

Genetic Divergence and Evolution of Reproductive Isolation in Eastern Mediterranean Water Frogs

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Abstract Water frogs [genus *Pelophylax* (*Rana*)] that occur around the eastern Mediterranean Sea provide an opportunity to study early stages of speciation. The geography of the eastern Mediterranean region has changed dramatically since the Middle Miocene as a result of motions of adjoining lithospheric plates and regional-scale vertical crustal motions (uplift and subsidence). For several hundred thousand years between 6 and 5 million years ago (Mya), the Mediterranean basin was isolated from the Atlantic Ocean, and became desiccated (the Messinian Salinity Crisis; MSC). Geological data suggest that the endemic water frog lineage on Cyprus was isolated by the flooding of the Mediterranean basin by salt water at the end of the MSC, circa 5.5–5.3 Mya. This suggests a rate of uncorrected genetic

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divergence of approximately 1.1% per million years (My). Divergence time estimates based on this rate are in good agreement with the chronology of events in the history of crustal deformation and landscape development in the eastern Mediterranean region.

Despite a high similarity in morphology, eastern Mediterranean water frogs show considerable genetic divergence, indicating the existence of several evolutionary species at varied levels of differentiation. Based on two mitochondrial (mt) genes (ND2 and ND3), several lineages have been identified: *Pelophylax bedriagae*, *P. cretensis*, *P. epeiroticus*, *P. ridibundus* (Europe), six Anatolian lineages, all provisionally subsumed under the name *P. cf. bedriagae*, and a distinct lineage restricted to Cyprus. Genetic data from transition zones in eastern Greece/western Anatolia, south-western Anatolia, and south-eastern Anatolia, in concert with the results of female choice experiments, indicate that antihybridization mechanisms are only weakly developed in eastern Mediterranean water frogs. Genetic incompatibility, as expressed by average hatching rate of heterospecific crosses, increases with genetic divergence measured by uncorrected distance estimated from mtDNA sequences. Hatching rates of heterospecific crosses show an extremely high variability, however, and viable F1 hybrids originated from almost all crosses. We conclude that speciation in eastern Mediterranean water frogs follows the allopatric model and has been closely associated with the geodynamic evolution of the Mediterranean since the Middle Miocene (i.e., since ~11 Mya).

Keywords Water frogs · *Pelophylax* (Rana) · Eastern mediterranean · Genetic diversity · Geology · Divergence time · Genetic incompatibilities · Antihybridization mechanisms · Speciation

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1 Introduction

Species are real entities in nature, originating in an interminable process called speciation, but their history is difficult to reconstruct because of many unknown parameters and events. Speciation is an adaptive process driven either by natural or by sexual selection; it involves complex genetic, ecological, and behavioral mechanisms. Several modes of speciation have been proposed: (1) allopatric speciation including bottleneck-induced speciation (reviewed by Carson and Templeton 1984), (2) sympatric speciation (reviewed by Bolnick and Fitzpatrick 2007), and (3) speciation via hybridization (e.g., Bullini 1994, Seehausen 2004; Baak and Rieseberg, 2007). In almost all cases, however, conventional evolutionary forces such as mutation, selection, and drift lead to distinct lineages of ancestor-descendant populations with specific gene pools and distinct evolutionary tendencies.

Speciation usually begins with the accumulation of genetic differences between isolated populations. If such differentiated populations come into secondary contact, the differences between them may favor the evolution of antihybridization and anticompetition mechanisms (Remington 1968), which reduce wasteful matings between the newly sympatric populations and decrease the competition between them. Neither speciation itself nor the establishment of these sequelae to speciation necessarily depends on the degree of genetic divergence of two lineages; single genes or gene complexes, rather than genomes as a whole, may be the units of speciation (e.g., Wu 2001).

Water frogs [genus *Pelophylax* (*Rana*)] distributed around the eastern Mediterranean Sea are an ideal group of vertebrates for studying speciation processes, especially in their early stages, because they represent a genetically and phylogenetically complex biological radiation comprising different levels of molecular and organismal differentiation. Furthermore, the geomorphological complexity of the region allows for analysis of the effects of geological processes and environmental change on genetic diversification and speciation. In particular, the formation of mountain ranges such as the Northern Anatolian Mountains (the Pontide Mountains, parallel to the Black Sea coast) and the Taurus Mountains (from south-west Anatolia, along the Mediterranean coast to the northern part of south-east Anatolia), the uplift of the Anatolian plateau between these mountain ranges, and the development of other more localized mountain ranges in uplifting blocks bounded by active fault zones may have had a considerable impact on the current distribution and genetic diversity of water frogs and other organismal groups in this area. Cyclic climate change, including the repeated glaciations in the last ~3 My at high northern latitudes in Europe and western Asia (e.g., Ehlers and Gibbard 2007), probably reduced the size of populations and isolated them in southern refugial areas (Hewitt 2000, 2004). In addition, contact zones between distinct genetic lineages can be used to test for the effects of allopatric divergence upon secondary contacts.

Eastern Mediterranean water frogs occur on many islands, separated from the mainland by effective salt-water barriers. Because frog skin is readily permeable to the ions in salt water, these barriers prevent exchange of individuals between island

and mainland populations. Water frogs have been introduced into many European countries (e.g., Schmeller et al. 2007; Holsbeek et al. 2008; Ohst 2008) but, provided that no individuals were introduced onto the islands by humans or arrived via rafting (e.g., Vences et al. 2003; Heinicke et al. 2007), and provided that the original populations on the adjacent mainlands have not been replaced by other lineages, the age of salt-water barriers isolating pairs of water frog populations should correspond to the minimum time that such pairs of populations have been genetically isolated (Beerli et al. 1996).

During a 6-year study, we have estimated, for several population pairs, the amount of genetic divergence using different sets of molecular markers, and have analyzed the role of genetic incompatibility and antihybridization mechanisms for speciation in eastern Mediterranean water frogs. The frogs studied represent at least four known and several cryptic undescribed species: one species of the *Pelophylax ridibundus* group and *P. epeiroticus* in the Peloponnese, *P. cretensis* (Crete), *P. bedriagae* (The Levant), a form restricted to Cyprus, and six more-or-less differentiated *ridibundus*-like forms distributed in Anatolia and on some Mediterranean islands. These latter six forms and the Cypriot form have provisionally been subsumed under the name *P. cf. bedriagae* (e.g., Plötner et al. 2001, Haefeli et al., unpublished) because their systematic status is not yet clear (Akin et al., in press).

2 Materials and Methods

2.1 Taxon Sampling

Frogs were collected at different localities in Anatolia and Greece including the islands Chios, Crete, Cyprus, Evvoia, Ikaria, Karpathos, Kerkyra (Corfu), Kythira, Lesbos, Rhodos, Samos, and Zakynthos (Table 1). Detailed locality information was published by Beerli et al. (1996), Haefeli (2005), Akin (2007), Plötner et al. (2001, 2009), and Akin et al. (2010).

2.2 Estimation of Genetic Divergence

Genetic divergence was estimated using three independent datasets: allozymes (products of up to 33 independent loci; Beerli et al. 1996, and unpublished data), nucleotide and amino acid sequences of two mitochondrial genes (NADH dehydrogenase subunits 2 and 3 or ND2 and ND3; e.g., Plötner et al. 2008, Akin et al., in press), and nucleotide sequences of serum albumin intron 1 (SAI1) and a non-LTR chicken repeat 1-like retrotransposon (*Rana*CR1) that was detected in SAI1 of water frog species (Plötner et al. 2009).

Table 1 Genetic distances between population pairs estimated on the basis of nucleotide (nu) and amino acid (aa) sequences of two mitochondrial genes (ND2, ND3), and by protein electrophoretic data (D^*_{Nei} , Beerli et al. 1996). Minimum times of geological isolation are compared with putative split times estimated from mean p distances using a rate of uncorrected genetic divergence of 1.1% per My. In parentheses: number of individuals/number of haplotypes, *cre*: *P. cretensis* (from Demati, Kastelli, Lavris, and Skinias), *cf. bed*: *P. cf. bedriagae*, *epc*: *P. epeiroticus*, *per*: *P. perezi*, *sah*: *P. saharicus*, *kur*: *P. kurtmuelleri* (individuals from the Peloponnese originated from the following populations: Kalanistra, Kaminiia, Kavasilas, Lake Stimfalias, Nea Manolada, and Skala)

Population pair	Estimated split time (ND2 + ND3) [Mya]	Minimum time of geological isolation [Mya]	Population pair	p Distance (nu) [%]			p Distance (aa) [%]			D^*_{Nei}
				Min	Mean±SD	Max	Min	Mean±SD	Max	
Spain (1/1) Crete (9/7)	14.8 9.7–13.4	5.3 5.3 or 10.0	<i>per</i> – <i>sah</i>	16.18	16.33±0.114	16.47	9.17	9.28±0.156	9.39	0.550
			<i>cre</i> – <i>cf. bed</i>	10.53	10.71±0.135	10.89	4.38	4.60±0.215	4.81	0.584
			<i>cre</i> – <i>kur</i>	11.18	11.44±0.178	11.69	5.25	5.47±0.220	5.69	0.698
Cyprus (3/2)	5.6–6.0	5.3	<i>cre</i> – <i>epc</i>	14.60	14.74±0.114	14.89	7.88	7.99±0.156	8.10	0.530
			<i>cf. bed</i> – <i>cf. bed</i>	6.39	6.46±0.070	6.53	3.49	3.60±0.156	3.71	–
			<i>bed</i>							
North Cyprus (2/1)	1.0–3.6	5.3	<i>cf. bed</i> – <i>cf. bed</i>	6.17	6.17±0.000	6.17	3.49	3.60±0.156	3.71	–
			<i>bed</i>							
			<i>cf. bed</i> – <i>bed</i>	6.61	6.85±0.357	7.26	2.63	3.06±0.345	3.50	–
Karpathos (3/1)	0.43–1.3	1.8–3.0	<i>cf. bed</i> – <i>cf. bed</i>	1.09	1.09±0.000	1.09	1.53	1.53±0.000	1.53	–
			<i>bed</i>							
			<i>cf. bed</i> – <i>cf. bed</i>	3.92	4.02±0.112	4.14	3.06	3.06±0.000	3.06	–
Rhodos (2/1)	Fethiye, Kas, Antalia (4/4) Akçapınar (4/3)	1.8–3.0	<i>bed</i>							
			<i>cf. bed</i> – <i>cf. bed</i>	1.38	1.42±0.050	1.45	0.87	0.98±0.156	1.09	0.26
			<i>bed</i>							
Rhodos (2/1)	Fethiye, Kas, Antalia (4/4) Akçapınar (4/3)	1.8–3.0	<i>cf. bed</i> – <i>cf. bed</i>	0.36	0.47±0.094	0.58	0.44	0.55±0.156	0.66	–
			<i>bed</i>							
			<i>cf. bed</i> – <i>cf. bed</i>	1.38	1.42±0.050	1.45	0.87	0.98±0.156	1.09	0.18
Rhodos (2/1)	Fethiye, Kas, Antalia (4/4) Akçapınar (4/3)	1.8–3.0	<i>bed</i>							
			<i>cf. bed</i> – <i>cf. bed</i>	0.36	0.47±0.094	0.58	0.44	0.55±0.156	0.66	–
			<i>bed</i>							

(continued)

Table 1 (continued)

Population pair		Estimated split time (ND2 + ND3) [Mya]	Minimum time of geological isolation [Mya]	Population pair	p Distance (nu) [%]		p Distance (aa) [%]		D* _{Nei}
					Min	Mean±SD	Max	Min	Max
Andros (3/1)	Aliartos (3/3)	0.24–1.53	0.2–0.45	cf. <i>bed</i> – cf. <i>bed</i>	0.29	0.36±0.075	0.44	–	– 0.01
	Evvoia (3/2)			cf. <i>bed</i> – cf. <i>bed</i>	0.22	0.33±0.156	0.44	0.0	0.11±0.156 0.22 0.03
	Özbasi (3/1)			cf. <i>bed</i> – cf. <i>bed</i>	1.60	1.60±0.000	1.60	0.88	0.88±0.000 0.88 –
	Samos (2/2)			cf. <i>bed</i> – cf. <i>bed</i>	1.68	1.68±0.000	1.68	1.10	1.21±0.156 1.32 0.06
	Peloponnese (10/6)			<i>kur</i> – <i>kur</i>	0.15	0.26±0.090	0.36	0.22	0.33±0.156 0.44 0.02
Kythira (3/1) Chios (3/1)	Foça (3/3)	0.16–0.90	0.012–0.025	cf. <i>bed</i> – cf. <i>bed</i>	0.29	0.65±0.317	0.87	0.0	0.22±0.220 0.44 –
				<i>bed</i>					
Evvoia (3/2) Kerkyra (2/1)	Aliartos (3/3) Igoumenitsa, Ioannina, Aitolikon (6/4)			<i>kur</i> – <i>kur</i> <i>epe</i> – <i>epe</i>	0.15 0.36	0.45±0.178 0.99±1.028	0.65 2.18	0.0 0.44	0.11±0.156 0.22 0.04 0.88±0.615 1.31 –
	Edremit (2/2)			cf. <i>bed</i> – cf. <i>bed</i>	0.80	0.94±0.115	1.09	0.22	0.33±0.156 0.44 –
	Özbasi (3/1)			cf. <i>bed</i> – cf. <i>bed</i>	0.51	0.51±0.000	0.51	0.22	0.33±0.156 0.44 –
	Peloponnese (10/6)			<i>kur</i> – <i>kur</i>	0.0	0.18±0.135	0.36	0.0	0.22±0.220 0.44 –

DNA sequences were aligned using CLUSTAL V (Higgins et al. 1992). For sequence statistics, the programs MEGA4 (Tamura et al. 2007) and DnaSP 5.0 (Librado and Rozas 2009) were used. Regression analyses were performed using Statgraphics Plus 4.1 (Statistical Graphics, StatPoint, Herndon, Virginia). Monophyletic groups were identified by Bayesian inference using the program MrBayes (Ronquist and Huelsenbeck 2003; Huelsenbeck and Ronquist 2005), with a GTR + I + G model of sequence evolution. For the phylogenetic tree, we augmented the sequences of Mediterranean water frogs (including *P. bedriagae*, the endemic Cypriot lineage, and all the Anatolian lineages of the *P. ridibundus* group) with sequences of *P. epeiroticus* and *P. cretensis* as outgroups.

2.3 *Estimation of Rates and Times of Divergence Using a Non-constant Molecular Clock*

BEAST (Drummond and Rambaut 2007), which takes all samples into account and allows for variable mutation rates among species lineages, was used with the mtDNA data to compare four geological scenarios and to re-estimate the divergence rate used for pairwise estimates (Akin et al., in press). In the Bayesian framework of BEAST, the times of internal nodes of the phylogeny can be associated with prior distributions that reflect prior knowledge about the time of speciation events associated with that node. We explored the power of our data to distinguish between four alternative scenarios concerning the putative isolation time of Crete with wide (half of the mean) and narrow (0.3) standard deviations:

1. Crete9w; isolation time = 9.0 ± 4.5 Mya,
2. Crete9n; isolation time = 9.0 ± 0.3 Mya,
3. Crete5w; isolation time = 5.3 ± 2.65 Mya,
4. Crete5n; isolation time = 5.3 ± 0.3 Mya.

The isolation time of Cyprus was set to 5.3 ± 0.3 Mya in all scenarios. BEAST was run for each scenario using a relaxed clock model that allows for differences between evolutionary rates among lineages; the rates were drawn from a lognormal prior distribution assuming that rates are not correlated. Each scenario was run five times with a burn-in of 10^6 iterations and 10^7 sampled iterations; subsequently, the five replicates were combined in Tracer (<http://tree.bio.ed.ac.uk/software/tracer/>) and analyzed; marginal likelihood estimates were then used to calculate Bayes factors (Kass and Raftery 1995), which were used to compare the different geological scenarios. Mutation rate was estimated using the TN93 model with site rate variation.

2.4 *Crossing Experiments*

To assess the amounts of genetic incompatibility between taxa, artificial inter- and intraspecific crosses were performed using standard methods (e.g., Berger et al.

1982, 1994). After reaching the free-swimming stage (stage 25; Gosner 1960) tadpoles were counted and hatching rates were calculated.

2.5 Bioacoustic Investigations

Recordings of advertisement calls were made in an artificial pond using a directional microphone (SONY ECM 707) and analyzed with Avisoft SASLab Pro version 4.2. Eleven call parameters were measured on the audio spectrograms and oscillograms (Haefeli 2005; Haefeli et al., unpublished). Five call parameters (call duration, pulse groups per call, duration of pulse groups, pulses per pulse group, interval between pulse groups) were directly compared with data published by H. Schneider and collaborators (reviewed by Plötner 2005). Temperature-dependent call parameters were predicted for a temperature of 20 °C, using the regression models published by Joermann et al. (1988), Schneider (1997), and Schneider and Sinsch (1992).

2.6 Female Choice Experiments

Female choice experiments were performed with 29 females from Crete (*P. cretensis*) and 26 lake frog females (*P. kurtmuelleri*) from the Peloponnese, following the experimental design developed by Roeseli and Reyer (2000). Two inwardly-facing speakers were placed 97 cm from the center of the arena on radii with an angle of 120° between them; the speakers were 160 cm apart. The basin was divided into 24 equal-sized sectors. Females were individually exposed to alternating vocalizations, with one loudspeaker playing the call of *P. cretensis*, the other the call of *P. kurtmuelleri* from the Peloponnese. The position of the females in the arena was registered every 7 s for 12 min. The overall interest of females was measured by the relative time a female spent in either loudspeaker sector, compared to the value expected under a random distribution. Haefeli (2005) provided additional details.

3 Results and Discussion

3.1 Genetic Diversity of Eastern Mediterranean Water Frogs

The extensive genetic diversity detected in eastern Mediterranean water frogs indicates the existence of several distinct lineages that may represent several evolutionary species (sensu Wiley 1978, 1981). Besides the main mitochondrial haplogroups (MHGs) specific for *P. epeiroticus* and *P. cretensis*, six additional

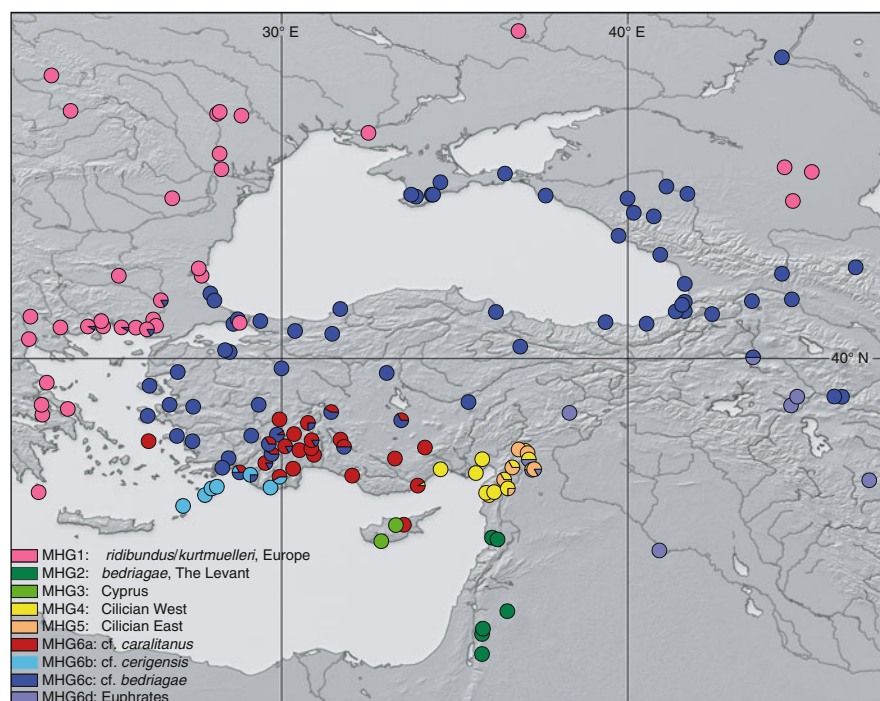
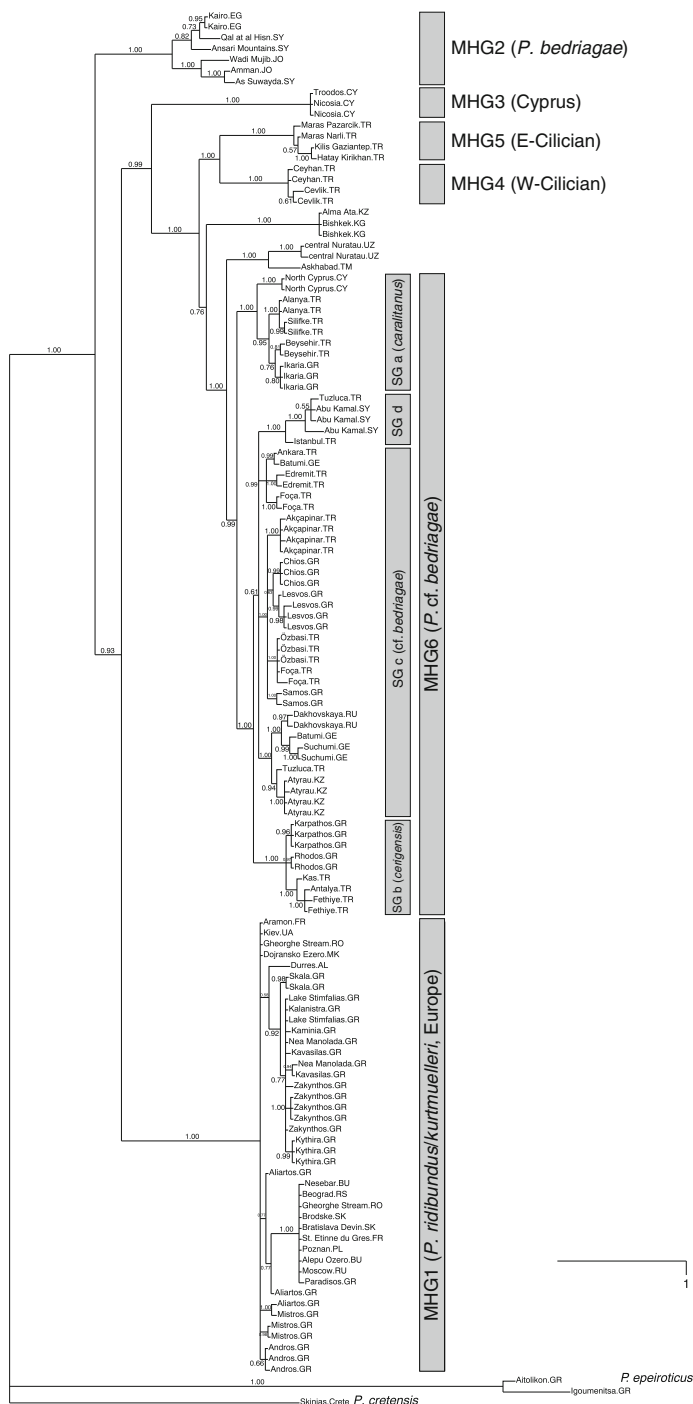


Fig. 1 Distribution of the main ND2/ND3 haplogroups (MHG) and subgroups of water frogs (genus *Pelophylax*) in the eastern Mediterranean region. MHGs of *P. cretensis* and *P. epeiroticus* are not plotted.

MHGs can be distinguished among eastern Mediterranean water frogs (Akin et al., in press). These MHGs are defined by pairwise uncorrected p distances $>3.5\%$; they are supported by specific geographic distribution (Fig. 1) and clade probabilities of 1.0 (Fig. 2).

MHG1 is composed of European *P. ridibundus*, including *P. kurtmuelleri* from the Balkan Peninsula. MHG2 comprises haplotypes of *P. bedriagae*, a species distributed in the Levant (Plötner 2005); haplotypes of this group are found in western Syria, Jordan, and the Nile delta. MHG3 comprises haplotypes from Cyprus. MHG4 haplotypes occur primarily in the Cilician plain of south-east Turkey, but also east of the Amanos Mountains, whereas MHG5 haplotypes only occur east of the Amanos Mountains. MHG4 and MHG5 represent sister clades with a sequence divergence of approximately 3.7%. Haplotypes of MHG6 are distributed from north-eastern Greece (Akin et al., in press; Hotz et al., in preparation) to central Russia; they are also found in Iran and Syria (Plötner et al. 2001, unpublished data). In some areas, MHG6 occurs syntopically with haplotypes of other groups, for example, with MHG4 in the western Cilician plain, with MHG5 in the eastern Cilician plain (Akin et al., in press), and with MHG1 in north-eastern Greece (Hotz et al., in preparation).



MHG6 can be divided into four subgroups (6a–d). Subgroup 6a is specific for a form that, because of particular morphological, karyological, bioacoustic, and enzymological characteristics (e.g., Jdeidi 2000; Alpagut Keskin and Falakalı Mutaf 2006), was assumed to represent a separate species, *P. caralitanus* (Jdeidi 2000; Jdeidi et al. 2001; Plötner 2005). Haplotypes of this group are distributed in south-western and south-central Anatolia, but are also found on the island of Ikaria and in northern Cyprus. They occur syntopically at the boundaries of their range with individuals carrying other haplotypes: with subgroup 6b to the south-west, with subgroup 6c to the north and west, and with MHG4 to the southeast (Akin et al., in press).

Subgroup 6b is characteristic of water frogs from Karpathos and Rhodos. Based on protein electrophoretic data, frogs inhabiting these islands were described as a separate species (*P. cerigensis*) by Beerli et al. (1994). Haplotypes of this subgroup are not restricted to Karpathos and Rhodos, however, but are also found in the coastal region of south-western Anatolia (Ohst 2001; Akin et al., in press).

Subgroup 6c has a wide distribution from eastern Greece in the west, beyond Anatolia to the Caspian Sea in the east, including Atyrau, the type locality of *P. ridibundus* on the northern shore of the Caspian Sea (Akin et al., in press; Plötner and Litvinchuk, unpublished data). The sequence divergence between haplotypes from Atyrau and haplotypes of Central European and Balkan lake frogs (MHG1) is about 6.5%, an indication that lake frogs from Atyrau and Central Europe are not conspecific. Haplotypes of subgroup 6c were also detected on several Mediterranean islands near the Anatolian coast, for example Chios, Lesbos, and Samos.

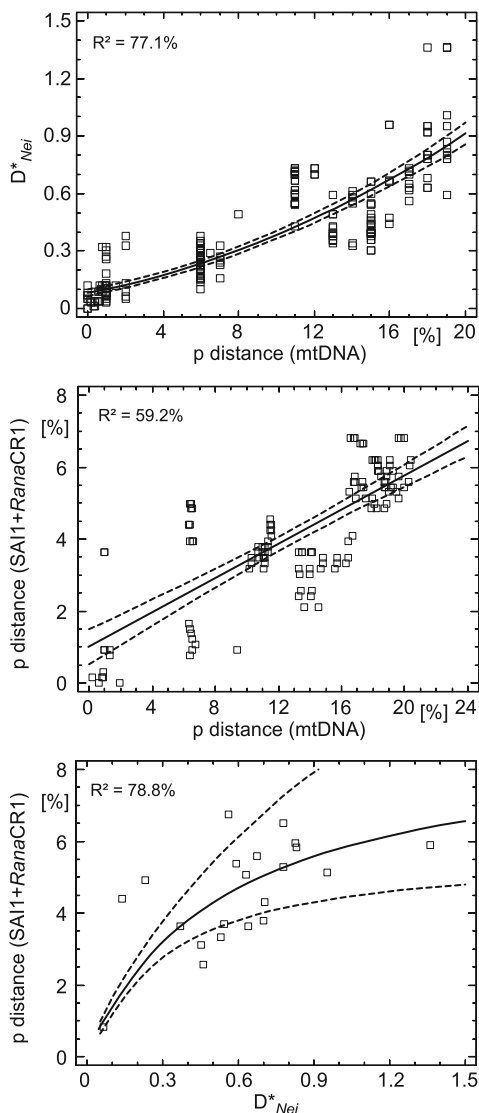
Haplotypes of subgroup 6d are found in the Tigris and Euphrates catchments of north-eastern Syria, eastern Anatolia and western Iran (Akin et al., in press; Plötner, unpublished data).

3.2 Genetic Divergence Between Population Pairs

Genetic divergence between selected population and species pairs (Table 1), calculated on the basis of protein electrophoretic data (Beerli et al. 1996), sequences of the mitochondrial genes ND2 and ND3 (Plötner et al. 2001; Akin et al., in press;

Fig. 2 (continued) Phylogenetic relationships of eastern Mediterranean water frogs estimated with Bayesian inference. The tree is based on 1,378 base pairs from the protein coding mitochondrial genes ND2 and ND3. The values at the branches are posterior probabilities for the clade to the right of that branch. The scale bar represents one expected mutation per site. *Pelophylax epeiroticus* and *P. cretensis* were used as outgroups. MHG Main haplogroup, SG subgroup. Country abbreviations (ISO-Code): AL Albania; BU Bulgaria; CY Cyprus; EG Egypt; FR France; GE Georgia; GR Greece; JO Jordan; KG Kyrgyzstan; KZ Kazakhstan; MK Macedonia; PL Poland; RO Romania; RS Republic Serbia; RU Russia; SK Slovakia; TM Turkmenistan; TR Turkey; UA Ukraine; UZ Uzbekistan

Fig. 3 Relationships between genetic distances based on mitochondrial (mt) DNA (ND2+ND3), nuclear (n) DNA of serum albumin intron 1 (SAI1) and *Rana*CR1, and protein electrophoretic data. Uncorrected p distances were calculated for both mtDNA and nuDNA. For protein electrophoretic data, modified Nei distances (D^*_{Nei}) were estimated as proposed by Hillis (1984). *Above* Uncorrected p distance (mtDNA) vs D^*_{Nei} .
 $D^*_{Nei} = (0.28 + 3.35 \cdot p_{mtDNA})^2$, $r = 0.88$,
 F-ratio = 709.8, $p < 0.01$.
Middle: p_{mtDNA} vs p_{nDNA} .
 $p_{nDNA} = 0.01 + 0.24 \cdot p_{mtDNA}$, $r = 0.77$,
 F-ratio = 191.0, $p < 0.01$.
Below D^*_{Nei} vs p_{nDNA} .
 $p_{nDNA} = 1/(11.2 + 6.06/D^*_{Nei})$, $r = 0.89$,
 F-ratio = 70.6, $p < 0.01$.
 Solid line regression line.
 Broken lines 95% confidence limits

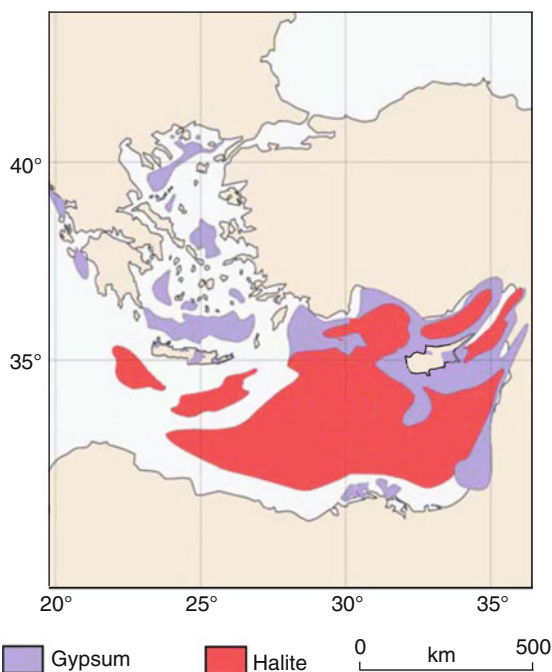


Plötner unpublished data), and sequences of the nuclear markers SAI1 and *Rana*CR1 (Plötner et al. 2009) are significantly correlated with each other (Fig. 3).

We envisage that environmental changes associated with the Messinian Salinity Crisis (MSC) had a significant effect on the development of the genetically distinct frog populations in the study region. Until ~ 6 Mya the Mediterranean Sea was connected to the Atlantic, the connection being via shallow seaways across southern Spain and northern Morocco, not via the Strait of Gibraltar, which did not yet

exist (e.g., Krijgsman et al. 1999a). Uplift of Spain and Morocco gradually closed these seaways, so that around 6 Mya (5.96 Mya according to Krijgsman et al. 1999b) the Mediterranean basin became isolated from the Atlantic Ocean. Because evaporation was no longer balanced by inflow from the Atlantic, the Mediterranean basin became desiccated at this time, marking the start of the MSC. Isolation was initially intermittent (during global sea-level minima that were contemporaneous with maxima of high-latitude glaciation, restricted at this time to the Antarctic) but became complete by ~ 5.6 Mya (at 5.59 Mya according to Krijgsman et al. 1999b); with complete isolation, what is now the Mediterranean sea floor (in places ~ 3 km or more below modern sea-level) was covered by vast playas (salt pans) in which salts such as halite (NaCl) and gypsum (CaSO_4) were precipitated (Fig. 4). This was an extremely hostile environment, with estimated surface temperatures as high as $\sim 80^\circ\text{C}$; frogs could not have survived there. These conditions probably lasted for less than 100,000 years, however, and were followed (at 5.50 Mya according to Krijgsman et al., 1999b) by partial flooding of the Mediterranean basin (known as the “Lago-Mare” stage of the MSC), which created a low-salinity lacustrine realm that lasted circa 200,000 years. It has been proposed that the influx of freshwater to create the Lago-Mare paleo-lake was a consequence of temporary capture, by the Mediterranean basin with its reduced base-level, of the river Danube (e.g., Kvasov 1983). Alternative explanations, relating to increased rainfall in surrounding regions as a result of global climate change, have also been put forward (e.g., van der Laan et al. 2006; Hilgen et al. 2007). The Lago-Mare phase ended when,

Fig. 4 Map of the eastern Mediterranean basin showing the distribution of evaporite deposits from the Messinian salinity crisis, modified from part of Fig. 1 of Hilgen et al. (2007). Modern coastlines are shown for location purposes and do not correspond to contemporaneous coastlines; the map does not attempt to restore changes in the relative positions and shapes of parts of the region caused by post-Messinian plate motions and related crustal deformation



apparently as a result of headward erosion of rivers in the westernmost Mediterranean (e.g., Loget et al. 2005), the Atlantic Ocean breached into the Mediterranean basin through what is now the Strait of Gibraltar and rapidly restored its water level to that of the global ocean. We adopt as the timing of this flooding event 5.33 Mya, from Krijgsman et al. (1999b), Hilgen et al. (2007), and others.

The environmental conditions during the Messinian in and around Cyprus were described in detail by Robertson (1998). During the evaporitic phase of the MSC, this island was largely surrounded by playas (Fig. 4); during the subsequent Lago-Mare phase, waters of the paleo-lake covered these areas. Sediments of the Lago-Mare facies are found both offshore of Cyprus in boreholes and onshore in low-lying parts of the island; the latter deposits reflect subsequent uplift of the island, the water surface during the Lago-Mare phase having been far below modern sea-level. The Kyrenia (Beşparmak) mountain range of northern Cyprus is structurally connected to the Misis mountain range east of Adana in southern Turkey by the Misis-Kyrenia Fault Zone (MKFZ), which forms part of the boundary between the Turkish and African plates (Fig. 5). This fault zone became active around the beginning of the MSC (e.g., Robertson et al. 2004; Westaway et al. 2008) and forms a significant linear ridge, both onshore in Cyprus and offshore to the northeast. As depicted schematically in Fig. 4, it appears to have protruded above the level of evaporite deposition during the MSC. We infer that it also remained above water level during the subsequent Lago-Mare phase, enabling frogs to migrate from Anatolia into Cyprus; this frog population became isolated when the normal sea-level was restored.

One may contrast this situation with that of Crete. As indicated in Fig. 4, Crete was surrounded by Messinian evaporite depocenters, parts of the island being low enough at the time (before subsequent uplift took place) to be covered by evaporite. Cosentino et al. (2007) have shown that much of Crete was subsequently inundated during the Lago-Mare phase. There is no evidence, however, of any adjoining



Fig. 5 Map of the eastern Mediterranean region, showing the boundaries (red lines) of the African (AF), Arabian (AR), Eurasian (EU), and Turkish (TR) plates. MKFZ: Misis-Kyrenia Fault Zone

geological structure, analogous to the MKFZ for Cyprus, that could permit migration of frogs to or from Crete at this time; Crete was evidently an island, surrounded by tens or hundreds of kilometres of open salt water in all directions. The local frog population (*P. cretensis*) probably became isolated before the MSC and survived its evaporitic phase, presumably in what are now the highest parts of the island, which were well above the contemporaneous playa level.

We conclude that the geological data strongly support a Messinian origin for Cypriot frogs and a potentially older isolation for frogs on Crete. The lower age bound for the isolation of Cyprus is the time of the end-MSC flooding at about 5.33 Mya; its upper bound is 5.50 Mya, the start of the Lago-Mare phase of the MSC. The Cyprus frogs (MHG3) differ in homologous positions of their ND2 +ND3 genes from Anatolian frogs (MHG6) by approximately 6%. The rate of development of uncorrected genetic divergence can thus be estimated as between 1.09% per My (6.0%/5.50 My) and 1.13% per My (6.0%/5.33 My) or approximately 1.1% per My.

3.3 Estimation of Confidence Limits for Divergence Times of Basic Lineages Using a Nonconstant Molecular Clock

The BEAST analysis (2.3) revealed only small statistical differences between the four geological scenarios; most credibility intervals of the time of divergence for particular clades overlap (Akin et al., in press). Crete appears to have become isolated before Cyprus but the posterior distributions of the divergence times overlap considerably. The scenario with strongly peaked prior distributions for the isolation of Crete and Cyprus achieved the highest marginal log likelihood (CRETE9n: $-10,511.035 \pm 0.164$), but the other scenarios produced very similar values: CRETE9w has $-10,511.474 \pm 0.177$, CRETE5w has $-10,511.623 \pm 0.183$, and CRETE5n has $-10,514.081 \pm 0.182$. The largest difference among scenarios is 3.046 log likelihood units; using Kass and Raftery's (1995) guidelines, this difference suggests that the scenarios CRETE5n and CRETE9n are different. The other scenarios, however, are only 0.588 and 0.439 log likelihoods units apart from CRETE9n, suggesting that they cannot be rejected. Selecting among the four scenarios, using Kass and Raftery's (1995) approach, results in probabilities of 0.445, 0.287, 0.247, and 0.021 for CRETE9n, CRETE9w, CRETE5w, and CRETE5n, respectively. Thus, although the CRETE9n scenario (involving isolation of Crete circa 9 Mya and Cyprus circa 5.3 Mya) is the most probable scenario considered, other possible scenarios (e.g., isolation of Crete earlier than Cyprus, but not circa 9 Mya) cannot be ruled out. The same is true for the separation of the western Mediterranean species pair *P. saharicus* (North Africa) and *P. perezii* (Iberian Peninsula) which is suggested to have occurred before 5.3 Mya (Table 1; Akin et al., in press). The present data are sufficient to reveal the order of the divergence events, but insufficient for exact correlation of geological events with species divergences.

BEAST also calculates a posterior estimate of the average expected mutation rate. The average mutation rate estimate for the best model was 0.00943 mutations per site per My with a coefficient of variation of 1.112 indicating a standard deviation of 0.0105. A standard deviation of the magnitude of the mean suggests considerable mutation rate heterogeneity among lineages in the phylogeny. A comparison using Bayes factors of the strict clock model versus the relaxed clock model with independent evolutionary rates and no divergence time constraints shows considerable support for the non-clock model (Akin et al., in press).

3.4 *Evolution of Anatolian Water Frog Populations*

Our sequence data are insufficient to choose among the four alternative scenarios for isolation of Crete from mainland areas, but they provide relatively strong support for the divergence sequence of the mitochondrial lineages themselves. The application of a constant divergence rate, however, offers plausible hypotheses on historical events that influenced the evolution of genetic diversity in Mediterranean water frogs, especially in Anatolian populations. If the divergence rate of 1.1% per My is applied to all Anatolian frogs, several lineages appear to have diverged between approximately 4 and 1 Mya (5.3–1.6 Mya, according to the BEAST analysis used by Akin et al., in press). The Cilician clades MHG4 and 5 may have split from the rest of the Anatolian populations (MHG6) first, with a divergence of about 4.0% from MHG6 populations and 3.7% from each other; these values correspond to divergence times of approximately 3.6 and 3.4 Mya, respectively (5.3–3.5 and 4.4–1.6 Mya, according to the BEAST analysis). Populations with haplotypes of MHG6 were probably isolated from the rest of the Anatolian populations by uplift of the central Taurus Mountains during the Late Miocene and Pliocene (Jaffey and Robertson 2005), although the timing of this uplift is not well constrained. The Cilician clades were probably separated from each other by the uplift of the Amanos Mountains, which began at ~3.7 or ~3.6 Mya as a result of a change in the pattern of plate motions in the region (Seyrek et al. 2008a; Westaway et al. 2008).

The observed rates of divergence imply a time window of 2.0–1.4 My (1.6–1.1 My, according to a more sophisticated TrN + G model of sequence evolution used by Akin et al., in press) for diversification of the subgroups (a–d) of MHG6, so the radiation of these Anatolian lineages appears to have taken place around the Pliocene–Pleistocene boundary. This was a time of significant global cooling (e.g., Head and Gibbard 2005; Ehlers and Gibbard 2007), the boundary being currently defined at the first influx of cold-climate marine mollusc and ostracod taxa into the Mediterranean Sea, as recorded in sediments in southern Italy (e.g., Aguirre and Pasini 1985). In Britain, a dramatic increase in fluvially-transported sediment occurred at this time (e.g., Rose et al. 2002), thought to reflect a reduction in vegetation cover and, as a result, increased transport of sediments into rivers (e.g., Bridgland and Westaway 2007a, b). Resulting from more rapid erosion

consistent with the climate change around this time, increased rates of uplift occurred in some regions, for instance north of the Black Sea (e.g., Bridgland and Westaway 2007b), on the northern Arabian Platform in south-eastern Turkey and adjacent parts of Syria (Demir et al. 2007, 2008; Westaway et al. 2009), and in the eastern Taurus Mountains (Seyrek et al. 2008b). These processes may have reduced distributional ranges and enhanced the isolation of different lineages (Akın et al., in press).

The subgroups of MHG6 have largely contiguous geographical ranges of varying sizes, but the first three forms (cf. *caralitanus*, cf. *cerigensis*, and cf. *bedriagae*) are all found in south-western Anatolia. South-western Anatolia has often been proposed as a glacial refugium (e.g., Kosswig 1955; Schmidler 1998) because of the presence of relict or endemic taxa, including the tree *Liquidambar* (Öztürk et al. 2008), the fishes *Aphanius* (Hrbek et al. 2002) and *Pseudophoxinus* (Hrbek et al. 2004), the salamander *Lyciasalamandra* (Veith and Steinfartz 2004), the mountain frog *Rana tavasensis* (Veith et al. 2003), and the snake *Vipera (ursinii) anatolica* (Joger et al. 1992). The presence, sometimes syntopically, of these closely related Anatolian water frog subgroups in this region probably indicates that two or more subgroups of MHG6 found refuge here during Pleistocene glaciations.

3.5 Genetic Incompatibilities

Genetic incompatibilities and conflicts of single genes, in conjunction with ecological factors, may result in lowered viability or fertility of hybrids or of the offspring of hybrids (e.g., Uzzell and Ashmole 1970; Guex et al. 2001, 2002; Orr 2005). The amount of genetic incompatibility can often be estimated by crossing experiments. The results of such experiments, however, have to be interpreted with caution because artificial crosses may differ from hybridization in nature in respect to fitness parameters such as hatching success, vitality, and fertility of hybrids. Among the factors that may have influenced the results of our crossing experiments, the long time of transport (up to 4 weeks) of parental individuals, artificial hormone treatment of females, and environmental conditions in the laboratory (e.g., food composition or water chemistry) could have had significant effects on the results. Although all crosses were performed with the same method, the physiological responses to artificial treatment were probably different among individuals and species. From the free-swimming stage (stage 25; Gosner 1960) to the onset of metamorphosis, water quality, density of individuals, and food supply are important parameters that influence the performance of larvae. In captivity, juvenile water frogs often suffer from bacterial and fungal infections resulting in high mortality, especially during and immediately after metamorphosis. We therefore mainly considered the hatching rate (stage 25) as a parameter of genetic incompatibility (Table 2), although it is known from former crossing experiments that viability of F₁ hybrids in water frogs is a weak estimator for genetic incompatibilities (e.g.,

Table 2 Hatching rates (minimum, geometric mean, maximum) of intra- and interspecific water frog crosses and uncorrected p distances (p) estimated for the forms crossed on the basis of two mitochondrial genes (ND2 and ND3). *cre*: *P. cretensis*, *cf. bed*: *P. cf. bedriagae* (Anatolia), *epe*: *P. epeiroticus*, *les*: *P. lessonae*, *kur*: *P. kurtmuelleri* (Balkan), *shq*: *P. shqipericus*, n : number of crosses

Female		Male		n	Hatching Rate [%]			p [%]
Species	Origin	Species	Origin		Min	Mean	Max	
Interspecific crosses								
cre	Ski	cf. bed	Akç	12	13.2	48.8	98.8	10.68
cf. bed	Akç	cre	Ski	4	22.5	46.1	88.9	
cre	Ski	kur	Ska	12	6.9	42.7	99.4	11.36
kur	Kav, Lec, Ska	cre	Ski	5	58.1	75.5	94.1	11.40
cre	Ski	epe	Kil, Lec	3	21.3	42.6	67.8	14.72
epe	Kil, Lec	cre	Ski	7	15.9	45.9	97.5	
cf. bed	Sil	cre	Ski	1	–	89.8	–	10.93
cf. bed	Sil	cf. bed	Cyp (N)	1	–	98.5	–	1.09
cf. bed	Cyp (N)	cf. bed	Sil	1	–	34.8	–	
les	Rog	epe	Igo, Lec	3	18.0	39.8	91.4	16.84
les	Rog	kur	Kav	1	–	66.9	–	15.13
les	Poz	shq	Vir	3 ^a	31.7	59.2	94.6	8.52
shq	Vir	les	Poz	3 ^a	89.4	91.4	91.7	
les	Poz	cf. les	Pia	6 ^a	25.5	62.7	96.2	4.93
cf. les	Pia	les	Poz	3 ^a	56.0	92.1	99.2	
Intraspecific crosses								
cre	Ski	cre	Ski	1	–	99.3	–	0
epe	Ker	epe	Igo	2	16.4	47.9	73.9	0.40
kur	Ska	kur	Ska	1	–	95.0	–	0
kur	Kav	kur	Zak	1	–	96.0	–	0.18

Localities: Akç: Akçapinar (Turkey); Cyp (N): North Cyprus (Turkey); Igo: Igoumenitsa (Greece); Kav: Kavasilas (Greece); Ker: Kerkyra (Greece); Kil: Kilini (Greece); Lec: Lechena (Greece); Pia: Piana di Catania (Italy, Sicily); Poz: Poznań (Poland); Rog: Rogaczewo (Poland); Sil: Silifke (Turkey); Ska: Skala (Greece); Ski: Skinias (Crete); Vir: Virpazar (Montenegro); Zak: Zakynthos (Greece)

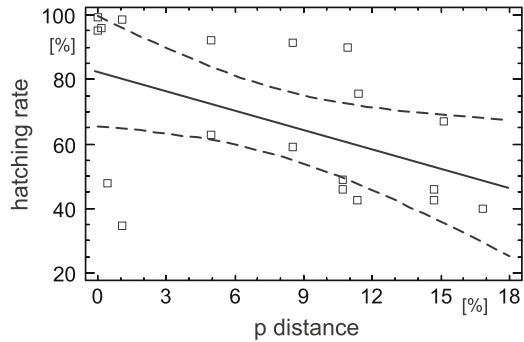
^aData from Berger et al. (1994)

Günther 1973; Kawamura and Nishioka 1979, 1986; Berger et al. 1982, 1994); fertility of F_1 hybrids and the viability of progeny from backcrosses between F_1 hybrids and their parental species are more appropriate parameters to reveal genetic incompatibilities in water frogs.

Hatching rates of both intra- and interspecific crosses of different combinations showed extremely high variability; the values obtained for the combination *P. cretensis* female x *P. ridibundus* male, for example, ranged from 6.9 to 99.4%.

The significant negative but relatively weak correlation between the average hatching rate and the uncorrected pairwise genetic distance estimated from mtDNA (Fig. 6) probably reflects gradual genetic divergence of populations in allopatry. The R^2 statistic indicates that the model as fitted explains only about 25% of the variability in hatching rate; prediction of hatching rates for certain distance (p) values seems to be error prone. This is also seen if the proposed linear regression model is used to estimate the theoretical p value at which the hatching rate is zero

Fig. 6 Relationship between hatching rate (Table 2) and uncorrected p distance estimated from the mt genes ND2 and ND3 ($r = -0.50$, F-ratio = 5.61, $p < 0.05$). Solid line regression line. Broken lines 95% confidence limits



(ca. 41%). Such high divergence values are not observed, even between species that are completely isolated reproductively. For example, the ND2 genes of Central European *P. ridibundus* and the Palearctic brown frog species *Rana temporaria* show a sequence divergence of approximately 24%. Similar values (~21%) were obtained between the mtDNA sequences (ND2+ND3) of western Palearctic water frogs and the eastern Palearctic water frog species *P. nigromaculatus* and *P. plancyi* (Plötner and Ohst, unpublished data). As shown by several crossing experiments, the genomes of eastern Palearctic water frogs are almost incompatible with those of western Palearctic water frog species; there is usually complete isolation by F_1 hybrid inviability and sterility in crosses between the two groups (Kawamura et al. 1972; Kawamura and Nishioka 1979). These data clearly indicate that around a divergence level of 20%, ND genes of ranid frogs are saturated by multiple substitutions. Even at lower levels of divergence, mtDNA sequence divergence seems to be a rather inappropriate parameter to predict the degree of genetic incompatibility in western Palearctic water frogs.

Relatively high hatching rates seen in certain heterotypic crosses between genetically distinct species (e.g., *P. ridibundus* \times *P. cretensis*) indicate that individual genomes can be more or less compatible. Natural interspecies F_1 hybrids, when observed, may originate from single exceptional crosses. This assumption is supported by the results of interspecies crosses, for example between *P. nigromaculatus* from Japan and *P. lessonae* from Luxembourg (Kawamura et al. 1972; Kawamura and Nishioka 1979). Normally, almost all embryos resulting from such crosses die before hatching. A few hybrids, however, were able to complete metamorphosis and some even reached maturity. Similar results were obtained by Berger et al. (1994), who performed interspecies water frog crosses, $F_1 \times F_1$ crosses, and backcrosses between F_1 hybrids and their parental species. For example, hatching success of crosses between *P. shqipericus* and *P. epeiroticus* varied between circa 74 and 97%. Two backcrosses, one to each parental species (using an F_1 hybrid individual from a *P. shqipericus* \times *P. epeiroticus* cross that had a hatching rate of 74.4%), resulted in hatching rates of 42.6 and 97.3%.

If the high reproduction rate of female water frogs is taken into consideration (one female can lay several hundred to several thousand eggs per breeding season; e.g., Berger and Uzzell 1980), only a few successful interspecific crosses could be

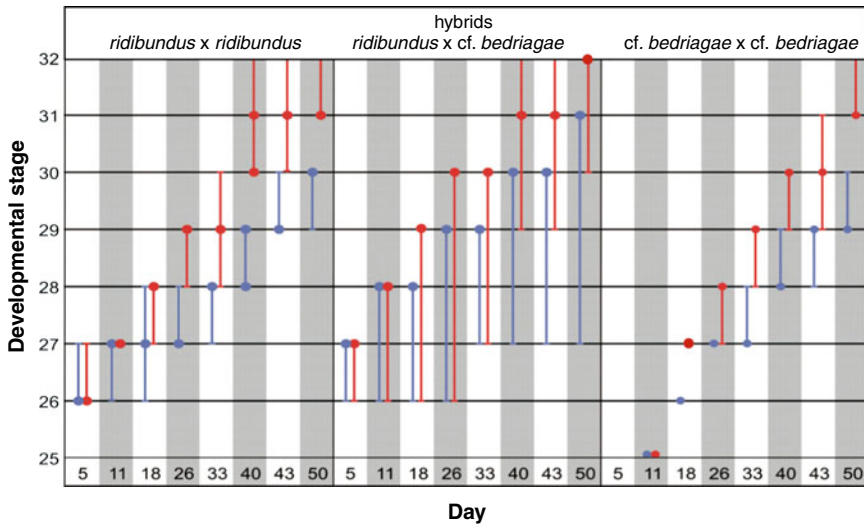


Fig. 7 Developmental stages (Gosner 1960) of F_1 larvae from homo- and heterospecific experimental crosses at different times after fertilization. *Red line* larvae kept in the laboratory. *Blue line* larvae kept outdoors. Points = mode. From Ohst (2008), modified

sufficient to maintain the occurrence of F_1 hybrids in areas of sympatry. The role of individual genome compatibility for hybrid fitness was also demonstrated by an experiment in which the development of F_1 individuals resulting from homo- and heterospecific crosses was analyzed under different environmental conditions (Ohst 2008). Compared to the homotypic progeny obtained from crosses of Central European *P. ridibundus* (MHG1) and of Anatolian lake frogs (MHG6c), F_1 larvae from a heterospecific cross between a Central European *P. ridibundus* female and an Anatolian *P. cf. bedriagae* male exhibited a greater heterogeneity in rate of larval development (Fig. 7). Whether hybrid inviability and sterility in western Palearctic water frogs is caused by single genes or is an additive effect of different loci remains to be investigated.

3.6 Antihybridization Mechanisms

Antihybridization mechanisms (also known as prezygotic or premating isolating mechanisms) serve to prevent the combination of two genetically incompatible genomes; they evolve because hybrids with maladapted diploid genomes have a lower fitness than their parental species. Genetic incompatibility between two or more lineages is often caused by the accumulation of genetic differences in allopatry. In contrast, antihybridization mechanisms, which minimize wastage of gametes as a result of heterospecific matings (e.g., Coyne and Orr 1998), are favored by

selection only when interbreeding occurs; they are, therefore, a sequela to speciation that is necessary to establishing sympatry of genetically incompatible lineages (Remington 1968).

In anuran amphibians, antihybridization mechanisms often include various elements of courtship and mating behavior, for example mating or advertisement calls of males that enable females to discriminate between conspecific and heterospecific males. Antihybridization mechanisms are only adaptive under sympatry. Nevertheless, they, or at least elements of them, may have already evolved in allopatry as indicated by the correlation between differences in advertisement calls and p distances (Fig. 8; Haefeli et al., unpublished).

While for many frog species, the mating call is known to be a major determinant of pair formation and thus syngamy (reviewed by Schneider and Sinsch 2007), its role as an integral element of antihybridization mechanisms in water frogs is not fully understood. For example, preliminary mate choice experiments with females from Crete and the Peloponnese, which represent genetically distinct species (*P. cretensis* and *P. kurtmuelleri*) with specific mating calls (Fig. 9), did not reveal a female preference for mating calls of conspecific males (Haefeli 2005; Haefeli et al., unpublished; Fig. 10). Despite the presence of female choice for mating, males can cause competition and sexual coercion because of highly male-biased operational sex ratios at crowded breeding sites (Bergen et al. 1997).

Although the behavior of females in such choice experiments is possibly biased by the artificial environments and/or the physiological conditions of the animals, field observations have also shown that quite distinct mating calls do not always prevent heterospecific matings and hybridization in areas of sympatry, for instance between *P. kurtmuelleri* and *P. epeiroticus* (Hotz and Uzzell 1982) or between

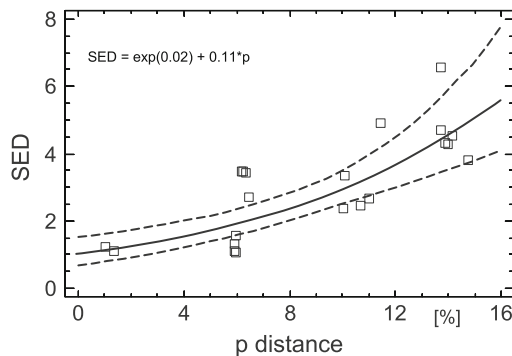


Fig. 8 Correlation between the Standard Euclidean Distance (SED) calculated on the basis of five mating call parameters of different water frog species (data from Joermann et al. 1988; Akef and Schneider 1989; Schneider and Sinsch 1992; Schneider and Haxhiu 1994; Schneider 1997, 1999; Haefeli 2005) and the uncorrected p distance obtained from the mt genes ND2 and ND3 of the same species ($r = 0.79$, F ratio = 31.72, $p < 0.01$). The R^2 statistic indicates that the model as fitted explains 62.5% of the variability in SED after transforming to a logarithmic scale to linearize the model. Solid line regression line. Broken lines 95% confidence limits

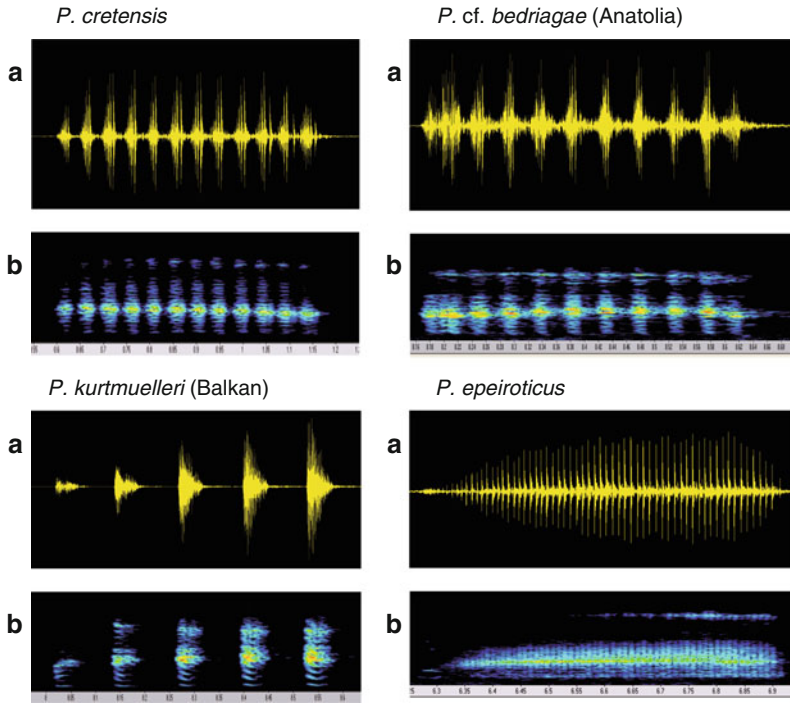
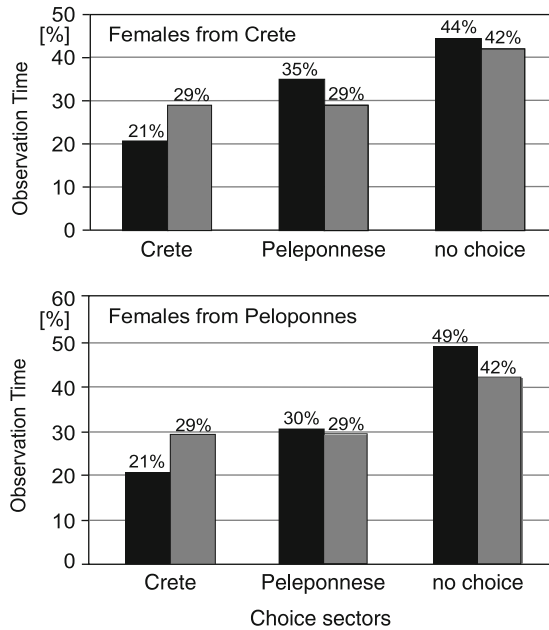


Fig. 9 Wave forms (a) and audio spectrograms (b) of mating calls recorded from *P. cretensis*, *P. cf. bedriagae* (Turkey), *P. kurtmuelleri* from the Peloponnese, and *P. epeiroticus* recorded at water temperatures of 26 °C (*cretensis*), 27 °C (*cf. bedriagae*), 28 °C (*kurtmuelleri*), and 26.5 °C (*epeiroticus*). From Haefeli (2005)

P. ridibundus and *P. lessonae* (Günther et al. 1991). Similarly, genetic investigations of two distinct groups with different mating calls in a transition zone, extending across north-eastern Greece and European Turkey, indicate nearly random mating among frogs in an area of secondary contact near the river Nestos (Beerli 1994; Hotz et al., in preparation). Similar observations have also been made in transition zones in Anatolia (Akin et al. 2010), such as in south-western Anatolia (MHG4 and 6) and east of the Amanos Mountains (MHG4, 5, and 6), where mtDNA haplotypes from distinct lineages are found syntopically in many populations. These observations are also supported by other findings in south-western Anatolia; individuals with the *caralitanus*-specific orange-colored ventral maculation have haplotypes of either subgroup 6b or 6c; and individuals with a black-spotted or white-colored venter may have *caralitanus*-specific haplotypes of subgroup 6a (Akin et al. 2010).

On the other hand, the low frequency of natural hybrids in western Greece, where *P. epeiroticus* and *P. kurtmuelleri* share the same habitats (e.g., Hotz and Uzzell 1982; Schneider et al. 1984; Berger et al. 1994), or in the Danube delta where *P. ridibundus* and *P. lessonae* occur sympatrically (Günther et al. 1991),

Fig. 10 Percentage of observation time that females of *P. cretensis* ($n = 26$) and of Balkanic *P. kurtmuelleri* ($n = 29$) spent in loudspeaker areas defined as conspecific and heterospecific and in no choice (indifferent) areas. Black bars observed values, gray bars expected values under random distribution. From Haefeli (2005)



speaks in favor of effective antihybridization mechanisms probably linked with the mating system, including mating calls. This assumption is consistent with empirical observations in western Greece where *P. epeiroticus* and *P. kurtmuelleri* occur syntopically but form separate choruses in the same aquatic habitat and at the same time. The choruses contained only single individuals of the foreign species and calling of *P. epeiroticus* choruses did not stimulate the calling activity of *P. kurtmuelleri* choruses (Kordges 1988). Similar observations were made by Günther (1982) in Lake Skutari (Montenegro) where *P. shqipericus* lives syntopically with *P. kurtmuelleri*.

3.7 Implications for Water Frog Systematics

Our genetic data suggest several new hypotheses on water frog systematics as a basis for future investigations. Most surprisingly, although all four individuals from the type locality of *P. ridibundus* (Atyrau, Kazakhstan) possessed mtDNA typical of the Anatolian clade (Fig. 2), they were all heterozygous for the nuclear markers SAI1 and *Rana*CR1, with one allele characteristic of Anatolian water frogs, the other of Central European lake frogs (Plötner et al. 2009). We therefore hypothesize that the northern Caspian Sea region represents a transition zone where different lineages have come into secondary contact and randomly interbreed.

As our molecular data clearly demonstrate, Anatolian water frogs and *P. bedriagae* from the Levant are not conspecific as proposed by Schneider and

co-workers (e.g., Schneider and Sinsch 1999). A sequence divergence in the mitochondrial ND2 and ND3 genes of approximately 6% (Ohst 2001; Akın et al., in press) and significant differences in the non-coding markers SAI1 and *Rana*CR1 (Plötner et al. 2009) clearly indicate that *P. bedriagae* and Anatolian water frogs represent distinct evolutionary species. Similarly, the Cilician MHG4 and 5 together represent a distinct clade separate from both *P. bedriagae* and the Anatolian (MHG6) lineages (Fig. 2). It is not yet clear, however, whether the observed degree of differentiation between MHG4, 5 and 6 (3.7–4.0%) warrants species status for the Cilician populations, jointly or separately.

On the other hand, the small genetic divergence values observed among the four subgroups (a–d) of the huge Anatolian MHG6 (1.5–2.2%) suggest that the subgroups are not distinct species. For instance, although populations belonging to subgroup b of MHG6 were described as a separate species under the name of *P. cerigensis* because of the presence of unique protein-coding alleles in Karpathos and Rhodos populations (Beerli et al. 1994), the samples used in this analysis were not fully representative of populations of that subgroup; specimens from the neighboring Anatolian populations from the coastal parts of the Antalya and Muğla provinces (Turkey), with exactly or very similar mitochondrial haplotypes, were not included in this study. The overall divergence between *cerigensis* and neighboring Anatolian subgroups does not exceed 1.3% (Table 1). The validity of *P. cerigensis* as a distinct species is not supported by our findings.

Similarly, although several authors (Jdeidi 2000; Jdeidi et al. 2001; Plötner 2005) have suggested raising *caralitanus* (MHG6a) to species status based on particular morphological, karyological, bioacoustic, and enzymological characteristics (e.g., Jdeidi 2000; Alpagut Keskin and Falakalı Mutaf 2006), its observed genetic distances to related forms and the extensive apparent hybridization with neighboring water frog populations (Akın et al. 2010) indicate that this suggestion was probably premature. Overall, despite, for example, Frost's (2008) treatment of the above-mentioned taxa as distinct species, the evolutionary relationships within Anatolian lineages require a taxonomic revision.

Two different lineages are represented on Cyprus, one closely related to Anatolian (MHG6) frogs of subgroup a, and the other (MHG3) unique to the island and of older origin. The genetic distinctness (ca. 6–7% pairwise distance; Table 1) and well-supported monophyly (Fig. 2) of the latter lineage confirms a separate species status for that group (Plötner et al. 2001; Plötner 2005). This hypothesis is also supported by sequence data obtained from SAI1 and *Rana*CR1 (Plötner and Akın, unpublished results). The same is true for Balkan lake frogs (Plötner et al. 2009), which were originally separated from Central European *P. ridibundus* on the basis of bioacoustic traits and named *Rana balcanica* by Schneider et al. (1993). Because morphological, protein electrophoretic, and mtDNA data have revealed only small differences between Balkan and Central European lake frogs, the systematic status of Balkan frogs has been questioned (e.g., Beerli et al. 1996; Plötner 1998, 2005; Plötner and Ohst 2001). Differences between the genomes of Balkan and Central European lake frogs are also expressed by crossing experiments with *P. lessonae*: while interspecies crosses between Central European *P. ridibundus*

and *P. lessonae* lead to hybridogenetic hybrids, crosses between Balkan lake frogs and *P. lessonae* result in non-hybridogenetic hybrids that are almost all sterile (Hotz et al. 1985; Berger et al. 1994). The results of crosses between Central European and Balkan lake frogs (Berger 1999, and unpublished data) also support the hypothesis that the two forms represent distinct species: backcross individuals (F_1 hybrids \times parental species) are sterile and the F_2 progeny are not viable. We therefore propose to recognize the Balkan frogs as a separate species for which the name *Pelophylax kurtmuelleri* Gayda, 1940 is available (Dubois and Ohler 1994).

3.8 Conclusions and Prospects

Genetic divergence among eastern Mediterranean water frogs, including differences in advertisement calls, appears to be the result of gradual genetic divergence in allopatry, closely associated with the geodynamic evolution of the Mediterranean since the Middle Miocene (i.e., since circa 11 Mya). Dispersal between populations has been prevented by the development of localized mountain ranges, waterless plateaux, and salt-water barriers that have originated as a result of plate motions, regional uplift and subsidence, and environmental changes such as the MSC. In addition, cyclic climatic events during the last 3 My further restricted the migration pattern and resulted in subdivision among populations, especially in the eastern Mediterranean region. We hypothesize three main factors to be important in speciation of eastern Mediterranean water frogs: (1) accumulation of genetic differences during periods of allopatry in glacial refugia and via subdivision of populations by the development of localized mountain barriers or regional uplift; (2) natural selection and adaptation imposed by different environments; and (3) stochastic effects associated with colonization and an extreme reduction in population size. The latter factor is thought to play an important role for several island populations (e.g., Rhodos and Karpathos) while the first and second factors appear to promote the evolution of most mainland populations. The extreme morphological stasis of the eastern Mediterranean water frogs suggests low adaptive phenotypic evolution. Despite this high similarity in morphology, they show considerable genetic divergence (1.5–6.9%), indicating the existence of several cryptic species with varied degrees of differentiation.

Hybridization, observed, for example, in eastern Greece and western Anatolia (Hotz et al., in preparation) and in the western Peloponnese (Hotz and Uzzell 1982), can be interpreted as results of secondary contacts between already divergent lineages. Under sympatry, one can expect a more rapid divergence of traits associated with antihybridization mechanisms, for example, mating signals. Because differences in advertisement calls have typically not diverged more rapidly than genetic traits (Fig. 8), natural selection, and drift in allopatry are apparently the main forces for the evolution of antihybridization mechanisms in water frogs, rather than sexual selection. Upon secondary contact, however, isolating mechanisms might be reinforced (Dobzhansky 1940), as seen in the sympatric species

P. kurtmuelleri/*P. epeiroticus* which have quite different mating calls (Fig. 9): *P. epeiroticus* mating calls are characterized by many very short pulse groups with few pulses per group (cf. Schneider et al. 1984) whereas mating calls of Balkan *P. kurtmuelleri* are characterized by a few relatively long pulse groups with many pulses per group (Schneider et al. 1984, 1993; Schneider and Sinsch 1992). We do not know, however, whether antihybridization mechanisms, genetic incompatibilities, or both cause the low frequency of hybrids between these species. Future work will also elucidate the role of ecological and demographic factors in causing speciation in Mediterranean water frogs.

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