Genetic isolation and calibration of an average protein clock in western Palearctic water frogs of the Aegean region

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Chapter 1

Introduction

The phylogenetic history of a group of organisms is interesting in its own right, but is of critical importance for any comparative or evolutionary study of that group, whether in morphology or physiology, ecology or behavior (cf. Harvey and Pagel 1991). 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 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 genetical information, such as species composition (Busack 1986, Legendre 1986, Avise 1992, Oosterbroek and Arntzen 1992), migratory pattern (Bowen et al. 1989), expansion directions (Cavalli-Sforza et al. 1993), ecological adaptations (cf. Grant 1986), and parasites (Tirard et al. 1992).

In 1962, Zuckerkandl and Pauling described a molecular clock based on amino acid replacement. They escaped the problem of scarcity of comparative fossil data because all

data are obtained from the extant taxa studied. This framework has rapidly expanded from amino acid replacement to nucleotide substitutions (Kimura 1983). This molecular clock is thought to be paced by neutral mutations and to be a stochastic process similar to radioactive decay, rather than a metronomical process (Kimura 1968, 1969, 1983; Uzzell and Corbin 1971; Wilson et al. 1977, 1987; Gillespie 1984; Takahata 1987). The albumin clock, as observed by microcomplement fixation immunology (reviewed by Maxson and Maxson 1986), was one of the first supports for the neutral theory, which states that most allelic variation found in natural populations is selectively neutral rather than adaptive (Kimura 1968; cf. also Lewontin 1974). Genes such as that for glutamine synthetase (Pesole et al. 1991) have also been suggested to define reliable clocks, but other studies falsified the generality of a standardized clock for all genes and species (for example, by Gillespie 1991, Scherer 1990). The observed discrepancies led to recognition of groups of "fast evolving" and conservative genes (Sarich 1977). Most studies for calibrating molecular clocks used averages over long time spans (hundreds of My) with a rather low density of data points along the time axis (several examples were summarized by Scherer 1990). The estimation of the divergence time is crucial and often not reliable (cf. Avise and Aquadro 1982). Moreover, when using short time spans the microphyletic structure must be well known to prevent heterologous comparisons. Many phylogenetic studies focusing on populations or species use some averaged clock for estimating the number of changes on a phylogenetic tree, using indices like Nei's (1972, 1978) or Cavalli-Sforza and Edwards' (1967) genetic distances. Testing for the existence of a molecular clock requires an exact timing of geological divergence because at least one independently established event has to be used for its estimation (Busack 1986). It is expected that a molecular clock is easier to calibrate in a relatively narrow time unit, with known relationships among the taxa under study, and with well-dated isolation times.

Frogs, like other amphibians, are unable to cross small 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 thus provides a measure of the minimum time over which such pairs of populations have diverged genetically. If we can determine from geology the duration of isolation and by genetic studies the amount of genetic divergence, we can estimate the rate of genetic divergence and therefore the pace of an average molecular clock. The western Palearctic water frogs occurring around the Aegean Sea provide an ideal group for such a study. Because the geological record of the Eastern Mediterranean Sea is relatively well known (Appendix 1 p. 61), 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 population pairs in this data set form a nested set containing several points spanning between 10,000 y and 5 My. An average molecular clock and its confidence limits are presented. Phylogenetic analyses of five species of the Aegean region (two of which are newly revealed by this study) and of the four other known species of the group are presented. Application of the estimated protein clock rate to date speciation events in several taxon pairs of the group suggests a cluster of speciation events after the Messinian period 5 My ago. Phylogenetic reliability of the set of 31 loci used was confirmed by a randomization study. The data obtained on phylogenetic relationships and divergence rates help to establish a reliable historical biogeographic concept for the western Palearctic water frogs and are important for the 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). Comparisons of the estimated divergence rates with those of other organismal groups in the same area can later provide a direct test of the molecular clock hypothesis.

Chapter 2

Methods and animals

Western Palearctic water frogs were collected from 21 localities around the Aegean Sea (Fig. 1, Table 1, details of collecting sites in Appendix 3 p. 71). As often as possible, localities sampled were situated in well-watered river plains, on the assumption that large populations living there would not have undergone recent bottlenecks. This was not possible on the islands of Andros, Samos and Karpathos; even during the winter rainy season, normally wet places (rivers, ponds and puddles) were dry, and no large populations could be located. The whole Aegean region has suffered from drought in the last years (a comparison between the the rainfall data 1987-1991 with the data from 1951-1986 shows a signifcant drop of about 15%; $P=0.005$, $F_{1, 218}=8.035$).

Biochemical analysis

Each frog was heparinized by injecting 0.1 - 0.5 ml of a Na-heparin (~20 mg / ml in 0.7% NaCl) solution into the abdominal cavity, and then anesthetized with 3-aminobenzoic acid ethyl ester (MS-222). Blood was collected by cutting the aorta, and plasma and erythrocytes were separated by short pulses in a microfuge and then separately frozen

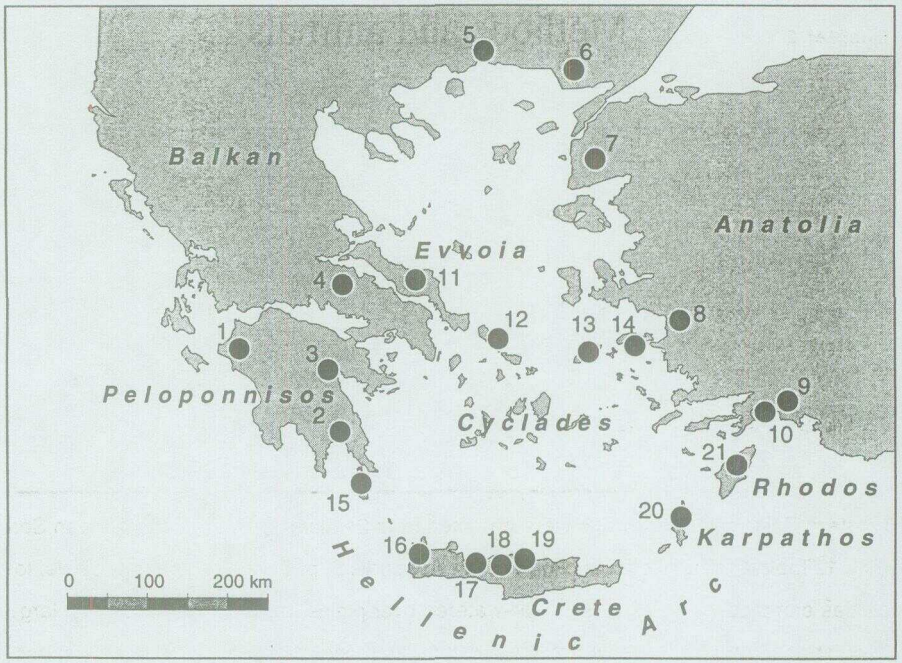

Fig. 1. Localities in the Aegean region from which frogs were sampled. 1 Nea Manolada, 2 Skala, 3 Argos, 4 Aliartos, 5 Paradisos, 6 Monastiraki, 7 Ezıne, 8 Selçuk, 9 Akçapınar, 10 Marmaris, 11 Evvoia, 12 Andros, 13 Ikaria, 14 Samos, 15 Kithira, 16 Kastelli, 17 Petros River, 18 Lavris, 19 Iraklion, 20 Karpathos, 21 Rhodos.

at -70 °C. Samples of skeletal muscle, liver, kidney, and heart were removed and frozen at -70 °C. The carcasses were frozen and stored at -20 °C.

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) [E.C. 2.7.4.3], albumin (ALB), aspartate aminotransferase (sAAT and mAAT) [E.C. 2.6.1.1], s-adenosyl-l-homocysteine hydrolase (AHH) [E.C. 3.3.1.1], carbonate dehydratase (CA-2) [E.C. 4.2.1.1], creatine kinase (CK-A) [E.C. 2.7.3.2], carboxylesterases (EST-5 and EST-

Table 1. List of localities, species and sample sizes. Taxa were identified by electrophoresis. The localities are described in Appendix 3 (p. 71) and mapped in Fig. 1. Locality numbers correspond to those in Fig. 1. KR taxon = Karpathos/Rhodos taxon.

Geographical entity				Taxon	Sampled animals
Region		Nr.	Locality		
Europe and adjacent islands	Peloponnisos	1	Nea Manolada	*R. epeirotica*	12
				R. ridibunda	1
		2	Skala	*R. ridibunda*	13
		3	Argos	*R. ridibunda*	10
	Attika	4	Aliartos	*R. ridibunda*	13
	Thrakia	5	Paradisos	*R. ridibunda*	13
		6	Monastiraki	*R. ridibunda*	13
	Evvoia	11	Mistros	*R. ridibunda*	10
	Andros	12	Andros	*R. ridibunda*	3
	Kithira	15	Kithira (Agia Pelagia)	*R. ridibunda*	7
Asia minor and adjacent islands	Anatolia	7	Ezıne	*R. bedriagae*	11
		8	Selçuk	*R. bedriagae*	16
		9	Akçapınar	*R. bedriagae*	14
		10	Marmaris	*R. bedriagae*	5
	Ikaria	13	Ikaria	*R. bedriagae*	11
	Samos	14	Samos (Idhrousa)	*R. bedriagae*	4
Isolated islands	Crete	16	Kastelli	Cretan taxon	11
		17	Petros river	Cretan taxon	2
		18	Lavris (Geropotamos river)	Cretan taxon	6
		19	Iraklion	Cretan taxon	7
	Karpathos	20	Olympos	KR taxon	9
	Rhodos	21	Archipolis	KR taxon	14

6) [E.C. 3.1.1.-, l-methyl-l-umbelliferyl acetate as substrate], fructose-biphosphatase (FDP-1 and FDP-2) [E.C. 3.1.3.11], glucose dehydrogenase (GCDH) [E.C. 1.1.1.118], glucose-6-phosphate isomerase (GPI) [E.C. 5.3.1.9], glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [E.C. 1.2.1.12], glycerol-3-phosphate dehydrogenase (αGDH) [E.C. 1.1.1.8], guanine deaminase (GDA) [E.C. 3.5.4.3], isocitrate dehydrogenase (mIDH and

sIDH) [E.C. 1.1.1.42], lactate dehydrogenase (LDH-A and LDH-B) [E.C. 1.1.1.27], malate dehydrogenase (sMDH and mMDH) [E.C. 1.1.1.37], mannose-6-phosphate isomerase (MPI) [E.C. 5.3.1.8], unidentified muscle proteins (MPR-1 and MPR-3), one peptidase (PEP-A [3.4.-], l-valyl-l-leucine as substrate), phosphoglucomutase (PGM-2) [E.C. 5.4.2.2], phosphogluconate dehydrogenase (6PGDH) [E.C. 1.1.1.44], and superoxide dismutase (sSOD) [E.C 1.15.1.1].

About a third of each tissue sample was homogenized with a glass pestle and diluted with homogenizing buffer (Wright et al. 1980). The ground samples were centrifuged at 12 krpm in a Sarstedt MH 2-K for 10 min. Both ground and unground samples were re-frozen at -70 °C for later use.

Enzymes were separated using standard starch gel electrophoresis (Uzzell and Berger 1975, Harris and Hopkinson 1976, Hotz 1983, Richardson et al. 1986, Murphy et al. 1990). Enzymes were localized on 1 mm thick gel slices with standard chromogenic methods. For most assays, gels were stained in 1.25% agar overlays, but gels for detecting AAT, GAPDH, and EST/CA activity were stained in solutions; to detect esterase activity with UV, reagents were applied to the gel as wet filter paper overlays. Each stained gel was photographed.

Albumin and the unspecified muscle proteins were separated on polyacrylamide gels. For large gels, methods described by Hotz (1983) were used, but most separations were made on minigels, using methods described by Harlow and Lane (1988). 10 µl of diluted serum (2 µl serum in 8 µl 0.7% NaCl) or crushed muscle tissue (5 mg in 5 µl 0.7% NaCl) were placed in each of the 21 2-mm wide pockets. Proteins were separated vertically at 120 V (5-10 mA) for at least 3 h, without cooling. They were stained for 30 minutes with Coomassie Blue Fast R dissolved in methanol, water and glacial acetic acid (5:5:1), and destained until the background was clear with the same solution without Coomassie Blue.

For each locus, all electromorphs observed for any taxon were calibrated by using samples of other water frog taxa or localities on the same gels. Alleles are named by lowercase letters (Hotz and Uzzell 1982); new alleles found were assigned letters not previously used, continuing with the next available alphabetical letter after those used by Hotz (1983) and Hotz and Uzzell (1983).

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 (Hotz, Uzzell, and Berger in prep.). Tissues (oocytes, liver, heart, and kidney) were homogenized in STE buffer (0.25 M sucrose, 0.03 M tris, 0.1 M EDTA, pH 7.4) and centrifuged at 3.5 krpm (SS34 rotor) for 5 min, the supernatant was centrifuged at 12 krpm (SS34) for 20 min, the resulting pellets redissolved in 2-3 vols STE and subjected to a 0.9 M/1.5 M sucrose step gradient (26 krpm, SW41 rotor for 1 h.). The fraction at the 0.9 M/1.5 M interphase containing mitochondria was collected and centrifuged in ~20 ml of 25 mM tris, 5 mM $MgCl_2$, 25 mM KCl, pH 7.4 at 12 krpm (SS34) for 20 min. The resulting pellet was redissolved and the organelles lyzed in ~1 vol of the same solution containing 1% Triton X-100 and 0.01% β-mercaptoethanol (Graf 1989). After centrifugation at 12 krpm (Sarstedt MH 2-K) for 10 min, the supernatant containing mitochondrial proteins was stored at -70 °C for later electrophoresis.

Data analysis

The data were analyzed using two different approaches.

Frequency approach:

Calculation of heterozygosity, Hardy-Weinberg equilibrium and genetic distance measures proposed by Nei (1972, 1978) and Cavalli-Sforza and Edwards (1967) were carried out with BIOSYS-1 (Swofford and Selander 1989). Hillis' (1984) modified Nei

distance was calculated with my own program. The genetic distances obtained were used to construct phenograms (*kitsch* and *contml*) using Felsenstein's (1993) PHYLIP 3.5c package. The linear regression and ANOVA calculations followed Sokal and Rohlf (1981) using Mathematica 2.2 (Wolfram Research 1993, Wolfram 1991). The ANCOVA was done with the GLM procedure of SAS Institute (1988). The analysis of the reliability of the number of loci studied was done with a randomization approach (own program): (1) 100 data sets for randomized combinations each of 5, 10, 15, 25, and 30 loci were generated; (2) with these data sets the modified Nei (1972) distance (Hillis 1984) D^*_{Nei} was calculated and then (3) analyzed with *kitsch*, using (4) the minimal sum of squares as a measure of the phylogenetic information quality of the locus groups.

Allele count approach:

Cladistic analysis was carried out using PAUP 3.1.1 (Swofford 1993) and MacClade 3.01 (Maddison and Maddison 1992).

Taxa used

The following taxa were studied (for locality list see Fig. 1 and Table 1, data on collecting sites are given in Appendix 3 p. 71): *Rana ridibunda* Pallas 1771, Europe; for the Anatolian relative of *Rana ridibunda* I use the name *Rana bedriagae* Camerano 1882; *Rana epeirotica* Schneider, Sofianidou, and Kyriakopoulou-Sklavounou 1984; an undescribed Cretan taxon, and an undescribed taxon living on Karpathos and Rhodos (Beerli et al., submitted).

The following taxa and populations were used for comparison: *Rana perezi* Seonae 1885 from Tarifa (Spain), *Rana saharica* Boulenger 1913 from Asilah (Morocco), *Rana lessonae* Camerano 1882 from Poznan-Zurawinjec (Poland) and Frauenfeld (Switzerland), *Rana shqiperica* Hotz, Uzzell, Günther, Tunner, and Heppich 1987 from Virpazar (Montenegro), *Rana epeirotica* from Aitolikon (northwestern Greece), and *Rana ridibunda* from Poznan-Fabianowo (Poland).

Chapter 3

Species and populations in space

Geographical distribution of alleles

The frequencies of the electrophoretically detectable alleles of 31 structural loci are summarized in Table 2. Twenty-nine of the loci examined (96%) have more than one allele; AK and LDH-A are invariant. Generally, the more alleles found per locus, the more private alleles (cf. Slatkin 1985) are present (Table 3). Hypervariable loci such as esterases and ALB have only a small fraction of alleles in common between taxa. Many loci have at least one common allele that is found in more than one taxon. Most of the samples showed a good agreement with the expectation of the Hardy-Weinberg equilibrium (HWE); among the few loci not in equilibrium were: GDA at Akçapınar, Aliartos, Ikaria, Samos, and Tarifa; GAPDH at Ezine; EST-6 at Virpazar; MPI at Marmaris and Virpazar; PGM-2 at Skala; and CK-A at Tarifa. In each of these cases, a heterozygote deficiency was observed. This may reflect sampling error; the standard test for HWE, as programmed in BIOSYS-1, can fail if the sample size is small and/or some genotypic frequencies are low (Guo et al. 1993). The different taxa have different numbers of private

Table 2. Electrophoretically detected allele frequencies for 29 variable genic loci. Taxa were identified by electrophoresis. Alleles are named by lowercase letters. 2 additional loci were invariable among the samples studied (AK:*b*; LDH-A:*a*). Genomes = number of genomes sampled. - = allele absent. Study localities are mapped in Fig. 1. The following localities are not mapped: Aitolikon (Greece) is about 50 km north of Patras; Frauenfeld (Switzerland) and Poznan (Poland) are in Middle Europe; Virpazar (Montenegro), in the western Balkan Peninsula near the Albanian border; Tarifa (southern Spain); and Asilah (northwestern Morocco). All taxa belong to the genus *Rana*. Cretan taxon = undescribed Cretan taxon, KR taxon = undescribed taxon on Karpathos and Rhodos, *shq* = *Rana shqiperica*, *per* = *Rana perezi*, *sah* = *Rana saharica*.

Taxon		*Cretan taxon*				epeirotica		lessonae		KR taxon		*bedriagae* Anatolia						*ridibunda* Europe											shq	per	sah
Locality		Iraklion	Kastelli	Lavris	Petros river	Aitolikon	Nea Manolada	Frauenfeld	Poznan	Karpathos	Rhodos	Akçapınar	Ezine	Marmaris	Selçuk	Ikaria	Samos	Aliartos	Monastiraki	Paradisos	Andros	Evvoia	Argos	Nea Manolada	Skala	Kithira	Poznan	Virpazar	Tarifa	Asilah
Genomes		14	20	12	4	38	24	30	26	18	30	30	22	10	32	22	8	26	26	26	6	20	20	2	26	18	28	44	26	24
sAAT	*c*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.07	-	-	-
	e	-	-	-	-	0.95	1.00	-	-	-	0.10	1.00	1.00	1.00	0.97	1.00	1.00	0.96	0.95	1.00	1.00	1.00	0.89	1.00	1.00	1.00	0.93	1.00	0.58	1.00
	f	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.04	0.05	-	-	-	-	-	-	-	-	-	-	-
	g	-	-	-	-	0.03	-	1.00	1.00	-	-	-	-	-	0.03	-	-	-	-	-	-	-	0.11	-	-	-	-	-	0.35	-
	k	-	-	-	-	0.03	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	l	-	-	-	-	-	-	-	-	1.00	0.90	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	m	1.00	1.00	1.00	1.00	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	n	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.08	-
mAAT	*a*	-	-	-	-	-	-	1.00	1.00	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	b	1.00	1.00	1.00	1.00	1.00	1.00	-	-	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
sACO	*a*	-	-	-	-	-	-	-	-	-	-	0.04	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	b	-	-	-	-	-	-	1.00	1.00	1.00	1.00	0.96	1.00	1.00	1.00	1.00	1.00	0.96	0.96	1.00	1.00	0.95	1.00	1.00	0.94	1.00	1.00	1.00	-	1.00
	c	-	-	-	-	0.87	1.00	-	-	-	-	-	-	-	-	-	-	0.04	0.04	-	-	0.05	-	-	-	-	-	-	1.00	-
	d	1.00	1.00	1.00	1.00	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	e	-	-	-	-	0.13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.06	-	-	-	-	-
mACO	*a*	-	-	-	-	-	-	-	0.04	-	0.08	0.33	0.50	-	0.62	1.00	1.00	-	0.88	0.17	-	-	-	-	-	-	0.29	-	-	-
	b	-	-	-	-	1.00	1.00	1.00	0.96	1.00	0.92	0.50	0.50	1.00	0.38	-	-	1.00	0.12	0.83	1.00	1.00	1.00	1.00	1.00	1.00	0.71	-	1.00	-
	c	1.00	1.00	1.00	1.00	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.00	-	-
	d	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.00
	e	-	-	-	-	-	-	-	-	-	-	0.17	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AHH	*a*	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	-	0.77	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	-	-
	b	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.00	0.62
	c	-	-	-	-	-	-	-	-	1.00	0.23	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	d	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.38
ALB	*a*	-	-	-	-	-	-	1.00	1.00	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	b	-	-	-	-	-	-	-	-	1.00	1.00	0.44	-	0.33	0.13	0.05	-	-	0.12	0.08	-	-	1.00	1.00	1.00	1.00	-	1.00	-	-
	c	-	-	-	-	-	-	-	-	-	-	0.50	1.00	0.67	0.80	0.95	1.00	1.00	0.88	0.92	1.00	1.00	-	-	-	-	-	-	-	-
	e	-	-	-	-	1.00	1.00	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	j	-	-	-	-	-	-	-	-	-	-	0.06	-	-	0.07	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 2. (Continued) Electrophoretically detected allele frequencies for 29 variable genic loci. Taxa were identified by electrophoresis. Alleles are named by lowercase letters. 2 additional loci were invariable among the samples studied (AK:*b*; LDH-A:*a*). Genomes = number of genomes sampled. - = allele absent. Study localities are mapped in Fig. 1. The following localities are not mapped: Aitolikon (Greece) is about 50 km north of Patras; Frauenfeld (Switzerland) and Poznan (Poland) are in Middle Europe; Virpazar (Montenegro), in the western Balkan Peninsula near the Albanian border; Tarifa (southern Spain); and Asilah (northwestern Morocco). All taxa belong to the genus *Rana*. Cretan taxon = undescribed Cretan taxon, KR taxon = undescribed taxon on Karpathos and Rhodos, *shq* = *Rana shqiperica*, *per* = *Rana perezi*, *sah* = *Rana saharica*.

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Genomes		14	20	12	4	38	24	30	26	18	30	30	22	10	32	22	8	26	26	26	6	20	20	2	26	18	28	44	26	24
	k	1.00	1.00	1.00	1.00	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	l	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.00	-
	m	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.00
CA -2	*a*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.67	0.83
	b	-	-	-	-	-	-	-	-	-	0.04	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	c	-	-	-	-	-	-	-	-	-	0.86	1.00	0.95	1.00	0.88	0.32	0.17	0.50	0.58	0.12	1.00	0.89	0.40	0.50	0.08	-	1.00	-	0.33	0.17
	d	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.94	1.00	0.04	-	0.05	-	0.12	0.68	0.83	0.50	0.42	0.88	-	0.11	0.60	0.50	0.92	1.00	-	-	-	-
	e	-	-	-	-	-	-	-	-	-	0.07	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.30	-	-
	f	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.70	-	-
	h	-	-	-	-	-	-	-	0.06	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CK -A	*a*	-	-	-	-	-	-	0.40	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	b	1.00	1.00	1.00	1.00	1.00	1.00	0.60	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	-	0.92	-
	c	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.00
	d	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.08	-
EST-5	*a*	-	-	-	-	1.00	1.00	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	b	-	-	-	-	-	-	-	-	-	-	0.50	0.50	-	0.50	1.00	0.67	-	0.75	0.64	-	-	-	-	-	-	-	-	-	-
	c	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.00	-	-
	d	-	-	-	-	-	-	1.00	1.00	0.93	0.92	0.50	0.50	1.00	0.50	-	0.33	1.00	0.25	0.36	1.00	1.00	1.00	1.00	1.00	1.00	1.00	-	1.00	1.00
	e	-	-	-	-	-	-	-	-	0.07	0.08	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	f	1.00	1.00	1.00	1.00	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
EST-6	*a*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.12
	b	-	-	-	-	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.83	1.00	0.88
	c	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.17	-	-
	d	1.00	1.00	1.00	1.00	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
FDP-1	*a*	1.00	1.00	1.00	1.00	1.00	1.00	-	-	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.88	1.00	1.00	1.00	1.00	0.94	1.00	1.00	1.00	1.00	-	1.00	1.00
	b	-	-	-	-	-	-	1.00	1.00	-	-	-	-	-	-	-	-	0.08	-	-	-	-	0.06	-	-	-	-	-	-	-
	c	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.00	-	-
	d	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.04	-	-	-	-	-	-	-	-	-	-	-	-
FDP-2	*a*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.04	-	-	-	-	-	-	-	-	-	-	-	-

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Genomes		14	20	12	4	38	24	30	26	18	30	30	22	10	32	22	8	26	26	26	6	20	20	2	26	18	28	44	26	24
	b	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	-	1.00	1.00	1.00	1.00	1.00	1.00	0.96	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	c	-	-	-	-	-	-	-	-	-	1.00	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
GAPDH	*a*	-	-	-	-	-	-	1.00	1.00	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	b	-	-	-	-	-	-	-	-	-	-	-	0.10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.00	1.00
	c	-	-	-	-	-	-	-	-	-	-	-	0.15	-	0.07	-	-	-	0.28	0.08	-	-	-	-	-	-	-	-	-	-
	d	1.00	1.00	1.00	1.00	1.00	1.00	-	-	1.00	1.00	0.95	0.75	1.00	0.93	1.00	1.00	1.00	0.72	0.92	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	-	-
	e	-	-	-	-	-	-	-	-	-	-	0.05	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
GDA	*a*	-	-	-	-	0.08	1.00	1.00	1.00	-	-	-	-	-	0.13	-	-	-	-	-	-	-	-	-	0.05	-	-	0.17	-	-
	b	-	-	-	-	0.92	-	-	-	-	-	0.23	0.91	-	0.20	-	0.50	0.58	0.95	1.00	1.00	0.90	1.00	1.00	0.95	1.00	1.00	0.83	0.25	1.00
	c	-	-	-	-	-	-	-	-	-	-	0.23	0.09	0.30	0.67	0.14	-	0.33	-	-	-	0.10	-	-	-	-	-	-	-	-
	d	1.00	1.00	1.00	0.25	-	-	-	-	1.00	1.00	0.54	-	0.70	-	0.86	0.50	0.08	0.05	-	-	-	-	-	-	-	-	-	0.75	-
	e	-	-	-	0.75	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
GCDH	*a*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.00
	b	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.00	-
	c	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	-	-
αGDH	*a*	-	-	-	-	-	-	-	-	-	-	0.29	0.20	-	-	-	0.38	1.00	0.85	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	-	-	-
	b	1.00	1.00	1.00	1.00	1.00	0.77	1.00	1.00	1.00	1.00	0.71	0.80	1.00	1.00	1.00	0.62	-	0.15	-	-	-	-	-	-	-	-	1.00	-	-
	c	-	-	-	-	-	0.23	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.00	1.00
GPI	*a*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.11	-	0.05	-	0.08	-	-	-	1.00	-	-	0.39	-	-	-
	d	-	-	-	-	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.89	1.00	0.95	1.00	0.92	1.00	1.00	1.00	-	1.00	0.94	0.61	1.00	-	-
	e	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.00	1.00
	f	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.06	-	-	-	-
	h	1.00	1.00	1.00	1.00	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
sIDH	*b*	-	-	-	-	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.97	-	-
	d	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.00
	e	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.00	-
	f	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.03	-	-
	g	1.00	1.00	1.00	1.00	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
mIDH	*b*	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	-	0.80	0.67	0.90	1.00	0.97	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	-

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Genomes		14	20	12	4	38	24	30	26	18	30	30	22	10	32	22	8	26	26	26	6	20	20	2	26	18	28	44	26	24
	c	-	-	-	-	-	-	-	-	1.00	0.20	0.30	0.10	-	0.03	-		-	-	-	-	-	-	-	-	-	-	-	-	-
	d	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.00
	e	-	-	-	-	-	-	-	-	-	-	0.03	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
LDH-B	*a*	-	-	-	-	-	-	-	-	1.00	1.00	0.36	0.15	-	0.25	-	0.50	1.00	0.31	0.85	1.00	0.95	1.00	1.00	1.00	1.00	1.00	-	-	-
	b	-	-	-	-	-	-	0.30	0.35	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	c	-	-	-	-	-	-	-	-	-	-	0.64	0.85	1.00	0.75	1.00	0.50	-	0.69	0.15	-	0.05	-	-	-	-	-	-	-	-
	d	1.00	1.00	1.00	1.00	1.00	1.00	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.00	1.00	-
	e	-	-	-	-	-	-	0.70	0.65	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	h	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.00
sMDH	*a*	1.00	1.00	1.00	1.00	1.00	1.00	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	b	-	-	-	-	-	-	1.00	1.00	1.00	1.00	0.97	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.90	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	d	-	-	-	-	-	-	-	-	-	-	0.03	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	e	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.10	-	-	-	-	-	-	-
mMDH	*a*	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.96	1.00
	b	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.04	-
MPI	*a*	-	-	-	-	-	-	-	-	-	0.23	0.82	0.73	0.75	1.00	0.95	1.00	1.00	0.69	0.50	1.00	1.00	1.00	1.00	1.00	1.00	0.64	-	-	-
	b	-	-	-	-	1.00	1.00	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	c	-	-	-	-	-	-	-	-	-	-	*0.11*	*0.27*	-	-	-	-	-	*0.31*	*0.50*	-	-	-	-	-	-	*0.36*	-	-	-
	d	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.88	-	-
	e	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.07	-	-
	h	-	-	-	-	-	-	1.00	1.00	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.02	-	-
	j	-	-	-	-	-	-	-	-	-	-	0.07	-	0.12	-	0.05	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	k	-	-	-	-	-	-	-	-	-	-	-	-	0.12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	l	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.62	-
	m	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.38	-
	n	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.96
	o	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.04
	p	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.02	-	-
	q	1.00	1.00	1.00	1.00	-	-	-	-	1.00	0.77	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

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Locality		Iraklion	Kastelli	Lavris	Petros river	Aitolikon	Nea Manolada	Frauenfeld	Poznan	Karpathos	Rhodos	Akçapınar	Ezine	Marmaris	Selçuk	Ikaria	Samos	Aliartos	Monastiraki	Paradisos	Andros	Evvoia	Argos	Nea Manolada	Skala	Kithira	Poznan	Virpazar	Tarifa	Asilah
Genomes		14	20	12	4	38	24	30	26	18	30	30	22	10	32	22	8	26	26	26	6	20	20	2	26	18	28	44	26	24
MPR-1	*b*	1.00	1.00	1.00	1.00	-	-	1.00	1.00	0.94	1.00	-	1.00	0.20	0.78	0.05	0.25	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	-	-
	c	-	-	-	-	1.00	1.00	-	-	-	-	1.00	-	0.80	0.22	0.95	0.62	-	-	-	-	-	-	-	-	-	-	-	1.00	1.00
	d	-	-	-	-	-	-	-	-	0.06	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	e	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.12	-	-	-	-	-	-	-	-	-	-	-	-	-
MPR-3	*a*	-	-	-	-	-	-	1.00	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	b	1.00	1.00	1.00	1.00	1.00	1.00	-	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
PEP-A	*a*	-	-	-	-	-	-	-	-	-	-	-	0.06	-	-	-	0.33	-	0.04	-	-	1.00	-	-	-	-	-	-	-	-
	b	-	-	-	-	1.00	0.67	1.00	1.00	1.00	1.00	1.00	0.94	1.00	1.00	1.00	0.67	1.00	0.96	1.00	1.00	-	1.00	1.00	1.00	1.00	1.00	1.00	-	-
	c	1.00	1.00	1.00	1.00	-	0.33	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.00	-
	d	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.00
6PGDH	*c*	-	-	-	-	-	-	1.00	1.00	-	-	-	0.09	-	-	-	-	-	-	-	-	-	0.70	-	-	-	-	1.00	1.00	-
	d	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.00	0.08	0.19	1.00	1.00	0.30	1.00	1.00	1.00	0.68	-	-	-
	e	1.00	1.00	1.00	1.00	-	-	-	-	1.00	1.00	1.00	0.91	1.00	1.00	1.00	1.00	-	0.92	0.81	-	-	-	-	-	-	0.32	-	-	-
	f	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.00
	g	-	-	-	-	1.00	1.00	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PGM-2	*a*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.05	-	-	-	-	-	-	-
	b	-	-	-	-	-	-	-	-	-	-	-	0.77	-	0.53	0.77	-	-	0.77	0.15	-	-	0.30	0.50	0.79	1.00	0.07	1.00	-	-
	c	-	-	-	-	-	-	1.00	1.00	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	d	1.00	1.00	1.00	1.00	1.00	1.00	-	-	1.00	1.00	0.93	0.23	1.00	0.47	0.23	1.00	1.00	0.23	0.85	1.00	1.00	0.65	0.50	0.21	-	0.93	-	1.00	1.00
	h	-	-	-	-	-	-	-	-	-	-	0.07	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
sSOD	*a*	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	-
	c	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.00

alleles (Table 3): in *Rana epeirotica* five private alleles were found, six in *Rana ridibunda*, seven in *Rana shqiperica*, eight each in the Cretan taxon and *Rana perezi*, nine each in *Rana lessonae* and *Rana bedriagae*, 14 in *Rana saharica*, and in the KR taxon four on Karpathos and five on Rhodos.

Table 3. Private alleles found in the investigated taxa and populations.

Taxon	Locality	Private alleles		
		#	fixed	segregating
Cretan taxon	*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	ALB:*e*, EST-5:*a*, MPI:*b*, 6PGDH:*g*	sAAT:*k*
	Nea Manolada			-
Rana lessonae	Frauenfeld	9	mAAT:*a*, ALB:*a*, GAPDH:*a*, PGM-2:*c*	CK-A:*a*, LDH-B:*b,e*, MPR-3:*a*
	Poznan			CA-2:*h*, LDH-B:*b,e*
KR taxon	Karpathos	4	sAAT:*l*, AHH:*c*	EST-5:*e*, MPR-1:*d*
	Rhodos	5	FDP-2:*c*	sAAT:*l*, AHH:*c*, EST-5:*e*, CA-2:*b*
Rana bedriagae	Akçapınar	9	-	sACO:*a*, mACO:*e*, ALB:*j*, mIDH:*e*, sMDH:*d*, MPI:*j*, PGM-2:*h*
	Ezine		-	-
	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*
	Poznan		-	sAAT:*c*
Rana shqiperica	Virpazar	8	FDP-1:*c*, EST-5:*c*	CA-2:*f*, EST-6:*c*, sIDH:*f*, MPI:*d,e,p*
Rana perezi	Tarifa	8	ALB:*l*, GCDH:*b*, sIDH:*e*	sAAT:*n*, CK-A:*d*, mMDH:*b*, MPI:*l,m*
Rana saharica	Asilah	14	mACO:*d*, ALB:*m*, CK-A:*c*, GCDH:*a*, sIDH:*d*, mIDH:*d*, LDH-B:*h*, PEP-A:*d*, 6PGDH:*f*, sSOD:*c*	AHH:*d*, EST-6:*a*, MPI:*n, o*

Allele distribution on the mainlands

Although *Rana ridibunda* and *Rana bedriagae* were considered a single species until recently, the differences between these two taxa are evident: they have different private alleles (Table 3) and for several loci there is a rapid spatial change of allele frequencies (Fig. 2 and Table 2) between Paradisos and Monastiraki in easternmost Greece: four out

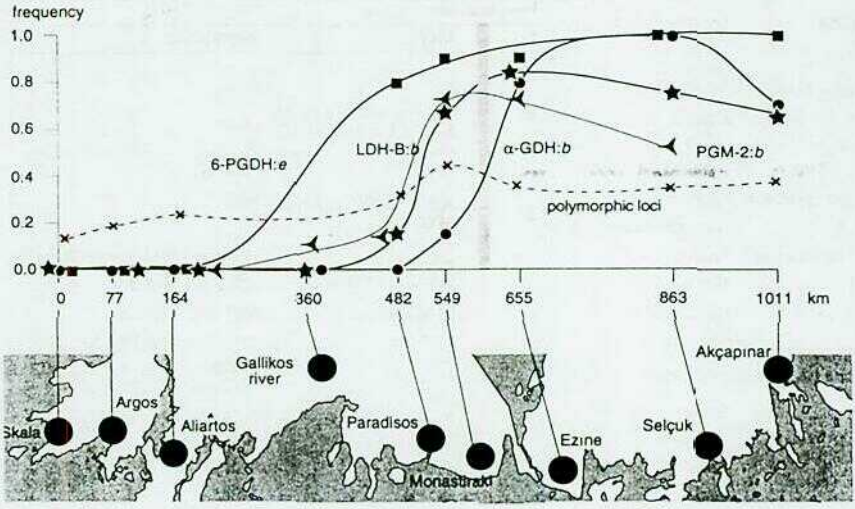

Fig. 2. Changes of frequencies of the alleles characterizing Anatolian populations along a transect around the Aegean Sea. The locality Gallikos river (Hotz and Uzzell, unpublished data) is included for some loci. The frequency changes along the transect were approximated with Bezier curves. The map is distorted along the transect, so that the localities are on a straight line (Fig. 1 provides an undistorted map). Locus and alleles names correspond to those in Table 2. The gray bar indicates the salt water barrier between Europe and Anatolia at the Dardanelles and Bosporus.

of 31 loci examined showed marked changes of allele frequencies between these two localities. The allele frequencies do not change, as might be expected, at the Dardanelles and Bosporus (which form a salt water barrier between Europe and Anatolia), but rather in Thrakia in eastern Greece. The other loci have either too many alleles to permit a reli-

able estimate of the frequency change along this transect, or *Rana ridibunda* and *Rana bedriagae* share the same major alleles (but may have rare alleles in a few populations).

The allele distributions in the other populations of *Rana ridibunda* and *Rana bedriagae* on the mainlands of Europe and Anatolia, respectively, are more homogeneous. No population is strongly different from its neighbors.

Allelic compositions on islands

Island populations showed a reduced number of alleles per locus (Table 4). On all islands, *Rana ridibunda* and *Rana bedriagae* always shared the most common alleles of the nearest population on the mainlands and never had a private allele (Table 4); on

Table 4. Examples of change in allele diversity between islands and their adjacent mainland. The distances D are measured over salt water from one locality to the other; bold values mark the most common alleles on the mainland; if other alleles are present, these have a minor frequency or are rare. There is a possible bias caused by the relatively low sample sizes, which may have prevented my finding very rare additional alleles on the mainland or on islands. Taxa: *bed* = *Rana bedriagae*, *rid* = *Rana ridibunda*, *KR* = Karpathos/Rhodos taxon, *CRE* = Cretan taxon.

Locality	Taxon	D [km]	Area [km²]	sAAT	α-GDH	GPI	LDH-B	sMDH	MPI	6PGDH	PGM-2
Anatolia	bed	0	-	**eg**	**ab**	d	ac	**bd**	acjk	**ce**	**bdh**
Samos	bed	5	476	e	**ab**	d	ac	b	a	e	d
Ikaria	bed	20	255	e	b	**ad**	c	b	aj	e	**bd**
Anatolia	bed	0	-	**eg**	ab	d	ac	bd	acjk	ce	**bdh**
Rhodos	KR	18	1398	**el**	b	**d**	**a**	**b**	**aq**	**e**	**d**
Karpathos	KR	67	301	**l**	b	**d**	**a**	**b**	**q**	**e**	**d**
Crete	CRE	120	8259	m	b	n	**d**	a	**q**	e	d
Peloponnisos	rid	0	-	**eg**	a	**ad**	a	be	**ac**	**cd**	**abd**
Kithira	rid	10	278	e	a	**df**	a	b	a	d	d
Crete	CRE	120	8259	m	b	n	**d**	a	**q**	e	d
Attika	rid	0	-	**ef**	a	**ad**	a	b	a	d	**d**
Evvoia	rid	<1	3654	e	a	d	ac	b	a	d	d
Andros	rid	6	380	e	a	d	a	b	a	d	d

Kithira, the widespread but rare allele GPI:*f* was found (1 heterozygous animal in a sample of 7). The LDH-B:*c* allele found in Evvoia (1 heterozygous animal in a sample of 10) is known also from *Rana ridibunda* in Thrakia and Central Europe (in Slovenia it is as common as LDH-B:*a*). In contrast the taxa on Crete and on Rhodos and on Karpathos are rather different from the taxon on the Anatolian mainland (Table 2, Table 3), but they are not directly comparable with the taxa on the adjacent mainlands because of their different history (Beerli et al., submitted). The Crete populations have private alleles for four of the eight loci illustrated in Table 4. On the islands Karpathos and Rhodos, at the loci MPI and LDH-B the alleles *q* and *a*, respectively, are fixed or predominant. The allele LHD-B:*a* is rare to infrequent in Anatolia, and MPI:*q* is shared only with the Cretan taxon. The allele sAAT:*l* is private.

Genic variability among taxa measured with polymorphism and heterozygosity

Substantial differences in heterozygosity values (H) between the species were detected (Table 5). The Cretan taxon is polymorphic for only one (GDA) of 31 examined loci (3.5%), compared with 58.1% polymorphic loci in *Rana bedriagae* and 58.1% in *Rana ridibunda*. The fractions of polymorphic loci of *Rana epeirotica* (16.1%), *Rana lessonae* (16.1%), *Rana shqiperica* (16.1%), *Rana perezi* (19.4%), and *Rana saharica* (12.9%) are similar and intermediate. The taxa studied can thus be separated into three groups:

- a highly variable *Rana ridibunda* and *Rana bedriagae* group
- a group with intermediate variability: *Rana epeirotica*, *Rana lessonae*, *Rana perezi*, *Rana saharica*, and *Rana shqiperica*
- the Cretan taxon with low variability

Table 5. Genetic variability at 31 loci in the 22 populations studied. Values are means ± standard errors. All values were calculated with BIOSYS-1 (Swofford and Selander 1989). Taxa were determined electrophoretically. Taxa: *bed* = *Rana bedriagae*, *rid* = *Rana ridibunda*, *epe* = *Rana epeirotica*, KR = Karpathos/Rhodos taxon, CRE = Cretan taxon.

Region	Population	Taxon	Average sample size per locus	Mean number of alleles per locus	Percentage of loci polymorphic[a]	Mean heterozygosity	
						Direct count	Hardy-Weinberg expected[b]
Anatolia	Akçapınar	bed	12.0 ± 0.7	1.5 ± 0.1	38.7	0.143 ± 0.045	0.159 ± 0.048
	Marmaris	bed	4.2 ± 0.2	1.2 ± 0.1	12.9	0.062 ± 0.031	0.059 ± 0.028
	Selçuk	bed	12.8 ± 1.0	1.4 ± 0.1	35.5	0.114 ± 0.039	0.132 ± 0.043
	Ezine	bed	9.3 ± 0.5	1.4 ± 0.1	38.7	0.126 ± 0.039	0.132 ± 0.041
Northern Greece	Aliartos	rid	10.4 ± 0.6	1.3 ± 0.1	22.6	0.025 ± 0.011	0.052 ± 0.025
	Paradisos	rid	11.4 ± 0.5	1.3 ± 0.1	32.3	0.101 ± 0.032	0.092 ± 0.028
	Monastiraki	rid[c]	10.9 ± 0.7	1.5 ± 0.1	45.2	0.118 ± 0.031	0.126 ± 0.032
Peloponnisos	Nea Manolada	epe	9.7 ± 0.6	1.1 ± 0.0	6.5	0.009 ± 0.009	0.029 ± 0.021
	Argos	rid	8.6 ± 0.5	1.2 ± 0.1	19.4	0.056 ± 0.025	0.063 ± 0.027
	Nea Manolada	rid	1.0	1.1	6.5	0.065 ± 0.045	0.065 ± 0.045
	Skala	rid	9.6 ± 0.8	1.1 ± 0.1	12.9	0.015 ± 0.007	0.023 ± 0.013
	Evvoia	rid	8.6 ± 0.4	1.1 ± 0.1	12.9	0.020 ± 0.010	0.019 ± 0.010
European islands	Andros	rid	2.7 ± 0.1	1.0	0.0	0.0	0.0
	Kithira	rid	7.8 ± 0.4	1.0	3.2	0.004	0.004
Anatolian islands	Samos	bed	3.5 ± 0.2	1.3 ± 0.1	22.6	0.097 ± 0.042	0.119 ± 0.041
	Ikaria	bed	9.2 ± 0.5	1.2 ± 0.1	22.6	0.042 ± 0.018	0.050 ± 0.021
Crete	Rhodos	KR	13.4 ± 0.6	1.3 ± 0.1	22.6	0.048 ± 0.017	0.059 ± 0.021
	Karpathos	KR	6.8 ± 0.4	1.1	6.5	0.009 ± 0.006	0.009 ± 0.006
	Iraklion	CRE	5.7 ± 0.4	1.0	0.0	0.0	0.0
	Kastelli	CRE	7.4 ± 0.7	1.0	0.0	0.0	0.0
	Lavris	CRE	5.0 ± 0.3	1.0	0.0	0.0	0.0
	Petros River	CRE	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

b. Unbiased estimate (Nei 1978)

c. The high proportion of polymorphic loci is consistent with differences in allele frequencies in the transect Akçapınar - Skala (Fig. 2), which suggests a gene flow from *Rana bedriagae* into *Rana ridibunda* in northeastern Greece, and perhaps in the reverse direction in northwestern Anatolia.

The KR-taxon is heterogeneous: on Rhodos a rather high genetic variability was detected (polymorphic loci: 22.6%, heterozygosity = 0.048), but on Karpathos the percentage of polymorphic loci is low (6.5%) and a heterozygosity of only H=0.009 was found.

Genic variation on the mainland

Given the large number of available alleles in Anatolia (Table 2 and Table 3), it is to be expected that the populations on Anatolia would show a high degree of heterozygosity (Table 5). The values for the Akçapınar population are interesting because they are higher than those observed in other Mendelian water frog species. This study provides no explanation for this high value, because Akçapınar is the southeasternmost population investigated and a transect along the southern coast of Anatolia has not yet been analyzed. The localities Monastiraki and Paradisos also show high heterozygosity values and a high degree of polymorphism. This region apparently represents a hybrid zone between *Rana ridibunda* (west) and *Rana bedriagae* (east), and alleles typical for both cooccur in the same populations and individuals (cf. Fig. 2). The heterozygosity of the population at Aliartos is lower than that of populations on the northern Peloponnisos and Paradisos; in contrast, the degree of polymorphism decreases on the transect along the sea coast from Paradisos to Skala.

Genic variation on the islands in relation to the adjacent mainlands

Island populations have lower heterozygosity values than populations on the adjacent mainland (Table 5). Genetic variability on the islands is not correlated with area, elevation, or mean rainfall (Table 6). The data visualized in Fig. 3 indicate a correlation with the distance to the nearest mainlands. The reduction in the heterozygosity on the islands relative to the mainland is similar in Anatolia and in Europe. Problematic in this correla-

tion analysis is the low sample size of only eight islands; this gives individual populations a high influence on the test of significance and the correlation values.

Table 6. Heterozygosity of island populations versus geographical parameters. The values of the mean rainfall regard the last 15-30 y (Hellenic National Meteorological Service, pers. comm.) and they are from the nearest available meteorological station: Samos, 1978-1991; Souda near Evvoia, 1955-1991; Kalamata on the Peloponnisos, 1958-1991; Rhodos, 1955-1991; Karpathos 1971-1991; Iraklion on Crete, 1955-1991. The distance D is measured over salt water. Taxa: *bed* = *Rana bedriagae*, *rid* = *Rana ridibunda*, *KR* = Karpathos/Rhodos taxon, *CRE* = Cretan taxon.

Island	Taxon	D from mainland [km]	Area [km^2]	Elevation [m]	Mean rainfall [mm]		Heterozygosity [H]
					Summer	Winter	
Samos	*bed*	5	476	1160	81	620	0.097
Ikaria	*bed*	20	255	~1200	~81	~620	0.042
Evvoia	*rid*	<1	3654	1743	72	572	0.020
Andros	*rid*	6	380	944	~72	~572	0.000
Kithira	*rid*	10	278	506	~125	~667	0.004
Rhodos	*KR*	18	1398	1215	48	656	0.048
Karpathos	*KR*	67	301	1215	47	401	0.009
Crete	*CRE*	120	8259	2456	69	421	0.005

Genetic isolation by geographical distance

The dissimilarity table of the populations (Table 7) shows variation over a wide range ($0.0 \leq$ modified Nei's standard distance $D^*_{Nei} \geq 1.3$). The differences between the modified D^*_{Nei} proposed by Hillis (1984) and Nei's standard distance D_{Nei} (Nei 1972) in my data set are very small (maximal difference = 0.013, mean difference = 0.005). I prefer the modified D^*_{Nei}, however, because this index is more robust in comparison between taxa with very different degrees of polymorphism, as encountered in this data set.

The Crete population is markedly distant genetically from all other populations, because of the high number of private alleles (Table 3). The differences between the Karpathos and Rhodos populations are rather small and their genetic distance from other taxa in

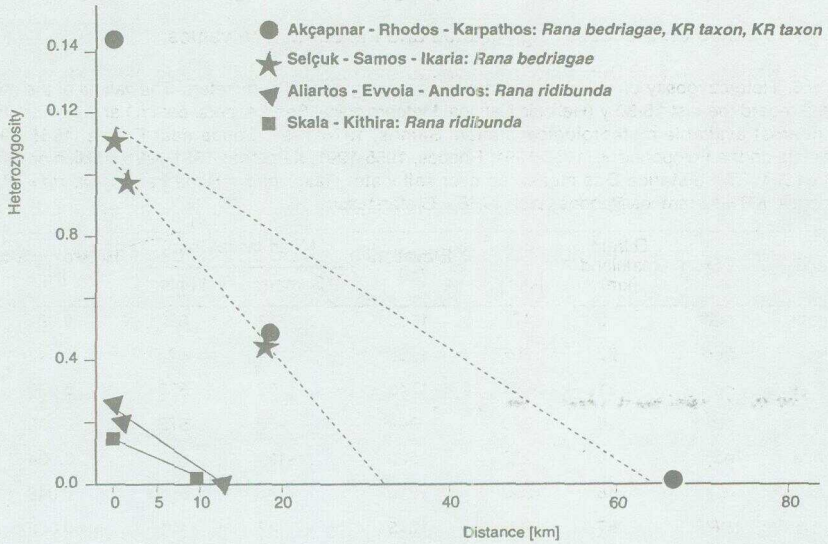

Fig. 3. Heterozygosity in mainland-island transects: the transects are direct connections between the mainland sites and the adjacent islands. The distance is the net distance over salt water. The starting point is on the mainland. The lines give a rough approximation of the reduction of heterozygosity and are not regression lines.

the region are not as high as those between the Cretan taxon and other taxa, but they are still higher than comparisons between *Rana ridibunda* and *Rana bedriagae*.

Comparing the D^*_{Nei} with geographical distance (D_{Geo}) for all mainland populations of *Rana ridibunda* and *Rana bedriagae*, which are closely related sister taxa, we can fit a linear relationship (Fig. 4). This regression is less well estimated for the single regions alone (Europe: $0.018 + 0.0002\ D_{Geo}$, Anatolia: $0.016 + 0.0002\ D_{Geo}$) because of the small numbers of populations sampled. Pooling all mainland populations, the parameters of the linear regression can be estimated: $D_{Nei} = (0.011 \pm 0.014) + (0.0003 \pm 0.00003)\ D_{Geo}$ (values $\pm \tau_{0.05\,,\,53} \times$ std. dev, $R^2_{adj} = 0.87$). The intercept is not statistically different

from 0.0, as expected, but the slope is. For all islands, a similar regression holds: D_{Nei} = (0.014 ± 0.038) + (0.0004 ± 0.0001) D_{Geo} (values ± $\tau_{0.05,48}$, R^2_{adj} = 0.537). Again the intercept is not significantly different from 0.0, but the slope is. The confidence limits for the islands are rather broad because of the limited number of islands. The groups mainland vs. mainland and islands vs. mainlands do not differ significantly, but the difference between mainland vs. mainland and islands vs. islands is slightly significant (ANCOVA: comparing D_{Nei} between the groups with the covariable D_{Geo}). Despite the problems with the significance levels caused by the small number of data points and their slight differences, there is a trend for steeper slopes in the island groups. The different groups with their regression lines and 95% confidence limits are shown in Fig. 4. Qualitatively, there is no difference between the single mainland regions and the pooled mainland populations. The difference between mainland and island populations suggests an isolating process different from the simple "isolation by distance" model. The salt water barrier between mainlands and islands effectively reduces dispersal and thus the rate of gene flow.

Table 7. 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' (1967) chord distance (lower left). KR taxon = Karpathos/Rhodos taxon, *shq* = *R.shqiperica*, *per* = *R. perezi*, *sah* = *R. saharica*.

Species		Cretan taxon				*epeirotica*		*lessonae*		KR taxon		*bedriagae* Anatolia						*ridibunda* Europe										*shq*	*per*	*sah*
	Locality	Iraklion	Kastelli	Lavris	Petros river	Aitolikon	Nea Manolada	Frauenfeld	Poznan	Karpathos	Rhodos	Akçapınar	Ezine	Marmaris	Selçuk	Ikaria	Samos	Allartos	Monastiraki	Paradisos	Andros	Evvoia	Argos	Nea Manolada	Skala	Kithira	Poznan	Virpazar	Tarifa	Asilah
Cretan taxon	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	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
	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	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
	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	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
	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	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
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	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
	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	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
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	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
	Poznan	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	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
KR taxon	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	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
	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	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
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	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
	Ezine	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	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
	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	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
	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	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
	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	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
	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	-	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
ridibunda	Allartos	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	-	0.14	0.07	0.01	0.04	0.06	0.08	0.07	0.08	0.04	0.40	0.63	0.80
	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	0.31	-	0.07	0.14	0.18	0.16	0.18	0.15	0.16	0.12	0.34	0.77	0.89
	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	0.23	0.17	-	0.08	0.12	0.10	0.13	0.11	0.12	0.07	0.38	0.68	0.83
	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	0.13	0.31	0.24	-*	0.03	0.07	0.09	0.09	0.10	0.03	0.39	0.62	0.78
	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	0.19	0.33	0.28	0.17	-	0.10	0.12	0.12	0.13	0.06	0.44	0.62	0.78
	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	0.23	0.32	0.26	0.24	0.28	-	0.06	0.04	0.05	0.06	0.29	0.58	0.80
	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	0.25	0.34	0.28	0.26	0.30	0.21	-	0.05	0.05	0.05	0.36	0.65	0.81
	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	0.23	0.31	0.26	0.25	0.29	0.15	0.18	-	0.00	0.08	0.30	0.70	0.85
	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	0.27	0.32	0.28	0.28	0.32	0.18	0.20	0.08	-	0.09	0.30	0.72	0.87
	Poznan	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	0.21	0.26	0.20	0.17	0.24	0.23	0.21	0.24	0.27	-	0.36	0.63	0.78
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	0.52	0.48	0.50	0.52	0.54	0.46	0.50	0.47	0.46	0.50	-	0.79	1.04
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	0.61	0.64	0.63	0.61	0.61	0.60	0.62	0.63	0.64	0.61	0.67	-	0.55
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	0.67	0.68	0.68	0.66	0.66	0.67	0.67	0.68	0.69	0.66	0.73	0.58	-

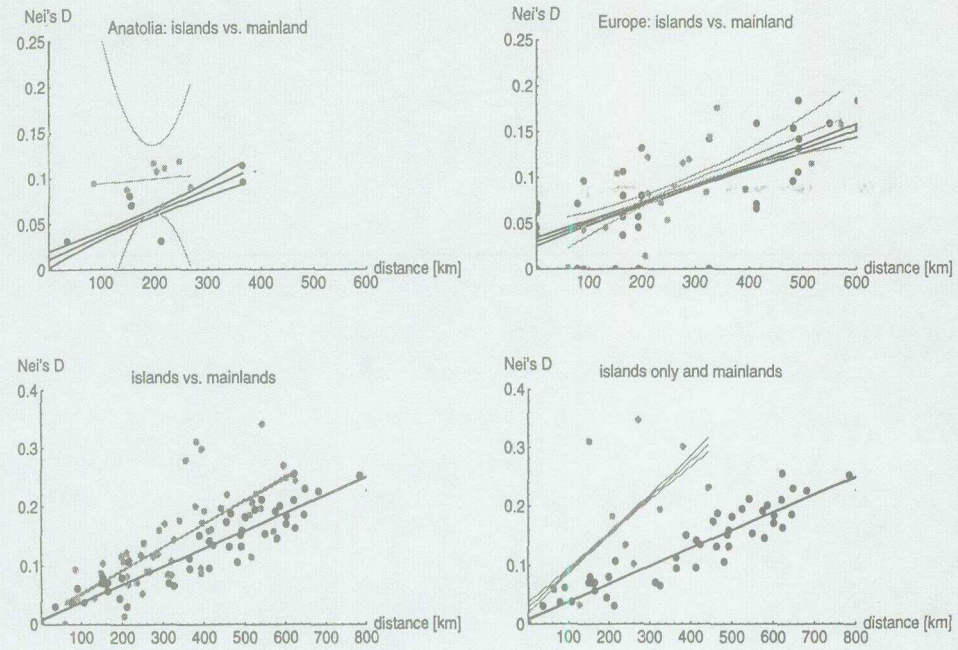

Fig. 4. *Scatterplots of geographic distance versus the within-taxon genetic distance (modified Nei's standard distance) and their linear fit for Rana ridibunda and Rana bedriagae, respectively, in the different mainland regions: top left: comparison between islands and mainland Anatolia, top right: comparison between islands and mainland Europe, bottom left: Rana bedriagae and Rana ridibunda pooled in comparison with the island populations; bottom right: comparison between islands inter se and the pooled mainlands Europe and Anatolia. All regression lines are drawn with 95% confidence limits for the slopes (barely visible for some slopes). Islands are in gray.*

Chapter 4

Phylogenetic analysis

The genetic distance data (Table 7, Table 8) were used to construct phylogenetic trees (Fig. 5, Fig. 6; kitsch, Felsenstein 1993). In these analyses, all populations were placed in their correct species or taxon clade, except *"Rana ridibunda"* from Monastiraki in Thrakia in northeastern Greece, which is grouped together with the Anatolian taxon *Rana bedriagae* (apparently a result of alleles introgressed from Anatolia; cf. Fig. 2). The island populations cluster with those on the adjacent mainland. The islands Crete, Karpathos and Rhodos, isolated for a longer time, branch off earlier (in character space) from the tree. Pooling the localities for each taxon does not change the general branching pattern (Fig. 6). All taxa keep their position on the tree. In all trees examined the outgroup (consisting of *Rana perezi* and *Rana saharica*) is consistently separated from the ingroup, but only in the maximum likelihood (ML) analysis (contml, Felsenstein 1993) the two species form a sister clade.

The ML-approach produces population and taxon trees (Fig. 7, Fig. 8), that are in most branching patterns equal to the kitsch trees, except for the positions of *Rana epeirotica* and *Rana perezi*. In the ML tree, *Rana epeirotica* is a sister taxon of the Cretan taxon, in

Table 8. Genetic distances between the studied species. Genetic distances used are the modified Nei standard distance (Nei 1972, Hillis 1984) in the upper right, and the Cavalli-Sforza and Edwards (1967) chord distance in the lower left.

Taxon	Rana bedriagae	Cretan taxon	Rana epeirotica	Karpathos taxon	Rhodos population	Rana lessonae	Rana perezi	Rana ridibunda	Rana ridibunda Poland	Rana saharica	Rana shqiperica
Rana bedriagae	-	0.584	0.359	0.317	0.231	0.523	0.63	0.144	0.161	0.82	0.35
Cretan taxon	0.599	-	0.53	0.544	0.548	0.895	0.799	0.655	0.698	1.355	0.661
Rana epeirotica	0.493	0.574	-	0.491	0.487	0.572	0.58	0.379	0.425	0.981	0.456
Karpathos taxon	0.444	0.585	0.564	-	0.119	0.507	0.73	0.274	0.303	0.95	0.491
Rhodos population	0.381	0.588	0.558	0.286	-	0.509	0.697	0.232	0.207	1.01	0.436
Rana lessonae	0.557	0.694	0.598	0.572	0.572	-	0.855	0.454	0.519	1.271	0.496
Rana perezi	0.608	0.671	0.588	0.651	0.639	0.684	-	0.64	0.629	0.545	0.785
Rana ridibunda	0.255	0.620	0.506	0.442	0.393	0.538	0.611	-	0.031	0.803	0.334
Rana ridibunda Poland	0.312	0.636	0.535	0.463	0.385	0.576	0.614	0.170	-	0.782	0.362
Rana saharica	0.667	0.775	0.708	0.707	0.712	0.765	0.584	0.671	0.661	-	1.041
Rana shqiperica	0.483	0.626	0.545	0.563	0.533	0.559	0.665	0.476	0.498	0.727	-

contrast to the kitsch tree, in which it is a sister taxon of the *"Rana shqiperica, Rana ridibunda, Rana bedriagae,* KR taxon" cluster. The consistent separation of *Rana ridibunda* and *Rana bedriagae* in the kitsch tree breaks down in the ML tree (Fig. 8). An obvious continuous transition between these two taxa appears. This transition is the result of an allele frequency change between the localities Paradisos and Monastiraki. The ML tree suggests an even broader transition zone between Paradisos and Selçuk (cf. Fig. 2). The approximate confidence limits, produced by the *contml* program, indicate that the observed branching pattern of the clades separating *Rana epeirotica*, Cretan taxon, and the *"Rana lessonae, Rana shqiperica, Rana ridibunda, Rana bedriagae,* KR taxon" cluster is not significant.

The divergent evolution of populations on the longer-isolated islands Crete, Karpathos and Rhodos is also supported by a cladistic tree (Fig. 9). The cladistic species tree

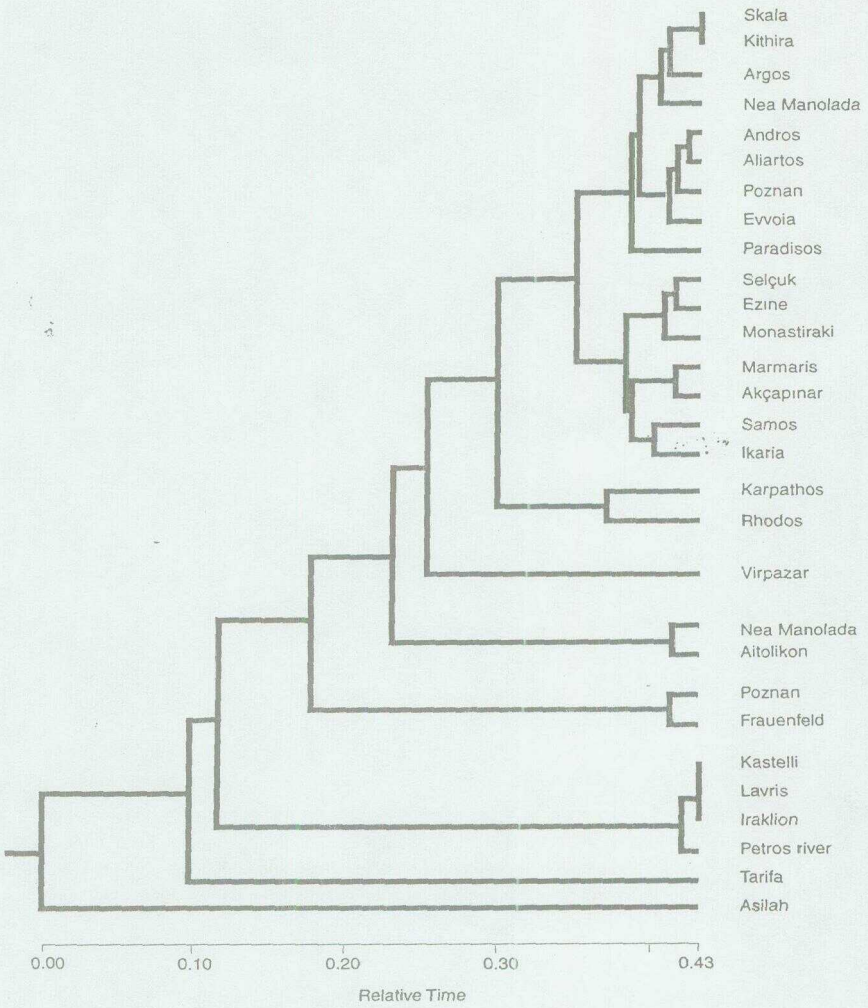

Fig. 5. Phylogenetic tree of the localities. The tree was constructed with the kitsch program 3.5c (Felsenstein 1993), the genetic distance measure used was Hillis' (1984) modification of Nei's (1972) standard distance: 89,295 trees were examined; sum of squares for the best tree found= 29.74, with average percent standard deviation =19.16.

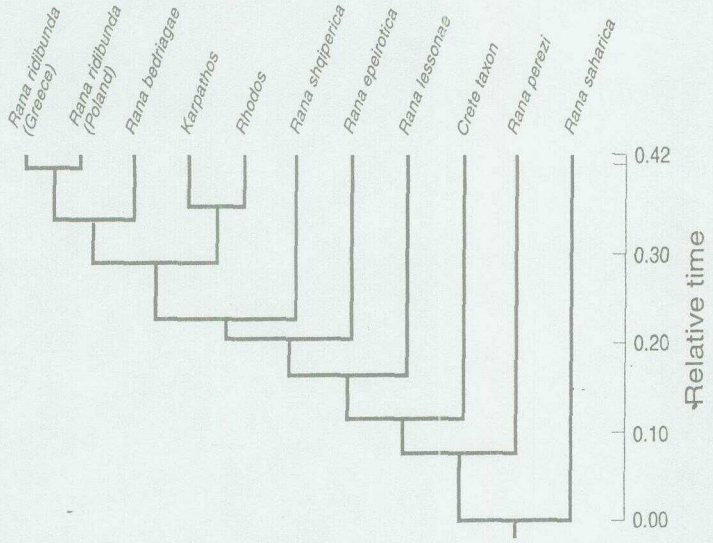

Fig. 6. Phylogenetic tree of the taxa used. The tree was constructed with the kitsch program 3.5c (Felsenstein 1993), the genetic distance measure used was Hillis' (1984) Modification of Nei's (1972) standard distance: 9971 trees were examined, sum of squares of the best tree found = 2.67, with average percent standard deviation = 15.7.

(Fig. 10) does not exactly copy the branching pattern of the kitsch- or the ML-tree. The unambiguous changes along some branches tend to define the same groups as in the other analyses, but the branching cannot be collapsed to a pattern similar to that in the frequency trees. All taxa except *Rana ridibunda* and *Rana bedriagae* are well defined with at least one unambiguous change.

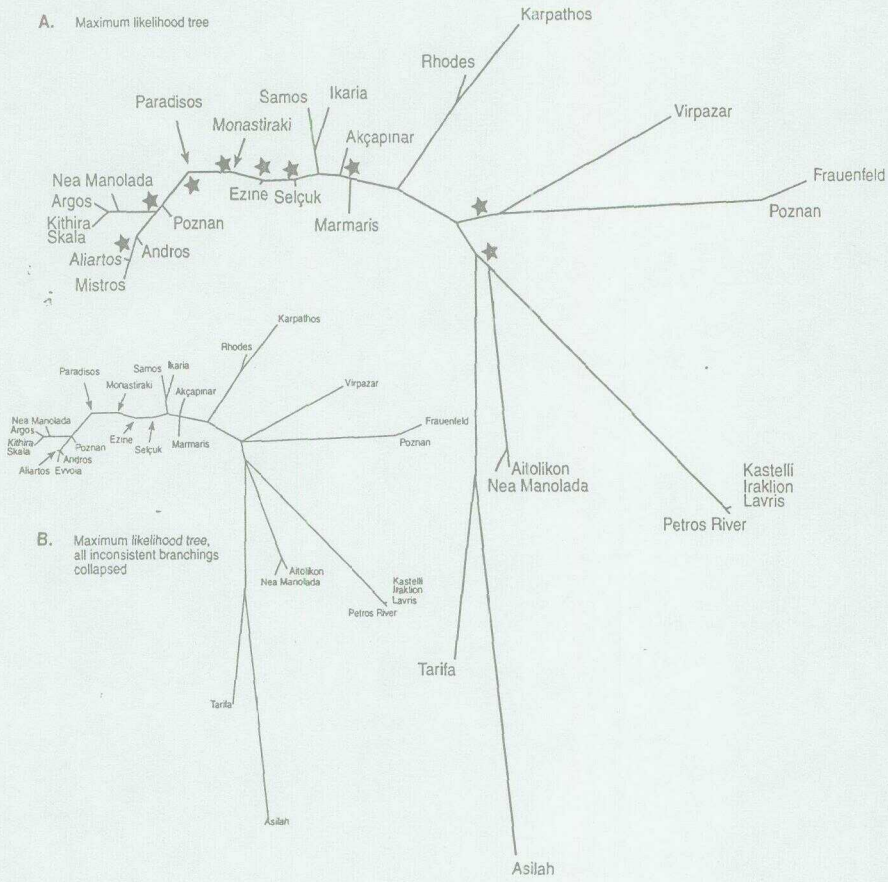

Fig. 7. Maximum likelihood tree (*contml*, Felsenstein 1993): (A) Stars indicate branches where the confidence limits suggest other possible branchings. The distance from Tarifa and Asilah to the next branching equal 0.079 and 0.161, respectively, in terms of square-root transformations of the allele frequencies (this measure is similar to Cavalli-Sforza and Edwards' (1967) chord distance). The inset (B) shows the most conservative interpretation of the confidence limits, collapsing to zero length all branches with possible other arrangements.

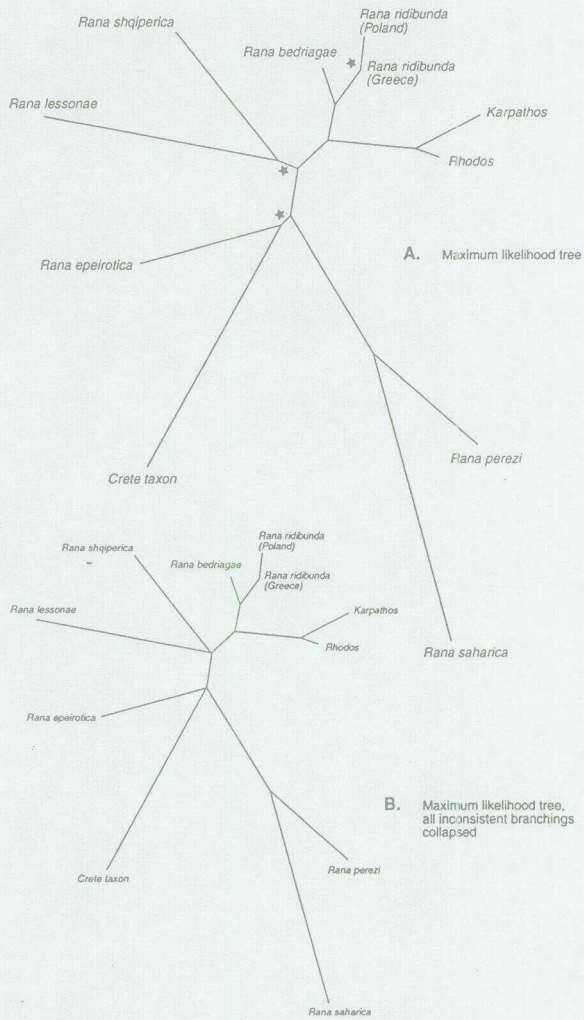

Fig. 8. Maximum likelihood tree (*contml*, Felsenstein 1993) of the taxa used: (A) The stars indicate branches where the confidence limits suggest possible other branchings. The distance from *Rana perezi* and *Rana saharica* to the next branching equals 0.078 and 0.161, respectively, in terms of square-root transformations of the allele frequencies (this measure is similar to Cavalli-Sforza and Edwards' (1967) chord distance). The lower graph (B) shows the most conservative interpretation of the confidence limits, collapsing to zero length all branches with possible other arrangements.

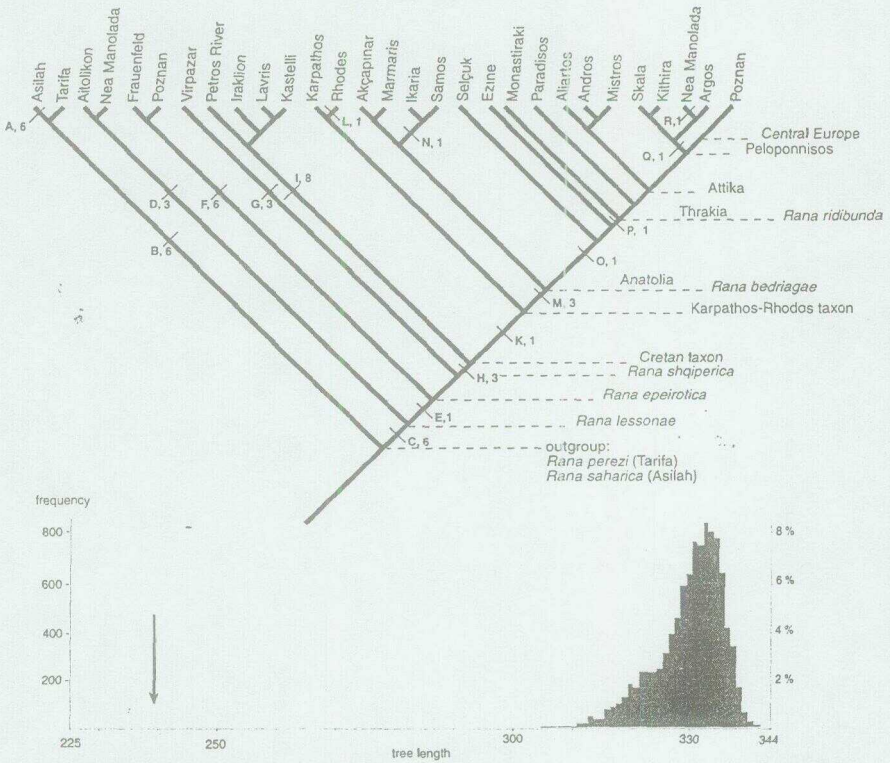

Fig. 9. Cladistic tree constructed with MacClade 3.0: The branching pattern is an intelligent guess, because each branching not supported with an unambiguous change can generate a whole family of equally parsimonious trees. 31 characters (loci) were used. Tree length: 239 (minimal possible: 225; maximal possible: 344), consistency index (CI) = 0.96, retention index (RI) = 0.91, rescaled consistency index (RC) = 0.87. The frequency chart gives the distribution of 10,000 random equiprobable trees; the shape of the distribution (mean=330, standard deviation = 6.1, g_1=-1.112, g_2= 1.576) indicates that a tree with a length of 239 (arrow) is among the shortest trees (no shorter tree was found with PAUP 3.1.1: heuristic, random addition approach). Unambiguous changes: **A.** mACO:*b*→*d*, CK-A:*b*→*c*, mIDH:*b*→*d*, LDH-B:*d*→*h*, 6PGHD:*c*→*f*, sSOD:*a*→*c*; **B.** αGDH:*c*↔*b*, AHH:*b*↔*a*, CA-2:c/a↔*d*, GDA:*b*↔*d*, GAPDH:*b*↔*d*, GPI:*e*↔*d*; **C.** αGDH:*c*↔*b*, AHH:*b*↔*a*, CA-2:c/a↔*d*, GDA:*b*↔*d*, GAPDH:*b*↔*d*, GPI:*e*↔*d*; **D.** EST-5:*d*→*a*, sMDH:*b*→*a*, 6PGDH:*c*→*g*; **E.** MPR-1:*c*→*b*; **F.** sAAT:*e*→*g*, mAAT:*b*→*a*, FDP-1:*a*→*b*, GAPDH:*d*→*a*, LDH-B:*d*→*e/b*, PGM-2:*d*→*c*; **G.** EST-5:*d*→*c*, FDP-1:*a*→*c*, PGM-2:*d*→*b*, ; **H.** GDA:*a*→*d*, MPI:*h*→*q*, 6PGDH:*c*→*e*; **I.** sAAT:*e*→*m*, Alb:*b*→*k*, EST-5:*d*→*f*, EST-6:*b*→*d*, GPI:*d*→*h*, sIDH-1:*b*→*g*, sMDH:*b*→*a*, PEP-A:*b*→*c*; **K.** LDH-B:*d*→*a*; **L.** FDP-2:*b*→*c*; **M.** Alb:*b*→*c*, CA-2:*d*→*c*, MPI:*q*→*a*; **N.** mACO:*b*→*a*; **O.** GDA:*d*→*b*; **P.** αGDH:*b*→*a*; **Q.** ALB:*c*→*b*, **R.** GPI:*d*→*a*.

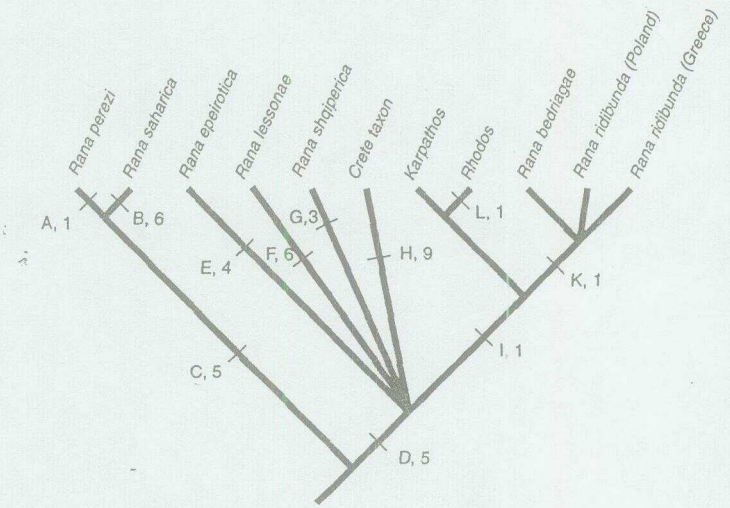

Fig. 10. Cladistic species tree constructed with MacClade 3.01 (Maddison and Maddison 1992) and tested with PAUP 3.1.1 (Swofford 1993). Unambiguous changes: **A.** sACO:*b→c*; **B.** mACO:*b→d*, CK-A:*b→c*, mIDH:*b→d*, LDH-B:*d→h*, 6PGHD:*c→f*, sSOD:*a→c*; **C.** αGDH:*c↔b*, AHH:*b↔a*, GAPDH:*b↔d*, GPI:*e↔d*, MPR-1:*c↔b*; **D.** αGDH:*c↔b*, AHH:*b↔a*, GAPDH:*b↔d*, GPI:*e↔d*, MPR-1:*c↔b*; **E.** ALB:*b→e*, EST-5:*d→a*, MPR-1:*b→c*; **F.** sAAT:*e→g*, mAAT:*b→a*, ALB:*b→a*, FDP-1:*a→b*, GAPDH:*d→a*, PGM-2:*d→c*; **G.** mACO:*b→c*, EST-5:*d→c*, FDP-1:*a→c*, PGM-2:*d→b*; **H.** sAAT:*e→m*, sACO:*b→d*, mACO:*b→c*, Alb:*b→k*, EST-5:*d→f*, EST-6:*b→d*, GPI:*d→h*, sIDH-1:*b→g*, PEP-A:*b→c*; **I.** LDH-B:*d→a*; **K.** MPI:*q→c/a*; **L.** FDP-2:*b→c*.

Chapter 5

How reliable are the loci used?

Number of alleles per locus: "fast" and "slow" evolvers

The number and distribution of alleles per locus across taxa provides information about the phylogenetic usefulness of the locus. Loci with no variation cannot indicate a phylogenetic substructure. Loci with only private alleles can provide information only in grouping populations of one species together, but are useless for grouping the end point taxa into higher taxonomic units. For the overall data set, most of the loci have three to five alleles (mean = 4.1, standard deviation = 1.7, n=31). The mean number of private alleles per locus is 2.2 (standard deviation = 1.6, n=31). Table 9 provides a summary of the number of alleles per locus and of the frequency of private alleles. The number of private alleles can be used as an indicator of the evolutionary rate for each locus, and the loci can be subdivided into slow (private allele ≤1), medium (2 ≤ private alleles ≤4), and fast evolvers (private alleles > 4). The division in three groups is according to the limits given by the standard deviations. It is arbitrary to use only the private alleles as this indicator, however, because the total number of alleles per locus also yields some information about the evolutionary rate; loci with a low number of alleles are normally considered as

Table 9. Number of alleles per locus. Bold loci are considered to be either slow or fast evolvers, respectively. The table is ordered according to the fraction of private alleles. Taxa: K = Karpathos, R = Rhodos, *rid* = *Rana ridibunda*, *epe* = *Rana epeirotica*, *bed* = *Rana bedriagae*, *shq* = *Rana shqiperica*, *per* = *Rana perezi*, *les* = *Rana lessonae*, CRE = Cretan taxon, *sah* = *Rana saharica*; E = estimated class of evolutionary rate: s = slow, m = medium, f = fast

Loci	K	R	*rid*	*epe*	*bed*	*shq*	*per*	*les*	CRE	sah	Total alleles	Private alleles	Fraction of private alleles	E
MPR-3	1	1	1	1	1	1	1	1	1	1	1	0	0.00	s
LDH-A	1	1	1	1	1	1	1	1	1	1	1	0	0.00	s
αGDH	1	1	2	2	2	1	1	1	1	3	3	0	0.00	s
AK	1	1	1	1	1	1	1	1	1	1	1	0	0.00	s
GDA	1	1	3	2	4	2	2	1	2	1	5	1	0.20	s
PEP-A	1	1	2	2	2	1	1	1	1	1	4	1	0.25	s
CA-2	1	4	2	1	2	2	2	2	1	2	7	2	0.29	m
LDH-B	1	1	2	1	2	1	1	2	1	1	6	2	0.33	m
sACO	1	1	2	2	2	1	1	1	1	1	5	2	0.40	m
6PGDH	1	1	3	1	2	1	1	1	1	1	5	2	0.40	m
mACO	1	2	3	1	3	1	1	2	1	1	5	2	0.40	m
G3PDH	1	1	3	1	2	1	1	1	1	1	5	2	0.40	m
MPR-1	2	1	1	1	3	1	1	1	1	1	4	2	0.50	m
FDP-1	1	1	2	1	1	1	1	2	1	1	4	2	0.50	m
sSOD	1	1	1	1	1	1	1	1	1	1	2	1	0.50	s
mMDH	1	1	1	1	1	1	2	1	1	1	2	1	0.50	s
mAAT	1	1	1	1	1	1	1	1	1	1	2	1	0.50	s
sMDH	1	1	2	1	2	1	1	1	1	1	4	2	0.50	m
mIDH	1	2	1	1	2	1	1	1	1	1	4	2	0.50	m
PGM-2	1	1	3	1	3	1	1	1	1	1	5	3	0.60	m
FDP-2	1	1	2	1	1	1	1	1	1	1	3	2	0.67	m
GCDH	1	1	1	1	1	1	1	1	1	1	3	2	0.67	m
MPI	1	2	2	1	4	4	2	1	1	2	14	10	0.71	f
CK-A	1	1	1	1	1	1	2	2	1	1	4	3	0.75	m
EST-6	1	1	1	1	1	2	1	1	1	2	4	3	0.75	m
AHH	1	2	1	1	1	1	1	1	1	1	4	3	0.75	m
GPI	1	1	1	1	1	2	1	1	1	1	5	4	0.80	m
sIDH	1	1	1	1	1	2	1	1	1	1	5	4	0.80	m
ALB	2	2	2	1	2	1	1	1	1	1	6	5	0.83	f
EST-5	2	2	2	1	2	1	1	1	1	1	6	5	0.83	f
sAAT	1	2	3	3	3	1	3	1	1	1	8	7	0.88	f
Total	34	41	54	37	56	39	38	36	32	36	137	76	0.56	-
Private	4	5	7	5	9	7	8	9	8	14	76	-	-	-
Fraction of private alleles	0.12	0.12	0.13	0.14	0.16	0.18	0.21	0.3	0.3	0.39	0.55	-	-	-

slow evolvers. For example, the loci CA-2 and LDH-B, with a total of more than 5 alleles each, have only a low number of private alleles. Problematic in this context are the loci α-GDH, LDH-B, and sMDH: these have the same common alleles in more than one species. Is there a possibility of some conserved old alleles at these loci?

How reliable are 31 loci for phylogenetic interpretation?

Using a randomizing study, I tested the convergence to the best phylogenetic information I can obtain with protein electrophoresis. The values presented in Fig. 11 suggest a good approximation with 31 loci to the maximal information attainable with protein electrophoresis. Between 20 and 25 loci, the sum of squares converges to a value of ~30. Electrophoretic estimates with a minimum of about 20 loci will therefore produce consistent results. Some values in the 5 loci case suggest an even lower sum of squares, but the standard deviation in the 5 loci case is much higher than in the 25 loci case or in the real data (cf. Fig. 5); it is expected that the variation is dependent on the number of populations, as well as the number of alleles per locus, both of which cannot be estimated a priori.

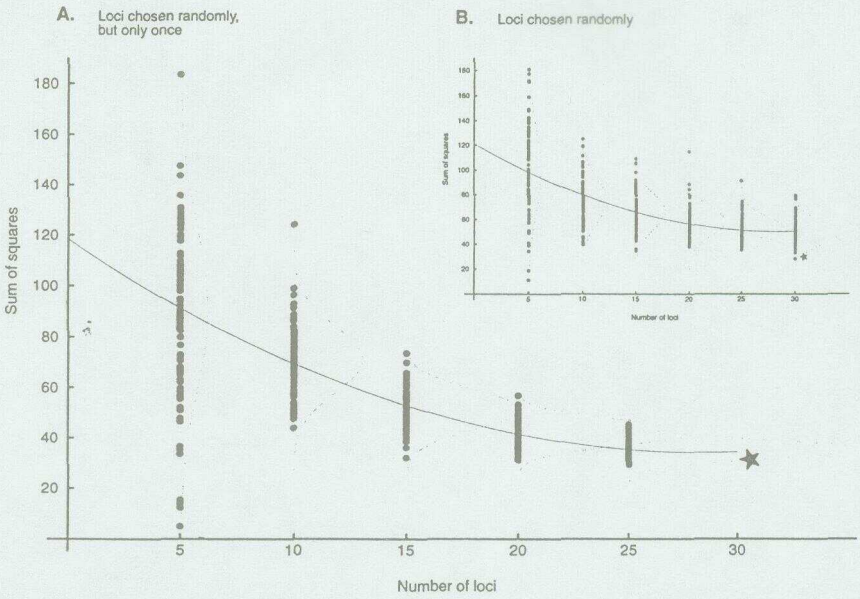

Fig. 11. Reliability of the phylogenetic information obtained by protein electrophoresis of 31 loci. Out of the data set (31 loci, 29 populations) 100 new data sets were generated by randomization with (B) and without (A) withdrawal for each group of 5, 10, 15, 20, 25, and 30 loci (only in B), respectively. With each of these new data sets the modified Nei distance was calculated, and these distance matrices were analyzed with kitsch (Felsenstein 1993). The sum of squares is a measure of quality in this phylogenetic analysis; lower values indicate better trees. The star marks the original data set. The regression line is (A) $118.4 - 5.9\,x + 0.1\,x^2$, (B) $121.2 - 5.0\,x + 0.1\,x^2$. The stippled lines to the right of each group of points indicate the relative frequency of the sum of square values for each group approximated with a normal distribution; their heights are relative and unrelated to the X-axis.

Chapter 6

Species and populations in time: Calibrating the protein clock

Known geological isolation times are presented in Table 10. The genetic distance per million years varies strongly among the pairs compared, but a closer look shows that the localities on more distantly isolated islands give similar values and only the two connections Samos-Anatolia and Evvoia-Aliartos (Attika) show exceptionally high values. Comparing the localities rather than the means between the localities (and taxa), a striking linear pattern appears (Fig. 12). It is clear from the data that the regression line is strongly affected by the low genetic distances of populations that are not isolated at present, and by the large distance between the Cretan taxon and all others. The genetic distances and the geological isolation time of Rhodos relative to the other populations fit the scenario quite well. The isolation of Karpathos is not well dated (cf. Appendix 1 p. 61). Using an isolation time of 3 My (middle Pliocene), the linearity is not significant (α=0.05). Linearity is confirmed for all isolation values between 2.0 and 2.7 My. The linear relationship is D^*_{Nei} = (0.027±0.027) + (0.114±0.008) • isolation time [My] (with Karpathos 3 My; $\tau(\alpha=0.05,df=65)=2.00$, $R^2_{adj}=0.93$), D^*_{Nei} =(0.034±0.023) + (0.115±0.007) • isolation

Table 10. Calibration of a molecular protein clock with different geological data. The calibration used only pairs with known geological divergence time. bed = *Rana bedriagae*, rid = *Rana ridibunda*, epe = *Rana epeirotica*, CRE = Cretan taxon, KR = Karpathos/Rhodos taxon.

Species combination	Mainland	Island	Last connection by land relative and absolute in 1000 y BP		Genetic distance[a] (D*Nei)	Genetic distance per million years
bed-bed	Selçuk	Samos	Würm	13	0.09	6.9
bed-bed	Selçuk	Ikaria	Riss/Mindel	200	0.09	0.5
bed-bed	Samos	Ikaria	Riss/Mindel	200	0.06	0.3
rid-rid	Aliartos	Evvoia	Würm	13	0.04	3.1
rid-rid	Aliartos	Andros	Riss/Mindel	200	0.01	0.05
rid-rid	Evvoia	Andros	Riss/Mindel	200	0.03	0.15
rid-rid	Skala	Kithira	Riss/Mindel	200	0.002	0.01
bed-CRE	Anatolia	Crete	Messinian	5,000	0.58	0.12
rid-CRE	Europe	Crete	Messinian	5,000	0.70	0.14
epe-CRE	Europe	Crete	Messinian	5,000	0.53	0.11
KR-CRE	Crete	Karpathos	Messinian	5,000	0.54	0.11
KR-CRE	Crete	Rhodos	Messinian	5,000	0.55	0.11
bed-KR	Anatolia	Karpathos	Pliocene	1,800-3,000	0.32	0.17-0.11
bed-KR	Europe	Karpathos	Pliocene	1,800-3,000	0.27	0.15-0.09
bed-KR	Anatolia	Rhodos	early Pleistocene	1,800	0.23	0.13
bed-KR	Europe	Rhodos	early Pleistocene	1,800	0.23	0.13

a. The values of D^*_{Nei} (Nei 1972, Hillis 1984) for the comparisons of Crete and Karpathos are means.

time [My] (without Karpathos, $\tau(\alpha=0.05,df=57)=2.00$, $R^2_{adj}=0.95$). These regression lines give an average of 0.14 D^*_{Nei} /My and 0.15 D^*_{Nei} /My, respectively. For the estimation of unknown divergence times I used the procedure described in Hillis and Moritz (1990) and got the following regression (Fig. 12): isolation time [My] = (-0.04±0.22) + (8.146±0.548) • D^*_{Nei} ($\tau(\alpha=0.05,df=65)=2.00$, $R^2_{adj}=0.93$). This gives a mean divergence rate of 8.2 My / D^*_{Nei}. The influence of the "fast evolvers" is detectable: without the loci sAAT, EST-5, Alb, and MPI, the regressions are D^*_{Nei} = (0.018±0.024) + (0.099±0.007) • isolation time [My], with 0.10 D^*_{Nei} /My and isolation time [My] = (-0.014±0.23) + (9.397±0.647) • D^*_{Nei}, with 9.4 My / D^*_{Nei}.

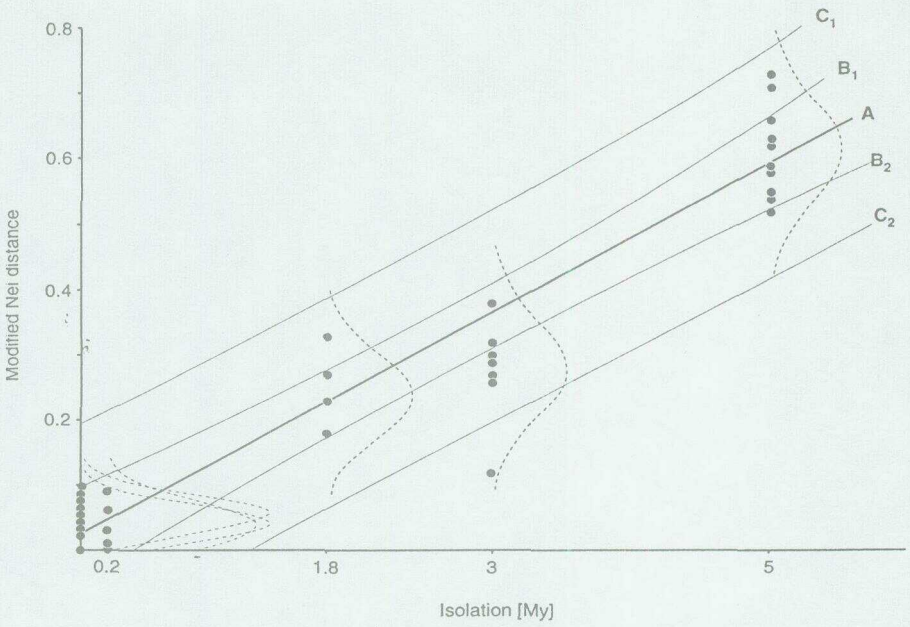

Fig. 12. Genetic distance (D^*_{Nei}) versus geological isolation time. Regression line (A: (-0.04±0.22) + (8.146 0.548) · isolation time); B1 and B2 are the bounds of the 95% confidence limits of the regression line; C1 and C2 are the bounds of the 95% confidence limit for the prediction of time based on genetic data (cf. Sokal and Rohlf 1981). The dashed lines provide the frequency distribution of the genetic distances approximated whith a normal distribution. The height of of the frequency distribution is relative and not correlated with the X-Axis.

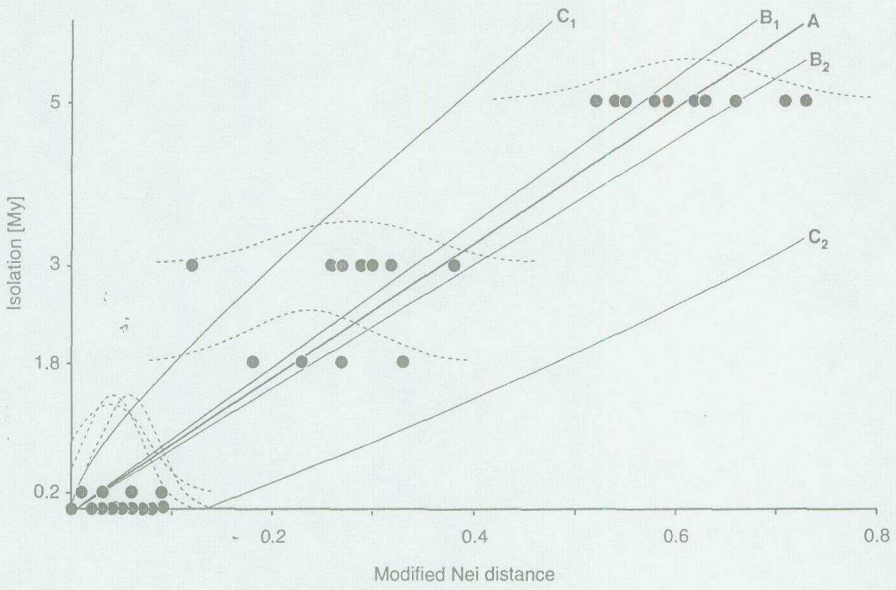

Fig. 13. *Geological isolation time versus genetic distance (D^*_{Nei}).* Regression line (A: (-0.04±0.22) + (8.146 0.548) · isolation time); B1 and B2 are the bounds of the 95% confidence limits of the regression line; C1 and C2 are the bounds of the 95% confidence limit for the prediction of time based on new genetic distance data (cf. Hillis and Moritz 1990). The dashed lines provide the frequency distribution of the genetic distances approximated whith a normal distribution. The height of of the frequency distribution is relative and not correlated with the Y-Axis.

Chapter 7

Discussion

Geographical isolation of the taxa

In a polymorphic species, populations are slightly different from each other, resulting in different within-species genetic distance values. The further the populations are apart from each other, the greater are these differences. This isolation by distance is related to the dispersal ability of the individuals (Singh and Rhomberg 1987a). If the migration possibilities are reduced, for example by the separation of an island population by salt water, the genetic distance increases faster per geographic distance unit than on the mainland, where there are no physiological barriers to dispersal (Fig. 4).

Differences in numbers of species between islands and the adjacent mainland are well documented (Mac Arthur and Wilson 1967). In this study, I detect similar reductions in heterozygosity and in number of alleles in populations on islands compared with those on the adjacent mainland. This fall in variability may have several causes. Due to bottlenecks, the following scenarios are possible:

1. *Vicariance*: an old, widely distributed species becomes subdivided during a sea level change into populations that are isolated on several islands. Severe bottlenecks

caused by loss of fresh water or other ecological catastrophes may subsequently reduce the number of alleles in such small isolated populations by random genetic drift, and lead to total homozygosity if the population size remains small for a prolonged period.

2. Introduction of frogs by humans, which results in a severe initial bottleneck (founder effect).

3. Colonization: Water frogs have problems with salt water, such that their colonization ability in the Aegean region is reduced. As a result of a hypothetical limited dispersal across the sea, only the most frequent alleles may have reached the islands.

Discrimination between these three scenarios is possible for many island populations. Scenario (3) is very improbable for water frogs, which are unable to live in salt or brackish water for a long time. Although G. Nascetti (pers. comm.) observed a living adult water frog on a piece of wood floating in the sea five kilometers off the coast of Italy, this does not guarantee a safe arrival on an island, where the surf on a sandy or stony shoreline may provide an additional barrier. Moreover, simultaneous arrival of at least one female and one male on an island is required to found a population. In the populations of the islands situated on the continental shelf, recent migration cannot be detected or ruled out by the present data set, because only common alleles were found. Rare alleles can be used as markers of migration patterns (Slatkin 1985, Barton and Slatkin 1986, Singh and Rhomberg 1987a), but none were found on shelf islands, except on Kithira and Samos. These rare alleles on these two islands have no counterpart, however, on the adjacent mainland sample. 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 would expect to find some remnants of any introduction from the Peloponnisos or Attika at least on the islands Samos and Ikaria. These islands cluster with the mainland of Anatolia, however, in all phylogenetic analyses (Fig. 5,

Fig. 8, Fig. 9). Introduction by humans (2) can be ruled out for the islands of the Hellenic arc. The Cretan taxon and the KR taxon have several private alleles not found elsewhere, and their genetic dissimilarity values suggest differentiation at the species level (Beerli et al., submitted). All these populations have reduced heterozygosity, except Rhodos. Scenario (1) is well supported by geological data, which is the precondition for an interpretation of the molecular clock. I hypothesize the following sequence of events: An ancient water frog species (or more than one) was widespread in the Aegean region in the Miocene. The islands of the Hellenic arc, isolated during the middle Miocene, were connected with the bigger surrounding landmasses during the Messinian. In this geologically short period (ca. 1 My), water frogs migrated onto the Hellenic arc. After the salinity crisis, five millions year ago (Appendix 1 p. 61), the water frog populations on Crete became permanently isolated and evolved independently from the others. The isolation of water frog populations on Karpathos is not so well estimated, but they were separated from Rhodos at least at the end of the Pliocene. The population on Rhodos has several alleles in common with the mainland and share private alleles with the Karpathos population. This suggests a rather late separation of the two islands Karpathos and Rhodos. Here we encounter an inconsistency, because the phylogenetic history inferred from my data set (Fig. 5, Fig. 6) is not concordant with the geological history (Fig. 14). For the

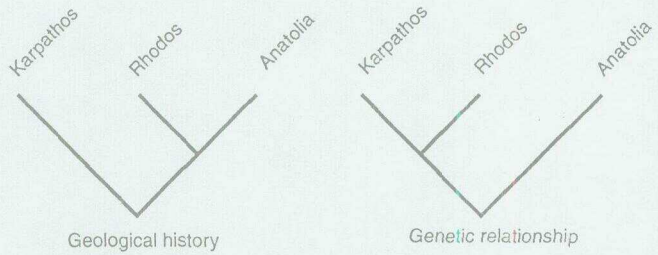

Fig. 14. Comparison between geological evolution and genetic evolution for the islands Karpathos, Rhodos and the adjacent mainland.

Rhodos population, the alleles shared only with the Karpathos population point to a common history. The discrepancy may reflect isolation of the southwestern region of ancient Anatolia, caused by a dispersal barrier for water frogs (e. g. a desert). After the separation of Karpathos from the mainland, the conditions changed and frogs from central or northern Anatolia invaded, resulting in a mixing of alleles from the KR taxon and *Rana bedriagae*: Rhodos has a high fraction of polymorphic loci (22.6%) compared to all other islands. Alternatively, alleles unique to Karpathos and Rhodos could have become extinct (or rare and remained undetected) on the Anatolian mainland. The resolution of this discrepancy requires additional molecular data and a reliable confirmation of the geologically dated record for this region.

In the Pliocene, eastern Europe and Anatolia were certainly inhabited by a very widespread taxon, an ancestor of *Rana ridibunda* and *Rana bedriagae*, and, in more restricted westernmore areas, ancestors of *Rana shqiperica* and of *Rana epeirotica*. During the last glaciation period, the Würm (28'000 - 10'000 y BP), the northern parts of the Aegean region contained forest tundra similar to that of today's Siberia (Frenzel et al. 1992). Fossils of reindeer found in the plain of the Danube (Herre 1986) suggest a climate unsuited for water frogs also at the northern border of the Aegean Sea. Today, the northernmost populations of water frogs (*Rana lessonae* of the Swedish coast; cf. Sjögren 1991) are outside of the distribution range of the reindeer. This suggests at least two refugia during the Würm: Peloponnisos and the south coast region of Anatolia. The large numbers of polymorphic loci in *Rana ridibunda* and *Rana bedriagae* suggest a subsequent mixing of some of these refugial gene pools. The Anatolian *Rana bedriagae* had a largely independent history at least since the end of the Würm, because of the barriers of Dardanelles and Bosporus, and the mountains of the Caucasus in the east. During the Pleistocene, the drainage of the Black Sea changed several times. This could explain that alleles typical for *Rana bedriagae* are found in *Rana ridibunda* in Thrakia. *Rana epeirotica* and

Rana shqiperica apparently survived the harsh glaciation periods in the lowlands of the Adriatic coast.

Calibration of the protein clock

The detection of interlocus differences in divergence rates that has led to the distinction of fast- and slow- evolvers is not a falsification of the molecular clock for protein-coding genes, because a reasonably constant evolutionary rate is assumed for comparable genes or comparable averages rather than across whole genomes. The differences do, however, call for consideration of differential rates occurring in a data set. The present study contains 13% of fast-evolving loci, based on the number of private alleles (Table 9). That these loci contain ALB and an esterase locus (EST-5) is consistent with Sarich's (1977) finding that blood proteins and secreted enzymes such as esterases evolve at a faster pace than others; a second esterase locus (EST-6), however, appears to have evolved at a medium rate. The fraction of fast-evolvers in my data set is comparable to or slightly lower than that in most similar studies, so that a comparison with rates reported by others appears unhampered.

As shown by the results presented, the regression line of genetic distance versus geological isolation is defined by a relatively small time interval, zero to five millions years. The regression line, of course, is only valid over this time range. The regression model used was not forced through the zero point, as most other studies suggest in their graphs (summarized by Hillis and Moritz 1990, Scherer 1990). This constraint is not necessary, especially given the found correlation of genetic distance with geographic distance (Fig. 4; Singh and Romberg 1987b). Neighboring conspecific populations are thus expected to be separated by small or zero genetic distance values.

The errors in evolutionary rates determined from genetic dissimilarities are in part a result of the general lack of independent information about times of speciation (Avise and Aquadro 1982). Hillis and Moritz (1990) could not locate any applicable data for the esti-

mation of the confidence limits for an allozyme clock. They go so far as to say that estimates of divergence times based on D_{Nei} are no better than arbitrary guesses. Using the nested set of geologically datable divergence times available for the Aegean region, however, my data apparently yield a reliable estimate of an allozyme clock and its confidence limits for the mentioned time interval and species group. A test of the obtained clock is possible within the same animal group, by comparing taxa that were not used for its calibration. The species pair *Rana perezi* and *Rana saharica* is separated by the Strait of Gibraltar, with the geologically known isolation time of 5 million years. Their observed genetic distance of D^*_{Nei} = 0.55 and D_{Nei} = 0.56 (this study) or D_{Nei} = 0.59 (Busack 1986) is close to the expected value calculated with the obtained regression: D^*_{Nei}= 0.597. This independent test of the calibration enhances our confidence in estimating the divergence time of the sympatric species pair *Rana epeirotica* and *Rana ridibunda* and other water frog species pairs where no geological dating of their separation is possible (Table 11). All speciation events estimated with the obtained protein clock fall in the range between the middle Pliocene and the Messinian, except for the more closely related pair *Rana ridibunda* and *Rana bedriagae*; so with the possible exception of this pair these events apparently were not induced by separations related to glaciations during the Pleistocene. Was this series of speciation events in this frog group triggered by a dramatic ecological change caused by the "salinity crisis" in the Messinian? If so, it is predicted that a similar clustering of speciation events in the time of about 5 My ago will 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 rates found in the present study (8.146 My / D^*_{Nei}) in the mid-range of their values (cf. Hillis and Moritz 1990), between the rates reported for reptiles (5 - 18 My / D_{Nei}), plethodontid salamanders (14 My / D_{Nei}), and birds (5 My / D_{Nei}). These differences confirm, as stated by Hillis and Moritz

Table 11. Estimation of the divergence times of species pairs with geologically unknown isolation times, assuming the calculated protein clock of this study: isolation time [My] = 8.146 · D^*_{Nei}. The mean values are given with the 95% confidence limits, minimal and maximal isolation are approximated using the confidence limits C1 and C2 in Fig. 12.

Species	D^*_{Nei}	Mean isolation [My]	Minimal isolation [My]	Maximal isolation [My]
Rana ridibunda - Rana bedriagae	0.144	1.2±0.1	0.1	2.3
Rana ridibunda - Rana epeirotica	0.359	2.9±0.2	1.1	4.7
Rana ridibunda - Rana shqiperica	0.334	2.7±0.2	1.0	6.5
Rana ridibunda - Rana lessonae	0.454	3.7±0.2	1.6	5.7
Rana ridibunda - Rana perezi	0.640	5.2±0.4	2.7	7.8
Rana shqiperica - Rana epeirotica	0.456	3.7±0.2	1.6	5.7
Rana shqiperica - Rana lessonae	0.496	4.0±0.3	1.8	6.3
Rana epeirotica - Rana lessonae	0.572	4.7±0.3	2.3	7.0

(1990), that the molecular clock must be calibrated for the species group of interest and the transfer of the calibration to other species groups requires caution.

Most of the calibration studies have used a very large interval of divergence time, up to 1000 My, for determining the pace of the molecular clock; for relatively fast-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 1993). Moreover, the clock may be disturbed over time intervals longer than 100 My by occasional major changes in functional constraints (Wilson et al. 1987) that may reflect unpredictable environmental catastrophes (cf. Gould and Eldredge 1977). My calibration largely circumvents these problems because the estimation is limited to a time interval spanning only the last 5 My. The reliability of the estimate is reflected in its linearity and the relatively small confidence limits (Fig. 12, Fig. 13).

At a later stage the estimated divergence rates can be compared with those of other groups of organisms, for which salt water is a dispersal barrier as well, in the same area

with its well known geological data; this will provide a direct test of the molecular clock hypothesis.

Chapter 8

Summary

Reliable estimates of phylogenetic relationships and divergence times are a crucial requirement for confident inferences in many evolutionary genetic and population biology studies, yet are typically hampered by scarcity and uncertainty of the fossil record. Frogs, like other amphibians, are freshwater animals that are unable to cross salt water barriers because their skin is readily permeable to both salt and water. The independently (geologically) estimated age of salt water barriers that separate related frog populations thus provides a measure of the minimum date of genetic divergence between pairs of such populations.

The Aegean region provides an ideal model area for measuring the relationship between amount of genetic divergence and time of spatial isolation, because the geology is well known, a nested set of isolation times between 10,000 y and 5 My is available, and the region is inhabited by the genetically well-investigated group of western Palearctic water frogs (*Rana esculenta* complex). Dispersal between populations on each of the two mainlands (Europe and Anatolia) can easily occur. All islands situated on the continental shelf (in this study: Samos, Evvoia) were connected by land to the adjacent mainland

several times during the Pleistocene cold phases. The greatest fall in the sea level (200 m below current level) during the maximal glaciation also raised land bridges between the islands Ikaria and probably between Kithira and Andros and their adjacent mainlands. The islands of Crete, Karpathos, and Rhodos have been isolated since approximately the end of the Miocene (5 My), the middle Pliocene (roughly 3 My), and the beginning of the Pleistocene (1.8 My), respectively.

Protein electrophoresis of 31 genic loci revealed the following phylogenetic structure calculated with a maximum likelihood procedure: (((((((*Rana ridibunda* [Europe], *Rana bedriagae* [Anatolia]), unnamed Karpathos taxon [including Rhodos]), *Rana shqiperica*), *Rana epeirotica*), *Rana lessonae*), unnamed Cretan taxon), (*Rana perezi*, *Rana saharica*)). This structure is also supported by a least square algorithm (except for the grouping of *Rana perezi* and *Rana saharica*) and by a cladistically derived tree. In all cases samples from the shelf islands are sister taxa to the adjacent mainland populations.

The number of loci used in this study (31) is phylogenetically informative, as shown by a randomization study: the sum of squares of the least square algorithm converges to a value near to the minimum possible between 20 and 25 loci and to that obtained with the complete locus set.

Within-species comparisons between the genetic distance and the geographical distance revealed a positive correlation and support an "isolation by distance" model.

Using pairs of neighboring populations, a linear relationship between geographical isolation time and genetic distance was found: $D^*_{Nei} = (0.027 \pm 0.027) + (0.114 \pm 0.008) \cdot$ isolation time [My], isolation time [My] $= (-0.04 \pm 0.22) + (8.146 \pm 0.548) \cdot D^*_{Nei}$, corresponding to an average divergence rate ("molecular clock") of 0.14 D^*_{Nei} / 1 My and 8.1 My / 1 D^*_{Nei}. This rate is slightly higher than that of previous estimates reported for protein electrophoretic data, but the value is conservative since relatively few of the loci used are "fast evolvers" (13%; sAAT, ALB, EST-5, MPI). Removing the "fast evolvers" from the

analysis results in 9.4 My / 1 D^*_{Nei}. A test in the outgroup *Rana perezi* and *Rana saharica* (isolated since 5 My by the Strait of Gibraltar) fits relatively well into the calibration: observed genetic Nei distance D^*_{Nei} = 0.55, D_{Nei} = 0.56, expected D^*_{Nei} = 0.60. Connecting the isolation time scale with the branching pattern of the phylogenetic tree suggests a rapid sequence of speciation events after the Messinian (5 My), possibly triggered by the rapid ecological changes accompanying the salinity crisis (deep desiccated basin model) of the Mediterranean Sea.

The data obtained on phylogenetic relationships and genic divergences will be important for our studies aiming at an evolutionary understanding of the initiation of clonal reproduction occuring in hybrids of this group of frogs. The estimated divergence rates can subsequently be compared with those of other groups of organisms in the same area, for which salt water is a dispersal barrier as well, and will thus provide the basis for a direct test of the molecular clock hypothesis.

Chapter 9

Zusammenfassung

Verlässliche Schätzungen der phylogenetischen Verwandtschaft und der Divergenzzeiten sind für die schlüssige Beantwortung vieler evolutionsgenetischer und populationsbiologischer Fragen unerlässlich. Diese Arbeiten werden aber üblicherweise durch das Fehlen einer vollständigen Fossilreihe erschwert. Frösche, wie alle Amphibien, können Salzwasserbarrieren nicht überwinden, da ihre Haut für Wasser und Salz gleichermassen permeabel ist. Die geologische Schätzung der Alter der Salzwasserbarrieren, die Froschpopulationen voneinander trennen, gibt daher ein Mass für die minimale Zeit seit der Trennung von Populationspaaren.

Die Ägäis bietet ein ideales Modell für die Ermittlung der Abhängigkeit zwischen genetischer Divergenz und geografischer Isolation, da die Geologie gut bekannt ist, verschiedene Isolationszeiten zwischen 10'000 y und 5 My vorhanden sind, und die Region durch die genetisch gut untersuchte Wasserfroschgruppe (*Rana esculenta*-Komplex) bewohnt wird. Migration zwischen Populationen auf dem Festland, in Europa und in Anatolien, ist ohne Probleme möglich. Alle Inseln auf dem Kontinentalschelf (in dieser Studie: Samos, Euböa) waren während der Kaltphasen des Pleistozäns

mehrmals mit dem benachbarten Festland verbunden. Der grösste Rückgang des Meeresspiegels während der maximalen Vereisung hat Landbrücken zwischen Ikaria und möglicherweise Kithira und Andros und dem benachbarten Festland ergeben. Die Inseln Kreta, Karpathos und Rhodos sind ungefähr seit dem Ende des Miozäns (5 My), dem Mittleren Pliozän (~3 My) und dem Beginn des Pleistozäns (~1.8 My) isoliert.

Proteinelektrophorese von 31 Loci zeigte die folgende phylogenetische Struktur, ermittelt durch einen maximum likelihood-Algorithmus: (((((*Rana ridibunda* [Europa], *Rana bedriagae* [Anatolien]), unbenanntes Karpathos-Taxon [Rhodos eingeschlossen]), *Rana shqiperica*), *Rana epeirotica*), *Rana lessonae*), unbenanntes Kreta-Taxon), (*Rana perezi*, *Rana saharica*)). Diese Struktur ist auch durch eine least square-Analyse und eine kladistische Analyse unterstützt. In allen Analysen waren die Inselpopulationen Schwestertaxa der benachbarten Festlandpopulationen.

Die Anzahl der benutzten Loci (31) ist phylogenetisch informativ: eine Studie mit zufälliger Auswahl der Loci zeigte, dass sich zwischen 20 und 25 Loci die Summe der Quadrate der least square-Analyse auf einen Wert konvergiert, der nahe dem möglichen Minimum und dem mit der vollständigen Locusmenge erhaltenen Wert liegt.

Vergleiche zwischen der genetischen Distanz (modifierte Nei-Distanz D^*_{Nei}; Nei 1972, Hillis 1984) und der geografischen Distanz zwischen Populationspaaren ergab eine positive Korrelation; dies unterstützt ein "isolation by distance" Modell.

Mit benachbarten Populationspaaren wurde eine lineare Relation zwischen der geographischen Isolationszeit und der genetischen Distanz gefunden: D^*_{Nei} = (0.027±0.027) + (0.114±0.008) • Isolationszeit in My [Wert±95%-Vertrauensintervall], Isolationszeit in My = (-0.04±0.22) + (8.146±0.548) • D^*_{Nei}. Diese Regressionen korrespondieren mit einer mittleren Divergenzrate ("molecular clock") von 0.14 D^*_{Nei} / 1 My und 8.1 My / D^*_{Nei}. Die Rate ist etwas höher als frühere durch Enzymelektrophorese geschätzte Werte. Der Wert ist aber konservativ, da relativ wenige "fast evolver" Loci

(13%; sAAT, ALB; EST-5, MPI) benutzt wurden. Werden diese aus der Analyse entfernt, sinkt die Rate auf 9.4 My / D^*_{Nei}. Ein Test mit der outgroup *Rana perezi* und *Rana saharica* (seit 5 My durch die Strasse von Gibraltar voneinander isoliert) lässt sich gut in die Kalibrierung einpassen: beobachtete genetische Distanz D^*_{Nei}=0.55, D_{Nei}=0.56, erwartete D^*_{Nei}= 0.59. Ein Vergleich der Isolationszeiten mit dem Verzweigungsmuster des phylogenetischen Baumes schlägt eine Anhäufung von Artbildungsereignissen nach dem Messinian (5 My) vor; dies ist möglicherweise eine Folge rapider Änderung der ökologischen Bedingungen, die durch die "Salinity crisis", die Austrocknung des Mediterranen Beckens, verursacht wurde.

Die erhaltenen Daten der phylogenetischen Verwandtschaft und die genetischen Divergenzraten werden für das evolutionsbiologische Verständnis des Ursprungs der klonalen Fortpflanzung in Hybriden dieser Wasserfroschgruppe wichtig sein. Die geschätzten Divergenzraten können später mit denen von anderen Tiergruppen in der gleichen Region, für die Salzwasser auch eine Barriere darstellt, verglichen werden. Dies wird Daten für einen direkten Test der Hypothese einer molekularen Uhr liefern.

Appendix 1

Geological events

Geological events in the Mediterranean region were used to determine the time of isolation between mainlands and islands. This appendix together with Table 12, summarizes the geological history of the region. For the Oligocene to the Pliocene, I used Steininger and Rögl's (1984) relative and absolute time scale; for the Pleistocene, I used van Eysinga's (1975) scales.

The collision of the African and Arabian plate with the Eurasian plate caused drastic changes in the landscape in southern Europe: in the $25 \cdot 10^6$yr (25 My) since the Oligocene, the Mediterranean Sea has changed its shape frequently. Connections to the Indo-Pacific Ocean were more than once closed and reopened. The connection to the Paratethys (pre-Black Sea) in middle Europe closed in the middle Miocene (15 My), the eastern connection moved from the pathway in the region of the Caucasus mountains to that in the region of the Bosporus (Steininger and Rögl, 1984: Figs. 2-7). In the late Miocene (about 11 My), the Balkan Peninsula and Anatolia had shapes similar to the present ones and were separated by a water connection between the Mediterranean and

Black Seas. The islands of the Hellenic arc (Crete and Karpathos) already existed and were isolated from the bigger land masses (Biju-Duval et al. 1977: plate 8).

The Messinian (latest Miocene) brought a dramatic change to the whole Mediterranean region. Evaporites, such as gypsum, halite, anhydrite, were deposited during this time, and mostly occur in the deepest parts of the Mediterranean basin. Evaporites sediment only in shallow and warm water (*sabkha conditions*). In some places, these evaporite layers are very thick (300-500 m). Because of this evidence, Hsü and his co-workers have proposed the "deep dry basin model" (Hsü 1972, Hsü et al. 1977): The Strait of Gibraltar closed about 6 My ago and the Mediterranean Sea in its deepest parts became a big salt pan. The Mediterranean islands were mountains in a steppe or desert, so that overland migration between islands and from the mainlands became possible. The Black Sea drained into this vast basin and left some brackish lakes in the Aegean region (the Lago Mare stage, Hsü and Giovanoli 1979). About 5.0 My ago, the Strait of Gibraltar reopened and the basin was filled from the Atlantic Ocean in about 1000 yr. Although this model is widely accepted in the scientific community, it has also been disputed (the shallow basin model of Fabricius and Hieke 1977, Fabricius et al. 1985, Sonnenfeld 1985). The first of these alternative models suggests a submarine topology similar to today's. The other models require a rapid subsidence of the submarine plains, with rates of 1 m / 1000 yr over the last 5 My in the Ionian Sea (Fabricius and Hieke 1977).

Many of the details of the geological history are not necessary for my study. I primarily need to know whether or not the islands of the Hellenic arc were connected by land to each other and to the mainland during the Messinian, and at what period they subsequently became isolated again.

All of the Aegean islands became united with the mainland during the Messinian. When the Mediterranean basin refilled at the end of the Messinian, Crete (which is surrounded by deep waters) became permanently isolated both from Anatolia to the east and

Peloponnisos to the west, as well as from the rest of the Hellenic arc. Although Crete was partly inundated and subdivided into a group of islands during the early Pliocene, it was uplifted later in the Pliocene and has remained emergent but isolated ever since.

The post-Messinian history of the other islands in the Hellenic arc (Kithira, Karpathos and Rhodos) is less clear. Their renewed isolation from each other and from the mainland depends not only on the refilling of the Mediterranean basin when the Strait of Gibraltar reopened, but also on complex tectonic movements in the region.The Hellenic arc has been rising for about 13 My because of the pressure of the Anatolian plate towards the west and subduction of the African plate under the European (Angelier 1979, Le Pichon and Angelier 1979). Because of these processes, the size of the Aegean Sea has increased about 33% and the floor of the Aegean Sea has subsided, while the Hellenic arc was pushed toward south (Angelier 1979, Le Pichon and Angelier 1979). The Pliocene changes in sea level that inundated some parts of Crete also affected other islands (Meulenkamp 1985). Kithira, for example, was submerged in the early Pliocene, and did not re-emerge until the later Pliocene (about 0.5 My ago), thus reinforcing the isolation of Crete from Peloponnisos during most of the Pliocene.

To the east, Karpathos and Kasos were above sea level and united in a single landmass at the beginning of the Pliocene, as evidenced by a micromammal fauna recovered from the Ruscinian (early Pliocene). The fauna was diverse, containing 5 different species documented by only 20 fossil specimens (Daams and van de Weerd 1980), which suggests that Karpathos had a connection to the mainland at about this time.

No geological data are available to show an early Pliocene separation of Karpathos from Rhodos, but Kuss (1975) described two endemic Pleistocene deer species from Kasos and Karpathos and discussed their differences from the deer present on Rhodos and Crete at this time. These data suggest the following scenario: Karpathos was isolated during the Miocene and connected to the Anatolian mainland in the early Pliocene. Its

connection to Rhodos was broken at some time during the middle or late Pliocene; Karpathos has remained isolated ever 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 remained isolated since then.

In the Pleistocene, all of today's islands were in place. The Hellenic arc continued to rise, and the central Aegean Sea continued to subside. Crete, Karpathos and Rhodos remained totally isolated, but because of sea level fluctuations associated with the Pleistocene glaciations, the history of the remaining Aegean islands is complex. The sea level varied between a level similar to that of today and one 200 m lower (Fig. 15). This eustatic sea level change is related to the magnitude of the worldwide glaciations. The greatest Neogene glaciation event was the Mindel period and probably with a similar magnitude the Riss period, and the eustatic sea level dropped about 200m below the present sea level (R. Hantke, U.-Radtke, K. Hsü personal communication). Only the sea level change of the Würm glaciation is exactly recorded: 18000 yr ago, the sea level stood 121±5 m below present sea level (Fairbanks 1989).

The islands of Samos and Evvoia are isolated only by a shallow salt water barriers (less than 80 m deep) from their nearby mainlands. These seaways dried up during each of the several Pleistocene glacial periods, forming a landbridge between Samos and Anatolia and between Evvoia and the Balkan peninsula. The two major mainlands Europe and Asia minor 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, such that these two islands were in contact at least during the Riss period. The isolation of Andros and of Kithira is more problematic. Each is 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, Udías 1985) and vertical movement can occur in

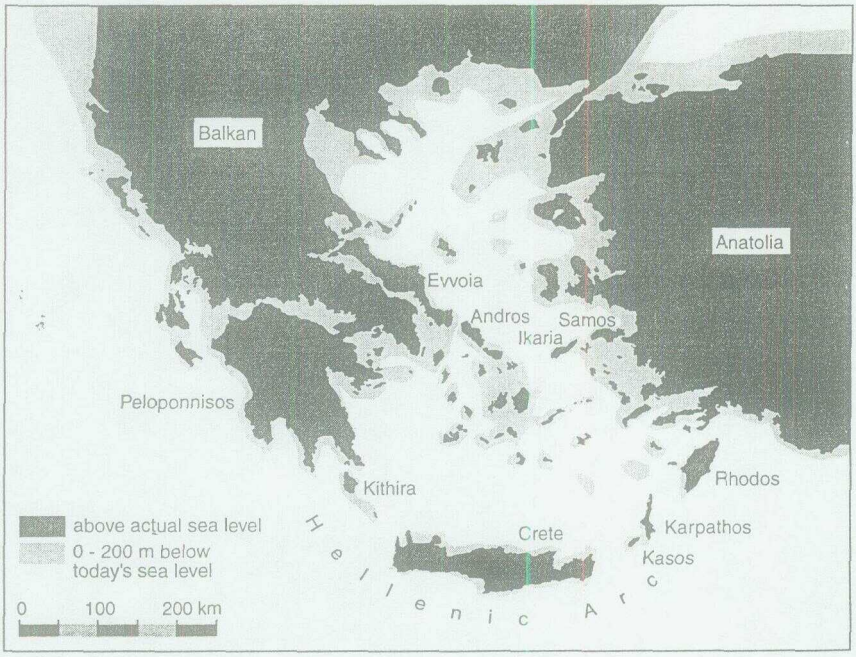

Fig. 15. Map of the Aegean region, showing approximate water depths. Areas surrounded by sea water less than 200 m deep may have been united during the peak of the Riss glaciation. Kithira and Andros are separated by narrow water gaps only slightly more than 200 m deep; it is possible that they also were united with adjacent land masses during the Riss.

rapid spurts (uplift or subsidence of 10 m per event are possible). For this reason, given the closeness of Kithira to the Peloponnisos or Andros to Evvoia and the relatively shallow seas between these two islands and adjacent landmasses, there is no compelling evidence for total isolation of Andros or Kithira during the Pleistocene.

Table 12. Geological time table for the Mediterranean sea with comments to the situation in the Aegean region. The absolute and relative times are given in Steininger and Rögl (1984); for the description of the different events, Angelier (1979), Daams and van de Weerd (1980), Fairbanks (1989), Hsü (1972), Le Pichon and Angelier (1979), Meulenkamp et al. (1972), Meulenkamp (1985) have been used.

Time scale			Mediterranean events	Aegean events
absolute [My BP]	relative: main periods	relative: sub-periods		
25 - 6.5	late Oligocene - late Miocene		Collision of African with the European continental plate. Different openings and closings of the connections with the Paratethys and the Indo-Pacific. In the late Miocene the connections are finally *permanently* closed (Pacific) or fixed (Black Sea).	The Hellenic arc is formed, the pressure of the Anatolian plate drives the arc southwest.
6.5 - 5.0	late Miocene	Messinian	Closing of the Strait of Gibraltar. The Mediterranean Sea dried up or at least partly dried up. The deeper parts formed salt pans.	Connection of all former islands with the mainland. Free terrestrial migration from European to Anatolian and/or to the African Plate. The salt pans in the present day abyssal plains possibly limited the migration routes.
5.0 - 3.4	Pliocene	early	The Strait of Gibraltar was permanently reopened and the Mediterranean sea was filled again. Some parts subsided and some were elevated.	The Aegean region subsided and some islands (e.g. Kithira) or parts (Crete, Karpathos) were inundated. Connection of Karpathos and Rhodos to the Anatolian mainland.
3.4 - 1.8		late		Rupture of the connection between Karpathos (probably 3 MY), Rhodos (probably 1.8 MY) and the Anatolian mainland
1.8 -0.5	Pleistocene	Biber Donau Günz	Older glaciations. During the maximal stands, the worldwide sea level fell to an unknown level.	Connection of Evvoia and Samos with their adjacent mainlands very probable. Connection of Anatolia with Europe.
0.5 - 0.4		Mindel	Thought to be the maximal glaciation, with a fall in the sea level to -200 m below the actual value.	The Cyclades including Andros form a landmass in the Aegean Sea and are probably connected to the Greek mainland via Evvoia. Kithira is probably connected with the Peloponnisos. The islands Ikaria, Samos and Evvoia are connected with their adjacent mainlands. Connection of Anatolia with Europe.
0.3 - 0.1		Riss I+II	Great glaciation with a fall in the sea level to to more than -120 m below the actual value. Probably similar in magnitude to Mindel glaciation.	Connection of Evvoia and Samos with their adjacent mainlands. Connection of Anatolia with Europe. Connection of Ikaria, Andros and probably Kithira with the adjacent mainland.
0.028 - 0.010		Würm	Last glaciation, fall in sea level to -121 ± 5 m	Connection of Evvioa, Samos and Anatolia with Europe.
0.011 - 0.0	Holocene		-	-

Appendix 2

Taxonomy and nomenclature

The view of the phyletic radiation of the western Palearctic water frogs has changed substantially in the last two decades. The check list of Mertens and Wermuth (1960) recognized two species in this group. Genetic data obtained in evolutionary studies evoked by hemiclonal reproduction of natural hybrid lineages have resulted among others in the recognition of *Rana perezi* and *Rana saharica* as distinct species (Graf et al. 1977, Uzzell 1982), and in the discovery of *Rana shqiperica* and of *Rana epeirotica* (Hotz and Uzzell 1982, Tunner and Heppich 1982).

The present study has revealed the existence of at least two additional taxa in the Aegean region. *Rana ridibunda* was thought to occur as the only water frog taxon throughout the Aegean region, including many islands (Wettstein 1953, 1957). Here I use the species status for three additional population groups: Crete; Karpathos and Rhodos; and Anatolia.

That the water frogs on Crete are genetically differentiated at the species level is indicated by protein electrophoretic data (Table 2, Table 8, e. g. Fig. 8): their lowest genetic distances to any other water frog species are similar to or higher than genetic distances

between taxa pairs that are known by sympatry not to be conspecific (*Rana ridibunda* and *Rana lessonae*, *Rana shqiperica*, *Rana epeirotica*, respectively), and they have a high number of private alleles (Table 3).

The water frogs distributed from Anatolia to northern Egypt, previously included in *Rana ridibunda*, have recently been proposed to constitute a separate species *Rana levantina* on the basis of their advertisement call (Schneider et al. 1992). This name, however, clearly is a junior synonym of *Rana esculenta* var. *bedriagae* Camerano 1882 from southwestern Syria. The species status of this taxon is consistent with but not required by genetic data (present study; Beerli, Hotz, and Uzzell, unpublished data). I therefore operationally use the name *Rana bedriagae* for the Anatolian water frogs.

The populations of Karpathos and Rhodos have lower genetic distances to their genetically and geographically closest mainland relative than does the Cretan taxon (Table 2, Table 8). Nevertheless, in all my cladistic and phenetic analysis of the electrophoretic data they cluster together and are the most divergent member of the *Rana ridibunda* stock (*Rana ridibunda*, *Rana bedriagae*; Fig. 5, Fig. 8). Problematic is the discrepancy between geological history and phylogenetic relationship of the Karpathos and Rhodos populations (Fig. 14). I here use the name Karpathos/Rhodos taxon for these frogs; if *Rana bedriagae* is recognized as a species distinct from *Rana ridibunda*, then the Karpathos/Rhodos taxon, which is genetically more distinct, should also be recognized.

A further species has recently been proposed for the Balkan frogs previously known as *Rana ridibunda*, again based on advertisement calls (Schneider et al. 1993). I regard this taxon as not valid, because genetic data based on protein electrophoretic, microcomplement fixation, and mtDNA analyses (present study; Hotz, Uzzell, Spolsky, and Beerli, unpublished) reveal no distinction from central European and more eastern populations of *Rana ridibunda*. (Moreover, if the Balkan taxon were distinct, then the new name proposed would again be a junior synonym of an already available name, *Rana rubiconda*

kurt-mülleri Gayda 1940, from northern Albania, and the species should be referred to as *Rana kurtmuelleri*.)

The Cretan taxon and the Karpathos population will be described at the species level (Beerli et al., submitted).

Appendix 3

Descriptions of the sampling localities

The localities are listed separately for mainlands and islands. Locality description record: Name used in this text (*local name*), coordinates in degrees and minutes, a description of the wider situation of the locality, a description of the locality itself.

Mainland

Europe (Peloponnisos, Attika, Thrakia)

1. Nea Manolada (Νεα Μανολαδα)

   Coordinates: 38°01' N / 21°19' E

   In the coastal plains of the northwestern Peloponnisos, about 60 km southwest of the town Patras (Πατρα). Below the bridge at the end of the village Nea Manolada. Small water puddle with a few reeds. The frogs were hiding under the stone-filled wire baskets.

2. Skala (Σκαλα)

   Coordinates: 36° 53' N / 22°42' E

In the plains of the river Evrotas (Ευρωτας) east of Githio (Γυθειο), Peloponnisos.

Slowly flowing irrigation canal with aquatic plants; scattered bushes on the bankside.

The frogs were sitting on the water plants.

3. Argos (Αργο)

Coordinates: 37°37' N / 22°45' E

In the plains of Argos (Αργο), Peloponnisos.

Slowly flowing irrigation canal with aquatic plants, the banks are very steep.

4. Aliartos (Αλιαρτος)

Coordinates: 38°26' N / 23°04' E

In the plains of Boiotia (Βοιωτια) north of Athina (Athens).

Slowly flowing irrigation canal with aquatic plants.

5. Paradisos (Παραδισος)

Coordinates: 41°03' N / 23°45' E

In the plain of the river Nestos (Νεστος) in Thrakia (Θρακια), northeastern Greece.

Shallow river, below a dam near the main road Kavala (Καβαλα) - Komotini (Κομοτηνη), the banks are partly natural or built with stone-filled wire baskets.

6. Monastiraki (Μοναστιρακι)

Coordinates:40°50' N / 26°5' E

In the plain of the river Evros (Εβρος), the border river between Turkey and Greece.

Vegetationless irrigation canal near the road from Alexandropolis (Αλεξανδρουπολι) to the Turkish border.

Anatolia

7. Ezine

Coordinates: 39°48' N / 26°20' E

Northwest Anatolia at a small river.

Shallow vegetationless banks of the river.

8. Selçuk

Coordinates: 28°4' N / 27°27' E

Between Torbalı and Selçuk, south of Izmir in the west of Anatolia at a river.

Shallow puddles shadowed by bushes near the banks of the river.

9. Akçapınar

Coordinates: 37°3' N / 28°32' E

Smooth hilly landscape in the southwest of Anatolia.

Channel along the road Marmaris - Fethiye. The banks are occupied by reeds, floating plants grow in the water.

10. Marmaris

Coordinates: 36°50' N / 28°17' E

Steep coast

Small stream in the middle of Marmaris, the sewer of the village. The frogs live at the edge of the beach.

Islands

Most Aegean islands are very dry (mean total rainfall per year: 300 mm). On most islands, the original forests have been destroyed and replaced by a dry hard-leaf shrubland (Macchia or Garigue type). Open water places are relatively rare and restricted to shaded areas in small steep valleys or relict forests or only have water in the early spring. The water is intensively used for irrigation of fields, greenhouses and lawns. The wastewater then flows back into the natural channels of the rivers, leading to considerable degradation of the aquatic habitat.

Crete (Κρητη)

11.Kastelli (Καστελλι)

Coordinates: 35°30' N / 23°43' E

In western Crete in a small plain with some small streams. They all dry up before reaching the beach. Two shadowed rivulets strongly overgrown with bushes and trees. The frogs live side by side with Caspian water turtles (*Mauremys caspica*).

12.Petros River (Ποταμος Πετρος)

Coordinates: 35°23' N / 24°23' E

Small river valley.

Shallow places shortly before the mouth of the river. This site does not dry up, even in summer. At the border is a pump station for irrigation water. Floating plants grow on the water surface.

13.Lavris (Λανρις)

Coordinates: 35°26' N / 24°45' E

Valley of the river Geropotamos (Γεροποταμος).

At the mouth of the river. This place does not dry up and farmers take water for their fields here. The banks are grown with reed, water plants are floating on the water surface.

14.Iraklion (Ιρακλειο)

Coordinates: 35°22' N / 25°7' E

Smooth hilly landscape.

River in the town Iraklion strongly polluted with sewage (the water is black with foam). No vegetation alongside or in the water.

15.Karpathos (Καρπαθος)

Coordinates: 35°44' N / 27°12' E

Dry treeless valley near Olympos (Ολυμπος) in the northern part of the island Karpathos. The stream is mostly dry with a stony bed. In spring there are some puddles covered by cruciferous plants. The frogs sit in these plants. The sewage of the village Olympos flows into the riverbed.

16. Rhodos (Ροδος)

Coordinates: 36°15' N / 28°2' E

Hilly landscape in the center of the island Rhodos near Archipolis (Αρχιπολις) with several small streams and brooks.

a) Relatively fast-flowing shallow rivulet with steep banks (erosional edges) and gravel banks overgrown with bushes.

b) Same river as a) 3 km south of first sampling locality. Landscape with woods, the river divides here into several smaller branches and gives way to stony islands with bushes and trees.

17. Kithira (Κυθηρα)

Coordinates: 36°22' N / 22°57' E

Small valley in the north of the island Kithira near the village Agia Pelagia (Αγια Πελαγια).

Mostly dry river with puddles. The banks are occupied by reeds, bushes and trees.

18. Andros (Ανδρος)

Coordinates: 37°53' N / 24°51' E

Treeless valley, ca. 700 m above sea level

Vegetationless artificial water basin near the village Vourkoti (Βουρκωτη).

19. Evvoia (Εννοια)

Coordinates: 38°30' N / 23°52' E

Valley in the center of the island Evvoia, southwest of the village Mistros (Μιστρος).

The riverbed is partly dry, the banks are grown with old trees.

20. Samos (Σαμος)

Coordinates: 37°48' N / 26°43' E

Forested valley with a small rivulet in the center of the island Samos near the village Idhrousa (Υδρουσα).

Shadowed dry rivulet with some puddles.

21. Ikària (Ικαρια)

Coordinates: 37°37' N / 26°11' E

Valley in the north of the island Andros.

Small valley with a small fast-flowing stream. The whole valley is shaded by old trees.

The frogs live in small puddles.

Appendix 4

Electrophoretic phenotypes and allele distribution

All enzymes and their electrophoretically detectable alleles are reported in this appendix. Indistinguishable electromobility at any locus of any species was treated as reflecting translation products of the same allele and coded with the same letter. Alleles in addition to those reported by Hotz (1983) and Hotz and Uzzell (1983), were named with the next available letters in the alphabet.

For each locus, the inferred subunit structure and the tissues used for scoring are given. All alleles reported for the first time in this manuscript are underscored. The alleles are listed in decreasing anodal mobility. Geographic and taxonomic distribution of the alleles can be inferred from Table 2.

Aconitate hydratase (sACO and mACO) [Enzyme Commission 4.2.1.3]

sACO

Monomer; muscle, liver

a, *b*, *c*, *d*, *e*

mACO

Monomer; muscle, liver

a, b, c, d, e

Adenylate kinase (AK) [E.C. 2.7.4.3]
Monomer (H. Hotz pers. comm.); muscle

a

s-Adenosyl-l-homocysteine hydrolase (AHH) [E.C. 3.3.1.1]
Monomer; liver

a, b, c, d

Albumin (ALB)
Monomer; serum

j, c, a, e, k, b, l, m

Aspartate aminotransferase (sAAT and mAAT) [E.C. 2.6.1.1]
sAAT

Dimer; liver

l, c, e, f, m, g, n

mAAT

Dimer; liver

a, b

Carbonate dehydratase (CA-2) [E.C. 4.2.1.1]
Monomer; liver

a, b, c, d, e, f, g, h

Creatine kinase (CK-A) [E.C. 2.7.3.2]
Dimer; muscle, serum

c, a, d, b

Carboxylesterases (EST-5 and EST-6) [E.C. 3.1.1.-]

EST-5

Subunit structure unknown; liver

a, *b*, *c*, *d*, *e*, *f* (probably a null-allele)

EST-6

Subunit structure unknown; *liver, muscle*

a, *b*, *c*, *d*

Fructose-biphosphatase (*FDP-1 and FDP-2*) [E.C. 3.1.3.11]

FDP-1

Dimer; liver

a, *b*, *c*, *d*

FDP-2

Dimer; muscle

a, *b*, *c* (probably a null-allele)

Glucose dehydrogenase (GCDH) [E.C. 1.1.1.118]

Subunit structure unknown, not onomeric (H. Hotz *pers. comm.*); liver

a, b, c

Glucose-6-phosphate *isomerase* (GPI) [E.C. 5.3.1.9]

Dimer; liver, muscle

a, b, d, e, f

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [E.C. 1.2.1.12]

Tetramer; liver

a, b, c, d, e

Glycerol-3-phosphate dehydrogenase (αGDH) [E.C. 1.1.1.8]

Dimer; liver

a, b, c

Guanine deaminase (GDA) [E.C. 3.5.4.3]
Dimer; liver

a, b, c, d, e

Isocitrate dehydrogenase (mIDH and sIDH) [E.C. 1.1.1.42]
sIDH

Dimer; liver

d, e, b, f, g

mIDH

Dimer; liver

c, b, e

Lactate dehydrogenase (LDH-A and LDH-B) [E.C. 1.1.1.27]
LDH-A

Tetramer (Hotz 1983); liver

a

LDH-B

Tetramer; muscle, liver

a, b, c, d, e, h

Malate dehydrogenase (sMDH and mMDH) [E.C. 1.1.1.37]
sMDH

Dimer; muscle

d, e, a, b

mMDH

Dimer; muscle

a, b

Mannose-6-phosphate isomerase (MPI) [E.C. 5.3.1.8]
Monomer; muscle, liver

j, a, b, c, o, l, g [q], m, n, k, h, d, e, p

Muscle proteins (MPR-1, MPR-3)
MPR-1

Monomer; muscle

d, b, e, c

MPR-3

Monomer (unpublished data); muscle

b, a

Peptidase (PEP-A [3.4.-])
Dimer; liver

a, b, c, e

Phosphoglucomutase (PGM-2) [E.C. 5.4.2.2]
Monomer; muscle

a, b, c, d, h

Phosphogluconate dehydrogenase (6PGDH) [E.C. 1.1.1.44]
Dimer; liver

c, d, e, g, f

Superoxide dismutase (sSOD) [E.C 1.15.1.1]
Dimer (Hotz 1983); liver

a, c

Chapter 10

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Chapter 11

Literature cited

Angelier, J. 1979. Néotectonique de l'arc Egéen. Soc. Géol. Nord. 3: 1 - 417.

Avise, J. C. 1992. Molecular population structure and the biogeographic history of a regional fauna: a case history with lessons for conservation biology. Oikos 63: 62 - 76.

Avise, J. C. and C. F. Aquadro. 1982. A comparative summary of genetic distances in the vertebrates. Evol. Biol. 15: 151 - 158.

Barton N. H. and M. Slatkin. 1986. A quasi - equilibrium theory of the distribution of rare alleles in a subdivided population. Heredity 56: 409 - 415.

Beerli, P., H. Hotz, H. Tunner, S. Heppich, and T. Uzzell. 1993. Two new water frog species from the Aegean islands Crete and Karpathos (Amphibia, Salientia, Ranidae). Submitted to Notulae Naturae, Acad. Nat. Sci. Philadelphia.

Biju - Duval, B., J. Dercourt, and X. Le Pichon. 1977. From the Tethys ocean to the Mediteranean Seas: a plate tectonic model of the evolution of the western alpine system. In: Biju, B. and L. Montadert (eds.). International symposium of the Mediterranean basins, Split 25 - 29 October 1976. Editions - Technip, Paris. pp. 143 - 164, 8 plates.

Bowen, B.W., A. B. Meylan, and J. C. Avise. 1989. An odyssey of the green sea turtle: Ascension Island revisited. Proc. Natl. Acad. Sci. USA 86: 573 - 576.

Busack S. D. 1986. Biogeographic analysis of the herpetofauna separated by the formation of the Strait of Gibraltar. National Geographic Research 2(1): 17 - 36

Cavalli - Sforza L. and A. W. F. Edwards 1967. Phylogenetic analysis: Models and estimation procedures. Evolution 21: 550 - 570

Cavalli - Sforza L., P. Menozzi, and A. Piazza. 1993. Demic expansion and human evolution. Science 259: 639 - 646.

Daams, R. and A. V. Van der Weerd. 1980. Early Pliocene small mammals from the Aegean island of Karpathos (Greece) and their paleogeographic significance. Geologie en Mijnbouw 59 (4): 327 - 331.

Dermitzakis, M. D. 1990. The evolution of the Aegeis during the Late Cenozoic. Geologica Balcanica, 20.3: 3 - 16

Fabricius, F. H. , K. Braune, G. Funk, W. Hieke, and J. Schmolin. 1985. Plio - Quaternary sedimentation and tectonics in the Ionian area: clues to the recent evolution of the Mediterranean. In: Stanley, D. J. and F. C. Wezel (eds.). Geological Evolution of the Mediterranean Basin. Springer, New York, 293 - 305.

Fabricius, F. H., and W. Hieke 1977. Neogene to Quaternary development of the Ionian basin (Mediterranean): considerations based on a "dynamic shallow basin model" of the Messinian salinity event. In: Biju, B. and L. Montadert (eds.). International symposium of the Mediterranean basins, Split 25 - 29 October 1976. Editions - Technip, Paris. 391 - 400.

Fairbanks, R. G. 1989. A 17'000 - year glacio - eustatic sea level record: influence of glacial melting rates on the Younger Dryas event and deep - ocean circulation. Nature 342: 637 - 642.

Felsenstein, J. 1993. PHYLIP (Phylogeny Inference Package) version 3.5c. Computer program and manual distributed by the author.

Frenzel, B., Pécsi, M., Velichko, A.A. (eds.) 1992. Atlas of paleoclimates and paleoenvironments of the northern hemisphere: Late Pleistocene - Holocene. Geographical Research Institute, Hungarian Academy of Sciences, Budapest and Gustav Fischer Verlag, Stuttgart, 153 pp.

Gillespie, J. H. 1991. The causes of molecular evolution. Oxford University Press, New York, Oxford. xiv + 336 pp.

Gould, S. J., and N. Eldredge. 1977. Punctuated equilibria: the tempo and mode of evolution reconsidered. Paleobiology 3: 115 - 151.

Graf, J. - D., F. Karch, and M. - C. Moreillon. 1977. Biochemical variation in the *Rana esculenta* complex: A new hybrid form related to *Rana perezi* and *Rana ridibunda*. Experientia 33(12): 1582 - 1584.

Graf, J. - D. 1989. Sex linkage of malic enzyme in *Xenopus laevis* established. Experientia 45: 194 - 196.

Graf, J - D., and M. Polls Pelaz. 1989. Evolutionary genetics of the *Rana esculenta* complex In: Dawley R. M. and P. Bogart (eds.). Evolution and Ecology of Unisexual Vertebrates, New York State Museum Bulletin 466: 289 - 301.

Grant, P. P. R. 1986. The evolution and ecology of Darwin's finches. Princeton University press, Princeton, XIV + 458 pp.

Guo, S. W. and E. A. Thompson. 1992. Performing the exact test of Hardy - Weinberg proportion for multiple alleles. Biometrics 48: 361 - 372.

Harlow, E. and D. Lane. 1988. Antibodies. A laboratory manual. New York, Cold Spring Harbor Laboratory, XIII + 726 pp.

Harris, H., and D. A. Hopkinson. 1976 et seq. Handbook of Enzyme Electrophoresis in Human Genetics. North - Holland, Amsterdam.

Harvey, P. H. and M. D. Pagel. 1991. The comparative method in evolutionary biology. Oxford series in ecology and evolution 1. Oxford University Press, Oxford: VIII + 239 pp.

Herre, W. 1986. *Rangifer tarandus* (Linnaeus, 1758) - Ren, Rentier. In: Niethammer, J. and F. Krapp (eds.). Handbuch der Säugetiere Europas. Band 2/II. Aula - Verlag, Wiesbaden: 198 - 216.

Hillis D. M. 1984. Misuse and modification of Nei's genetic distance. Systematic Zoology 33(2): 238 - 240.

Hillis, D. M. and C. Moritz. 1990. An overview of applications of molecular systematics. In: Hillis, D.M., Moritz, C. (eds.): Molecular systematics. Sinauer Associates, Inc. Sunderland, 502 - 515.

Hotz, H. 1983. Genic diversity among water frog genomes inherited with and without recombination. Dissertation Universität Zürich 1983, 136 pp.

Hotz, H. and T. Uzzell. 1982. Biochemically detected sympatry of two water frog species: two different cases in the Adriatic Balkans (Amphibia, Ranidae). Proc. Acad. Nat. Sci. Philadelphia 134: 50 - 79.

Hotz, H. and T. Uzzell. 1983. Interspecific hybrids of *Rana ridibunda* without germ line exclusion of a parental genome. Experientia 39: 538 - 540.

Hotz, H., T. Uzzell, and L. Berger. in prep. Linkage groups in western Palearctic water frogs support evolutionary conservation of protein - coding genes.

Hsü, K.J. 1972. Origin of saline giants: a critical review after the discovery of the Mediterranean evaporites. Earth Sci. Rev. 8: 371 - 396.

Hsü, K.J. and F. Giovanoli. 1979. Messinian event in the Black Sea. Palaeogeography, Palaeoclimatology, Palaeoecology 29: 75 - 93.

Hsü, K.J., L. Montadert, D. Bernoulli, M. B. Cita, A. Erickson, R. E. Garrison, R. B. Kidd, F. Mèlierés, C. Müller, and R. Wright. 1977. History of Mediterranean salinity crisis. Nature 267: 399 - 403.

Kimura, M. 1968. Evolutionary rate at the molecular level. Nature 217: 624 - 626.

Kimura, M. 1969. The rate of molecular evolution considered from the standpoint of population genetics. Proc. Natl. Acad. Sci. USA 63: 1181 - 1188.

Kimura, M. 1983. The neutral theory of molecular evolution. Cambridge University Press, Cambridge, xv + 367 pp.

Kuss, S. E. 1975. Die pleistozänen Hirsche der ostmediterranen Inseln Kreta, Kasos, Karpathos und Rhodos (Griechenland). Ber. Naturf. Ges. Freiburg i. Br. 65: 25 - 79.

Legendre, P. 1986. Reconstructing biogeographic history using phylogenetic - tree analysis of community structure. Syst. Zool. 35: 68 - 80.

Le Pichon, X. and J. Angelier. 1979. The Hellenic arc and trench system: a key to the neotectonic evolution of the eastern Mediterranean area. Tectonophysics 60: 1 - 42.

Lewontin, R.C. 1974. The genetic basis of evolutionary change. Columbia University Press, New York and London: 346 pp.

Mac Arthur, R. H. and E. O. Wilson. 1967. Theory of Island Biogeography. Monographs in population biology, Princeton University Press, Princeton: xi + 203 pp.

Maddison W. P. and D.R. Maddison. 1992. MacClade: analysis of phylogeny and character evolution (Version 3). Sinauer Associates, Sunderland, Mass. xi + 398 pp. + computer program.

Maxson, R. D. and L. R. Maxson. 1986. Micro - complement fixation: a quantitative estimator of protein evolution. Mol. Biol. Evol. 3: 375 - 388.

Mertens, R., and H. Wermuth. 1960. Die Amphibien und Reptilien Europas. Waldemar Kramer, Frankfurt a. M., XI + 264 pp.

Meulenkamp, J. E. 1985. Aspects of the late Cenozoic evolution of the Aegean region. In: Stanley, D. J. and F. C. Wezel (eds.). Geological Evolution of the Mediterranean Basin. Springer, New York, 307 - 321.

Meulenkamp, J. E., Ed F.J. De Mulder, and A. van de Weerd. 1972. Sedimentary history and paleogeography of the late Cenozoic of the island Rhodos. Z. Deutsch. Geol. Ges. 123: 541 - 553.

Murphy, R.W., J. W. Sites Jr., D. G. Buth, and C. H. Haufler. 1990. Proteins I: Isozyme electrophoresis. In: Hillis, D.M. and C. Moritz (eds.). Molecular systematics. Sinauer Associates, Inc. Sunderland, 45 - 126.

Nei, M. 1972. Genetic distance between populations. American Naturalist 106(949): 283 - 292.

Nei, M. 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. Genetics 89: 583 - 590.

Oosterbroek, P. and J. W. Arntzen. 1992. Area - cladograms of circum - Mediterranean taxa in relation to Mediterranean palaeogeography. J. Biog. 19: 3 - 20.

Pesole G., M. P. Bozzetti, C. Lanave, G. Preparata, and C. Saccone. 1991. Glutamine synthetase gene evolution: A good molecular clock. Proc. Natl. Acad. Sci. USA 88: 522 - 526.

Richardson, B.J., P. R. Baverstock, and M. Adams. 1986. Allozyme Electrophoresis. Academic Press. Sydney. XII + 279 pp.

Sarich, V. M. 1977. Rates, sample sizes, and the neutrality hypothesis for electrophoresis in evolutionary studies. Nature 265. 24 - 28.

SAS Institute. 1988. SAS/STAT User's Guide, Release 6.03 Ed. SAS Institute Inc., Cary, NC, USA

Scherer, S. 1990. The protein molecular clock. In: Hecht, M.K., B. Wallace and R.J. Macintyre (eds.). Evolutionary Biology 24: 83 - 106.

Schneider, H., U. Sinsch, and E. Nevo. 1993. The lake frog in Israel represent a new species. Zoologischer Anzeiger 1/2: 97 - 106.

Schneider, H., U. Sinsch, T. S. Sofianidou. 1993. The water frogs of Greece, bioacustic evidence for a new species. Zeitschrift für zoologische Systematik und Evolutionsforschung 31: 47 - 63

Singh R. S. and L. R. Rhomberg. 1987a. A comprehensive study of genic variation in natural populations of *Drosophila melanogaster*. I. Estimates of gene flow from rare alleles. Genetics 115:313 - 322.

Singh R. S. and L. R. Rhomberg. 1987b. A comprehensive study of genic variance in natural populations of *Drosophila melanogaster*. II. Estimates of heterozygosity and patterns of geographic differentiation. Genetics 117: 255 - 271.

Sjögren, P. 1991. Extinction and isolation gradients in metapopulations: the case of the pool frog (*Rana lessonae*). Biol. J. Linn. Soc. 42(1/2): 135 - 147.

Slatkin, M. 1985. Rare alleles as indicators of gene flow. Evolution 39(1): 51 - 65.

Sokal R. R. and F. J. Rohlf. 1981. Biometry. Freeman and Company, New York. XVIII + 859 pp.

Sonnenfeld, P. 1985. Models of upper Miocene evaporite genesis in the Mediterranean region. In: Stanley, D. J. and F. C. Wezel (eds.). Geological Evolution of the Mediterranean Basin. Springer, New York, 323 - 346.

Steininger, F. F. and F. Rögl. 1984. Palaeography and palinspastic reconstruction of the Neogene of the Mediterranean and Parathetys. In: Dixon, J. E. and A. H. F. Robertson. The Geological Evolution of the Eastern Mediterranean. Blackwell Scientific Publications, Oxford, 659 - 668.

Swofford D. L. 1993. PAUP: Phylogenetic Analysis Using Parsimony, Version 3.1.1. Computer program distributed by the Illinois Natural History Survey, Champaign, Illinois.

Swofford D. L. and R. B. Selander. 1989. Biosys - 1: a Computer Program for the Analysis of Allelic Variation in Population Genetics and Biochemical Systematics, Release 1.7. Computer program distributed by the Illinois Natural History Survey, Champaign, Illinois

Takahata, N. 1987. On the overdispersed molecular clock. Genetics 116: 169 - 179.

Tirard, C., P. Berrebi, A. Raibaut, and F. Frenaud. 1992. Parasites as biological markers: evolutionary relationships in the heterospecific combination of helminths (monogeans) and teleosts (Gadiadae). Biol. J. Linn. Soc. 47: 173 - 182.

Tunner, H.G. and S. Heppich. 1982. A genetic analysis of water frogs from Greece: evidence for the existence of a cryptic species. Zeitschrift für zoologische Systematik und Evolutionsforschung 20(3): 209 - 223.

Udías, A. 1985. Seismicity of the Mediterranean Basin. In: Stanley, D. J. and F. C. Wezel (eds.). Geological Evolution of the Mediterranean Basin. Springer, New York, 55 - 63.

Uzzell, T. 1982. Immunological relationship of western Palearctic water frogs (Salientia: Ranidae). Amphibia Reptilia 3(2/3): 135 - 143.

Uzzell, T. and K.W. Corbin. 1971. Fitting discrete probability distributions to evolutionary events. Science 172(3988): 1089 - 1096.

Uzzell, T. and L. Berger 1975. Electrophoretic phenotypes of *Rana ridibunda*, *Rana lessonae* and their hybridogenetic associate, *Rana esculenta*. Proc. Acad. Nat. Sci. Philadelphia 127(2):13 - 24.

van Eysinga, F. W. B. (compiler) 1975. Geological time table, 3rd ed., Elsevier, Amsterdam.

Wettstein, O. 1953. Herpetologia aegaea. Sitz. Ber. akad. wiss. Wien, math. nat. Kl. Abt. I 162(9/10):-651 - 833.

Wettstein, O. 1957. Nachtrag zu meiner Herpetologia aegaea. Sitz. Ber. akad. wiss. Wien, math. nat. Kl. Abt. I 166(374): 10 - 164, 8 plates.

Wilson, C. A., S. S. Carlson, and T. J. White. 1977. Biochemical evolution. Ann. Rev. Biochem. 46: 573 - 639.

Wilson, C. A., H. Ochman, and E. M. Prager.1987. Molecular time scale for evolution. Trends Genet. 3: 241 - 247.

Wolfram Research. 1993. Mathematica, computer program (NeXT release 2.2). Wolfram Research, Inc. Champaign, Illinois.

Wolfram S. 1991. Mathematica: a system for doing mathematics by computer (2nd ed.). Addison Wesley, Redwood City, California. xxii + 961 pp.

Wright, D. A., C. M. Richards, and G. W. Nace. 1980. Inheritance of enzymes and blood proteins in the leopard frog, *Rana pipiens*: three linkage groups established. Biochem. Genet. 18(5/6): 591 - 616.

Zuckerkandl, E. and L. Pauling. 1962. Molecular disease, evolution and genic heterogeneity. In: M. Kasha and B. Pullmann (eds.). Horizons in Biochemistry. Academic press, New York, 189 - 225.

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