

Balancing a Cline by Influx of Migrants: A Genetic Transition in Water Frogs of Eastern Greece

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Abstract

Variation patterns of allozymes and of ND3 haplotypes of mitochondrial DNA reveal a zone of genetic transition among western Palearctic water frogs extending across northeastern Greece and European Turkey. At the western end of the zone, allozymes characteristic of Central European frogs known as *Pelophylax ridibundus* predominate, whereas at the eastern end, alleles characteristic of western Anatolian water frogs (*P. cf. bedriagae*) prevail. The ND3 haplotypes reveal 2 major clades, 1 characteristic of Anatolian frogs, the other of European; the European clade itself has distinct eastern and western subclades. Both the 2 major clades and the 2 subclades overlap within the transition zone. Using Bayesian model selection methods, allozyme data suggest considerable immigration into the Nestos River area from eastern and western populations. In contrast, the ND3 data suggest that migration rates are so high among all locations that they form a single panmictic unit; the best model for allozymes is second best for mitochondrial DNA (mtDNA). Nuclear markers (allozymes), which have roughly 4 times as deep a coalescent history as mtDNA data and thus may reflect patterns over a longer time, indicate that eastern and western refugial populations have expanded since deglaciation (in the last 10 000 years) and have met near the Nestos River, whereas the mtDNA with its smaller effective population size has already lost the signal of partitioning into refugia.

Key words: *allozymes, Bayes factors, gene flow, hybridization, migration, mitochondrial DNA, model selection, Pelophylax, sympatry, water frogs*

Secondary contact zones between previously isolated, genetically diverged groups of closely related organisms provide opportunities for examining a variety of evolutionary processes, including amounts of genetic exchange between populations, selection against hybrids, and speciation. In most studies, however, hybrid zones have been investigated after a certain degree of reproductive isolation, such as reduced fitness or fertility of F_1 hybrids, has developed (reviewed by Howard et al. 2003). Analysis of hybrid zones between well-diverged yet more or less interfertile lineages is necessary to explore the mechanisms and diversity of speciation, especially the amount of genetic incompatibility of distinct lineages and species.

A genetic transition zone in northeastern Greece and European Turkey among water frogs of the genus *Pelophylax*

(Beerli 1994; Sinsch and Eblekamp 1994; Sofianidou et al. 1994) was first revealed by west–east differences in male advertisement calls (Schneider and Sinsch 1992; Schneider et al. 1993; personal observation). Based on their bioacoustic and electrophoretic data, Schneider et al. (1993) and Sinsch and Eblekamp (1994) concluded that the transition zone occurs between 2 reproductively isolated species and that the 2 species are sympatric at the Nestos River, near the center of the transition zone.

We here report protein electrophoretic data and mitochondrial DNA (mtDNA) sequence data for samples along this transition zone. We looked for coincidental distributions of differences in allele frequencies, which would confirm the presence of 2 species. We examined patterns of migration across the transition zone, looking for evidence of stability.

Low migration rates in the contact zone and other parts of the transect and increased numbers of homozygosities would indicate the presence of 2 distinct species. Simple neutral differentiation, in contrast, might result in either high or low rates of migration, depending on geography and local isolation.

Material and Methods

We use the name *Pelophylax ridibundus* (Pallas 1771) [TSN 775195; Integrated Taxonomic Information System on-line database, <http://www.its.gov>] for most of the lake frogs in eastern Greece, even though bioacoustic data reported for *Rana balcanica* Schneider and Sinsch 1993 [TSN 776493], a junior synonym of *Pelophylax kurtmuelleri* (Gayda 1940) [TSN 775188], suggest that many of these frogs are *P. kurtmuelleri*. For Anatolian frogs of the *P. ridibundus* group, we use the name *Pelophylax* cf. *bedriagae*; this includes frogs called *Rana ridibunda* by Schneider et al. (1993) or *Rana bedriagae* (Camerano 1882) [TSN 775178] by Schneider and Sinsch (2001).

Frogs for electrophoresis (283 individuals) were collected at 25 localities (Figure 1; Table 1); mtDNA sequence data are available for subsamples from 18 of these 112 individuals. Frogs were collected over many years for different studies; not all allozyme loci have been scored for all frogs. The samples chosen for mtDNA analysis were those that seemed most pertinent to the present study.

Protein electrophoresis used standard procedures (Hotz and Uzzell 1982; Beerli et al. 1996; Hotz et al. 1997). We examined products of 23 loci: aspartate aminotransferase (sAAT and mAAT, Enzyme Commission 2.6.1.1); aconitate hydratase (sACO and mACO, EC 4.2.1.3); adenosine deaminase (ADA, EC 3.5.4.4); adenylate kinase (AK-2, EC 2.7.4.3); albumin (ALB); creatine kinase (CK-A, EC 2.7.3.2); fructose-1,6-diphosphatase (FDP-1 and FDP-2, EC 3.1.3.11); glyceraldehyde-3-phosphate dehydrogenase (GAPDH, EC 1.2.1.12); α -glycerophosphate dehydrogenase (α GDH, EC 1.1.1.8); glucose-6-phosphate isomerase (GPI, EC 5.3.1.9); isocitrate dehydrogenase (mIDH and sIDH, EC 1.1.1.42); L-lactate dehydrogenase (LDH-A and LDH-B, EC 1.1.1.27); malate dehydrogenase (mMDH and sMDH, EC 1.1.1.37); mannose-6-phosphate isomerase (MPI, EC 5.3.1.8); 6-phosphogluconate dehydrogenase (PGDH, EC 1.1.1.44); phosphoglucomutase (PGM-2, EC 5.4.2.2, formerly 2.7.5.1); and superoxide dismutase (sSOD, EC 1.15.1.1). Alleles are named by lowercase letters in an established system (cf. Hotz and Uzzell 1982, Beerli 1994, Hotz et al. 1997 for designations).

Nucleotide sequences were determined for the mitochondrial gene NADH dehydrogenase subunit 3 (ND3; 340 bp), using methods specified by Plötner et al. 2008. Sequences are stored in the EMBL database under accession numbers HE855736–HE855847. Relations among the sequences were examined with RAXML (Stamatakis et al. 2005).

Both allozyme and mtDNA data were analyzed using the program MIGRATE 3.1.7 (Beerli 2006; Beerli and Palczewski 2010). Eleven localities (5–10, 12–14, 16, 21) at which at least 3 ND3 sequences were available and the allozyme data were

judged sufficiently complete were chosen to look for potential gene flow across the transect zone. Other localities were omitted for a variety of reasons: too little sequence data, too little allozyme data (too few individuals or too few loci for the available individuals), too far from the zone of genetic change. Localities 9 and 10, which are very close to each other in the same river drainage, were pooled into 1 population, as were localities 13 and 14. The geographic distances between the 9 resulting populations (localities 5, 6, 7, 8, pooled 9 and 10, 12, pooled 13 and 14, 16, and 21) were used to set the mutation-scaled migration rates to a consistent distance unit. To reduce the number of parameters, these 9 populations were analyzed using modified stepping stone migration models (Figure 2). This resulted in a maximum of 17 parameters (1 population size parameter and 16 interpopulation migration parameters).

MIGRATE was run using the Bayesian mode with a Metropolis-coupled heating scheme using 1 cold and 3 heated chains; each of 4 chains was run, after burn-in of 100 000 steps, for a total of 100 million steps per locus, distributed on a 98-CPU computer cluster in 100 replicates, to estimate parameters. During the course of the run, roughly 50 million genealogies for each locus were visited, resulting in 1.15 billion genealogies for the allozyme data set and 50 million for the mtDNA data set. In contrast to the standard Metropolis–Hastings method applied to the genealogies, the parameters were estimated using slice sampling (Neal 2003), which typically delivers parameters that are less autocorrelated than standard Metropolis–Hastings proposals.

Preliminary analyses revealed that our data sets do not have enough power to resolve the parameter values for all 72 possible migration connections and all 9 effective population sizes.

Multilocus allozyme data and mtDNA sequence data often yield multimodal posterior distributions with complex migration models resulting in parameter estimates that are inconsistent from run to run regardless of run-lengths; we have used these as indicators of failure to resolve parameters for complex models.

Nine different migration models that enforce particular biogeographic patterns were analyzed (Figure 2): I) individuals move randomly west or east through the transect; II) individuals move only to the west; III) individuals move only to the east; IV) individuals move to the center of the transect (towards localities 9 and 10) from both west and east; models V to VIII pool localities to the west of the transect center (6, 5, 7, 8, and 9) and localities to the east of the transect center (12, pooled 13 and 14, 16, 21); V) a stepping stone model with bidirectional migration; VI) migration only to the west; VII) migration only to the east; VIII) migration only to the central population (pooled 9 and 10); and IX) all localities form a single panmictic population.

These models were then compared using Bayes factors (Jeffreys 1961, Kass and Raftery 1995), which allow comparing and ordering of nonnested models, such as ours, taking into account the information content of the data and the complexity of the model. In addition, model probabilities (Bayes factor model weights; Kass and Raftery 1995) were

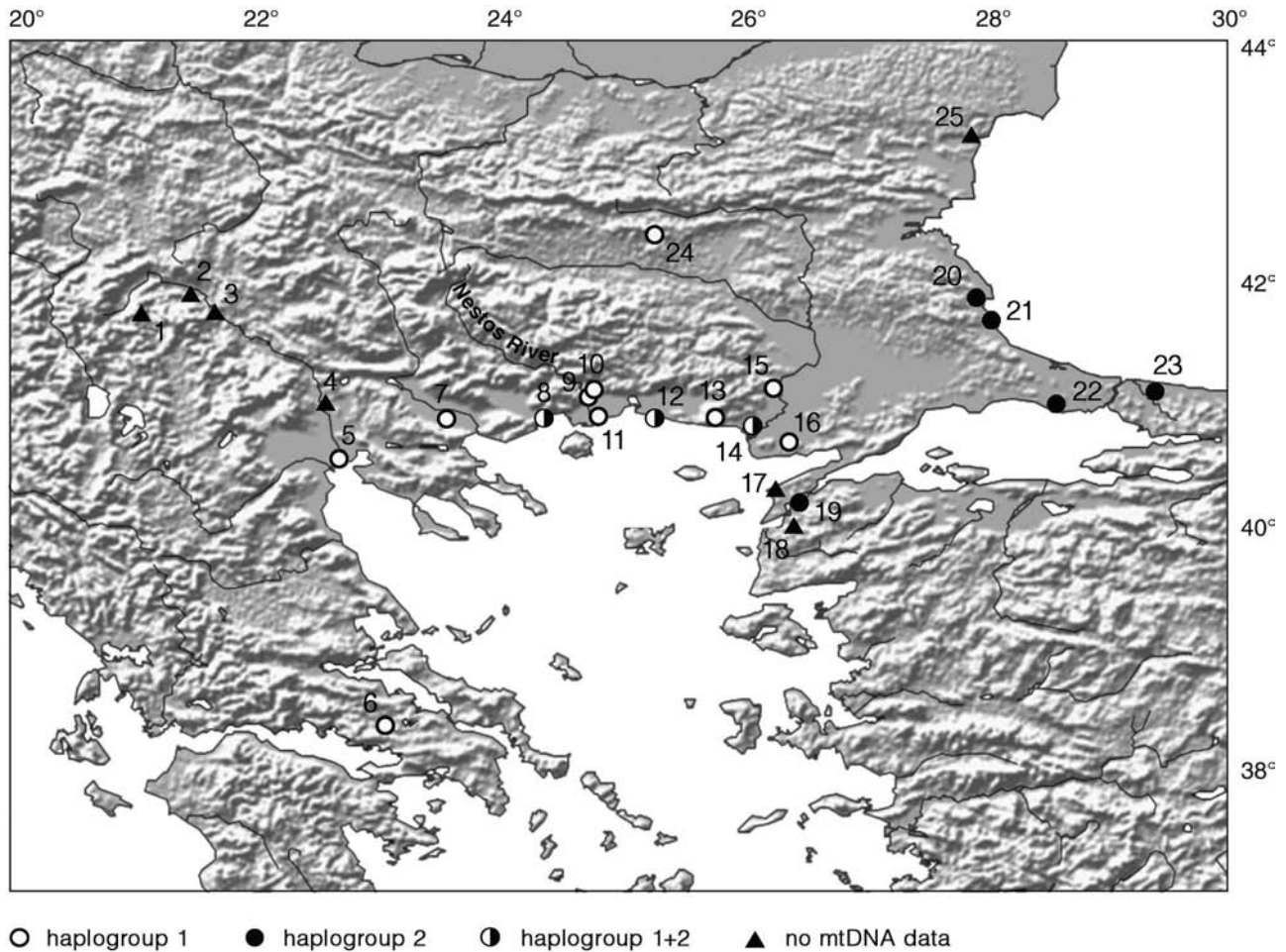


Figure 1. Map of the collecting localities; numbers correspond to localities in Materials and Methods. At least partial allozyme data are available from each locality. Distribution of *Pelophylax ridibundus* and *P. cf. bedriagae* mtDNA in our samples is indicated.

also calculated. These model probabilities are equivalent to Akaike weights (Burnham and Anderson 2002). Bayes factors and marginal likelihoods, in contrast to Akaike's information criterion or Bayesian information criterion, depend on the distribution of the likelihoods and not only on the maximum likelihood.

Bayes factors are dependent on the accurate calculation of marginal likelihoods. In fact, we can consider the marginal likelihood as a relative Bayes factor. The calculation of marginal likelihoods is difficult in standard Markov chain Monte Carlo (MCMC) programs. Currently, the best approaches depend on running multiple Markov chains at different temperatures and use "Thermodynamic integration" (or path sampling) to calculate an estimate of the marginal likelihood. This method was introduced by Gelman and Meng (1998). Thermodynamic integration has proved to be an accurate estimator of the marginal likelihood overcoming the poor performance of the widely used harmonic mean estimator. Lartillot and Philippe (2006) introduced the thermodynamic integration in phylogeny. Beerli and Palczewski (2010), Fan et al. (2011), and Xie et al. (2011) showed that the

thermodynamic integration estimator is delivering accurate estimates of the marginal likelihood that overcome the problems of the harmonic mean estimator. Beerli and Palczewski (2010) implemented thermodynamic integration for comparison of population genetics models in MIGRATE.

To look for the presence of 2 sympatric water frog species near the Nestos River, we examined the individual multilocus genotypes for 7 polymorphic loci for 33 frogs from 4 localities (Keramoti [8], Toxotai [9], Paradisos [10], Mangana [11]) in this region. For comparison, we also examined the individual multilocus genotypes for 6 polymorphic loci for 31 water frogs of 2 species from near Igoumenitsa in western Greece.

In addition, we also used the program Structurama 2.0 (Huelsenbeck and Andolfatto 2007) to assign individuals from the presumptive center of the cline (the locations Paradisos and Toxotai on the Nestos River). The prior distribution for the population numbers used was an exponential with mean of 1.0 (in the program this is equivalent to a gamma distribution with parameters a and b of 1.0 and 1.0). We ran the program for 10 000 000 steps. Multiple runs consistently delivered similar

Table 1 Origin and number (*N*) of individuals for which allozymes and mtDNA were investigated

No.	Population	Coordinates	Location	<i>N</i>	
				Allozymes	mtDNA
Macedonia					
01	Skopje-2	41.75°N/21.1°E	Stream 15 km SE Skopje, SW of road 1 Skopje-Thessaloniki	41	0
02	Skopje-1	41.9°N/21.5°E	Stream 10 km SE Skopje, at road 1 Skopje-Thessaloniki	25	0
03	Skopje-3	41.75°N/21.7°E	Stream near Rzanicanı, ca. 25 km S Skopje, at road to Titov Veles	20	0
Greece					
04	Gallikos	41.0°N/22.6°E	Ponds near river Gallikos, plain N of Thessaloniki	24	0
05	Kymina	40.55°N/22.7°E	Ditch near Kymina, N of highway Thessaloniki-Athinai (W of intersection to Gevgelija)	7	5
06	Aliartos	38.37°N/23.10°E	E Greece mainland N of Athinai. Channels in plain of river Kifisos (between Lamia and Athinai), near road E92, ca. 5 km NNE Aliartos	13	3
07	Ivira	40.9°N/23.6°E	Stream near river Strymon, N of Ivira, ca. 70 km W of Kavala	7	5
08	Keramoti	40.9°N/24.4°E	Stream near Keramoti, W of river Nestos delta	9	9
09	Toxotai	41.08°N/24.78°E	Ditch ca. 1 km SW Toxotai, E of Nestos River	4	4
10	Paradisos	41.1°N/24.8°E	River Nestos at road E55, ca. 14 km WSW Xanthi, 1 km E Paradisos	13	12
11	Mangana	40.92°N/24.84°E	Pond 2.8 km SW of Mangana, coastal plain E of river Nestos delta	7	7
12	Pagouria	40.9°N/25.3°E	Stream near Pagouria, between Alexandroupolis and Komotini	10	8
13	Lefkes	40.9°N/25.8°E	Near Lefkes, WNW of Alexandroupolis	10	8
14	Monastiraki	40.85°N/26.10°E	Stream near road E55 near Monastiraki, ca. 22 km ESE Alexandroupolis	13	12
15	Dadia	41.14°N/26.28°E	Ditch few km E of Dadia, 0.65 km W of road Alexandroupolis-Orestias	9	9
Turkey					
16	Ipsala	40.7°N/26.4°E	Streams near Ipsala, near border between Turkey and Greece	11	9
17	Gelibolu	40.3°N/26.3°E	Stream 1–2 km N Gelibolu, European side of Dardanelles	14	0
18	Umurbey	40.0°N/26.45°E	River Umurbey, Anatolian side of Dardanelles	16	0
19	Bayramdere	40.2°N/26.5°E	Stream near Bayramdere, Anatolian side of Dardanelles	8	6
20	İgneada	41.88°N/27.94°E	Stream at road 565, 3.3 km W İgneada, Black Sea coast	1	1
21	Kıyıköy	41.7°N/28.05°E	Pool at road between Vize and Kıyıköy, near Black Sea coast	5	4
22	Bü Çekmece	41.0°N/28.6°E	East shore of the lake Bü Çekmece Gölü, ca. 1 km N of Bü Çekmece, 30 km W Istanbul	2	2
23	Ömerli	41.1°N/29.4°E	Stream at road 20 near Ömerli, 35 km SW Sile, 34 km E Istanbul	4	2
Bulgaria					
24	Kazanlık	42.4°N/25.3°E	Pools in Tundza River plain S of Kazanlık, ca. 30 km NW Stara Zagora	6	6
25	Kamcija	43.2°N/27.9°E	River Kamcija, region of Staro Orjachovo, ca. 30 km S Varna	4	0
Sum				283	112

results. Changing the parameters of the prior distribution (e.g., changing to a mean of 2.0) did not change the inferred assignment or inferred number of populations.

Results

Allele Frequency Analyses

Allele frequencies for 15 variable loci are presented in Table 2 and Appendix 1. Numbers of loci examined for each locality, together with the fraction polymorphic and the mean heterozygosity, are included in Appendix 1. Four alleles are here newly designated. ADA *a*: This is the predominant allele in European frogs related to *P. ridibundus*. FDP-1 *e*: A rare allele

in *P. ridibundus* stock frogs from NE Greece. The mobility of its product on continuous tris–citrate gels is anodal of that of the predominant allele *a*. SIDH *b*: A widespread but rare allele in Anatolian frogs. The mobility of its product on continuous tris–citrate gels is anodal of that of the predominant allele *b*. LDH-B *k*: A rare allele in frogs of the *P. ridibundus* group in NW Anatolia. The mobility of its product on continuous tris–citrate gels is intermediate between that of the common alleles *a* and *c* of European frogs of the *P. ridibundus* group.

An overall west–east transition in allele frequencies is clearly evident, but there is considerable fluctuation, and not all loci show the same geographic location of major allele frequency changes (Appendix 1). Patterns for 4 loci with more complete data (α GDH, LDH-B, PGM-2, PGDH) are shown

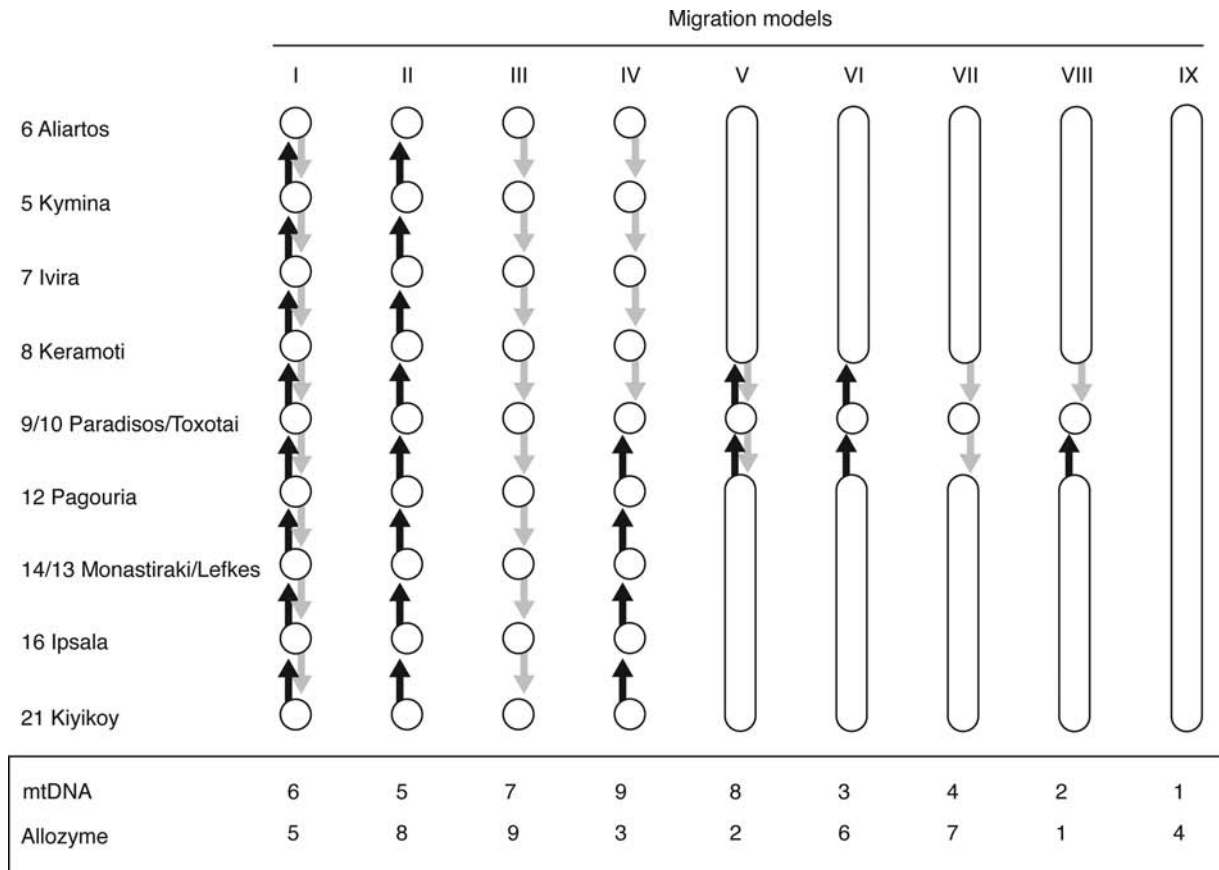


Figure 2. Comparison of migration models for allozyme and mtDNA data. Numbers by locality names correspond to those on the map (Figure 1); Paradisos and Toxotai (localities 9 and 10) are close to the Nestos River and the center of the transition zone. The models are described in Material and Methods. The numbers in the box represent model ranks based on Bayes factors calculated with MIGRATE (Beerli and Palczewski 2010); the log marginal likelihood values are tabulated in Supplementary Table 1.

in Figure 3. A 3-point moving average of the data reveals steep changes in allele frequencies for α GDH, LDH-B, and PGM-2 between Keramoti and Pagouria (Figure 1, localities 8–12), but the cline for PGDH is shifted to the west. There is no evidence of a gradient in number of polymorphic loci or mean heterozygosity across the transition zone, nor is a region of increased variability detectable (Appendix 1).

At the 4 localities near the Nestos River, there is no evidence of a significant association of alleles of the various loci in individuals (Table 2). In strong contrast, there is marked nonrandom association of alleles at the various loci in individuals from Igoumenitsa, where sympatry of 2 distinct species is known. For example, each individual homozygous for the allele ALB *e* was also homozygous for the alleles GPI *d*, LDH-B *d*, PGDH *g*, and PGM-2 *d* and had the MPI alleles *b* or *d*. This group of individuals represents the species *P. epeiroticus* [TSN 775185]; the majority of the others are *P. kurtmuelleri*. Two individuals were highly heterozygous and at each locus had 1 allele each of *P. epeiroticus* and of *P. kurtmuelleri*. These are interpreted as F₁ hybrids between these 2 species.

An additional analysis of the multilocus data set of the population Paradisos/Toxotai using the program Structurama

2.0 (Huelsenbeck and Andolfatto 2007) assigned all individuals to a single population. The most probable number of populations was also 1 (with probability 0.67); the probability of 2 populations was 0.27. These probabilities are dependent on the prior distribution and also depend on the size and variability of the data set. In contrast to sequence or microsatellite data, allozyme data are not very variable, allowing considerable uncertainty in a population assignment.

mtDNA Haplotype Analysis

ND3 sequences for 112 individuals examined from 18 localities across the transect form 2 quite distinct, well-supported groups (Figure 4, bootstrap support 100%). One corresponds to European *P. ridibundus/kurtmuelleri* mtDNA group (Plötner et al. 2001, 2008); it was observed in almost all samples from Greece and Bulgaria. The other group resembles ND3 sequences seen in the *P. cf. bedriagae* lineage from northwestern Anatolia. In our samples, the 2 types differ by at least 16 bp (4.7%). The Anatolian type alone was found at 3 localities in European Turkey: Igneada (locality 20: 1 individual), Kiyiköy (21: 5), and Bü Çekmece (22: 2). The 2 types coexist

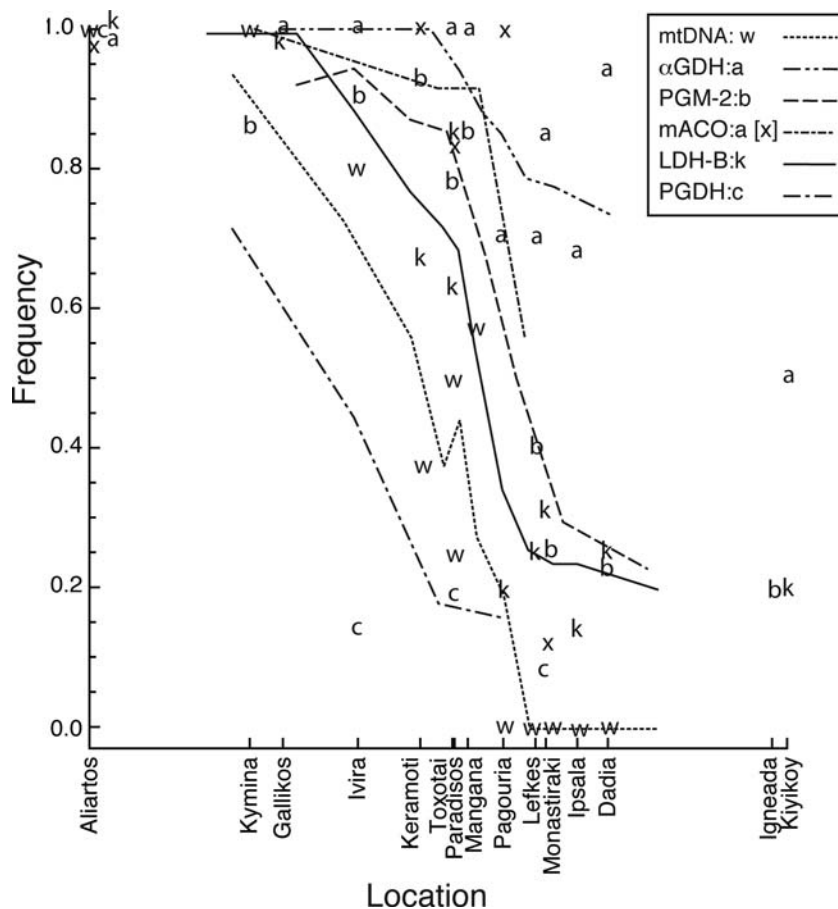


Figure 3. Frequency of alleles more common in western Greece at 5 allozyme loci along a transect from west to east in northeastern Greece and northwestern Turkey. Frequencies of 2 subtypes of *P. ridibundus* ND3 are also indicated. The lines are based on 3-point running averages.

at 3 localities in eastern Greece (Keramoti, Pagouria, and Monastiraki: localities 8, 12, and 14), the Anatolian type in lower frequencies (Monastiraki: 2 individuals, 17%; Pagouria: 1, 13%; Keramoti: 1, 11%). All other European localities contained only European *P. ridibundus*-type mtDNA. Only *P. cf. bedriagae* mtDNA was found at the Anatolian localities Ömerli (locality 23: 2 individuals) and Bayramdere (19: 6).

The 2 mtDNA types (European and Anatolian) are not associated with allozyme genotypes. For example, for the loci α GDH (with “western” allele a and “eastern” allele b) and LDH-B (with “western” allele a and “eastern” allele c; Appendix 1), the 2 individuals with Anatolian mtDNA from Monastiraki (locality 14) had, respectively, the genotypes a/b and a/a and c/c and a/c; the individual from Pagouria (12) had a/a and c/c; and the individual from Keramoti (8) had a/a and a/a. Likewise, at the localities in European Turkey where we found only Anatolian mtDNA (Igneada, Kiyiköy, Bü Çekmece; localities 20, 21, 22) both “eastern” and “western” allozyme alleles occurred (Appendix 1).

There is minor variation within both *P. ridibundus* and *P. cf. bedriagae* mtDNA. No geographic pattern is obvious for the *P. cf. bedriagae* sequences, within which sequence divergences of 0–4 bp (0–1.2%) occur. There is, however, a west–east

gradient in the *P. ridibundus* sequences (cf., Figure 4): all individuals from 6 eastern localities (Pagouria, Lefkes, Monastiraki, Dadia, Ipsala, and Kazanlık; localities 12–16, 24) and excepting the 2 frogs from Monastiraki (14) and 1 each from Keramoti (8) and from Pagouria (12) with *P. cf. bedriagae* mtDNA have ND3 sequence differences of 0–2 bp. (0–0.58%) from each other; frogs from the far west (Kymina and Aliartos; localities 5–6) have sequence differences of 0–4 bp (0–1.8%) from each other. ND3 sequences for these eastern and western localities differ by 3–7 bp (0.88–2.06%). Frogs from geographically intermediate localities, mostly near the Nestos River (Ivira, Keramoti, Toxotai, Paradisos, and Mangana; localities 7–11), fall into both groups. Three frogs from Mangana (locality 11), for example, have sequence differences of 0–1 bp (0–0.29%) from sequences from Kymina (5) and 1–3 bp (0.29–0.88%) from sequences from Aliartos (6), but differences of 2–4 bp (0.59–1.18%) from the eastern sequences. Three other frogs from Mangana have sequences that differ by 0–1 bp (0–0.29%) from sequences from the 6 eastern localities, but by 3–4 bp (0.89–1.18%) from sequences from Kymina and by 2–6 bp (0.59–1.76%) from sequences from Aliartos. Results for frogs from Toxotai, Keramoti, Paradisos, and Ivira are similar.

Table 2 Individual genotypes at variable protein loci in water frogs from 4 localities of the Nestos River area and from a locality near Igoumenitsa (western Greece) where 2 water frog species are sympatric. Alleles are named with lowercase letters. To facilitate overview, homozygotes are indicated by single letters. Missing data are blank

Field number	Taxon	ALB	GAPDH	GPI	LDH-B	MPI	PGDH	PGM-2
Paradisos								
12923	<i>P. ridibundus</i>	c	d	d	a c	a c	d e	d
12924	<i>P. ridibundus</i>	c	d	d	a	a c	d	d
12925	<i>P. ridibundus</i>	c	d	d	a	a c	e	d
12926	<i>P. ridibundus</i>	c	d	d	a	a	d e	d
12927	<i>P. ridibundus</i>	c	c d	d	a c	c	d e	b d
12928	<i>P. ridibundus</i>	c	d	d	a	a c	e	b d
12929	<i>P. ridibundus</i>	c	d	d	a	c	e	d
12930	<i>P. ridibundus</i>	c	c d	d	a	a c	e	d
12931	<i>P. ridibundus</i>	c	d	d	a	a c	e	d
12932	<i>P. ridibundus</i>	b c	d	d	a	a c	e	b d
12933	<i>P. ridibundus</i>	b c	d	a d	a c	a	e	b d
12934	<i>P. ridibundus</i>	c	d	a	c	c	e	d
12935	<i>P. ridibundus</i>	c	d	a d	a c	a c	e	d
Keramoti								
17412	<i>P. ridibundus</i>		d		a c	a		d
17413	<i>P. ridibundus</i>		d	a d	a c	a		b d
17435	<i>P. ridibundus</i>	c	d	d	a	a c		b d
17436	<i>P. ridibundus</i>	b c	d	d	a c	a c		d
17437	<i>P. ridibundus</i>		d	d	a			d
17438	<i>P. ridibundus</i>		d	d f	a c	a c		d
17439	<i>P. ridibundus</i>		d	a d	a c	a c		d
17440	<i>P. ridibundus</i>	b	a d	d	a c	a		b
17441	<i>P. ridibundus</i>	b c	d	d	a	a		b d
Toxotai								
22707	<i>P. ridibundus</i>			d	a c	a c		
22708	<i>P. ridibundus</i>			d	a c	a	e	
22709	<i>P. ridibundus</i>			d	a	a c		
22710	<i>P. ridibundus</i>			d	a c	a c	e	
Mangana								
22720	<i>P. ridibundus</i>		d	d	a	a c	e	
22721	<i>P. ridibundus</i>		d	a d	a c	a c	e	
22722	<i>P. ridibundus</i>		d	a d	c		e	
22723	<i>P. ridibundus</i>		d	a d	c	a c	e	
22724	<i>P. ridibundus</i>			d	a	a	e	
22725	<i>P. ridibundus</i>		c d	d f	a		e	
22726	<i>P. ridibundus</i>		d	a d	a c		e	
Igoumenitsa								
623	<i>P. epeiroticus</i>	e		d	d	b	g	d
632	<i>P. epeiroticus</i>	e		d	d	b	g	d
636	<i>P. epeiroticus</i>	e		d	d	b	g	d
638	<i>P. epeiroticus</i>	e		d	d	b	g	d
642	<i>P. epeiroticus</i>	e		d	d	b d	g	d
645	<i>P. epeiroticus</i>	e		d	d	b		d
646	<i>P. epeiroticus</i>	e		d	d	b	g	d
649	<i>P. epeiroticus</i>	e		d	d	b d	g	d
650	<i>P. epeiroticus</i>	e		d	d	b	g	d
651	<i>P. epeiroticus</i>	e		d	d	b	g	d
652	<i>P. epeiroticus</i>	e		d	d	b	g	d
626	Hybrid	b e		a d	a b	a b	d g	d
641	Hybrid	b e		d	a d	a b	d g	d
624	<i>P. ridibundus</i>	b		d	a	a b	d	d
625	<i>P. ridibundus</i>	b		a d	a	a b	d	b d
627	<i>P. ridibundus</i>	b		d	a	a b	d	d
628	<i>P. ridibundus</i>	b		d	a	a	c d	d
629	<i>P. ridibundus</i>	b		a	a	a b	d	d
630	<i>P. ridibundus</i>	b		a d	a	a	d	b d
631	<i>P. ridibundus</i>	b		d	a	a	d	b d
633	<i>P. ridibundus</i>	b		d	a	a b	d	b
634	<i>P. ridibundus</i>	b		a d	a	a	d	d
635	<i>P. ridibundus</i>	b		a d	a	b	d	b d
637	<i>P. ridibundus</i>	b		d	a	a	d	b d
639	<i>P. ridibundus</i>	b		a d	a	a	d	b d
640	<i>P. ridibundus</i>	b		d	a	a	d	b d
643	<i>P. ridibundus</i>	b		a	a	a	d	d
644	<i>P. ridibundus</i>	b		a b	a	a	d	b d
647	<i>P. ridibundus</i>	b		a	a	a	d	d
648	<i>P. ridibundus</i>	b		b d	a	a	d	d
653	<i>P. ridibundus</i>	b		a b	a	a b	d	d

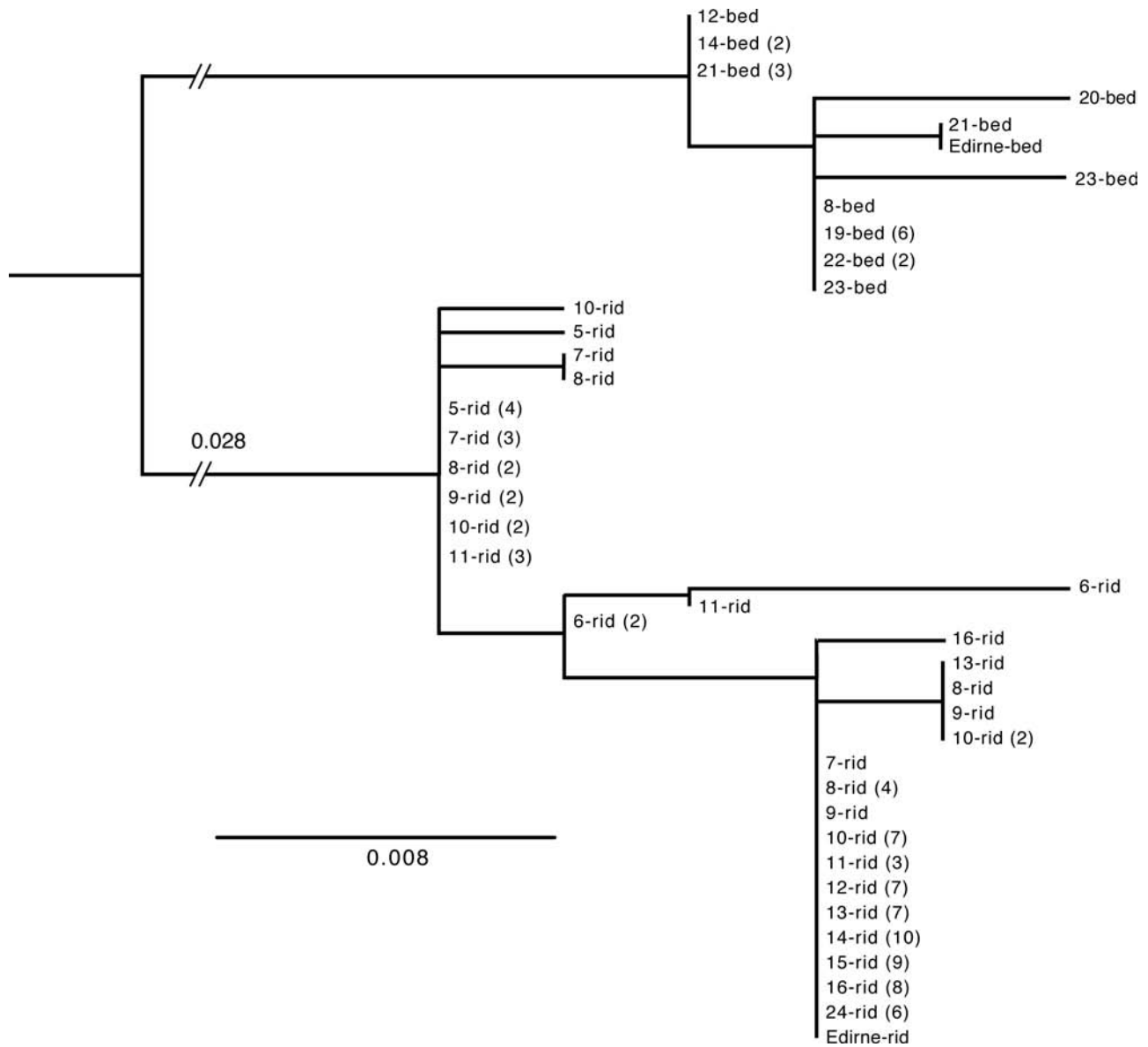


Figure 4. RAxML tree based on unique mitochondrial ND3 sequences of water frogs across a genetic transition in northern Greece and European Turkey. Tree rooted at midpoint. Sequences are identified by numbers corresponding to the 18 localities with mtDNA data; these are given in Materials and Methods and correspond to those of Figure 1. Number of individuals with a given sequence provided in parentheses if greater than 1.

There are thus “eastern” and “western” *ridibundus*-like ND3 sequences in the transect area, but just as for the European and Anatolian mtDNA groups, there is no significant association of “eastern” *ridibundus*-like ND3 sequences with “eastern” allozyme alleles, nor is there any association of “western” *ridibundus*-like ND3 sequences with “western” allozyme alleles.

Population Model Comparison

For the mtDNA data, the estimated mutation-scaled effective population size is 0.002, and the magnitude of the mutation-scaled migration rates is high, when using a modified

stepping stone model (Figure 2). Multiplying the mutation-scaled effective population size with the mutation-scaled immigration results in high number of effective immigrants per generation, suggesting that the number of immigrants for each of the populations is much larger than 1 per generation and that the transect localities form a panmictic unit. Indeed, the model comparison (Figure 2; see Supplementary Table 1 online) favors model IX (all localities form a panmictic population) over the other models tested. Using Bayes factor weights (Kass and Raftery 1995) to calculate the model probability, models I–VIII have near zero probability, whereas IX has a probability of 1.0.

For the allozyme data, the 9 migration models tested (Figure 2) had very different log marginal likelihoods. These allow an unequivocal ordering of the models and choice of the best model among those tested (Figure 2; see Supplementary Table 1 online). Model VIII, in which migrants move toward the Nestos River from both east and west, has much greater support than any other model tested. Models VII and IX have probabilities close to zero, whereas model VIII has a probability of 1.0.

The best model (VIII) treats locations to the east and to the west of the Nestos River as single panmictic populations, and so does not allow us to investigate potential isolations among western and among eastern locations. The best 9-population model (IV), which might allow investigation of more local isolation, estimated the mutation-scaled migration rates between all neighboring locations to be of the same magnitude (between 200 and 450), thus indicating no geographic barrier between any of the sampling locations.

Discussion

Geographic Patterns of Molecular Markers

Our allozyme data reveal an overall east–west transition in allele frequencies, generally with change most rapid near the Nestos River in the central part of the transition zone. There is, however, considerable variability in regions of maximal change (Figure 3; Appendix 1), and alternative alleles for many of these loci are observed both to the west and to the east of the Nestos River (Appendix 1). There is no apparent association of particular alleles in multilocus genotypes for individuals from near the Nestos River (Table 2). Frequencies of polymorphic alleles, obtained from 3 of our samples (localities 6, 10, and 14; the last 2 near the Nestos River), could not be shown to depart from Hardy–Weinberg equilibrium; there is no significant association of alleles at individual loci within individuals (Beerli 1994). These findings of random association of alleles into genotypes in the Nestos River region are corroborated by Structurama's assignment of all individuals from Paradisos and Toxotai to a single rather than 2 populations. This also suggests that they are members of a randomly mating, panmictic population.

There is extensive variation in ND3 sequences along the transect zone and we observed 2 major groups of sequences, 1 characteristic of northwest Anatolian *P. cf. bedriagae*, the other characteristic of Central European *P. ridibundus*. Sympatry of these 2 mitochondrial haplotypes occurs in the eastern part of the transect (Figure 1) although the Anatolian type is apparently scarce and spotty in the transition zone. It also occurs at Edirne in western European Turkey (Akin et al. 2010), where 1 individual among 5 had the Anatolian haplotype. Lymberakis et al. (2007), utilizing partial cytochrome *b* and 16S rRNA mtDNA sequences, reported 2 quite different sequence types from Dadia in eastern Greece, a single individual of each. One of these sequences is nested deeply within their *P. ridibundus*–*P. kurtmuelleri* group, whereas the sequence from the other individual is nested deeply within their *P. bedriagae* group. We detected only the *ridibundus* type

in our sample of 9 individuals from near Dadia. This broad geographic overlap of the Anatolian and European ND3 haplotypes does not support the existence of 2 biological species of *Pelophylax*, sympatric in the Nestos River region, especially because there is no marked association of these 2 mitochondrial types with allozyme alleles, either across the area of sympatry or near the Nestos River.

The frequency of the 2 different subgroups of *P. ridibundus* ND3 sequences also changes along the transect between western and eastern ends, with the center of change near the Nestos River, where both subgroups occur in nearly equal frequencies. This pattern resembles that for several allozyme loci (Figure 3; Appendix 1). Again, however, there is no noticeable association of these subgroup sequences with allozyme alleles. ND3 sequences corresponding to these 2 subgroups also occur in northern and Central Europe, where they are intermixed with no geographic patterning (Plötner et al. 2008).

One Species or Two?

Bioacoustic data collected by Schneider and Sinsch (1992) and Schneider et al. (1993) and allozyme data collected by Sofianidou et al. (1994) and Sinsch and Eblenkamp (1994) were interpreted as demonstrating that 2 largely allopatric species are present in a transition zone in northeastern Greece, with an area of sympatry in the Nestos River region. Male advertisement calls of frogs from the western end of the transition zone were ascribed to *R. balcanica* (a junior synonym of *P. kurtmuelleri*), whereas calls from frogs from the eastern end were ascribed to *P. ridibundus* (Schneider and Sinsch 1992; Schneider et al. 1993). An area of sympatry in the Nestos River region was identified based on analysis of 18 mating call series recorded at Toxotes and Neo Erasmio. These calls collectively did not match calls recorded either west or east of the Nestos area; but a discriminant analysis assigned calls of 10 series to *R. balcanica* (= *P. kurtmuelleri*) and calls of 8 series to *R. ridibunda* (= *P. cf. bedriagae*). Probably this, more than their allozyme data, convinced them that there are 2 sympatric species in the Nestos River region. Regardless, the ways in which parameters of advertisement calls of frogs from the eastern end of the zone deviate from parameters of calls of western frogs make the eastern frog calls more like calls characterizing western Anatolian frogs, *P. cf. bedriagae* (Joermann et al. 1988; Haefeli 2005).

Two allozyme loci that were reportedly diagnostic of the 2 taxa in the transition zone (ADA, Sofianidou et al. 1994; GPI, Sinsch and Eblenkamp 1994) clearly are not so in our data set. In our data, ADA is monomorphic for all of the 98 frogs scored from 14 localities (19 frogs from 3 localities in the Nestos River region, 17 from 3 localities west of it, 62 from 8 localities east of it). ADA, thus, cannot be used to distinguish 2 species. GPI is, in contrast, polymorphic in our data set (Appendix 1). One allele (*b*) is very rare, occurring as a heterozygote in 1 individual from near Skopje; another (*f*) is uncommon. Of the 2 more common alleles, *a* (which probably corresponds to allele 33 of Sinsch and Eblenkamp 1994) occurs, although often infrequently, in many of the western

populations, whereas *d* (which probably corresponds to Sinsch and Eblenkamp's allele 100) is more common in eastern populations. In our data, however (Appendix 1), these 2 alleles do not distinguish 2 species. Similar variability was in fact observed in "GPI-1" by Sinsch and Eblenkamp (1994). Only their allele 100 occurred in *P. ridibundus* from Valtos and from the Nestos River (Neo Erasmo), but their *R. balcanica* (= *P. kurtmuelleri*) sample from near the Axios River (Gefyra) had both alleles 100 (60%) and 33 (40%).

It is clear that our allozyme data and ND3 sequence data do not support the idea of 2 reproductively isolated species of *Pelophylax* across the transect zone, with sympatry near the Nestos River; our evidence that there is random association of allozyme alleles with each other or with ND3 haplotypes suggests that interbreeding among the water frogs in this region is random. This is also suggested by the Structurama results. Schneider et al.'s (1993) analysis of calls of frogs recorded near the Nestos River possibly suggests that the change between voice types in the transition zone is more abrupt than it is for ND3 sequences and allozyme allele frequencies and possibly convinced them that there are 2 sympatric species in the Nestos River region. It is difficult, however, to see how such a situation could persist, given the random mating among frogs near the Nestos River.

Patterns of Migration across the Transect

MIGRATE analysis of the allozyme data indicates an influx of genetic material into the center of the transition zone from both east and west. The estimated magnitudes of gene flow into the center of the zone are similar, suggesting that the cline is either fixed at its current location or only moving slowly. The migration rates among sample locations are high, but, in contrast to the mtDNA data set, a panmictic single-population model is not favored. The best model among those tested pools all locations east and all locations west of the center of the cline (Nestos River) so that only 3 populations are considered in the MIGRATE analysis. Among these 3-population models (Figure 2, models V–VIII), the model with migration rates toward the center of the cline (Model VIII) is strongly favored with a probability of 1.0, again using all 9 tested models as a basis.

Analysis of the ND3 sequences strongly suggests that mitochondrial transmission is not reduced across the transect and that there is little or no difference between the localities from the east or the west in this respect. Comparison of several models reveals that a model that treats all localities in the MIGRATE transect mtDNA data set as a single panmictic population has by far the highest marginal likelihood and its model probability is 1.0 using all the 9 tested models as a basis for comparison.

The migration patterns of mtDNA and nuclear markers are contradictory: the former indicates either different dispersal rates of females or else a selective sweep of a particular mtDNA variant (Smith and Haigh 1974), whereas the pattern of the nuclear markers may suggest that selection in the hybrid zone is acting to enforce the cline. We do not have data to show that the cline is fixed by reduced fitness of individuals in the

hybrid zone; that our samples from localities 6, 10, and 14 do not depart from Hardy–Weinberg equilibrium indicates that there is little selection on particular allelic combinations and little reduction in fitness. For a moving cline we would expect that the gene flow pattern would indicate migration from 1 end toward the center in the direction of cline movement, but on the other side of the cline we would expect migration patterns in all directions or at least a pattern that does not show increased migration toward the center of the cline.

The most recent common ancestor (MRCA) of a single population for nuclear genes occurred roughly 4 times the effective population size ($4N_e$) generations in the past, whereas the time of the MRCA for mitochondria occurred about N_e generations in the past (Palumbi et al. 2001). The frogs that we observe today probably are descendents of frog populations that were separated during the last glacial period but could potentially have remixed during the last 10 000 years. With a generation time of about 2 to 3 years (Günther 1990; Plötner 2001), this represents about 5000 generations. Effective frog population sizes (N_e) of a few thousand individuals are not unreasonable. For nuclear markers, an N_e larger than 1250 might allow the MRCA to have occurred more than 10 000 years ago, thus allowing the isolation signature to persist; whereas for the mtDNA marker, an N_e larger than 5000 would be required for the signature of isolation to persist. Additional mtDNA and multilocus nuclear DNA data may shed light on the contradiction among the population models, but ultimately evaluation of fitness parameters along the transect will be required to resolve the puzzle.

A potential separation into a western (southern Greece) and an eastern refugium (Black Sea coast or Anatolia) during the last glaciation perhaps allowed changes of allele frequencies in these refugia. After the cold period, the populations expanded east and west and met in the region of the Nestos River where we see the admixed populations. The separation obviously did not lead to reproductive isolation as the different refugial types seem to mix without problems. These expansion routes probably are reflected in the model ranking for the allozyme data, which suggest that a model with movement toward the Nestos River should be preferred over all other models.

The transition zone in eastern Greece discussed here differs from the transition zone detected in Italian water frogs in Calabria (Santucci et al. 1996) in having greater breadth and a lower geographic concordance of allele frequencies among the various loci. The transition in Calabria more resembles classical tension zones such as that between *Bombina bombina* and *Bombina variegata* (Szymura 1983; Szymura et al. 1985; Szymura and Barton 1986, 1991; Fijarczyk et al. 2011). The transition zone in eastern Greece, in contrast, is more similar to the transition zone along the Adriatic coast of Italy, which is also long (from coastal central Italy to the southern Po valley) and differs across loci in the location of allele frequency changes (Uzzell and Hotz 1979; Hotz 1983, unpublished data).

In summary, our data on both allozymes and mtDNA sequences demonstrate that, rather than there being 2 reproductively isolated species of water frogs sympatric near the

Nestos River, there is a broad zone of hybridization across Greece and Turkey north of the Aegean Sea between 2 genetically distinct stocks. Our data also show that there is widespread introgression of previously diverged northwestern Anatolian mtDNA lineages into water frog populations of European Turkey and easternmost Greece. This introgression is consistent with the broad east–west trends in both allozyme allele frequencies and ND3 haplotypes across northeastern Greece, with a region of more rapid change near the Nestos River.

Supplementary Material

Supplementary material can be found at <http://www.jhered.oxfordjournals.org/>.

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Appendix 1 Allele frequencies for 15 variable protein-coding loci in the genetic transition zone. The localities are basically listed from west to east. N: total sample size; #: number of individuals scored per locus. Alleles are named with lowercase letters. Eight additional loci were invariant in these samples (mAAT *b*, ADA *a*, AK-2 *a*, CK-A *b*, mIDH *b*, sMDH *b*, mMDH *a*, sSOD *a*); they are omitted from the table but are included in fraction of polymorphic loci and mean heterozygosity. The localities are keyed to the map (Figure 1) by number

No.	Locality	N	sAAT					sACO					mACO					ALB					FDP-I					FDP-2				
			n	c	e	f	l	n	b	c	n	a	b	n	a	b	c	d	n	a	b	c	d	e	n	a	b	c	d	e	n	a
1	Skopje-2	41	41	0.04	0.94	0.02	—	0	0	0.67	0.30	0.02	0	23	0.67	0.30	0.02	0	0	0.88	0.08	0.04	—	13	0.88	0.08	0.04	—	13	0.04	0.96	
2	Skopje-1	25	25	0.02	0.98	—	—	0	0	0.42	0.58	—	0	6	0.42	0.58	—	0	0	1.00	—	—	—	0	1.00	—	—	—	0	—	1.00	
3	Skopje-3	20	20	—	1.00	—	—	0	0	0.58	0.43	—	0	20	0.58	0.43	—	0	0	0.94	0.06	—	—	0	0.94	0.06	—	—	0	—	1.00	
4	Gallikos	24	24	—	0.94	—	0.06	0	0	0.05	0.59	0.36	0	11	0.05	0.59	0.36	0	0	1.00	—	—	—	0	1.00	—	—	—	0	—	1.00	
5	Kymina	7	0	—	—	—	—	0	0	0.75	0.25	—	1	4	0.75	0.25	—	1	0	0.93	—	—	0.07	0	0.93	—	—	0.07	—	—	1.00	
6	Aliartos	13	13	—	0.96	0.04	—	13	0.96	0.04	13	—	1.00	11	—	1.00	—	13	0.88	0.08	0.04	—	13	0.88	0.08	0.04	—	13	0.04	0.96		
7	Ivira	7	0	—	—	—	—	0	0	0.50	0.42	0.08	0	6	0.50	0.42	0.08	0	0	1.00	—	—	—	0	1.00	—	—	—	0	—	1.00	
8	Keramoti	9	7	—	1.00	—	—	5	1.00	—	5	—	1.00	4	0.50	0.50	—	3	1.00	—	—	—	2	1.00	—	—	—	2	—	1.00		
9	Toxotai	4	0	—	—	—	—	0	0	0.50	0.50	—	0	4	0.50	0.50	—	4	1.00	—	—	—	0	1.00	—	—	—	0	—	1.00		
10	Paradisos	13	11	—	1.00	—	—	13	1.00	—	6	0.17	0.83	13	0.08	0.92	—	10	1.00	—	—	—	13	1.00	—	—	—	13	—	1.00		
11	Mangana	7	0	—	—	—	—	0	0	0.83	0.17	—	0	0	0.83	0.17	—	7	0.93	—	—	—	0	0.93	—	—	—	0	—	1.00		
12	Pagouria	10	10	—	1.00	—	—	10	1.00	—	5	—	1.00	6	0.83	0.17	—	9	0.94	0.06	—	—	0	0.94	0.06	—	—	0	—	1.00		
13	Lefkes	10	2	—	1.00	—	—	10	1.00	—	0	—	1.00	1	1.00	—	—	9	1.00	—	—	—	0	1.00	—	—	—	0	—	1.00		
14	Monastiraki	13	11	—	0.95	0.05	—	13	0.96	0.04	13	0.88	0.12	12	0.13	0.88	—	5	1.00	—	—	—	13	1.00	—	—	—	13	—	1.00		
15	Dadia	9	0	—	—	—	—	0	0	0.88	0.12	—	0	0	0.88	0.12	—	9	1.00	—	—	—	0	1.00	—	—	—	0	—	1.00		
16	Ipsala	11	6	—	1.00	—	—	11	1.00	—	1	1.00	—	0	1.00	—	—	7	1.00	—	—	—	0	1.00	—	—	—	0	—	1.00		
17	Gelibolu	14	2	—	1.00	—	—	0	0	1.00	—	—	0	0	1.00	—	—	3	1.00	—	—	—	0	1.00	—	—	—	0	—	1.00		
18	Umurbey	16	1	—	1.00	—	—	0	0	1.00	—	—	0	0	1.00	—	—	0	1.00	—	—	—	0	1.00	—	—	—	0	—	1.00		
19	Bayramdere	8	0	—	—	—	—	8	1.00	—	1	—	1.00	0	1.00	—	—	4	1.00	—	—	—	0	1.00	—	—	—	0	—	1.00		
20	Ignrada	1	0	—	—	—	—	0	0	1.00	—	—	0	0	1.00	—	—	0	1.00	—	—	—	0	1.00	—	—	—	0	—	1.00		
21	Kiviköy	5	0	—	—	—	—	0	0	1.00	—	—	0	5	—	1.00	—	0	1.00	—	—	—	0	1.00	—	—	—	0	—	1.00		
22	Bü Çekmece	2	0	—	—	—	—	0	0	0.50	0.50	—	0	1	0.50	0.50	—	0	1.00	—	—	—	0	1.00	—	—	—	0	—	1.00		
23	Ömerli	4	0	—	—	—	—	0	0	1.00	—	—	0	0	1.00	—	—	0	1.00	—	—	—	0	1.00	—	—	—	4	—	1.00		
24	Kazanlık	6	0	—	—	—	—	0	0	1.00	—	—	0	6	—	1.00	—	6	1.00	—	—	—	0	1.00	—	—	—	0	—	1.00		
25	Kamecja	4	0	—	—	—	—	2	1.00	—	2	1.00	—	2	1.00	—	—	3	1.00	—	—	—	0	1.00	—	—	—	0	—	1.00		

Appendix I (Continued)

No.	Locality	N	GAPDH			αGDH			GPI			sIDH			LDH-A			LDH-B				
			n	c	d	n	a	b	n	a	b	n	a	b	n	a	b	n	a	b	c	k
1	Skopje-2	41	0			41	1.00		41	0.43	0.01	0.55	0.01	41	1.00		41	0.99	0.01			
2	Skopje-1	25	0			25	1.00		25	0.38		0.60	0.02	25	1.00	0.02	25	1.00				
3	Skopje-3	20	0			20	1.00		20	0.43		0.53	0.05	0			0	1.00				
4	Gallikos	24	0			24	1.00		24	0.13		0.88		19	1.00		24	1.00	0.02	23	0.98	0.02
5	Kymina	7	6		1.00	7	1.00		7	0.14		0.86		7	1.00		7	1.00		7	1.00	
6	Aliartos	13	8		1.00	13	1.00		13	0.08		0.92		13	0.90	0.10	13	1.00		13	1.00	
7	Ivira	7	5		1.00	7	1.00		5	0.10		0.90		7	1.00		6	1.00		7	1.00	
8	Keramoti	9	9	0.06	0.94	7	1.00		8	0.13		0.81	0.06	8	1.00		8	1.00		9	0.67	0.33
9	Toxotai	4	1		1.00	4	1.00		4			1.00		4	1.00		4	1.00		4	0.63	0.38
10	Paradisos	13	13	0.08	0.92	13	1.00		13	0.08		0.92		13	1.00		13	1.00		13	0.85	0.15
11	Mangana	7	6	0.08	0.92	6	1.00		6	0.33		0.67		7	1.00		7	1.00		7	0.57	0.43
12	Pagouria	10	7	0.21	0.79	10	0.70	0.30	7	0.07		0.93		10	1.00		10	1.00		10	0.20	0.80
13	Lefkes	10	7	0.07	0.93	10	0.70	0.30	7	0.07		0.93		10	1.00		10	1.00		10	0.25	0.75
14	Monastiraki	13	9	0.28	0.72	13	0.85	0.15	13			1.00		13	1.00		13	1.00		13	0.31	0.69
15	Dadia	9	9	0.06	0.94	9	0.94	0.06	6			0.92	0.08	9	1.00		9	1.00		6	0.25	0.75
16	Ipsala	11	3		1.00	11	0.68	0.32	9			1.00		11	1.00		11	1.00		11	0.14	0.86
17	Gelibolu	14	0			3	0.17	0.83	12	0.04		0.92	0.04	2	1.00		2	1.00		14	0.21	0.79
18	Umurbey	16	0			1	1.00		15			1.00		1	1.00		1	1.00		16	0.22	0.72
19	Bayramdere	8	8	0.31	0.69	8	0.56	0.44	5			1.00		8	0.94	0.06	8	1.00		8	0.06	0.94
20	Igneda	1	0			0			1			1.00		0			1	1.00		1		1.00
21	Kiyiköy	5	0			5	0.50	0.50	4			1.00		5	1.00		5	1.00		5	0.20	0.80
22	Bü Çekmece	2	0			2	0.25	0.75	0			1.00		0			2	1.00		2		1.00
23	Ömerli	4	3		1.00	2	0.50	0.50	1	0.50		0.50		0			4	1.00		4		1.00
24	Kazanlık	6	6	0.08	0.92	6	0.75	0.25	6	0.25		0.75		6	1.00		6	1.00		6	0.58	0.42
25	Kamcaja	4	1	1.00		4	0.38	0.63	2			1.00		4	1.00		4	1.00		4	0.38	0.63

Appendix I (Continued)

No.	Locality	N	MPI			PGDH						PGM-2						N Loci	Fraction polymorphic	Mean observed heterozygosity
			n	a	b	c	n	c	d	e	n	a	b	d	g					
1	Skopje-2	41	41	0.77	—	0.23	0.02	0.93	0.05	41	—	0.15	0.85	—	17	0.412	0.108			
2	Skopje-1	25	25	0.84	0.04	0.12	0.04	0.92	0.04	25	—	0.14	0.86	—	17	0.412	0.101			
3	Skopje-3	20	0	—	—	—	—	—	—	20	—	0.08	0.93	—	9	0.333	0.139			
4	Gallikos	24	24	0.85	—	0.15	—	1.00	—	24	—	0.08	0.90	0.02	15	0.400	0.087			
5	Kymina	7	6	0.83	—	0.17	—	1.00	—	7	—	—	1.00	—	17	0.176	0.053			
6	Aliartos	13	13	1.00	—	—	—	1.00	—	13	—	—	1.00	—	22	0.273	0.039			
7	Ivira	7	7	0.71	—	0.29	—	0.14	0.86	7	—	0.07	0.93	—	17	0.235	0.114			
8	Keramoti	9	8	0.75	—	0.25	—	—	—	9	—	0.28	0.72	—	22	0.273	0.129			
9	Toxotai	4	4	0.63	—	0.38	—	—	1.00	0	—	0.15	0.85	—	13	0.154	0.128			
10	Paradisos	13	13	0.50	—	0.50	—	0.19	0.81	13	—	—	—	—	21	0.381	0.102			
11	Mangana	7	4	0.63	—	0.38	—	—	1.00	0	—	0.60	0.40	—	13	0.385	0.128			
12	Pagouria	10	10	0.55	—	0.45	—	0.20	0.80	10	—	0.75	0.25	—	22	0.409	0.140			
13	Lefkes	10	10	0.45	—	0.55	—	—	1.00	10	—	0.77	0.23	—	21	0.286	0.108			
14	Monastiraki	13	13	0.69	—	0.31	—	0.08	0.92	13	—	—	—	—	20	0.500	0.133			
15	Dadia	9	9	0.67	—	0.33	—	—	1.00	0	—	—	—	—	13	0.385	0.109			
16	Ipsala	11	11	0.68	0.05	0.27	—	—	1.00	11	—	0.64	0.36	—	21	0.190	0.098			
17	Gelibolu	14	14	0.93	—	0.07	—	—	1.00	14	0.04	0.89	0.07	—	19	0.263	0.140			
18	Umurbey	16	16	0.88	—	0.13	—	—	1.00	16	—	0.81	0.19	—	15	0.200	0.243			
19	Bayramdere	8	7	0.93	—	0.07	—	—	1.00	8	—	1.00	—	—	20	0.250	0.076			
20	İğneada	1	1	1.00	—	—	—	—	—	0	—	—	—	—	6	0	0			
21	Kiyıköy	5	5	0.50	—	0.50	—	—	1.00	5	0.10	0.70	0.20	—	16	0.250	0.127			
22	Bü Çekmece	2	2	1.00	—	—	—	—	—	2	—	0.50	0.50	—	10	0.300	0.211			
23	Ömerli	4	4	0.88	—	0.12	—	—	—	4	—	0.63	0.38	—	12	0.333	0.119			
24	Kazanlık	6	6	0.25	—	0.75	—	—	1.00	6	—	0.58	0.42	—	18	0.250	0.139			
25	Kamçija	4	4	0.50	—	0.50	—	—	1.00	4	—	0.75	0.25	—	20	0.167	0.121			

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