Conservation Genetics (2006) 7:185–195
DOI 10.1007/s10592-006-9124-y

The declining Spadefoot toad, *Pelobates fuscus* (Pelobatidae): paleo and recent environmental changes as a major influence on current population structure and status

Christophe Eggert[1,*], Dan Cogălniceanu[2], Michael Veith[3], Georg Dzukic[4] & Pierre Taberlet[5]

[1]*Laboratory of Alpine Ecology, CNRS UMR 5553, Université de Savoie, CISM, F-73376, Le Bourget du Lac Cedex, France;* [2]*Faculty of Biology, Department of Ecology, Bucharest University, Splaiul Independenţei 91-95, 050095, Bucharest, Romania;* [3]*Institut für Zoologie, Abteilung Ökologie, Johannes Gutenberg-Universität Mainz, Saarstraße 21, D-55099, Mainz, Germany;* [4]*Institute for Biological research "Sinisa Stankovic" Bulevar despota Stefana 142, 11060, Belgrade FR, Serbia;* [5]*Laboratory of Alpine Ecology, CNRS UMR 5553, Université Joseph Fourier, BP 53, F-38041, Grenoble Cedex 9, France (*Corresponding author: E-mail: eggert@univ-savoie.fr)*

Received 5 July 2005; accepted 27 November 2005

Key words: amphibian decline, Dryas, global changes, low polymorphism, mtDNA, nested clade phylogeographic analysis, *Pelobates fuscus*, Spadefoot toad

Abstract

Populations of the European Spadefoot toad (*Pelobates fuscus*) have experienced recent declines all over Europe, but these appear to be more intense in north and western Europe. Due to the toad's fossorial nature and specific habitat requirements, environmental conditions have played a major role in structuring current populations. We examined the phylogeographic structure in *P. fuscus* from 16 localities throughout Europe using mitochondrial cytochrome *b* gene sequence analysis. Sequence divergence among haplotypes was low ($0.54 \pm 0.15\%$). Three very closely related haplotypes occupy northern and western parts of Europe whereas 12 others were observed among samples from south-eastern Europe, including the Balkans. Our results suggest that toads only recently colonized the northern and western parts of Europe following glacial retreat. This expansion probably took place in steppic-like areas during the younger Dryas cold interval, about 12,900–11,500 years ago. Restricted gene flow with an isolation-by-distance population structure characterises a major part of its distribution range. Based on our results we suggest that the northern and western lineages should be considered as distinct conservation units, while the south-eastern populations from the refugial areas, where nearly all genetic polymorphism occurs and populations appear less vulnerable, should receive special attention.

Introduction

Climatic changes during the Pleistocene had a profound impact on the distributional and genetic patterns of many species living in temperate zones (Hewitt 2000). During the coldest periods many Palaearctic species were confined to southern refugia, subsequently expanding their ranges again in warmer periods with retreat of the ice. Evidence for range expansions and contractions can be detected in current population genetic structures and used have been used to reconstruct population history (e.g. Avise 1992; Taberlet et al. 1998). Lower genetic diversity typifies recently colonized areas more so than refugial areas, mainly due to repeated founding events over colonizing routes

(Hewitt 1996). Because amphibians are ectothermic and adapted to specific climates, they are known to be very sensitive to environmental conditions and have even provided reliable information concerning palaeoenvironmental conditions (Bailon and Rage 1992). Therefore, palaeoenvironmental fluctuations could lead to population expansion or isolation in amphibian species that could be inferred from current population genetic structure (e.g. Barber 1999; Riberon et al. 2001).

Three species of the Spadefoot toad genus *Pelobates* occur in Europe (*P. cultripes, P. fuscus* and *P. syriacus*) and a fourth in north-western Africa (*P. varaldii*). The Spadefoot toads have narrow habitat requirements, due to their fossorial behaviour (Shpun et al. 1993; Martinez-Rica 1997; Nöllert 1997). Toads inhabit preferentially sandy areas, in which they burrow during daytime by digging with metatarsal tubercles of their hind limbs. They breed in permanent or temporary ponds and the larval period lasts for 2–4 months. European Spadefoot toads *Pelobates fuscus*, although widely distributed across Europe (Nöllert 1997), shows a patchy distribution restricted to specific friable soils (e.g. Nöllert 1997; Kuzmin 1999). The species is strictly protected by both the Bern Convention and Habitat Directive 92/43/EEC, is included in most local Red Data Book lists (Nöllert 1997; Kuzmin 1999), and is the object of many local conservation efforts. It is nevertheless considered a least concern species by the last IUCN assessment due to its wide range and presumably large populations (Baillie et al. 2004). Recently a cryptic species, representing populations in the former Soviet Union, was detected with DNA flow cytometry (Borkin et al. 2001).

Declining amphibian populations have a worldwide occurrence, with various causes hypothesised to explain the phenomenon (e.g. Gardner 2001; Collins and Storfer 2003). Spadefoot toad population declines have been observed or suspected over major portions of their geographic range (Nöllert 1997). Declines were particularly striking in the western part of their range, where population extinctions were clearly documented during the last century (see Lescure 1984; Parent 1985; Dubois 1998 for France, Rappė 1982; Perczy 1994 for Belgium, Pelt and Van Bree 1965 for Netherlands, Fog et al. 1997 for Denmark, Gislen and Kauri 1959; Berglund 1998 for Sweden), but also in the north-eastern and eastern parts (Kuzmin 1999) or in the south-eastern parts in Serbia (Džukić et al. 2005). As an example of local extirpation, toads disappeared from 98% of the breeding ponds over a 45 year period in Denmark (Fog et al. 1997). The fact that strong regression has occurred mainly in this part of the species distribution has been singled out as an important observation in need of scientific explanation (Dubois 1998).

Because of their specific habitat requirements, we argue that environmental conditions strongly influence Spadefoot toad population dynamics. We assume that palaeoenvironmental conditions, especially repartition of palaeobiomes, have played a major role in structuring current populations, making the European Spadefoot toad a good candidate for illustrating the contraction–expansion model (Taberlet and Cheddadi 2002). Furthermore, we hypothesise that toads from declining areas represent a distinct lineage. For the present study, we compared samples from the declining area with samples from other areas where large populations still persist. We focused on a sequence of cytochrome *b* (mtDNA) to investigate current phylogeographic structure and estimate the impacts of palaeoenvironmental changes.

Material and methods

We obtained samples from 16 localities throughout Europe. Since this species is strictly protected, rare and secretive, collection of large samples has generally been precluded. Tissue samples in ethanol came from a variety of sources: adults killed by road traffic, clipped toes, larvae or larval tailtips and museum specimens (Figure 1; Table 1).

DNA was isolated from skin or muscle using a standard proteinase K digestion protocol (Qiagen^{TM}) for animal tissues, except that centrifugation was performed after digestion (12,000 g, 3-min) to eliminate undigested tissue. Sample extracts 2 (μl) were amplified using PCR with cytochrome *b* primers H15915 (Irwin et al. 1991) and L15162 (Taberlet et al. 1992) in a final volume of 25 μl (200 μM of primers, 2 mM of $MgCl_2$, 1 unit of *Taq* Gold^{TM}, Perkin Elmer polymerase). A 10-min initial denaturation at 95°C was followed with 45 thermal cycles of 1-min denaturation at 95 °C, 1-min annealing at 50 °C and 3-min

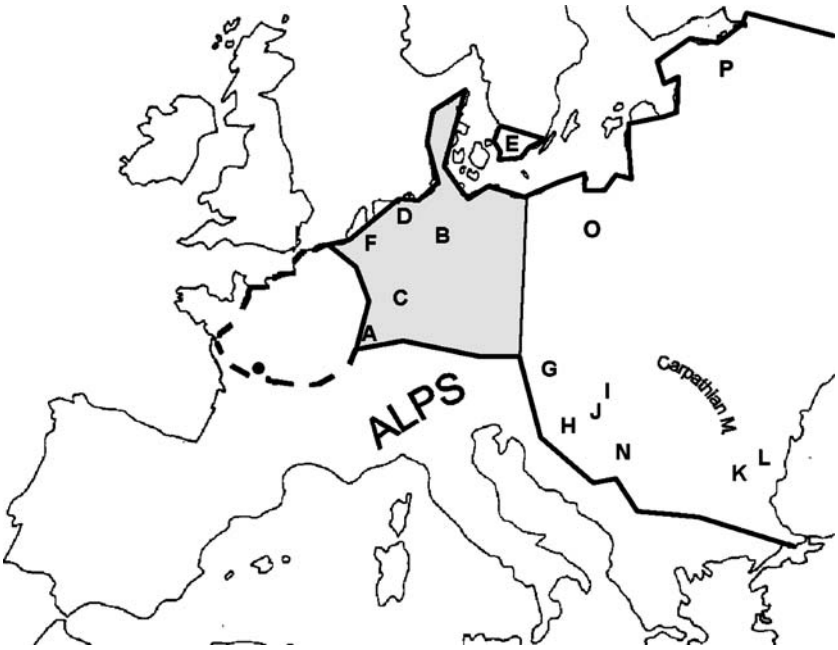

Figure 1. Map of the sampled locations for *Pelobates f. fuscus*. Population codes correspond to the localities listed in Table 1. Population M was too close to population N to be shown. The dotted line indicates 19th century western edge of expansion (Lescure 1984); the solid line indicates current expansion (Nöllert 1997). The isolated black point in France represents an extant remnant population. The shaded area reveals region where current population declines have been documented (see text for references).

Table 1. List of samples used in this study with population codes, locality, sample sizes, mitochondrial haplotypes and nucleotide diversity Π of Nei and it standart deviation

Population code	Country	Locality	Latitude	Longitude	n	Haplotypes	Π	SD (Π)
A	France	Saint-Avold	49°08 N	6°43 E	5	W14	0	0
B	Germany	Hannover	*Circa* 52°20 N	9°40 E	2	W14	0	0
C	Germany	Mainz	49°50 N	8°20 E	3	W14	0	0
D	Germany	Emsland-Werlte	52°50 N	7°40 E	1	W14	–	–
E	Sweden	South Scania	*Circa* 56°10 N	13°40 E	1	W14	–	–
F	Netherlands	Nijmegen-Ewijk	*Circa* 51°50 N	5°35 E	2	W13 W14	0.00142	0.00071
G	Austria	Vienna	48°15 N	16°24 E	12	W2 (n = 4); W6 (n = 7); W7	0.038	0.00142
H	Hungary	Nagybajom	46°26 N	17°28 E	1	W1	–	–
I	Hungary	Dabas-Gyon	47°09 N	19°18 E	1	W4	–	–
J	Hungary	Kunadacs	*Circa* 46°04 N	19° E	1	W6	–	–
K	Romania	Bucharest	44°29 N	25°58 E	11	W1; W8 (n = 2); W9 W11 (n = 6); W12	0.00313	0.00073
L	Romania	Brăila	44°47 N	27°49 E	4	W2; W10; W11 (n = 2)	0.00475	0.00148
M	Serbia	Kladovo	*Circa* 45°40 N	19°40 E	2	W1	0	0
N	Serbia	Deliblato	45°50 N	19°50 E	3	W3 (n = 2); W6	0.00427	0.00214
N	Serbia	Hrastovaca	46°09 N	19°41	4	W5 (n = 3); W6	0.00427	0.00214
O	Polonia	Torun	53°01 N	18°37 E	2	W14; W15	0.00142	0.00071
P	Estonia	Piirissaar	58°22 N	27°29 E	5	W14	0	0

extension at 72 °C. PCR products were purified on QiaQuick PCR columns. Sequencing was performed with a Perkin Elmer ABI PRISM^{TM} 377 automated DNA sequencer in 5% LongRanger^{TM} gels (FMC) after preparation with ABI PRISM^{TM} Dye Terminators Sequencing Ready Reaction Kit

(Perkin Elmer). Fragment sequences were aligned using the SEQUENCE NAVIGATOR program (version 1.0.1, Perkin Elmer). The sequences are deposited in EMBL, GenBank and DDBJ (Accession numbers DQ333357 to DQ333373).

With MODELTEST version 3.06 (Posada and Crandall 1998) we determined the HKY substitution model as the best supported by our data (hLRT test with $\ln L = -1069.2742$; base frequencies: $\pi_A = 0.2377, \pi_C = 0.3150, \pi_G = 0.1309, \pi_T = 0.3165$ with equal rates for all sites and the number of invariable sites $I = 0$). Phylogenetic relationships were determined by the neighbour-joining method using PAUP* (version 4) (Swofford 1998), with 2000 bootstrap replicates to assess topology robustness. *P. syriacus* from Deliblato (Serbia) and *P. cultripes* from Argeles-sur-Mer (France) were used for outgroup rooting. Average molecular distances and substitution rates between *P. fuscus* haplotypes and outgroups as well as among *P. fuscus* haplotypes were calculated with MEGA 3 (Kumar et al. 2003), with standard errors calculated for 2000 bootstrap replicates.

Nucleotide diversity Π of Nei (1987), the average number of nucleotide differences per site between sequences, was calculated for all samples with $n > 1$ using DnaSP version 4.10.3 (Rozas et al. 2003). We compared nucleotide diversity among geographic groups of populations using the non-parametric Mann and Whitney *U*-test.

In order to free our phylogeographic interpretation of *P. fuscus* haplotype distribution from *a priori* hypotheses of lineage evolution, we applied a nested clade phylogeographic analysis, NCPA (Templeton et al. 1995; Templeton 2004). It links the evolutionary position of haplotypes within a minimum spanning tree with their geographic location via rigorous testing of different causes of geographical association of haplotypes. A 95% plausible set of all haplotype linkages in an unrooted haplotype tree (program TCS of Clement et al. 2000) provided the basis for nested clade definition following the basic nesting rules as described in Templeton et al. (1987). Geographical distances (in kilometers) were calculated from geographical co-ordinates. D_c (X) quantifies the average distances of single clade X haplotypes from their respective geographical centre; whereas D_n (X) quantifies the nested clade distances of all nested clade X haplotypes from their geographical centre. Differences of $(I - T)D_c$ and $(I - T)D_n$ quantify equivalent measures for tip (T) and interior (I) nested clades. All measures were calculated using GeoDis (Posada et al. 2000). This program also calculates tests for geographic association as described by Templeton and Sing (1992). The distribution of distance measures under the null hypothesis of no geographic association was determined after 10,000 random permutations of clades against sampling locations and recalculating the distances after each permutation (Templeton et al. 1995). This allows testing for significantly larger (^{L}) and smaller (^{S}) distances for each clade within a group of nested clades with respect to the null hypothesis. We interpreted NCPA results using the inference key provided at the GeoDis homepage (http://inbio.byu.edu/Faculty/kac/crandall_lab/geodis.htm; inquiry date 14th of July 2004). This inference key addresses some recent criticism to NPCA (Knowles and Maddison 2002).

Results

The alignment of a 702 bp fragment of *cytochrome b* sequenced for 60 individuals was straightforward; there were no indels, and all sequences were translated without nonsense codons. A total of 15 mitochondrial haplotypes was recovered, with 17 (2.4%) variable sites (Table 2). Levels of divergence among *P. fuscus* haplotypes were quite low (HKY molecular distance: 0.0054 ± 0.0015), whereas molecular divergence between *P. fuscus* and outgroup species was 0.1728 ± 0.0232 for *P. syriacus* and 0.1932 ± 0.0253 for *P. cultripes*. All samples from the north-western populations (codes A to F) shared the same haplotype (W14), except for one individual from locality F showing one nucleotide sequence mutation haplotype (W13). W14 was also found in Poland, together with W15 (only one substitution difference). A total of 14 distinct haplotypes was found for individuals from populations G to P. Most phylogenetic relationships among haplotypes were poorly resolved (Figure 2).

Nucleotide diversity of populations ranged from 0 to 0.00427. Populations from formerly glaciated or permafrost areas in the north-west and north (A–F, O and P) showed a significant lower Π than those from south-eastern Europe (*U*-test; $U = 4$, $Z = 2.24179$, $P < 0.05$).

While haplotype W12 appears to be basal in the NJ tree, it becomes a terminal haplotype in the minimum spanning tree (Figure 3). When rooted with additional *P. fuscus* haplotypes from Italy and eastern Europe (Crottini et al. unpublished), the wide-spread W14 becomes the root of the minimum spanning tree, making clades 1-6 and 2-1 interior clades, which has consequences for NPCA interpretation.

Significant deviation from random association of haplotypes and localities is indicated only for clades 1-1, 2-1 (which covers the whole study area), and the total clade. For all other clades the outcome is inconclusive. However, interpreting clade 1-1 also gives no result. The pattern observed for clade 2-1 in the rooted tree can be explained by restricted gene flow with isolation-by-distance. Level 2 clades probably have spread over western Europe in a process of long distance colonisation (total clade; Table 4). Alternatively, each subclade of the total clade may have expanded its area after past fragmentation.

Discussion

Nucleotide divergence among haplotypes

This study revealed a surprisingly low level of sequence divergence between haplotypes ($0.54 \pm 0.15\%$). In amphibians, intraspecific sequence divergence ranges from 0 to 16.2% (review in Ribéron et al. 2001). Avise et al. (1998) reported for amphibians and reptiles a median value of 3.1% in mtDNA (except for the control region) sequence divergence between phylogroup pairs (i.e. at intraspecific level). Slow mtDNA clocks have also been suggested for some poikilotherms (Johns and Avise 1998; Avise et al. 1998), but it is possible that the rate of mtDNA evolution in Spadefoot toads is unusually slow. Nevo and Beiles (1991) reported that among amphibians *Pelobates* species have among the lowest levels of allozyme heterozygosity and polymorphism. Based on an estimated rate of mtDNA divergence in some amphibians of 0.7–0.8%/Myear (see Ribéron et al. 2001), the low level of diversity in the studied samples of *P. fuscus* could result from Pleistocene divergence, about 1.4–0.18 Myear ago. If however substitution rates are lower in *Pelobates*, then divergence among *P. fuscus* lineages may well have started prior to the Pleistocene.

Historical biogeography

A low level of intraspecific polymorphism at higher latitudes, particularly in areas glaciated during the Pleistocene cold periods, and a higher level of genetic variation in areas that corresponded to refugia is a trend described in many species (Taberlet 1998). Limited mitochondrial

Table 2. Variable sites from the aligned 702 base pair sequences

Base position	Haplotypes																
	16	79	88	97	136	148	184	202	250	292	307	331	433	481	505	574	694
W1	T	T	T	C	A	T	C	T	T	C	C	T	A	C	G	T	C
W2	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.
W3	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.
W4	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.
W5	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.
W6	.	.	.	.	.	.	.	.	.	T	.	.	.	.	A	.	.
W7	.	.	.	.	.	.	.	.	.	T	.	.	G	.	A	.	.
W8	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	C	.
W9	.	C	.	.	.	.	.	.	.	T	.	.	.	.	.	C	.
W10	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	C	T
W11	C	.	.	.	.	.	.	.	.	T	.	.	.	.	.	C	.
W12	C	.	.	.	.	.	.	.	.	T	.	.	.	T	.	C	.
W13	.	.	.	T	G	.	.	.	C	T	.	C	.	.	.	.	.
W14	.	.	.	T	G	.	.	.	.	T	.	C	.	.	.	.	.
W15	.	.	.	T	G	.	T	.	.	T	.	C	.	.	.	.	.

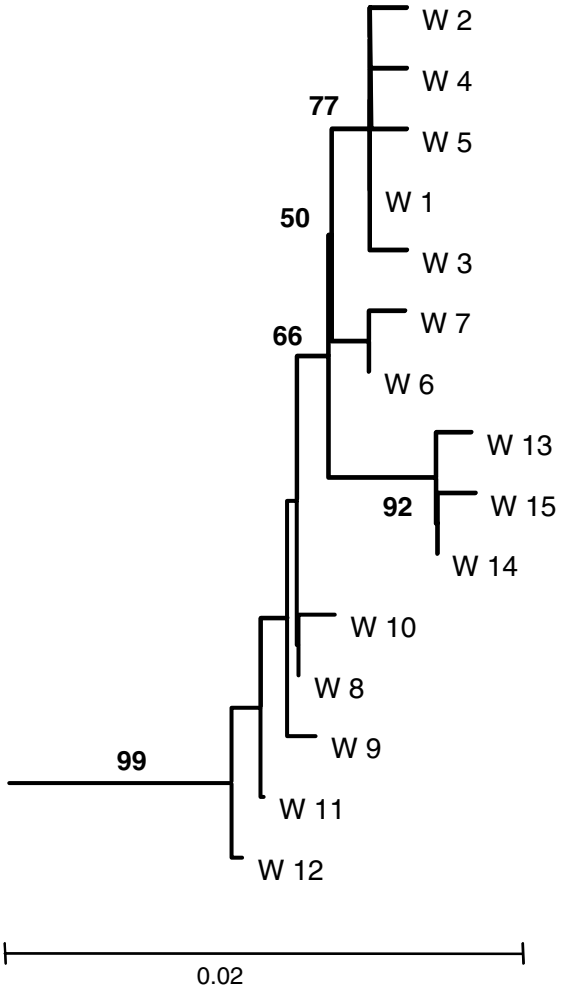

Figure 2. Neighbour joining tree of *P. fuscus* haplotypes; the root was located using *P. syriacus* and *P. cultripes* as outgroups; only bootstrap support values ≥50 are given.

variation over a wide area usually suggests a recent range expansion from a single source area (e.g. Phillips 1994; Barber 1999; Phillips et al. 2000). In this very specialized species, landscape features are known to strongly influence the dispersal and abundance of populations. Fossil data from the Quaternary suggest fluctuations in *Pelobates* population abundance. During the Neolithic period remains of *Pelobates fuscus* were scarce in a southern France locality, but became the most abundant species during the medieval period, in a more agricultural landscape (Bailon and Rage 1992). Range extensions were noticed by Kauri (1946) in north Estonia following deforestation and expansion of agricultural landscape associated with pond cleaning practices. Andreone et al. (1993) reported that Italian Spadefoot toads (*Pelobates fuscus insubricus*) prefer the alluvial and morainal plains, and that colonization was faster in regions with morainal debris from glaciers. Also Meissner (1970) argued that river banks with alluvial sand deposits are the most suitable Spadefoot toad habitat. Kauri (1946) observed that glacial drift areas promoted population spreading in northern countries. Steppic-like areas with short vegetation are more likely used by toads, while adults seem to avoid shrub-covered areas (Eggert 2002).

The present north-western populations probably originated during a range expansion in the younger Dryas cold and dry interval (12,900–11,500 year bp). There are several facts that support this hypothesis. (I) During this period dry steppe and steppe-tundra replaced, mainly in loessic soil, the previously extended woodland covered much of Europe (Figure 4; Frenzel et al. 1992). (II) From the debate about the natural or the introduced origin of Spadefoot toads in Sweden, the natural origin hypothesis was retained (Gislen 1938). Swedish toads could not have descended from Pleistocene populations (120,000–80,000 years), because Scandinavia was mostly covered by ice for the last time 22,500 to about 15,000 years ago (Adams and Faure 1997). During Dryas southern Sweden was free of ice and connected for the last time to Denmark. As Gislen and

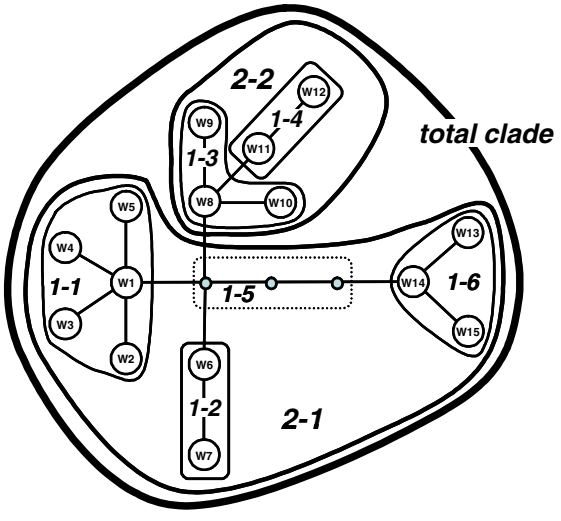

Figure 3. Minimum spanning tree and nested clade structure.

Table 3. NCA result for clades with significant geographic structure; *I* = interior clade, *T* = tip clade; χ^2 = observed chi-square; 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; the root was set to W14 via multiple outgroup haplotypes

Clade		χ^2	P_{χ^2}	D_c	D_n	$(I-T)/D_c$	$(I-T)/D_n$
1-1	*I*/*T*	60	< 0.001			−95.92	−105.85
W1	*I*			115.97	132.62		
W2	*T*			0.00	58.98		
W3	*T*			0.00	94.79		
W4	*T*			0.00^S	23.07^S		
W5	*T*			466.16^L	468.25^L		
1-2		0.41	1.000			131.05	−95.83
W6	*I*			131.05	135.06		
W7	*T*			0.00	230.88		
1-3		4.00	0. 500			0.0	−3.17
W8	*I*			0.00	71.83		
W9	*T*			0.00	71.83		
W10	*T*			0.00	78.17		
1-4		0.32	1.000			74.86	8.52
W11	*I*			74.86	74.61		
W12	*T*			0.00	66.09		
1-6		19.89	0.264			492.63	44.80
W13	*T*			0.00	466.05		
W14	*I*			492.63	491.84		
W15	*T*			0.00	428.03		
2-1		63.76	<0.001			348.06^L	211.89^L
1-1	*T*			137.87^S	497.30^S		
1-2	*T*			138.61^S	410.48^S		
1-6	*I*			486.24	672.46^L		
2-2		0.012	1.000			0.931	0.543
1-3	*I*			74.86	74.69		
1-4	*T*			73.93	74.15		
Total clade		50.22	<0.001			501.59^L	-360.24^S
2-1	*I*			575.75	570.31^S		
2-2	*T*			74.32^S	930.56^L		

Table 4. Result of the inference key

Clade	Inferred phylogeographic scenario
Clade 1-1	$1\text{-}2_a\text{-}3_b$-5-6-not applicable
Clade 2-1	$1\text{-}2_a$-3-4-NO → restricted gene flow with isolation by distance
Total clade	$1\text{-}2_{a,d}\text{-}3_{a,b,c}$-5-$6_a$-13-YES → long distance colonisation possibly coupled with subsequent fragmentation [a]or past fragmentation followed by range expansion

[a] Subsequent fragmentation is not indicated due to only one mutation between clades 2-1 and 2-2.

Kauri (1959) suggested, toads probably reached Sweden via Denmark 8–9,000 years ago, before the land connection broke. (III) After being unsuitable for herpetofauna during the last glaciation, which ended around 10,000 years ago, Britain was separated from the continent around 7,000 year ago, but Spadefoot toads have never been found in Great Britain (Lambeck 1997; Gleed-Owen 2000). We assume that population expansion reached this part of western Europe after the connection was severed, unlike *Triturus vulgaris* and *T. cristatus*, originating from the

Balkan refuge (Kalezić 1984; Crnobrnja et al. 1997), managed to colonize Britain and even Ireland (*T. vulgaris*). The specific habitat requirements of *P. fuscus* which definitely limits their rate of dispersal and choice of migration routes, might explain their absence from the British Isles. It comes a bit surprising that our rigorous testing for historical processes without *a priori* assumptions did not select a 'contiguous range expansion' model for the wide-spread clade 1-6. This clade exhibits the typical pattern of an expanding population, with one ancestral wide-spread haplotype (W14) and few locally restricted haplotypes (W13 and W15) (Avise 2000). Based on outgroup rooting of the minimum spanning tree with *P. fuscus* haplotypes from Italy and Russia (Crottini et al. unpublished) the north-western clade 1-6 (haplotypes W13, W14 and W15) is ancestral to all other haplotypes. This unusual pattern of north–south descent with a haplotype that currently is found in formerly glaciated areas as ancestor to haplotypes in the presumed glacial refuge argues for a more comprehensive analysis that covers the whole range of *P. fuscus*. Nevertheless, occurrence of the *P. fuscus* clade 1-6 in a previously glaciated area does not necessarily mean that it survived there. This striking result may therefore indicate that (I) in this special case NCPA identified a wrong hypothesis as being significant due to incomplete geographical sampling (type II error; see Knowles and Maddison 2002), (II) that the north-western lineage in fact survived in southern or south-eastern European refugium and postglacially reached the areas now north of their descendants, or that (III) *P. fuscus* is another example for a species that survived glaciations in more northern cryptic refugia (e.g. Stewart and Lister 2001). For the time being we are not able to distinguish between theses variants, although the significantly lower nucleotide diversity of northern and north-eastern populations is indicative of the second hypothesis.

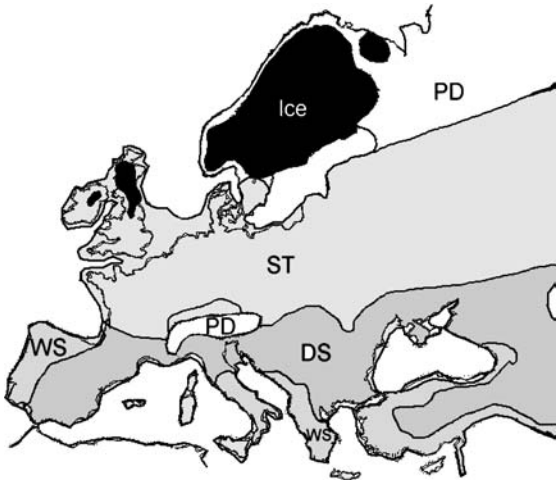

Figure 4. Dryas paleobiomes map (12,900–11,500 years ago) after Adams and Faure (1997). PD: Polar dessert, ST: Steppe-tundra, WS: Wooded steppe, DS: Dry steppe.

Fossil data suggest several expansion–regression events in western Europe. For example, fossil remains that resemble specimens of *P. fuscus* in both size and morphology were described from the Pliocene and Early Pleistocene of Poland (Mlynarski 1962), and from the Pleistocene of Romania (Venczel 1989) and Czechoslovakia (Roček 1988). Spadefoot toads populations reached the south of France during Pleistocene (Riss III–Wurm II, 120,000–80,000 years, Bailon 1991), which corresponds to the end of the Eemian interglacial period, with a rapid cooling about 110,000 years ago. A strong regression likely occurred in central Europe during the next glaciation event, but spadefoots were again present in southern France since at least the Neolithic period (Bailon and Rage 1992). This suggests that spadefoots were relatively constant inhabitants in central and eastern Europe, sometimes even during glaciations.

Conservation implications

Spadefoot toads from north-western populations, including all the declining area, represent a distinct lineage and therefore should be considered as an evolutionary significant unit (ESU, Ryder 1986; Moritz 1994a). This ESU's current repartition may be the result of a *circa* younger Dryas biological process. Our data were consistent with the local observations of Spadefoot toad populations spreading in open landscapes (Kauri 1946; Bailon and Rage 1992) and highlight the influence of climatic and landscape changes in toad population expansion process. Since 8,000 years ago temperate forest had returned to most of Europe and loessic areas became gradually less abundant (Frenzel et al. 1992). Phylogeographic data suggests that because of their limited dispersal opportunities due to the narrow habitat requirements of adults, Spadefoot toads are especially

vulnerable to environmental changes. Even so recent *P. fuscus* population decline may be the result of many proximate causes that could have various and complex connections with global environmental changes (Kiesecker et al. 2001). Metapopulation processes have critical influence on Spadefoot toad population viability (Eggert 2000; Hels 2002) and habitat fragmentation, resulting from human activity or natural environmental changes, may significantly participate in decline of the toad by further limiting their dispersal. It is noteworthy that very low mtDNA polymorphism was observed, especially in areas where populations have suffered recent strong declines. Nevertheless nuclear DNA polymorphisms remain to be studied, since the survival of anuran populations could be hindered by low genetic variability and inbreeding depression (e.g. Rowe et al. 1999). The observed mtDNA low polymorphism, associated with the observation of a lower female fecundity in the border of the declining area (Eggert, *unpublished data*), underline the need of a study on nuclear genetic diversity. Nevertheless we suggest that future conservation efforts in the declining area should include management of genetic diversity.

Long-term conservation policies, devoted on genetic diversity management (see Moritz 1994b), should clearly consider Balkan region, while almost all mitochondrial diversity was found in southern refugial areas, underscoring the high conservation value of those areas. Anyway the biogeographical uniqueness of the Balkan peninsula is indisputable, with ca. 28% of amphibians and 21% of reptiles being endemic (Dzukic and Kalezic 2004). Identifying areas where populations are more likely to survive is now more urgent, not only for short term conservation goals, but for the long term persistence through climatic oscillations.

Acknowledgements

This study was mainly supported by the French Office National des Forêts and Electricité de France. One of the author (G.D.) is partly supported by the Ministry of Science, Technology and Development of Serbia (project title ''Integrative study of amphibians and reptiles of the central Balkans'', grant No. 1623). We thank C. LeBihan (ONF), R. Guyétant, and A. Ribéron for assistance and R. Jehle, E. Ripfl, A. Bitz, B. Ujvari, M. Puky, A. Stumpel, V. Lacoste and K. Grossenbacher for providing us with tissue samples.

References

Adams JM, Faure H(eds.) (1997) *QEN members. Review and Atlas of Palaeovegetation: Preliminary land ecosystem maps of the world since the Last Glacial Maximum*, Oak Ridge National Laboratory, TN, USA http://www.esd.ornl.gov/ern/qen/adams1.html.

Andreone F, Fortina R, Chiminello A (1993) *Natural History, Ecology and Conservation of the Italian Spadefoot Toad, Pelobates fuscus insubricus*, Societa Zoologica La Torbiera, Novara.

Avise JC (1992) Molecular population structure and the biogeographic history of a regional fauna: a case history with lessons for conservation biology. *Oikos*, **63**, 62–76.

Avise JC (2000) *Phylogeography, The History and Formation of Species*, Harvard University Press, Cambridge.

Avise JC, Walker D, Johns GC (1998) Speciation durations and Pleistocene effects on vertebrate phylogeography. *Proc. Roy. Soc. Lond. B*, **265**, 1707–1712.

Baillie JEM, Hilton-Taylor C, Stuart SN (eds.) (2004) *2004 IUCN Red List of Threatened Species A Global Species Assessment*. IUCN, Gland, Switzerland and Cambridge, UK, 191 pp.

Bailon S (1991) Amphibiens et reptiles du Pliocène et du Quaternaire de France et d'Espagne: Mise en place et évolution des faunes. PhD Thesis, Université de Paris VII, France.

Bailon S, Rage JC (1992) Amphibiens et Reptiles du Quaternaire. Relations avec l'homme. *Mém. Soc. géol. Fr.*, **160**, 95–100.

Barber PH (1999) Patterns of gene flow and population genetic structure in the canyon treefrog, *Hyla arenicolor* (Cope). *Mol. Ecol.*, **8**, 563–576.

Berglund B (1998) Projekt Lökgroda 1993–1996. Länsstyrelsen i Skane län (ed.). Malmö, Sweden.

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.

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

Collins JP, Storfer A (2003) Global amphibian declines: sorting the hypotheses. *Diversity Distrib.*, **9**, 89–98.

Crnobrnja-Isailović J, Džukić G, Krstić N, Kalezić ML (1997) Evolutionary and paleogeographical effects on the distribution of the *Triturus cristatus* superspecies in the central Balkans. *Amphibia-Reptilia*, **18**, 321–332.

Dubois A (1998) Mapping European amphibians and reptiles: collective inquiry and scientific methodology. *Alytes*, **15**, 176–204.

Džukić G, Beskov V, Sidorovska V, Cogălniceanu D, Kalezić M (2005) Historical and contemporary ranges of the Spadefoot toads (*Pelobates* spp., Amphibia, Anura) in the Balkan Peninsula. *Acta Zoologica Cracoviensia*, **48**, 1–9.

Dzukic G, Kalezic M (2004). The biodiversity of amphibians and reptiles in the Balkan peninsula. In: *Balkan Biodiversity* (ed. Griffiths HI et al.), pp. 167–192. Kluwer Academic Publishers.

Eggert C (2000) Le déclin du Pélobate brun (*Pelobates fuscus*, Amphibien Anoure): Apport de la phylogéographie et de la dynamique de population à sa compréhension. Implications pour sa conservation. PhD thesis, University of Savoie, France.

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

Fog K, Schmedes A, de Lasson DR (1997) *Nordens padder og krybdyr*, En felthåndbog, Gads Forlag, København.

Frenzel B, Pecsi B, Velichko AA (eds.) (1992) *Atlas of Palaeoclimates and Palaeoenvironments of the Northern Hemisphere*, INQUA/Hungarian Academy of Sciences, Budapest.

Gardner T (2001) Declining amphibian populations: a global phenomenon in conservation biology. *Anim. Biodiv. Conserv.*, **24**, 25–44.

Gislen T (1938) On the history of evolution and distribution of the European Pelobatids. *Zoogeographica*, **3**, 119–131.

Gislen T, Kauri H (1959) Zoogeography of the Swedish amphibians and reptiles with notes on their growth and ecology. *Acta Vert.*, **1**, 195–398.

Gleed-Owen CP (2000) Subfossil records of *Rana* cf. *lessonae*, *Rana arvalis* and *Rana* cf. *dalmatina* from Middle Saxon (c. 600–950 AD) deposits in eastern England: evidence for native status. *Amphibia-Reptilia*, **21**, 57–65.

Hels T (2002) Population dynamics in a Danish metapopulation of Spadefoot toads (*Pelobates fuscus*). *Ecography*, **25**, 303–313.

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

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

Irwin DM, Kocher TD, Wilson AC (1991) Evolution of the cytochrome *b* gene mammals. *J. Mol. Evol.*, **32**, 128–144.

Johns GC, Avise JC (1998) A comparative summary of genetic distances in the vertebrates from the mitochondrial cytochrome *b* gene. *Mol. Biol. Evol.*, **15**, 1481–1490.

Kalezić ML (1984) Evolutionary divergences in the smooth newt, *Triturus vulgaris* (Urodela, Salamandridae): electrophoretic evidence. *Amphibia-Reptilia*, **5**, 221–230.

Kauri H (1946) Die Verbreitung der Amphibien und Reptilien in Estland. *Kungl. Fysiografiska Sallskapets I Lund Forhandlingar.*, **16**, 167–186.

Kiesecker JM, Blaustein AR, Belden LK (2001) Complex causes of amphibian population declines. *Nature*, **410**, 681–684.

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

Kumar S, Tamura K, Jakobsen IB, Nei M (2003) *MEGA3: Molecular Evolutionary Genetics Analysis software*, Arizona State University, Tempe, Arizona, USA.

Kuzmin SL (1999) *The Amphibians of the Former Soviet Union*, Pensoft, Sofia, Moscow.

Lambeck K (1997) Sea-level change along the French Atlantic Channel coasts since the time of the Last Glacial Maximum. *Palaeogeo. Palaeoclim. Palaeoeco.*, **129**, 1–22.

Lescure J (1984) La répartition passée et actuelle des Pélobates (Amphibiens Anoures) en France. *Bull. Soc. Herp. Fr.*, **29**, 45–59.

Martinez-Rica JP (1997) General features of European climate and vegetation consequences on the distribution of herpetofauna. In: *Atlas of Amphibians and Reptiles in Europe* (eds. Societas Europaea Herpetologica, Muséum National d'Histoire Naturelle), pp. 17–21. IEGB/SPN, Paris.

Meissner K (1970) Obligatorisches Lernen im Funktionskreis der Vergrabehandlung von *Pelobates fuscus fuscus* Laur. (Anura). Ein Beitrag zur Ethometrie des Appetenzverhaltens. *Zool. Jb. Physiol.*, **75**, 423–469.

Mlynarski M (1962) Notes on the amphibian and reptilian fauna of the Polish Pliocene and Early Pleistocene. *Acta Zool. Cracov.*, **7**, 177–194.

Moritz C (1994a) Defining "evolutionary significant units" for conservation. *Trends Ecol. Evol.*, **9**, 373–375.

Moritz C (1994b) Applications of mitochondrial DNA analysis in conservation: a critical review. *Mol. Ecol.*, **3**, 401–411.

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

Nevo E, Beiles A (1991) Genetic diversity and ecological heterogeneity in amphibian evolution. *Copeia*, **1991**, 565–592.

Nöllert A (1997) *Pelobates fuscus* (Laurenti, 1768). In: *Atlas of Amphibians and Reptiles in Europe* (eds. Societas Europaea Herpetologica, Muséum National d'Histoire Naturelle), pp. 110–111. IEGB/SPN, Paris.

Parent G (1985) Précisions sur la répartition du Pelobate brun, *Pelobates fuscus* (Laurenti, 1768), en France. *Alytes*, **4**, 52–60.

Pelt FL, Van Bree PJH (1965) Enkele aantekeningen over de knoflookpad, *Pelobates fuscus* (Laurenti, 1768) in Nederland. *Overdr. Nat. Hist. Maandbl.*, **54**, 58–65.

Perczy C (1994) Le site à Pelobates de la région Wallonne. *Les cahiers des Réserves Naturelles – RNOB.*, **7**, 105–108.

Phillips CA (1994) Geographic distribution of mitochondrial DNA variants and the historical biogeography of the spotted salamander Ambystoma maculatum. *Evolution*, **48**, 597–607.

Phillips CA, Suau G, Templeton A (2000) Effects of Holocene climate fluctuation on mitochondrial DNA variation in the ringed salamander, *Ambystoma annulatum*. *Copeia*, **2000**, 542–545.

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 analysis of the geographical distribution of genetic haplotypes. *Mol. Ecol.*, **9**, 487–488.

Rappè G (1982) Nieuwe gegevens over het voorkomen van *Pelobates fuscus* (Laurenti) (Anura, Pelobatidae) in België. *Biol. Jb. Dodonaea*, **50**, 255–259.

Riberon A, Miaud C, Grossenbacher K, Taberlet P (2001) Phylogeography of the Alpine salamander, *Salamandra atra* (Salamandridae) and the influence of the Pleistocene climatic oscillations on population divergence. *Mol. Ecol.*, **10**, 2555–2560.

Roček Z (1988) List of fossil amphibians of Czechoslovakia. *Acta zool. Cracov.*, **31**, 513–540.

Rowe G, Beebee TJC, Burke T (1999) Microsatellite heterozygosity, fitness and demography in natterjack toads *Bufo calamita*. *Anim. Cons.*, **2**, 85–92.

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

Ryder OA (1986) Species conservation and systematics: the dilemma of subspecies. *Trends Ecol. Evol.*, **1**, 9–10.

Shpun S, Hoffman E, Nevo E, Katz U (1993) Is the distribution of *Pelobates syriacus* related to its limited osmoregulatory capacity?. *Comp. Biochem. Physiol.*, **105A**, 135–139.

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

Swofford DL (1998) *PAUP* Phylogenetic Analysis Using Parsimony (*and Other Methods), Version 4*, Sinauer Associates, Sunderland MA.

Taberlet P (1998) Biodiversity at the intraspecific level: the comparative phylogeographic approach. *J. Biotechnol.*, **64**, 91–100.

Taberlet P, Cheddadi R (2002) Quaternary refugia and persistence of biodiversity. *Science*, **297**, 2009–2010.

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

Taberlet P, Fumagalli L, Wust-Saucy AG, Cossons JF (1998) Comparative phylogeography and postglacial colonization routes in Europe. *Mol. Ecol.*, **7**, 453–464.

Templeton AR (2004) Statistical phylogeography: methods of evaluating and minimizing inference errors. *Mol. Ecol.*, **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.

Templeton AR, Sing CF (1992) A cladistic analysis of phenotypic association with haplotypes inferred from restriction endonuclease mapping and DNA sequence data. IV. Nested analyses with cladogram uncertainty and recombination. *Genetics*, **134**, 659–669.

Venczel M (1989) Data on the herpetofauna from fossil deposits of Burzău-Rîpa, Bihor County. *Crisia*, **19**, 761–771.