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Detection of Glacial Refugia and Post-Glacial Colonization Routes of Morphologically Cryptic Marsh Frog Species (Anura: Ranidae: *Pelophylax*) Using Environmental Niche Modeling

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Abstract: Studying the distribution of morphologically cryptic animal species is always a very difficult task. Because most marsh frog species (the *Pelophylax ridibundus* complex) are cryptic, we used molecular markers to identify them. Three marsh frog species (*P. ridibundus*, *P. kurtmuelleri* and *P. cf. bedriagae*) inhabit the northern part of Western Palearctic. We created a database of localities and built models of their modern distribution. These models showed that the most suitable habitats are on the north of the Mediterranean region for *P. cf. bedriagae*, temperate Europe for *P. ridibundus*, and the Balkan coastal areas for *P. kurtmuelleri*. The projection of the modern ecological niches under the late-Quaternary climatic conditions showed that the range of *P. kurtmuelleri* remained largely unchanged during the period, whereas the ranges of *P. cf. bedriagae* and especially *P. ridibundus* changed greatly over time. During the Last Glacial Maximum, the presumed range of *P. cf. bedriagae* covered a relatively large area in the north of the Mediterranean region and the south of European Russia. Glacial refugia of *P. ridibundus* were apparently located in the northern Balkans, the northern coast of the Black and Azov seas, and possibly in Western Europe. The northward long-distance post-glacial dispersal of *P. ridibundus* occurred from refugia in the northeastern Balkans and the Black-Azov seas region. Since the Late Pleistocene, suitable habitats for *P. cf. bedriagae* in southern Russia began to decline, but local habitats for *P. ridibundus* become more suitable. Therefore, a mosaic of populations consisting of these both species and their hybrids has now been found here.

Keywords: ecological modeling; West Palearctic; hybridization; asymmetric mitochondrial introgression; Holocene; Pleistocene



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1. Introduction

Analysis of species distribution is very important for studying the processes of speciation and the formation of ecological niches, as well as their changes under the influence of various environmental factors [1]. Ecological niche models of species distributions are becoming increasingly popular and accurate [2]. They are used to find the most suitable habitats for species and to predict the expansion and contraction of a species' range under climate change [3,4]. Models have been used to understand the ecological requirements, study niche segregation, and facilitate field work by predicting potential distribution areas and improving their conservation, and for many other purposes [5]. The modeling can also determine the boundaries of both past and future ranges of species [6–8]. For example, the question of how the Quaternary glaciations influenced changes in biota is of significant scientific interest, and modeling can reconstruct the dynamics of species distributions in the late Quaternary [9–11].

Marsh frogs of the *Pelophylax ridibundus* complex are a group widespread in the Western Palearctic from northwestern Spain, France, and Great Britain in the west to the Kamchatka peninsula in the east and from the outskirts of St. Petersburg (Russia) in the north to West China, Afghanistan, Pakistan, Saudi Arabia, North Syria, East Libya, Malta, and Sardinia in the south [12–16]. The frogs inhabit various biotopes including forest, bushland, meadow, forest-steppe, steppe, semi-desert, and desert and survive well in anthropogenic and agricultural landscapes. The complex composition has not yet been sufficiently studied. At the moment, it includes about a dozen described and undescribed taxa. According to phylogenetic relationships and distribution patterns, the complex can be divided into western and eastern groups [17,18]. The western group includes *P. ridibundus* (Pallas, 1771) and *P. kurtmuelleri* (Gayda, 1940). The eastern group consists of numerous species and lineages, namely *P. cypriensis* Plötner, Baier, Akn, Mazepa, Schreiber, Beerli, Litvinchuk, Bilgin, Borkin, and Uzzell, 2012, *P. caralitanus* (Arikan, 1988), *P. cerigensis* (Beerli, Hotz, Tunner, Heppich, and Uzzell, 1994), *P. persicus* (Schneider, 1799), *P. terentievi* (Mezhzherin, 1992), the Anatolian (“*P. cf. bedriagae*”), Cilician, Euphrates, Iranian, Balkhash, and Syrdarya lineages [17–23]. Because most of these species are morphologically cryptic, boundaries of their ranges are extremely insufficiently studied. A particular difficulty is that various species of the complex are actively introduced into new regions, because they are used for food, as laboratory animals, or carried by tadpoles when water bodies are artificially stocked with fish [24–26].

Phylogenetic relationships in this complex have been repeatedly studied [17–19,22,27–30]. However, only one environmental modeling study has been conducted that examined the range changes history of *P. kurtmuelleri* from the late Pleistocene to the present [31]. However, the main problem with this study was that the analysis was carried out using data on the distribution of mitochondrial DNA (mtDNA) haplotypes, which often, due to asymmetric introgression, provide highly distorted data on the position of the species’ range boundaries [32].

High spatial resolution paleoclimate surfaces for global land areas, such as WorldClim [33] and PaleoClim [34], provide a good opportunity for tracing shifts in range boundaries of various species during the late Quaternary. Amphibians and marsh frogs in particular are very suitable subjects for reconstructing their past distribution dynamics using ecological niche modeling because they are strongly associated with suitable terrestrial or semi-aquatic habitats and are not capable of long-distance migrations (like, for example, some birds). Using the method of the multiplex PCR for identification of cryptic marsh frog species, we studied vast areas of the East European Plain to identify patterns of their distribution and revealed here alleles of three marsh frog species (*P. ridibundus*, *P. kurtmuelleri* and *P. cf. bedriagae*). Therefore, the aims of our study were (1) to define recent ranges of these three species and (2) to identify the positions of their glacial refugia and post-glacial colonisation routes, using ecological niche modeling methods.

2. Materials and Methods

We used PCR-based analyses to identify three cryptic marsh frog species. In order to exclude the effect of mtDNA asymmetric introgression, we used only nuclear DNA (nuDNA) data for niche modeling. To determine species-specific nuDNA alleles, we analyzed the intron-1 of the nuclear serum albumin gene (SAI-1) fragments using the methods described by Hauswaldt et al. [35] and Ermakov et al. [36]. In total, we studied 389 specimens from 156 localities. Thus, the total database, including our and previously published nuDNA data, consists of 694 localities (Table S1). As it turned out, 240 populations were of hybrid origin and were excluded from the niche modeling analysis. “Pure” populations (>75% own alleles) were observed in 454 localities: 243 *P. cf. bedriagae*, 29 *P. kurtmuelleri*, and 182 *P. ridibundus*. Invasive populations beyond the native distributional ranges, as well as species and lineages closely related to *P. cf. bedriagae* (such as *P. caralitanus*, *P. cerigensis*, *P. persicus*, and others) were not included in our modeling analysis.

Studying the distribution of mtDNA haplotypes is very useful for analyzing changes in the past ranges, especially in hybridizing species [37]. Discordant patterns of introgression of mtDNA haplotypes and nuDNA alleles across a hybrid zone were observed in many species [38,39]. A variety of processes (e.g., natural selection associated with the environment or adaptive introgression, sex-biased dispersal, asymmetric reproductive isolation, and stochasticity associated with the increased effects of drift on maternally inherited mtDNA), can explain these discordances [40]. An asymmetric mtDNA introgression can often indicate changes in the position of the hybrid zone in the past. If one hybridizing species displaces another, their hybrid zone will move, and such hybrid zones can move significant distances [41].

We used the method described by Ermakov et al. [36] to separate the mtDNA (fragments of the subunit 1 of the cytochrome-c-oxidase gene) of *P. cf. bedriagae* from the other two marsh frog species. To identify haplotypes of *P. ridibundus* and *P. kurtmuelleri*, we used an endonuclease restriction analysis [33] or the multiplex PCR of the NADH dehydrogenase subunit 2 (ND2) gene fragments with two species-specific forward primers (for *P. ridibundus* ND2Pr 5'-TAT AAT CGC GGT GAC TGG GA-3' and for *P. kurtmuelleri* ND2Pk 5'-GGG TCA GGC TTG GAT TAA ATT TT-3') and a single reverse primer (ND2R1 5'-GCT TTT GGG CCC ATA CCC-3'). Summarizing all of our data and the previously published data [42–82], the total results included in our database of both the nuclear (nuDNA) and mtDNA gene analyses consisted of 1176 localities and 5234 specimens (Figure 1; Table S1).

As a tissue sample, we used a toe fixed in a 96% alcohol solution. The samples are stored in the tissue collections of the Institute of Cytology of Russian Academy of Sciences (St. Petersburg, Russia) and the Penza State University (Penza, Russia). For the extraction of DNA, we used the DNA-Extran-2 kit (Syntol, Moscow, Russia); for PCR, we used the 2X Taq M master mix green kit (Alkor Bio, St. Petersburg, Russia). The PCR mixture (50 µL) contained 50–100 ng of DNA, 0.5 µM of each primer, and 25 µL of 2X Taq M master mix green kit. The following method for the amplification ND2 gene species-specific fragments was used: initial denaturation for 15 min at 95 °C, followed by 36 cycles of 94 °C for 30 s, 64 °C for 30 s, 72 °C for 30 s, and a final extension for 10 min at 72 °C. This method allowed receiving PCR products with lengths of 811 bp for *P. ridibundus* and 431 bp for *P. kurtmuelleri*. We used an electrophoresis in polyacrylamide gel to determine the differences between amplified species-specific fragments.

To predict ecological niches, current and past (the late-Quaternary) ranges of three marsh frog species, we constructed species distribution models. We applied recent methodological recommendations to compute robust ecological niche models with MaxEnt 3.4.4 [2,83], such as occurrence filtering (with MaxEnt), using multiple combinations of model parameters (features and regularization multipliers), and multiple statistical criteria for model selection.

Altitude and 19 bioclimatic layers representative of the climatic data over ~1950–2000 were extracted from the WorldClim database (<http://www.worldclim.org>, accessed on 26 June 2023) to build the model under the current climatic conditions. Ten additional layers were considered: the aridity index (Global Aridity and Potential Evapo-Transpiration; <http://www.cgiar-csi.org/data/global-aridity-and-pet-database>, accessed on 26 June 2023), the global percent of tree coverage (https://github.com/globalmaps/gm_ve_v1, accessed on 26 June 2023), and eight land cover variables (spatial homogeneity of global habitat, broadleaf forests, needle leaf forests, mixed forests and shrubs, and barren, herbaceous, and cultivated vegetation; <https://www.earthenv.org/>, accessed on 26 June 2023). To consider topography in the model, two landscape layers were calculated with QGIS: terrain roughness and terrain roughness index. Layers had a 30 arc seconds spatial resolution, and all analyses were conducted under the WGS 84 projection with a species-specific mask covering to the area of occurrence of these three marsh frog species and adjacent regions (from 30° N to 63° N and 6° W to 62° E).

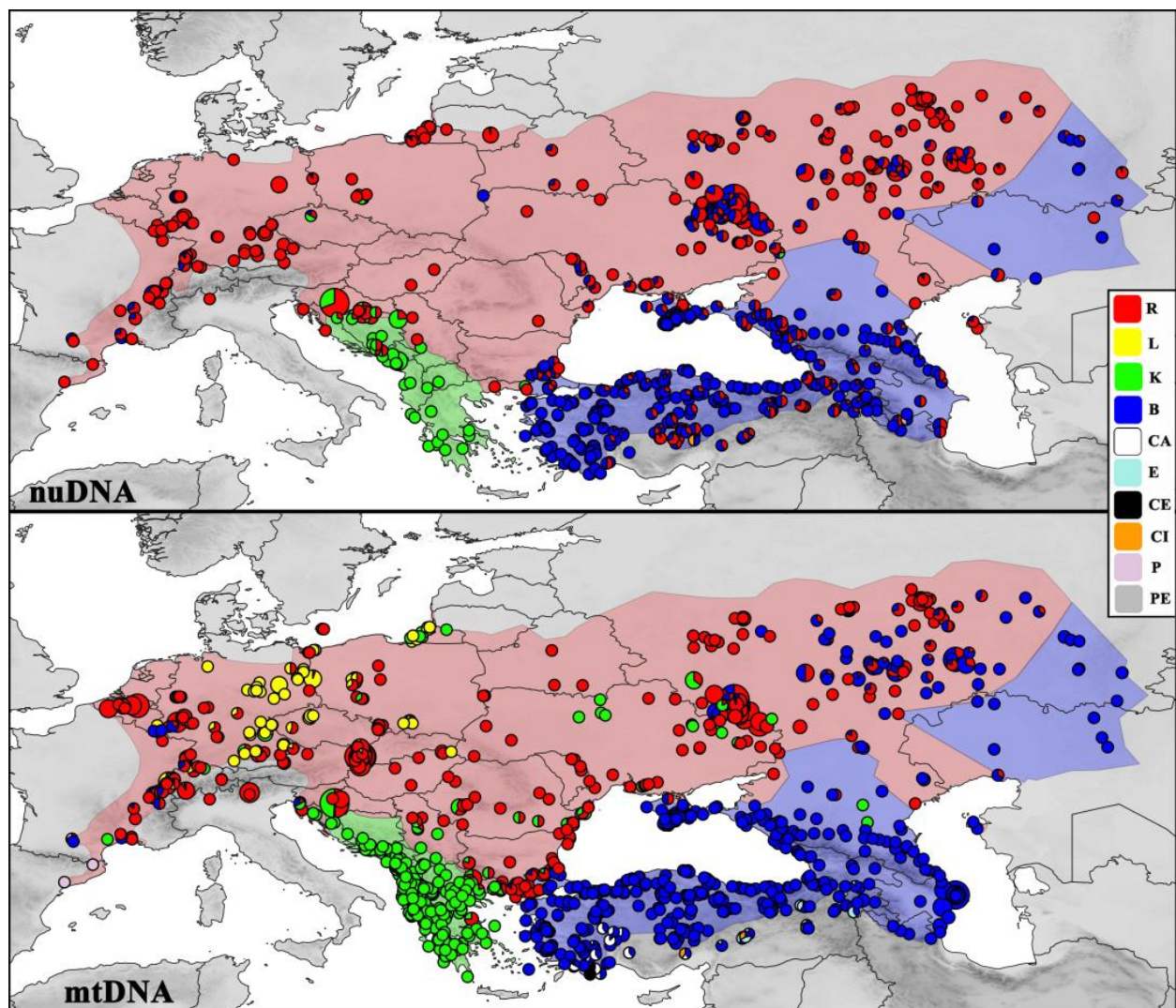


Figure 1. Distribution of alleles (nuDNA) and haplotypes (mtDNA) of water frog species in the Western Palearctic. The range of *P. cf. bedriagae* is designated by the blue area, *P. ridibundus* by the red area, and *P. kurtmuelleri* by the green area. B is *P. cf. bedriagae*, R is *P. ridibundus*, K is *P. kurtmuelleri*, L is *P. lessonae*, CA is *P. caralitanius*, CE is *P. cerigensis*, PE is *P. persicus*, P is *P. perezi*, E is the Euphrates lineage, and CI is the Cilician lineage. Pie diameter reflects the sample size. Other details are in Table S1.

Before generating the model, we calculated Pearson's correlation coefficients for all pairs of 19 bioclimatic variables using ENMTools [84] to eliminate predictor collinearity. For correlated pairs ($|r| > 0.75$), we excluded the variable that appeared the least biologically important for marsh frogs. The resulting dataset contained seven bioclimatic variables: Bio 2 (mean diurnal range; $^{\circ}\text{C} \times 10$), Bio 4 (temperature seasonality; $\text{CV} \times 100$), Bio 8 (mean temperature of wettest quarter; $^{\circ}\text{C} \times 10$), Bio 9 (mean temperature of driest quarter; $^{\circ}\text{C} \times 10$), Bio 14 (precipitation of driest month; mm), Bio 15 (precipitation seasonality; CV), and Bio 19 (precipitation of coldest quarter; mm). A jackknife analysis was used to estimate the relative contributions of variables to the MaxEnt model.

We ran the MaxEnt for 10 replicates with 30% random test percentage testing. For the calibration, we evaluated models created with distinct regularization multipliers (0.5 to 6 at intervals of 0.5) and feature classes (resulted from all combinations of linear, quadratic, product, threshold, and hinge response types). The number of background points was 20,000. The best parameter settings of statistical significance (partial ROC), predictive power (omission rates $E = 5\%$), and complexity level (AICc) obtained using the R package

kuenm [85] were applied to build the models. Additionally, the model performance was evaluated using the Area Under the Curve (AUC; ranging 0–1) and the True Skill Statistic (TSS; ranging from –1 to +1) of the 10-percentile training omission threshold [86]. The ClogLog output format (ranging 0–1) was chosen for processing the resulting maps [87]. The altitudes for each locality were extracted using the QGIS Point Sampling Tool Plugin (<https://plugins.qgis.org/plugins/pointssamplingtool/>, accessed on 26 June 2023).

To project the current ecological niches of three marsh frog species on climate conditions during the late Quaternary, we applied seven sets of uncorrelated bioclimatic layers (Bio 2, Bio 4, Bio 8, Bio 9, Bio 14, Bio 15, and Bio 19) with a 2.5 arc minutes spatial resolution. Bioclimatic layers representative of the climatic data over 4.2–0.3 ka (Late Holocene), 8.3–4.2 ka (Mid Holocene), 11.7–8.3 ka (Early Holocene), 12.9–11.7 ka (Late Pleistocene, Younger Dryas Stadial), 14.7–12.9 ka (Late Pleistocene, Greenland Interstade 1), 17.0–14.7 ka (Late Pleistocene, Heinrich Stadial 1), and ~21 ka (Last Glacial Maximum) were extracted from the PaleoClim database (<http://www.paleoclim.org/>, accessed on 26 June 2023).

3. Results

3.1. Current Distribution of Water Frog Species

The native range of *P. ridibundus* covers most of Europe from northeastern Spain and eastern France in the west to the eastern part of the Volga-Kama Region in Russia (Permskiy Kray and Bashkortostan) in the east, and from the ~55–57° N parallel in the north to the northern foothills of the Alps and Balkans, the northern coastal of the Black and Azov seas, and the Volga River delta in the south (Figure 1). Isolated populations are known in the Mangyshlak peninsula (West Kazakhstan), the southern foothills of the Alps (Switzerland) and Bornholm Island (Denmark). The species inhabits plains and foothills (median 113 m a.s.l.) from the Caspian Sea level (–27 m a.s.l.) up to ~1552 m a.s.l. in the French Alps. The majority of localities (98%) are scattered below 1000 m a.s.l.

It is important to note that “alien” nuDNA alleles of *P. kurtmuelleri* and *P. cf. bedriagae* were observed almost throughout the entire range of *P. ridibundus*. Additionally, in many populations of *P. ridibundus*, we found alien mtDNA haplotypes of various water frog species. Moreover, numerous populations of *P. ridibundus* in the east were characterized by only *P. cf. bedriagae* mtDNA haplotypes (Figure 1). Among the populations of *P. ridibundus*, the amount of nuDNA alleles and mtDNA haplotypes of *P. cf. bedriagae* was significantly higher in the east than in the west (nuDNA: $r = 0.230$, $df = 1.180$, $p = 0.002$; mtDNA: $r = 0.397$, $df = 1.164$, $p = 0.000$). Additionally, numerous populations of *P. ridibundus* in Central Europe were characterized by mtDNA haplotypes of *P. lessonae* (Camerano, 1882). Therefore, the number of these mtDNA haplotypes was significantly greater in the west than in the east ($r = 0.369$, $df = 1.164$, $p = 0.000$). Mitochondrial haplotypes of *P. perezi* (López-Seoane, 1885) were observed in southwestern populations (Spain) of *P. ridibundus* only.

The Balkan marsh frog (*P. kurtmuelleri*) inhabits Greece (except for the easternmost areas), Albania, Macedonia, Montenegro, southern Serbia, Bosnia and Herzegovina, and southern Croatia (Figure 1). Isolated populations are known in islands of Croatia, Montenegro, and Greece. The species inhabits foothills from the sea level up to ~802 m a.s.l. (in median 97 m a.s.l.). Most localities (83%) are scattered below 500 m a.s.l. In some cases, nuDNA alleles of the species (3–67%) were observed far beyond the species range within the populations of *P. ridibundus* (Table S1). For example, such localities were revealed in Russia, Lithuania, Poland, and the Czech Republic. Mitochondrial haplotypes of *P. kurtmuelleri* were widely scattered through the range of *P. ridibundus*, and were also found in Thracian populations of *P. cf. bedriagae* (Table S1).

The native range of *P. cf. bedriagae* covers the western and northern parts of Turkey and nearby regions of Greece, northwestern Iran, the Caucasus, the Crimea, western Kazakhstan, and some regions in the south of European Russia (Figure 1). Isolated populations are known in the Greek islands. The species was recorded on plains and foothills from the Caspian Sea level up to ~2273 m a.s.l. in Turkey (median 359 m a.s.l.). The majority of the

localities (82%) were scattered below 1500 m a.s.l. Several populations of the species were revealed in Ukraine and Russia, far beyond the species range (Table S1).

High admixtures of *P. cf. bedriagae* and “*P. ridibundus*” nuDNA alleles were observed in the southern part of the *P. cf. bedriagae* range in Turkey (Figure 1), but it should be noted that *P. caralitanus* and the Euphrates lineage are characterized by a presence of the same SAI-1 alleles as *P. ridibundus*. Also we revealed that among populations of *P. cf. bedriagae*, the amount of nuDNA alleles and mtDNA haplotypes of *P. ridibundus* was significantly higher in the north than in the south (nuDNA: $r = 0.172$, $df = 1.241$, $p = 0.007$; mtDNA: $r = 0.141$, $df = 1.241$, $p = 0.028$). Mitochondrial haplotypes of *P. kurtmuelleri* were only found in the north and west parts of the *P. cf. bedriagae* range, but *P. cerigensis*, *P. caralitanus*, *P. persicus*, and the Euphrates and Cilician lineages were observed in south only (Figure 1; Table S1).

3.2. Distribution Modeling of Species under the Current Environmental Conditions

To calibrate models, we assessed 372 replicates for each species. All of them were statistically significant when compared with a null model of random prediction. Of these significant models, no model met the omission criterion of 5%, and only a single (*P. cf. bedriagae*) model and two (*P. kurtmuelleri*) or three (*P. ridibundus*) models were statistically significant among the models meeting AICc criteria. Performance metrics for the parameter settings used for creating the final models are given in Table S2. Barren land, mean temperature of driest quarter (Bio 9), and mean temperature of wettest quarter (Bio 8) were the variables with the highest percentage contributions (39%, 31%, and 11%, respectively) among the parameters included in the *P. cf. bedriagae* model. The average AUC and TSS evaluations were 0.881 (SD = 0.014) and 0.419 (SD = 0.070) indicating a good predictive power of the final model. Of the parameters included in the *P. ridibundus* model, precipitation of driest month (Bio 14), habitat homogeneity, and cultivated vegetation were the variables with the highest percentage contributions (24%, 20%, and 16%, respectively). The average AUC and TSS evaluations were 0.918 (SD = 0.010) and 0.529 (SD = 0.028) indicating a high predictive power of the final model. Precipitation of the coldest quarter (Bio 19), mean temperature of driest quarter (Bio 9), and barren land were the variables with the highest percentage contributions (37%, 20%, and 12%, respectively) in the *P. kurtmuelleri* model. The average AUC and TSS evaluations were 0.981 (SD = 0.008) and 0.781 (SD = 0.076), indicating a high predictive power of the final model.

The averages of the selected model for *P. cf. bedriagae* identified areas with high levels of suitability across the Balkan, Anatolian, and Crimea peninsulas; most Mediterranean islands; the Caucasus; northern Iran; and some regions in the south of European Russia and northwestern Kazakhstan (Figure 2A). Some regions were also quite suitable (e.g., in North Africa, Western Europe, the Apennine peninsula, and Middle Asia), but native populations of the species were absent here. The suitability of habitats for *P. cf. bedriagae* declined toward the temperate zone of Europe. The model for *P. ridibundus*, in contrast, identified suitable areas in temperate Europe, as well as some regions in north of the Apennine, Balkan, and Crimea peninsulas and the Caucasus (Figure 2B). The model for *P. kurtmuelleri* identified suitable areas across the Balkan peninsula (Figure 2C). Some additional areas (e.g., in North Africa; Western Europe; the Apennine, Anatolian and Crimea peninsulas; most Mediterranean islands; the Caucasus; and North Iran), where native populations of *P. kurtmuelleri* are currently absent, displayed suitable habitats as well.

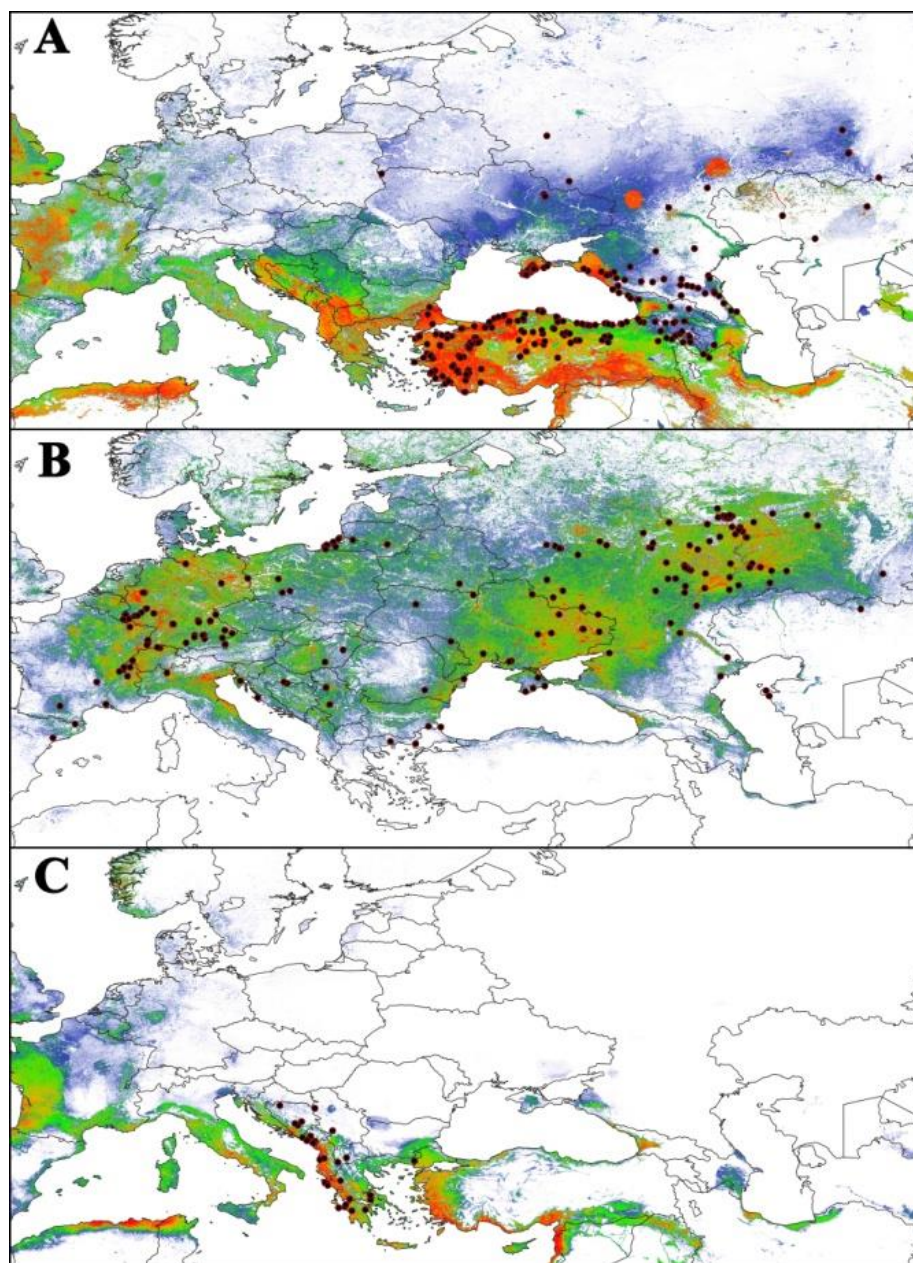


Figure 2. Average predictions of suitable areas for *Pelophylax cf. bedriagae* (A), *P. ridibundus* (B), and *P. kurtmuelleri* (C) distributions under the current climate conditions. All areas with red (high suitability), green (medium), and blue (low) colors likely represent suitable environmental conditions. Black dots are the localities of each species used in the construction of ecological niche models.

3.3. Distribution of Species at the Late-Quaternary

In order to consistently analyze shifts in the past range boundaries of three marsh frog species, we used projections of modern ecological niches onto climatic conditions of the late Quaternary. The presumed range of *P. cf. bedriagae* covered a relatively large territory during the Last Glacial Maximum (~21 ka), mostly coinciding with the recent range of the species' distribution (Figure 3). In addition, the species appears to have been more widely distributed in the Eastern Balkans, and areas with suitable conditions were even located along the Po River valley and the dry bottom of the northern Adriatic. During warming at the end of the late Pleistocene (17.0–11.7 ka), suitable areas for *P. cf. bedriagae* strongly contracted in the Caucasus but expanded in the western Balkans, Apennines, and even Western Europe (Figure 3). In the Holocene, the situation began to change in the opposite

direction. The potential range of *P. cf. bedriagae* in the west shrank, but in the east, the species' distribution became wider. In general, however, in the Holocene the boundaries of the range have not changed much.

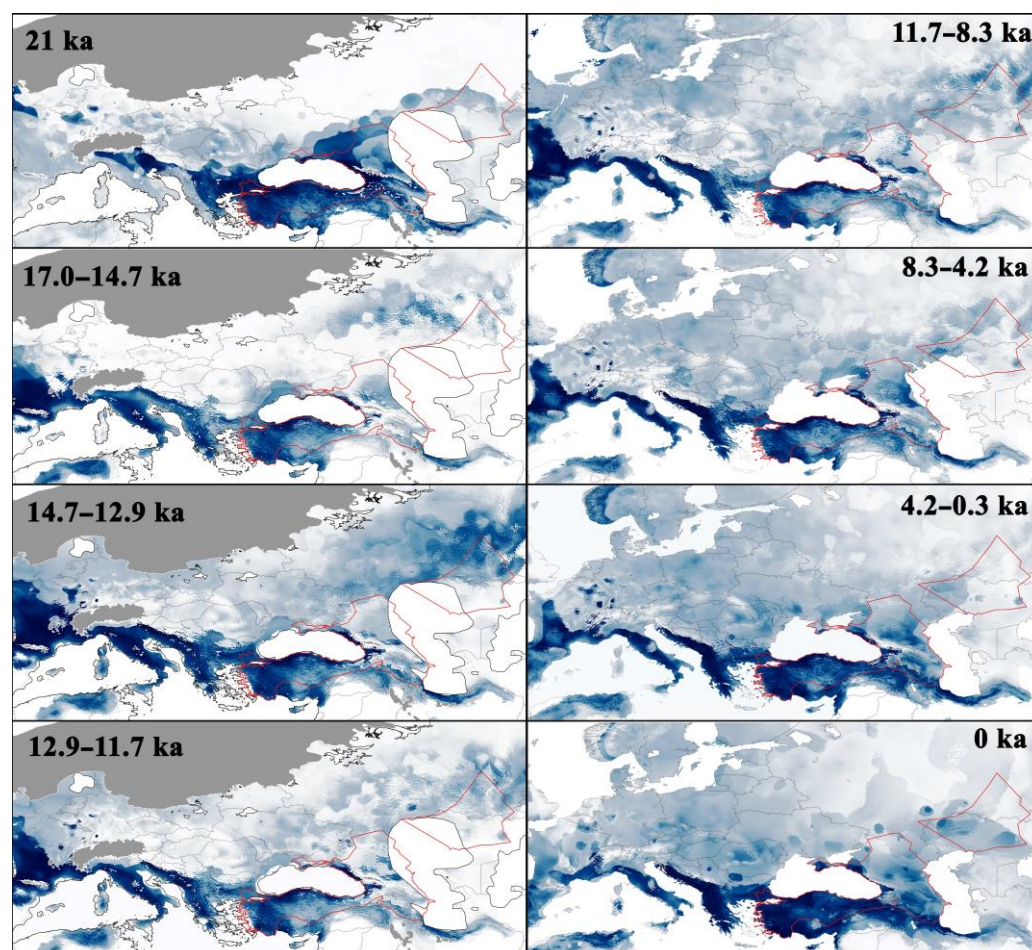


Figure 3. Predicted suitable regions for *Pelophylax cf. bedriagae* distribution under the Last Glacial Maximum (~21 ka), the end of the late Pleistocene (17.0–11.7 ka), and the Holocene (11.7–0 ka) climatic conditions. The degrees of habitat suitability (dark is high-suitable, and light is little-suitable) correlates with the intensity of the blue color. Territories covered by glaciers are in grey. The red lines indicate the limits of the current range of the species.

During the Last Glacial Maximum (~21 ka) the presumed range of *P. ridibundus* covered the northern Balkans, the northern Black-Azov seas region, the western Caucasus, and the south of European Russia, as well as perhaps some areas in Western Europe (Figure 4). During the warmer Heinrich Stadial 1 (17.0–14.7 ka), the presumed range limits of *P. ridibundus* were further reduced and covered only some areas in France, the northern Adriatic, the northern Balkans, and the northern Black-Azov Sea region (Figure 4). However, the northward expansion of species (especially in the Azov Sea region) already began at the end of the Pleistocene (14.7–11.7 ka). Since the beginning of the Holocene (11.7 ka), the range of *P. ridibundus* apparently began to rapidly expand northward and reached its recent margins around the border of the early and mid-Holocene (8.3 ka).

During the late Quaternary, the presumed range of *P. kurtmuelleri* was located in the Balkan coastal areas and remained largely unchanged during the period (Figure 5). Apparently, only the degree of habitat suitability in eastern Greece and the Dinaric Alps has changed repeatedly.

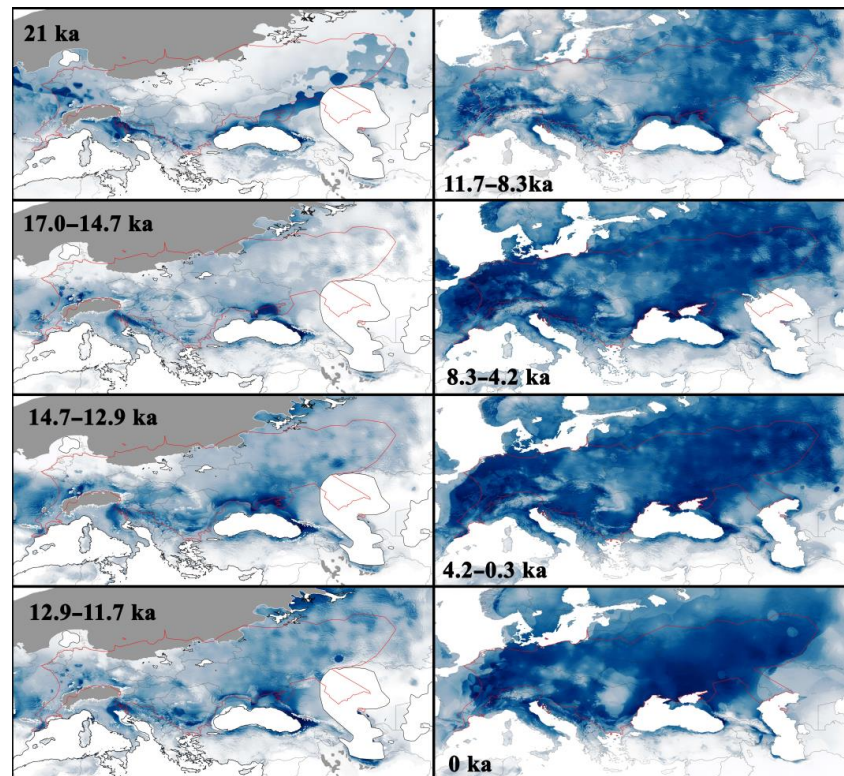


Figure 4. Predicted suitable regions for *Pelophylax ridibundus* distribution under the Last Glacial Maximum (~21 ka), the end of the late Pleistocene (17.0–11.7 ka), and the Holocene (11.7–0 ka) climatic conditions.

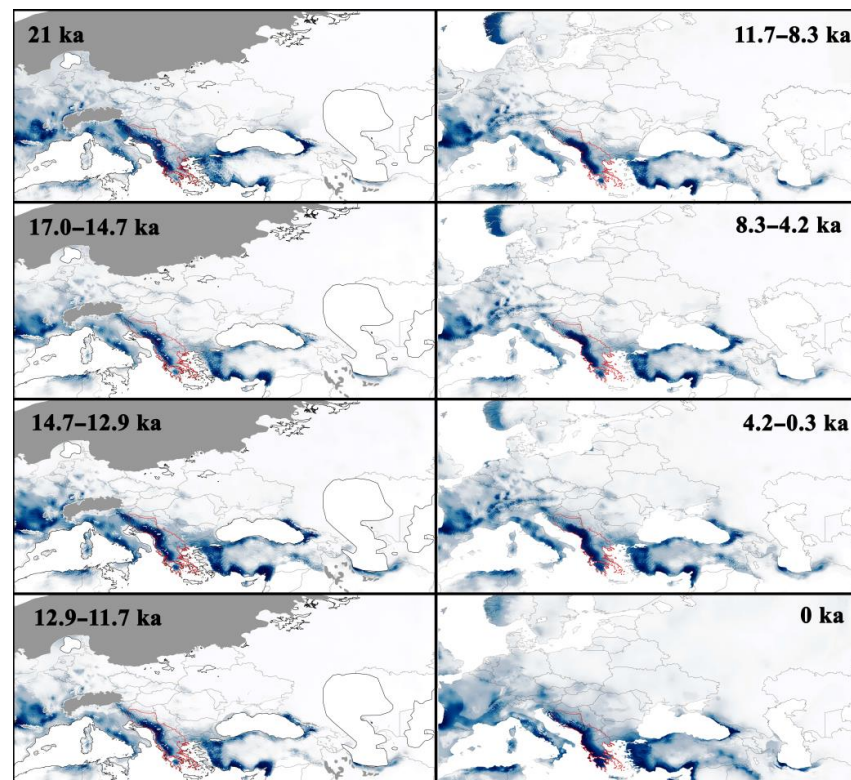


Figure 5. Predicted suitable regions for *Pelophylax kurtmuelleri* distribution under the Last Glacial Maximum (~21 ka), the end of the late Pleistocene (17.0–11.7 ka), and the Holocene (11.7–0 ka) climatic conditions.

4. Discussion

The recent range of *P. ridibundus* extends over a vast territory of temperate Europe (Figure 1). However, it should be noted that in many populations of the species, the introgression of nuDNA genes of closely related *P. cf. bedriagae* and *P. kurtmuelleri* was observed (Table S1). This may be due to the spread of nuDNA genes from populations located near hybrid zones and recent human-mediated introductions (over the whole range). In some regions, the widespread mtDNA introgression of *P. lessonae* and *P. perezi* genes has been observed and associated with the participation of local *P. ridibundus* in the hemiclinal reproduction of hybridogenous species, such as *P. esculentus* (Linnaeus, 1758) and *P. grafi* (Crochet, Dubois, Ohler, and Tunner, 1995), respectively [12,62]. Moreover, the emergence of some isolated populations of *P. ridibundus* outside the main species range (e.g., southern Switzerland) may be associated with hybridolysis in the *P. esculentus* populations [26,75].

In some parts of the range of *P. ridibundus*, we did not find own mtDNA, but only alien mtDNA (Figure 1; Table S1). For example, populations of the species in Kaliningrad province (the Baltic part of Russia) were characterized by only mtDNA haplotypes of *P. lessonae* and *P. kurtmuelleri*. It is important to note that the native range of *P. kurtmuelleri* is many hundreds of kilometers away from this area. This situation is likely related to the post-glacial expansion into the region of refugee populations of *P. ridibundus*, which are characterized by *P. kurtmuelleri* mtDNA. Most likely, such refugee populations of *P. ridibundus* were located in the northern Balkans. This assumption is also supported by our niche modeling data, which show that the Balkans were suitable for the habitat of *P. ridibundus* and *P. kurtmuelleri* in the Last Glacial Maximum and the end of the late Pleistocene (Figure 4). At the Younger Dryas Stadial (12.9–11.7 ka), the refugee populations of *P. ridibundus* could have spread north along the Carpathians, reaching the Baltic Region at the Early Holocene (Figure 6). Additional evidence of this post-glacial dispersal of *P. ridibundus* is provided by Early Holocene paleontological records in Moldova and western Ukraine [88]. The paleontological records dating from the end of Early Holocene are also known from northern Germany [89–91]. A similar post-glacial colonization route was considered for green toads of the genus *Bufo* Rafinesque, 1815 [32,92].

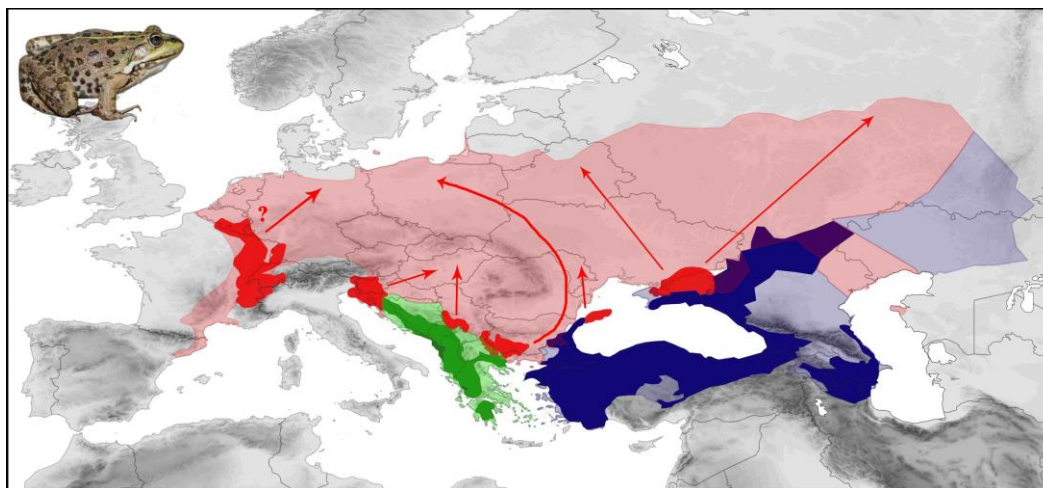


Figure 6. Dynamics of the ranges of *Pelophylax cf. bedriagae* (blue), *P. ridibundus* (red), and *P. kurtmuelleri* (green) during the Late-Quaternary. The approximate locations of glacial refugia are designated by darker blue, red and green areas. Routes of post-glacial colonization of *P. ridibundus* according to the LGM predictions and the distribution of nuclear and mitochondrial DNA markers (Figure 1) are indicated by red arrows. Photo credit: SNL (*P. ridibundus* from Moscow Province).

On the other side, nuDNA alleles of *P. cf. bedriagae* are particularly widespread in Moldova, Ukraine, and Russia (Figure 1; Table S1), which is difficult to explain through recent human-mediated introductions. These regions are quite far north from the hybrid

zones of *P. ridibundus* and *P. cf. bedriagae* in the Balkans, Crimea, and southern Russia [47,63,93]. This may be due to the post-glacial northward dispersal of marsh frogs along the shores of the Black and Azov seas from refugia located near the hybrid zones of *P. ridibundus* and *P. cf. bedriagae* in the north of the Balkans and the south of European Russia [53].

However, we assume that changes in the range boundaries of *P. ridibundus* and *P. cf. bedriagae* in the post-glacial period may have had the greatest impact on the recent distribution of nuDNA alleles and mtDNA haplotypes of these species, at least in the east of the *P. ridibundus* range. This hypothesis is confirmed by the distribution of mtDNA haplotypes. In many populations of *P. ridibundus* in the east of European Russia, haplotypes of *P. cf. bedriagae* predominated (Figure 1; Table S1). As a rule, such a situation occurs when one species is gradually replaced by another [41]. Indeed, according to our niche modeling data, refugia of *P. cf. bedriagae* could be located in the East Balkans, the Anatolian peninsula, the Caucasus, and south of European Russia. However, since the Heinrich Stadial 1 (17.0–14.7 ka) habitats suitable for *P. cf. bedriagae* in the south of European Russia began to decline (Figure 3) and vice versa; over time, living conditions for *P. ridibundus* become more suitable (Figure 4). Nevertheless, the process of replacing one species by another apparently did not proceed very quickly, and therefore we can observe mosaics of the populations of both *P. ridibundus* and *P. cf. bedriagae* and wide distribution of their hybrids in this region (Figure 1). This trend may be similar to that observed in other hybridizing amphibian species. For example, in tree frogs *Hyla arborea* (Linnaeus, 1758) and *H. orientalis* Bedriaga, 1890 hybrid zones located in the refugee zone in the Balkans are much narrower than those located outside them in Poland [10]. Perhaps we see a similar picture in these marsh frogs, where *P. ridibundus* and *P. cf. bedriagae*'s Balkan hybrid zone is much narrower than those located in southern Russia [63,93].

The northward post-glacial dispersal of *P. kurtmuelleri* from the Balkan refugium appears to have been blocked by competition and hybridization with two other marsh frog species living to the north and east. Therefore, the current native range of *P. kurtmuelleri* has not changed much since the late Pleistocene [31]. However, current human-mediated introductions of *P. kurtmuelleri* to other regions of southern Europe, some Mediterranean islands, and North Africa [12,24,94,95], which have quite suitable habitats according to our niche modeling analyses (Figure 2), have been very successful.

Although direct paleontological evidence does not yet exist, according to our niche modeling data, in addition to the traditional Mediterranean marsh frog refugia, such as the Balkan and Anatolian peninsulas and the Caucasus, which are common for most Western Palearctic amphibians [96–107], other extra-Mediterranean refugia could also exist [94,108–112]. During the Last Glacial Maximum (~21 ka), the refugee regions in the northern Black-Azov seas region and the south of European Russia were covered by steppe herb-shrub vegetation, with small groves mainly located in ravines [113,114]. Recently, such habitats have been quite suitable for the populations of marsh frogs in the steppes and deserts of southern Russia [115,116].

The existence of extra-Mediterranean refugia is also confirmed by data obtained on other amphibian species. Thus, the presumed refugia of *Pelobates vespertinus* (Pallas, 1771), *Lissotriton vulgaris* (Linnaeus, 1758), and *Bombina bombina* (Linnaeus, 1761) were located in the northern Black Sea region and the south of European Russia [117–121]. The presence of other extra-Mediterranean refugia (e.g., the Carpathians, the Codri Hills, and the Danubian Plain) was also assumed for *Triturus cristatus* (Laurenti, 1768), *T. dobrogicus* (Kiritzescu, 1903), *Lissotriton vulgaris* (Linnaeus, 1758), *Lissotriton montandoni* (Boulenger, 1880), *Pelobates vespertinus* (Pallas, 1771), *Bombina variegata* (Linnaeus, 1758), *Rana arvalis* Nilsson, 1842, and *R. temporaria* Linnaeus, 1758 [41,120–129].

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/d16020094/s1>, Table S1: Locality data partitioned according to nuDNA composition and mtDNA type (only populations within the native ranges of *P. cf. bedriagae*, *P. kurtmuelleri*, and *P. ridibundus*). Table S2: Performance metrics for parameter settings used for creating the present time model and relative contribution (%) of variables.

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