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GEOLOGICALLY DATED SEA BARRIERS CALIBRATE A PROTEIN CLOCK FOR AEGEAN WATER FROGS

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Abstract.—Reliable estimates of phylogenetic relationships and divergence times are a crucial requirement for many evolutionary studies, but are usually difficult because fossils are scarce and their interpretation is often uncertain. Frogs are fresh water animals that generally are unable to cross salt water barriers (their skin is readily permeable to both salt and water). The geologically determined ages of salt water barriers that isolate related frog populations thus provide an independent measure of the minimum date of genetic divergence between pairs of such populations. For the genetically well-studied western Palearctic water frogs (*Rana esculenta* group), the Aegean region provides an ideal area for determining the relationship between genetic divergence and time of spatial isolation, using a nested set of geologically determined isolation times (12,000 yr, 200,000 yr, 1.8 Myr, 2–3 Myr, and 5.2 Myr).

Using 31 electrophoretic loci for 33 pairs of neighboring frog populations, a linear relationship between geologically determined isolation time and Hillis' modified Nei genetic distance was found: D^*_{Nei} = (0.04 ± 0.01) + (0.10 ± 0.01) isolation time [Myr] corresponding to an average divergence rate ("molecular clock" pace) of 0.10 D^*_{Nei}/Myr (0.10 D_{Nei}/Myr). This rate is in the range of previous estimates reported for protein electrophoretic data; the value is conservative because relatively few of the loci used are "fast evolvers" (13%; sAAT, ALB, EST-5, MPI). Removing these fast evolvers from the analysis results in 0.08 D^*_{Nei}/Myr (0.08 D_{Nei}/Myr). The confidence limits for estimation of the divergence time given the genetic distance are large, but unusually narrow for this kind of study; they permit us to estimate divergence times during the Pliocene and Miocene. Few previous studies, including sequence analyses, have provided reasonable estimates of divergence time for the Pliocene. A test using the outgroup taxa *Rana perezi* and *Rana saharica* (also isolated for 5.2 Myr by the Strait of Gibraltar) fits the calibration well: observed genetic Nei distance D^*_{Nei} = 0.55, expected D^*_{Nei} = 0.56. The calculated divergence times, based on this absolute molecular clock, suggest a series of speciation events after the Messinian (5.2 Myr), possibly triggered by the rapid ecological changes accompanying the desiccation and refilling of the Mediterranean Basin.

Key words.—Aegean region, frogs, geology, molecular clock, phylogeny, protein electrophoresis, *Rana esculenta* group, rate of evolution.

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The phylogenetic history of a group of organisms is interesting in its own right, but is of critical importance for drawing general conclusions from any comparative or evolutionary study of that group, whether in morphology or physiology, ecology or behavior. Determining the correct history is hampered on the one hand by a general scarcity of fossils, and on the other by the multiplicity of equally or nearly equally good phylogenetic trees that can be generated from many data sets. This is particularly true of molecular data sets, in which the very abundance and objectivity of the data almost force us to conduct extensive or, where possible, exhaustive analyses. Several studies have tried to limit the number of alternative histories to be considered by using biological data other than morphological or genetic information, such as species composition (Busack 1986; Legendre 1986; Avise 1992; Oosterbroek and Arntzen 1992), migratory pattern (Bowen et al. 1989), ecological adaptations (cf. Grant 1986), and parasites (Tirard et al. 1992).

Based on the number of amino acid replacements in proteins of several mammalian species, Zuckerkandl and Pauling (1962) proposed that the rate of protein evolution provides us with a molecular clock. They avoided the problems of fossil data by using only extant taxa. This framework rapidly expanded from amino acid replacements in proteins to nucleotide substitutions in genes (Kimura 1983). Molecular clocks are thought to be paced by neutral mutations and to reflect a stochastic process analogous to radioactive decay rather than a metronomical process (e. g. Kimura 1968, 1969, 1983; Uzzell and Corbin 1971; Fitch and Langley 1976; Wilson et al. 1977, 1987; Takahata 1987).

Molecular clocks have been reported in a variety of proteins. The albumin clock, extensively applied to phylogenetic studies through microcomplement fixation immunology (reviewed by Maxson and Maxson 1986), is consistent with but does not require the neutral theory of evolution, which states that most allelic variation found in natural populations is selectively neutral rather than adaptive (Kimura 1968, 1983; cf. Lewontin 1974). Although some genes appear to provide reliable clocks (Pesole et al. 1991), other studies have demonstrated that there is no standardized clock for all genes and species (e.g., Scherer 1990; Gillespie 1991). Observed differences in divergence rate among proteins led to recognition of groups of rapidly evolving and conservative genes (Sarich 1977).

The estimation of divergence time is crucial in calibrating molecular clocks but often is not reliable (Avise and Aquadro 1982; Avise 1994). At least one independently timed event has to be used for calibrating any clock (Busack 1986). Moreover, when using short time spans, the microphyletic structure of the group studied must be well known to prevent heter-

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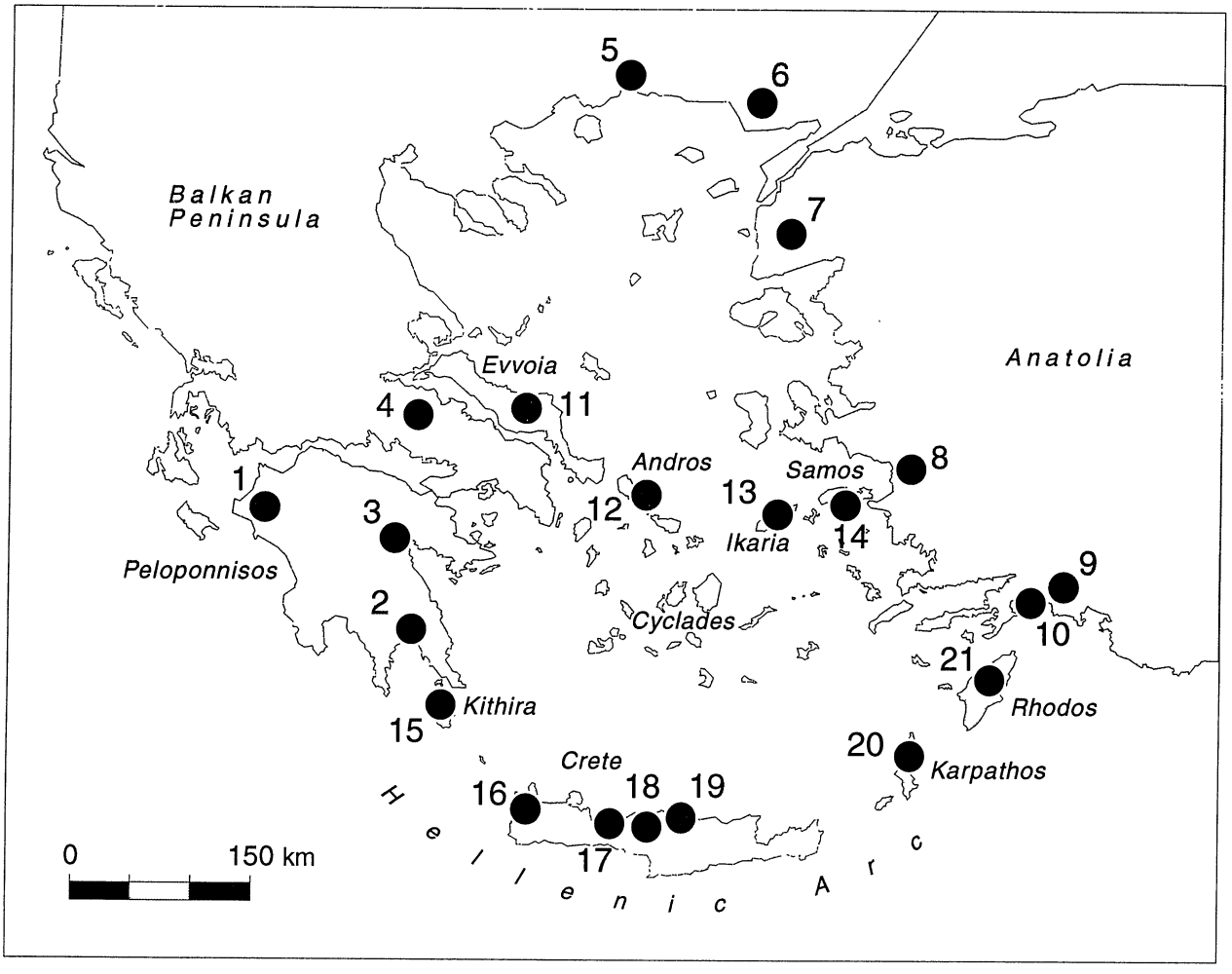

FIG. 1. Localities in the Aegean region from which frogs were sampled: (1) Nea Manolada; (2) Skala; (3 Argos; (4) Aliartos; (5) Paradisos; (6) Monastiraki; (7) Ezıne; (8) Selçuk; (9) Akçapınar; (10) Marmaris; (11) Evvoia; (12) Andros; (13) Ikaria; (14) Samos; (15) Kithira; (16) Kastelli; (17) Petros River; (18) Lavris; (19) Iraklion; (20) Karpathos; (21) Rhodos. *Rana epeirotica* was sampled in locality 1; *Rana ridibunda* in localities 1–6, 11, 12, 15; *Rana bedriagae* in 7–10, 13, 14; *Rana cretensis* in 16–19; *Rana cerigensis* in 20, 21.

ologous comparisons. A molecular clock with known relationships among the taxa under study and with well-dated isolation times will give the most accurate estimates, although extrapolation to other groups and beyond the time interval requires caution.

Frogs are usually unable to cross salt water barriers, because their skin is readily permeable to both salt and water. The age of salt water barriers isolating pairs of frog populations therefore provides a measure of the minimum time that such pairs of populations have been genetically isolated. If we can determine from geology the duration of isolation and from genetic studies the amount of genetic difference, we can estimate the rate of genetic divergence and therefore calibrate a "molecular clock." The western Palearctic water frogs (*Rana esculenta* group) in the Aegean region are an ideal group for such a study. Because the geological history of the eastern Mediterranean Sea is relatively well known, both the oldest and the most recent possible times of isolation can be estimated for many pairs of populations.

This study presents genetic data based on protein electrophoresis for 22 populations from 21 localities in the Aegean region, including eight islands and the surrounding mainlands of southern and eastern Greece and western Anatolia. Geologically known minimal divergence times of these population pairs form a nested set containing several points ranging between 12,000 yr and 5.2 Myr. From these data we have developed an average molecular clock with relatively narrow confidence limits. Phylogenetic analyses of five species of the Aegean region and of four other species of the group are presented. Application of the protein clock to estimate times of divergence from observed genetic distances in several taxon pairs of the group suggests a cluster of speciation events following the Messinian period 5 Myr ago. The data obtained on phylogenetic relationships and divergence rates help to establish a reliable historical biogeography for the western Palearctic water frogs. This framework is important for studies aiming at an evolutionary understanding of the initiation of clonal reproduction that characterizes widespread natural hybrid lineages of this group (reviewed by Graf and Polls Pelaz 1989).

TABLE 1. Genetic variability at 31 loci for the 22 populations studied. Localities are keyed to the map (Fig. 1) by numbers. Values are means ± SE. Taxa were identified by protein electrophoresis. *N* = number of sampled individuals. Taxa: bed = *Rana bedriagae,* rid = *Rana ridibunda,* epe = *Rana epeirotica,* cer = *Rana cerigensis,* cre = *Rana cretensis.*

Region	Locality	Taxon	*N*	Average number of individuals examined per locus	Mean number of alleles per locus	Percentage of loci polymorphic[a]	Mean heterozygosity: Direct count	Mean heterozygosity: Hardy-Weinberg expected[b]
Anatolia	9 Akçapınar	bed	14	12.0 ± 0.7	1.5 ± 0.1	38.7	0.143 ± 0.045	0.159 ± 0.048
	10 Marmaris	bed	5	4.2 ± 0.2	1.2 ± 0.1	12.9	0.062 ± 0.031	0.059 ± 0.028
	8 Selçuk	bed	16	12.8 ± 1.0	1.4 ± 0.1	35.5	0.114 ± 0.039	0.132 ± 0.043
	7 Ezıne	bed	11	9.3 ± 0.5	1.4 ± 0.1	38.7	0.126 ± 0.039	0.132 ± 0.041
Northern Greece	4 Aliartos	rid	13	10.4 ± 0.6	1.3 ± 0.1	22.6	0.025 ± 0.011	0.052 ± 0.025
	5 Paradisos	rid	13	11.4 ± 0.5	1.3 ± 0.1	32.3	0.101 ± 0.032	0.092 ± 0.028
	6 Monastiraki	rid[c]	13	10.9 ± 0.7	1.5 ± 0.1	45.2	0.118 ± 0.031	0.126 ± 0.032
Peloponnisos	1 Nea Manolada	epe	12	9.7 ± 0.6	1.1 ± 0.0	6.5	0.009 ± 0.009	0.029 ± 0.021
	3 Argos	rid	10	8.6 ± 0.5	1.2 ± 0.1	19.4	0.056 ± 0.025	0.063 ± 0.027
	1 Nea Manolada	rid	1	1.0	1.1	6.5	0.065 ± 0.045	0.065 ± 0.045
	2 Skala	rid	13	9.6 ± 0.8	1.1 ± 0.1	12.9	0.015 ± 0.007	0.023 ± 0.013
European islands	11 Evvoia	rid	10	8.6 ± 0.4	1.1 ± 0.1	12.9	0.020 ± 0.010	0.019 ± 0.010
	12 Andros	rid	3	2.7 ± 0.1	1.0	0.0	0.0	0.0
	15 Kithira	rid	9	7.8 ± 0.4	1.0	3.2	0.004	0.004
Anatolian islands	13 Samos	bed	4	3.5 ± 0.2	1.3 ± 0.1	22.6	0.097 ± 0.042	0.119 ± 0.041
	14 Ikaria	bed	11	9.2 ± 0.5	1.2 ± 0.1	22.6	0.042 ± 0.018	0.050 ± 0.021
	21 Rhodos	cer[d]	14	13.4 ± 0.6	1.3 ± 0.1	22.6	0.048 ± 0.017	0.059 ± 0.021
	20 Karpathos	cer	9	6.8 ± 0.4	1.1	6.5	0.009 ± 0.006	0.009 ± 0.006
Crete	19 Iraklion	cre	7	5.7 ± 0.4	1.0	0.0	0.0	0.0
	16 Kastelli	cre	11	7.4 ± 0.7	1.0	0.0	0.0	0.0
	18 Lavris	cre	6	5.0 ± 0.3	1.0	0.0	0.0	0.0
	17 Petros River	cre	2	1.9 ± 0.1	1.0 ± 0.0	3.2	0.016 ± 0.016	0.016 ± 0.016

[a] A locus is considered polymorphic if more than one allele was detected; for this data, this equals the P99 criteria.
[b] Unbiased estimate (Nei 1978).
[c] The high proportion of polymorphic loci is consistent with differences in allele frequencies in the transect Akçapınar-Skala, which suggest a genetic exchange from *Rana bedriagae* into *Rana ridibunda* in northeastern Greece, and perhaps in the reverse direction in northwestern Anatolia (Beerli 1994).
[d] The taxonomic status of the Rhodos population needs further study (Beerli et al. 1994).

MATERIAL AND METHODS

Western Palearctic water frogs were collected from 21 localities around the Aegean Sea (Fig. 1). Each frog was heparinized and then anesthetized with 3-aminobenzoic acid ethyl ester (MS-222). Samples of skeletal muscle, liver, kidney, heart, and plasma were removed and stored at −70°C. In the Aegean region, the following taxa were studied: *R. ridibunda* Pallas 1771 in the southern Balkan Peninsula; *R. bedriagae* Camerano 1882 in Anatolia (Dubois 1992; Beerli 1994); *R. epeirotica* Schneider, Sofianidou, and Kyriakopoulou-Sklavounou 1984; *R. cretensis* Beerli, Hotz, Tunner, Heppich, and Uzzell 1994; and *R. cerigensis* Beerli, Hotz, Tunner, Heppich, and Uzzell 1994. The following species and populations were used for comparison: *R. perezi* Seoane 1885 from Tarifa (Spain); *R. saharica* Boulenger 1913 from Asilah (Morocco); *R. lessonae* Camerano 1882 from Poznań Żurawiniec (Poland) and Frauenfeld (Switzerland); *R. shqiperica* Hotz, Uzzell, Günther, Tunner, and Heppich 1987 from Virpazar (Yugoslavia); *R. epeirotica* from Aitolikon (western Greece); and *R. ridibunda* from Poznań-Fabianowo (Poland).

Proteins examined were encoded by 31 structural gene loci, and include aconitate hydratase (sACO and mACO; Enzyme Commission 4.2.1.3), adenylate kinase (AK; EC 2.7.4.3), albumin (ALB), aspartate aminotransferase (sAAT and mAAT; EC 2.6.1.1), s-adenosyl-l-homocysteine hydrolase (AHH; EC 3.3.1.1), carbonate dehydratase (CA-2; EC 4.2.1.1), creatine kinase (CK-A; EC 2.7.3.2), carboxylesterases (EST-5 and EST-6; EC 3.1.1.-, l-methyl-l-umbelliferyl acetate as substrate), fructose-biphosphatase (FDP-1 and FDP-2; EC 3.1.3.11), glucose dehydrogenase (GCDH; EC 1.1.1.118), glucose-6-phosphate isomerase (GPI; EC 5.3.1.9), glyceraldehyde-3-phosphate dehydrogenase (GAPDH; EC 1.2.1.12), glycerol-3-phosphate dehydrogenase (a-GDH; EC 1.1.1.8), guanine deaminase (GDA; EC 3.5.4.3), isocitrate dehydrogenase (mIDH and sIDH; EC 1.1.1.42), lactate dehydrogenase (LDH-A and LDH-B; EC 1.1.1.27), malate dehydrogenase (sMDH and mMDH; EC 1.1.1.37), mannose-6-phosphate isomerase (MPI; EC 5.3.1.8), unidentified soluble muscle proteins (MPR-1 and MPR-3), one peptidase (PEP; EC 3.4.-, l-valyl-l-leucine as substrate), phosphoglucomutase (PGM-2; EC 5.4.2.2), phosphogluconate dehydrogenase (6PGDH; EC 1.1.1.44), and superoxide dismutase (sSOD; EC 1.15.1.1). Enzymes were separated using standard starch gel electrophoresis and localized on 1-mm-thick gel slices with standard chromogenic methods (Uzzell and Berger 1975; Wright et al. 1980; Hotz 1983; Richardson et al. 1986; Murphy et al. 1990). Albumin and the unspecified muscle proteins were separated on polyacrylamide gels (Hotz 1983; Harlow and Lane 1988). For each locus, all electrophoretic patterns observed for any taxon were compared by using samples of other water frog taxa or localities on the same gels. Alleles are named by lowercase letters following an established system (Hotz and Uzzell 1982). For pairs of enzyme loci encoding proteins with alternative subcellular localization (AAT, ACO, IDH, MDH, SOD), the translation products located in mitochondria were identified by comparing the observed electrophoretic patterns with those obtained from a tissue fraction enriched in mitochondrial proteins (Graf 1989; Hotz, Uzzell, and Berger unpubl.). Loci coding for cytosolic and mitochondrial enzymes are designated with the prefix s and m, respectively.

TABLE 2. Unique alleles found in nine taxa and 29 populations of western Palearctic water frogs, including the 22 populations studied for this report. Fixed alleles occur throughout a taxon, whereas segregating alleles are listed by population within taxa.

Taxon	Locality	Unique alleles		
		Number	Segregating	Fixed
Rana cretensis	Iraklion	8	—	sAAT:*m*, sACO:*d*, ALB:*k*, Est-5:*f*, Est-6:*d*, GPI:*h*, sIDH:*g*
	Kastelli		—	
	Lavris		—	
	Petros River		GDA:*e*	
Rana epeirotica	Aitolikon	5	sAAT:*k*	ALB:*e*, EST-5:*a*, MPI:*b*, 6PGDH:*g*
	Nea Manolada		—	
Rana lessonae	Frauenfeld	9	CK-A:*a*, LDH-B:*b,e*, MPR-3:*a*	mAAT:*a*, ALB:*a*, GAPDH:*a*, PGM-2:*c*
	Poznań		CA-2:*h*, LDH-B:*b,e*	
Rana cerigensis	Karpathos	4	EST-5:*e*, MPR-1:*d*	sAAT:*l*, AHH:*c*
	Rhodos	5	sAAT:*l*, AHH:*c*, EST-5:*e*, CA-2:*b*	FDP-2:*c*
Rana bedriagae	Akçapınar	9	sACO:*a*, mACO:*e*, ALB:*j*, mIDH:*e*, sMDH:*d*, MPI:*j*, PGM-2:*h*	
	Ezıne		—	
	Marmaris		MPI:*j,k*	
	Selçuk		ALB:*j*	
	Ikaria		MPI:*j*	
	Samos		MPR-1:*e*	
Rana ridibunda	Aliartos	6	sAAT:*f*, FDP-1:*d*, FDP-2:*a*	
	Monastiraki		sAAT:*f*	
	Paradisos, Andros, Evvoia, Nea Manolada, Skala, Kithira		—	
	Argos		sMDH:*e*, PGM-2:*a*	
	Poznań		sAAT:*c*	
Rana shqiperica	Virpazar	8	CA-2:*f*, EST-6:*c*, sIDH:*f*, MPI:*d,e,p*	FDP-1:*c*, EST-5:*c*
Rana perezi	Tarifa	8	sAAT:*n*, CK-A:*d*, mMDH:*b*, MPI:*l,m*	ALB:*l*, GCDH:*b*, sIDH:*e*
Rana saharica	Asilah	14	AHH:*d*, EST-6:*a*, MPI:*n,o*	mACO:*d*, ALB:*m*, CK-A:*c*, GCDH:*a*, sIDH:*d*, MIDH:*d*, LDH-B:*h*, PEP:*d*, 6PGDH:*f*, sSOD:*c*

The phylogenetic structure of allele frequencies was analyzed with CONTML (PHYLIP 3.5c; Felsenstein 1993) and the heterozygosity values were calculated with BIOSYS-1 (Swofford and Selander 1989). Hillis's (1984) modified Nei distance D^*_{Nei} (Nei 1972) was calculated with our own program. We used the modified Nei distance D_{Nei} because Nei's original distance is more affected by variation among loci in evolutionary rate (Hillis 1984), although for our data the modified and standard distances differ little. The weighted linear regression and ANOVA calculations followed Sokal and Rohlf (1981) and Snedecor and Cochran (1980), using Mathematica 2.2 (Wolfram 1991; Wolfram Research 1993).

GEOLOGICAL EVENTS

Geological events in the Mediterranean region were used to determine the time of isolation between the mainlands and islands. Geomorphological change in the Mediterranean region is driven by the collision of the African and Arabian plate with the Eurasian plate: In the late Miocene (about 11 Myr; cf. Van Eysinga 1975), the Balkan Peninsula, Anatolia, and the shapes of the islands Crete and Karpathos were similar to their present ones, and were separated from each other (Biju-Duval et al. 1977: pl. 8). In the Messinian (latest Miocene) the entire Mediterranean basin dried up, as a result of the closing of the Strait of Gibraltar (Hsü 1972; Hsü et al. 1977; Hsü and Giovanoli 1979). The Mediterranean islands then became mountains in a steppe or desert, so that overland migration between islands and from the mainlands was possible. Some 5.2 ± 0.1 Myr ago, the Strait of Gibraltar reopened and the basin was refilled from the Atlantic Ocean in about 1000 yr. Crete became permanently isolated, both from Anatolia to the east and Peloponnisos to the west, as well as from the rest of the Hellenic arc. Kithira was submerged in the early Pliocene, and did not reemerge until the late Pliocene (Meulenkamp 1985). Although Karpathos was isolated during the Miocene, it was connected through Rhodos to the Anatolian mainland in the early Pliocene (Daams and Van der Weerd 1980); Karpathos's connection to Rhodos was broken at some time during the middle or late Pliocene (cf. Kuss 1975), and it has remained isolated since. Rhodos itself remained connected to the Anatolian mainland until the late Pliocene or early Pleistocene, as shown by sedimentation and fossil data (Meulenkamp et al. 1972; Meulenkamp 1985; Dermitzakis 1990), but has been isolated since.

In the Pleistocene, all of today's islands were in place. Crete, Karpathos, and Rhodos remained completely isolated. During the cold and warm stages, the sea level varied between approximately today's level or slightly higher and 200 m lower (R. Hantke, U. Radtke, K. Hsü, pers. comm.). Only the sea level of the Würm glaciation is exactly recorded:

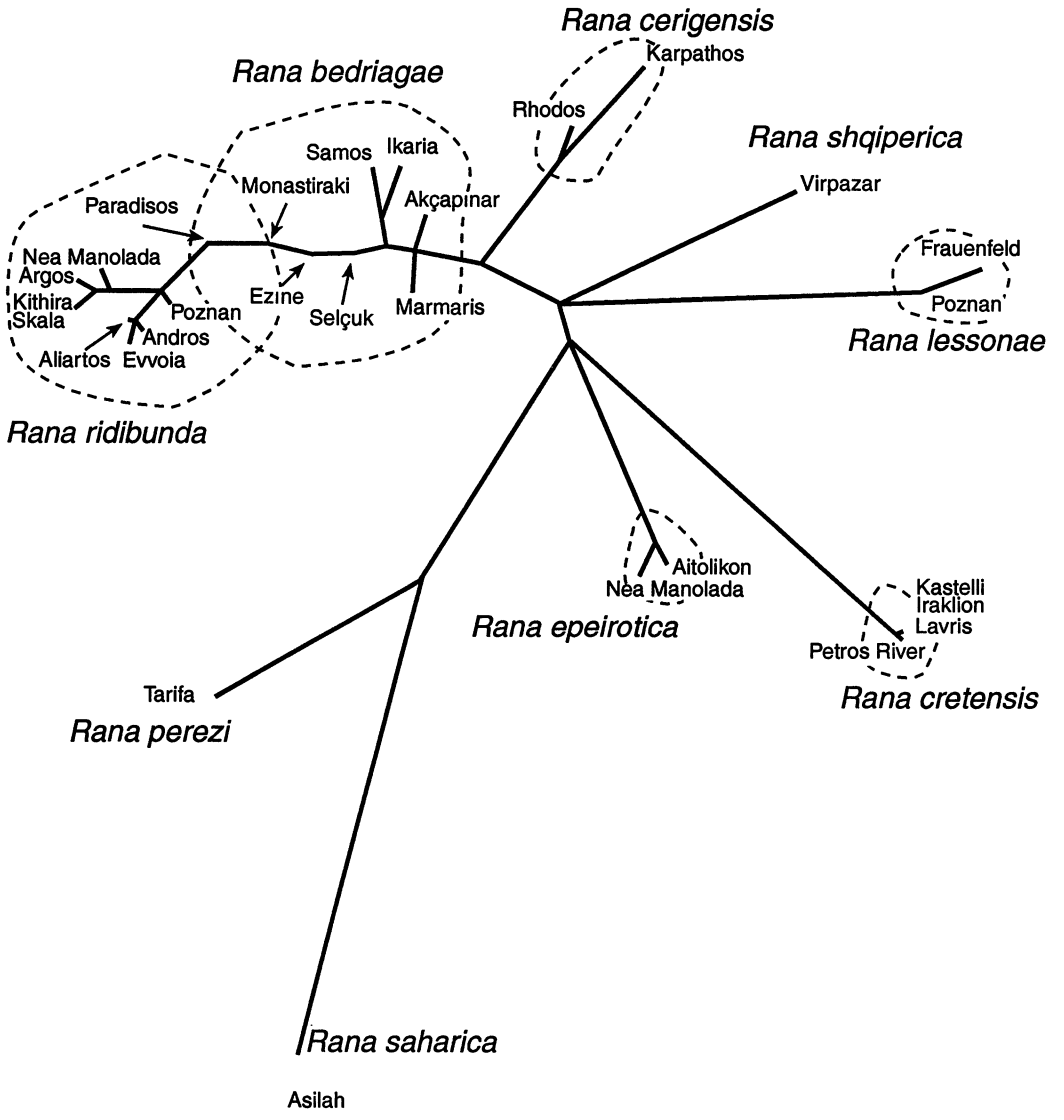

FIG. 2. Maximum likelihood tree (CONTML, Felsenstein 1993) of 29 localities based on allele frequencies of 31 loci. Populations from Tarifa and Asilah were used as outgroups in this study. The distances from the Tarifa and Asilah populations to their common node equal 0.079 and 0.161, respectively, in terms of square-root transformations of the allele frequencies; this measure is similar to Cavalli-Sforza and Edwards's (1967) chord distance. The most conservative interpretation of the confidence limits, collapsing to zero length all branches with other possible arrangements, is shown.

TABLE 3. Partition of 31 loci examined in Aegean water frogs into slowly, moderately, and rapidly evolving groups. Loci with more or fewer unique alleles than the range encompassed by mean number of unique alleles ± one standard deviation (2.5 ± 1.6) are considered to be rapidly and slowly evolving, respectively. Slowly evolving loci include MPR-3, LDH-A, GDA, α-GDH, AK, PEP, sSOD, mMDH, mAAT; rapidly evolving loci include MPI, ALB, EST-5, sAAT.

		Alleles				
				Unique alleles		
Group	Loci	Total number	Mean number per locus	Total number	% of alleles in group	% of all alleles
Slow	9	21	2.3	5	23.8	3.6
Medium	18	80	4.4	44	55.0	32.1
Fast	4	36	9.0	27	75.0	19.7
Total	31	137	4.4	76	55.5	55.5

18,000 yr ago, the sea level was 121 ± 5 m lower than at present (Fairbanks 1989). The islands of Samos and Evvoia are isolated from the nearby mainlands by salt water barriers less than 80 m deep. These seaways dried up during each of the several Pleistocene glacial periods, forming landbridges between Samos and Anatolia and between Evvoia and the Balkan peninsula. Europe and Anatolia are also separated from each other by a similar small and shallow salt water gap and for this reason multiple contact was possible. The sea floor between Ikaria and Samos is less than 200 m deep, so that these two islands were in contact at least during the Riss period (200,000 yr). The isolation of Andros and of Kithira is more problematic. Both islands are separated from adjacent land masses by very narrow gaps, but today the sea floor in these gaps is slightly deeper than 200 m. The whole Aegean region is tectonically very active (Le Pichon and

Angelier 1979) and vertical movement can occur in rapid spurts (uplifts or subsidences of 10 m per event are possible; Udías 1985). For this reason, given the closeness of Kithira to the Peloponnisos and Andros to Evvoia and the relatively shallow seas between these two islands and adjacent land masses, there is no compelling evidence for total isolation of Andros or Kithira during the Pleistocene.

Results

A summary of the electrophoretic data is presented in Tables 1 and 2. Twenty-nine of the loci examined (96%) have more than one allele; AK and LDH-A were invariant. Generally, the more alleles found per locus, the more unique alleles (cf. Slatkin 1985) are present (Table 2). Highly variable loci such as MPI and ALB share only a small fraction of alleles between taxa. Frequencies of alleles for most of the samples agreed with those expected at Hardy-Weinberg equilibrium; a few loci not in equilibrium are GDA at Akçapınar, Aliartos, Ikaria, Samos, and Tarifa; GAPDH at Ezıne; EST-6 at Virpazar; MPI at Marmaris and Virpazar; PGM-2 at Skala; and CK-A at Tarifa. In each case, a heterozygote deficiency was observed; this may reflect sampling error.

Island populations had fewer alleles per locus than mainland ones (Table 1; Beerli 1994). For all loci, *R. ridibunda* and *R. bedriagae* on islands always had the alleles most common in the nearest mainland population, never unique alleles (Table 2); on Evvoia and Kithira, two rare alleles found for LDH-B and GPI are also rare but widespread on the European mainland. Some populations of *R. ridibunda* and *R. bedriagae* are genetically close (Fig. 2; Paradisos, 5; Monastiraki, 6; and Ezine, 7). The high heterozygosity and polymorphism in populations at Paradisos and Monastiraki (Table 1), together with the presence in them of alleles common in Anatolian *R. bedriagae*, suggest a past or present hybrid zone between these two taxa (Beerli 1994; Hotz, Beerli, and Uzzell, unpubl.).

The phylogenetic analysis groups *R. cretensis* and *R. epeirotica* close to the outgroup used (*R. saharica* and *R. perezi*) and clusters *R. lessonae* and Rana shqiperica (Fig. 2). *Rana ridibunda* and *R. bedriagae* form a clade in which we detect a transition from southwestern Anatolian populations to the populations on the Peloponnisos. The topology of the maximum likelihood tree shown is consistent with the topology developed with other methods, except for differences in branch lengths and in intraspecific branching sequences (Beerli 1994).

The overall number of unique alleles for each locus was used to estimate evolutionary rate of change at that locus. Pairs of populations that have been isolated for long periods of time are expected to have distinct alleles at more loci than pairs of populations not or but recently isolated; this expectation is confirmed by our data (Table 2). At the same time, loci that evolve more rapidly are expected to have distinct alleles more often for any population pair, than loci that evolve more slowly. Because the 31 loci that we examined were compared over the same set of populations and taxa, the main contribution to total number of unique alleles per locus is differences in evolutionary rate. We therefore used the number of unique alleles per locus to divide the 31 loci into three arbitrary classes. The mean number of unique alleles per locus is 2.5, with standard deviation 1.6; those loci for which the number of unique alleles falls in the range 2 to 4 (approximately the mean ± one standard deviation) are considered to evolve at an intermediate rate; those with five or more unique alleles are considered to evolve rapidly, whereas those with but one or no unique allele are considered to evolve slowly. The different evolutionary rates in these classes of loci are also reflected, as expected, in the total number of alleles per locus: the mean total is highest in loci classified as rapidly evolving and lowest in those classified as slowly evolving (Table 3).

When the genetic divergence between localities (Tables 4 and 5) is compared with isolation time, a linear pattern appears (Fig. 3). The populations chosen for comparison (Table 5, Fig. 3) represent pairs that are geographically close. For comparison of populations isolated for 5.2 Myr, we pooled the conspecific populations on Crete, in Anatolia, and on the Peloponnisos to avoid inflating the number of nonindependent comparisons. This produced wider confidence limits (Fig. 3, C_1, C_2) than obtained when using localities as populations (D_1, D_2). The genetic distances and geological isolation time of Rhodos relative to the other populations are close to the regression line. The time of isolation is less well established for Karpathos, and when a figure of 3 Myr (middle Pliocene) is used, a linear regression does not fit (deviation of linear regression: $P = 0.024$, $F[4, 27]$); but varying the isolation dates within the boundaries determined by geomorphological data showed no deviation from linearity ($P > 0.05$) for isolation values for Karpathos between 1.8 and 2.85 Myr. Because the time of isolation of Karpathos is unclear, we tentatively accept a linear relationship for these data. The linear relationship is: $D^*_{Nei} = (0.04 \pm 0.01) + (0.10 \pm 0.01)$ isolation time [Myr] (coefficient ± SD, $R^2_{adj} = 0.95$). This gives a mean divergence rate of 0.10 D^*_{Nei}/Myr. The influence of the "fast evolver" is detectable: without the loci sAAT, EST-5, ALB, and MPI, the regression is $D^*_{Nei} = (0.04 \pm 0.01) + (0.08 \pm 0.01)$ isolation time, resulting in 0.08 D^*_{Nei}/Myr.

Discussion

Heterozygosity and number of alleles in populations on islands are reduced compared with those on the adjacent mainland. This loss in variability may have several causes, each associated with a population bottleneck. The following scenarios are possible

I. Vicariance.—An old, widely distributed species became subdivided during a sea level change into populations that were isolated on several islands. Severe population bottlenecks caused by loss of fresh water or other ecological catastrophes may subsequently have reduced the number of alleles in such small isolated populations by random genetic drift, and may have led to total homozygosity if the population size remained small for a prolonged period.

II. Introduction by Humans.—A few individual frogs were accidentally or intentionally introduced on one or more islands by humans, resulting in an initial population bottleneck

TABLE 4. Genetic distances among western Palearctic water frog species, estimated by protein electrophoresis using 31 loci. Coefficients are the modified Nei (1972; Hillis 1984) standard distance (upper right) and Cavalli-Sforza and Edwards's (1967) chord distance (lower left). Abbreviations: cer = *Rana cerigensis,* shq = *Rana shqiperica,* per = *Rana perezi,* sah = *Rana saharica.*

		cretensis				*epeirotica*		*lessonae*		cer		*bedriagae*					
Species	Locality	Iraklion	Kastelli	Lavris	Petros River	Aitolikon	Nea Manolada	Frauenfeld	Poznań	Karpathos	Rhodos	Akçapınar	Ezıne	Marmaris	Selçuk	Ikaria	Samos
cretensis	Iraklion	—	0.00	0.00	0.02	0.54	0.52	0.96	0.87	0.54	0.55	0.62	0.65	0.59	0.62	0.59	0.55
	Kastelli	0.00	—	0.00	0.02	0.54	0.52	0.96	0.87	0.54	0.55	0.62	0.65	0.59	0.62	0.59	0.55
	Lavris	0.00	0.00	—	0.02	0.54	0.52	0.96	0.87	0.54	0.55	0.62	0.65	0.59	0.62	0.59	0.55
	Petros River	0.11	0.11	0.11	—	0.54	0.52	0.96	0.87	0.58	0.59	0.66	0.65	0.63	0.62	0.63	0.58
epeirotica	Aitolikon	0.58	0.58	0.58	0.58	—	0.04	0.67	0.59	0.49	0.49	0.34	0.39	0.34	0.40	0.38	0.33
	Nea Manolada	0.57	0.57	0.57	0.57	0.17	—	0.62	0.55	0.50	0.49	0.37	0.45	0.35	0.41	0.39	0.37
lessonae	Frauenfeld	0.71	0.71	0.71	0.71	0.62	0.62	—	0.04	0.55	0.56	0.59	0.52	0.54	0.52	0.61	0.58
	Poznań	0.69	0.69	0.69	0.69	0.60	0.59	0.18	—	0.49	0.49	0.52	0.46	0.48	0.46	0.55	0.51
cerigensis	Karpathos	0.58	0.58	0.58	0.60	0.56	0.57	0.59	0.56	—	0.12	0.26	0.35	0.27	0.32	0.38	0.30
	Rhodos	0.59	0.59	0.59	0.60	0.56	0.56	0.59	0.56	0.29	—	0.18	0.25	0.18	0.23	0.33	0.27
bedriagae	Akçapınar	0.62	0.62	0.62	0.63	0.49	0.51	0.61	0.58	0.44	0.37	—	0.10	0.03	0.07	0.09	0.07
	Ezıne	0.62	0.62	0.62	0.62	0.51	0.54	0.57	0.54	0.47	0.41	0.27	—	0.11	0.03	0.11	0.11
	Marmaris	0.60	0.60	0.60	0.61	0.49	0.50	0.58	0.55	0.43	0.35	0.21	0.30	—	0.08	0.12	0.12
	Selçuk	0.61	0.61	0.61	0.61	0.50	0.51	0.56	0.53	0.45	0.38	0.23	0.16	0.24	—	0.09	0.09
	Ikaria	0.60	0.60	0.60	0.61	0.51	0.51	0.61	0.58	0.50	0.45	0.27	0.29	0.29	0.23	—	0.06
	Samos	0.59	0.59	0.59	0.59	0.49	0.51	0.60	0.57	0.46	0.41	0.24	0.26	0.30	0.25	0.23	—
ridibunda	Aliartos	0.63	0.63	0.63	0.63	0.50	0.52	0.57	0.54	0.46	0.41	0.35	0.33	0.35	0.33	0.43	0.33
	Monastiraki	0.62	0.62	0.62	0.62	0.52	0.55	0.59	0.56	0.48	0.42	0.29	0.15	0.35	0.22	0.29	0.23
	Paradisos	0.61	0.61	0.61	0.61	0.50	0.52	0.58	0.55	0.45	0.42	0.31	0.23	0.36	0.28	0.36	0.26
	Andros	0.65	0.65	0.65	0.65	0.51	0.54	0.59	0.56	0.49	0.42	0.35	0.31	0.36	0.34	0.45	0.35
	Evvoia	0.64	0.64	0.64	0.64	0.53	0.55	0.60	0.58	0.51	0.45	0.38	0.33	0.38	0.36	0.47	0.35
	Argos	0.64	0.64	0.64	0.64	0.50	0.53	0.54	0.51	0.45	0.40	0.38	0.34	0.39	0.36	0.46	0.38
	Nea Manolada	0.64	0.64	0.64	0.64	0.53	0.56	0.60	0.57	0.48	0.43	0.41	0.37	0.42	0.38	0.47	0.41
	Skala	0.64	0.64	0.64	0.64	0.50	0.53	0.56	0.53	0.45	0.42	0.40	0.35	0.41	0.36	0.45	0.39
	Kithira	0.65	0.65	0.65	0.65	0.51	0.54	0.57	0.54	0.46	0.44	0.42	0.37	0.43	0.38	0.46	0.40
	Poznań	0.64	0.64	0.64	0.64	0.52	0.55	0.60	0.57	0.46	0.39	0.32	0.28	0.35	0.32	0.43	0.34
shqiperica	Virpazar	0.63	0.63	0.63	0.63	0.54	0.56	0.58	0.55	0.56	0.53	0.52	0.47	0.51	0.48	0.51	0.52
perezi	Tarifa	0.67	0.67	0.67	0.68	0.60	0.59	0.70	0.68	0.65	0.64	0.60	0.62	0.59	0.63	0.64	0.62
saharica	Asilah	0.78	0.78	0.78	0.78	0.71	0.72	0.78	0.76	0.71	0.71	0.66	0.67	0.66	0.67	0.69	0.66

(founder effect). Ecological stress following such introduction may have resulted in additional population bottlenecks.

III. Overwater Colonization.—Because water frogs have problems with salt water, their ability to migrate to and between islands in the Aegean area is much reduced; even if rafting across salt water were possible in rare cases, any successful immigration would have been accompanied by severe founder effects, and ecological stress subsequently may have produced other population bottlenecks.

In both models II and III, the common mainland alleles are more likely to reach the islands and become fixed. It is less clear which alleles are likely to become fixed in model I: the most common alleles in the 'island' part of the range are more likely to be fixed, but because of geographic variation, these locally abundant alleles may not have been the most abundant 'mainland' ones.

Overwater colonization (scenario III) is improbable for water frogs: they cannot survive long in salt or even brackish water. Although a living adult water frog has been observed on a piece of wood floating in the sea 5 km off the coast of Italy (G. Nascetti, pers. comm. 1993), such unusual rafting does not guarantee safe arrival on shore, where surf and a stony or sandy shoreline provide additional barriers. Moreover, given external fertilization, simultaneous arrival of at least one female and one male on an island is required to found a population. For water frog populations on islands situated on the continental shelf, recent colonization cannot be ruled out by the present data set. Because only common alleles were found, the populations on the islands on the continental shelf are very similar to the populations on the adjacent mainland. Given the long-established sailing traditions of the ancient Greeks, we could recognize remnants of any introduction by the ancient Greeks from the Peloponnisos or Attika at least on the islands Samos and Ikaria. In all phylogenetic analyses, however, these islands cluster with mainland Anatolia (Fig. 2). Introduction by humans (scenario II) can be ruled out for the islands of the Hellenic arc (Crete, Karpathos, and Rhodos): *R. cretensis* and *R. cerigensis* have several unique alleles not found elsewhere, and their genetic dissimilarity values support differentiation at the species level (Beerli et al. 1994). Each of these populations, except the one on Rhodos, shows reduced heterozygosity. Scenario I, on the other hand, is entirely consistent with geological data, and provides the basis for our molecular clock.

The detection of interlocus differences in divergence rates that led to the distinction between rapidly and slowly evolving genes is not a falsification of the molecular clock. The differences do, however, call for consideration of differential rates occurring in a data set, which are probably sampled from a continuum rather than from well-separated discrete classes. Based on the number of unique alleles, 13% of the loci we examined are evolving rapidly. That these loci include

TABLE 4. Extended.

					ridibunda							
Aliartos	Monastiraki	Paradisos	Andros	Evvoia	Argos	Nea Manolada	Skala	Kithira	Poznań	shq Virpazar	per Tarifa	sah Asilah
0.67	0.66	0.60	0.73	0.72	0.68	0.70	0.71	0.73	0.70	0.66	0.80	1.36
0.67	0.66	0.60	0.73	0.72	0.68	0.70	0.71	0.73	0.70	0.66	0.80	1.36
0.67	0.66	0.60	0.73	0.72	0.68	0.70	0.71	0.73	0.70	0.66	0.80	1.36
0.68	0.66	0.60	0.73	0.72	0.68	0.70	0.71	0.73	0.70	0.66	0.85	1.36
0.36	0.43	0.35	0.39	0.43	0.36	0.42	0.38	0.39	0.40	0.44	0.59	0.95
0.41	0.49	0.40	0.44	0.48	0.41	0.47	0.43	0.44	0.46	0.49	0.57	1.01
0.51	0.60	0.53	0.55	0.60	0.45	0.57	0.50	0.50	0.57	0.54	0.92	1.36
0.45	0.53	0.47	0.49	0.54	0.39	0.51	0.44	0.44	0.50	0.48	0.83	1.24
0.31	0.39	0.28	0.34	0.38	0.27	0.33	0.29	0.30	0.30	0.49	0.73	0.95
0.25	0.31	0.26	0.24	0.29	0.22	0.27	0.27	0.28	0.21	0.44	0.70	1.01
0.16	0.13	0.15	0.16	0.20	0.19	0.23	0.23	0.24	0.14	0.40	0.56	0.78
0.15	0.04	0.11	0.14	0.17	0.17	0.20	0.18	0.19	0.13	0.30	0.69	0.85
0.17	0.19	0.19	0.17	0.21	0.21	0.25	0.26	0.27	0.17	0.41	0.54	0.78
0.15	0.07	0.14	0.16	0.19	0.20	0.23	0.21	0.22	0.15	0.34	0.69	0.86
0.28	0.11	0.20	0.31	0.35	0.31	0.34	0.30	0.30	0.29	0.39	0.71	0.92
0.16	0.09	0.10	0.18	0.19	0.20	0.25	0.22	0.23	0.17	0.40	0.65	0.79
—	0.14	0.07	0.01	0.04	0.06	0.08	0.07	0.08	0.04	0.40	0.63	0.80
0.31	—	0.07	0.14	0.18	0.16	0.18	0.15	0.16	0.12	0.34	0.77	0.89
0.23	0.17	—	0.08	0.12	0.10	0.13	0.11	0.12	0.07	0.38	0.68	0.83
0.13	0.31	0.24	—	0.03	0.07	0.09	0.09	0.10	0.03	0.39	0.62	0.78
0.19	0.33	0.28	0.17	—	0.10	0.12	0.12	0.13	0.06	0.44	0.62	0.78
0.23	0.32	0.26	0.24	0.28	—	0.06	0.04	0.05	0.06	0.29	0.58	0.80
0.25	0.34	0.28	0.26	0.30	0.21	—	0.05	0.05	0.05	0.36	0.65	0.81
0.23	0.31	0.26	0.25	0.29	0.15	0.18	—	0.00	0.08	0.30	0.70	0.85
0.27	0.32	0.28	0.28	0.32	0.18	0.20	0.08	—	0.09	0.30	0.72	0.87
0.21	0.26	0.20	0.17	0.24	0.23	0.21	0.24	0.27	—	0.36	0.63	0.78
0.52	0.48	0.50	0.52	0.54	0.46	0.50	0.47	0.46	0.50	—	0.79	1.04
0.61	0.64	0.63	0.61	0.61	0.60	0.62	0.63	0.64	0.61	0.67	—	0.55
0.67	0.68	0.68	0.66	0.66	0.67	0.67	0.68	0.69	0.66	0.73	0.58	—

ALB and an esterase locus (EST-5) is consistent with Sarich's (1977) finding that blood proteins and secreted enzymes such as esterases evolve more rapidly than others; a second esterase locus (EST-6), however, appears to have evolved at a moderate rate in our set. Because the 31 loci that we used include most of those commonly studied electrophoretically, the rates we observe are validly comparable to rates reported by others.

The regression line relating genetic distance to time of isolation is defined by a relatively small time interval, zero to five million years. The regression model we used does not force the regression line through the origin, in contrast to most other studies (summarized by Hillis and Moritz 1990; Scherer 1990). Forcing through the origin is not necessary and seems inappropriate for this study, given the correlation detected between genetic distance and geographic distance (Beerli 1994): neighboring conspecific populations are separated by small or zero genetic distance values.

The variation in evolutionary rates determined from genetic dissimilarities results in part from the general lack of independent information about times of speciation (Avise and Aquadro 1982). Data considered by Hillis and Moritz (1990) were too variable to calculate useful confidence limits for an allozyme clock. They concluded that estimates of divergence times based on Nei's genetic distance are no better than arbitrary guesses. Using the nested set of geologically dated divergence times available for the Aegean region, however, our data apparently yield a reliable estimate of an absolute protein clock with confidence limits for this time interval and species group that are unusually narrow for such studies.

A test of a uniform clock pace is possible within this frog group, by comparing taxa that were not used for its calibration. The species pair *R. perezi* and *R. saharica* is separated by the Strait of Gibraltar; their geologically known isolation time is thus 5.2 ± 0.1 Myr. The observed genetic distance between them, $D^*_{Nei} = 0.55$ and $D_{Nei} = 0.56$ (this study) or $D_{Nei} = 0.55$ (Busack 1986), is close to the expected value calculated with the regression that we obtained: $D^*_{Nei} = 0.56$. This independent test of the calibration enhances our confidence in estimating the divergence time of the sympatric species pair *R. epeirotica* and *R. ridibunda* and other water frog species pairs for which no geological dating of time of separation is possible (Table 6). The dates estimated for each of these speciation events all fall between the middle Pliocene and the Messinian, except for the closely related pair *R. ridibunda* and *R. bedriagae*; with the possible exception of this pair, therefore, these events apparently were not induced by separations related to glaciations during the Pleistocene. Was such a series of speciation events in this frog group triggered by dramatic ecological changes caused by the "salinity crisis" in the Messinian? If so, a

TABLE 5. Population pairs used to estimate divergence rates. Localities are keyed to the map (Fig. 1). Multiple localities indicate pooled samples. The taxa are: bed = *Rana bedriagae,* cer = *Rana cerigensis,* cre = *Rana cretensis,* epe = *Rana epeirotica,* rid = *Rana ridibunda.* Isolation is the minimal isolation time ± one standard deviation estimated with the age of salt water barriers; D^*_{Nei} is Nei's standard genetic distance with Hillis's modification (Nei 1972; Hillis 1984), D_{Nei} is Nei's standard genetic distance.

Isolation [My]	Population pair (locality number)	Taxon	Genetic distance	
			D^*_{Nei}	D_{Nei}
0	Akçapınar (9) - Marmaris (10)	bed	0.031	0.031
0	Akçapınar (9) - Selçuk (8)	bed	0.070	0.056
0	Ezıne (7) - Selçuk (8)	bed	0.031	0.028
0	Marmaris (10) - Selçuk (8)	bed	0.080	0.063
0	Aliartos (4) - Argos (3)	rid	0.062	0.063
0	Aliartos (3) - Paradisos (5)	rid	0.066	0.061
0	Argos (3) - Nea Manolada (1)	rid	0.057	0.054
0	Argos (3) - Skala (2)	rid	0.037	0.029
0	Monastiraki (6) - Paradisos (5)	rid	0.071	0.057
0	Nea Manolada (1) - Skala (2)	rid	0.045	0.043
0	Iraklion (19) - Lavris (18)	cre	0.000	0.000
0	Kastelli (16) - Petros River (17)	cre	0.022	0.018
0	Lavris (18) - Petros River (17)	cre	0.022	0.018
0.012 ± 0.001	Ezıne (7) - Monastiraki (6)	bed-rid	0.038	0.032
0.012 ± 0.001	Samos (14) - Selçuk (8)	bed	0.094	0.073
0.012 ± 0.001	Evvoia (11) - Aliartos (4)	rid	0.043	0.044
0.2 ± 0.05	Ikaria (13) - Samos (14)	bed	0.059	0.054
0.2 ± 0.05	Ikaria (13) - Selçuk (8)	bed	0.087	0.070
0.2 ± 0.05	Andros (12) - Aliartos (4)	rid	0.014	0.013
0.2 ± 0.05	Andros (12) - Evvoia (11)	rid	0.033	0.034
0.2 ± 0.05	Kythira (15) - Skala (2)	rid	0.001	0.002
1.8 ± 0.2	Rhodos (21) - Anatolia (8–10)	cer-bed	0.184	0.182
1.8 ± 0.2	Rhodos (21) - Ikaria (13)	cer-bed	0.333	0.320
1.8 ± 0.2	Rhodos (21) - Samos (14)	cer-bed	0.272	0.260
3	Karpathos (20) - Anatolia (8–10)	cer-bed	0.263	0.259
3	Karpathos (20) - Rhodos (21)	cer	0.119	0.110
3	Karpathos (20) - Ikaria (13)	cer-bed	0.377	0.373
3	Karpathos (20) - Samos (14)	cer-bed	0.300	0.300
5.2 ± 0.1	Crete (16–19) - Peloponissos (1–3)	cre-rid	0.690	0.684
5.2 ± 0.1	Crete (16–19) - Nea Manolada (1)	cre-epe	0.544	0.546
5.2 ± 0.1	Crete (16–19) - Rhodes (21)	cre-cer	0.573	0.578
5.2 ± 0.1	Crete (16–19) - Karpathos (20)	cre-cer	0.568	0.570
5.2 ± 0.1	Crete (16–19) - Anatolia (8–10)	cre-bed	0.598	0.583

similar clustering of speciation events in the earlier Pliocene should be observed in other organismal groups of this region as well.

Comparison with the compilation of genetic distances obtained by protein electrophoresis (Avise and Aquadro 1982) places the mean divergence rate found in the present study (0.10 D^*_{Nei}/Myr, 0.10 D_{Nei}/Myr) in the midrange of reported values (cf. Hillis and Moritz 1990), between the rates reported for reptiles (0.06–0.2 D_{Nei}/Myr), plethodontid salamanders (0.07 D_{Nei}/Myr), and birds (0.2 D_{Nei}/Myr). The rate of 0.07 D_{Nei}/Myr estimated by Sarich (1977) appears to be too slow for our water frog data; using this rate with our genetic distances results in large differences between estimated divergence times and the isolation times based on geological events. Immunological distance data (Hotz and Uzzell 1982; Uzzell 1982), using the calibration provided by Maxson et al. (1975), show an even larger variation in the divergence times (Table 6). This may be because ALB evolves rapidly (Table 3), with different rates in different lineages (cf. Uzzell et al. 1994; Uzzell and Hotz unpubl.), but needs further investigation. All these differences confirm, as noted by Hillis and Moritz (1990), that a molecular clock must be calibrated for the species group of interest and that transfer of the calibration to other species groups requires caution. Nevertheless, using distances based on mtDNA restriction fragment patterns (Hotz, Spolsky, and Uzzell unpubl.) with the calibration of Brown et al. (1979) produces very similar results; only the times for the pairs *R. lessonae-R. shqiperica* and *R. lessonae-R. epeirotica* differ appreciably from our estimates, but even these are in a similar range (Table 6). This suggests that, when properly calibrated, average clocks based on the nuclear and mitochondrial genomes yield similar estimates of divergence times.

Most calibration studies have used long intervals of divergence time, up to 1000 Myr, for determining the pace of molecular clocks. For relatively rapidly evolving parts of the genome, however, errors caused by multiple substitutions become a significant problem for large divergence times (cf. Hillis and Moritz 1990; Scherer 1990; Gillespie 1991). Moreover, a clock may be disturbed over time intervals longer than 100 Myr by occasional major changes in functional constraints (Wilson et al. 1987) that may reflect unpredictable environmental catastrophes (cf. Gould and Eldredge 1977). Our calibration is unlikely to be affected by these problems because the estimation is limited to a time interval spanning only the last 5 Myr. In fact, this study is, as far as we know, the first in which relatively narrow confidence limits of the molecular clock allow a reliable

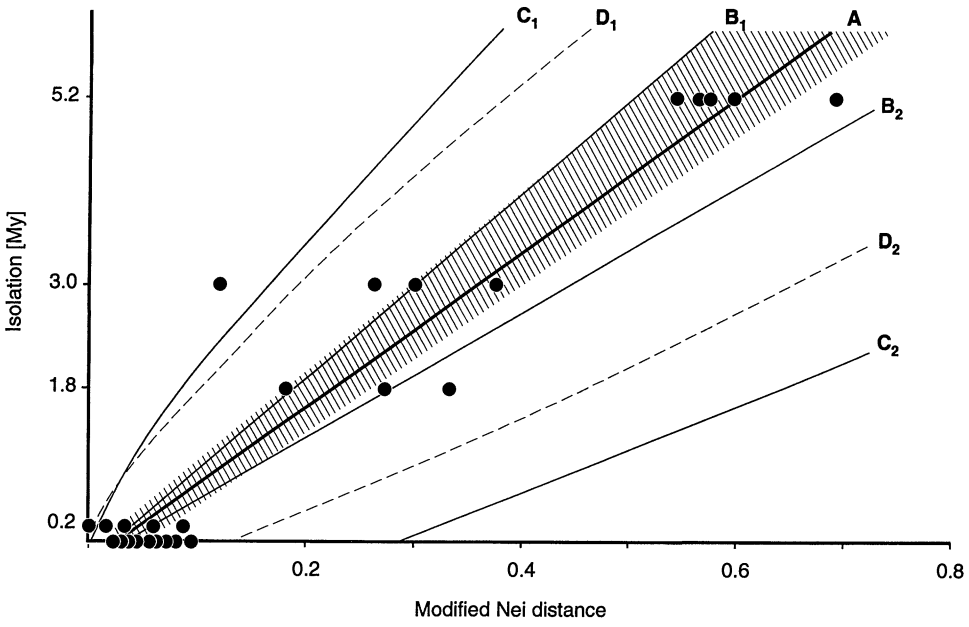

FIG. 3. Geographical isolation time versus genetic distance (D^*_{Nei}) for 33 population pairs. The rows of points, from top to bottom, involve populations on Crete; Karpathos; Rhodos; Ikaria, Andros, and Kithira; Evvoia and Samos; mainlands. The differences between the last two groups are not visible (see Table 5). Regression line A: (0.04 ± 0.01) + (0.10 ± 0.01) isolation time; B1 and B2 are the bounds of the 95% confidence limits of the regression line; C1 and C2 are the bounds of the 95% confidence limit for the prediction of time based on new genetic distance data (cf. Hillis and Moritz 1990). D1 and D2 are the confidence limits treating each locality on Anatolia, Crete, Peloponnissos as a population, resulting in 72 pairs. In the hatched area are 95% of all slopes generated by resampling the 33 pairs randomly 1000 times.

timing of divergences that occurred during the Pliocene. The graphs showing the relation between genetic divergence and isolation time given by Hillis and Moriz (1990; data of Britten 1986; Brown et al. 1979; and Prager et al. 1974) indicate that molecular clocks based on most current sequence and immunological data have confidence limits larger than or similar to ours. This suggests that protein electrophoresis is still competitive with sequence data for providing answers about temporal origin of species.

A direct comparison of different estimation methods of molecular clocks in water frogs of the Aegean region should increase our understanding of the differences presently shown by these methods. Comparisons with other animal groups for which salt water is a barrier using identical methods will provide a test of the uniformity of a molecular clock across groups of organisms.

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TABLE 6. Estimation of divergence times of species pairs of European water frogs with isolation times not inferable by geological data, assuming the divergence rate calculated in this study: 9.1 Myr/D^*_{Nei} (0.10 D^*_{Nei}/Myr). The mean values are given with their standard errors, minimal and maximal isolation are approximated using the confidence limits C1 and C2 in Figure 3. Other calibrations and data: electrophoresis: present data set, but using a rate of 14 Myr/D_{Nei} (Sarich 1977); albumin microcomplement fixation (MC'F): data from Hotz and Uzzell (1982) and Uzzell (1982), using a rate of 0.6 Myr/1 IDU (Maxson et al. 1975); mtDNA restriction fragment pattern (RFLP): data from Hotz, Spolsky, and Uzzell (unpubl. 21 enzymes) using a rate of 0.5 Myr/1% sequence divergence (Brown et al. 1979).

Species	D^*_{Nei}	Mean isolation ± SD [Myr]	Minimal isolation [Myr]	Maximal isolation [Myr]	Isolation time [Myr] with other calibrations and data		
					Electro-phoresis	Albumin MC'F	mtDNA RFLP
ridibunda-bedriagae	0.144	1.0 ± 0.1	0.0	2.7	2.0	—	—
ridibunda-epeirotica	0.359	3.0 ± 0.3	0.3	5.7	5.0	3.0	3.0
ridibunda-shqiperica	0.334	2.8 ± 0.3	0.2	5.4	4.7	1.8	3.4
ridibunda-lessonae	0.454	3.9 ± 0.4	0.8	7.0	6.4	5.7	3.3
ridibunda-perezi	0.640	5.6 ± 0.5	1.8	9.3	9.0	18.4	—
shqiperica-epeirotica	0.456	3.9 ± 0.4	0.8	7.0	6.4	—	3.7
shqiperica-lessonae	0.496	4.3 ± 0.4	1.0	7.5	6.9	9.0	2.4
epeirotica-lessonae	0.572	4.9 ± 0.5	1.4	8.5	8.0	12.6	3.5

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