ACADEMIC PRESS

Available online at www.sciencedirect.com

SCIENCE DIRECT®

Molecular Phylogenetics and Evolution 26 (2003) 310–327

MOLECULAR PHYLOGENETICS AND EVOLUTION

www.elsevier.com/locate/ympev

Climatic oscillations triggered post-Messinian speciation of Western Palearctic brown frogs (Amphibia, Ranidae)

M. Veith,[a,*] J. Kosuch,[a] and M. Vences[b]

[a] *Institut für Zoologie, Universität Mainz, Saarstraße 2 Mainz D-55099, Germany*
[b] *Institute for Biodiversity and Ecosystem Dynamics, PO Box 94766, Amsterdam 1090 GT, The Netherlands*

Received 2 July 2001; revised 12 July 2002

Abstract

Oscillating glacial cycles over the past 2.4 million years are proposed to have had a major impact on the diversity of contemporary species communities. We used mitochondrial and nuclear DNA sequence data to infer phylogenetic relationships within Western Palearctic brown frogs and to test the influence of Pliocene and Pleistocene climatic changes on their evolution. We sequenced 1976 bp of the mitochondrial genes 16S rRNA and cytochrome *b* and of the nuclear rhodopsin gene for all current species and subspecies. Based on an established allozyme clock for Western Palearctic water frogs and substitution rate constancy among water frogs and brown frogs, we calibrated a molecular clock for 1425 bp of the 16S and rhodopsin genes. We applied this clock to date speciation events among brown frogs. Western Palearctic brown frogs underwent a basal post-Messinian radiation about 4 million years ago (mya) into five major clades: three monotypic lineages (*Rana dalmatina*, *Rana latastei*, *Rana graeca*), an Anatolian lineage, and a lineage comprising *Rana italica*, *Rana arvalis*, and all Iberian taxa. Polytypic lineages radiated further in concordance with the onset of climatic oscillations ca. 3.2, 2.0, and 1.0–0.6 mya, respectively. The dated fossil record corroborates our paleobiogeographic scenario. We conclude that drastic climatic changes followed by successive temperature oscillations "trapped" most brown frog species in their southern European glacial refugia with enough time to speciate. Substantial dispersal was only possible during extensive interglacial periods of a constant subtropical climate.

Keywords: Phylogeny; Speciation; Vicariance; Glacials; Interglacials; Molecular clock; Palearctic brown frogs; *Rana*

1. Introduction

Species evolve either instantaneously or through three processes of gradual speciation: allopatric, sympatric, and parapatric speciation (Mayr and Ashlock, 1991). Despite recent empirical and theoretical evidence for sympatric speciation mechanisms (e.g., Dieckmann and Doebeli, 1999; Schliewen et al., 1994) the significance of parapatric and sympatric speciation is a controversial issue (e.g., Bush, 1994). Allopatric speciation is usually regarded the major process of species diversification (Coyne and Orr, 1989).

In an allopatric speciation scenario, climatic changes are often invoked to explain the break-up of distribution areas and subsequent evolution of separated lineages. In particular, the large series of climatic changes since the Pliocene has markedly shaped the present-day species communities of the Northern hemisphere (Blondel and Aronson, 1999). The Praetiglian cold stage, starting at 2.3–2.4 mya, brought to an end the long period of Tertiary forests with their rich floristic diversity in northwestern Europe (West, 1988). It was initiated by an abrupt glaciation of substantial scale in the Northern hemisphere, although it was preceded by small increases in ice volume at ca. 3.0 and 2.55 mya (Ruddiman and Raymo, 1988). Smaller climatic fluctuations followed throughout the Late Pliocene. Calcareous nannoplankton records indicate a relatively warm period with less pronounced temperature fluctuations during the early Pleistocene (Müller, 1985; Ruddiman and Raymo, 1988). During the last 700.000 years climatic oscillations, followed by the growth and retreat of the Arctic

* Corresponding author. Fax: +49-6131-3923731.
E-mail address: michael@oekologie.biologie.uni-mainz.de (M. Veith).

1055-7903/02/$ - see front matter © 2002 Elsevier Science (USA). All rights reserved.
PII: S1055-7903(02)00324-X

ice sheet, have been mainly the result of orbital eccentricity with a roughly 100 ka cycle (Milankovitch oscillations; Hays et al., 1976). This caused changes in insolation. Along with lesser variation in axial tilt (40 ka) and precession (23 ka) they produced a complex pattern of climatic oscillations (e.g., Ruddiman and Raymo, 1988).

Oscillating climatic shifts were greater towards the poles. Consequently, they initiated tremendous species range dynamics in Europe and Northern America (e.g., Coope, 1994). Pliocene and Pleistocene temperature changes often occurred very rapidly (Lowe and Walker, 1997), leading to a negative effect on gradual speciation in northern Europe (Dynesius and Jansson, 2000). In contrast, they probably promoted divergence and speciation in southern refugia (Taberlet et al., 1998).

Classical vicariance theory (Rosen, 1978) predicts that the phylogenetic relationships among taxa in an area should mirror successive rises of gene flow barriers among glacial refugia. Amphibians are ideal for testing such vicariance hypotheses, as has been repeatedly shown for the Mediterranean fauna (e.g., Oosterbroek and Arntzen, 1992; Taberlet et al., 1998). They disperse terrestrially; marine or even air-bound dispersal is almost impossible. Since each species individually responds to climatic changes (Hewitt, 1996), speciose taxa allow for a more differentiated evaluation of the effect of climatic deterioration or amelioration. Among Western Palearctic amphibians, the frog genus *Rana* represents the most specious genus. Of its 20 species, nine are comprised in the subgenus *Pelophylax* (Dubois, 1992), the so-called European water frogs. The remaining species represent the western members of the Palearctic subgenus *Rana*, the so-called brown frogs. So far, the distinction of Western and Eastern Palearctic brown frogs has simply been based on biogeographical grounds.

In contrast to the semi-aquatic water frogs, brown frogs are largely terrestrial, with an aquatic larval stage. They are usually found in montane habitats, and while some species are explicit generalists (e.g., *Rana tempo-*

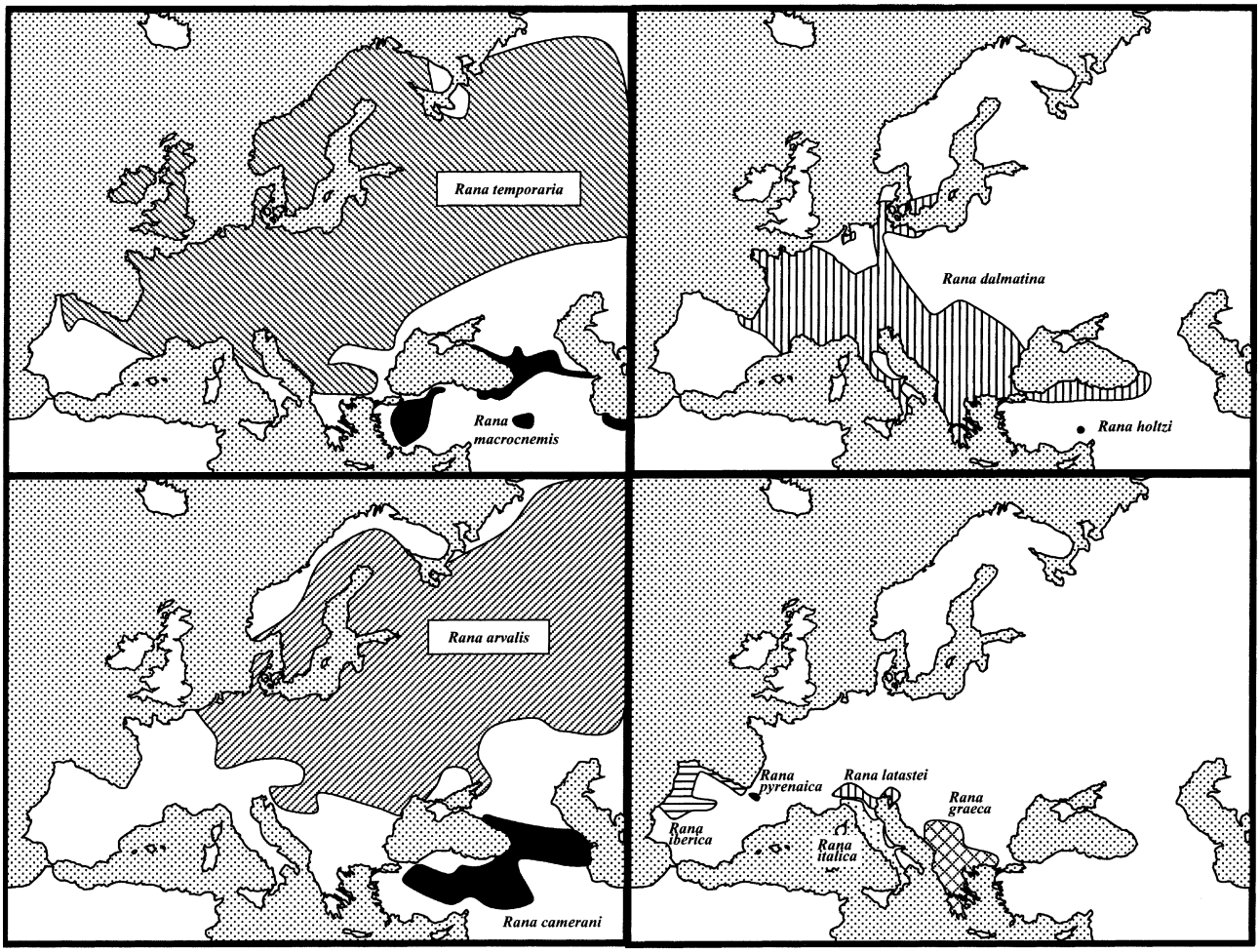

Fig. 1. Distributions of currently recognised species of Western Palearctic brown frogs (after Gasc et al., 1997; Demirsoy, 1996; Kuzmin, 1999). Upper left: *Rana temporaria* (hatched) and *R. macrocnemis* (black). Upper right: *R. dalmatina* (hatched) and *R. holtzi* (dot). Lower left: *R. arvalis* (hatched) and *R. camerani* (black). Lower right: Atlanto-Iberian and Mediterranean endemics: *R. iberica* (horizontally hatched), *R. pyrenaica* (black), *R. latastei* (vertically hatched), *R. italica* (dotted), and *R. graeca* (cross-hatched).

raria breeds in all kinds of stagnant and moderately running water and occurs from lowland habitats up to 2745 m a.s.l. in the Alps), others are specialists (e.g., *Rana graeca* is restricted to fast running mountain brooks of the southern Balkans and adjacent regions).

Western Palearctic brown frogs occur all over Europe and Asia Minor (Gasc et al., 1998; Baran and Atatür, 1998). With the exception of *R. graeca* Boulenger, 1891, *Rana italica* Dubois, 1985, and *Rana dalmatina* Bonaparte, 1840, they are absent from the Mediterranean climatic zone (as defined by Blondel and Aronson, 1999: Fig. 1.5), including islands (only *R. dalmatina* occurs on Sicily and some Dalmatian and Ionian islands; Lanza and Vanni, 1987). According to their distribution, they can be divided into four groups (Fig. 1): (i) wide-spread European species: *R. temporaria* Linnaeus, 1758; *R. dalmatina*; *Rana arvalis* Nilsson, 1842 (the latter with a strong affiliation to Asia); (ii) Ibero-Atlantic endemics: *R. iberica* Boulenger, 1879; *R. pyrenaica* Serra-Cobo, 1993; (iii) Mediterranean endemics: *R. graeca*; *R. italica*; *Rana latastei* Boulenger, 1879; and (iv) Minor Asian and Caucasian endemics: *Rana camerani* Boulenger, 1886; *R. holtzi* Werner, 1898; *R. macrocnemis* Boulenger, 1885.

The phylogeny of Western Palearctic brown frogs has repeatedly been studied using allozymes, although only for subsets of taxa (Arano et al., 1993; Green and Borkin, 1993; Mensi et al., 1992; Picariello et al., 1990). A comprehensive study that includes all currently known and accepted species is still lacking. Therefore, Barbadillo et al. (1997) summarised the scattered knowledge on the phylogenetic relationships of Western Palearctic brown frog species to infer a reconciled tree. However, *R. pyrenaica*, a recently described species (Serra-Cobo, 1993) was included in their tree without electrophoretic evidence. We therefore used recently published allozyme data (Veith et al., 2002) to propose another tree depicting the presumed relationships of Western Palearctic brown frogs (Fig. 2). This tree will serve as reference to test phylogenetic hypotheses: are Western Palearctic brown frogs monophyletic? If not, as suggested by Barbadillo et al. (1997), can we define major monophyletic lineages within Western Palearctic brown frogs?

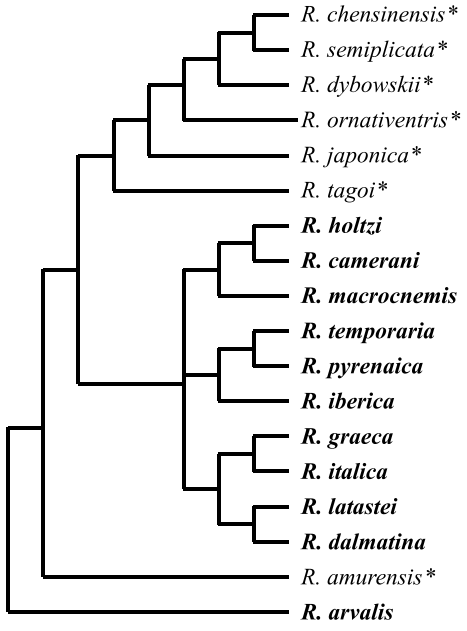

Fig. 2. Hypothesis on the phylogenetic relationships among Western Palearctic (in bold face) and some Eastern Palearctic (indicated by asterisks) brown frog species (modified after Barbadillo et al., 1997).

To link speciation events to paleoclimate we calibrated and applied a molecular clock. If in fact onsets of climatic oscillations were responsible for brown frog speciation (Dynesius and Jansson, 2000) we would expect a concentration of speciation events at the beginning of each oscillation interval rather than a random distribution of species splits across periods of climatic oscillations.

2. Materials and methods

2.1. Samples

We studied all Western Palearctic brown frog taxa that are currently acknowledged (Appendix A). For outgroup rooting we used *Rana* (*Pelophylax*) *bedriagae* from Turkey and the Nearctic *Rana* (*Aquarana*) *catesbeiana* which belong to related subgenera within the genus *Rana* (Dubois, 1992). All sequences generated for this study are deposited in GenBank (see Appendix A for Accession Numbers).

2.2. Preparation of DNA templates, PCR, and DNA sequencing

Tissues were obtained from either fresh, frozen, or ethanol preserved specimens. DNA was extracted using the QiAmp tissue extraction kits (Qiagen). PCR was used to amplify fragments of the mitochondrial 16S rRNA (two separate portions) and cytochrome *b* genes as well as for part of the nuclear rhodopsin gene. The primers and cycling procedures were:

(i) *16S-1*: 16SA (light chain; 5′-CGC CTG TTT ATC AAA AAC AT-3′) and 16SB (heavy chain; 5′-CCG GTC TGA ACT CAG ATC ACG T-3′) of Palumbi et al. (1991) amplified a ca. 560 bp section of the mitochondrial 16S ribosomal RNA gene. PCR cycling procedure was as follows. Initial denaturation step: 90 s at 94 °C; 33 cycles: denaturation 45 s at 94 °C, primer annealing for 45 s at 55 °C, extension for 90 s at 72 °C.

(ii) *16S-2*: 16L3 (light chain; 5′-AGC AAA GAH YWW ACC TCG TAC CTT TTG CAT-3′) modified

from Hedges (1994) and 16SA-H (heavy chain; reverse complement of 16SA) amplified a ca. 680 bp section of the mitochondrial 16S ribosomal RNA gene; PCR cycling procedure was the same as for 16S-1.

(iii) Cyt *b*: *L14841* (light chain; 5′-CTC CCA GCC CCA TCC AAC ATC TCA GCA TGA TGA AAC TTC G-3′), modified from Kocher et al. (1989), and CB3-H (heavy chain; 5′-GGC AAA TAG GAA GTA TCA TTC TG-3′), modified from Palumbi et al. (1991), amplified a ca. 700 section of the mitochondrial cytochrome *b* (cyt *b*) gene. PCR cycling procedure was as follows. Initial denaturation step: 120 s at 94 °C; 34 cycles: denaturation 45 s at 92 °C, primer annealing for 60 s at 50 °C, extension for 90 s at 72 °C.

Rhodopsin: rhod1-a (5′-ACC ATG AAC GGA ACA GAA GGY CC-3′) and rhod1-d (5′- GTA GCG AAG AAR CCT TCA AMG TA-3′) of Bossuyt and Milinkovitch (2000) amplified a ca. 380 bp section of the rhodopsin gene (rhod in Appendix A); PCR cycling procedure was as follows. Thirty-five cycles: denaturation 45 s at 94 °C, primer annealing for 45 s at 55 °C, extension for 60 s at 72 °C.

PCR products were purified using the Qiaquick purification kit (Qiagen). We sequenced single-stranded fragments on an ABI 377 automatic sequencer using Applied Biosystems standard protocols.

Obtained mitochondrial DNA sequences (lengths referring to the aligned sequences including gaps) comprised 540 and 643 bp (16S), and 465 bp (cyt *b*) homologous to base pair positions 4004–4565, 3278–3942, and 16420–16885 of the *Xenopus laevis* mitochondrial genome (Roe et al., 1985). The rhodopsin fragment was 327 bp long. We consistently achieved 1976 bp for all samples. Sequences were aligned automatically using the clustal option of the SEQUENCE NAVIGATOR software (Applied Biosystems). The 16S alignment was slightly refined by eye in the loop regions.

Partial sequences of the 16S rRNA (equivalent to our 16S-1 fragment) and the cytochrome *b* genes for five species of Eastern Palearctic brown frogs were accessible through GenBank (Appendix A). We aligned them to the homologous sequences of our samples to test for monophyly of Western Palearctic species.

To test for saturation of genes we plotted uncorrected *p* distances versus TrN distances for 16S, rhodopsin, and first, second and third codon positions of cytochrome *b* following the approach of Reed and Sperling (1999).

2.3. Phylogenetic analysis

We determined the number, nature, and distribution of base substitutions. To assess the amount of phylogenetic signal we generated 10^6 random trees and calculated the skewness (g_1) and kurtosis (g_2) of the resulting tree length distribution (with PAUP*; Swofford, 2001).

To gain confidence in a combined phylogenetic analysis of mitochondrial and nuclear gene fragments we tested for congruence among data partitions (Huelsenbeck et al., 1996). We used the implementation of the parsimony method of Farris et al. (1995) in PAUP* (100 replicates, heuristic search using the TBR branch swapping algorithm).

Conventional tree-building methods from nucleotide sequences can be unreliable when the base composition of taxa varies between sequences (Steel et al., 1993). Varying base compositions necessitate the application of the LogDet transformation that allows to consistently recover the correct tree when sequences evolve under simple asymmetric models that can vary between lineages (Lockhart et al., 1994). We therefore used PAUP* to perform χ^2 tests for equal base composition between sequences. Equal base composition between sequences would allow us to apply substitution models that fit our data best (as recently emphasised by Sullivan and Swofford, 2001).

We applied a hierarchical likelihood ratio test for the goodness-of-fit of nested substitution models (for ingroup taxa only). Using the program MODELTEST (Posada and Crandall, 1998) we calculated the test statistic $\delta = 2\log \Lambda$ with Λ being the ratio of the likelihood of the null model divided by the likelihood of the alternative model (for details see Huelsenbeck and Crandall, 1997). Due to the performance of multiple tests, we adjusted the significance level of rejection of the null hypothesis via the sequential Bonferroni correction to $\alpha = 0.01$ (Rice, 1989).

The data were subjected to four different methods of phylogenetic reconstruction: (i) neighbor joining (NJ) (Saitou and Nei, 1987) using the TrN + I + Γ substitution model; (ii) maximum parsimony with gaps treated

Table 1
Alignment statistics for fragments of the 16S rRNA, the cytochrome *b*, the rhodopsin gene and combined alignments; number of base pairs (bp), number of variable sites (vs), number of phylogenetically informative sites (pi); base frequencies (π_A, π_G, π_T, π_C), skewness (g_1), kurtosis (g_2). The transition-transversion ratio (ti/tv) is given for ingroups only, [1] used for molecular clock calibration only

Alignment	bp	vs	pi	π_A	π_G	π_T	π_C	g_1	g_2	ti/tv
16S	1184	317	182	0.374	0.183	0.243	0.236	0.69	0.49	4.82
Cytochrome *b*	465	169	132	0.239	0.162	0.306	0.292	0.65	0.34	6.11
Rhodopsin	327	23	14	0.268	0.178	0.308	0.246	0.81	0.66	2.85
16S + rhodopsin[1])	1511	354	204	0.321	0.210	0.245	0.223	0.68	0.45	4.63
16S + cyt *b* + rhodopsin	1976	509	328	0.318	0.176	0.255	0.251	0.67	0.40	5.20

as fifth character state; transitions and transversions were given equal weight; heuristic search with the TBR branch swapping algorithm; (iii) maximum likelihood (ML) analysis based on the $\mathrm{TrN}+I+\Gamma$ substitution model, (iv) Bayesian inference (Huelsenbeck et al., 2001; Rannala and Yang, 1996), which is similar to ML and is based upon the notion of posterior probabilities of a phylogenetic tree. With the exception of the Bayesian approach (MrBayes; Huelsenbeck and Ronquist, 2001), all analyses were done with PAUP* (Swofford, 2001). Robustness of NJ and MP tree topologies was tested by bootstrap analyses (Felsenstein, 1985), with 2000 replicates each (Hedges, 1992). Only bootstrap values $\geqslant 70\%$ indicate sufficiently resolved topologies (Huelsenbeck and Hillis, 1993), those between 50 and 70% were regarded as tendencies. Despite some reasonable criticism (Cao et al., 1998) but due to computational constraints, we used Quartet Puzzling (Strimmer and von Haeseler, 1996) with 2000 permutations to infer reliability values (which are usually slightly higher than bootstrap values; Cao et al., 1998) for ML tree topologies. We applied the Bayesian method using the general time reversible model of nucleotide substitution (GTR; Rodríguez et al., 1990; the TrN model is not available in MRBAYES) with a gamma shape parameter estimated for eight rate categories of equal weight from the data. We run four simultaneous Metropolis-coupled Monte-Carlo Markov chains for 500,000 generations. We sampled a tree every 100 generations and calculated a consensus topology for 4000 trees by omitting the first 1000 trees (burn-in).

For the combined analysis of Western and Eastern Palearctic brown frogs sequences (16S and cytochrome *b*) we applied the Bayesian method, using the same settings as outlined above.

2.4. Statistical test of competing phylogenetic tree hypotheses

A priori chosen tree topologies were tested against the alternative topology of the NJ tree using the non-parametric likelihood ratio test of Shimodaira and Hasegawa, 1999; likelihood settings: HKY85 substitution model, ti/tv ratio = 2, empirical base frequencies; one-tailed SH test using 1000 RELL bootstrap replicates) as implemented in PAUP* (Swofford, 2001). This modification of the Kishino–Hasegawa test (Kishino and Hasegawa, 1989) corrects for multiplicity of testing and appears to be very conservative in rejecting topologies as untrue (Whelan et al., 2001). To avoid overrepresentation of polytypic lineages we used a reduced data set with *Rana macrocnemis macrocnemis*, *R. m. tavasensis*, and *R. m. pseudodalmatina* for the clade of Anatolian mountain frogs and the respective nominotypic subspecies as representatives of *R. temporaria* and *R. arvalis*. We kept the topology of the NJ tree as constant as possible. For some hypotheses several alternative topologies had to be considered (trees are given in Table 2):

(1) Monophyly of all species living in the Iberian peninsula (*R. temporaria*, *R. pyrenaica*, and *R. iberica*).
(2) *Rana graeca* and *R. italica* form a monophylum; they had originally been regarded as conspecific (Dubois, 1985); two alternative topologies.
(3) *Rana iberica* is the sister taxon of *R. temporaria*; this is the original hypothesis of Barbadillo et al. (1997); two alternative topologies.
(4) *Rana arvalis* is ancestral to all other Western Palearctic taxa; this is assumed by Barbadillo et al. (1997) based on its $2n = 24$ chromosome number (all other Western Palearctic species: $2n = 26$).

2.5. Test for substitution rate constancy and molecular clock calibration

A test for substitution rate constancy (molecular clock test) was performed using TREE-PUZZLE (Schmidt et al., 2000), with *R. bedriagae* as the root for Western Palearctic brown frogs.

Concerns against the application of molecular clocks have repeatedly been published (e.g., Scherer, 1990), and substitution rate heterogeneity among taxa is obvious

Table 2
Shimodaira–Hasegawa test of alternative tree topologies against the NJ tree topology; the asterisk indicates topologies significantly worse than the best tree ($p < 0.05$)

Tree	$-\ln L$	Diff $-\ln L$	p
NJ tree	7386.70	0.67	0.882
1	7386.03	(Best tree)	
2a	7418.33	32.30	0.026*
2b	7416.78	30.75	0.031*
3a	7399.98	13.94	0.222
3b	7404.52	18.49	0.141
4	7402.07	16.04	0.167

NJ tree = (*P. bedriagae*, (*R. catesbeiana*, ((((*R. m. pseudodalmatina, R. m. tavasensis*), *R. m. macrocnemis*), ((*R. latastei, R. dalmatina*), *R. graeca*)), (((*R. pyrenaica, R. t. temporaria*), *R. a. arvalis*), (*R. italica, R. iberica*))))); tree 1 = (*P. bedriagae*, (*R. catesbeiana*, ((((*R. m. pseudodalmatina, R. m. tavasensis*), *R. m. macrocnemis*), ((*R. latastei, R. dalmatina*), *R. graeca*)), (*R. italica*, (((*R. pyrenaica, R. t. temporaria*), *R. a. arvalis*), *R. iberica*))))); tree 2a = (*P. bedriagae*, (*R. catesbeiana*, (((*R. m. pseudodalmatina, R. m. tavasensis*), *R. m. macrocnemis*), ((*R. latastei, R. dalmatina*), (*R. italica, R. graeca*))), (((*R. pyrenaica, R. t. temporaria*), *R. a. arvalis*), *R. iberica*)))); tree 2b = (*P. bedriagae*, (*R. catesbeiana*, ((((*R. m. pseudodalmatina, R. m. tavasensis*), *R. m. macrocnemis*), (*R. latastei, R. dalmatina*)), (((*R. pyrenaica, R. t. temporaria*), *R. a. arvalis*), ((*R. italica, R. graeca*), *R. iberica*))))); tree 3a = (*P. bedriagae*, (*R. catesbeiana*, ((((*R. m. pseudodalmatina, R. m. tavasensis*), *R. m. macrocnemis*), (*R. graeca*, (*R. latastei, R. dalmatina*))), (((*R. pyrenaica*, (*R. iberica, R. t. temporaria*)), *R. a. arvalis*), *R. italica*)))); tree 3b = (*P. bedriagae*, (*R. catesbeiana*, ((((*R. m. pseudodalmatina, R. m. tavasensis*), *R. m. macrocnemis*), (*R. graeca*, (*R. latastei, R. dalmatina*))), ((*R. pyrenaica, R. a. arvalis*), ((*R. iberica, R. t. temporaria*), *R. italica*))))); tree 4 = (*P. bedriagae*, (*R. catesbeiana*, (*R. a. arvalis*, ((((*R. m. pseudodalmatina, R. m. tavasensis*), *R. m. macrocnemis*), (*R. graeca*, (*R. latastei, R. dalmatina*))), ((*R. pyrenaica, R. t. temporaria*), (*R. iberica, R. italica*)))))).

(e.g., Martin et al., 1992; Mindel et al., 1996; Rand, 1994). Therefore, usage of a clock calibration from closely related taxa is considered more reliable (e.g., Caccone et al., 1997; Pook et al., 2000).

Trustworthy paleogeographic events that can unambiguously be attributed to species splits within Western Palearctic brown frogs are lacking (as outlined above, these species are almost completely missing on Mediterranean islands with their usually well known last connection to the mainland). Therefore, a molecular clock can only be inferred indirectly. Beerli (1994) calibrated an allozyme clock for Aegean and other Mediterranean water frogs (subgenus *Pelophylax* of *Rana*) to 8.14 my/D^*_{Nei}). His calibration can serve as a base line to calibrate a molecular clock. We compared pairwise allozyme genetic distances of Beerli (1994) to 16S and rhodopsin $\mathrm{TrN} + I_{0.5148} + \Gamma_{0.4549}$ distances among species pairs among water frogs (for samples see Appendix A). Methods of sequencing, alignment, and data analysis were the same as above. Cytochrome *b* was excluded from the molecular clock calibration since (i) transitions showed signs of saturation and (ii) two water frog species, *R. epeirotica* and *R. saharica*, repeatedly gave negative results in the PCR.

To test whether the selected gene fragments were suited for molecular clock calibration, we conducted relative rate tests (Takezaki et al., 1995) using PHYLTEST software (Kumar, 1996). All tests were performed with two of the different substitution models implemented in PHYLTEST, namely the Jukes–Cantor and the Kimura 2-parameter model. To test for possible rate heterogeneity between water frogs (as used for calibration) and brown frogs, we compared both groups using *Fejervarya limnocharis* (Dicroglossinae), a distantly related ranid, as an outgroup. Brown frogs were tested both together and separately (with the *R. macrocnemis* complex and the *R. temporaria* and *R. arvalis* subspecies subsumed in single clusters, respectively).

3. Results

Saturation plots of uncorrected sequence divergence against TrN distances are given in Fig. 3. Only the third codon position of cytochrome *b* showed clear evidence for saturation. Beginning saturation was indicated for the 16S rDNA, the rhodopsin gene and the first codon position of the cytochrome *b* gene. We therefore accepted the homoplastic noise related to saturation and kept all base positions for further analyses.

The partition homogeneity test revealed a lack of difference among data partitions ($P = 0.84$). Consequently, we merged gene fragments for further analyses.

The Tamura and Nei (1993) model fitted our combined alignment best ($-\ln L = 7823.99$), with the proportion of invariable sites ($I = 0.5148$) and the gamma distribution shape parameter ($\alpha = 0.4549$) estimated from the data ($\mathrm{TrN} + I + \Gamma$-model). We used empirical base frequencies and the substitution model of $\mathrm{rate}_{[\mathrm{A–G}]} = 9.69$ and $\mathrm{rate}_{[\mathrm{C–T}]} = 17.04$ (all other rates $= 1.0$), estimated from the data.

3.1. Test for monophyly of Western Palearctic brown frogs

In the combined analysis of partial 16S and cytochrome *b* sequences of 19 Western and five Eastern Palearctic brown frog taxa (Bayesian inference), Western Palearctic species came out as a monophylum with respect to Eastern Palearctic species. However, basal splits were poorly resolved (Fig. 4). In the NJ analysis (not shown), basal resolution was even worse. Consequently, monophyly of Western Palearctic species could not unambiguously be proven. This allowed us only to tentatively discuss the phylogenetic relationships among Western Palearctic species. We rather focussed on the evolution of monophyletic species groups within Western Palearctic brown frogs. This does not affect the dating of speciation events, which is based on anagenetic (pairwise sequence divergence), not on cladogenetic processes.

3.2. Sequence and tree statistics

Sequence statistics for the three gene fragments and for the combined alignment are given in Table 1. The g_1 statistics indicated that significant phylogenetic signal was present in all data sets.

All multiple samples of a taxon showed identical sequences. Five hundred and nine out of 1976 bp were variable in the combined alignment, with 328 being parsimony informative. Base frequencies clearly deviated from equal distribution. The average ti/tv ratio varied among genes and was 5.20 in the combined data set when considering only ingroup taxa. This 10-fold bias towards ti's indicated that they were at best slightly saturated among ingroups (see also Fig. 3).

The single most parsimonious tree required 1132 evolutionary steps. For 10^6 random trees we calculated the skewness $g_1 = 0.67$ and kurtosis $g_2 = 0.40$. Tree statistics were as follows: consistency index $\mathrm{CI} = 0.5716$, homoplasy index $\mathrm{HI} = 0.4284$, retention index $\mathrm{RI} = 0.6372$, and rescaled consistency index $\mathrm{RC} = 0.3642$. An adjusted consistency index that corrects for random effects in a data set of a given number of taxa was calculated to 0.4006 from the formula of Klassen et al. (1991): $\mathrm{CI}_{\mathrm{adjusted}} = \mathrm{CI} - \mathrm{CI}_{\mathrm{random}}$, with $\mathrm{CI}_{\mathrm{random}} = 2.9370 \times n_{\mathrm{taxa}}^{-0.9339}$.

3.3. Trees and topology tests

All phylogenetic analyses resulted in essentially the same topologies (only the NJ tree is shown in Fig. 5).

The following clades consistently emerged from all analyses with sufficient resolution when regarding only splits with bootstrap *p*-values $\geqslant 70\%$ as statistically reliable (Huelsenbeck and Hillis, 1993):

(i) A basal multifurcation into five lineages emerged: *R. graeca*, *R. dalmatina*, *R. latastei*, an Anatolian clade, and a clade comprising all remaining taxa (for the time being we name it "*Rana temporaria* species group"). *Rana graeca*, *R. dalmatina* and *R. latastei* form a significant monophylum in the ML and Bayesian trees.

(ii) The *Rana temporaria* species group immediately splits into five sub-lineages: *R. italica*, *R. iberica*, *R. arvalis*, *R. temporaria*, and *R. pyrenaica*; *R. arvalis*, *R. temporaria*, and *R. pyrenaica* form a monophylum; however, *R. pyrenaica* is the sister taxon to a (*R. arvalis R. temporaria*) clade in the Bayesian phylogeny (95% Bayesian posterior probability).

(iii) The polytypic *R. temporaria* is a monophylum (support: 100, 100, 94, and 100% Bayesian posterior probability, respectively) with *R. t. parvipalmata* standing basal to all other subspecies (support: 100, 100, 95, and 100%).

(iv) The polytypic *R. arvalis* is a monophylum (support: 100% each).

(v) Anatolian brown frogs form a monophylum (support: 100, 100, 97, and 100%, respectively), however, *R. macrocnemis* does not; a significant internal monophylum is formed by *R. m. macrocnemis*, *R. holtzi*, and *R. camerani* (support: 100% each).

Some weekly supported clades formed as follows:

(vi) *Rana iberica* and *R. italica* are sister taxa.

(vii) *Rana pyrenaica* and *R. temporaria* are sister taxa.

Neither monophyly of the species that occur in the Iberian Peninsula (tree 1) nor a sister relationship of *R. temporaria* and *R. iberica* (tree 2a and 2b) could be excluded (Table 2). Despite a fairly high bootstrap support for the *R. temporaria* species group, a position of *R. arvalis* basal to all other western Palearctic brown

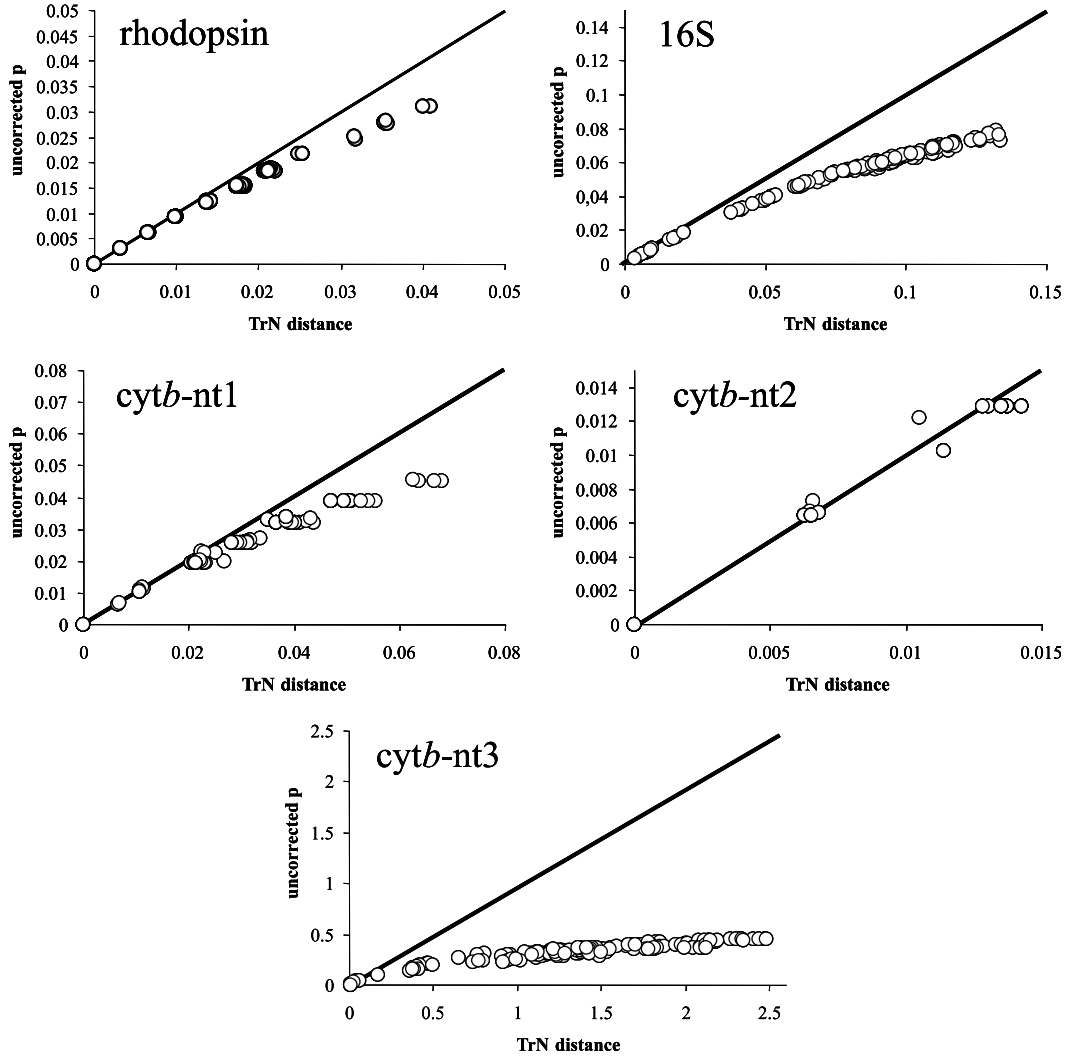

Fig. 3. Plot of uncorrected *p* distances of 16S, rhodopsin and different codon positions of cytochrome *b* (cyt *b*-nt1, cyt *b*-nt2, and cyt *b*-nt3) against corrected molecular (TrN) distances; deviations of points from the uncorrected *p* = TrN line suggest the degree of saturation for each data partition.

frogs was also supported by our data. In contrast, our data did not support a sister relationship of *R. graeca* and *R. italica*.

3.4. Calibration and application of a molecular clock

Enforcing a molecular clock and using *R. bedriagae* as the root resulted in a tree with a log-likelihood significantly worse than the more complex tree without a clock ($\log L_{\text{withoutclock}} = -7011.39$; $\log L_{\text{withclock}} = -7038.39$; the simpler, clock-like tree was rejected on a significance level of 5%). This is probably caused by the fact that only a small number of autapomorphic substitutions accumulated in the brook-dwelling *R. graeca* and *R. pyrenaica.*

Rate constancy among Western Palearctic brown frogs (we compared all taxa with each other, again defining the *R. macrocnemis* complex and the *R. temporaria* and *R. arvalis* subspecies as single lineages, and using *R. catesbeiana* as the outgroup) was not rejected at the 0.05% level in any of the 28 comparisons (PHYLTEST; Kumar, 1996).

Rate constancy between Western Palearctic brown frogs and water frogs was not rejected (at the 0.05 level) in any of the comparisons, although the rates of brown frogs were consistently slightly higher (up to 5%). In the overall comparison using Jukes-Cantor distances, the brown frog and water frog rates (La and Lb in PHYLTEST) were estimated as 0.0827 and 0.080 ($Z = 0.165$).

TrN + $I_{0.5623}$ + $\Gamma_{0.5129}$ distances and D^{*}_{Nei} (Nei's, 1972, standard genetic distance in the modification of Hillis, 1984) of Western Palearctic water frogs are significantly correlated ($r^2 = 0.602$, $p < 0.001$; Fig. 6). Time of

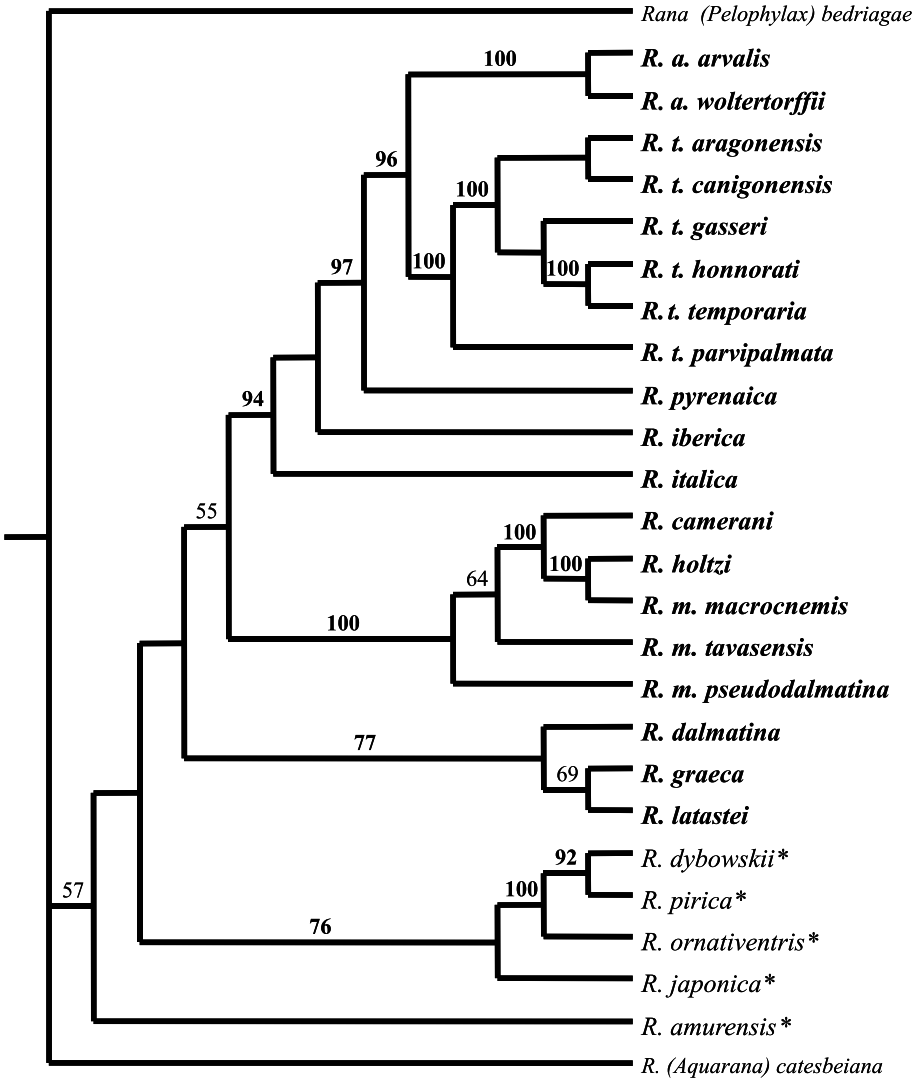

Fig. 4. Bayesian inference of phylogenetic relationships among Western and Eastern Palearctic brown frogs, based on partial sequences of the mitochondrial 16S rRNA and the cytochrome *b* genes; Bayesian posterior probabilities are given for 4000 trees; asterisks indicates Eastern Palearctic species.

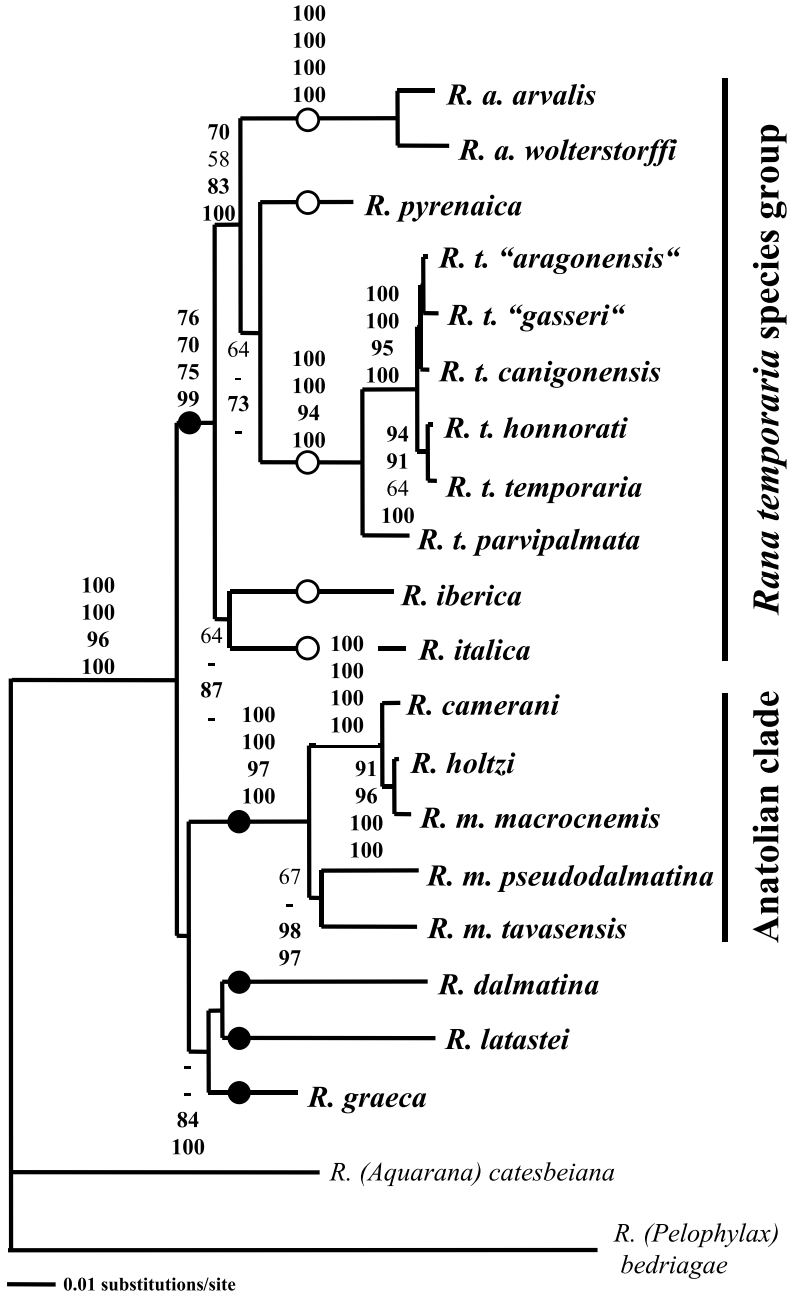

Fig. 5. Neighbor-joining tree (TrN + $I_{0.5148}$ + $\Gamma_{0.4549}$ substitution model) of 1976 bp of 16S, cytochrome *b* and rhodopsin for all species and subspecies of Western Palearctic brown frogs. Bootstrap supports are given for NJ (upper value; 2000 replicates), MP (upper half; 2000), ML (lower half; 2000), and Baysian inference (lower; 4000). Closed circles indicate brown frog lineages that evolved after a basal radiation. Open circles indicate lineages within the *R. temporaria* species group.

separation was therefore calculated from TrN distances using the following equation: time-of-separation$_{mean}$ = $0.198 + 8.189 \cdot D^{*}_{Nei} \cdot$ TrN, with $1D^{*}_{Nei} = 8.14$ million years (my) being Beerli's (1994) calibration of the allozyme clock. The lower and upper 95% confidence limits (CI) of the calibration were calculated from time-of-separation$_{-95\%CI}$ = $(0.198 + 1.96SD) + (8.189 - 1.96SD) \cdot 8.14my \cdot$ TrN, and time-of-separation$_{+95\%CI}$ = $(0.198 - 1.96SD) + (8.189 + 1.96SD) \cdot 8.14my \cdot$ TrN. We subsequently used this calibration to discuss splits among Western Palearctic brown frogs.

The basic radiation among Western Palearctic brown frogs can be dated to the post-Messinian, ca. 4.0 million years ago (mya) (Table 3). The upper 95% CI just approaches the time of the Messinian salinity crisis; a Messinian origin of the earliest splits thus can not be completely ruled out, although it is unlikely. It can be taken for granted that the diversification into the major

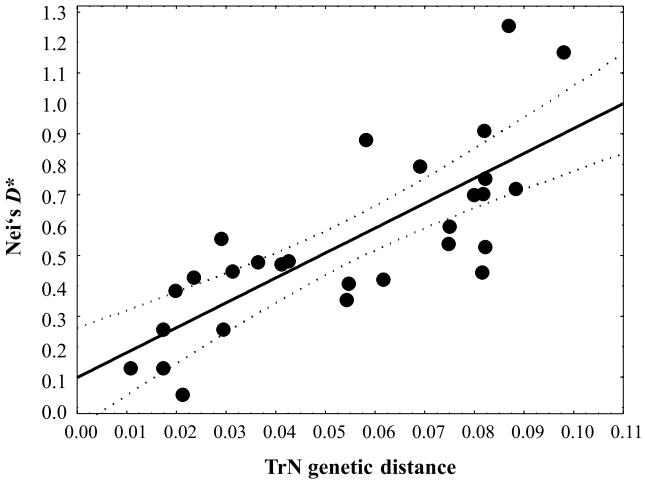

Fig. 6. Calibration of a 16S clock for Western Palearctic water frogs; allozyme distances were taken from Beerli (1994). Dashed lines indicate the 95% CI of the regression line. Regression parameters are as follows: slope ± SD = 8.189 ± 1.306, intercept ± SD = 0.198 ± 0.079; $r^2 = 0.602$, $p < 0.001$.

lineages was completed before the onset of the first Pleistocene glaciations. Starting with the pronounced glaciation about 3.0 mya at the Early Pliocene/Late Pliocene boundary, the *R. temporaria* species group (as named above) rapidly split into five lineages (*R. arvalis*, *R. pyrenaica*, *R. temporaria*, *R. iberica*, and *R. italica*). In concordance with the onset of further sever glaciation around 2.4 mya the Anatolian lineage further radiated (*R. m. macrocnemis*, *R. m. tavasensis*, and *R. m. pseudodalmatina*). About 1 mya, *R. temporaria* has split into two genetically well-differentiated lineages: the Iberian *R. t. parvipalmata* and a clade of all remaining subspecies. Simultaneously, *R. arvalis* has split into its currently recognised subspecies. A simultaneous diversification of the Anatolian clade into *R. camerani*, *R. holtzi*, and *R. m. macrocnemis* and of *R. temporaria* can be dated to the onset of the present cycle of climatic oscillations ca. 600.000 years ago.

4. Discussion

Our molecular data clearly indicate that Western Palearctic brown frogs underwent a basal radiation into at least five distinct lineages about four million years ago. Well-supported sub-lineages comprise the Anatolian taxa (*R. macrocnemis R. camerani*, and *R. holtzi*) and most Western Mediterranean species (*R. italica*, *R. iberica*, *R. pyrenaica*, and *R. temporaria*, including *R. arvalis*), respectively. This scenario supports the allozyme-based assumption of a Plio-Pleistocene diversification of Western Palearctic brown frogs (Mensi et al., 1992) rather than a Miocene origin of basal lineages as assumed by Oosterbroek and Arntzen (1992). The evolutionary relationships within Anatolian taxa contradict current systematics and call for a taxonomic revision (Appendix B). The polytypic *R. arvalis* and *R. temporaria* constitute valid monophyletic lineages.

4.1. Are Western and Eastern Palearctic brown frogs monophyletic lineages?

The masked brown frog morphotype that is common even among semi-terrestrial frogs of other families (e.g., Leptodactylidae and Myobatrachidae; Green and Borkin, 1993) makes a morphological delimitation of monophyletic assemblages problematic, if not impossible. Therefore, Eastern and Western Palearctic brown frogs do not necessarily define monophyletic lineages.

Table 3
Estimated time of divergence (my = million years) among brown frog lineages; mean time of divergence from *n* pairwise comparisons and the lower and upper 95% CI of the calibration are given; lower and upper 95% CI of the total estimate are gained from $\text{lower}_{cal} - 1.96\text{SD}$ and $\text{upper}_{cal} + 1.96\text{SD}$

Split	*n*	Average time of divergence (my) using pairwise comparisons			Total 95% CI of the time of divergence (my)		
		$\text{Lower}_{cal} \pm \text{SD}$	$\text{Mean}_{cal} \pm \text{SD}$	$\text{Upper}_{cal} \pm \text{SD}$	Lower_{total}	Mean_{total}	Upper_{total}
Pelophylax to *Rana*	20	6.62 ± 0.22	9.32 ± 0.32	12.01 ± 0.43	6.19	9.32	12.85
Rana to *Aquarana*	19	5.01 ± 0.24	7.00 ± 0.39	8.84 ± 0.46	4.54	7.00	9.74
Basal split within brown frogs	106	2.96 ± 0.32	3.99 ± 0.34	5.02 ± 0.45	2.33	3.99	5.90
Basal split within *R. temporaria* species group	39	2.40 ± 0.29	3.18 ± 0.43	3.96 ± 0.56	1.83	3.18	5.06
R. a. arvalis to *R. a. wolterstorffi*	1	0.92	1.03	1.13	0.92	1.03	1.13
R. t. parvipalmata to other *R. temporaria* ssp.	5	0.98 ± 0.07	1.12 ± 0.10	1.25 ± 0.13	0.84	1.12	1.50
Among *R. temporaria* ssp. but without *R. t. parvipalmata*	10	[a]	0.58 ± 0.11	[a]	0.36	0.58	0.80
Major Anatolian lineages	7	1.62 ± 0.14	2.04 ± 0.20	2.46 ± 0.27	1.35	2.04	2.99
Within *R. m. macrocnemis* lineage	3	[a]	0.60 ± 0.12	[a]	0.36	0.61	0.84

[a] A calculation of lower and upper 95% CI's of D^*_{Nei} was not possible due to low levels of TrN; it resulted in lower 95% CIs > upper 95% CIs, which is an artefact of the non-zero intercept of the regression equation.

One Western Palearctic species, *R. arvalis*, is geographically intermediate and overlaps with Western (e.g., *R. dalmatina*, *R. temporaria*) and Eastern (*R. amurensis*) species. It is this species that was placed basal to the remaining Western Palearctic brown frogs and to two clades of eastern species in the consensus tree of Barbadillo et al. (1997; see Fig. 2). This may appear justified by its $2n = 24$ chromosome number, which it shares only with some Eastern Palearctic species like *R. chensinensis*, *R. dybowskii*, *R. ornativentris*, and *R. pirica* (King, 1990; Miura et al., 1995; Odierna et al., 2001; but see Green and Borkin, 1993, for discussion of species boundaries within these groups). However, paraphyly of the $2n = 24$ species was already indicated by an allozyme analysis of Eastern and Western brown frogs (Green and Borkin, 1993), with *R. arvalis* and *R. amurensis* standing basal to a mixed clade of Western and Eastern Palearctic species. We found strong evidence that *R. arvalis*, the $2n = 24$ species, is member of the *R. temporaria* species group. This adds support to the assumptions of Green and Borkin (1993) that a $2n = 24$ genotype evolved convergently among Palearctic brown frogs.

4.2. Timing of speciation among Western Palearctic brown frogs

Speciation within several European amphibian genera can be attributed to the end of the Messinian salinity crisis (e.g., *Salamandra*, water frogs of the genus *Rana*, subgenus *Pelophylax*; see Veith et al., 1998; Beerli, 1994). A sudden re-flooding of the formerly desiccated Mediterranean basin at 5.9–5.5 mya (Riding et al., 1998) separated many amphibian populations that had spread throughout the basin during the Messinian.

The Messinian salinity crisis was associated with drastic changes towards a cooler and arid climate in the circum-Mediterranean (Hsü et al., 1977). Nevertheless, dispersal of amphibians throughout the basin was possible even for montane taxa (e.g., *Salamandra*; Lanza and Vanni, 1987; Veith et al., 1998). To date, almost all European amphibian genera are present on larger Mediterranean islands. Only spade-foot toads (*Pelobates*), the widespread newt genus *Triturus*, and brown frogs are restricted to a few coastal islands that they have colonised in recent times (Lanza and Vanni, 1987). Brown frogs that had dispersed during the Messinian crisis should have found suitable habitats on large islands such as Corsica, Sardinia, or Crete. It is therefore astonishing that they are currently absent. Two explanations seem plausible: (i) brown frogs in fact reached Mediterranean islands during the Messinian, but subsequently went extinct; (ii) brown frogs did not live in the circum-Mediterranean during the Messinian salinity crisis. Two lines of evidence advocate the second hypothesis. First, apart from a single Holocene record of *R.* cf. *temporaria* on Corsica (Vigne, 1985), no fossil brown frogs are known from Mediterranean islands (Holman, 1998). The oldest fossil records that can be attributed to present day species (e.g., *R. temporaria*, *R.* cf. *arvalis*, *R.* cf. *latastei*, *R.* cf. *dalmatina*) are known from the late Pliocene (Roçek and Rage, 2000, and references cited therein). Second, brown frogs are montane or subalpine species that prefer cold and moist habitats and breed in lowland habitats only towards northern latitudes. The arid climate that dominated the circum-Mediterranean during the salinity crisis (Hsü et al., 1977) would have forced an ancestral brown frog to retreat to isolated high elevation habitats. If so, a Messinian split of major lineages should have been the result. However, according to our estimate, brown frogs diverged in the post-Messinian, about 4 mya. Bianco (1990) discussed a similar scenario for primary freshwater fish of the Mediterranean basin. Like Baranescu (1983), he postulated that most of the present-day genera reached Central Europe from eastern Asia during the Miocene. Most of them were then established in Europe in the Middle Miocene, however, they were apparently absent in Mediterranean peninsular regions and NW Africa, or occurred after the Messinian.

From where Western Palearctic brown frogs originated remains still open. Boehme (2001) recently published a fossil record that may link Western Palearctic brown frogs to eastern Asia. However, a final solution of this question necessitates the inclusion of Eastern and Western Palearctic brown frogs as well as numerous Asian genera of the subfamily Raninae.

But what accounted for brown frog radiation during Pliocene? Considering that most modern brown frogs are cold-adapted (the single exception is *R. latastei*), we require a moist climate during the Pliocene that allowed for a substantial range expansion of an ancestral brown frog. In fact, a long-lasting subtropical period of about 1 my during the Early Pliocene (Müller, 1985; Maldonado, 1985) with only minor climatic oscillations should have allowed for range expansion of any amphibian groups, including brown frogs. Eustatic sea-level changes induced the first periods of cooling 4 mya and ca. 3 mya (Wilson et al., 1999). A strong pan-oceanic drop of temperature in combination with a substantial growth of the Arctic ice sheet marked the onset of a series of glaciations in the late Pliocene, ca. 2.4 mya (Müller, 1985; Wilson et al., 1999). These post-Messinian climatic fluctuations almost perfectly meet our estimates of major speciation times in brown frogs, ca. 4, 3, and 2.4 mya (Table 3).

4.3. Oscillation per se may have promoted speciation processes in Western Palearctic brown frogs

It seems that speciation of Western Palearctic brown frogs was triggered by the onset of glacials with cycles of

repeated cold (stadials) and warm (interstadials) periods. However, why did single stadials of interstadials did not have the same effect? It is obvious that despite ongoing climatic oscillations since the Late Pliocene and throughout the Quaternary, a substantial subdivision of extant brown frog lineages into more sublineages did not occur (several biochemical and molecular studies on intraspecific genetic variation of brown frogs are in line with this: Capula, 1991; Arano et al., 1993; Rafinski and Babik, 2000; Veith et al., 2002).

The large and regularly occurring (on a major interval of ca. 100,000 years) temperature changes during the Quaternary and before may have had a negative effect on gradual speciation following vicariance (Dynesius and Jansson, 2000). Especially towards northern latitudes, species range dynamics caused by climatic oscillations favoured ecological generalism and vagility, which promote high regional population densities and wide distributions. Vagility enhances gene flow, and this predates differentiation that had evolved in isolation. This may well explain the low degree of differentiation among populations of widespread species such as *R. arvalis* (Rafinski and Babik, 2000) and *R. dalmatina* (unpublished data).

More specialised species like the southern mountain endemics (e.g., *R. graeca*, *R. iberica*, and *R. pyrenaica*) were probably "trapped" in their glacial refugia. Fast climatic oscillations left little time for range expansions. Evidence comes from paleontology. Pleistocene species-specific brown frog records mostly lie within the species' present distribution (Fig. 7). The only exceptions are *R. arvalis*, *R. dalmatina*, and *R. graeca* of which single fossils are found outside their current distribution. But why did southern endemics never escape from their refugia, though they apparently persisted for at least 2.5 my? Hewitt (1996) developed a scenario for grasshopper distribution as a consequence of climatic oscillations that may also hold for montane brown frog endemics. Following a climatic amelioration, a montane brown frog population has two ways to react: (i) it can move upthe mountain (altitudinal reaction), or (ii) it can migrate northward (latitudinal reaction). Both these

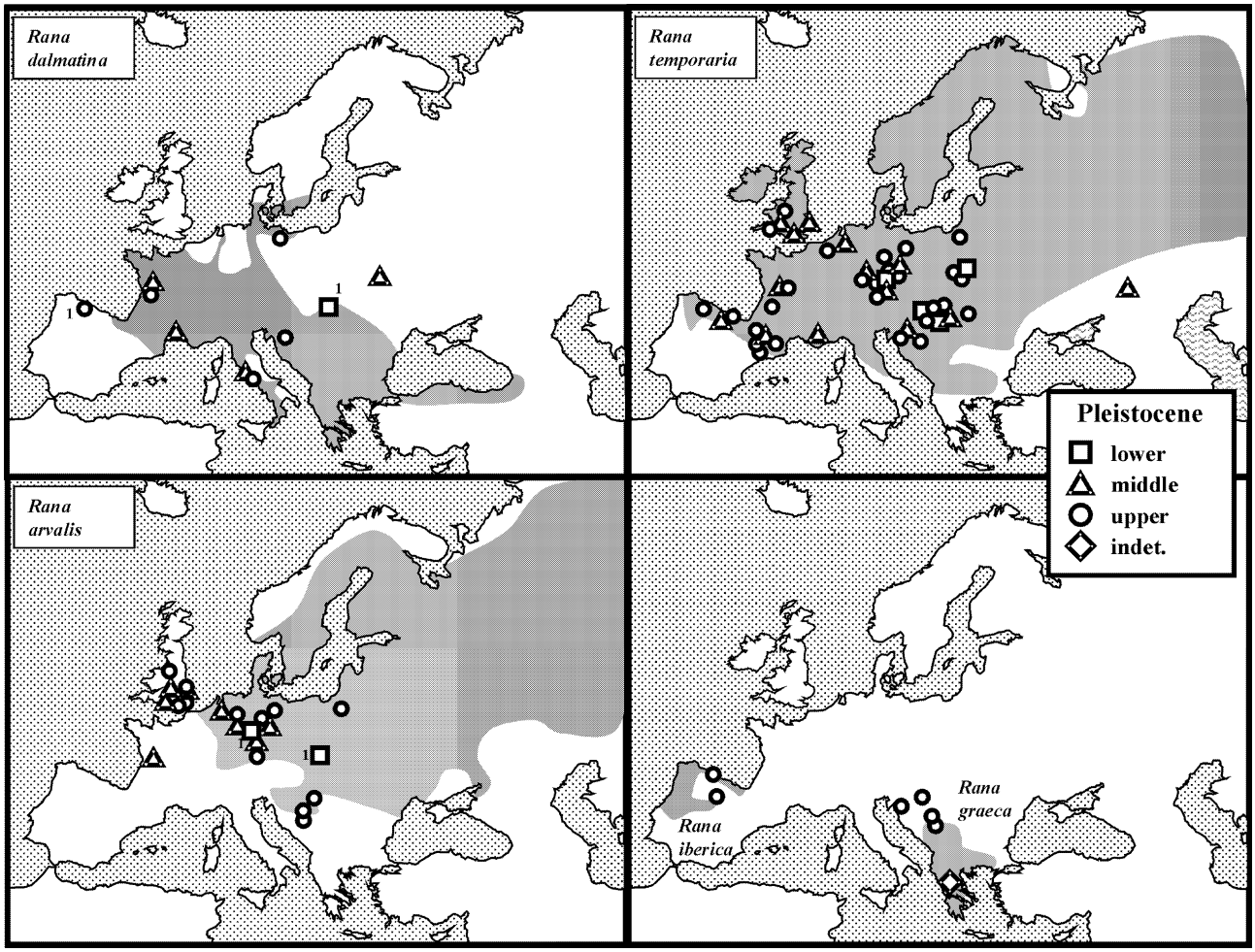

Fig. 7. Current distribution (grey) and Pleistocene fossil records (symbols) of five Western Palearctic brown frog species (according to Holman, 1998); geographically close fossil sites are pooled; [1]) lower Pleistocene records that are only affiliated as c.f. to the respective taxon.

"subpopulations" have three principle ways to respond to a troublesome subsequent cooling (Coope, 1994): (i) they can evolve out of trouble, (ii) they can move out of trouble, (iii) they may become extinct. Only island species have to endure environmental changes on the spot. Temperature probably changed during oscillations with great rapidity and intensity (e.g., the transition from the last glaciation to the present day interglacial occurred within one human lifetime; Dansgaard et al., 1989). Assuming that rapid changes were common (Wilson et al., 1999: p. 113), options (i) and (ii) did not exist for latitudinally responding brown frog populations. Consequently they went extinct, and with them all adaptations they may have evolved through the colonisation process. In contrast, populations that had shifted altitudinally were able to escape extinction by simply moving up and down the mountains due to short distances between different temperature zones. Any species that was able to survive the first change could, by adopting the same strategy, survive succeeding ones. As a consequence, and even if substantial latitudinal range expansions may have occurred, populations evolved in their refugia as if they had constantly been isolated.

Rana temporaria does not fit this model. It is the only species that was recorded from ice-free areas during full glacial times (Holman, 1998). Today, its northern distribution is more or less delimited by forest-free tundras (Gasc et al., 1998). Therefore, its glacial refugia may well have been in the close vicinity of the east–west extended southern tundra line or even close to the Arctic ice-sheet. Increasing evidence from tree charcoal and mammalian fossils suggest that southern refugia were supplemented by multiple cryptic refugia in Central and Northern Europe during the Pleistocene (Steward and Lister, 2001). This may also explain why *R. temporaria* harbours the largest degree of genetic variation among Western Palearctic brown frogs (e.g., Reh and Seitz, 1990).

4.4. Concluding remarks

All lines of evidence advocate a post-Messinian speciation of brown frogs, originating from an ancestor that had invaded Europe from Asia during the Early Pliocene. Subsequent major radiations among Western Palearctic brown frogs seem to be triggered by the onsets of drastic climatic oscillations. It becomes obvious that such climatic oscillations marked simultaneous speciation events in different lineages (Fig. 8). Tempo and length of cold periods seem to have been as important as the strength of the initial oscillation. Even if the ice-sheets and permafrost did not reach the Mediterranean (which in principle was established geologically and climatically some 3.2 mya; Blondel and Aronson, 1999), its marked effect on the Mediterranean

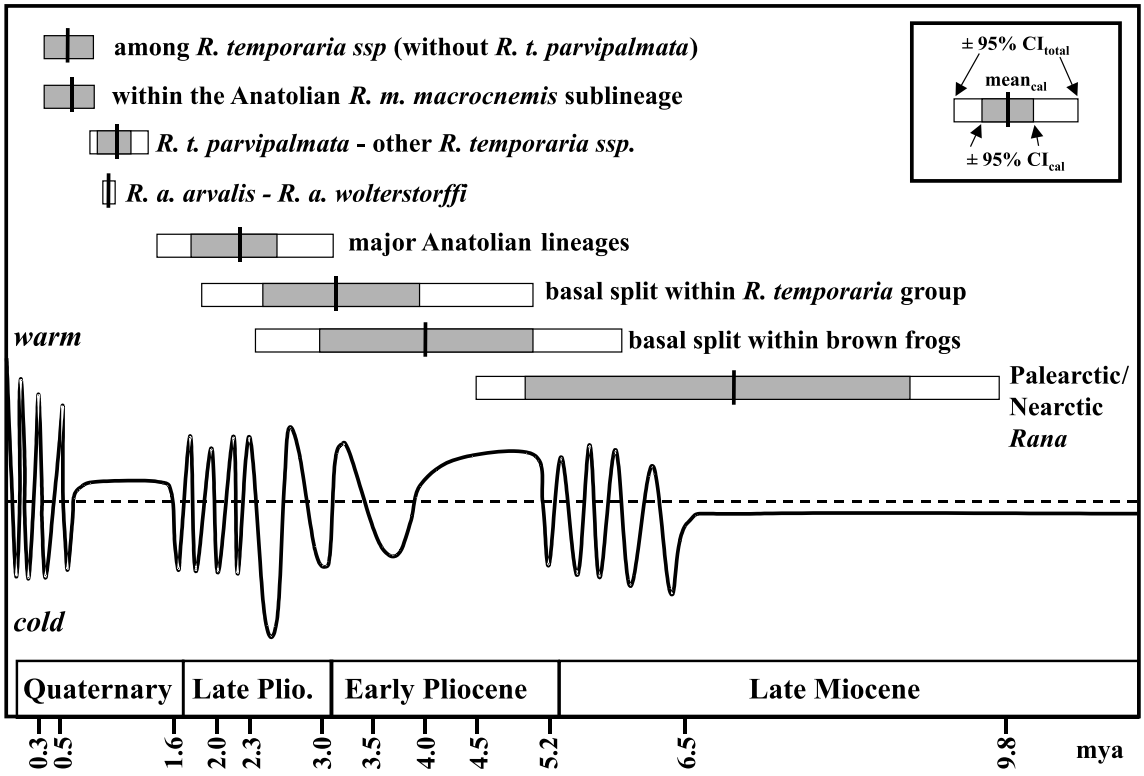

Fig. 8. Inference of fluctuation of surface-water temperature during the late Miocene to the Recent in the Mediterranean (after Müller, 1985) based on calcareous nannoplankton records, and estimated time of speciation in different brown frog lineages. Minor climatic oscillations also occurred on a regular scale during periods of stable nannoplankton records in the Early Quaternary and the Early Pliocene (Ruddiman and Raymo, 1988; Wilson et al., 1999).

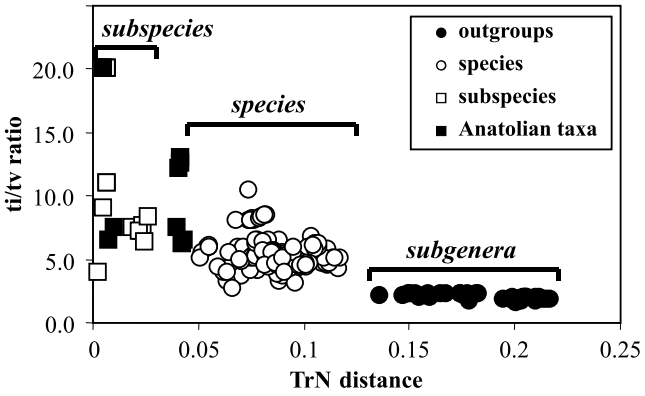

Fig. 9. A two-dimensional plot of pairwise ti/tv ratios against $\mathrm{TrN} + I_{0.5148} + \Gamma_{0.4549}$ molecular distances; comparisons with no transversions (ti/tv ratio $= \infty$) were set to 20.

climate must have been responsible for a retreat of brown frog distribution ranges. Afterwards, populations diverged under isolation, and the oscillation process itself trapped them in their refuges and prevented differentiation from being predated by secondary contact. Dispersal was possible in the comparatively warm phases of climatic stability during the Early Pliocene and the Early Quaternary. Although most present-day brown frogs are rather cold-adapted mountain species, long-lasting sub-tropical climatic conditions probably allowed their ancestor to disperse throughout major parts of Europe and especially along the northern Mediterranean. Thus, Western Palearctic brown frogs may serve as an outstanding example of a geographically nested set of taxa on different taxonomical levels that evolved through an interplay of drastic initial climatic changes and their successive oscillations.

Acknowledgments

We are grateful to Toni Amann, Alain Dubois, Lutz Fromhage, David Frynta, Kurt Grossenbacher, Hemmo Nickel, Jörg Plötner, Richard Podloucky, Dirk Schmeller, Josef Schmidtler, and David Tarkhnishvili for providing tissue or DNA samples. Katrin Böning-Gaese, Jes Johannesen, Andreas Kiefer, Oliver Lange, Jakob Müller, Thomas Schmitt, Jutta Schneider, David Tarkhnishvili, and two anonymous reviewers improved a former version of the manuscript. Thanks to Renata Platenberg for linguistic improvement.

Appendix A

Sample locations, voucher numbers, and GenBank Accession Numbers of the taxa studied (CPU, Charles University Prague, Czech Republic; FMNH, Field Museum of Natural History, Chicago, USA; MNHN, Muséum National d'Histoire Naturelle, Paris, France; ZFMK, Zoologisches Forschungsinstitut und Museum Alexander Koenig, Bonn, Germany; ZMB, Zoologisches Museum Berlin, Germany; ZSM, Zoologische Staatssammlung München, Germany); all sequences were obtained new with the exception of [1])

Taxon	*n*	Sample locality	Voucher number	GenBank Accession Numbers		
				16S-1/16S-2	cyt *b*	rhod
Western Palearctic brown frogs						
R. arvalis arvalis Nilsson, 1842	2	Lower Saxony/ Germany	vouchers not preserved	AY147938	AY147958	AY147988
R. arvalis wolterstorffi Fejérváry, 1919	1	Neusiedlersee surroundings, Austria	voucher not preserved	AY147939	AY147959	AY147989
R. camerani Boulenger, 1886	1	Tabatskuri/ Georgia (type locality)	private collection of D. Tarkhnishvili	AY147940	AY147960	AY147990
R. dalmatina Bonaparte, 1840	1	Drover Heide/ Germany	ZSM 88/2001	AY147941	AY147962	AY147992
R. graeca Boulenger, 1891	1	Metsovo/Epirus Mts./Greece	private collection of J.F. Schmidtler	AY147942	AY147963	AY147993
R. holtzi Werner, 1898	2	Karagöl/ Turkey (type locality)	private collection of J.F. Schmidtler	AY147943	AY147964	AY147994
R. iberica Boulenger 1879	1	Salas, Asturias/Spain	ZFMK 68875	AY147944	AY147965	AY147995

Appendix A (*continued*)

Taxon	*n*	Sample locality	Voucher number	GenBank Accession Numbers		
				16S-1/16S-2	cyt *b*	rhod
R. italica Dubois, 1985	1	Naples/Italy	voucher not preserved	AY147945	AY147966	AY147996
R. latastei Boulenger, 1879	2	Campagna, Seseglio 2 km SW Chiasso/Italy	vouchers not preserved	AY147946	AY147967	AY147997
R. macrocnemis macrocnemis Boulenger, 1885	2	Uludag/Turkey (type locality)	private collection of J.F. Schmidtler	AY147947	AY147968	AY147998
R. macrocnemis tavasensis Baran and Atatür, 1986	1	Akdag near Tavas/ Turkey (type locality)	MNHN 2000.660 and 2000.661	AY147949	AY147970	AY148000
R. macrocnemis pseudodalmatina Eiselt and Schmidtler, 1971	1	Mazandaran province/Iran	CUP AMPH/IRA/ 013; see Frynta et al. (1997)	AY147948	AY147969	AY147999
R. pyrenaica Serra-Cobo, 1993	2	Zuriza, Aragón/ Spain	ZFMK 65447-65448	AY147950	AY147971	AY148001
R. temporaria "aragonensis" Palanca-Soler, Rodriguez Vieites and Suárez Martínez, 1998	1	Respomuso, type locality/Spain	ZFMK 65430	AY147951	AY147972	AY148002
R. temporaria canigonensis Boubée, 1833	1	Mt. Canigou/ France (type locality)	MNHN 1997.4347	AY147952	AY147973	AY148003
R. temporaria "gasseri"	1	surroundings of Gerde/France	MNHN 1997.4910	AY147953	AY147974	AY148004
R. temporaria honnorati Héron-Royer, 1881	2	Faillefeu Bas Granges and Le Brusquet/France	vouchers not preserved	AY147954	AY147975	AY148005
R. temporaria parvipalmata Seoane, 1885	1	Serra de Capelada/Spain (vicinity of type locality)	ZSM 88/2001	AY147955	AY147976	AY148006
R. temporaria temporaria Linnaeus, 1758	3	Koblenz/ Germany	ZFMK 69883-69885	AY147956	AY147977	AY148007
Eastern Palearctic brown frogs						
Rana amurensis	1	Mongolia	not available	AB058885[1])	AF205094[1])	
Rana dybowskii	1	Japan (Tsushima)	not available	AB058873[1])	AF077399[1])	
Rana japonica	1	Japan (Hiroshima)	not available	AB058876[1])	AF077395[1])	
R. ornativentris	1	Japan (Aomori)	not available	AB058875[1])	AF077400[1])	
R. pirica	1	Japan (Sapporo)	not available	AB058872[1])	AF077398[1])	
water frogs						
R. bedriagae Camerano, 1882	2	Marmaris/ Turkey	vouchers not preserved	AY147937	AY147957	AY148008

Appendix A (*continued*)

Taxon	*n*	Sample locality	Voucher number	GenBank Accession Numbers		
				16S-1/16S-2	cyt *b*	rhod
R. lessonae Camerano, 1882	1	Italy	voucher not preserved	AY147982		AY148012
R. epeirotica Schneider, Sofianidou and Kyriakopoulou-Sklavounou, 1984	1	Greece	ZMB 47711	AY147981		AY148011
R. perezi Seoane, 1885	1	Narbonne/ France	private collection of Dirk Schmeller	AY147985		AY148015
R. ridibunda Pallas, 1771	1	Greece	ZMB 49267	AY147983		AY148013
R. saharica Boulenger, 1913	1	Bizente/Tunisia	private collection of Dirk Schmeller	AY147984		AY148014
R. cerigensis Beerli, Hotz, Heppich and Uzzell, 1994	1	Rhodes/Greece	voucher not preserved	AY147979		AY148009
R. cretensis Beerli, Hotz, Heppich and Uzzell, 1994	1	Crete/Greece	voucher not preserved	AY147980		AY148010
outgroups						
R. catesbeiana Shaw, 1802	1	frog farm/Java	private collection of Michael Veith	AY147978	AY147961	AY147991
Fejervarya limnocharis Gravenhorst, 1829	1	Sidikalang, Sumatra/ Indonesia	FMNH 256768	AY147986		AY147991

Appendix B. Taxonomic conclusions

The subspecies *Rana m. tavasensis*, *R. m. pseudodalmatina*, and the (*R. m. macrocnemis*, *R. camerani*, and *R. holtzi*) clade are basal lineages within the Anatolian clade. In contrast, the species *R. holtzi* and *R. camerani* form a monophylum with *R. m. macrocnemis*. This makes *Rana macrocnemis* paraphyletic. On the basis of 16S haplotypes of Anatolian mountain frogs from 40 populations, Veith et al. (in press) already showed that morphological assignment of species status to populations is impossible and has led to much confusion. This calls for a taxonomic revision.

In doing so, we (i) intend to stay as close as possible to current taxonomy and (ii) to apply taxonomic categories in accordance to other brown frog lineages.

$\mathrm{TrN} + I_{0.5148} + \Gamma_{0.4549}$ distances among Anatolian brown frogs fall into two categories: species and subspecies (Fig. 9). The latter refers to comparisons among *R. m. macrocnemis*, *R. camerani*, and *R. holtzi*. We therefore regard them as conspecific: *R. macrocnemis macrocnemis*, *R. m. camerani*, and *R. m. holtzi*. This opinion was repeatedly advocated by various authors (reviewed in Veith et al., in press). Species level differentiation, although at the lower end within brown frogs, separates three lineages to which we assign species rank: *R. macrocnemis*, *R. tavasensis*, and *R. pseudodalmatina*.

References

Arano, B., Esteban, M., Herrero, P., 1993. Evolutionary divergence of the Iberian brown frogs. Ann. Sci. Nat. Zool. 14 (13), 49–57.

Baran, I., Atatür, M.K., 1998. Turkish herpetofauna (amphibians and reptiles). Ankara.

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

Barbadillo, L.J., García-Paris, M., Sanchíz, B., 1997. Orígenes y relaciones evolutivas de la herpetofauna Ibérica. In: Pleguezuelos, J.M. (Ed.), Distribución y biogeografía de los anfibos y reptiles en Espana y Portugal. Monografías de Herpetología, vol. 3. Granada, pp. 47–100.

Beerli, P., 1994. Genetic isolation and calibration of an average protein clock in western Palearctic water frogs of the Aegean region. Ph.D. Thesis, Zürich.

Bianco, P.G., 1990. Potential role of the paleohistory of the Mediterranean and Paratethys basin on the early dispersal of Euro-Mediterranean freshwater fishes. Ichthyol. Explor. Freshwaters 1, 167–184.

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

Boehme, M., 2001. The oldest representative of a brown frog (Ranidae) from the Early Miocene of Germany. Acta Pal. Polonica 46, 119–124.

Bossuyt, F., Milinkovitch, C.M., 2000. Convergent adaptive radiation in Madagascan and Asian ranid frogs reveal covariation between larval and adult traits. Proc. Natl. Acad. Sci. USA 97 (12), 6585–6590.

Bush, G.L., 1994. Sympatric speciation in animals: new wine in old bottles. Trends Ecol. Evol. 9, 285–288.

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

Cao, Y., Adachi, J., Hasegawa, M., 1998. Comment on the Quartet Puzzling method for finding maximum-likelihood tree topologies. Mol. Biol. Evol. 15, 87–89.

Capula, M., 1991. Allozyme variation in *Rana latastei* populations (Amphibia: Ranidae) from Northern Italy and Istria (NW Yugoslavia): biogeographic inferences from electrophoretic data. Zool. Anz. 227, 1–12.

Coope, G.R., 1994. The response of insect faunas to glacial-interglacial climatic fluctuations. Philos. Trans. R. Soc. London B 344, 19–26.

Coyne, J.A., Orr, H.A., 1989. Two rules of speciation. In: Otte, D., Endler, J.A. (Eds.), Speciation and Its Consequences. Sinauer, Massachusetts, pp. 180–207.

Dansgaard, W., White, J.W.C., Johnson, S.J., 1989. The abrupt termination of the Younger Dryas event. Nature 339, 532–533.

Demirsoy, A., 1996. Amfibiler. Ankara.

Dieckmann, U., Doebeli, M., 1999. On the origin of species by sympatric speciation. Nature 400, 354–357.

Dubois, A., 1985. Notes sur les grenouilles brunes (groupe de *Rana temporaria* Linné, 1758). IV. Note préliminaire sur *Rana graeca* Boulenger, 1891. Alytes 4, 135–138.

Dubois, A., 1992. Notes sur la classification des Ranidae (Amphibiens Anoures). Bull. Mens. Soc. Linn. Lyon. 61, 305–352.

Dynesius, M., Jansson, R., 2000. Evolutionary consequences of changes in species' geographical distribution driven by Milankovitch climate oscillations. Proc. Natl. Acad. Sci. USA 97, 9115–9120.

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

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

Frynta, D., Moravec, J., Chihakova, J., Sadlo, J., Hodkova, Z., Kaftan, M., Kodym, P., Kral, D., Pitule, V., Sejne, L., 1997. Results of the Czech Biological Expedition to Iran. Part 1: Notes on the distribution of amphibians and reptiles. Acta Soc. Zool. Bohem. 61, 3–17.

Gasc, J.-P., Cabela, A., Crnobrnja-Isailovic, J., Dolmen, D., Grossenbacher, K., Haffner, P., Lescure, J., Martens, H., Martínez-Rica, J.P., Maurin, H., Oliveira, M.E., Sofianidou, T.S., Veith, M., Zuiderwijk, A., 1997. Atlas of amphibians and reptiles in Europe. Societas Europaea Herpetologica and Muséum national d'Histoire naturelle, Paris.

Green, D.M., Borkin, L.J., 1993. Evolutionary relationships of Eastern Palearctic brown frogs, genus *Rana*: paraphyly of the 24-chromosome species group and the significance of chromosome number change. Zool. J. Linn. Soc. 109, 1–25.

Hays, J.D., Imbrie, J., Shackleton, N.J., 1976. Variation in the Earth's orbit: pacemaker of the ice age. Science 194, 1121–1132.

Hedges, S.B., 1992. The number of replications needed for accurate estimation of the bootstrap *P* value in phylogenetic studies. Mol. Biol. Evol. 9, 366–369.

Hedges, S.B., 1994. Molecular evidence for the origin of birds. Proc. Nat. Acad. Sci. USA 91, 2621–2624.

Hewitt, G.M., 1996. Some genetic consequences of ice ages, and their role in divergence and speciation. Biol. J. Linn. Soc. 58, 247–276.

Hillis, D.M., 1984. Misuse and modification of Nei's genetic distance. Syst. Zool. 3, 238–240.

Holman, J.A., 1998. Pleistocene Amphibians and Reptiles in Britain and Europe. Oxford University Press, New York/Oxford.

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

Huelsenbeck, J.P., Bull, J.J., Cunningham, C.W., 1996. Combining data in phylogenetic analysis. TREE 11, 152–158.

Huelsenbeck, J.P., Crandall, K.A., 1997. Phylogeny estimation and hypothesis testing using maximum likelihood. Ann. Rev. Ecol. Syst. 28, 437–466.

Huelsenbeck, J.P., Hillis, D.M., 1993. Success of phylogenetic methods in the four-taxon case. Syst. Biol. 42, 247–264.

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

Huelsenbeck, J.P., Ronquist, F., Nielsen, R., Bollback, J.P., 2001. Bayesian inference of phylogeny and its impact on evolutionary biology. Science 294, 2310–2314.

King, M., 1990. Animal Cytogenetics, vol.4: Chordata 2: Amphibia. Borntraeger, Berlin/Stuttgart.

Kishino, H., Hasegawa, M., 1989. Evaluation of the maximum likelihood estimate of the evolutionary tree topologies from DNA sequence data, and the branching order in Hominoidea. J. Mol. Evol. 29, 170–179.

Klassen, G.J., Mool, R.D., Locke, A., 1991. Consistency indices and random data. Syst. Zool. 40, 446–457.

Kocher, T.D., Thomas, W.K., Meyer, A., Edwards, S.V., Pääbo, S., Villablanca, F.X., Wilson, A.C., 1989. Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. Proc. Natl. Acad. Sci. USA 86, 6196–6200.

Kumar, S., 1996. PHYLTEST: Phylogeny Hypothesis Testing Software. The Pennsylvania State University, Pennsylvania.

Kuzmin, S.L., 1999. The Amphibians of the Former Soviet Union. Pensoft, Sofia/Moscow.

Lanza, B., Vanni, S., 1987. Hypotheses on the origin of the Mediterranean island batrachofauna. Bull. Soc. Zool. France 112, 179–196.

Lockhart, P.J., Steel, M.A., Hendy, M.D., Penny, D., 1994. Recovering evolutionary trees under a more realistic model of sequence evolution. Mol. Biol. Evol. 11, 605–612.

Lowe, J.J., Walker, M.J.C., 1997. Reconstructing Quaternary Environments, second ed. Longman, London.

Maldonado, A., 1985. Evolution of the Mediterranean basins and a detailed reconstruction of the Cenozoic paleooceanography. In: Margalef, R. (Ed.), Key Environments: Western Mediterranean. Pergamon Press, Oxford, pp. 17–59.

Martin, A.P., Naylor, G.J.P., Palumbi, S.R., 1992. Rates of mitochondrial DNA evolution in sharks are slow compared with mammals. Nature 357, 153–155.

Mayr, E., Ashlock, P.D., 1991. Principles of Systematic Zoology, second ed. McGraw-Hill, New York.

Mensi, P., Lattes, A., Marcario, B., Salvidio, S., Giacoma, C., Baletto, E., 1992. Taxonomy and evolution of European brown frogs. Zool. J. Linn. Soc. 104, 293–311.

Mindel, D.P., Knight, A., Baer, C., Huddlestone, C.J., 1996. Slow rates of molecular evolution in birds and the metabolic rate and body temperature hypothesis. Mol. Biol. Evol. 13, 422–426.

Miura, I., Nishioka, M., Borkin, L., Wu, Z., 1995. The origin of the brown frogs with $2n = 24$ chromosomes. Experientia 51, 179–188.

Müller, C., 1985. Late Miocene to recent Mediterranean biostratigraphy and paleoenvironments based on calcareous nannoplankton. In: Stanley, D.J., Wezel, F.-C. (Eds.), Geological Evolution of the Mediterranean Basin. Springer, New York, pp. 475–481.

Nei, M., 1972. Genetic distance between populations. Am. Nat. 106, 283–292.

Odierna, G., Aprea, G., Arribas, O., Capriglione, T., 2001. A chromosomal study of the Pyrenean endemic brown frog species,

Rana pyrenaica, and various Italian populations of *R. dalmatina* and *R. italica*. Folia Zool. 50, 55–63.

Oosterbroek, P., Arntzen, J.W., 1992. Aera-cladograms of Circum-Mediterranean taxa in relation to Mediterranean paleogeography. J. Biogeography 19, 3–20.

Palumbi, S., Martin, A., Romano, S., McMillan, W.O., Stice, L., Grabowski, G., 1991. The simple fool's guide to PCR, Version 2. Honolulu, Hawai.

Picariello, O., Scillitani, G., Cretella, M., 1990. Electrophoretic data supporting the specific rank of the frog *Rana graeca italica* Dubois, 1985. Amph.-Rept. 11, 189–192.

Pook, C.E., Wüster, W., Thorpe, R.S., 2000. Historical biogeography of the western rattlesnake (Serpentes: Viperidae: *Crotalus viridis*), inferred from mitochondrial DNA sequence information. Mol. Phyl. Evol. 15, 269–282.

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

Rafinski, J., Babik, W., 2000. Genetic differentiation among northern and southern populations of the moor frog *Rana arvalis* Nilsson in central Europe. Heredity 84, 610–618.

Rand, D.M., 1994. Thermal habitat, metabolic rate and the evolution of mitochondrial DNA. TREE 9, 125–131.

Rannala, B., Yang, Z., 1996. Probability distribution of molecular evolutionary trees: a new method for phylogenetic inference. J. Mol. Evol. 43, 304–311.

Reed, R.D., Sperling, F.A.H., 1999. Interaction of process partitions in phylogenetic analysis: an example from the swallowtail butterfly genus *Papilio*. Mol. Biol. Evol. 16, 286–297.

Reh, W., Seitz, A., 1990. The influence of land-use on the genetic structure of populations of the common frog *Rana temporaria*. Biol. Cons. 54, 239–249.

Rice, W.R., 1989. Analyzing tables of statistical tests. Evolution 43, 223–225.

Riding, R., Braga, J.C., Martin, J.M., Sánchez-Almazo, I.M., 1998. Mediterranean Messinian salinity crisis: constraints from a coeval marginal basin, Sorbas, south-eastern Spain. Marine Geol. 146, 1–20.

Roçek, Z., Rage, L.-C., 2000. Tertiary anura of Europe, Africa, Asia, North America, and Australia. In: Heatwole, H., Carrol, R.L. (Eds.), Amphibian Biology, vol. 4, Palaeontology. Surrey Beatty & Sons, USA, pp. 1332–1337.

Rodríguez, F., Oliver, J.F., Marín, A., Medina, J.R., 1990. The general stochastic model of nucleotide substitution. J. Theoret. Biol. 142, 485–501.

Roe, B.A., Din-Pow, M., Wilson, R.K., Wong, J.F., 1985. The complete nucleotide sequence of the *Xenopus leavis* mitochondrial genome. J. Biol. Chem. 260, 9759–9774.

Rosen, D.E., 1978. Vicariant pattern and historical explanation in biogeography. Syst. Zool. 27, 159–313.

Ruddiman, W.F., Raymo, M.E., 1988. Northern hemisphere climate régimes during the past 3 ma: possible tectonic connections. Philos. Trans. R. Soc. London B 318, 411–430.

Saitou, N., Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4, 406–425.

Scherer, S., 1990. The protein molecular clock. In: Hecht, M.K., Wallace, B., Mcintyre, R.J. (Eds.), Evolutionary Biology, vol. 24, pp. 83–106.

Schliewen, U.K., Tautz, D., Pääbo, S., 1994. Sympatric speciation suggested by monophyly of crater lake cichlids. Nature 368, 629–632.

Schmidt, H.A., Strimmer, K., Vingron, M., von Haeseler, A., 2000. TREE-PUZZLE, version 5.0. München.

Serra-Cobo, J., 1993. Descripción de una nueva especie europea de rana parda (Amphibia, Anura, Ranidae). Alytes 11, 1–15.

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

Steel, M.A., Lockhart, P.J., Penny, D., 1993. Confidence in evolutionary trees from biological sequence data. Nature 364, 440–442.

Steward, J.R., Lister, A.M., 2001. Cryptic northern refugia and the origin of the modern biota. TREE 16, 608–613.

Strimmer, K., von Haeseler, A., 1996. Quartet puzzling: a quartet maximum likelihood method for reconstructing tree topologies. Mol. Biol. Evol. 13, 964–969.

Sullivan, J., Swofford, D.L., 2001. Should we use model-based methods for phylogenetic inference when we know that assumptions about among-site rate variation and nucleotide substitution pattern are violated? Syst. Biol. 50, 723–729.

Swofford, D.L., 2001. Paup*: Phylogenetic Analysis Using Parsimony and Other Methods, Version 4.0b8a. Sinauer Associates, Sunderland/Massachusetts.

Taberlet, P., Fumagalli, L., Wust-Saucy, A.-G., Cosson, J.-F., 1998. Comparative phylogeography and postglacial colonization routes in Europe. Mol. Ecol. 7, 453–464.

Takezaki, N., Rzhetsky, A., Nei, M., 1995. Phylogenetic test of the molecular clock and linearized trees. Mol. Biol. Evol. 12, 823–833.

Tamura, K., Nei, M., 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzee. Mol. Biol. Evol. 10, 512–526.

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., Vences, M., Rodriguez Vieites, D., Nieto Roman, S., Palanca-Soler, A., 2002. Genetic differentiation and population structure within the Spanish *Rana temporaria* complex (Amphibia: Ranidae). Folia Zool., 51, in press.

Veith, M., Schmidtler, J.F., Kosuch, J., Baran, I., Seitz, A., in press. Paleoclimatic changes explain Anatolian mountain frog evolution: a test for alternating, vicariance and dispersal events. Mol. Ecol.

Vigne, J.-D., 1985. Les amphibiens du Quarternaire d'Araguina-Sennola Bonfacio: présence d'une grenouille rousse dans les niveaux préhistorique de Corse. Bull. Soc. Herp. France 35, 13–16.

West, F.R.S., 1988. The record of the cold stage. Philos. Trans. R. Soc. London B 318, 505–522.

Whelan, S., Liò, P., Goldman, N., 2001. Molecular phylogenetics: state-of-the-art methods for looking into the past. Trends Genet. 17, 262–272.

Wilson, R.C.L., Drury, S.A., Chapman, J.L., 1999. The Great Ice Age. The Open University, London and New York.