Available online at www.sciencedirect.com
ScienceDirect
Molecular Phylogenetics and Evolution 44 (2007) 115–125
MOLECULAR PHYLOGENETICS AND EVOLUTION
www.elsevier.com/locate/ympev

Mitochondrial phylogeography of *Rana* (*Pelophylax*) populations in the Eastern Mediterranean region

P. Lymberakis [a,1], N. Poulakakis [a,b,*,1], G. Manthalou [a,b], C.S. Tsigenopoulos [c], A. Magoulas [c], M. Mylonas [a,b]

[a] *Natural History Museum of Crete, University of Crete, Knosos Av., P.O. Box 2208, 71409 Irakleio, Crete, Greece*
[b] *Department of Biology, University of Crete, Vassilika Vouton, P.O. Box 2208, 71409 Irakleio, Crete, Greece*
[c] *Institute of Marine Biology and Genetics, Hellenic Centre for Marine Research, Thalassocosmos, Gournes Pediados, P.O. Box 2214, 71003 Irakleio, Crete, Greece*

Received 4 July 2006; revised 12 January 2007; accepted 8 March 2007
Available online 27 March 2007

Abstract

Phylogenetic relationships in the western fraction of *Rana* (*Pelophylax*) have not been resolved till now, even though several works have been devoted to the subject. Here, we infer phylogenetic relationships among the species distributed in the area of the Eastern Mediterranean, comparing partial mitochondrial DNA sequences for the cytochrome *b* and 16S rRNA genes. The obtained molecular data clearly indicate that Western Palearctic water frogs underwent a basal radiation into at least 3 major lineages (the *perezi*, the *lessonae*, and the *rindibunda/bedriagae* lineages) advocating an upper Miocene speciation. Moreover, we consider that within the *rindibunda/bedriagae* lineage, *R.* (*P.*) *ridibunda*, *R.* (*P.*) *epeirotica*, *R.* (*P.*) *cretensis*, *R.* (*P.*) *bedriagae*, *R.* (*P.*) *cerigensis* and *R.* (*P.*) *kurtmuelleri* were differentiated from a common ancestor through a series of vicariant and dispersal events, during the last ~5 Mya, even though the specific rank of some taxa may be questionable, such as *R. cerigensis* in respect with *R. bedriagae* and *R. kurtmuelleri* in respect with *R. ridibunda*.

Keywords: *Rana*; *Pelophylax*; Phylogeny; Phylogeography; Cytochrome *b*; 16S rRNA; East Mediterranean

1. Introduction

Phylogeography is considered the bridge between population genetics and phylogenetic systematics (Avise et al., 1987; Avise, 2000), and has contributed considerably to our understanding of what factors have influenced population structure and species divergence (Avise, 1994). Dispersal and vicariance have been identified as the principal mechanisms shaping biogeographic patterns (Stace, 1989). Amphibians are a key group for historical biogeography because they are often thought to be unable to disperse over saltwater barriers (Brown and Guttman, 2002; Bossuyt and Milinkovitch, 2001; Duellman and Trueb, 1986; Inger and Voris, 2001; Meirte, 1999). The age of salt water barriers provides a measure of the minimum time that such pairs of populations have been genetically isolated. Therefore, amphibians are considered to be excellent models for vicariance scenarios as explanation for general biogeographic patterns, and major biogeographic hypotheses have been influenced by the occurrence of endemic amphibians on islands or continents (Bossuyt and Milinkovitch, 2001; Brown and Guttman, 2002; Duellman and Trueb, 1986; Richards and Moore, 1996; Worthy et al., 1999).

The complex geological history of the East Mediterranean region, and especially that of the Hellenic area with multiple geological events of land fragmentations and connections

* Corresponding author. Address: Department of Biology, University of Crete, Vassilika Vouton, P.O. Box 2208, 71409 Irakleio, Crete, Greece. Fax: +30 2810 393 270.
E-mail address: poulakakis@nhmc.uoc.gr (N. Poulakakis).
[1] These authors contributed equally to this work.

1055-7903/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.ympev.2007.03.009

during the late Tertiary, has likely contributed to the diversification and pattern of distribution of many terrestrial animals (Beerli et al., 1996; Douris et al., 1998; Parmakelis et al., 2003, 2005, 2006a,b; Poulakakis et al., 2003, 2005a,b,c). Located at the margin of the Eurasian and African plates, the Hellenic area of the East Mediterranean region has experienced tremendous geological alterations since the late Tertiary, including multiple events of land connections (Creutzburg, 1963; Dermitzakis, 1990; Dermitzakis and Papanikolaou, 1981; Meulenkamp, 1985; Steininger and Rögl, 1984). Such connections offered opportunities for biological dispersal, while submergence of land bridges brought about distributive isolation among related taxa. If this was a major factor influencing the formation of the rich fauna of the Hellenic area, then the phylogenetic relationships of terrestrial taxa should reflect these paleogeographic events.

In his review on the superfamily Ranoidea, Dubois (1992) proposed a provisional taxonomical classification for the family Ranidae and, particularly, the genus *Rana*. He described the difficulties he confronted referring especially to the great number of species, currently estimated to over 250 (Hillis and Wilcox, 2005), and their correspondingly great distribution. He proposed the division of the genus in 33 subgenera, concluding that his proposed hypothesis may serve as a starting point for future research. One of the subgenera that Dubois (1992) delimited, namely *Pelophylax*, currently comprises 26 taxa, which are collectively referred to as ''Palaearctic Water Frogs''. *Pelophylax* is one of the most studied taxa within *Rana* and many works have been published since the study of Dubois (1992), including, among others, those of Beerli et al. (1994, 1996), Casola et al. (2004), Crochet et al. (1995), Dubois and Ohler (1994), Günther (1998), Plötner and Ohst (2001), Plötner et al. (2001) and Sumida et al., 2000). The majority of these works explore relationships among taxa of the western fraction of *Pelophylax* as contrasted to the far eastern one. Still, phylogenetic relationships even within the western fraction of *Pelophylax* are yet to be resolved (Plötner and Ohst, 2001).

In the present study, *Rana* (*Pelophylax*) specimens were collected from several localities of the Eastern Mediterranean region, and the DNA sequences were obtained from the cytochrome *b* (cyt *b*) and 16S rRNA (16S) genes to infer the phylogenetic relationships of these species. The sequences for these two genes were combined with previously published sequences in order to (a) examine the validity of the current taxonomy, (b) produce a historical interpretation of the species' distribution and (c) evaluate alternative models of the biogeographic history of *Rana* (*Pelophylax*) in this area.

2. Materials and methods

2.1. DNA extraction, amplification and sequencing

Specimens were collected from various geographic locations around the Eastern Mediterranean region (Table 1 and Fig. 1) and voucher specimens ($n = 52$) were deposited at the Natural History Museum of Crete (NHMC), Crete, Greece. Total genomic DNA was extracted from small pieces of muscle using standard methods (Sambrook et al., 1989). Two target genes were selected for molecular phylogenetic analysis. A partial sequence (~560 bp) of mitochondrial cyt *b* gene was amplified with the primers L14850 and H15410 (Tanaka et al., 1994). Furthermore, an approximately 500 bp fragment of the mitochondrial 16S rRNA gene was amplified using the universal primers 16Sar and 16Sbr (Palumbi, 1996).

Amplification of all sequences involved an initial cycle of denaturation at 94 °C for 5 min, and 35 subsequent cycles of 94 °C for 30 s, 47 °C for 30 s and 72 °C for 1 min, using single *Taq* DNA polymerase (Minotech, IMBB, Crete). PCR products were purified with the QIAquick PCR purification kit (Qiagen). Single stranded sequencing of the PCR product was performed using the Big-Dye Terminator (v3.1) Cycle Sequencing kit on an MJ Basestation automated sequencer following the manufacturer's protocol. Primers used in cycle sequencing were those used in PCR amplification. The mtDNA genes were sequenced in both directions for all taxa. In addition, 24 sequences of *Rana* spp. (both cyt *b* and 16S) were retrieved from GenBank and included in the phylogenetic analysis (for details see Table 1). *Rana catesbeiana* was used as an outgroup.

2.2. Alignment and genetic divergence

The alignment of the concatenated cyt *b* and 16S sequences was performed with Clustal X (Thompson et al., 1997), and corrected by eye. The data matrix and the tree are available from TreeBase (Accession No. SN3205 and matrix Accession No. 13696). Alignment gaps were inserted to resolve length differences between sequences, and positions that could not be unambiguously aligned were excluded. Cytochrome *b* sequences were translated into amino acids prior to analysis and did not show any stop codons. Sequence divergences were estimated using PAUP* (v.4.0b10, Swofford, 2002) and a saturation analysis was performed in DAMBE (Xia and Xie, 2001). GenBank Accession Numbers for the sequences obtained are: DQ474129–180 for cyt *b* and DQ474181–232 for 16S rRNA (see Table 1).

2.3. Phylogenetic analyses

Phylogenetic inference analyses were conducted using maximum parsimony (MP), maximum likelihood (ML), and Bayesian inference (BI) methods. Nucleotides were used as discrete, and ratiounordered characters. To examine whether the sequences from the two genes should be combined in a single analysis, a partition homogeneity test (Farris et al., 1995), was run in PAUP* and significance was estimated by 1000 repartitions.

Table 1
List of the specimens of *Rana* used in molecular analyses

Code	Species	Locality	Museum No.	Accession No. cyt *b*	Accession No. 16S
Rkurt1	*R. kurtmuelleri*	Greece, Macedonia (Kerkini L.)	NHMC 80.2.11.3	DQ474156	DQ474208
Rkurt2	*R. kurtmuelleri*	Greece, Macedonia (Kerkini L.)	NHMC 80.2.11.4	DQ474157	DQ474209
Rkurt3	*R. kurtmuelleri*	Greece, Plastira L.	NHMC 80.2.11.5	DQ474158	DQ474210
Rkurt4	*R. kurtmuelleri*	Greece, Prespa L.	NHMC 80.2.11.7	DQ474159	DQ474211
Rkurt5	*R. kurtmuelleri*	Greece, Evvoia Isl.	NHMC 80.2.11.20	DQ474164	DQ474216
Rkurt6	*R. kurtmuelleri*	Greece, Plastira L.	NHMC 80.2.11.22	DQ474165	DQ474217
Rkurt7	*R. kurtmuelleri*	Greece, Zagori Mt.	NHMC 80.2.11.25	DQ474166	DQ474218
Rkurt8	*R. kurtmuelleri*	Greece, Ossa Mt.	NHMC 80.2.11.26	DQ474167	DQ474219
Rkurt9	*R. kurtmuelleri*	Greece, Ossa Mt.	NHMC 80.2.11.27	DQ474168	DQ474220
Rkurt10	*R. kurtmuelleri*	Greece, Kythira Isl.	NHMC 80.2.11.28	DQ474169	DQ474221
Rkurt11	*R. kurtmuelleri*	Greece, Pamisos R.	NHMC 80.2.11.35	DQ474170	DQ474222
Rkurt12	*R. kurtmuelleri*	Greece, Messologi	NHMC 80.2.11.36	DQ474171	DQ474223
Rkurt13	*R. kurtmuelleri*	Greece, Nestani	NHMC 80.2.11.37	DQ474172	DQ474224
Rkurt14	*R. kurtmuelleri*	Greece, Platykampos	NHMC 80.2.11.40	DQ474173	DQ474225
Rkurt15	*R. kurtmuelleri*	Greece, Skyros Isl.	NHMC 80.2.11.45	DQ474174	DQ474226
Rkurt16	*R. kurtmuelleri*	Greece, Sarantaporos	NHMC 80.2.11.47	DQ474175	DQ474227
Rkurt17	*R. kurtmuelleri*	Greece, Arta	NHMC 80.2.11.12	DQ474176	DQ474228
Rrid1	*R. ridibunda*	Greece, Thrace (Kotili)	NHMC 80.2.11.8	DQ474160	DQ474212
Rrid2	*R. ridibunda*	Greece, Thrace (Nestos R.)	NHMC 80.2.11.9	DQ474161	DQ474213
Rrid3	*R. ridibunda*	Greece, Thrace (Therma)	NHMC 80.2.11.10	DQ474162	DQ474214
Rrid4	*R. ridibunda*	Greece, Thrace (Dadia)	NHMC 80.2.11.11	DQ474163	DQ474215
Rrid5	*R. ridibunda*	East Europe	—	**AY043049**	**AY043067**
Rrid6	*R. ridibunda*	Ukraine	—	**AB029945**	n.a.
Rbed1	*R. bedriagae*	Syria 1 Al Jaboul L.	NHMC 80.2.99.1	DQ474129	DQ474181
Rbed2	*R. bedriagae*	Syria 2 Hawaig Gorge	NHMC 80.2.99.3	DQ474130	DQ474182
Rbed3	*R. bedriagae*	Syria 3 Krak des Chevaliers	NHMC 80.2.99.6	DQ474131	DQ474183
Rbed4	*R. bedriagae*	Syria 4 Lattakia beach	NHMC 80.2.99.7	DQ474132	DQ474184
Rbed5	*R. bedriagae*	Syria 5 Qal' at Al Rahbeh castle	NHMC 80.2.99.8	DQ474133	DQ474185
Rbed6	*R. bedriagae*	Syria 6 Maquam Assayedh	NHMC 80.2.99.10	DQ474134	DQ474186
Rbed7	*R. bedriagae*	Greece, Lesvos Isl.	NHMC 80.2.99.18	DQ474135	DQ474187
Rbed8	*R. bedriagae*	Cyprus Cha river, springs	NHMC 80.2.99.19	DQ474136	DQ474188
Rbed9	*R. bedriagae*	Cyprus Cha river, springs	NHMC 80.2.99.20	DQ474137	DQ474189
Rbed10	*R. bedriagae*	Greece, Chios Isl.	NHMC 80.2.99.22	DQ474138	DQ474190
Rbed11	*R. bedriagae*	Greece, Macedonia (Dadia)	NHMC 80.2.99.21	DQ474139	DQ474191
Rbed12	*R. bedriagae*	Greece, Astypalaia Isl.	NHMC 80.2.99.23	DQ474140	DQ474192
Rbed13	*R. bedriagae*	Greece, Astypalaia Isl.	NHMC 80.2.99.24	DQ474141	DQ474193
Rbed14	*R. bedriagae*	Turkey, Antalya	—	**AY014392**	**AF215422**
Rbed15	*R. bedriagae*	Turkey, Marmaris	—	**AY147957**	**AY147937**
Rcer1	*R. cerigensis*	Greece, Karpathos Isl.	NHMC 80.2.110.1	DQ474142	DQ474194
Rcer2	*R. cerigensis*	Greece, Karpathos Isl.	NHMC 80.2.110.4	DQ474143	DQ474195
Rcer3	*R. cerigensis*	Greece, Karpathos Isl.	NHMC 80.2.110.5	DQ474144	DQ474196
Rcer4	*R. cerigensis*	Greece, Rodos Isl.	—	n.a.	**AF215420**
Rcer5	*R. cerigensis*	Greece, Rodos Isl.	—	n.a.	**AF147979**
Rcret1	*R. cretensis*	Greece, Crete Isl. (Lentas)	NHMC 80.2.46.1	DQ474145	DQ474197
Rcret2	*R. cretensis*	Greece, Crete Isl. (Petres R.)	NHMC 80.2.46.8	DQ474146	DQ474198
Rcret3	*R. cretensis*	Greece, Crete Isl. (Irakleio)	NHMC 80.2.46.9	DQ474147	DQ474199
Rcret4	*R. cretensis*	Greece, Crete Isl. (Geropotamos R.)	NHMC 80.2.46.12	DQ474148	DQ474200
Rcret5	*R. cretensis*	Greece, Crete Isl. (Bramiana L.)	NHMC 80.2.46.13	DQ474149	DQ474201
Rcret6	*R. cretensis*	Greece, Crete Isl. (Kaloudiana L.)	NHMC 80.2.46.15	DQ474150	DQ474202
Rcret7	*R. cretensis*	Greece, Crete Isl. (Partira L.)	NHMC 80.2.46.17	DQ474151	DQ474203
Rcret8	*R. cretensis*	Greece, Crete Isl. (Giofyros R.)	NHMC 80.2.46.18	DQ474152	DQ474204
Rcret9	*R. cretensis*	Greece, Crete	—	n.a.	**AF215423**
Rcret10	*R. cretensis*	Greece, Crete	—	n.a.	**AY147980**
Rep1	*R. epeirotica*	Greece, Peloponnesos (Lisimaxia L.)	NHMC 80.2.109.1	DQ474153	DQ474205
Rep2	*R. epeirotica*	Greece, Peloponnesos (Kalogria)	NHMC 80.2.109.2	DQ474154	DQ474206
Rep3	*R. epeirotica*	Greece, Peloponnesos (Strofilia)	NHMC 80.2.109.4	DQ474155	DQ474207
Rep4	*R. epeirotica*	Greece	—	n.a.	**AY147981**
Rsah1	*R. saharica*	Tunis, Ickeul Mt.	NHMC 80.2.126.1	DQ474177	DQ474229
Rsah2	*R. saharica*	Tunis, 4 km before Sedjenan	NHMC 80.2.126.2	DQ474178	DQ474230
Rsah3	*R. saharica*	Tunis, Tamerza oasis	NHMC 80.2.126.3	DQ474179	DQ474231
Rsah4	*R. saharica*	Tunis, Bizente	—	n.a.	**AY147984**
Rsah5	*R. saharica*	Tunis	—	n.a.	**AF215426**

(*continued on next page*)

Table 1 (*continued*)

Code	Species	Locality	Museum No.	Accession No. cyt *b*	Accession No. 16S
Rles1	*R. lessonae*	Belgium	—	**AY057099**	**AY322276**
Rles2	*R. lessonae*	Italy	—	**AY043051**	**AY147982**
Rles3	*R. lessonae*	Italy, Firenze	—	**AB029942**	n.a.
Rles4	*R. lessonae*	Luxemburg	—	**AB029943**	n.a.
Rles5	*R. lessonae*	Britain	—	**AB084047**	n.a.
Resc	*R. esculenta*	Ukraine	—	**AB029944**	n.a.
Rper	*R. perezi*	France	—	**AY043052**	**AY147985**
Rnigr	*R. nigromaculata*	China	—	**AY315757**	**AY322278**
Rfuk	*R. fukienensis*	Taiwan	—	**AB029941**	n.a.
Rpla1	*R. plancyi*	Korea	—	**AF205088**	n.a.
Rpla2	*R. plancyi*	Korea	—	**AF274930**	n.a.
Rporoz1	*R. porozi*	Japan	—	**AB029940**	n.a.
Rporoz2	*R. porozi*	Japan	—	**AB029938**	n.a.
Rcat	*R. catesbeiana*	Greece, Crete Isl.	NHMC 80.2.13.1	DQ474180	DQ474232

NHMC: Natural History Museum of Crete; Acceccion No.: GenBank Accession Numbers. Sequences retrieved from GenBank are indicated by bold.

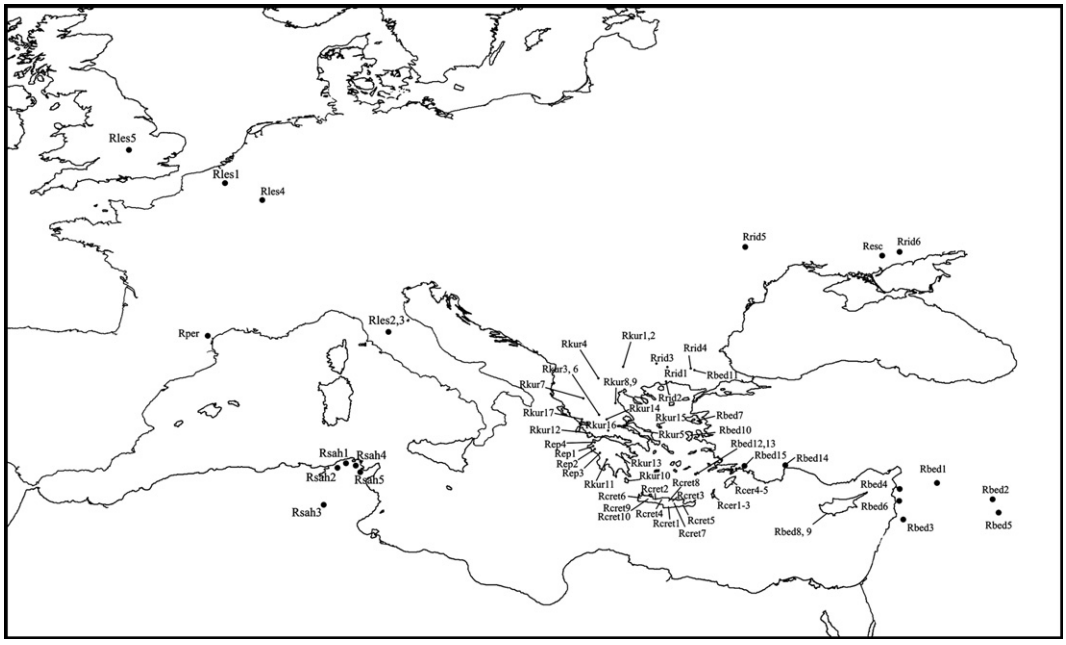

Fig. 1. Sample localities. Rbed, *R. bedriagae*; Rcret, *R. cretensis*; Rcer, *R. cerigensis*; Rep, *R. epeirotica*; Rkur, *R. kurtmuelleri*; Rrid, *R. ridibunda*; Rles, *R. lessonae*; Resc, *R.kl. esculenta*; Rper, *R. perezi*; and Rsah, *R. saharica*. Codes correspond to those in Table 1. Note that *R. berdriagae* is reported for the first time in Europe and on Euphrates in Syria.

2.3.1. Maximum parsimony

Maximum parsimony analysis was performed with PAUP*, with heuristic searches using stepwise addition and performing tree-bisection reconnection (TBR) branch swapping (Swofford et al., 1996). Confidence in the nodes was assessed by 1000 bootstrap replicates (Felsenstein, 1985) with random addition of taxa. Branch support was assessed from decay indices calculated for all internal branches of the tree (Bremer, 1994). Decay indices were calculated using AutoDecay (ver. 5.0) (Eriksson, 2001) for Windows.

2.3.2. Maximum likelihood

Maximum likelihood analyses (Felsenstein, 1981) were conducted using PAUP*. We used Modeltest 3.7 (Posada and Crandall, 1998) to select the best fitting model under the Akaike Information Criterion (AIC, Akaike, 1974), since this method is able to simultaneously compare multiple nested or non-nested models, assess model selection uncertainty, and allow for the estimation of phylogenies and model parameters using all available models (Posada and Buckley, 2004). Heuristic ML searches were performed with 10 replicates of random sequence addition and TBR branch swapping, using the search strategy of Swofford et al. (1996) and Sullivan et al. (2005) based on successive-approximations approach, as follows. First, a set of topologies that will provide reasonable estimates of model parameters is identified through an initial tree search conducted using an approximate approach such as neighbor-joining or parsimony. This set of initial topologies is then

used to select a model. The process of model selection also provides initial estimates of the parameters of the selected models, and a second tree search is conducted using likelihood as the optimality criterion with a fully defined model of substitution (i.e., parameters of the substitution model are fixed to the previously estimated values). If the ML tree is not a subset of the trees found by the initial search, the new tree should be used as the starting tree for a subsequent iteration. Model parameters are then re-optimized on the new tree, and a second search is conducted; the process continues until the same tree is found in two successive iterations.

2.3.3. Bayesian inference

Bayesian inference (BI) was performed with the software MrBayes (v3.1; Ronquist and Huelsenbeck, 2003). This version of MrBayes is able to analyze multiple gene regions simultaneously with differing substitution parameters, allowing for a combined analysis of both datasets. The AIC was used in order to determine the most accurate model of molecular evolution for each DNA region (cyt *b* and 16S). The analysis was performed with four runs and four chains for each run for 10^7 generations and the current tree was saved to file every 100 generations. This procedure generated an output of 10^5 trees. A plot of log-likelihood scores of sample points against generation showed that stationarity was achieved after the first 10^5 generations, thus the first 10^3 trees were ultimately discarded as a conservative measure to avoid the possibility of including random, sub-optimal trees. A majority rule consensus tree ('Bayesian' tree) was then produced from the posterior distribution of trees, and the posterior probabilities calculated as the percentage of samples recovering any particular clade (Huelsenbeck and Ronquist, 2001), where probabilities $\geqslant 95\%$ indicate significant support. Two further independent Bayesian analyses were run so that global likelihood scores, individual parameter values, topology and nodal support could be compared to check for local optima.

2.3.4. Testing alternative hypotheses

We tested the hypothesis that *R. bedriagae* is monophyletic. A tree with *R. bedriagae* constrained to be monophyletic was generated with MrBayes, and compared with our optimal topology using the Shimodaira–Hasegawa (SH) test (Goldman et al., 2000; Shimodaira and Hasegawa, 1999) as implemented in PAUP* and employing RELL bootstrap with 1000 replicates.

2.4. Calibration of molecular clock and estimation of divergence times

A log-likelihood ratio test was used to examine the clock-like evolution of sequences in the combined data set by calculating a χ^2 statistic (Likelihood Ratio Test, LRT) based on ML values with and without rate constancy enforced ($\chi^2 = 2 \times [(-\ln L_{\mathrm{CLOCK}}) - (-\ln L_{\mathrm{UNCONSTRAINED}})]$, df = number of terminal nodes—2; Felsenstein 1981). The LRT was negative (LRT = 228.6, df = 74, p 0.05), therefore a clock-like evolution of the involved sequences could not be assumed.

Time estimates and their confidence intervals were calculated from the genes-specific rate using a non-parametric rate smoothing (NPRS) analysis with the recommended Powell algorithm as implemented in the software r8s (v.1.7 for Linux) (Sanderson, 1997, 2003). This method uses a penalty function to smooth the speed of rate change among lineages, thereby relaxing the molecular clock assumption. For calibration points we used two well dated paleogeographical events, the isolation of the island of Crete from neighboring mainland (Peloponnesos) and the isolation of Kasos and Karpathos islands from the island of Rhodes. The first event, which was happened at 5–5.5 Mya (Dermitzakis, 1990; Meulenkamp, 1985) or 5.2 Mya (after Beerli et al., 1996), corresponds to the divergence of *R. cretensis*, and the second, which was happened at 3–3.5 Mya (Daams and Van Der Weerd, 1980), corresponds to the divergence of *R. cerigensis*. Mean divergence times and confidence intervals were obtained for each node based on the procedure described in the r8s software manual (http://ginger.ucdavis.edu/r8s). We generated 100 bootstrap data matrices using Seqboot of Phylip (Felsenstein, 2004) and used these to generate 100 MP phylograms with the same topology but differing branch lengths in PAUP*, which were then read into r8s, estimating ages for all trees and summarizing age distributions for each node of interest (Eriksson, 2003).

3. Results

Of the 1055 sites examined, there were 185 variable cyt *b* sites, of which 157 were parsimony informative (210 and 177, respectively, when the outgroup were included in the analysis) and 107 variable 16S sites, 82 of which were parsimony informative (126 and 85, respectively, including outgroups). For cyt *b*, ingroup sequence divergence (between group means) ranged from 2.5 to 24.1%, while for 16S sequence divergence ranged between 0.4 and 11.3% (Table 2). Saturation analysis did not show any kind of saturation (figure now shown).

A partition homogeneity test indicated no conflicting phylogenetic signals between the datasets ($p = 0.971$), and the mtDNA genes were analyzed together. Tree length distribution, determined from random sampling of 10^6 unweighted trees, was significantly skewed to the left ($g1 = -0.48$), suggesting a strong phylogenetic signal in the data ($p < 0.01$; Hillis and Huelsenbeck, 1992). For the phylogenetic analyses, a data set of 76 combined sequences, including outgroup, were used. All analyses (MP, ML, and BI) were congruent regarding the branching order of the deeper clades, differing only in the relationships within these clades (*i.e.*, the relationships of the specimens of *R. cretensis* from Crete Island).

Table 2
Sequence divergences (%) among the main mtDNA subclades of *Rana* for cyt *b* (HKY + I + G model of evolution, below diagonal) and 16S rRNA (GTR + G model of evolution, above diagonal)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1. *R. bedriagae*—Turkey/Greece	—	1.1	1.8	0.6	2.8	2.8	3.5	4.0	5.5	n/c	9.6	7.5	8.2	13.7
2. *R. bedriagae*—Syria	3.8	—	1.8	1.0	2.8	2.6	3.4	4.0	5.8	n/c	9.1	7.2	8.4	14.2
3. *R. bedriagae*—Cyprus	3.3	3.5	—	2.0	2.8	3.7	4.4	5.0	6.0	n/c	10.4	8.9	9.0	15.6
4. *R. cerigensis*	1.7	4.1	4.1	—	1.5	2.1	3.3	3.8	5.3	n/c	9.5	7.3	8.7	14.0
5. *R. kurtmuelleri*	7.5	8.2	7.7	7.5	—	0.4	3.7	3.8	5.7	n/c	8.5	6.2	7.9	14.1
6. *R. ridibunda*	7.5	8.9	8.0	8.2	2.5	—	3.0	4.5	6.8	n/c	10.1	7.0	9.5	16.4
7. *R. cretensis*	12.1	12.6	12.5	13.2	12.4	12.1	—	2.6	5.9	n/c	8.6	6.7	9.2	15.0
8. *R. epeirotica*	10.6	11.0	11.8	10.9	8.6	9.1	10.3	—	6.7	n/c	9.2	7.5	8.6	15.4
9. *R. lessonae*	13.9	16.1	16.5	13.7	16.4	16.6	15.7	15.9	—	n/c	11.3	9.1	10.4	16.0
10. *R. esculenta*	15.0	16.9	17.5	14.7	15.8	16.3	15.4	16.1	1.3	—	n/c	n/c	n/c	n/c
11. *R. saharica*	19.5	20.4	20.4	18.9	18.9	19.6	20.1	19.3	21.2	20.0	—	7.6	8.8	16.7
12. *R. perezi*	15.0	17.3	16.4	15.3	16.8	16.8	21.7	13.6	19.1	19.7	20.0	—	8.5	14.3
13. Far Eastern group	21.7	23.0	21.8	21.4	21.5	22.1	21.2	21.5	20.5	20.2	21.6	24.1	—	12.4
14. Outgroup	23.7	24.3	24.0	22.7	25.4	24.6	22.2	24.1	22.6	24.1	24.8	22.1	19.9	—

No values were calculated (n/c) where no data was available.

Equally weighted parsimony analysis of the 262 parsimony-informative characters (239 for the ingroup) produced more than 10,000 most-parsimonious trees with a length of 791 steps (consistency index, CI = 0.569 and retention index, RI = 0.888). The number of equally parsimonious solutions was due to terminal branch swapping, particularly among specimens belonging to the same species and coming from the same or geographically proximal populations, such as the specimens of *R. kurtmuelleri*, *R. bedriagae*, *R. cretensis*, etc. Furthermore, when only one sequence from each of the mentioned branches was included in the analysis under the criterion of MP, resulted in a single parsimonious tree, which has the same topology as that MP produced from the large data set.

Maximum likelihood analysis under the General Time Reversible (GTR, Rodriguez et al., 1990) + I + G model for all data set (cyt *b* and 16S rRNA) resulted in a topology ($\ln L = -5357.36$) which is consistent with the parsimony one (final parameters estimates: base frequencies A = 0.29, C = 0.27, G = 0.17, T = 0.27, gamma distribution parameter $a = 0.6983$, invariable sites $P_{inv} = 0.4589$, and rate matrix A/C = 1.21, A/G = 8.08, A/T = 1.90, C/G = 0.43, C/T = 11.15, and G/T—1.000).

Bayesian inference under the Hasegawa–Kishino–Yano (HKY, Hasegawa et al., 1985) + I + G model for cyt *b* and General Time Reversible (GTR) + G model for 16S rRNA resulted in a topology with mean $\ln L = -5553.10$. Identical topologies were recovered for each of the 4 runs with the full dataset and the 50% majority-rule consensus tree of the 99×10^3 trees remaining after burn-in are presented in Fig. 2.

In all analyses, two very well supported allopatric basal clades of water frogs were identified (Fig. 2), corresponding to different groups of species and/or to separate geographic regions throughout the Palearctic region: the Far Eastern (clade A) and the Western (clade B) types. The Far Eastern clade (93/91/0.99 = NJ bootstrap value/MP bootstrap value /BI posterior probability, respectively) could be further subdivided into two subclades. The phylogenetic relationships of this group have been discussed by Sumida et al. (2000) and will not be discussed here.

The Western clade (100/99/1.00) could be subdivided into six subclades: (a) B1 with *R. saharica* from Tunis and *R. perezi* from Spain (81/78/0.97), (b) B2 with *R. lessonae* from central Europe and *R. esculenta* from Ukraine (100/100/1.00), (c) B3 with *R. cretensis* from the island of Crete, Greece (100/100/1.00), (d) B4 with *R. epeirotica* from Peloponessos, Greece (100/100/1.00), (e) B5 with *R. bedriagae* from Greece, Turkey, Syria and Cyprus and *R. cerigensis* from the islands of Rhodos and Karpathos (99/99/1.00), and (f) B6 with *R. kurtmuelleri* from Greece and *R. ridibunda* from Northeastern Greece and Eastern Europe (100/99/1.00).

The NPRS method applied to 100 bootstrapped phylograms produced divergence times and confidence intervals (Fig. 2). According to the calibration reference points, the diversification of the water frogs in Western Palearctic region occurred at 10 Mya during the late Miocene, whereas the speciation within the *ridibunda/bedriagae* lineage occurred the last 5–6 Mya.

4. Discussion

The results of the present study revealed a well-resolved phylogeny and identified a number of haplotype clades which, based on the observed levels of sequence divergence (Table 2), represent long-separated lineages and diverse evolutionary histories within water frogs. The obtained molecular data clearly indicate that Western Palearctic water frogs underwent a basal radiation into at least 3 major lineages (or six subclades, see Fig. 2).

In the *perezi* lineage, *R. perezi* and *R. saharica* form a sister group (B1 in the phylogenetic tree) and are clearly separated from the other European species. This pattern is also inferred in Beerli et al. (1996) and Plötner and Ohst (2001), although in the latter study, the sister group

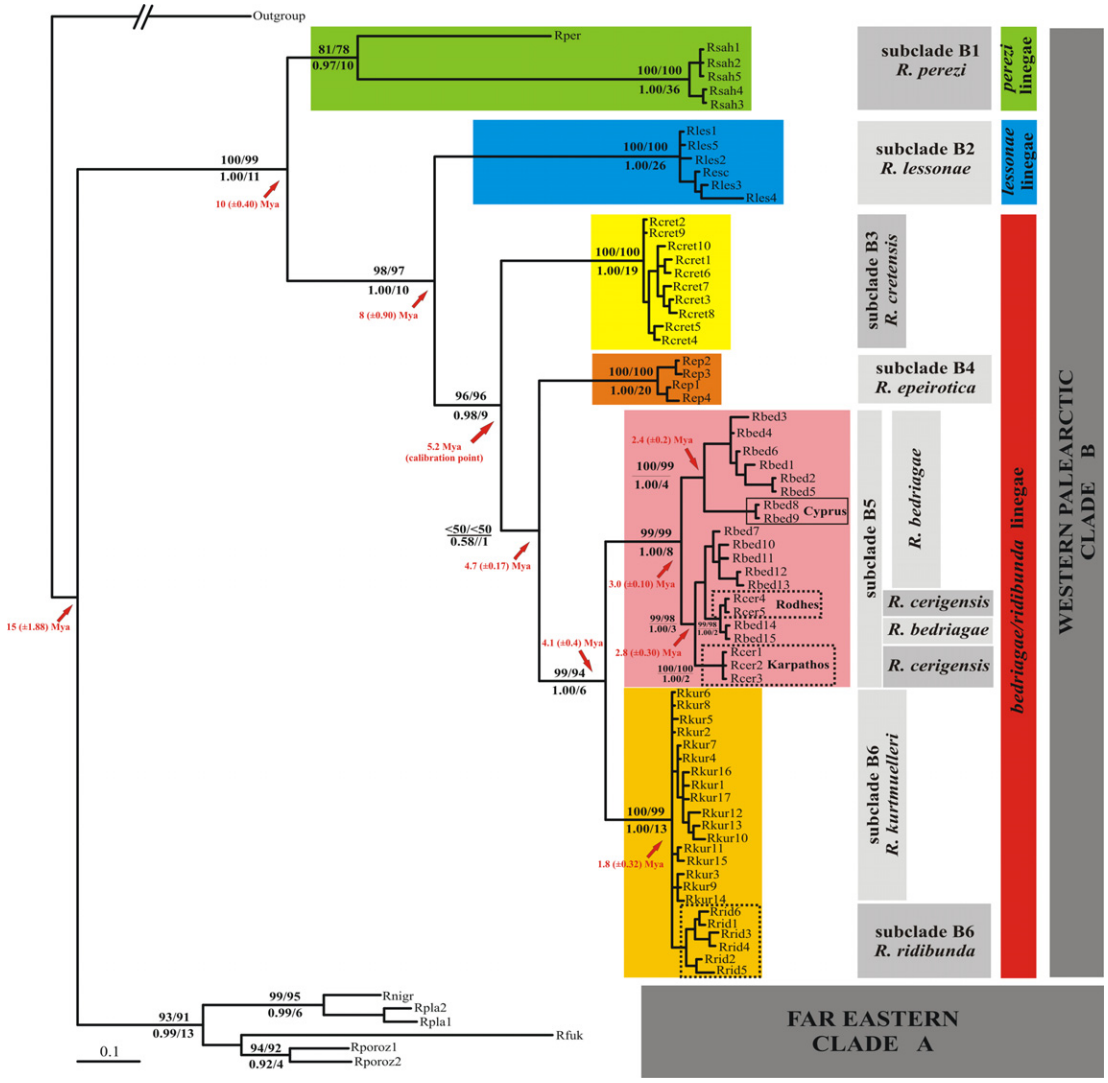

Fig. 2. Bayesian Inference (BI) tree of the haplotypes of the ten *Rana (Pelophylax)* spp. included in the analyses. A sequence of *Rana catesbeiana* was used as outgroup taxon. Neighbor joining (NJ), maximum parsimony (MP), and maximum likelihood (ML) produced trees with the same topology with regard to the major lineages. Numbers above branches are bootstrap values on NJ and MP greater than 50% based on 1000 replicates. Numbers below branches are posterior probabilities values of BI and decay indices.

relationship is ambiguous. On the other hand, it is completely different from what Casola et al. (2004) presented, since in the latter study *R. saharica* appears to be distinct from all other Western Palearctic water frog species, but *R. perezi* branches off before *R. epeirotica* and after *R. cretensis*. However, the latter authors stated that the elements they compared were probably not orthologous, which renders their sequences inadequate for reconstructing phylogenetic histories.

The rest of the western species fall into either the pool frog lineage (the *lessonae* lineage, B2 in the phylogenetic tree) with 100% statistical support, or the Balkan–Anatolian frog lineage (the *ridibunda/bedriagae* lineage) that comprise the species *R. cretensis, R. epeirotica, R. bedriagae, R. cerigensis, R. kurtmuelleri* and *R. ridibunda*.

The *lessonae* lineage includes the species *R. lessonae* and *R. esculenta*, and most likely *R. shqiperica* (west Albania and Montenegro) and *R. bergeri* (Italian peninsula), since these are considered to be closely related to *R. lessonae* (Beerli et al., 1996; Plötner and Ohst, 2001). The firm placing of *R. esculenta* (edible frog) within *R. lessonae* (pool frog) was expected, since *R. esculenta* is a hybridogenetically reproducing hybrid between *R. lessonae* and *R. ridibunda*, an issue that has been extensively discussed earlier (Casola et al., 2004; Hotz et al., 1992; Monnerot et al., 1985; Sumida et al., 2000; Spolsky and Uzzell, 1984 and references therein).

In the *ridibunda/bedriagae* lineage (or Balkan–Anatolian), the Cretan frogs (*R. cretensis*, B3) branch off first, followed by the Epeirus water frogs (*R. epeirotica*, B4). However, phylogenetic analyses failed to resolve this relationship (no statistical support: <50/<50/0.58). Based on genetic distances (Table 2), *R. cretensis* is closer to the western Greek *R. epeirotica* (10.3% in cyt *b* and 3.0% in 16S). Nevertheless, although the differences in genetic distances between *R. cretensis, R. epeirotica* and the other Balkan–Anatolian species,

including the geographically nearest mainland taxa of *R. ridibunda*, *R. bedriagae*, *R. cerigensis*, and *R. kurtmuelleri*, confirm their species status, these differences are too small to determine reliably their closest relative.

Finally, *R. ridibunda* and *R. kurtmuelleri* are sister species (B6) and represent a sister group (99/94/1.00) to the *R. bedriagae* and *R. cerigensis* (B5). In B5, *R. bedriagae* appears to be paraphyletic with respect to *R. cerigensis*, since *R. bedriagae* specimens from Turkey and east Aegean islands clustered with *R. cerigensis* specimens from the islands of Karpathos and Rhodes (bootstrap 99/97/1.00) and not with the other specimens of *R. bedriagae* from Syria, and Cyprus (100/99/1.00). Given that the SH test ($p < 0.001$) provided further support of this assertion, the above results confirmed the necessity of revision of the existing taxonomy of this species, i.e., *R. cerigensis* could be consider as a newer synonym of *R. bedriagae*.

In all phylogenetic trees, Syrian and Cypriote populations have a sister clade relationship both clustering in strongly supported respective clades. This is in disagreement with what Plötner and Ohst (2001) and Plötner et al. (2001) stated, since in these studies the Cypriot lineage branched off earlier than *R. ridibunda* and *R. bedriagae*; and the observed divergence between Cypriot water frogs and frogs from surrounding mainland (Turkey and Syria) were in the same range as between *R. bedriagae* and *R. ridibunda*. Based on these data, the above authors suggested that the Cyprus populations do not belong to *R. bedriagae*, but to a different undescribed species. However, in our study a strong relationship of the Cypriot lineage with Syria was observed, a connection that has been suggested in another phylogeographic study of the skink lizard genus *Ablepharus* (Poulakakis et al., 2005a). In addition, the genetic distances between the Cypriot and other *R. bedriagae* lineages (Turkey, East Aegean Islands and Syria) was 3.3–3.5% for cyt *b* and 1.8% for 16S rRNA (both within the range of intraspecific variation), whilst the corresponding distances between *R. bedriagae* and *R. ridibunda* were 7.5–8.9% for cyt b and 2.6–3.7% for 16S, indicating that Cypriot populations belong to *R. bedriag*ae, having a different evolutionary history the last 3 Mya (see below in phylogeography).

In B6, the relationships among *R. ridibunda* and *R. kurtmuelleri* are considered as unresolved, because of the low bootstrap support (<50%). However, the topology of the phylogenetic tree, the small genetic distances (within the range of intraspecific variation) and the insignificant morphological differentiation between *R. kurtmuelleri* and *R. ridibunda* (Schneider et al., 1993) reinforce the opinion of some taxonomists, who are reluctant to acknowledge *R. kurtmuelleri* as a distinct taxon (but see Schneider et al., 1993).

4.1. Historical biogeography

The origin of Western Palearctic water frogs still remains unanswered. Baranescu (1983) postulated that most of the present day aquatic genera reached Europe from Eastern Asia during the Miocene. Most of them were then established in Europe in the middle Miocene. Western and Eastern Palearctic water frogs define monophyletic lineages (Fig. 2). Based on the estimated time, they diverged about 15 (±1.88) Mya during the middle Miocene. This period fits well with what Baranescu (1983) reported for the origin of most European aquatic genera, while is much older than the divergence time (6–10 Mya) reported by of Sumida et al. (2000). However, the latter authors estimated this date using a rate of 1.1–1.2% per My for the 12S rRNA, which is evaluated based on the anuran *Bufo* mtDNA sequence divergence of 1.38% (Macey et al., 1998), and assuming that the sequences studied the latter researchers (ND1, tRNAs, and ND2) probably evolved slightly faster, on average, than the 12S rRNA gene used by Sumida et al. (2000). Kosuch et al. (2001) stated that based on the work of Beerli et al. (1996) and their assumed clock rate, the divergence between *R. bedriagae* and *R. saharica* can be calculated at 10 My before present, which means that the divergence between the Eastern and Western groups dated before 10 My. Rates assumed for the 16S and 12S rRNA genes in other amphibians such as newts (Caccone et al., 1997) and true salamanders (Veith et al., 1998) range between 0.4 and 0.7% My^{-1}. If these rates of divergence are used, and given that the mean nucleotide sequence divergence between the Far Eastern and the Western water frogs of Sumida et al., was 10.12% for 12S rRNA, then the estimated time of divergence between these groups of water frogs range from 14.5 to 25.3 Mya. It is widely accepted that a molecular clock with known relationships among the taxa under study and with well-dated isolation times will give the most accurate estimates, nevertheless, extrapolation to other groups and beyond the time interval requires caution (Beerli et al., 1996), since observations of rates of molecular evolution varying widely within and among lineages are abundant (Gillooly et al., 2005). It should be more accurate if a rate of evolution for the 12S rRNA gene of *Rana* is used. When an attempt was made to calibrate a molecular clock for the 12S rRNA gene of *Rana* using the 31 sequences of *R. ridibund*a and *R. bedriagae* from Greece and Turkey and the 2 sequences of *R. cretensis* from Crete (Plötner et al., 2001), and the separation of Crete from the mainland at 5–5.5 Mya (Dermitzakis, 1990; Meulenkamp, 1985) or 5.2 Mya (after Beerli et al., 1996), a rate of 0.44–0.54% Mya^{-1} was estimated. Given that the mean nucleotide divergence is 10.12% (Sumida et al., 2000), these rates produced a time of divergence ranging from 18.7 to 23 Mya. Almost all recently published phylogeographic articles used tree-based rather than distance based estimates of divergence. When one seeks to estimate time of divergence in a produced phylogenetic tree using a distance-based method, it is imperative that the sequences evolve in a clock-like manner. In the present study, there was not a homogeneous clock-like rate for the tree produced by the *Rana* sequences. In the studies of Plötner et al. (2001) and Sumida et al. (2000), there was no test for homogeneous clock-like rate. In the present study, when a Likelihood Ratio Test (LRT) was performed

on the data of Plötner et al. (2001) the null hypothesis that the rate of evolution is homogeneous among all branches in the phylogeny was rejected (LRT = 59.21, df = 31, p 0.05). This result demonstrates that the use of a distance-based method in the estimation of time divergence on the data of Plötner et al. (2001) is inappropriate. The same was found for the data of Sumida et al. (2000). Consequently, the deviation in the estimation of time divergence between the Eastern and Western lineage of water frogs using tree-based (present study) and distance-based (Sumida et al., 2000) methods could be due to the fact that the rates of substitution vary significantly among branches and a molecular clock is inappropriate.

The tree topology and the estimated divergence times advocate an upper Miocene speciation of Western water frogs, and perhaps an ancestor that had invaded Europe from Asia, gave rise to the three different lineages (Fig. 2). The *perezi* lineage branched off first before 10 (±0.40) Mya, and expanded into the Iberian Peninsula and probably North Africa, where the closely related species *R. saharica* exists. The diversification of the *lessonae* lineage started before 8 (±0.9) My in the upper Miocene. It appears very likely that *R. lessonae* had a glacial refuge in the Adriatio-Mediterranean region (Günther, 1997) from which it expanded, marginally reaching the Balkans, where the distinct but closely related species *R. shqiperica* exists.

The speciation within the third lineage (the *ridibunda/bedriagae lineage*) was dated at the end of Miocene and during the Pliocene, coinciding with the end of the Messinian salinity crisis and the drastic climatic changes of Pliocene towards a cooler and arid climate in the Mediterranean. Speciation within several European amphibian genera can be attributed to this phenomenon, the true salamanders (Veith et al., 1998) and brown frogs (Veith et al., 2003a,b) being such examples.

The ability of water frogs to migrate to and between islands is much reduced. All examples (except Comoro frogs *Mantidactylus* n.sp., Vences et al., 2003) of amphibians present on oceanic islands of fully volcanic origin refer to non-endemic species of presumed or demonstrated human translocation origin (*Ptychadena mascareniensis* and *B. gutturalis* on Mauritius and Reunion; *Hyla meridionalis* and *R. perezi* on the Canary archipelago; *Triturus carnifex* on Madeira; *Eleutherodactylus, Dendrobates* and *Bufo* sp. on Hawaii; and *Scinax* on Galapagos (Kraus et al., 1999; Snell and Rea, 1999; Staub, 1993).

Within the Balkan–Anatolian frog lineage (the *ridibunda/bedriagae* lineage), there are two very interesting cases, regarding the dispersal ability of the water frogs. The island of Astypalaia has been separated from the Eastern Aegean islands as well as from Asia Minor before 9 Mya, due to the formation of the Mid Aegean trench (12–9 Mya), and remained connected to continental Greece for a long period of time, via the Cyclades island group (Dermitzakis and Papanikolaou, 1981). Assuming that the *Rana* population on the island has originated vicariantly, one would expect a *R. ridibunda* or *R. kurtmuelleri* related population. However, according to the phylogenetic tree the Astypalaia population appears to have a sister-group relationship with East Aegean–Turkey populations of *R. bedriagae*, indicating that the lineage of Astypalaia originated recently from the East Aegean–Turkey lineages by human translocation or overseas dispersal. Astypalaia is a small island with limited fresh water areas. The establishment of the Mediterranean climate around 3.2 Mya (Blondel and Aronson, 1999) and the numerous arid periods that ensued could have caused the disappearance of fresh water resources of the island and the extinction of the ancestral form of *R. ridibunda* or *R. kurtmuelleri* populating them. Therefore, the appearance of water frogs on an isolated island such as Astypalaia could be either a random dispersal event (island sweepstakes) from the nearby Asia Minor or a human translocation.

The case of Cyprus is different, because Cyprus is an oceanic island with an unclear geological history. Cyprus originated from the raised seabed and is geologically and biogeographically one of the most isolated Mediterranean islands (Moores et al., 1984). Some geologists noted that Cyprus has never been connected to any mainland (Hadjisterkotis, 1993; Sondaar, 1977, 1986), whereas others suggested that the animals could have arrived by a means of a land bridge, when the island was joined to the nearby mainland (Küss, 1973; Hsü, 1983; Palikarides, 1997). The suggestion that Cyprus was conected to the mainland was also based on the presence of non-Mediterranean vegetation (Zohary, 1973). These elements are not only clear evidence of Cyprus's past connections with Syria and southern Anatolia, but also support the assumption that a land bridge has persisted long enough to allow the penetration of biota. The mtDNA data presented here supports a very strong relationship of Cyprus with Syria, a connection that has been suggested in the phylogeographic study of the Snake-eyed skink lizard (Poulakakis et al., 2005a). Given the limited ability of water frogs for overseas dispersal, the Cyprus–Anatolia connection hypothesis seems legitimate and confirms the opinion of Zohary (1973). Consequently, the colonization of Cyprus by the ancestral form of *Rana* took place 2.4 (±0.20) Mya, possibly enabled by the land bridge connecting the island with Anatolia, indicating that overland dispersal is a conceivable explanation for the presence of water frogs on Cyprus.

Acknowledgments

We are grateful to Dr. K. Sotiropoulos for providing Rana specimens from Peloponnesos (Rkurt11, Rkurt13, Rep2) and Cyprus (Rbed8, Rbed9) and Mr. P. Georgiakakis for collecting samples from Northeastern Greece (Rrid4, Rrid11).

References

Akaike, H., 1974. A new look at the statistical model identification. IEEE Trans. Automat. Contr. 19, 716–723.

Avise, J.C., 1994. Molecular Markers. Natural History and Evolution. Chapman & Hall, New York.

Avise, J.C., 2000. Phylogeography. The History and Formation of Species. Harvard University Press.

Avise, J.C., Arnold, J., Ball, R.M., Bermingham, E., Lamb, T., Neigel, J.E., Reeb, C.A., Saunders, N.C., 1987. Intraspecific phylogeography: the mitochondrial DNA bridge between population genetics and systematics. Ann. Rev. Ecol. Syst. 18, 489–522.

Baranescu, P., 1983. On the affinities and derivation of the aquatic fauna of high Asia. Rev. Roum. Biol. Biol. Anim. 28, 97–101.

Beerli, P., Hotz, H., Tunner, H.G., Heppich, S., Uzzell, T., 1994. Two new water frog species from the Aegean islands Crete and Karpathos, Amphibia, Salientia, Ranidae. Notulae Naturae, Acad. Nat. Sci. Philadelphia 470, 1–9.

Beerli, P., Hotz, H., Uzzell, T., 1996. Geologically dated sea barriers calibrate a protein clock for Aegean water frogs. Evolution 50 (4), 1676–1687.

Blondel, J., Aronson, J., 1999. Biology and Wildlife of the Mediterranean Region. Oxford University Press, New York.

Bossuyt, F., Milinkovitch, M.C., 2001. Amphibians as indicators of Early Tertiary ''Out-of-India'' dispersal of vertebrates. Science 292, 93–95.

Bremer, K., 1994. Branch support and tree stability. Cladistics 10, 295–304.

Brown, R.F., Guttman, S.I., 2002. Phylogenetic systematics of the *Rana signata* complex of Philippine and Bornean stream frogs: reconsideration of Huxley's modification of Wallace's line at the Oriental–Australian faunal zone interface. Biol. J. Linn. Soc. 76, 393–461.

Caccone, A., Milinkovitch, M.C., Sbordoni, V., Powell, J.R., 1997. Mitochondrial DNA rates and biogeography in European newts, genus *Euproctus*. Syst. Biol. 46, 126–144.

Casola, C., Marracci, S., Bucci, S., Ragghianti, M., Mancino, G., Hotz, H., Uzzell, T., Guex, G.-D., 2004. A hAT-related family of interspersed repetitive elements in genomes of western Palaearctic water frogs. J. Zool. Syst. Evol. Res. 42, 234–244.

Creutzburg, N., 1963. Paleogeographic evolution of Crete from Miocene till ourdays. Cretan Annals (15/16), 336–342.

Crochet, P.-A., Dubois, A., Ohler, A., Tunner, H., 1995. *Rana* (*Pelophylax*) *ridibunda* Pallas, 1771, *Rana* (*Pelophylax*) *perezi* Seoane, 1885 and their associated klepton (Amphibia, Anura): morphological diagnoses and description of a new taxon. Bull. Mus. Natl. Hist. Nat. Paris 17 (1–2), 11–30.

Daams, R., Van Der Weerd, A.V., 1980. Early Pliocene small mammals from the Aegean island of Karpathos (Greece) and their paleogeographic significance. Geol. Mijnbouw 59 (4), 327–331.

Dermitzakis, D.M., 1990. Paleogeography, geodynamic processes and event stratigraphy during the Late Cenozoic of the Aegean area. Accademia Nazionale de. Lincei 85, 263–288.

Dermitzakis, D.M., Papanikolaou, D.J., 1981. Paleogeography and geodynamics of the Aegean region during the Neogene. Ann. Geol. Pays Hellen. 30, 245–289.

Douris, V., Cameron, R.A.D., Rodakis, G.C., Lekanidou, R., 1998. Mitochondrial phylogeography of the land snail Albinaria in Crete: long-term geological and short-term vicariance effects. Evolution 52, 116–125.

Dubois, A., 1992. Notes sur la classification des Ranidae (Amphbiens Anoures). Bull. mens. Soc. linn. Lyon 61 (10), 305–352.

Dubois, A., Ohler, A., 1994. Frogs of the subgenus *Pelophylax* (Amphibia, Anura, Genus *Rana*): a catalogue with comments on name-bearing types, complete synonymies, proposed common names, and maps showing all type localities. Zool. Poloniae 39, 139–204.

Duellman, W.E., Trueb, L., 1986. Biology of Amphibians. McGraw–Hill, New York.

Eriksson, T., 2001. AutoDecay ver. 5.0, program distributed by the author. Bergius Foundation, Royal Swedish Academy of Sciences, Stockholm.

Eriksson, T., 2003. The r8s bootstrap kit. Computer software distributed by the author. Bergius Foundation, Royal Swedish Academy of Sciences, Stockholm. URL: http://www.bergianska.se/forskning_software.html.

Farris, J.S., Kaellersjo, M., Kluge, A.G., Bults, C., 1995. Testing significance of incongruence. Cladistics 10, 315–319.

Felsenstein, J., 1981. Evolutionary trees from DNA sequences: a maximum likelihood approach. J. Mol. Evol. 17, 368–376.

Felsenstein, J., 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39 (4), 783–791.

Felsenstein, J., 2004. Inferring Phylogenies. Sinauer Associates, Sunderland, Massachusetts.

Gillooly, J.F., Allen, A.P., West, G.B., Brown, J.H., 2005. The rate of DNA evolution: effects of body size and temperature on the molecular clock. Proc. Natl. Acad. Sci. USA 102 (1), 140–145.

Goldman, N., Anderson, J.P., Rodrigo, A.G., 2000. Likelihood-based tests of topologies in phylogenetics. Syst. Biol. 17, 652–670.

Günther, R., 1997. *Rana lessonae*. In: Gasc, J.P., Cabela, A., Crnobrnja-Isailovic, J., Dolmen, D., Grossenbacher, K., Haffner, P., Lescure, J., Martens, H., Martinez Rica, J.P., Maurin, H., Oliveira, M.E., SoWanidou, T.S., Veith, M., Zuiderwijk, A. (Eds.), Atlas of Amphibians and Reptiles in Europe. Societas Europaea Herpetologica and Museum National d' Histoire Naturelle, IEGB/SPN., Paris, pp. 148–149.

Günther, R., 1998. Probleme der Abgrenzung und taxonomischen Bewertung von neuen Formen der westpaläarktischen Wasserfrosch-Gruppe (Amphibia: Anura: Ranidae). Zool. Abh. Mus. Tierk. Dresden 50 (9), 99–108.

Hadjisterkotis, E., 1993. The Cyprus mouflon *Ovis gmelini ophion*, management conservation and evolution. Ph.D. thesis. McGill University, Montreal.

Hasegawa, M., Kishino, H., Yano, T., 1985. Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. J. Mol. Evol. 22, 160–174.

Hillis, D.M., Huelsenbeck, J.P., 1992. Signal, noise, and reliability in molecular phylogenetic analysis. J. Hered. 83, 189–195.

Hillis, D.M., Wilcox, T.P., 2005. Phylogeny of the New World true frogs (Rana). Mol. Phyl. Evol. 34 (2), 299–314.

Hotz, H., Beerli, P., Spolsky, C., 1992. Mitochondrial DNA reveals formation of nonhybrid frogs by natural matings between hemiclonal hybrids. Mol. Biol. Evol. 9 (4), 610–620.

Hsü, K.J., 1983. The Mediterranean was a desert. A Voyage of the Glomar Challenger. Princeton University Press, Princeton.

Huelsenbeck, J.P., Ronquist, F.P., 2001. MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17, 754–755.

Inger, R.F., Voris, H.K., 2001. The biogeographical relations of the frogs and snakes of Sundaland. J. Biogeogr. 28, 863–891.

Kosuch, J., Vences, M., Dubois, A., Ohler, A., Bohme, W., 2001. Out of Asia: mitochondrial DNA evidence for an Oriental origin of tiger frogs, genus *Hoplobatrachus*. Mol. Phyl. Evol. 21 (3), 398–407.

Kraus, F., Campbell, E.W., Allison, A., 1999. Eleutherodactylus frog introductions to Hawaii. Herpetol. Rev. 30, 21–25.

Küss, S., 1973. Die pleistozaenem Saeugetierfaunen der ostmediterranen Inseln. Ihr Alter und ihre Herkunft. Beriche der Naturforscheden Gesellschaft zu Freiburg im Breisgau 63, 49–71.

Macey, J.R., Schultell, J.A., Larson, A., Fang, Z., Wang, Y., Tuniyev, B.S., Papenfuss, T.J., 1998. Phylogenetic relationships of toads in the *Bufo bufo* species group from the Eastern Escarpment of the Tibetan plateau: a case of vicariance and dispersal. Mol. Phylogenet. Evol. 9, 80–87.

Meirte, D., 1999. Batraciens. In: Louette, M. (Ed.), La faune terrestre de Mayotte. Royal Museum for Central Africa, Tervuren, Belgium, pp. 114–135.

Meulenkamp, J.E., 1985. Aspects of the Late Cenozoic Evolution of the Aegean Region. In: Stanley, D.J., Wezel, F.C. (Eds.), Geological Evolution of the Mediterranean Basin. Springer, New York, pp. 307–321.

Monnerot, M., Dubois, A., Tunner, H., 1985. Mitochondrial DNA polymorphism among *Rana ridibunda*, *Rana lessonae* and *R. kl. esculenta*: preliminary study. Alytes 4, 101–112.

Moores, E.M., Robinson, P.T., Malpas, J., Xenophontos, C., 1984. A model for the origin of the Troodos Massif, Cyprus, and other Mideast Ophiolites. Geology 12 (8), 500–503.

Palikarides, A., 1997. Mediterranean, the youngest of the seas. To periodikon 565, 80–85.

Palumbi, S., 1996. Nucleic Acids II: The Polymerase Chain Reaction. In: Hillis, D.M., Moritz, C., Mable, B.K. (Eds.), Molecular Systematics. Sinauer, Sunderland, MA, pp. 205–248.

Parmakelis, A., Spanos, E., Papagiannakis, G., Louis, C., Mylonas, M., 2003. Mitochondrial DNA phylogeny and morphological diversity in the genus *Mastus* (Beck, 1837): a study in a recent (Holocene) island group (Koufonisi southeast Crete). Biol. J. Linn. Soc. 78, 383–399.

Parmakelis, A., Pfenninger, M., Spanos, L., Louis, C., Mylonas, M., 2005. Inference of a radiation in *Mastus* (Gastropoda, Pulmonata, Enidae) on the island of Crete. Evolution 59 (5), 991–1005.

Parmakelis, A., Stathi, I., Spanos, L., Papagiannakis, G., Louis, C., Mylonas, M., 2006a. Phylogeography of *Iurus dufoureius* (Brulle, 1832) (Scorpiones, Iuridae). J. Biogeogr. 33 (2), 251–260.

Parmakelis, A., Stathi, I., Chatzaki, M., Simaiakis, S., Spanos, L., Louis, C., Mylonas, M., 2006b. Evolution of *Mesobuthus gibbosus* (Brullé, 1832) (Scorpiones: Buthidae) in the North-eastern Mediterranean region. Mol. Ecol. 15, 2883–2894.

Plötner, J., Ohst, T., 2001. New hypotheses on the systematics of the Western Palearctic water frog complex (Anura, Ranidae). Mitt. Mus. Nat. kd. Berl., Zool. Reihe 77, 5–21.

Plötner, J., Ohst, T., Böhme, W., Schreiber, R., 2001. Divergence in mitochondrial DNA of Near Eastern water frogs with special reference to the systematic status of Cypriote and Anatolian populations (Anura, Ranidae). Amphibia-Reptilia 22, 397–412.

Posada, D., Buckley, T.R., 2004. Model selection and model averaging in phylogenetics: advantages of Akaike information criterion and Bayesian approaches over likelihood ratio tests. Syst. Biol. 53, 793–808.

Posada, D., Crandall, K.A., 1998. Modeltest: testing the model of DNA substitution. Bioinformatics 14, 817–818.

Poulakakis, N., Lymberakis, P., Antoniou, A., Chalkia, D., Zouros, E., Mylonas, M., Valakos, E., 2003. Molecular phylogeny and biogeography of the wall-lizard *Podarcis erhardii* (Squamata: Lacertidae). Mol. Phyl. Evol. 28, 38–46.

Poulakakis, N., Lymberakis, P., Tsigenopoulos, C.S., Magoulas, A., Mylonas, M., 2005a. Phylogenetic relationships and evolutionary history of snake-eyed skink *Ablepharus kitaibelii* (Sauria: Scincidae). Mol. Phyl. Evol. 34 (2), 245–256.

Poulakakis, N., Lymberakis, P., Valakos, E., Pafilis, P., Zouros, E., Mylonas, M., 2005b. Phylogeography of Balkan wall lizard (*Podarcis taurica*) and its relatives inferred from mitochondrial DNA sequences. Mol. Ecol. 14 (8), 2433–2443.

Poulakakis, N., Lymberakis, P., Valakos, E., Zouros, E., Mylonas, M., 2005c. Phylogenetic relationships and biogeography of *Podarcis* species from the Balkan Peninsula, by Bayesian and maximum likelihood analyses of mitochondrial DNA sequences. Mol. Phyl. Evol. 37 (3), 845–857.

Richards, C.M., Moore, W.S., 1996. A phylogeny for the African tree-frog family Hyperoliidae based on mitochondrial rDNA. Mol. Phyl. Evol. 5, 522–532.

Rodriguez, F., Oliver, J.F., Marin, A., Medina, J.R., 1990. The general stochastic model of nucleotide substitutions. J. Theor. Biol. 142, 485–501.

Ronquist, F., Huelsenbeck, J.P., 2003. MRBAYES 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19, 1572–1574.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor NY.

Sanderson, M.J., 1997. A nonparametric approach to estimating divergence times in the absence of rate constancy. Mol. Biol. Evol. 14, 1218–1231.

Sanderson, M.J., 2003. r8s: inferring absolute rates of molecular evolution, divergence times in the absence of a molecular clock. Bioinformatics 19 (2), 301–302.

Schneider, H., Sinsch, U., Sofianidou, T.S., 1993. The water frogs of Greece—bioacoustic evidence for a new species. Z. Zool. Syst. Evol. Forsch. 31, 47–63.

Shimodaira, H., Hasegawa, M., 1999. Multiple comparisons of log-likelihoods with applications to phylogenetic inference. Mol. Biol. Evol. 16, 1114–1116.

Snell, H.L., Rea, S., 1999. El Nino 1997–1998 en Galapagos: se puede estimar 120 anos de variaciones climaticas con estadisticas de 34? In: Ospina, P., Munoz, E. (Eds.), Informe Galapagos 1998–1999. Fundacion Natura, Quito, Ecuador, pp. 65–71.

Sondaar, P.Y., 1977. Insularity and its effect on mammal evolution. In: Hecht, M.K., Goody, P.L., Hecht, B.M. (Eds.), Major Patterns in Vertebrate Evolution. Plenum Publ. Co., New York, pp. 671–707.

Sondaar, P.Y., 1986. The island sweepstakes. Nat. History 95, 91–98.

Spolsky, C., Uzzell, T., 1984. Natural interspecies transfer of mitochondrial DNA in amphibians. Proc. Natl. Acad. Sci. USA 81, 5802–5805.

Stace, C.A., 1989. Dispersal versus vicariance—no contest. J. Biogeogr. 16, 201–202.

Staub, F., 1993. Fauna of Mauritius and associated flora. Precigraph, Mauritius.

Steininger, F.F., Rögl, F., 1984. Paleogeography and palinspastic reconstruction of the Neogene of the Mediterranean and Paratethys. In: Dixon, J.E., Robertson, A.H.F. (Eds.), The Geological Evolution of the Eastern Meidterranean. Blackwell Scientific Publications, pp. 659–668.

Sullivan, J., Abdo, Z., Joyce, P., Swofford, D.L., 2005. Evaluating the performance of a successive-approximations approach to parameter optimization in maximum-likelihood phylogeny estimation. Mol. Biol. Evol. 22 (6), 1386–1392.

Sumida, M., Ogata, M., Nishioka, M., 2000. Molecular phylogenetic relationships of pond frogs distributed in the Palearctic region inferred from DNA sequences of mitochondrial 12S ribosomal RNA and cytochrome *b* genes. Mol. Phyl. Evol. 16 (2), 278–285.

Swofford, D.L., 2002. PAUP*: Phylogenetic Analysis Using Parsimony, * and Other Methods. 4.0b10a. Sinauer Associates, Sunderland, Massachusetts.

Swofford, D.L., Olsen, G.J., Waddel, P.J., Hillis, D.M., 1996. Phylogenetic inference. In: Hillis, D.M., Moritz, C., Mable, B.K. (Eds.), Molecular Systematics. Sinauer, Sunderland, MA, pp. 407–514.

Tanaka, T., Matsui, M., Takenaka, O., 1994. Estimation of phylogenetic relationships among Japanese Brown frogs from mitochondrial cytochrome *b* gene (Amphibia, Anura). Zool. Sc. (Tokyo) 11, 753–757.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G., 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tool. Nucleic Acids Res. 24, 4876–4882.

Veith, M., Steinfartz, S., Zardoya, R., Seitz, A., Meyer, A., 1998. A molecular phylogeny of true salamanders (family Salamandridae) and the evolution of terrestriality of reproductive modes. J. Zool. Syst. Evolut. Res. 36, 7–16.

Veith, M., Kosuch, J., Vences, M., 2003a. Climatic oscillations triggered post-Messinian speciation of Western Palaearctic brown frogs (Amphibia, Ranidae). Mol. Phyl. Evol. 26 (2), 310–327.

Veith, M., Schmidtler, J.F., Kosuch, J., Baran, I., Seitz, A., 2003b. Palaeoclimatic changes explain Anatolian mountain frog evolution: a test for alternating vicariance and dispersal events. Mol. Ecol. 12 (1), 185–199.

Vences, M., Vieites, D.R., Glaw, F., Brinkmann, H., Kosuch, J., Veith, M., Mayer, A., 2003. Multiple overseas dispersal in amphibians. Proc. R. Soc. Lond. B 270, 2435–2442.

Worthy, T.H., Anderson, A.J., Molnar, R.E., 1999. Megafaunal expression in a land without mammals: the first fossil faunas from terrestrial deposits in Fiji (Vertebrata: Amphibia, Reptilia, Aves). Senck. Biol. 79, 237–242.

Xia, X., Xie, Z., 2001. DAMBE: data analysis in molecular biology and evolution. J. Hered. 92, 371–373.

Zohary, M., 1973. Geobotanical foundations of the Middle East. Gustav Fischer Verlag, Stuttgart.