Molecular Ecology (2007) doi: 10.1111/j.1365-294X.2007.03274.x

Fossorial but widespread: the phylogeography of the common spadefoot toad (*Pelobates fuscus*), and the role of the Po Valley as a major source of genetic variability

ANGELICA CROTTINI,*† FRANCO ANDREONE,† JOACHIM KOSUCH,‡§ LEO J. BORKIN,¶ SPARTAK N. LITVINCHUK,** CHRISTOPHE EGGERT†† and MICHAEL VEITH‡‡

*Universitá degli Studi di Milano, Dipartimento di Biologia, Sezione di Zoologia e Citologia, Via Celoria 26, 20133 Milano, Italy, †Museo Regionale di Scienze Naturali, Sezione di Zoologia, Via G. Giolitti, 36, 10123 Torino, Italy, ‡Universität Mainz, Institut für Zoologie, Abteilung Ökologie, Saarstraße 21, 55099 Mainz, Germany, §Universität Trier, Fachbereich VI Biogeographie, 54286 Trier, Germany, ¶Department of Herpetology, Zoological Institute, Russian Academy of Sciences, Universitetskaya nab. 1, 199034 St. Petersburg, Russia, **Institute of Cytology, Russian Academy of Sciences, Tikhoretsky pr. 4, 194064 St. Petersburg, Russia, ††Laboratoire d'Ecologie Alpine, CNRS UMR 5553, Université de Savoie, CISM, 73376 Le Bourget du Lac cedex, France, ‡‡Present address: Institute for Biodiversity and Ecosystem Dynamics (IBED), University of Amsterdam, Kruislaan 318, 1098 SM Amsterdam, The Netherlands*

Abstract

***Pelobates fuscus* is a fossorial amphibian that inhabits much of the European plain areas. To unveil traces of expansion and contraction events of the species' range, we sequenced 702 bp of the mitochondrial cytochrome *b* gene. To infer the population history we applied phylogeographical methods, such as nested clade phylogeographical analysis (NCPA), and used summary statistics to analyse population structure under a neutral model of evolution. Populations were assigned to different drainage systems and we tested hypotheses of explicit refugial models using information from analysis of molecular variance, nucleotide diversity, effective population size estimation, NCPA, mismatch distribution and Bayesian dating. Coalescent simulations were used as *post hoc* tests for plausibility of derived or *a priori* assumed biogeographical hypotheses. Our combination of all approaches enabled the reconstruction of the colonization history and phylogeography of *P. fuscus* and confirmed a previous assumption of the existence of two major genetic lineages within *P. fuscus*. Using the Afro-European vicariance of *Pelobates cultripes* and *Pelobates varaldii* and applying Bayesian dating we estimated the divergence of these phylogeographical lineages to the Pliocene. We suggest the existence of three different glacial refugia: (i) the area between the Caspian and Black Seas as the origin for the expansion of the 'eastern lineage'; (ii) the Danube system as a centre of diversity for part of the 'western lineage'; (iii) the Po Valley, the largest centre of genetic variability. This fits the hypothesis that climatic fluctuation was a key event for differentiation processes in *P. fuscus*.**

Keywords: mismatch distribution, nucleotide diversity, *Pelobates*, phylogeographical analysis, postglacial range expansion, summary statistics

Received 24 September 2006; revision accepted 11 December 2006

Introduction

Cyclic Pleistocene climatic changes, the so-called Milankovitch oscillations (Milankovitch 1941) deeply affected the distribution of temperate zone biota (Hewitt 2000). Species colonization from glacial refugia into formerly uninhabitable areas occurred repeatedly, and populations that had successfully settled in previously glaciated areas faced rapid climatic deterioration at the onset of the next period of glaciation (Dansgaard *et al*. 1989). Depending on their mobility and ecology, these populations either moved southward to suitable areas or went extinct (Coope 1994).

Correspondence: Angelica Crottini, Fax: +39-02-50314781; E-mail: tiliquait@yahoo.it

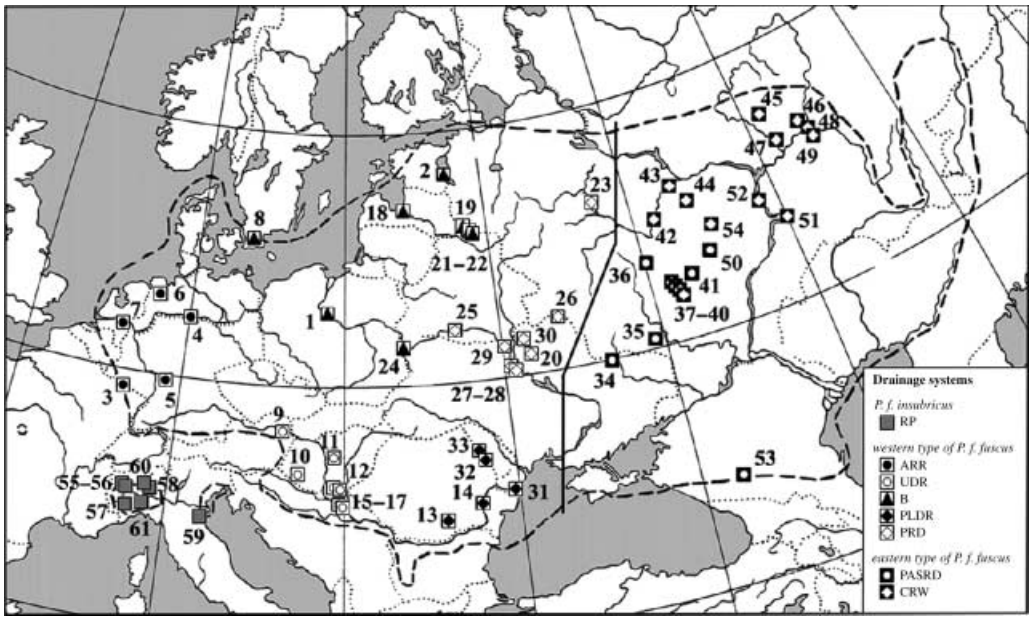

Fig. 1 Distribution of *Pelobates fuscus* (dashed line) and sample locations; for population numbers see Appendix. The border between the 'western' and 'eastern' genome types of *P. fuscus* is marked by a solid line. ARR (Atlantic/River Rhine), RP (River Po), UDR (Upper Danube River), PLDR (Pontic/Lower Danube River), B (Baltic drainage systems, PRD (Pontic/River Dnepr), PASRD (Pontic/Asow Sea and River Don) and CRW (Caspian/River Wolga).

The expansions and contractions of species' distribution ranges leave traces in the gene pools of current populations that can be used for history reconstruction (Avise 2000). In general, populations that have recently colonized areas show a lower genetic variability than those living in refugia, exhibiting low effective population sizes due to the population bottleneck of colonization (founder effect). This is mainly due to the nearly continuous diffusion events during colonization history (Hewitt 1996). On the other hand, past vicariance may be mirrored by a spatial segregation of lineages.

Traditionally, patterns of species distribution are interpreted with reference to historical biogeographical events and the spatial distribution of characters is often interpreted in terms of vicariance, with dispersal usually not detectable from simple character mapping (Avise 2000). Recently, the study of geographically structured populations is increasingly being addressed by two principal approaches (Hey & Machado 2003): phylogeographical methods such as nested clade phylogeographical analysis (NCPA, Templeton 2004) depict population histories from trees, while summary statistics, which are fundamentally mathematical, analyse population structure under a neutral model of evolution. Both approaches have their limitations (reviewed by Hey & Machado 2003), but their combination has been successfully applied in recent studies (e.g. Vila *et al*. 2005). Finally, coalescent simulation can be used as a *post hoc* test for plausibility of derived or *a priori* assumed biogeographical hypotheses by implementing a stochastic process of sequence evolution within a given population history (Knowles & Maddison 2002; Rosenberg & Nordberg 2002; Carbone & Kohn 2004; Knowles 2004).

The genus *Pelobates* (Wagler, 1830) is well suited for phylogeographical analysis, since it includes fossorial species generally not very mobile over long distances (Eggert 2002). The common spadefoot toad, *Pelobates fuscus* (Laurenti, 1768), is one of four species currently belonging to this genus. According to García-París *et al*. (2003), *Pelobates syriacus* (Boettger, 1889) is the sister species of the lineage that includes *Pelobates cultripes* (Cuvier, 1829) and *Pelobates varaldii* (Pasteur & Bons, 1959). *Pelobates fuscus* is a wide-ranging European species (Fig. 1; Nöllert 1990, 1997; Andreone *et al*. 1993; Borkin 1998, 1999), with two accepted subspecies. The range of *Pelobates fuscus fuscus* covers a huge territory from the plains of northeastern France east to western Siberia, and from southern Sweden to the north to the northern Balkans, northern Caucasus and the northwestern Kazakhstan steppes to the south. In contrast, *Pelobates fuscus insubricus* Cornalia, 1873, occupies a small isolated area in the Po Valley in northern Italy.

Recently, a cryptic speciation was suggested in *P. fuscus*, based on differences in the amount of nuclear DNA (Borkin *et al*. 2001, 2005), later confirmed by allozyme data (Borkin *et al*. 2001, 2003; Khalthurin *et al*. 2003). Despite their morphological similarities (Lada *et al*. 2005), two distinct *P. fuscus* types were identified: an 'eastern' group

with a larger amount of DNA, and a 'western' group with a smaller amount. The ranges of the DNA amounts in the two groups did not overlap. The Italian spadefoot toad, assigned to the subspecies *P. f. insubricus*, has not yet been studied. However, its geographical distribution suggests an inclusion within the 'western' type. It is historically distributed throughout the whole Po Valley, from the south of Switzerland (at present probably extinct; Andreone *et al*. 1993) in the north to the Po Delta in the south (Mazzotti & Rizzati 2001; Mazzotti *et al*. 2002), as well as in the western part of the Balkan Peninsula, including Slovenia and Croatia (no recent records; Džukić *et al*. 2005). It seems unlikely that *P. f. fuscus* and *P. f. insubricus* overlap geographically, although Bruno *et al*. (1974) suggested a possible zone of overlap (not yet identified) in northeastern Italy.

Previous phylogeographical analysis of the westernmost and Pannonian populations of the 'western' genome type using the same molecular marker showed an unusual pattern of north–south descent (Eggert *et al*. 2006), suggesting the existence of a northern European area having played a key role as glacial refugium. This, although not common, has already been proposed for other taxa (Stewart & Lister 2001; Kotlík *et al*. 2006). Unfortunately, neither eastern populations of the 'western' genome type nor populations of *P. f. insubricus* were included in this analysis, leaving open the possibility of this peculiar scenario simply being the result of incomplete taxon sampling.

We herein analysed *P. fuscus* populations from its entire distribution range using a part of the cytochrome *b* (cyt *b*) gene. The objectives were to determine (i) whether the north–south pattern of descent is corroborated by a complete geographical sampling, (ii) whether the Italian populations belong to the 'western' clade, and (iii) whether mtDNA variation supports the idea of the 'western' and 'eastern' genome types being distinct species. Finally, using the Afro-European vicariance of *P. cultripes* and *P. varaldii* (García-París *et al*. 2003; Veith *et al*. 2006) and applying Bayesian dating we inferred a time frame for *P. fuscus* evolution.

Materials and methods

Sample localities/tissue sampling

One hundred and fifty-six specimens (90 belonging to the 'western lineage', 37 to the 'eastern' and 29 to Italy) sampled from 61 different localities covering almost the entire species' range were analysed (Fig. 1; Appendix). Ethanol-stored tissue samples were obtained from recent field collections, donations of several researchers and zoological collections, adults killed by road traffic, toes clipped and larvae. Cyt *b* sequences for 17 populations had already been published by Eggert *et al*. (2006).

Populations were assigned to different drainage systems: Atlantic/River Rhine (ARR), River Po (RP), Upper Danube River (UDR; Pannonian Plain), Pontic/Lower Danube River (PLDR), Baltic drainage systems (B), Pontic/River Dnepr and the upper part of River Wolga (point 23) (PRD), Pontic/Azov Sea and River Don (PASRD) and Caspian/River Wolga (CRW). ARR, B, PRD and CRW live in areas that were covered by ice or at least by permafrost during the maximum of the Würm glaciation (Blondel & Aronson 1999). At that time, the areas of RP and PLDR were covered by boreal forest, whereas the UDR area was partially covered by prairies (Flindt 1971). Since the recent distribution of *Pelobates fuscus* is confined to areas south of the permafrost, this makes RP, PLDR, UDR and PASRD potential refugia during the ice ages. With the exception of population 53, all populations of PASRD live in areas formerly covered by ice or by permafrost.

We used homologous sequences of the three other *Pelobates* species (*P. cultripes*, *P. syriacus* and *P. varaldii*) and of *Spea bombifrons* (Cope 1863) and *Spea multiplicata* (Cope 1863) for hierarchical outgroup rooting (GenBank Accession nos DQ333373, DQ333372, EF191042, EF191043 and EF191044).

DNA extraction and sequencing

DNA was extracted from skin or muscle tissue using the High Pure PCR Template Preparation Kit of Boehringer Mannheim GmbH following the protocol of Vogelstein & Gillespie (1979). We sequenced a fraction of the mitochondrial cyt *b* gene, homologous to positions 14676–15378 of the *P. cultripes* complete mitochondrial genome (Gissi *et al*. 2006). We used primers L15162 (light chain; 5′-GCAAGCTTCTACCATGAGGACAAATATC-3′) (Taberlet *et al*. 1992) and H15915 (heavy chain; 5′-AACTGCAGTCATCTCCGGTTTACAAGAC-3′) (Irvin *et al*. 1991). For polymerase chain reaction (PCR) we used 0.2 μL Ready to Go PCR Beads (Pharmacia Biotech), adding 4 μL DNA sample, 1 μL of each primer (20 pmol/μL) and of 19 μL distilled water. PCR cycling procedure was as follows: initial denaturation step: 600 s at 94 °C; 35 cycles: denaturation 60 s at 92 °C, annealing 60 s at 50 °C, extension 90 s at 72 °C. PCR products were purified using the High Pure PCR Product Purification kit of Boehringer Mannheim GmbH.

We sequenced single-stranded fragments of the L-strand using an automatic sequencer (ABI PRISM 377 DNA Sequencer, PerkinElmer Applied Biosystems, Inc.) in a 4.5% polyacrylamide denaturing gel, after preparation with ABI PRISM Big-Dye Terminator Cycle Sequencing Ready Reaction kit (PerkinElmer Applied Biosystems, Inc.).

Sequence alignment and statistics

We consistently sequenced 702 bp for all specimens. Sequences were aligned automatically using the CLUSTAL option of the SEQUENCE NAVIGATOR software (ABI PRISM,

PerkinElmer Applied Biosystems, Inc.). The alignment was subsequently checked by eye. Sequences are deposited in GenBank. We determined the number, nature and distribution of base substitutions using PAUP* 4.b10 software (Swofford 2002).

Nucleotide diversity

Nucleotide diversity (Π) of Nei (1987), the average number of nucleotide differences per site between sequences, was calculated for pooled samples within drainage systems and as an average across samples of drainage systems (the latter only for samples with $n > 1$) using DNASP. We compared nucleotide diversity within genome types between populations from glaciated and nonglaciated areas using the Mann and Whitney *U*-test: ARR, B, PDR vs. RP, UDR, PLDR and PASRD vs. CRW, respectively.

Phylogenetic analyses

Using MODELTEST and following the Akaike information criterion (AIC) (version 3.06; Posada & Crandal 1998), we determined the best-fitting substitution model from a set of 56 models. Phylogenetic relationships of unique haplotypes were determined according to the neighbour-joining method (NJ) applying TrN + I substitution model using PAUP* 4.b10 (Swofford 2002) and to Bayesian inference using MRBAYES, version 3.1 (Ronquist & Huelsenbeck 2003). The Bayesian analysis was run with Markov chain Monte Carlo (MCMC) ngen = 4 000 000, nchains = 4 and sumt burnin = 80 000 to obtain one consensus tree. Two *Spea* species were retained as outgroups. In the NJ topology, robustness of clades was estimated by 2000 bootstrap replicates (Felsenstein 1985).

Group averages of TrN molecular distances (see below) between and among *P. fuscus* haplotype groups and outgroups were calculated with MEGA3 (Kumar *et al*. 2004).

Nested clade phylogeographical analysis

Templeton *et al*. (1995) proposed the use of a nested clade analysis (NCA; later renamed 'NCPA', Templeton 2004) to infer the pattern of potential historical and recurrent processes that may have shaped a species' current distribution range. This approach considers and distinguishes among an array of contemporary (e.g. restricted gene flow) and historical (e.g. past fragmentation, range expansion, or colonization) processes. We first constructed a haplotype network based on statistical parsimony using TCS (version 1.21; Clement *et al*. 2000). We then converted this haplotype network into a nested clade design (Templeton *et al*. 1987), a nested hierarchy of haplotype associations with an increasing clade level reflecting an increasing temporal dimension. Distance measures for any clade X on any level were calculated using GEODIS (version 2.4; Posada *et al*. 2000). The distribution of these distance measures was tested under the null hypothesis of no geographical association, and the history of current haplotype distribution for clades with nonrandom association was inferred using the revised inference key of Templeton (2004). Geographical measures (in kilometres) were calculated from geographical coordinates.

Rooting of minimum spanning (MS) trees for NCPA is strongly recommended (see inference key; Templeton 2004). It adds a temporal hierarchy to the clade design that allows for a better definition of tip (descendant) vs. interior (ancestral) status of haplotypes/clades. Since no *a priori* root was available, we estimated the root of the *P. fuscus* haplotype network from the outgroup-rooted NJ tree that as root has *Spea* sp.

Population fragmentation

We analysed the molecular variance distribution within and among drainage systems for both genome types separately at three hierarchical levels: population (*P*), drainage system (*D*) and lineage (*T*). We applied an analysis of molecular variance (AMOVA; Excoffier *et al*. 1992) using the ARLEQUIN software of Schneider *et al*. (2001). We estimated three variance components and fixation indices: within populations (V_{PT}), among populations within drainage systems (V_{PD}) and among drainage systems (V_{DT}). Significance was determined by 1023 permutations. Pons & Petit (1996) showed that ordered haplotypes may harbour information about their own history, depending on their phylogenetic background. We therefore conducted the AMOVA for unordered alleles.

Mismatch distribution

Episodes of population growth and decline may leave characteristic signatures in the distribution of pairwise nucleotide differences of populations (mismatch distribution; Rogers & Harpending 1992). Under the assumption of the infinite-sites model (Kimura 1971) an observed mismatch distribution can be compared to expectations under different demographic population histories, such as constant population size over time or growth-decline models. There is reason to assume that *Pelobates* populations postglacially expanded within refugial drainage systems and out of refugia into formerly glaciated areas (Eggert *et al*. 2006). We therefore compared empirical mismatch distributions for drainage systems with expectations under a growth-decline model using the DNASP software, version 4.10.3 (Rozas *et al*. 2003). Since we are aware of limitations of this approach (different processes may produce similar mismatch patterns resulting in partially unrealistic assumptions; Rogers & Harpending 1992; Excoffier 2004), we simply use it to search for supportive arguments for inferred phylogeographical scenarios.

Estimation of divergence times

A likelihood-ratio test for substitution rate constancy among *Pelobates* lineages, using *Spea multiplicata* as outgroup and applying the TrN substitution model, rejected the more simple clock-like tree (–log L = 4084.28) when compared to the more complex (no clock) tree (–log L = 3268.51) (analysis performed with TREE-PUZZLE, version 5.0 by Schmidt *et al*. 2000).

We therefore applied Bayesian molecular dating (Thorne *et al*. 1998) to haplotype splits using the MULTIDIVTIME package of Thorne & Kishino (2002). This method uses a probabilistic model to quantify changes of substitution rates over time under the relaxed assumption of substitution rate variation between branches. The MULTIDIVTIME package is only designed for the F84 substitution model (Felsenstein 2004); however, Böhning-Gaese *et al*. (2006) recently showed that different parameter-rich substitution models might produce branch lengths that are highly correlated. The use of F84 instead of the TrN model should therefore not significantly alter species' age estimates.

We used default settings of MULTIDIVTIME as recommended by Rutschmann (2005) except for the following parameters: rtrate = 0.015, rtratesd = 0.015, rttm = 12.0, rttmsd = 12.0, bigtime = 20.0; except rtrate and rtratesd all in million years. We defined the vicariance of Europe from Africa following the end of the Messinian salinity crisis 5.33 ± 0.02 million years ago (Ma) (Krijgsman *et al*. 1999) for calibration. This geological event is thought to be responsible for a number of speciation events in amphibians (e.g. Busack 1986; Fromhage *et al*. 2004), among them *Pelobates varaldii* and *P. cultripes* (García-París *et al*. 2003; Veith *et al*. 2006). To avoid over-representation of *P. fuscus* compared to other species (Yoder & Yang 2004), we selected a single representative haplotype for each major haplotype lineage within *P. fuscus* (see result section).

We repeated molecular dating four times with different random seeds to guarantee that the results of each run did not deviate too much. This assures that program settings are appropriate (Rutschmann 2005).

Test of explicit refugial models

Hypothesis testing has become a central issue of phylogeographical analyses (Knowles & Maddison 2002; Knowles 2001, 2004). However, hypotheses must be statistically tractable and at the same time biologically meaningful (Knowles 2004).

We used information from AMOVA, nucleotide diversity, effective population size, NCPA, mismatch distribution and Bayesian dating to derive a hypothesis to be tested under coalescence theory (see below; Figs 2b and 7). Alternatively, we tested a second geographical hypothesis, plausible in the light of current haplotype distribution (Fig. 2a). Both models assume the existence of two glacial refugia, however, with different geographical patterns of postglacial range expansion.

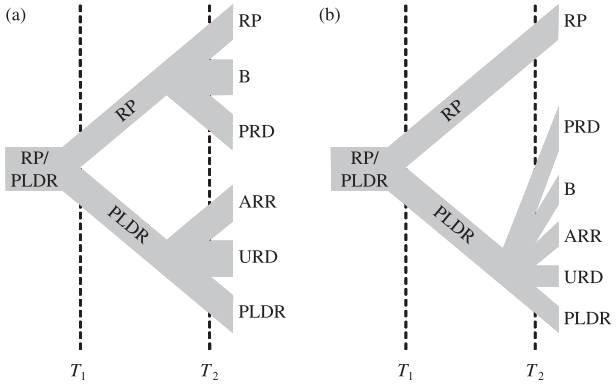

Fig. 2 Two hypotheses on the evolution of ancient *Pelobates fuscus* populations of the western lineage into two sublineages at T_1; the sublineages survived in different glacial refugia (RP and PLDR), from where they colonized further drainage systems at T_2; hypothesis (b) is derived on combined evidence from our phylogenetic and population genetic analyses (see Fig. 7).

We used the coalescence tree simulation approach of MESQUITE (version 1.3; Maddison & Maddison 2006) to model evolutionary histories of the haplotype tree within population trees specified according to our biogeographical hypotheses. We tested two alternative hypotheses of postglacial dispersal (Fig. 2) against the null hypothesis of fragmentation of a single widespread source population during the last glacial maximum. No biogeographical hypotheses were tested for the 'eastern lineage' because of its simple geographical structure.

The MS network and the NJ tree consistently indicate a basal bifurcation within the 'western lineage'. This pattern may correspond to a first historical event at time T_1 (Fig. 2) that forced ancient spadefoot toad populations into two refugia (RP and PLDR), from where later on multiple lineages colonized parts of the formerly glaciated European continent (Fig. 2).

In principle, we assume that *P. fuscus* started to retreat from glaciated and permafrost areas as soon as the climate became colder. This is supported by palaeontological data (Holman 1998; Eggert *et al*. 2006). Based on skeletochronological data (Eggert & Guyetant 1999) we set the generation time to 2 years (age at maturity), an estimate that is the same for *P. cultripes* (Leclair *et al*. 2005).

We tested for divergence between the two putative refugial populations for every 100 000 years within the 95% credibility limits of our Bayesian time estimate for the basal split within the 'western lineage'. Oxygen isotope profiles (Shackleton *et al*. 1990) make it plausible to start *c*. 850 000 years ago (T_1) since climatic oscillations before 850 000 years ago were less severe, with a more or less regular interval of one major cold period every 100 000 years

(Imbrie *et al*. 1984). We assumed the latest expansion process out of refugia to have started after the last ice age *c*. 12 000 yr BP (T_2). This results in eight different time intervals between T_1 and T_2. Initial refugium age was always set to 5000 generations. Based on our estimates of effective population sizes (see below) we regard the UDR population as having expanded postglacially into this area, although we initially classified it as a potential refugial population that lives in an area that was covered by prairie during the last glacial maximum. We chose Slatkin & Maddison's (1989) '*s*' and Maddison's (1997) deep coalescence cost '*dcc*' for test statistics. The statistic '*s*' quantifies the degree of discord between the gene tree and the subdivision into populations by counting the number of parsimony steps in this character on the gene tree, which can be interpreted as the minimum number of migration events between the populations. The statistic '*dcc*' quantifies the number of extra gene lineages to be introduced to fit the gene tree on the population tree (assuming it is due to incomplete lineage sorting; Maddison & Maddison 2006). Both test statistics were estimated for 10 000 random coalescence trees and compared to the empirical NJ haplotype tree within a population tree that reflects our null hypothesis.

For coalescence simulation, we used the substitution model estimated with MODELTEST. We set the overall effective population size (N_e) to empirical population sizes and constrained the refugial effective population sizes to a size proportional to the relative N_e of the population sampled from the putative refugium. All postglacial effective population sizes were constrained to equal proportions of their respective refugial effective population size of the total N_e (following Carstens *et al*. 2005). Empirical estimates of N_e were computed using Beerli's (2002) MIGRATE-N (version 2.1.3). We first estimated theta ($\theta = 2N_e\mu$) under Bayesian inference, which allows reliable results even for low-information data sets such as single locus data sets (Beerli 2006), and then calculated N_e assuming $\mu = 1.0 \times 10^{-7}$ (Carstens *et al*. 2005).

To discern the sensitivity of the deep coalescence test statistic in accepting our null hypotheses of allopatric fragmentation, we ran 10 000 coalescence simulations each for every combination of T_1 = 50 000, 200 000, 350 000 and 500 000 generations and effective population sizes of 1000, 10 000, 100 000 and 1 000 000.

Table 1 Distribution of haplotypes among populations and number of individuals sharing the same haplotype; for population codes see Appendix

Haplotype	Populations	Number of specimens	GenBank Accession no.
W1	10, 13, 15, 33	5	DQ333357
W2	9, 14	5	DQ333358
W3	16	2	DQ333359
W4	11	1	DQ333360
W5	17	3	DQ333361
W6	9, 12, 16, 17	10	DQ333362
W7	9	1	DQ333363
W8	13	2	DQ333364
W9	13	1	DQ333365
W10	14	1	DQ333366
W11	13, 14	8	DQ333367
W12	13	1	DQ333368
W13	7	1	DQ333369
W14	1, 2, 3, 4, 5, 6, 7, 8, 19, 21, 22, 24, 28, 30	29	DQ333370
W15	1	1	DQ333371
W16	31, 32	3	EF133829
W17	60	1	EF133830
W18	61	1	EF133831
W19	55, 56, 57	7	EF133832
W20	57	1	EF133833
W21	60	1	EF133834
W22	58, 60	4	EF133835
W23	58	2	EF133836
W24	57, 58, 59	10	EF133837
W25	18, 19, 20, 21, 22, 25, 26, 27, 29	13	EF133838
W26	57	2	EF133839
W27	26	2	EF133840
W28	23	1	EF133841
E1	39, 40, 42, 43, 44, 45, 48, 49, 50, 51, 52, 53	19	EF133842
E2	35	1	EF133843
E3	51	1	EF133844
E4	45, 47	3	EF133845
E5	43	1	EF133846
E6	44	1	EF133847
E7	41	1	EF133848
E8	35, 36, 38	4	EF133849
E9	46, 54	4	EF133850
E10	34	1	EF133851
E11	37	1	EF133852

Results

Alignment characteristics, nucleotide diversity and molecular divergence

Alignment of sequences was straightforward since there were no gaps, and translation into amino acids indicated neither nonsense nor stop codons. We identified a total of 39 haplotypes (for GenBank Accession nos see Table 1), 72 sites out of 702 (10.3%) were variable, with 54 being parsimony informative. Empirical base frequencies were $\pi_A = 0.238$, $\pi_C = 0.312$, $\pi_G = 0.131$ and $\pi_T = 0.319$. Considering only the 'western lineage' haplotypes the variable sites were 39 (and 25 were parsimony informative), while in the 'eastern lineage' 12 sites were variable and only one was parsimony informative.

The McDonald & Kreitman (1991) test, performed with DNASP, version 4.10.3 (Rozas *et al*. 2003), proved neutrality

Table 2 Diversity statistics by drainage systems for *Pelobates fuscus*; number of localities (n_{loc}), individuals (n_{ind}) and haplotypes (n_{hap}), mean estimated theta (θ), the effective population size (N_e), the proportion of local population sizes from the overall population size (N_e percentage), the probability of capturing the deepest coalescence (*P*), Nei's (1987) nucleotide diversity 'Π' for pooled and nonpooled samples, Harpending's (1994) raggedness statistics '*r*' and Ramos-Onsins' & Rozas' (2002) 'R_2' are shown. nc, not calculated

Drainage	n_{loc}	n_{ind}	n_{hap}	θ	N_e	N_e percentage	*P*	Π_{pooled}	sd (Π_{pooled})	Π_{mean}	sd (Π_{mean})	*r*	R_2
Western lineage													
RP	7	29	9	0.00477	47 700	0.278	0.93	0.00975	0.00121	0.0227	0.0507	0.0401	0.171
ARR	5	13	2	0.00005	500	0.003	0.86	0.00142	0.00142	0.0004	0.0007	nc	nc
UDR	7	24	7	0.00058	5 800	0.034	0.92	0.00339	0.00058	0.0031	0.0021	0.1134	0.0938
B	8	23	3	0.00112	11 200	0.065	0.92	0.01045	0.0045	0.0715	0.0778	0.4444	0.4088
PRD	8	14	4	0.00061	6 100	0.036	0.87	0.00855	0.00367	0.0004	0.0007	0.3333	0.3385
PLDR	5	19	8	0.01	100 000	0.584	0.90	0.00519	0.00124	0.0026	0.0024	0.0689	0.1405
Total	40	122	28		171 300	1.000	0.98	0.01234	0.00104			0.0118	0.1026
Eastern lineage													
PASRD	6	8	5	0.01	100 000	0.983	0.78	0.00342	0.00092	0.00285	—	0.26	0.1633
CRW	15	29	7	0.00017	1 700	0.017	0.93	0.00285	0.00043	0.0008	0.0008	0.7755	0.0583
Total	21	37	11		101 700	1.000	0.95	0.00332	0.0005			0.03405	0.0521

Table 3 Number of substitutions (above diagonal) and molecular TrN + I distances (below diagonal) within and among lineages and species of *Pelobates*; standard errors are derived from 1000 bootstrap replicates

Lineage	Eastern	Western	*P. fuscus*	*P. syriacus*	*P. cultripes*	*P. varaldii*	*Spea*
Eastern	2.3 ± 0.7 0.0033 ± 0.0009	38.5 ± 6.5					
Western	0.0582 ± 0.0101	8.8 ± 1.0 0.0128 ± 0.0027					
P. fuscus				104 ± 12	112 ± 12	111 ± 12	163 ± 13
P. syriacus			0.1754 ± 0.0239		102 ± 12	98 ± 11	173 ± 14
P. cultripes			0.1866 ± 0.0242	0.1681 ± 0.0222		72 ± 9	167 ± 13
P. varaldii			0.1874 ± 0.0250	0.1640 ± 0.0215	0.1158 ± 0.0166		167 ± 13
Spea			0.2851 ± 0.0277	0.3094 ± 0.0313	0.2912 ± 0.0286	0.2956 ± 0.0287	

of the sequenced fragment (test statistics NI = 2.805; Fisher's exact test: *P* value (two tailed) = 0.422165).

Nucleotide diversity within the 'western lineage' (Table 2) was not significantly different between populations from glaciated and nonglaciated areas, either when UDR was or was not considered as a potential refugium. This result also held when populations from 'western' and 'eastern lineages' were pooled (Mann and Whitney *U*-test; $P > 0.05$ in all tests).

Substitution rates and molecular TrN + I distances show that the level of divergence between the 'eastern' and 'western lineage' is *c*. three times lower than that between currently accepted *Pelobates* species (Table 3). Within the 'western lineage', haplotype differentiation is more than three times higher than within the 'eastern lineage'.

Phylogeny and haplotype distribution

According to the Akaike information criterion, MODELTEST selected the TrN + I model of Tamura & Nei (1993), with $\pi_A = 0.2413$, $\pi_C = 0.3122$, $\pi_G = 0.1273$, $\pi_T = 0.3192$, $rate_{(A–G)} = 45.4554$, $rate_{(C–T)} = 23.3009$, and a proportion of invariable sites I = 0.7841. The base pair composition shows the characteristic anti-G bias of mitochondrial genes that is not found in nuclear genes (Zhang & Hewitt 1996).

Haplotypes formed two distinct clusters (Fig. 3) that correspond to the 'western' and 'eastern' genome types of Borkin *et al*. (2001, 2003). We will refer to them here as 'western lineage' and 'eastern lineage'. Among the 'western lineage', 13 haplotypes come from the Balkans, 9 from the Po Valley, with only 6 being widespread from France to the european Russia (Appendix). All *Pelobates fuscus insubricus* sequences are grouped together in a sub-branch of the 'western lineage' (bootstrap *P* = 77%; herein named the '*f. insubricus*-sublineage'), which turned out to be the sister group of the '*f. fuscus*-sublineage'.

Italian haplotypes were unequivocally attributed to the 'western lineage', although they turned out to be exclusive of northern Italy. None of the European haplotypes, including the widespread W14 and W25, occurs in Italy, but the (W25, W27, W28) subclade within the '*f. insubricus*-sublineage' (bootstrap *P* = 89%) is entirely non-Italian and occurs in the B and PRD drainage systems.

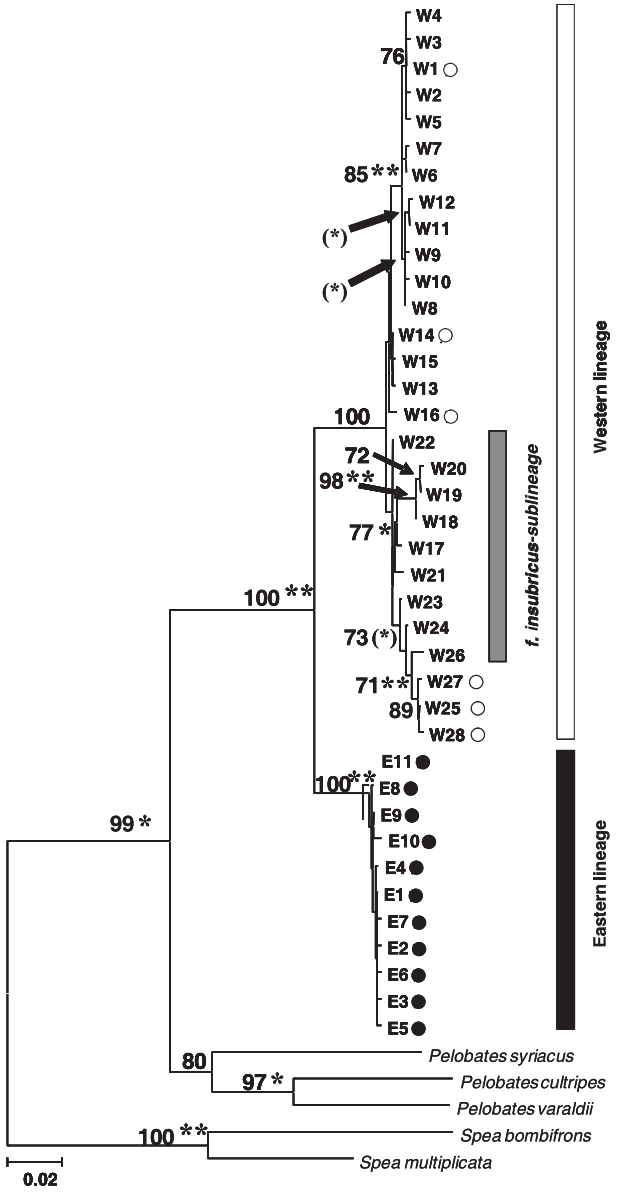

Fig. 3 NJ tree for 39 *Pelobates fuscus* haplotypes based on 702 bp of the cyt *b* gene and the TrN + I substitution model; haplotypes from populations with known genome type are marked with open ('western' genome type) and filled ('eastern' genome type) circles; only bootstrap values = 70% are reported.

Haplotypes of the '*f. fuscus*-sublineage' show a distinct geographical pattern. W14 is widespread across drainage systems ARR and B, with the closely related W13 and W15 found within its range. Haplotypes W1-W12 are restricted to the southern part of the '*f. fuscus* sublineage's range, occurring in PLDR and UDR. W25 and its close relatives W27 and W28 are confined to the easternmost part of the '*f. fuscus*-sublineage's distribution. They intermingle geographically with the W13-W15 haplogroup across PRD drainage system, although not within populations (but note the small samples sizes). Populations polymorphic for both groups of haplotypes are confined to the easternmost part of B (populations 19, 21 and 22).

The 'eastern lineage' shows much lower haplotype diversity as compared to the 'western lineage', and the only widespread haplotype is E1.

Minimum spanning trees and haplotype distribution

The MS trees for the 'western' and 'eastern lineages' were fully resolved (Figs 4 and 5). However, because of their pronounced sequence divergence they did not link together in a single network. In the western network the most widespread haplotype is W14 that, because of its position close to the presumed root of the tree (Templeton *et al*. 1995), should be the ancestor to all haplotypes of the '*f. fuscus*-sublineage' (Fig. 4).

W14 and W25 are both widespread as a result of range expansion or of a widespread historic distribution. The last late glacial from 22 000 until 13 000 yr BP produced very cold and dry conditions throughout Europe. Large ice sheets were present over much of northern Europe with dry open 'steppe tundra' and polar desert covering areas not occupied by ice sheets and sparse grassland or semidesert in most of southern Europe, with permafrost extending across most of Europe down to central France (Frenzel *et al*. 1992). For that reason (i) the wide occurrence of W14 across B, ARR and PRD drainage systems could be explained by a recent expansion out of a refugium where it had persisted since its origin, while (ii) W25 is descendant of W24, restricted to Italy. A radiation of this group out of Italy should have at least partially affected the present range of the haplotype group occurring in the Danube system. Since traces of W25 are left neither in Italy nor in UDR, the species might have gone extinct in this area, with an ensuing range expansion of another haplolineage (W1-W12).

W26 (geographically restricted to a single Italian locality south of Turin) is a descendant of the widely distributed W25, suggesting (i) a second colonization of the Po Valley, or (ii) a loss of the ancestor of this haplolineage, maybe during the last glaciations.

Within the Danube system (UDR and PLDR with W1-W12 plus W16), there is a clear indication of two subsequent radiations: a (W1, W6 and W8) radiation from an unsampled or even extinct ancestor, followed by several local radiations.

In the RP system, W22 and W17 appear to be the two ancient haplotypes. There is no obvious pattern of local geographical arrangement, except that W17 and W22 are locally restricted to the upper Ticino River drainage system and that W24, the ancestor of the W25 radiation into PRD and B is the only haplotype occurring close to the Adriatic Sea.

In the 'eastern lineage' (Fig. 5) haplotype E1, assumed to be the ancestor, is distributed over the whole lineage range,

Table 4 NCPA result for clades with significant geographical structure

Clade		χ^2	P_{χ^2}	D_c	D_n	(I–T)/D_c	(I–T)/D_n
'Western lineage'							
W1-1	I/T	64.00	< 0.001			115.87	40.42
W1	I			327.76	310.97		
W2	T			466.16^L	467.88^L		
W3	T			0.00	104.26		
W4	T			0.00	141.06		
W5	T			0.00^S	95.70^S		
W1-3		4.00	0.502			0.0	–3.17
W8	I			0.00	71.83		
W9	T			0.00	71.83		
W10	T			0.00	78.17		
W1-4		0.32	1.000			74.86	8.52
W11	I			74.86	74.61		
W12	T			0.00	66.09		
W1-5		0.41	1.000			131.05	–95.83
W6	I			131.05	135.06		
W7	T			0.00	230.88		
W1-6		29.93	0.400			702.72	254.87
W13	T			0.00	838.06		
W14	I			702.72	703.16		
W15	T			0.00	58.52		
W1-12		1.90	1.000			11.06	–23.19
W19	I			11.06	12.90		
W20	T			0.00	36.09		
W1-13		0.60	1.000			17.17	7.39
W21	T			0.00	8.41		
W22	I			17.17	15.80		
W1-18		3.00	0.331				
W27	T			0.00	238.45		
W28	T			0.00	156.82		
W2-1		0.012	1.000			0.93	0.54
W1-3	I			74.86	74.69		
W1-4	T			73.93	74.15		
W2-2		10.09	0.350				
W1-1	T			248.97	230.62		
W1-5	T			138.61	166.83		
W2-3		33.00	0.012			609.60^L	–245.16^S
W1-6	I			682.21^S	712.56^S		
W1-7	T			72.61^S	957.72^L		
W2-6		9.00	0.109			–14.45	17.30
W1-11	I			0.00	41.84		
W1-12	T			14.45	24.54		
W2-7		0.80	1.000				
W1-14	I			0.00	160.95		
W1-15	I			164.091	165.89		
W2-8		29.35	0.176			212.05	–426.54^S
W1-16	I			354.16	362.48		
W1-17	T			0.00	1884.77		
W1-18	T			189.47	423.77		
W3-1		32.44	< 0.001			139.09	–208.03^S
W2-1	T			74.32^S	465.95^L		
W2-2	I			213.41	257.92		
W3-2		40.00	< 0.001				
W2-3	I			747.17	757.89		
W2-4	I			14.24^S	964.72^L		
W3-3		26.94	< 0.001			–236.58	970.88^L
W2-7	I			165.44^S	1460.38^L		
W2-8	T			402.02^S	489.50^S		

Table 4 *Continued*

Clade		χ^2	P_{χ^2}	D_c	D_n	(I–T)/D_c	(I–T)/D_n
W3-4		10.00	0.036			–29.49	52.18^L
W2-5	I			0.00	84.54^L		
W2-6	T			29.49	32.35^S		
Total W		298.63	< 0.001			373.02^S	95.98
W3-1	T			292.43^S	489.35^S		
W3-2	I			773.82	819.18		
W3-3	T			664.93	921.58^L		
W3-4	T			38.88^S	1060.95^L		
'Eastern lineage'							
E1-2		158.67	0.313			293.83	41.12
E1	I			409.99	407.30		
E2	T			0.00	584.25		
E3	T			0.00	250.90		
E4	T			47.73^S	455.78		
E5	T			0.00	302.80		
E6	T			0.00	206.16		
E7	T			0.00	227.54		
E8	T			127.51^S	414.44		
E9	T			301.33	318.53		
Total E		71.00	0.123			388.34	–107.77
E1-1	T			0.00	258.03		
E1-2	I			388.34	386.33		
E1-3	T			0.00	730.17		

I, interior clade; T, tip clade; χ^2, observed chi-squared; P_{χ^2}, probability of random χ^2 (10 000 permutations) being greater or equal to observed χ^2; D_c, distance within clade; D_n, distance within nested clade; (I–T)/D, interior vs. tip clade distances; significant values: ^{S}, smaller than mean distance; ^{L}, larger than mean distance.

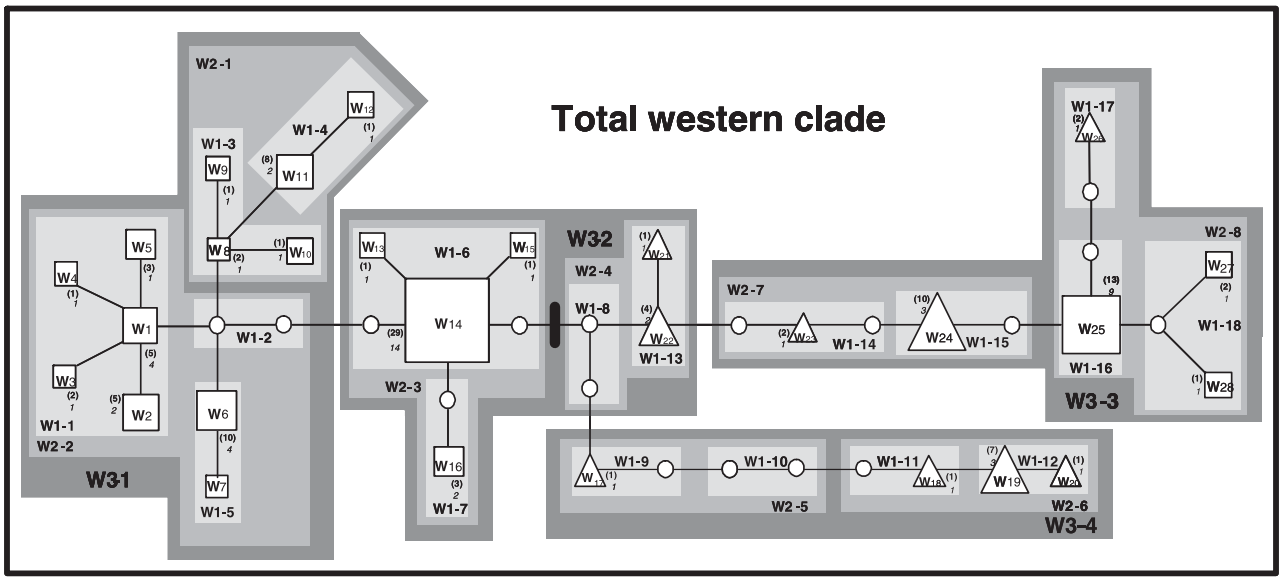

Fig. 4 Minimum spanning tree and combined nested clade design of 28 *Pelobates fuscus* haplotypes of the 'western lineage' based on 702 bp of the cyt *b* gene; small open circles correspond to hypothetical haplotypes (not found or extinct), triangles refer to Italian haplotypes; the black bar between W2-3 and W2-4 marks the position of the root as inferred from the NJ tree. Size of squares and triangles is proportional to the number of individuals (in parentheses) carrying a given haplotype. Single numbers, in italics, show the number of populations in which a haplotype occurs.

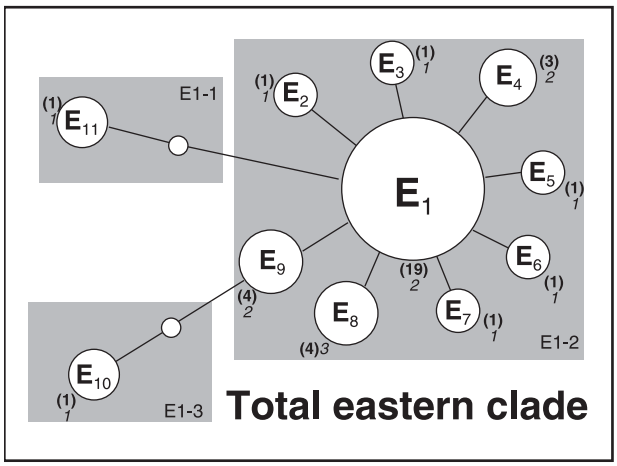

Fig. 5 Minimum spanning tree and combined nested clade design of 11 *Pelobates fuscus* haplotypes of the 'eastern lineage', based on 702 bp of the cyt *b* gene; small open circles correspond to hypothetical haplotypes (not found or extinct). Size of circles is proportional to the number of individuals (in parentheses) carrying a given haplotype. Single numbers, in italics, show the number of populations in which a haplotype occurs.

with E10 representing the most derived haplotype. All other haplotypes are locally restricted. It therefore seems that the range of the 'eastern lineage' arose from a rather recent radiation of E1.

NCPA and Bayesian dating

For Bayesian dating, we used W12 and W26 from the 'western lineage', which, according to the MS tree (Fig. 4), accumulated the largest number of substitutions with respect to the inferred root, while the 'eastern lineage' was represented by E10, the most derived haplotype of this lineage (Fig. 5). Our four repeated Monte Carlo simulations converged at similar time estimates for all nodes, with an average deviation of only 3.7% across all nodes.

The nested clade design sums up to four clade levels in the 'western lineage' and, because of its lower level of divergence, only two clade levels in the 'eastern lineage'. Bayesian molecular dating estimated the time of divergence between them at 2.42 ± 1.40 Ma (lower and upper 95% credibility interval = 0.81–6.15 Ma).

The nested contingency analysis indicates a significant association between clade structure and geographical location only for seven clades of the 'western lineage', mainly on higher clade levels (Table 4).

At the total western clade level, the idea of an initial past fragmentation between the '*f. insubricus*-' and the '*f. fuscus*-sublineages' is corroborated by the NCPA (Table 5), with at least an Italian and a Danube lineage emerging from a vicariance event *c.* 0.55 ± 0.46 Ma (lower and upper 95% credibility interval: 0.11 and 1.69 Ma, respectively) and by the observation that between these two lineages there is no haplotypic overlapping.

Contiguous range expansion within clade W3-3 from the Po Valley (clade W2-7) into large parts of Eastern Europe (W2-8) occurred later. It comprises the W23-W28 haplotypes and the widespread ancestral (internal) haplotypes W24-W25 and the locally derived (terminal) haplotypes W26-W28. Theoretically, this range dispersion was then followed by a second dispersion from Eastern Europe back into the Po Valley within clade W2-8, although this hypothesis is not supported by our NCPA. While these theoretical dispersion events were taking place outside Italy, populations living in the Po Valley (W3-4) were further fragmented into subpopulations in the upper parts of different Po tributaries (W2-5 and W2-6).

In the UDR and PLDR the significant outcome for clade W2-3 was restricted gene flow with some long-distance dispersal, while subclade W1-7 with the single haplotype W16 is restricted to PLDR near the Danube Delta, clade W1-6 has spread over central Europe, with a widespread haplotype W14 and its locally restricted descendant haplotypes W13 and W15.

Although phylogeographical scenarios such as recent range expansion with local isolation appear to be likely for several first-order clades, none of them is supported by the NCPA (Table 5).

Table 5 Inference of phylogeographical scenarios using the revised inference key of Templeton (2004)

Clade	Chain of inference	Inference
W1-1	1-2_a-3_b-5-6	Not applicable
W2-3	1-19-20-2_d-$3_{a,b,c}$-5-6 (insufficient genetic resolution to discriminate between range expansion/colonization and restricted dispersal/gene flow)-7-YES	Restricted gene flow/dispersal but with some long distance dispersal
W3-1	1-2_a-$3_{a,b,d}$-5	Not applicable
W3-2	1-19-20-2-NO tip/interior status can be determined	Inconclusive outcome
W3-3	1-2-11_b-range expansion-12-NO	Contiguous range expansion
W3-4	1-19-NO	Allopatric fragmentation
Total W	1-$2_{a,d}$-$3_{a,b}$-5-15-NO (only Ravenna in between)	Past fragmentation

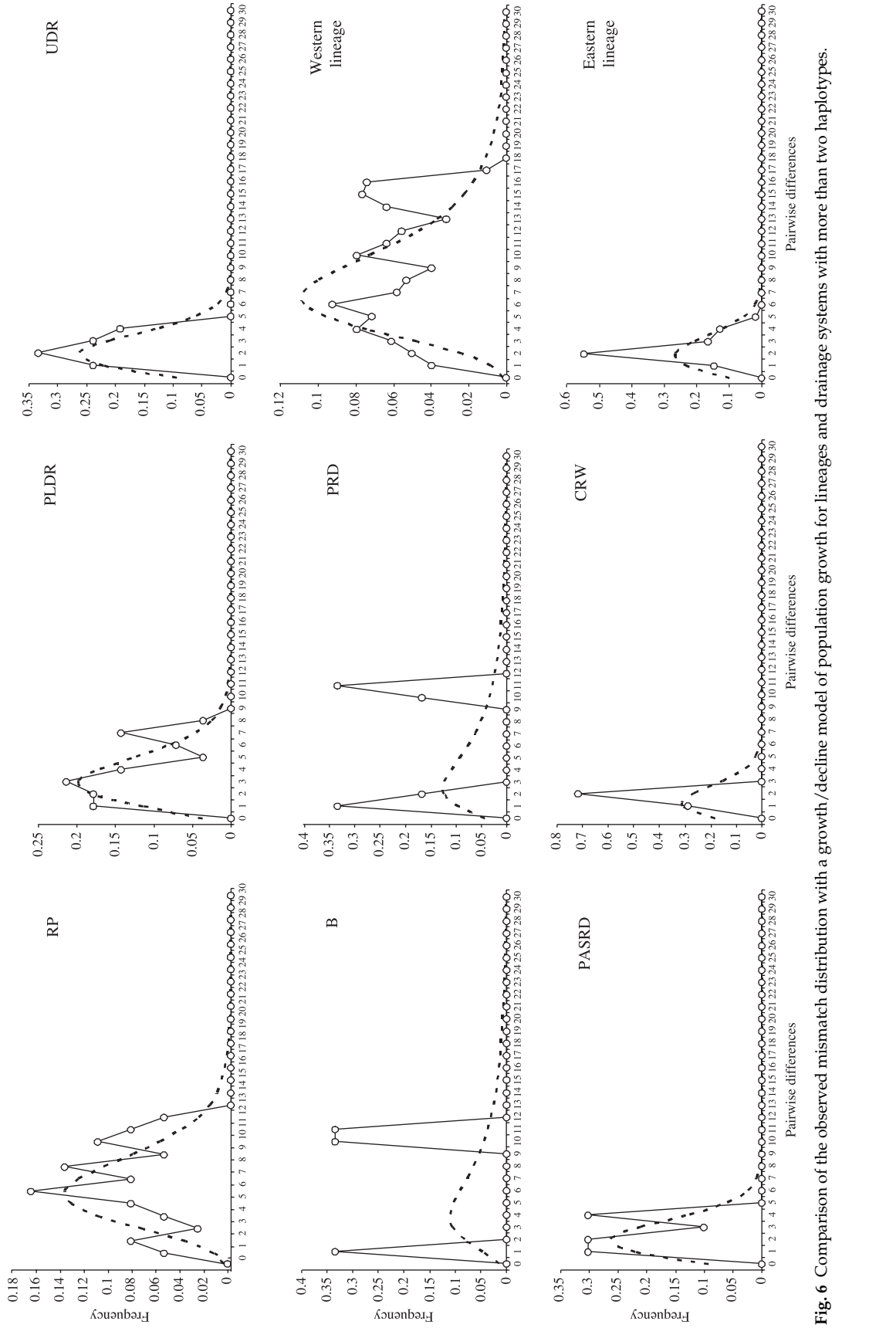

Fig. 6 Comparison of the observed mismatch distribution with a growth/decline model of population growth for lineages and drainage systems with more than two haplotypes.

Table 6 Partitioning of molecular variance within and among drainage systems; significance of variance components and fixation indices was tested after 1023 permutations; d.f., degrees of freedom; significance levels '*P*' are given for variance components and fixation indices simultaneously

Source of variation (XY)	d.f.	Variance components $V_{(XY)}$	Percentage of variation	Fixation index (F_{XY})	*P*
Western lineage					
Among drainage systems (DT)	5	0.11545	24.19	0.24187	< 0.001
Among populations within drainage systems (PD)	34	0.11774	24.66	0.32534	< 0.001
Within populations (PT)	79	0.24415	51.15	0.48852	< 0.001
Total	118	0.47734			
Eastern lineage					
Among drainage systems (DT)	1	0.02260	5.58	0.05581	> 0.05
Among populations within drainage systems (PD)	34	0.14791	36.53	0.38691	< 0.01
Within populations (PT)	79	0.23438	57.89	0.42113	< 0.001
Total	118	0.47734			

Analysis of molecular variance

Half of the molecular variance of the 'western lineage' is hosted within populations (51%; Table 6). The remaining variance is equally distributed among and within drainage systems. Fixation indices also indicate substantial structuring among drainage systems and are in line with variance components. There is no significant variation among drainage systems within the 'eastern lineage', whereas again a major proportion of molecular variance is distributed within populations.

Mismatch distribution

The raggedness of mismatch distributions for lineages and drainage systems shows three principle patterns (Fig. 6): (i) congruence with a distribution under a growth/decline model in UDR, CRW and the entire 'eastern lineage' is indicative of a recent population expansion; (ii) ragged distributions that in general fit the distribution expected under a growth/decline model can be explained by repeated fluctuations in population size that lasted over different amounts of time (see degree of pairwise differences), as is expected for fluctuating populations within refugia: RP, PLDR, the entire 'western lineage' and PASRD; (iii) disruptive empirical distributions, which can be explained best by immigration of distantly related lineages: PRD and B.

Test for population history

Effective population sizes of RP, PLDR and PASRD are large compared to all other drainage systems (Table 2). This may indicate that they persisted over a long period, whereas the other populations went through bottlenecks, potentially during postglacial range expansions.

Coalescence simulations of the gene history within our test hypotheses for the population histories did allow the rejection of the null hypothesis for synchronous postglacial allopatric fragmentation of a continuously distributed ancestral population (Table 7). Our sensitivity analysis, conducted over a wide range of effective population sizes, clearly showed that we must invoke effective population sizes of 1 000 000 or more to accept the null hypothesis of allopatric fragmentation of a formerly widespread population being the origin of the populations in the current drainage systems (Table 8).

Discussion

Evolutionary levels of divergence

Our mitochondrial DNA sequence data support the results of Borkin *et al.* (2001, 2003, 2004) and Khalthurin *et al.* (2003) that *Pelobates fuscus* consists of two distinct and divergent lineages. Their molecular differentiation is about three times lower than that among currently defined biological *Pelobates* species and corresponds to the degree of allozyme divergence that Borkin *et al.* (2003) and Khalthurin *et al.* (2003) found between *P. fuscus* and *Pelobates syriacus* and between the 'eastern' and 'western' genome types, respectively.

However, none of the 20 polymorphic allozyme loci in *P. fuscus* turned out to be diagnostic for either genome type. Three populations of the 'eastern' genome type, two of which are close to the presumed contact zone of both types (our 20 and 26), clustered with the 'eastern' type populations. This, in a straightforward interpretation, is indicative of introgression. Considering the relative age of both forms as inferred from Bayesian dating (2.42 ± 1.40 Ma) this would inevitably necessitate the persistence of large effective populations, thereby preventing genetic drift from fixing single

Table 7 Coalescence simulations (10 000 trees) of 24 two-refugia hypotheses for the western lineage haplotype trees based on Fig. 2. Two alternative centres of origin (RP or PLDR) were assumed for Fig. 2(a). Eight different separation times T_2 were assumed. The number of years and generations (gen), observed (obs), mean value and range for Slatkins and Maddisons '*s*' (1989) and for Maddison's (1997) deep coalescence cost (*dcc*) are shown. Significance levels *P* for observed '*s*' and '*dcc*' being larger than simulated values are given simultaneously. Observed values for '*s*' and '*dcc*' under the null hypothesis of allopatric fragmentation were 11 and 14, respectively

	T_1 (years)	T_1 (gen)	*s* mean	*dcc* mean	*P*
Figure 2a					
RP	850 000	425 000	10.70	15.27	> 0.05
	750 000	375 000	10.66	15.28	> 0.05
	650 000	325 000	10.67	15.40	> 0.05
	550 000	275 000	10.65	15.36	> 0.05
	450 000	225 000	10.66	15.53	> 0.05
	350 000	175 000	10.67	15.79	> 0.05
	250 000	125 000	10.67	16.27	> 0.05
	150 000	75 000	10.88	17.52	> 0.05
PLDR	850 000	425 000	10.67	15.28	> 0.05
	750 000	375 000	10.67	15.25	> 0.05
	650 000	325 000	10.67	15.36	> 0.05
	550 000	275 000	10.67	15.41	> 0.05
	450 000	225 000	10.68	15.54	> 0.05
	350 000	175 000	10.66	15.73	> 0.05
	250 000	125 000	10.72	16.26	> 0.05
	150 000	75 000	10.87	17.53	> 0.05
Figure 2b					
PLDR	850 000	425 000	10.41	10.63	> 0.05
	750 000	375 000	10.42	10.65	> 0.05
	650 000	325 000	10.40	10.68	> 0.05
	550 000	275 000	10.40	10.78	> 0.05
	450 000	225 000	10.41	10.90	> 0.05
	350 000	175 000	10.41	11.15	> 0.05
	250 000	125 000	10.48	11.67	> 0.05
	150 000	75 000	10.64	12.94	> 0.05

alleles at previously polymorphic loci. For a maternally inherited haplotypic locus, the average number of generations until fixation equals the effective population size. Even if we invoke the lower 95% credibility limit of our Bayesian time estimate for the split between the 'eastern' and 'western' lineages (0.81 Ma), this would render effective population sizes that far exceed our estimates. Hence, the conservation of an ancient polymorphism at 20 loci in both lineages as a source of shared alleles can be ruled out.

Whether the 'eastern' and 'western' genome types of *P. fuscus* deserve to be considered as separate species is still a matter of debate (Borkin *et al*. 2001, 2004; Crochet & Dubois 2004). Considering (i) the low levels of molecular divergence between both genome types, and (ii) the evidence for introgression, it is too hasty to assign species status to them. On the other hand, Veith *et al*. (2006) emphasized overall low levels of molecular divergence among *Pelobates* species as compared to other western Palaearctic anurans, possibly because of their fossorial lifestyle. We therefore recommend analysis of populations from an area where both forms co-occur to see if reproductive isolation in syntopy may justify their treatment as two distinct species as Borkin *et al*. (2001, 2004) suggested.

Phylogeographical history of the 'eastern lineage'

The geographical distribution of the 'eastern' mtDNA lineage meets that of the 'eastern' genome type of Borkin *et al*. (2003). It is sparsely differentiated, with a central haplotype E1 giving rise to mostly local haplotypes which, with only two exceptions, evolved only one substitution away from this widespread ancestor. This intuitively indicates a radiation of E1, with descendant haplotypes having recently evolved. Their restriction to local populations (private alleles) argues for restricted gene flow among populations. Strong differences of effective population sizes among drainage systems with N_e-PASRD >> N_e-CRW and the low molecular variance among them strongly argues for a recent expansion out of PASRD northwards into CRW. NCPA did not uncover this scenario.

Phylogeographical history of the 'western lineage'

The phylogeographical history of the 'western lineage' is much more complicated than that of the 'eastern lineage' (Fig. 7). It starts with a basal vicariance followed by a pattern of range expansions within and away from drainage systems, as well as recent gene flow among the drainage systems.

A major split of the 'western' genome type into an Italian '*f. insubricus*-' and a Pontic '*f. fuscus*-sublineage ('c' in Fig. 7) at *c*. 0.55 Ma is corroborated by the NJ and the MS tree and was also inferred by the NCPA. This necessitates a previous range expansion from the Pontic area into the RP region ('b'), although NCPA does not explicitly support this.

Large effective population sizes of RP and PLDR support the idea that both functioned as refugia during periods of deteriorated climate. The pronounced raggedness of the mismatch distribution for RP population gives some indication of recurrent growth/decline cycles of population size. Such a demographic population expansion in a non-subdivided population may give a similar imprint on a mismatch distribution as on a range expansion in a subdivided population (Excoffier 2004). Although it is sound to invoke the consecutive glacials and interglacials of the last 500 000 years as having shaped this oscillation in population size, direct evidence for this does not exist.

All other drainage systems harbour populations with small effective sizes. Such a pattern is typically observed in populations that went through a population bottleneck (e.g. through founder events). In ARR, B, PRD and UDR it

Table 8 Sensitivity analysis for the deep coalescent test statistics (*dcc*) with varying effective population sizes, N_e. A $P < 0.001$ (printed in bold) indicates that the null hypothesis of allopatric fragmentation cannot be rejected

		N_e							
		Figure 2a				Figure 2b			
	T_2	1000	10 000	100 000	1 000 000	1000	10 000	100 000	1 000 000
dcc mean	50 000	0	0	13	**38**	0	0	9	**28**
	200 000	0	0	11	**29**	0	0	8	22
	350 000	0	0	12	**27**	0	0	8	20
	500 000	0	0	11	**26**	0	0	8	19
dcc average	50 000	0.00	0.35	13.24	**37.78**	0	0.133	9.170	**28.010**
	200 000	0.00	0.40	11.53	**28.87**	0	0.136	7.900	22.312
	350 000	0.00	0.33	11.58	**26.94**	0	0.137	7.800	19.845
	500 000	0.00	0.32	11.28	**26.13**	0	0.143	7.771	18.690
P	50 000	> 0.999	> 0.999	> 0.05	**< 0.001**	> 0.99	> 0.99	> 0.99	**< 0.001**
	200 000	> 0.999	> 0.999	> 0.05	**< 0.001**	> 0.99	> 0.99	> 0.99	> 0.05
	350 000	> 0.999	> 0.999	> 0.05	**< 0.001**	> 0.99	> 0.99	> 0.99	> 0.05
	500 000	> 0.999	> 0.999	> 0.05	**< 0.001**	> 0.99	> 0.99	> 0.99	> 0.05

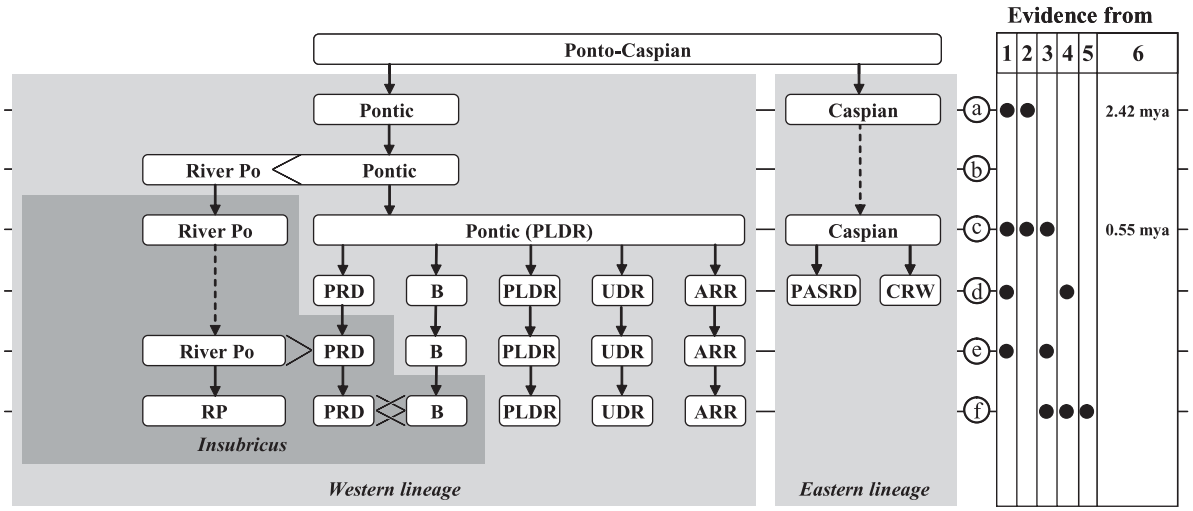

Fig. 7 Hypothesis on the evolution of the eastern and western lineages of *Pelobates fuscus* and the evidence from (1) phylogenetic analysis (2) the minimum spanning tree (3) NCPA (4) mismatch distributions and (5) AMOVA; Bayesian dating (6) for two splits are given; (a) vicariance of a sister lineage into a Caspian and a Pontic lineage; (b) range expansion from the Pontic into the Adriatic area; (c) vicariance of a Pontic and an Adriatic lineages; (d) postglacial range expansion from PLDR into PRD, B, UDR and ARR and from PASRD into CRW; (e) long distance dispersal of haplotype W25 from the Lower Po Valley into PRD; (f) dispersal from PRD into B and vice versa.

probably mirrors postglacial range expansion out of refugia ('d' in Fig. 7). The genealogy of haplotypes and the NJ tree argue for PLDR as the source population for postglacial colonization of formerly glaciated areas of northern and northwestern Europe. Evidence comes from the fact that in the NJ tree W16, a haplotype restricted to PLDR, represents the sister subclade of all other '*f. fuscus*' haplotypes that form regionally exclusive subclusters in the MS tree.

Long-distance dispersal is the only plausible explanation for the occurrence of haplotypes W25, W27 and W28, descendants of the Italian lineage, in northeastern drainage systems. Divergence within this tip lineage is rather recent. NCPA supports this scenario on the third clade level (W3-3) as 'contiguous range expansion'. Further support comes from the bimodality of the mismatch distributions of PRD and B with two very distinct levels of divergence, a pattern that can hardly be explained by within-group diversification.

Gene flow among PRD and B (event 'f' in Fig. 7) is mirrored by the AMOVA, with the same supportive

arguments coming from the mismatch distribution. Haplotype W14, mainly present in ARR and B, is found in PRD, while W25 from PRD entered B. Both are the most widespread haplotypes of their respective group, indicating dispersal throughout large areas. However, it still remains illusive why the PRD populations are fixed for the respective haplotypes of the two different lineages, while in B they intermingle in several single populations. However, for the time being we cannot rule out a sampling artefact.

The 'western lineage' of *P. fuscus* apparently experienced a dynamic population history within and out of refugia. Indeed *P. fuscus* prefers lateral pools along river branches as breeding sites. Riparian corridors along rivers with temporary ponds are very good areas for facilitating population dispersal. This kind of one-dimensional river-bound dispersion may speed up colonization time, and in a short span of time (such as the case here) may largely widen the distributional area of a lineage.

Phylogeographical history of the 'f. insubricus-sublineage'

The high molecular variability and the pattern of haplotype ancestry found in the RP are in line with the theory that glacial refugia are centres of biodiversity (Surget-Groba *et al*. 2002). But neither phylogenetic analysis nor NCPA were able to infer a clear geographical picture of haplotype evolution of the *'f. insubricus*-sublineage'. Given the fact that (i) this sublineage originated *c*. 0.5 Ma, giving rise to two separate sublineages with unclear geographical arrangement, but with a hypothetical common ancestor (extinct or not sampled), and that (ii) its distribution area is relatively small, with many populations already extinct (Andreone *et al*. 2004), it may not be surprising that the recurrent climatic changes during the late Pleistocene with probable oscillating species ranges might have obscured any previously existing geographical pattern. A similar erosive effect of consecutive range expansions and restrictions on geographical patterns of genetic variation has been described from *Salamandra salamandra* on the Iberian Peninsula (Steinfartz *et al*. 2000).

Discordance of analytical approaches

Inclusion of eastern European and Italian samples as well as our combination of phylogenetic, phylogeographical and summary statistical analyses significantly modified the pattern of a north–south descent of haplotypes within the 'western lineage' as suggested by Eggert *et al*. (2006). Because of their position in the MS tree, the PLDR/UDR haplotype group still emerges as a descendant of the widespread central European W14. However, this is contradicted by the phylogenetic analysis where W16 from the presumed Pontic refugium is the sister clade of the entire W1-W15 clade. Effective population size and mismatch distribution also suggest that the current northwestern European distribution of W13-W15 is the result of a postglacial range expansion. In this respect even the discordance of the NJ and MS tree in placing W16 or W14, respectively, at the root of the '*f. fuscus*-sublineage' poses no problem since both may have originated from the Danube Delta, and the overall polarity of both trees is not affected.

Discordance between the MS and the NJ trees is not restricted to the position of the W13-W15 clade. The MS tree places W25 as ancestor of W26. This renders a relocation of W26 from formerly glaciated areas back to the Po Valley, a scenario not supported by further analyses. The hypothesis of W26 being an intra-Italian descendant of W24 as suggested by the phylogenetic tree is more parsimonious since it only necessitates the initial out-of-Italy dispersal of W25. This makes us hypothesize that problems of NCPA in detecting the true history of populations (Knowles & Maddison 2002) may at least partially stem from complicated genealogies inferred by the MS tree, the necessary input for NCPA, and not from the NCPA itself.

Palaeogeographical reconstructions for the spadefoot toad distribution — a summary

Despite all uncertainties associated with phylogeographical analysis, our data suggest the existence of three different glacial refugia of *Pelobates fuscus*: (i) the area between the Caspian and Black Seas as the origin of the expansion of the 'eastern lineage'; (ii) the Danube system as a centre of diversity for part of the 'western lineage'; (iii) the Po Valley, the largest centre of genetic variability.

'Western' and 'eastern lineages' originated from a vicariance event that took place between the end of Miocene and Lower Pleistocene (6.2–0.8 Ma). This is consistent with the estimate of Khalthurin *et al*. (2003) of 2.2 Ma inferred from allozyme data. Considering our results, range expansion of the 'western lineage' follows a complex scenario: (i) survival of a small population in the Pontic area followed by a colonization event; (ii) Lower to Middle Pleistocene vicariance of an Italian and a Danube sublineage *c*. 1.7–0.1 Ma; the Italian sublineage colonized the easternmost area of the 'western lineage'; in conjunction with that this sublineage underwent a second allopatric fragmentation that gave rise to the current situation of two syntopic and distinct Italian sublineages; (iii) the Lower Danube lineage colonized the whole Upper Danube plain. Hence, Pliocene and Pleistocene climatic oscillations left their imprint in the haplotype evolution of *P. fuscus*, characterized by successive vicariance and dispersal events.

Implications for conservation

The populations from northern Italy, currently attributed to *Pelobates fuscus insubricus*, are almost three times less differentiated from 'western' *Pelobates fuscus fuscus* populations

than are the two genome types from each other. So far, they are clustered within the 'western lineages', and there is no evidence of a clear distinctness. In contrast, the RP appears as a major source of genetic variation for the 'western lineage', with even ancient haplotypes being conserved until today. More studies are needed to ascertain whether the Italian populations need to maintain taxonomic and nomenclatorial distinctness, and we prefer to maintain the nomenclature as it stands, that is *P. f. fuscus* for the western 'extra-Italian' sublineage, *P. f. insubricus* for the Italian populations, but we suggest *Pelobates fuscus vestertinus* (Crochet & Dubois 2004) for the 'eastern lineage'. Regardless of any nomenclature rearrangement that might occur, it is clear that the Po Valley acted as a major source of genetic differentiation and that therefore conservation of the Italian populations must necessarily be maintained or even reinforced.

Acknowledgements

The study was supported by the RFBR.05-04-48403 and 05-04-48815. Many people helped us in collecting specimens and tissue samples of *Pelobates fuscus* in Italy, Russia, Belarus, Azerbaijan, and Ukraine. Among these, we wish to thank P. Eusebio Bergò, F. Bernini, S. Scali, A. Gentilli, S. Mazzotti, V. Mercurio, D.V. Skorinov, D.A. Shabanov, S.A. Ryabov, M.V. Pestov. Thanks to Renata Platenberg for linguistic corrections. We thank three previous anonymous reviewers; their comments helped to substantially improve the paper.

References

Andreone F, Fortina R, Chiminello A (1993) Natural history, ecology and conservation of the Italian spadefoot toad, *Pelobates fuscus insubricus*. *Società Zoologica 'la Torbiera'. Scientific Reports*, **2**, 1–96.

Andreone F, Eusebio Bergò P, Bovero S, Gazzaniga E (2004) On the edge of extinction? The spadefoot *Pelobates fuscus insubricus* in the Po Plain, and a glimpse at its conservation biology. *Italian Journal of Zoology*, **71** (Suppl. 1), 61–72.

Avise JC (2000) *Phylogeography. The history and formation of species*. Harvard University Press, Cambridge, Massachusetts.

Beerli P (2002) *MIGRATE: Documentation and Program, Part of LAMARCK, version 1.5*. Available at http://evolution.genetics.washington.edu/lamarc.html.

Beerli P (2006) Comparison of Bayesian and maximum-likelihood inference of population genetic parameters. *Bioinformatics*, **22**, 341–345.

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

Böhning-Gaese K, Caprano T, van Ewijk K, Veith M (2006) Range size: disentangling current traits, phylogenetic, and biogeographic factors. *American Naturalist*, **167**, 555–567.

Borkin LJ (1998) Amphibians. In: *The Encyclopedia of Nature of Russia: Amphibians and Reptiles* (Ananjeva N, Borkin LJ, Darevsky IS, Orlov NL), pp. 19–174. ABF, Moscow, Russia [in Russian].

Borkin LJ (1999) Distribution of amphibians in North Africa, Europe, Western Asia, and the former Soviet Union. In: *Patterns of Distribution of Amphibians A Global Perspective* (ed. Duellman WE), pp. 329–420. Johns Hopkins University Press, Baltimore, Maryland.

Borkin LJ, Litvinchuk SN, Rosanov JM, Milto KD (2001) Cryptic speciation in *Pelobates fuscus* (Anura, Pelobatidae): evidence from DNA flow cytometry. *Amphibia-Reptilia*, **22**, 387–396.

Borkin LJ, Litvinchuk SN, Rosanov JM *et al*. (2003) New data on the distribution of two cryptic forms of the common spadefoot toad (*Pelobates fuscus*) in eastern Europe. *Russian Journal of Herpetology*, **10**, 115–122.

Borkin LJ, Litvinchuk SN, Rosanov YuM, Skorinov DV (2004) On cryptic species (an example of amphibians). *Zoologicheskii Zhurnal*, **83** (8), 936–960 [in Russian; full English version –. *Entomological Review*, **84** (Suppl. 1), 575–598].

Borkin LJ, Litvinchuk SN, Rosanov JM, Džukić G, Kalezić ML (2005) Genome size variation in the Balkan anurans. In: *Herpetologia Petropolitana* (eds Ananjeva N, Tsinenko O), pp. 16–19. Societas Europaea Herpetologica, St. Petersburg, Russia.

Bruno S, Burattini E, Casale A (1974) Il rospo bruno del Cornalia *Pelobates fuscus insubricus* (Cornalia, 1873) (Amphibia, Anura, Pelobatidae). *Atti IV Simposio Nazionale Sulla Conservazione Della Natura*, **3**, 33–55.

Busack SD (1986) Biochemical and morphological differentiation in Spanish and Moroccan populations of *Discoglossus* and the description of a new species from Southern Spain (Amphibia, Anura, Discoglossidae). *Annals of Carnegie Museum*, **55**, 41–61.

Carbone I, Kohn L (2004) Inferring process from pattern in fungal population genetics. *Applied Mycology and Biotechnology*, **4**, 1–30.

Carstens BC, Degenhardt JD, Stevenson AL, Sullivan J (2005) Accounting for coalescent stochasticity in testing phylogeographical hypotheses: modelling Pleistocene population structure in the Idaho giant salamander *Dicamptodon aterrimus*. *Molecular Ecology*, **14**, 255–265.

Clement M, Posada D, Crandall KA (2000) TCS: a computer program to estimate gene genealogies. *Molecular Ecology*, **9**, 1657–1659.

Coope GR (1994) The response of insect faunas to glacial-interglacial climatic fluctuations. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, **344**, 19–26.

Crochet P-A, Dubois A (2004) Recent changes in the taxonomy of European amphibians and reptiles. In: *Atlas of Amphibians and Reptiles in Europe* (eds Gasc JP *et al*.), 2nd edn, p. 498. Societas Europaea Herpetologica & Muséum National d'Histoire Naturelle, Paris, France.

Dansgaard W, White JWC, Johnson SJ (1989) The abrupt termination of the Younger Dryas event. *Nature*, **339**, 532–533.

Džukić G, Beškov V, Sidorovska V, Cogălniceanu D, Kalezić LM (2005) Historical and contemporary ranges of the spadefoot toads *Pelobates* spp. (Amphibia: Anura) in the Balkan Peninsula. *Acta Zoologica Cracoviensia*, **48A** (1–2), 1–9.

Eggert C (2002) Use of fluorescent pigments and implantable transmitters to track a fossorial toad (*Pelobates fuscus*). *Herpetological Journal*, **12**, 69–74.

Eggert C, Guyetant R (1999) Age structure of a spadefoot toad *Pelobates fuscus* (Pelobatidae) population. *Copeia*, **1999**, 1127–1130.

Eggert C, Cogălniceanu D, Veith M, Džukić G, Taberlet P (2006) The declining spadefoot toad, *Pelobates fuscus* (Pelobatidae): paleo and recent environmental changes as a major influence on current population structure and status. *Conservation Genetics*, **7**, 185–195.

Excoffier L (2004) Patterns of DNA sequence diversity and genetic structure after a range expansion: lessons from the infinite-island model. *Molecular Ecology*, **13**, 853–864.

Excoffier L, Smousse P, Quattro J (1992) Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics*, **131**, 479–491.

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

Felsenstein J (2004) PHYLIP *(Phylogeny Inference Package), version 3.6*. Distributed by the Author. Department of Genome Sciences, University of Washington, Seattle, Washington.

Flindt RF (1971) *Glacial and Quaternary Geology*. John Wiley & Sons, New York.

Frenzel B, Pécsi M, Velichko AA (1992) *Atlas of Paleoclimates and Paleoenvironments of the Northern Hemisphere, Late Pleistocene — Holocene*. Geographical Research Institute, Hungarian Academy of Sciences, Budapest, Gustav Fischer Verlag, Budapest, Stuttgart.

Fromhage L, Vences M, Veith M (2004) Testing alternative vicariance scenarios — the case of western Mediterranean Discoglossidae (Amphibia: Anura). *Molecular Phylogenetics and Evolution*, **31**, 308–322.

García-París M, Buchholz DR, Parra-Olea G (2003) Phylogenetic relationships of Pelobatoidea re-examined using mtDNA. *Molecular Phylogenetics and Evolution*, **28**, 12–23.

Gissi C, San Mauro D, Pesole G, Zardoya R (2006) Mitochondrial phylogeny of Anura (Amphibia): a case study of congruent phylogenetic reconstruction using amino acid and nucleotide characters. *Gene*, **366** (2), 228–237.

Harpending HC (1994) Signature of ancient population growth in a low-resolution mitochondrial DNA mismatch distribution. *Human Biology*, **66**, 591–600.

Hewitt DM (1996) Some genetic consequences of the ice ages, and their role in divergence and speciation. *Biological Journal of the Linnean Society*, **58**, 247–276.

Hewitt DM (2000) The genetic legacy of the Quaternary ice ages. *Nature*, **405**, 907–913.

Hey J, Machado CA (2003) The study of structured populations — new hope for a difficult and divided science. *Nature Reviews, Genetics*, **4**, 535–543.

Holman JA (1998) *Pleistocene Amphibians and Reptiles in Britain and Europe*. Oxford University Press, New York.

Imbrie J, Hays JD, Martinson DG *et al.* (1984) The orbital theory of Pleistocene climate: support from a revised chronology of the marine $\delta^{18}O$ record. In: *Milankovitch and Climate* (eds Berger A, Imbrie J, Hays JD, Kukla G, Saltzman B), pp. 269–305. Dordrecht, Reidel, Norwell, Massachusetts.

Irvin DM, Kocher TD, Wilson AC (1991) Evolution of the cytochrome *b* gene mammals. *Journal of Molecular Evolution*, **32**, 128–144.

Khalthurin MD, Litvinchuk SN, Borkin LJ, Rosanov JM, Milto KD (2003) Genetic variation in two cryptic forms, with different genome size, of the common spadefoot toad, *Pelobates fuscus* (Pelobatidae, Anura, Amphibia). *Tsitologiya*, **45**, 308–323 (in Russian).

Kimura M (1971) Theoretical foundation of population genetics at the molecular level. *Theoretical Population Biology*, **2**, 174–208.

Knowles LL (2001) Did the Pleistocene glaciations promote divergence? Tests of explicit refugial models in montane grasshoppers. *Molecular Ecology*, **10**, 691–701.

Knowles LL (2004) The burgeoning field of statistical phylogeography. *Journal of Evolutionary Biology*, **17**, 1–10.

Knowles LL, Maddison WP (2002) Statistical phylogeography. *Molecular Ecology*, **11**, 2623–2635.

Kotlík P, Deffontaine V, Mascheretti S, Zima J, Michaux JR, Searle JB (2006) A northern glacial refugium for bank voles (*Clethrionomys glareolus*). *Proceedings of the National Academy of Sciences, USA*, **103** (40), 14860–14864.

Krijgsman W, Hilgen FJ, Raffi I, Sierro FJ, Wilson DS (1999) Chronology, causes and progression of the Messinian salinity crisis. *Nature*, **400**, 652–655.

Kumar S, Tamura K, Nei M (2004) MEGA 3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Briefings in Bioinformatics*, **5**, 150–163.

Lada GA, Borkin LJ, Litvinchuk SN (2005) Morphological variation in two cryptic forms of the common spadefoot toad (*Pelobates fuscus*) from Eastern Europe. In: *Herpetologia Petropolitana* (eds Ananjeva N, Tsinenko O), pp. 53–56. Societas Europaea Herpetologica, St. Petersburg, Russia.

Leclair MH, Leclair R Jr, Gallant J (2005) Application of skeletochronology to a population of *Pelobates cultripes* (Anura: Pelobatidae) from Portugal. *Journal of Herpetology*, **39**, 199–207.

Maddison WP (1997) Gene trees in species trees. *Systematic Biology*, **46**, 523–536.

Maddison WP, Maddison DR (2006) MESQUITE: *A Modular System for Evolutionary Analysis, version 1.1* Available at http://mesquiteproject.org.

Mazzotti S, Rizzati E (2001) Prima segnalazione di *Pelobates fuscus insubricus* (Cornalia, 1873) nel Delta del Po ferrarese (Amphibia, Anura, Pelobatidae). *Annali del Museo Civico di Storia Naturale di Ferrara*, **4**, 137–142.

Mazzotti S, Penazzi R, Lizzio L (2002) Nuove segnalazioni di *Pelobates fuscus insubricus* Cornalia, 1873 nel sistema dei biotopi costieri del ravennate. *Quaderno di Studi e Notizie di Storia Naturale della Romagna*, **17**, 91–97.

Mc Donald JH, Kreitman M (1991) Adaptive protein evolution at the Adh locus in *Drosophila*. *Nature*, **351**, 652–654.

Milankovitch M (1941) Kanon der Erdbestrahlung und seine Anwendung auf das Eiszeitproblem. *Akad Royal Serbe*, **133**, 1–633. Translated into English as *Canon of Insolation and the Ice Age Problem.* Alven Global, 1998.

Nei M (1987) *Molecular Evolutionary Genetics*. Columbia University Press, New York.

Nöllert A (1990) *Die Knoblauchkröte, Pelobates fuscus*, 2nd edn. *A. Ziemsen Verlag* (Die Neue Brehm-Bücherei 561), Wittenberg, Lutherstadt, Germany.

Nöllert A (1997) *Pelobates fuscus* (Laurenti 1768). In: *Atlas of Amphibians and Reptiles in Europe* (eds Gasc JP *et al.*), 2nd edn, pp. 110–111. Societas Europaea Herpetologica & Muséum National d'Histoire Naturelle, Paris, France.

Pons O, Petit RJ (1996) Measuring and testing genetic differentiation with ordered versus unordered alleles. *Genetics*, **144**, 1237–1245.

Posada D, Crandall KA (1998) MODELTEST: testing the model of DNA substitution. *Bioinformatics*, **14**, 817–818.

Posada D, Crandall KA, Templeton AR (2000) GEODIS: a program for the cladistic nested clade analysis of the geographical distribution of genetic haplotypes. *Molecular Ecology*, **9**, 487–488.

Ramos-Onsins SE, Rozas J (2002) Statistical properties of new neutrality tests against population growth. *Molecular Biology and Evolution*, **19**, 2092–2100.

Rogers AR, Harpending H (1992) Population growth makes waves in the distribution of pairwise genetic differences. *Molecular Biology and Evolution*, **9**, 552–569.

Ronquist F, Huelsenbeck JP (2003) MRBAYES 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics*, **19**, 1572–1574.

Rosenberg NA, Nordberg M (2002) Genealogical trees, coalescent theory and the analysis of genetic polymorphisms. *Nature Reviews, Genetics*, **3**, 380–390.

Rozas J, Sánchez-Del Barrio JC, Messeguer X, Rozas R (2003) DNASP, DNA polymorphism analyses by the coalescent and other methods. *Bioinformatics*, **19**, 2496–2497.

Rutschmann F (2005) *Bayesian Molecular Dating Using PAML/MULTIDIVTIME: A Step-by-Step Manual, version 1.5 (July 2005)*. University of Zurich, Zurich, Switzerland.

Schmidt HA, Strimmer K, Vingron M, von Haeseler A (2000) *TREE-PUZZLE, version 5.0*. Maximum likelihood analysis for nucleotide, amino acid, and two state data. München, Germany.

Schneider S, Roessli D, Excoffier L (2001) *ARLEQUIN, version 2001. A Software for Population Genetics Data Analysis*. Genetics and Biometry Laboratory, Department of Anthropology, University of Geneva, Geneva, Switzerland.

Shackleton NJ, Berger A, Peltier WR (1990) An alternative astronomical calibration of the lower Pleistocene timescale based on OPD Site 677. *Transactions of the Royal Society of Edinburgh: Earth Sciences*, **81**, 251–261.

Slatkin M, Maddison WP (1989) A cladistic measure of gene flow inferred from phylogenies of alleles. *Genetics*, **123**, 603–613.

Steinfartz S, Veith M, Tautz D (2000) Mitochondrial sequence analysis of *Salamandra* taxa suggest old splits of major lineages and postglacial recolonizations of Central Europe from distinct source populations of *Salamandra salamandra*. *Molecular Ecology*, **9**, 397–410.

Stewart JR, Lister AM (2001) Cryptic northern refugia and the origins of modern biota. *Trends in Ecology & Evolution*, **16**, 608–613.

Surget-Groba Y, Heulin B, Ghielmi S, Guillaume C, Vogrin N (2002) Phylogeography and conservation of the populations of *Zootoca vivipara carniolica*. *Biological Conservation*, **106**, 365–372.

Swofford DL (2002) *PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods), version 4.b10*. Sinauer & Associates, Sunderland, Massachusetts.

Taberlet P, Meyer A, Bouvet J (1992) Unusual mitochondrial DNA polymorphism in two local populations of blue tit *Parus caerulus*. *Molecular Ecology*, **1**, 27–36.

Tamura K, Nei M (1993) Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Molecular Biology and Evolution*, **10**, 512–526.

Templeton AR (2004) Statistical phylogeography: methods of evaluating and minimizing inference errors. *Molecular Ecology*, **13**, 789–809.

Templeton AR, Boerwinkle E, Sing CF (1987) A cladistic analysis of phenotypic association with haplotypes inferred from restriction endonuclease mapping and DNA sequence data. I. Basic theory and an analysis of alcohol dehydrogenase activity in *Drosophila*. *Genetics*, **117**, 343–351.

Templeton AR, Routman E, Phillips CA (1995) Separating population structure from population history: a cladistic analysis of the geographical distribution of mitochondrial DNA haplotypes in the tiger salamander, *Ambystoma tigrinum*. *Genetics*, **140**, 767–782.

Thorne JL, Kishino H (2002) Divergence time and evolutionary rate estimation with multilocus data. *Systematic Biology*, **51**, 689–702.

Thorne JL, Kishino H, Painter IS (1998) Estimating rates of evolution and secondary structure. *Molecular Biology and Evolution*, **15**, 1647–1657.

Veith M, Fromhage L, Kosuch J, Vences M (2006) Evolution of Western Palearctic pelobatid and pelodytid frogs — a molecular perspective. *Contributions to Zoology*, **75**, 109–120.

Vila M, Vidal-Romaní JR, Björklund M (2005) The importance of time scale and multiple refugia: incipient speciation and admixture of lineages in the butterfly *Erebia triaria* (Nymphalidae). *Molecular Phylogenetics and Evolution*, **36**, 249–260.

Vogelstein B, Gillespie D (1979) Preparative and analytical purification of DNA from Agarose. *Proceedings of the National Academy of Sciences, USA*, **76** (2), 615–619.

Yoder A, Yang Z (2004) Divergence data for Malagasy lemurs estimated from multiple gene loci: genealogical and evolutionary context. *Systematic Biology*, **13**, 757–773.

Zhang D-X, Hewitt GM (1996) Nuclear integrations: challenges for mitochondrial DNA markers. *Trends in Ecology & Evolution*, **11**, 247–251.

This study is part of A.C.'s Master thesis. She is a PhD student at the University of Milan and her research interests focus on phylogeny, phylogeography and conservation of amphibians. M.V. is professor of animal systematics and zoogeography at the Institute for Biodiversity and Ecosystem Dynamics (IBED, University of Amsterdam) and his research is mainly focused on evolution and phylogeography of Western Palearctic animal species. F.A. prepared the action plan of *P. f. insubricus* for the EU and he is engaged in several conservation-orientated projects on amphibians. L.J.B. is working on systematics, zoogeography, speciation, ecology and conservation of amphibians and reptiles of Eurasia. C.E. has completed his PhD studying *P. fuscus* and has a strong interest in phylogeography, evolutionary biology and conservation of amphibians. S.N.L.'s research focuses mainly on speciation of amphibians of Eastern Europe. Now situated at the Trier University, J.K. mainly works on molecular systematics, phylogeography and ecology of anurans.

Appendix

Population code, drainage system, country code names and sample localities, sample size (*N*); geographical coordinates; mitochondrial haplotypes designation and Nei's (1987) nucleotide diversity (Π) are given (nc, not calculated)

Location	Drainage system	Country	Locality	*N*	Latitude	Longitude	Haplotypes	Π
1	B	PL	Torun	2	53°01′N	18°37′E	W14; W15	0.00142
2	B	EE	Peipsi lake island	5	58°22′N	27°29′E	W14	0
3	ARR	FR	Saint-Avold	5	49°08′N	06°43′E	W14	0
4	ARR	DE	Hanover	2	52°20′N	09°40′E	W14	0
5	ARR	DE	Mainz	3	49°50′N	08°20′E	W14	0
6	ARR	DE	Emsland-Werlte	1	52°50′N	07°40′E	W14	nc
7	ARR	NL	Nijmegen-Ewijk	2	51°50′N	05°35′E	W13; W14	0.00142
8	B	SE	Near Malmö	1	56°10′N	13°40′E	W14	nc
9	UDR	AT	Vienna	12	48°15′N	16°24′E	4xW2; 7xW6; 1xW7	0.00380
10	UDR	HU	Nagybajom	1	46°26′N	17°28′E	W1	nc
11	UDR	HU	Dabas-Gyon	1	47°09′N	19°18′E	W4	nc
12	UDR	HU	Kunadacs	1	46°04′N	19°00′E	W6	nc
13	PLDR	RO	Bucharest	11	44°29′N	25°58′E	1xW1; 2xW8; 1xW9; 6xW11; 1xW12	0.00313
14	PLDR	RO	Braila	4	44°47′N	27°49′E	1xW2; 1xW10; 2xW11	0.00475
15	UDR	CS	Kladovo	2	45°40′N	19°40′E	W1	0
16	UDR	CS	Deliblato	3	45°50′N	19°50′E	2xW3; 1xW6	0.00427
17	UDR	CS	Hrastovaca	4	46°09′N	19°42′E	3xW5; 1xW6	0.00427
18	B	LV	Riga	1	56°57′N	24°07′E	W25	nc
19	B	RU	Osyno	3	56°08′N	28°36′E	1xW14; 2xW25	0.01425
20	PRD	UA	Faivka	1	50°45′N	31°48′E	W25	nc
21	B	RU	Idritsa	2	56°20′N	28°54′E	W14; W25	0.01425
22	B	RU	Peschanka	3	56°09′N	28°33′E	1xW14; 2xW25	0.01425
23	PRD	RU	Simonovo	1	54°32′N	37°25′E	W28	nc
24	B	BY	Medno	3	51°52′N	23°45′E	W14	0
25	PRD	BY	Borovaya	3	52°17′N	27°40′E	W25	0
26	PRD	UA	Antonovka	3	51°52′N	33°29′E	1xW25; 2xW27	0.00142
27	PRD	UA	Chertoryi	1	50°28′N	30°32′E	W25	nc
28	PRD	UA	Koncha Zaspa	2	50°15′N	30°32′E	W14	0
29	PRD	UA	Chernobyl′	1	51°17′N	30°14′E	W25	nc
30	PRD	UA	Naumovka	2	50°53′N	31°32′E	W14	0
31	PLDR	UA	Vilkovo	2	45°24′N	29°35′E	W16	0
32	PLDR	MD	Kantemir	1	46°17′N	28°12′E	W16	nc
33	PLDR	MD	Ungeny	1	47°12′N	27°48′E	W1	nc
34	PASRD	UA	Kharkov	1	49°59′N	36°12′E	E10	nc
35	PASRD	RU	Rossosh	3	50°12′N	39°35′E	1xE2; 2xE8	0.00285
36	PASRD	RU	Bukhovoe	1	53°14′N	40°04′E	E8	nc
37	CRW	RU	Chistye Prudy	1	52°43′N	41°30′E	E11	nc
38	CRW	RU	Prokudskie Prudy	1	52°44′N	41°11′E	E8	nc
39	CRW	RU	Zarech′e	2	52°52′N	41°31′E	E1	0
40	CRW	RU	Bol′shaya Lipovitsa	2	52°33′N	41°20′E	E1	0
41	PASRD	RU	Orshevka	1	52°36′N	43°02′E	E7	nc
42	CRW	RU	Gus′-Zheleznyi	1	55°03′N	41°09′E	E1	nc
43	CRW	RU	Reshetikha	3	56°12′N	43°19′E	2xE1; 1xE5	0.00142
44	CRW	RU	Ichalki	3	55°37′N	44°44′E	2xE1; 1xE6	0.00142
45	CRW	RU	Kilmez′	4	57°02′N	51°21′E	2xE1; 2xE4	0.00142
46	CRW	RU	Boyarka	3	56°04′N	54°04′E	E9	0
47	CRW	RU	Krymskie Sludki	1	56°04′N	51°21′E	E4	nc
48	CRW	RU	Izhevsk	1	56°50′N	53°12′E	E1	nc
49	CRW	RU	Amzya	1	56°15′N	54°10′E	E1	nc
50	PASRD	RU	upper Khopyor River	1	52°49′N	44°27′E	E1	nc
51	CRW	RU	Nizhnee Sencheleevo	4	53°29′N	49°30′E	3xE1; 1xE3	0.00142
52	CRW	RU	Ul′yanovsk	1	54°18′N	48°52′E	E1	nc
53	PASRD	RU	Stavropol	1	45°03′N	41°57′E	E1	nc
54	CRW	RU	Yalga	1	54°11′N	45°10′E	E9	nc

Appendix *Continued*

Location	Drainage system	Country	Locality	*N*	Latitude	Longitude	Haplotypes	Π
55	RP	IT	Moncrava	2	45°29′N	07°54′E	W19	0
56	RP	IT	Cascinette	3	45°28′N	07°55′E	W19	0
57	RP	IT	Poirino/Favari/La Gorra	6	44°56′N	07°50′E	1xW20; 1xW24; 2xW19; 2xW26	0.01260
58	RP	IT	Cameri	11	45°27′N	08°37′E	2xW22; 2xW23; 7xW24	0.00380
59	RP	IT	Classe pinewood/ Bassa del Bardello	2	44°32′N	12°16′E	W24	0
60	RP	IT	Arsago Seprio	4	45°42′N	08°42′E	1xW17; 1xW21; 2xW22	0.00665
61	RP	IT	Belangero	1	44°54′N	08°12′E	W18	
Pelobates syriacus		AZ	Moshkhan	1	38°29′N	48°50′E	OS	
Pelobates cultripes		FR	Argelès-sur-Mer	1	42°33′N	03°02′E	OC	