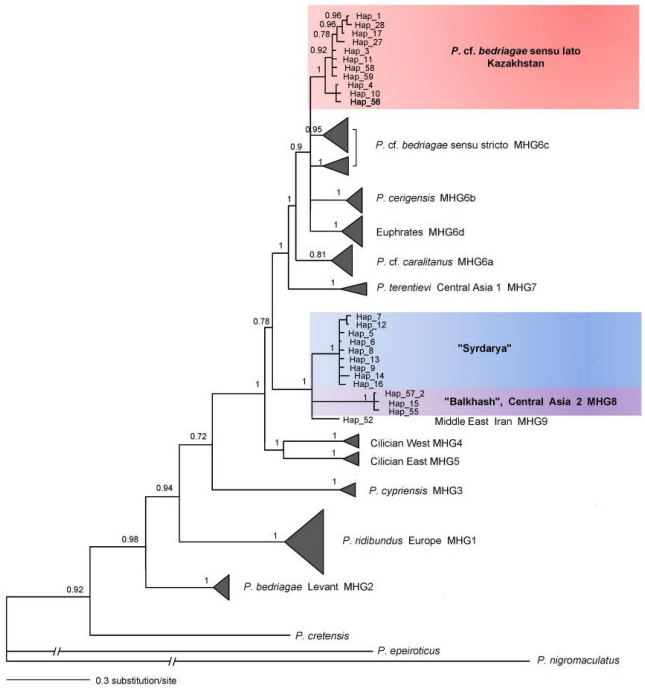

Figure 2. Bayesian phylogenetic tree of mtDNA (ND2 + COI genes), abbreviation of MHG (mitochondrial haplogroups) is in accordance with the data published by Akin et al., 2010. Posterior probabilities less than 0.70 are not shown.

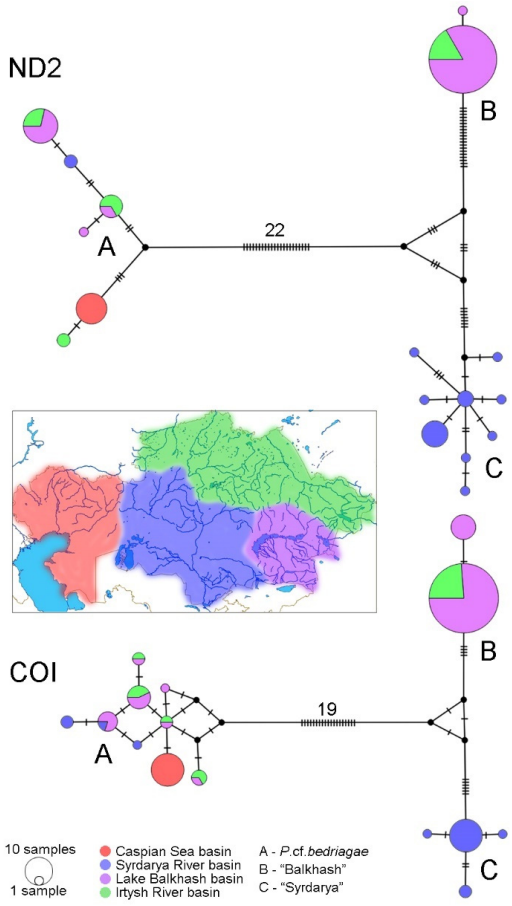

Figure 3. Median-joining haplotype networks of mtDNA lineages.

3.1.3. Nuclear DNA Phylogeny

Homozygous alleles of the SAI gene were processed in DnaSP [88,89]. The 26 haplotypes were generated and used to construct phylogenetic relationships. The best-fitting evolutionary model for the SAI data set was Jukes–Cantor [90], with the highest log likelihood score ln $L = -2274.71$.

Nuclear haplotypes of the studied marsh frogs were distributed among two main clades (Figure 4). The first well-supported clade (Hap 2–6, 11) seems to be conspecific to the Central Asian *P.* sp. *novum*. No differences between the Balkhash and Syrdarya lineages by the nuclear marker were found. This clade is sister to the Near East P. bedriagae and the Cilician marsh frog lineage. The second clade (Hap 1, 8–10, 13) contains haplotypes close to the Anatolian *P.* cf. *bedriagae* and the Karpathos *P. cerigensis*. In addition, two haplotypes (Hap 7, 12) were close to those of the European *P. ridibundus*. The haplotype network built only on the SAI sequences supported the division into marked clades and showed that in one third of the studied specimens (35% of the sample), there is a mismatch in the inheritance of mitochondrial and nuclear markers (Figure 4).

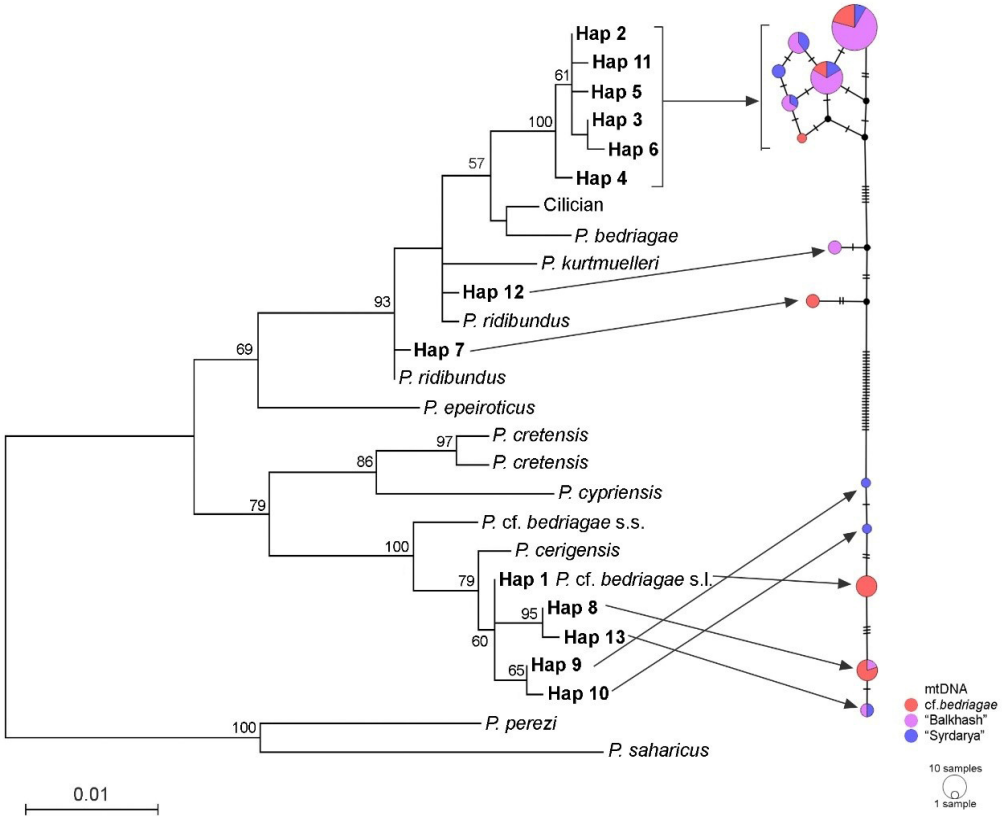

Figure 4. ML tree based on SAI sequences. Bootstrap supports lower than 50% are not shown on the tree. Median-joining haplotype network based on SAI of nDNA.

3.2. Distributional Pattern

Based on the nuclear DNA data, we revealed alleles of three species: the European *P. ridibundus*, the Anatolian *P.* cf. *bedriagae* and the Central Asian *P.* sp. *novum*. Alleles of the European *P. ridibundus* were found in only four localities (9% of studied localities). Homozygous alleles specific for the species were observed in two specimens from western Kazakhstan and a specimen from southeastern Kazakhstan (Figure 1, Table S2). Heterozygous alleles specific for the European P. ridibundus and the Anatolian *P.* cf. *bedriagae* were observed in four specimens from Atyrau City.

Alleles of the Anatolian *P.* cf. *bedriagae* were observed in 18 localities (41%) over the entirety of Kazakhstan, with the exception of the Syrdarya River drainage. Homozygous alleles

specific for the species were observed in 18 specimens from nine localities (Table S2). Heterozygous alleles specific for the Anatolian *P.* cf. *bedriagae* and the Central Asian *P.* sp. *novum* were observed in seven specimens from six localities in northeastern Kazakhstan.

Alleles of the Central Asian *P.* sp. *novum* were found in 33 localities (75%). Homozygous alleles specific for the species were observed in 44 specimens from 26 localities in southern and eastern Kazakhstan (Table S2). Heterozygous alleles specific for the Anatolian *P.* cf. *bedriagae* and the Central Asian *P.* sp. *novum* were observed in seven specimens from six localities in northeastern Kazakhstan.

Distribution of mtDNA haplotypes showed a similar pattern, except that haplotypes of the European *P. ridibundus* were not found (Figure 1).

3.3. Molecular Dating

According to a time-calibrated mtDNA tree, the marsh frogs of the Western and Eastern Palearctic diverged from their most recent common ancestor about 5.2 Mya (Figure S1).

Central Asian *P*. sp. *novum* (Balkhash, Syrdarya and Middle East lineages) were separated from the Cilician marsh frogs about 2.66 Mya (the Pliocene), whereas the split from *P. terentievi* (MHG7 Central Asia 1) occurred about 2.46 Mya. An approximate time of divergence within lineages of *P.* sp. *novum* took place about 1.57 Mya, which coincides with the Early Pleistocene epoch. The Syrdarya lineage separated from the Middle East lineage MHG9 about 1.17 Mya, while divergence within the Syrdarya clade occurred about 0.74 Mya. Within the Balkhash lineage the diversification took place at 0.28 Mya. The Anatolian lineage *P.* cf. *bedriagae* sensu lato diverged from the Euphrates lineage about 1.27 Mya, while the split on the main three subclades within this clade started at 1.1 Mya, respectively.

3.4. Genetic Structure and Demographic History According to mtDNA Data

The average p-distances between lineages of Central Asian marsh frogs and other lineages of *Pelophylax ridibundus* complex are given in the Table 1. The uncorrected pairwise distances between the Anatolian marsh frog and the Syrdarya and Balkhash lineages of *P.* sp. *novum* were 4.8% and 4.1%, respectively, and between last two lineages, was 2.7%.

Table 1. Uncorrected pairwise differences between lineages of marsh frogs of *P. ridibundus* complex based on ND2 gene sequences.

Group	1	2	3	4	5	6	7	8	9
2	0.047								
3	0.036	0.031							
4	0.067	0.079	0.070						
5	0.068	0.080	0.074	0.063					
6	0.061	0.079	0.073	0.072	0.075				
7	0.042	0.055	0.049	0.071	0.070	0.069			
8	0.013	0.046	0.036	0.064	0.066	0.061	0.040		
9	0.027	0.051	0.042	0.070	0.072	0.066	0.044	0.026	
10	0.036	0.027	0.019	0.069	0.069	0.075	0.046	0.036	0.042

1—*P.* cf. *bedriagae* (Anatolian sensu lato, Kazakhstan), 2—Balkhash lineage (=*P.* sp. *novum* = MHG8 Central Asia 2, Kazakhstan, NW China, Kyrgyzstan), 3—Syrdarya lineage (=*P.* sp. *novum*, Kazakhstan), 4—MHG1 (*P. ridibundus*, Central European), 5—MHG2 (*P. bedriagae*, Levant), 6—MHG3 (*P. cypriensis*), 7—MHG4-5 (Cilician West and East), 8—MHG6 (Anatolian forms, i.e., *P.* cf. *bedriagae* sensu stricto), 9—MHG7 (*P. terentievi* Central Asia 1, Iran, Turkmenistan, Uzbekistan), 10—MHG9 (Middle East, Southwest Iran).

The genetic diversity indices of the lineages are given in Table S3. The Anatolian *P.* cf. *bedriagae* and the Syrdarya lineages of *P*. sp. *novum* have a high level of haplotype diversity that does not differ statistically. The values of genetic variability decrease in

the direction of the Anatolian—Syrdarya—Balkhash lineages. The Balkhash lineage is characterized by minimal genetic variability compared to the other two ($p < 0.0001$).

For the Anatolian *P.* cf. *bedriagae*, the neutrality tests yielded insignificant positive values, indicating a lack of rare alleles or that the population experienced a bottleneck. The mismatch distribution analysis produced a multimodal graph, which indicates the stable population size over time (Figure 5, Table S3).

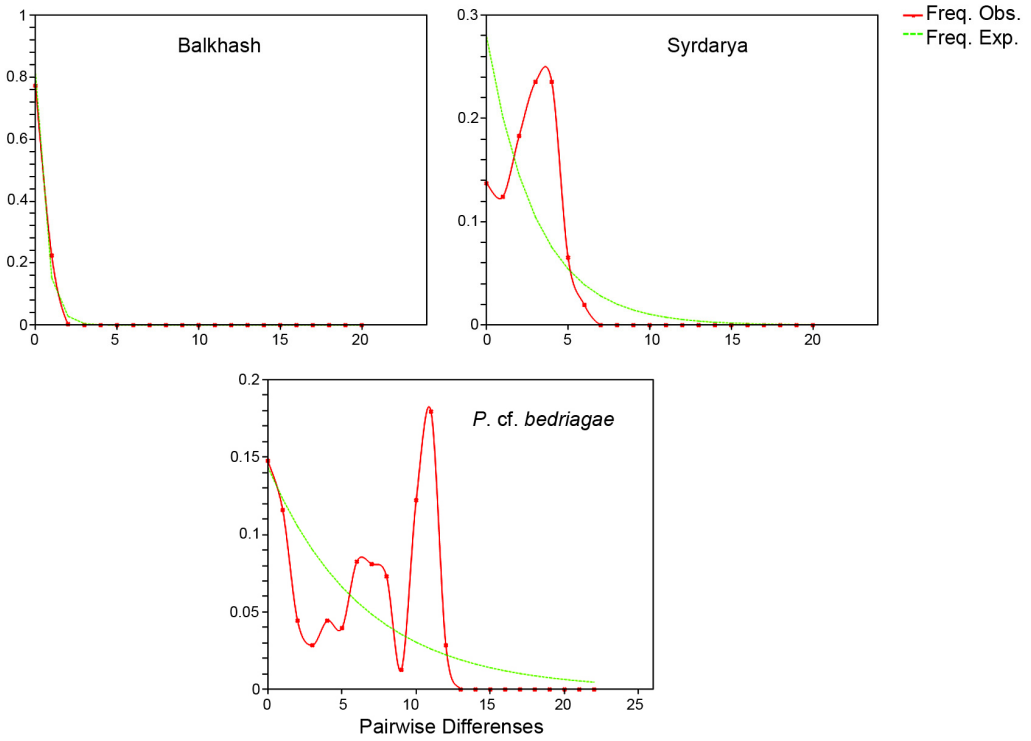

Figure 5. Mismatch distribution plots based on combined COI + ND2 of mtDNA datasets. Multimodal graph indicates demographic equilibrium and unimodal graphs often indicate population expansion.

The Syrdarya and Balkhash lineages of *P.* sp. *novum* showed insignificant negative values, which may suggest population stability over time. However, mismatch distribution analysis produced a unimodal graph in the Syrdarya lineage, suggesting past population expansion, while the Balkhash lineage went through recent population growth after a long period of stationary population.

4. Discussion

4.1. Species Distributional Ranges

Intensive sampling throughout Kazakhstan and adjacent regions of China allowed us to determine the local marsh frog species distributional ranges. Based on nDNA markers, it was established that representatives of three species (*P. ridibundus*, *P.* cf. *bedriagae* and *P.* sp. *novum*) inhabit the studied territory. In addition, according to mtDNA data, it was determined that *P.* sp. *novum* has an obvious geographical structuring and can be divided into the Syrdarya and Balkhash forms, which are strictly allopatric in Kazakhstan.

According to nuclear data, the "pure" native populations of *P. ridibundus* inhabit the western part of the Mangyshlak Peninsula only. However, the size of our sample (a single specimen) was too small to draw final conclusions. It should be noted that the mtDNA in this individual was inherited from *P.* cf. *bedriagae*. Introgressive combinations incorporating in the genome mtDNA of *P.* cf. *bedriagae* with nDNA of *P. ridibundus* are typical for populations inhabiting the Lower Volga River region, Crimea and Ciscaucasia [17,56–58,60,61].

According to previously published data [8,16,39], the "pure" native populations of the Anatolian *P.* cf. *bedriagae* were found in western Kazakhstan only. Mixed populations of *P. ridibundus* and *P.* cf. *bedriagae* (and presumably their hybrids) were observed in

the northeastern shore of the Caspian Sea (the Ural and Emba rivers drainages). This distribution of these two species in western Kazakhstan is very similar to that observed in the adjacent Lower Volga River region [17].

A single mixed population of *P. ridibundus* and *P.* sp. *novum* was observed in the Ketmen Ridge in southeastern Kazakhstan. The local invasive population of *P. ridibundus* could have arisen due to the accidental introduction of the species as a result of colonization of local fish farms with fish fry taken from Eastern Europe.

We found several "pure" populations of *P.* cf. *bedriagae* and mixed populations of *P.* sp. *novum* and *P.* cf. *bedriagae* (and their presumed hybrids) among numerous invasive populations located in eastern Kazakhstan (Figure 1). The distribution of *P.* cf. *bedriagae* in the region is associated with numerous accidental introductions associated with the stocking of reservoirs with fish and the release of these frogs which were used in local universities for educational purposes [42].

4.2. Balkhash and Syrdarya—mtDNA Clades of P. sp. *novum*

The uncorrected *p*-distance between Balkhash and Syrdarya sister clades is 3.1%. They are significantly differentiated from the marsh frogs from Cilician Turkey (5.5% and 4.9%, respectively) and from the Anatolian *P.* cf. *bedriagae* (3.7% and 4.6%).

The Balkhash lineage was first discovered in the cities of Almaty and Bishkek [8]. However, according to our data, these haplotypes are much more widespread in drainages of the Irtysh River and lakes Balkhash and Alakol (Figures 1 and 3), reaching the foothills of the Mongol Altay (86.47° E), thus inhabiting the eastern limits of the range of marsh frogs. We obtained haplotypes of the Syrdarya lineage for the first time. In Kazakhstan, marsh frogs of this lineage have so far been found only along the Syrdarya River from the headwaters to the estuary of the Aral Sea. As mentioned above, the isolation of the common ancestor of the Balkhash and Syrdarya lineages probably occurred in the Early Pleistocene (2.46 Mya), and their subsequent differentiation occurred in the Late Pleistocene. Apparently, factors were climatic conditions of the Akchagylian epoch (3.6–1.8 Mya) in the Late Pliocene–Early Pleistocene. This period was characterized by tectonic activities and a cooling aridization of the climate with formation of steppes and mountain landscapes [91,92]. For example, the ridges of the Northern and Central Tien Shan almost doubled during this period up to 2000–3500 m a.s.l. [93–95]. Moreover, transgression of the Caspian and Aral seas flooded over a vast territory of Central Asia, which could play a role of an isolating mechanism of the Syrdarya and Balkhash lineages [96,97].

4.3. Anatolian Marsh Frog (P. cf. *bedriagae)*

The Anatolian *P.* cf. *bedriagae* is one of the most widespread among the *P. ridibundus* complex. MtDNA haplotypes of the species in the eastern part of the range are assigned to northern Turkey, the Caucasus and Crimea [8,57,60]. They predominate over the haplotypes of the European *P. ridibundus* in Trans-Volga region of Russia [56]. In addition, *P.* cf. *bedriagae* is considered as an invasive species in many European countries: e.g., Italy, Belgium, France, Switzerland, Germany and Luxembourg [38,41,44,45,47–49]. Natural populations of the Anatolian *P*. cf. *bedriagae* were found in the western Kazakhstan region in the Caspian Sea drainage and Mugodzhary mountains. In the remaining territory of Kazakhstan, the Anatolian *P.* cf. *bedriagae* lives syntopically with the Balkhash lineage of *P.* sp. *novum*. In addition, it was found in two localities of the upper reaches of the Syrdarya River, in one of which, it co-occurs with the Syrdarya lineage of *P.* sp. *novum*. It is known that the introduced Anatolian *P.* cf. *bedriagae* can hybridize with native green frog species and displace them [44]. The rapid dispersal of *P.* cf. *bedriagae* over the range appears to be related to fishery activities [40,50].

4.4. Taxonomic and Conservation Implications

Owing to the continuous nature of evolution, species are inevitably undefinable as natural discontinuous units whenever the time dimension is taken into consideration.

Therefore, the most accurate way to reflect both the relativity of species and the duality of speciators in species delimitation is probabilistic [98]. Analysis of genomic landscapes of introgression across the hybrid zones of 41 pairs of frog and toad lineages in the Western Palearctic region showed that anuran speciation proceeds through a gradual accumulation of multiple barrier loci scattered across the genome [99]. Therefore, in the absence of direct evidence (e.g., hybridization experiments or study of hybrid zones), it was proposed to interpret genetic divergence estimates with respect to the overall probability to speciate P(S) [12]. Species were delimited based on the age of lineages, noting that cryptic Pleistocene lineages (younger than 2 Mya) are almost always conspecific [P(S) < 0.1], while Miocene lineages (older than 6 Mya) are almost always well isolated [P(S) > 0.9]. Lineages in between (Pliocene) fall into the so-called "grey zone of speciation", with an equal probability [P(S) = 0.5; about 4.3% by COI] to form narrow or wide hybrid zones, and thus, interspecific or intraspecific lineages, around 3 Mya. According to these data, all three main marsh frog lineages in Kazakhstan (*P. ridibundus*, *P.* cf. *bedriagae* and *P.* sp. *novum*), with a high degree of probability (P(S) $\geq$ 0.5), can be considered as independent species. Whereas the Syrdarya and Balkhash lineages of *P.* sp. *novum* do not reach the specific level, and, if their existence would be confirmed by other informative nuclear markers, they could be considered as distinct subspecies. However, it should be noted that mass introductions of various species of frogs can destroy isolating barriers between species and lead to despeciation, or vice versa, to the formation of homoploid hybridogenic species that can be better adapted to local conditions, which may ultimately lead to the displacement of local native species.

5. Conclusions

Central Asian marsh frogs are enigmatic amphibians that have not been fully studied on a genetic scale. The present study sheds light on the species composition of marsh frogs from Kazakhstan and Northwest China and allows us to trace the phylogeographical patterns of the potential speciation processes and the current phylogenetic relationships among all known local forms and species of the *Pelophylax ridibundus* complex. Based on a nuclear DNA marker, it was determined that three species (*P. ridibundus*, *P.* cf. *bedriagae* and *P.* sp. *novum*) inhabit the region. According to mitochondrial markers, three lineages (*P.* cf. *bedriagae* s.l. and the Central Asian *P.* sp. *novum* consisting of two Syrdarya and Balkhash lineages) were revealed. In populations of *P. ridibundus*, own mtDNA is replaced by that of *P.* cf. *bedriagae*. The inferred the Late Pliocene-Early Pleistocene origin of the Syrdarya and Balkhash lineages of *P.* sp. *novum* provides support for a vicariant process associated with the genesis of Northern and Central Tian-Shan Mountain ridges, severe aridification, and transgressions of the Aral-Caspian Basin.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/d14100869/s1, Figure S1: Divergence time of the matrilines of the marsh frogs *P. ridibundus* complex. The time shown next to node in italic (for the study lineages *P.* cf. *bedriagae,* Syrdarya and Balkhash of *P.* sp. *novum* in bold italic), Table S1: sampling information of mtDNA datasets; Table S2: sampling information of nDNA dataset; Table S3: molecular diversity indices and neutrality tests. References [8,9,39,43] are cited in the Tables S1 and S2.

Author Contributions: Conceptualization, T.N.D., O.A.E., X.G. and D.U.; Methodology, D.U., O.A.E. and X.G.; Software, D.U. and A.Y.I.; Validation, O.A.E. and X.G.; Formal analysis, D.U., R.X., J.L. and F.X.; Investigations, A.G.K. and I.I.A.; Resources, R.X., J.L., F.X., V.A.K., V.N.K. and F.S.; Data curation, T.N.D., O.A.E. and X.G.; Writing—original draft preparation, D.U.; writing—review and editing, O.A.E., S.N.L. and X.G.; Visualization, D.U.; Supervision, O.A.E. and X.G.; Project Administration, T.N.D.; Funding Acquisition, T.N.D. and X.G. All authors have read and agreed to the published version of the manuscript.

Funding: The work was supported by the grant project of the Ministry of Education and Science of the Republic of Kazakhstan "Genetic polymorphism and ecological plasticity as the basis of evolutionary welfare and progressive settlement of the marsh frogs of the *Pelophylax ridibundus*

complex in Kazakhstan" for 2020–2022 (No. AP08856275) and the Third Xinjiang Scientific Expedition Program (Grant No. 2021xjkk0600). S.N.L. was funded by RFBR.20-04-00918.

Institutional Review Board Statement: The study was conducted under the permition of the Committee of Forestry and Wildlife of the Ministry of Ecology and Natural Resources of the Republic of Kazakhstan (27-3-16/2983-CFW from 25.04.2022), and the Institutional Review Board (or Ethics Committee) of Republican State Enterprise on the Rights of Economic Management "Institute of Genetics and Physiology" of the Committee of Science (of the Ministry of Education and Science of the Republic of Kazakhstan (protocol №5, code 12-222 from 04.08.2022) for studies involving animals.

Data Availability Statement: All sequences in this study were deposited to the GenBank (ON796820-ON796944); (ON809573-ON809697); (ON856262-ON856335).

Acknowledgments: The authors are grateful to Chirikova M.A., Liu J.L., Eszhanov A.B., Gorelov R.A., Khamitov A.Zh., Mushtakov D.N., Nigmatullina A.K., Nurpeiskizi G., Pachin S.A., Pazylbekov M., Sayakova Z.Z., Shokaev V.A., Spirina T.V., Starikov S.V., Tarasovskaya N.E., Timoshenko A., and Titov S.V. for participation in field research and assistance in collecting material; Lukonina S.A. for assistance in lab analysis and two anonymous reviewers for their helpful comments on an earlier draft of this paper.

Conflicts of Interest: The authors declare no conflict of interest.

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