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Phylogeographic patterns of genetic diversity in the common spadefoot toad, *Pelobates fuscus* (Anura: Pelobatidae), reveals evolutionary history, postglacial range expansion and secondary contact

Spartak N. Litvinchuk · Angelica Crottini · Silvia Federici · Philip De Pous · David Donaire · Franco Andreone · Miloš L. Kalezić · Georg Džukić · Georgy A. Lada · Leo J. Borkin · Jury M. Rosanov

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Abstract Based on allozyme variation of 410 newly collected individuals from 52 localities, we reconstructed range-wide phylogeography of the widespread Western Palearctic anuran, *Pelobates fuscus*. To study genetic diversity, evolutionary history, postglacial range expansion and secondary contact zones, we used a multidisciplinary approach combining information from various genetic analyses and ecological niche modeling. We confirmed the presence of two main groups in *P. fuscus*, initially revealed by genome size variation. *Pelobates f. vespertinus* presents a monomorphic group, but two main groups can be identified in *P. f. fuscus*: an East European and a West European group. We suggest the

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S. N. Litvinchuk (✉) · J. M. Rosanov
Institute of Cytology of Russian Academy of Sciences,
Tikhoretsky pr. 4,
194064 St. Petersburg, Russia
e-mail: slitvinchuk@yahoo.com

A. Crottini
CIBIO, Centro de Investigação em Biodiversidade
e Recursos Genéticos, Campus Agrário de Vairão,
R. Padre Armando Quintas,
4485-661 Vairão, Portugal

S. Federici
ZooPlantLab, Dipartimento di Biotecnologie
e Bioscienze, Universitá degli Studi
di Milano-Bicocca, Piazza della Scienza, 2,
Milano, Italy

P. De Pous
Escola Tècnica Superior Enginyeria Agrària,
Departament Producció Animal (Fauna Silvestre),
University of Lleida, 125198 Lleida, Spain

P. De Pous
Institute of Evolutionary Biology (CSIC-UPF),
Passeig Maritim de la Barceloneta 37-49,
08003 Barcelona, Spain

P. De Pous
Society for the Preservation of Herpetological Diversity,
Oude Molstraat 2E,
2513 BB Den Haag, The Netherlands

D. Donaire
Calle Mar Egeo 7,
11407 Jerez de la Frontera, Cadiz, Spain

F. Andreone
Museo Regionale di Scienze Naturali, Via G.
Giolitti 36,
10123 Torino, Italy

M. L. Kalezić
Faculty of Biology, Institute of Zoology,
Studentski trg. 3,
Belgrade 11000, Serbia

G. Džukić
Institute for Biological Research,
29 Novembra 142,
Belgrade 11000, Serbia

existence of at least four different Last Glacial refugia for *P. fuscus*: (1) the area between the Caspian and Azov Seas as the origin for the expansion of *P. f. vespertinus*; (2) the northwestern part of the Black Sea area for the East European *P. f. fuscus*; (3) the southwestern part of the Pannonian Plain and (4) the Po Plain for the West European *P. f. fuscus*. The routes of postglacial range expansions from the refugia are discussed. We newly identified a hybrid zone between *P. f. fuscus* and *P. f. vespertinus*. The width of this zone is about 12.5 km. In light of these findings, the two subspecies of *P. fuscus* constitute distinct evolutionary lineages and merit recognition as separate species. Our data do not provide support for the validity of *P.f. insubricus*. We therefore propose to synonymize this subspecies with *P. f. fuscus.*

Keywords Allozymes · Ecological niche model · Genome size · Glacial refugium · Hybridization · *Pelobates vespertinus* · *Pelobates fuscus insubricus*

Introduction

Species with distributions extending from glacial refugia into postglacially colonized areas are good models to study the influence of paleoclimatic and paleogeographic events on distribution dynamics and speciation (Avise 2000; Hewitt 2000, 2011a). The classic "glacial refugia" theory attempts to explain most of these speciation events as a consequence of shifts in the distribution ranges toward the south during the glacial maxima, leading to subsequent allopatric isolation and genetic differentiation in southern refugia (Borkin 1984; Hewitt 2000). However, in amphibians, when dealing with dating estimates, most splits can be firmly placed in Pre-Pleistocene times (e.g., Babik et al. 2007; Hofman et al. 2007; Garcia-Porta et al. 2012). Therefore, the role ascribed to Pleistocene glacial cycles on speciation shifted from being thought of as a major cause to being relevant only in particular cases (Klicka and Zink 1997). The now generally held view is that phylogeographic structure mostly results from pre-Pleistocene events with some remolding related to populations fluctuations during the Quaternary (e.g., Nascetti et al. 2005; Fijarczyk et al. 2011; Stöck et al. 2012).

The colonization of northern European regions by species with lineages from different refugia has produced the secondary contact zones (Hewitt 2011b). Hybridization between interacting lineages provides useful information for systematists because the structure of contact zones provides insight into the extent of gene flow. The zones in which there is no selection against hybrid genotypes (a neutral hybrid zone) have width cline and discordant cline shapes and cline centers among loci (Endler 1977; Barton and Hewitt 1985). In contrast, when there is selection against hybrids, cline width is a function of the rate of dispersal into the hybrid zone and the strength of selection against hybrid genotypes. With strong selection, such zones will tend to be narrow, and clines among loci will tend to have concordant shapes and centers.

The common spadefoot toad, *Pelobates fuscus* (Laurenti 1768), is a widespread anuran species distributed from central France in the west to western Siberia and western Kazakhstan in the east (Borkin et al. 2002). Previous studies of genome size variation revealed two distinct groups (Borkin et al. 2001, 2002, 2003): a group with a relatively small genome size distributed in the west and a group with a relatively large genome size in the east. Studies of allozyme and mitochondrial DNA variations showed that these groups constitute two distinct evolutionary lineages (Borkin et al. 2003; Khalturin et al. 2003; Crottini et al. 2007). Despite a lack of morphological differentiation (Lada et al. 2005), several authors proposed to discriminate these groups as different subspecies or even species, western *P. f. fuscus* and eastern *P. f. vespertinus* (Pallas 1771), respectively (Borkin et al. 2004; Crochet and Dubois 2004; Crottini et al. 2007; Litvinchuk et al. 2008; Kwet and Nöllert 2009).

Based on morphological peculiarities, the populations of common spadefoot toads inhabiting the Po Plain in northern Italy were considered as a separate subspecies, *P. f. insubricus* Cornalia 1873. However, mitochondrial DNA variation showed that this subspecies formed a lineage within *P. f. fuscus* (Crottini et al. 2007).

The aims of the present study were: (1) to study wide-range phylogeographic patterns of genetic diversity of *P. fuscus* and propose an updated taxonomy; (2) to infer the Last Glacial refugia and possible routes of postglacial recolonization; and (3) to analyze the contact zone of *P. f. fuscus* and *P. f. vespertinus* in order to investigate the taxonomic status of these subspecies.

Materials and methods

Allozymes

A total of 410 individuals of *P. fuscus* (52 localities) were collected for the allozyme analysis. As outgroup, we included the remaining species in the genus *Pelobates* Wagler 1830: 9 individuals of *P. cultripes* (Cuvier 1829), 2 of *P. varaldii* Pasteur and Bons 1959 and 17 of *P. syriacus*

G. A. Lada
Tambov State University,
Internatsionalnaya 33,
Tambov 392000, Russia

L. J. Borkin
Zoological Institute of Russian Academy
of Sciences, Universitetskaya nab. 1,
St. Petersburg 199034, Russia

Boettger 1889. All specimens and samples were newly collected and analyzed. The sampling localities and sample details are provided in Table 1 and Fig. 1. *Pelobates fuscus* and *P. syriacus* were syntopic in some localities (Jagodina, Banatska Palanka and Gurbuki). We mostly collected larvae because their collection causes less damage to natural populations. Larvae were reared in the laboratory up to metamorphosis (the complete displacement of larval proteins). Each individual was anaesthetized by methoxyethane or submersion in a 1 % solution of 3-aminobenzoic acid ethyl ester (MS 222). After anesthesia, blood, muscles and liver were taken. Before allozyme analysis, collected tissues were stored at –80 °C. Genome size (the nuclear DNA content) was studied for each individual using DNA flow cytometry following the procedure described in Borkin et al. (2002).

Standard vertical polyacrylamide gel (6–8 %) electrophoresis was performed to analyze the genetic variation in 23 putative loci of allozymes and water-soluble proteins: aspartate transaminase (*Aat-1* and *Aat-2*; EC 2.6.1.1), creatine kinase (*Ck*; EC 2.7.3.2), esterase (*Est-2* and *Est-P*; EC 3.1.1.-), glycerol-3-phosphate dehydrogenase (*G3pdh-2*; EC 1.1.1.8), glucose-6-phosphate dehydrogenase (*G6pdh-1*; EC 1.1.1.49), glutamate dehydrogenase NAD (*Gtdh-1*; EC 1.4.1.2), glutamate dehydrogenase NADP (*Gtdhp-1* and *Gtdhp-2*; EC 1.4.1.4), L-iditol dehydrogenase (*Iddh-2*; EC 1.1.1.14), lactate dehydrogenase (*Ldh-1* and *Ldh-2*; EC 1.1.1.27), malate dehydrogenase (*Mdh-1* and *Mdh-2*; EC 1.1.1.37), 6-phosphogluconate dehydrogenase (*6Pgdh-1*; 1.1.1.44), phosphoglucomutase (*Pgm-1*; EC 5.4.2.2), superoxide dismutase (*Sod-1* and *Sod-2*; EC 1.15.1.1), muscle proteins (*Prot-1* and *Prot-2*), and hemoglobin (*Hem-1* and *Hem-2*). For electrophoresis, we used two buffers: Tris-citrate pH8.0 (for *Aat-1, Aat-2*, *G3pdh-2*, *G6pdh-1*, *Ldh-1, Ldh-2*, *Pgm-1*, *Sod-1* and *Sod-2*) and Tris-EDTA-borate pH8.3 (*Ck*, *Est-2, Est-P*, *Gtdh-1, Gtdhp-1, Gtdhp-2*, *Iddh-2*, *Mdh-1, Mdh-2*, *6Pgdh-1*, *Prot-1*, *Prot-2*, *Hem-1* and *Hem-2*). Other electrophoretic and staining procedures were performed following Khalturin et al. (2003).

Estimates of allele frequencies and genetic variability, i.e., observed and expected heterozygosity expressed as a percentage, percentage of polymorphic loci, average number of alleles per locus (all estimates were calculated for samples with ≥5 individuals) and Nei's (1978) unbiased genetic distance (D_{Nei}) were obtained using the software BIOSYS-2 (Swofford and Selander 1999). Allelic richness, a measure of genetic diversity that accounts for variable sample sizes through rarefaction, was calculated for all loci in all populations with FSTAT (ver. 2.9.3.2; http://www2.unil.ch/popgen/softwares/fstat.htm). Hardy-Weinberg equilibrium was evaluated for each locus by exact testing (Genepop; Rousset 2008). Fisher's exact test evaluated deviation from expected linkage equilibrium between each pair of loci in a sample. Genetic distances between populations (D_{Nei}) were used to construct an unweighted pair group method with an arithmetic mean (UPGMA) phenogram with TFPGA (Miller 1997), and we ran 1,000 bootstrap pseudo-replicates over loci to test reliability of the UPGMA phenogram. A neighbor-joining tree (NJ) constructed from Nei's (1978) unbiased genetic distances using Mega (ver. 5.05; Tamura et al. 2011) provided a distance-based phylogenetic reconstruction, known to perform well for allozyme data (Wiens 2000), and bootstrap analysis (1000 pseudo-replicates) was then performed using Phylip (ver. 3.6b; Felsenstein 2004).

Data for *P. fuscus* were ordered using multiple correspondence analysis (MCA) with population frequency data (further details are available in Garcia-Porta et al. 2012). This analysis was performed using Statistica (ver. 8.0; http://www.statsoft.com/).

We applied Structure (ver. 2.3.3; Pritchard et al. 2000; Falush et al. 2003) to assign individuals of *P. fuscus* to population groups. We ran five pseudo-replicates with 10^5 iterations after a burn-in of 5×10^4 steps using the correlated allele frequencies model. We estimated the smallest number of parental populations (K) to increase the number of K from two to ten hypothetical populations while monitoring their posterior probabilities (ln Pr($X|K$)) and variance (Pritchard *et al.* 2000) and using delta K method (Evanno et al. 2005) by Structure Harvester (Earl and vonHoldt 2012). K was evaluated from 1 to 10 and estimated assuming no a priori assignment of individuals to populations and using the admixture model.

Hierarchical analysis of molecular variance (AMOVA; Excoffier et al. 1992) was carried out using Arlequin (ver. 3.5.1.2; Excoffier and Lischer 2010), and significance was assessed by 1,023 permutations, with the aim of partitioning total genetic variation into three hierarchical levels: among-group, among-populations within-group and within-populations. Results from previous UPGMA and MCA analyses were used to define a priori three groups of *P. fuscus* (two groups of *P. f. fuscus* and one of *P. f. vespertinus*).

For the time calibration of the phylogenetic framework based on allozymes data, we used the linear interpolation method and the Afro-European vicariance of *P. cultripes* and *P. varaldii* (the Messinian Salinity Crisis at 5.33 Ma; Busack et al. 1985).

Mitochondrial DNA (mtDNA)

Nine specimens from five localities of *P. f. vespertinus*, 10 specimens from four localities of *P. f. fuscus* and 23 specimens sampled in ten localities across the contact zone between *P. f. fuscus* and *P. f. vespertinus* (Kursk Province, Russia) were newly analyzed. A finger tip, a part of the muscle or a part of the liver was cut from each collected individual and stored in 99 % ethanol. Total genomic DNA was extracted from the tissue samples using proteinase K digestion (10 mg/ml concentration) following the Bandi et al. (1994) protocol. To sequence a fragment of about 570 bp of the mitochondrial cyt *b* gene, we used the primers CBJ10933 5'-TATGTTCTACCATGAGGACAAATATC-3'

Table 1 Geographic location, sample size (n), average number of alleles per locus (*A*), mean allelic richness (*Ar*), percentage of polymorphic loci (*P*), observed and expected heterozygosity expressed as a percentage (H_o and H_e, respectively), mtDNA haplotypes (number of specimens is given in parentheses) and the nuclear DNA content (NC; mean ± SD; in pg) variation for the analyzed samples of *Pelobates*

Samples		Latitude	Longitude	Allozymes						mtDNA	NC
				n	*A*	*Ar*	*P*	H_o	H_e		
Pelobates fuscus vespertinus											9.34±0.07
1	Leninaul	44°12' N	46°01' E	4	–	1.03	–	–	–	–	9.21±0.03
2	Stupino	48°19' N	45°50' E	11	1.0	1.02	4.3	3.3	2.2	E13(1)	9.29±0.02
3	Dzhelga	48°18' N	46°10' E	9	1.0	1.02	4.3	1.2	1.9	–	9.28±0.08
4	Ural'sk	51°13' N	51°25' E	32	1.1	1.02	8.7	1.6	2.1	–	9.33±0.03
5	Borskoe	53°02' N	51°41' E	8	1.2	1.04	17.4	5.1	4.5	–	9.42±0.02
6	Timashevo	53°21' N	51°12' E	6	1.0	1.02	4.3	1.7	1.5	–	9.42±0.02
7	Nizhnee Sencheleevo	53°29' N	49°30' E	35	1.1	1.03	8.7	2.7	3.2	E1(3)+E3(1)	9.42±0.03
8	Simkino	54°15' N	46°10' E	9	1.1	1.04	8.7	1.9	3.6	–	9.31±0.03
9	Kamchatka	54°44' N	45°30' E	5	1.1	1.03	8.7	3.0	3.5	–	9.38±0.02
10	Andreevka	54°03' N	44°02' E	23	1.1	1.04	13.0	3.3	4.2	–	9.33±0.06
11	Kochelaevo	54°01' N	44°00' E	6	1.1	1.03	8.7	3.8	3.0	–	9.38±0.02
12	Potma	52°16' N	43°23' E	12	1.1	1.03	4.3	3.0	2.6	E1(1)	9.37±0.04
13	Rasskazan'	51°34' N	42°39' E	5	1.2	1.09	17.4	4.1	8.7	–	9.38±0.03
14	Passkazovo	52°40' N	41°52' E	9	1.1	1.05	13.0	4.8	4.7	–	9.35±0.03
15	Bolshaya Lipovitsa	52°33' N	41°20' E	10	1.0	1.00	0	0	0	E1(2)	9.44±0.04
16	Oksky Reserve	54°43' N	40°44' E	10	1.1	1.02	8.7	1.9	1.8	–	9.32±0.04
17	Krasnoe	49°55' N	37°46' E	6	1.1	1.04	8.7	7.2	4.5	–	9.26±0.01
18	Chervonyi Shakhtar	49°11' N	37°02' E	12	1.0	1.02	4.3	1.7	2.2	–	9.32±0.04
19	Liptsy	50°13' N	36°23' E	7	1.0	1.01	4.3	1.2	1.1	–	9.25±0.03
20	Gaydary	49°37' N	36°20' E	24	1.2	1.05	17.4	4.3	4.8	–	9.28±0.08
21	Karadag Reserve	44°57' N	35°14' E	5	1.1	1.06	13.0	4.3	5.6	E8(1)	9.35±0.04
Contact zone of Pelobates fuscus fuscus and Pelobates fuscus vespertinus											
22	Bobryshevo	51°13' N	36°19' E	1	–	1.09	–	–	–	E1(1)	9.36
23	Selihovi Dvori	51°35' N	36°05' E	4	–	1.07	–	–	–	E1(1)+E8(1)	9.30±0.04
24	Bushmenovo	51°09' N	35°55' E	5	1.2	1.08	17.4	9.6	8.2	E1(2)+E8(1)	9.16±0.02
25	Bobrava	51°05' N	35°49' E	7	1.3	1.10	26.1	8.7	9.7	E1(5)	9.17±0.04
26	Vanina	51°41' N	35°56' E	1	–	1.17	–	–	–	–	9.07
27	Ivanovsky	51°04' N	35°46' E	1	–	1.13	–	–	–	–	9.16
28	Loshakovka	51°04' N	35°44' E	1	–	1.13	–	–	–	W25(1)	9.19
29	Dolgiy Kolodez	51°06' N	35°46' E	2	–	1.14	–	–	–	E8(1)	9.02±0.04
30	Pervyi Zyabkin	51°43' N	34°59' E	2	–	1.05	–	–	–	–	8.95±0.04
31	Peschanoe	51°05' N	35°40' E	7	1.3	1.09	26.1	9.3	8.9	E1(3)+W25(1)+W30(1)	8.85±0.06
32	Guevo	51°07' N	35°18' E	1	–	1.00	–	–	–	W30(1)	8.80
33	Gaponovo	51°22' N	34°50' E	6	1.1	1.05	8.7	5.4	3.9	W30(1)E8(1)	8.90±0.02
34	Zabolotovka	51°21' N	34°21' E	4	–	1.04	–	–	–	E8(2)	8.89±0.03
Pelobates fuscus fuscus (Eastern Europe)											8.78±0.07
35	Skuratovo	54°11' N	37°43' E	11	1.1	1.03	13.0	3.9	4.1	–	8.85±0.03
36	Luchki	48°59′ N	34°06′ E	5	1.1	1.05	8.7	7.8	4.7	–	8.86±0.03
37	Bolshie Bubny	50°51' N	33°16' E	10	1.1	1.03	13.0	3.1	3.2	–	8.86±0.05
38	Khotymsk	53°24' N	32°35' E	6	1.1	1.02	8.7	2.0	2.0	–	8.82±0.02
39	Vitebsk	55°13' N	30°15' E	6	1.1	1.03	8.7	2.9	2.8	–	8.82±0.06
40	Bor	58°12' N	30°41' E	23	1.1	1.03	4.3	4.2	2.7	W14(1)	8.73±0.02
41	Luga	58°44' N	29°51' E	12	1.0	1.00	0	0.4	0.4	W14(1)	8.77±0.07
42	Koncha Zaspa	50°15' N	30°32' E	5	1.2	1.06	17.4	3.4	6.3	W14(2)	8.80±0.03

Table 1 (continued)

Samples		Latitude	Longitude	Allozymes						mtDNA	NC
				n	*A*	*Ar*	*P*	H_o	H_e		
43	Novaya Trojanda	50°21' N	31°15' E	4	–	1.06	–	–	–	–	8.75±0.03
44	Luninets	52°14' N	26°49' E	10	1.1	1.03	8.7	1.9	2.5	–	8.73±0.06
45	Tiraspol'	46°50' N	29°39'	6	1.1	1.05	13.0	6.4	5.0	–	8.74±0.07
Pelobates fuscus fuscus (Western Europe)											8.92±0.09
46	Strausberg	52°35' N	13°53' E	1	1.0	1.04	–	–	–	–	8.67
47	Aleksinac	43°33' N	21°42' E	2	–	1.07	–	–	–	–	8.83±0.06
48	Obrež	43°46' N	21°21' E	5	1.2	1.08	21.7	2.8	8.3	–	8.96±0.03
49	Banatska Palanka	44°51' N	21°20' E	4	–	1.06	–	–	–	–	8.94±0.05
50	Jagodina	43°59' N	21°16' E	1	–	1.04	–	–	–	–	8.76
51	Pancevo	44°52' N	20°40' E	3	–	1.10	–	–	–	–	9.01±0.02
52	Poirino	44°55' N	7°51' E	6	1.1	1.03	8.7	2.9	3.4	W19(6)	8.92±0.03
Pelobates cultripes											7.37±0.04
53	Chaparrito	36°38' N	5°53'W	7	1.1	1.05	13.0	5.1	4.8	–	7.36±0.04
54	Verdizela	38°35' N	9°09'W	2	1.0	1.00	–	–	–	–	7.40±0.01
Pelobates varaldii											
55	Mamora forest	34°02' N	6°43'W	2	–	1.03	–	–	–	–	7.28±0.04
Pelobates syriacus balcanicus											7.91±0.03
56	Jagodina	43°59' N	21°16' E	2	–	1.09	–	–	–	–	7.88±0.02
57	Banatska Palanka	44°51' N	21°20' E	5	1.3	1.09	26.1	7.0	9.4	–	7.91±0.03
58	Nacolec	42°00' N	21°28' E	3	–	1.10	–	–	–	–	7.94±0.01
Pelobates syriacus syriacus											
59	Gurbuki	42°37' N	47°36' E	7	1.0	1.01	4.3	0.6	0.6	–	8.17±0.01

and Cytb-c 5'-CTACTGGTTGTCCTCCGATTCATGT-3' (Bossuyt and Milinkovitch 2000). PCR reactions were performed in 20-µl reactions using 0.2 µl each of 0.2 mM primer, 2 µl of total dNTP (2 mM), 0.1 µl (0.5 U) of MasterTaq Eppendorf® and 2 µl 1× buffer including $MgCl_2$ at 1.5 mM and 14.5 µl of water. PCR conditions were as follows: 94 °C for 90 s, followed by 35 cycles of denaturation at 94 °C (30 s), annealing at 49.8 °C (45 s) and extension at 72 °C (90 s), followed by 10 min at 72 °C of final elongation.

The light strands were sequenced using an ABI3730XL by Macrogen Inc. Sequences were blasted in GenBank, checked by eye, edited, aligned using the BioEdit sequence alignment editor (ver. 7.0.5.3; Hall 1999). The alignment of all the processed samples was straightforward, and it did not require the inclusion of gaps to account for indels. All newly determined sequences have been deposited in GenBank accession nos. KC595564-KC595605.

Sequences obtained in this study were merged with all known *P. fuscus* cyt *b* haplotypes. Sequences were merged into haplotypes using the online application DNAcollapser (ver. 1.0; http://www.birc.au.dk/fabox/). Homologous sequences of the three other *Pelobates* species (*P. cultripes*, *P. syriacus* and *P. varaldii*) and of *Spea bombifrons* and *Spea multiplicata* were added to the alignment and used for hierarchical outgroup rooting (GenBank accession nos. AJ871086, DQ333372, EF191042, EF191043 and EF191044).

Bayesian analyses were performed in MrBayes (ver. 3.1.2; Ronquist and Huelsenbeck 2003). The GTR+G model was determined by AIC in jModeltest (Posada 2008) as the best-fitting model of substitution. We performed two runs of 10 million generations (started on random trees) and four incrementally heated Markov chains (using default heating values), sampling the Markov chains at intervals of 1,000 generations. Stabilization and convergence of likelihood values were checked by visualizing the log likelihoods associated with the posterior distribution of trees in Tracer (ver. 1.4; Rambaut and Drummond 2007) and occurred after about 3 million generations. The first 5 million generations were conservatively discarded, and 5,000 trees were retained post burn-in and used to generate the majority rule consensus tree.

The software TCS (ver. 1.21; Clement et al. 2000) was used to reconstruct the haplotype networks using the data published in Crottini et al. (2007) and the new sequences analyzed in this study (sequences of *P. cultripes*, *P. syriacus, P. varaldii, S.*

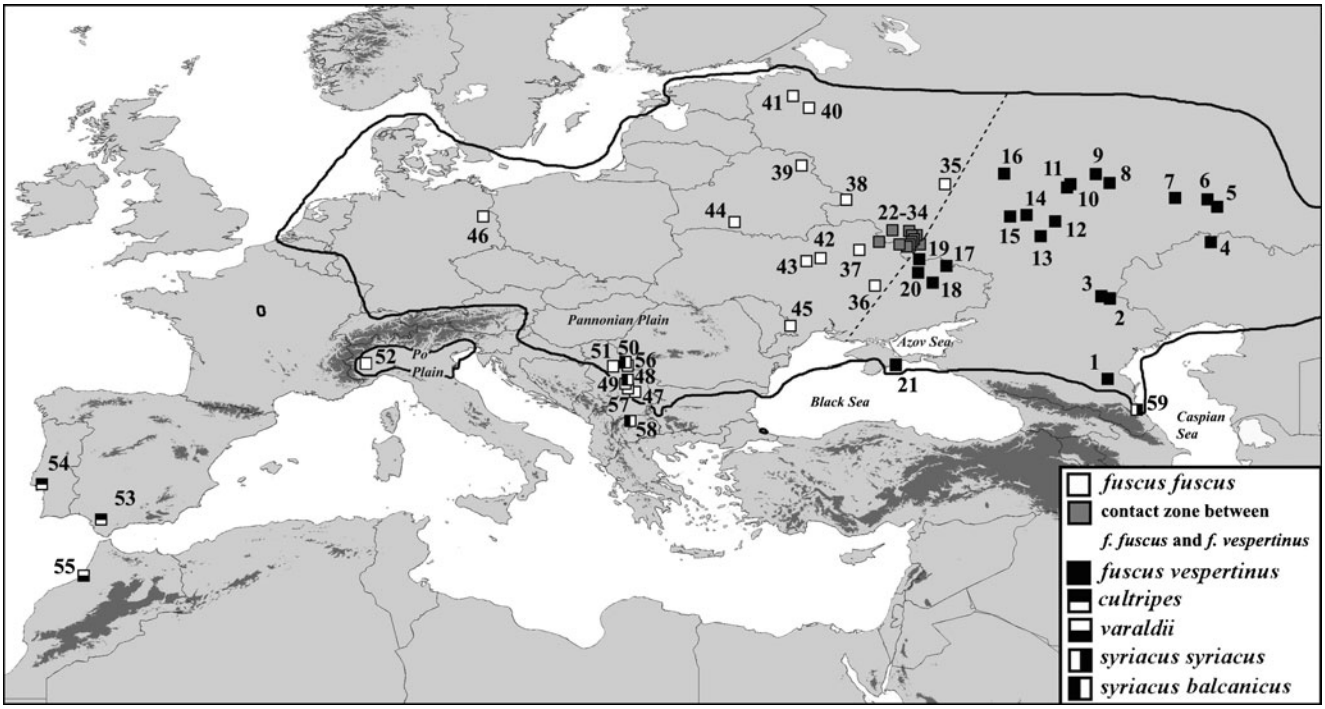

Fig. 1 Geographic locations of the 59 analyzed populations of *Pelobates*. The limit of the geographic distribution of *Pelobates fuscus* is indicated with the *solid line*. The *dashed line* indicates the presumptive position of the contact zone between *P. f. fuscus* and *P. f. vespertinus*. *Dark gray areas* show mountains with an altitude over 1,300 m above sea level. Localities are numbered as in Table 1. Localities 22–34 are from the Kursk Province (Russia)

bombifrons and *S. multiplicata* were excluded). This software applies the statistical parsimony method (Templeton et al. 1992) and calculates the number of pairwise nucleotide differences by which each haplotype pair differs, computes the probability of parsimony for pairwise differences until the probability exceeds 0.95 and estimates gene genealogies from DNA sequences.

Hybrid zone analysis

To identify the hybrid zone between *P. f. vespertinus* and *P. f. fuscus*, we estimated the proportion of membership of each population in *P. f. vespertinus* or *P. f. fuscus* clusters with Structure (with K=2), as described previously. The assignment test from New Hybrids (10^4 iterations; Anderson and Thompson 2002) was used to identify *P. f. fuscus*, *P. f. vespertinus* and their hybrids. We included 388 individuals of *P. fuscus* (locality nos. 1–45) in both analyses. Additionally, we used Hybridlab (ver. 1; Nielsen et al. 2006) to simulate "known" hybrid individuals based on the data from parental genotypes, and then we ran New Hybrids with the simulated data in order to assess whether the real data have the necessary power to support the main inference. The criteria used to classify individuals as belonging to a parental subspecies were $P>0.8$ in Structure and $P>0.9$ in New Hybrids. Individuals were considered "intermediate" when they had a Structure score of $0.2\leq P\leq 0.8$ and a pooled hybrid and backcross hybrid score in New Hybrids of $P\geq 0.1$ (Arntzen and Wielstra 2010).

For the cline analysis based on allozyme data, we calculated the mean proportion of membership of each sampled locality to each cluster identified by the Structure analysis. These data were used to estimate a multilocus cline from which levels of introgression across the contact zone could be inferred. The center of the cline is the point where the frequency of alleles switches above 0.5. The multilocus cline width was calculated as the inverse of the maximum of the slope of the cline curve (Szymura and Barton 1986).

Ecological niche modeling (ENM)

Distribution of *P. fuscus* subspecies were modeled using 4,147 georeferenced localities: 463 *P. f. fuscus* and 783 *P. f. vespertinus* localities georeferenced by us (assembled from various literature sources) and 2,901 additional localities for *P. f. fuscus* from Gbif (http://data.gbif.org), the European Environmental Agency (EEA, http://eunis.eea.europa.eu/) and Džukić et al. (2008). Duplicated localities were removed by ENMTools (ver. 1.3; Warren et al. 2010). The easternmost localities of *P. f. fuscus* and the westernmost localities *P. f. vespertinus* were determined based on previously published (Borkin et al. 2001, 2002, 2003) and our unpublished genome size data.

For the contemporary niche predictions, we used the 19 bioclimatic variables from the WorldClim data set (ver. 1.4; http://www.worldclim.org) at 2.5 arc min spatial resolution and converted to 10 arc min (to include in the analysis localities from 10×10 km UTM maps) using a bilinear interpolation in ArcGIS (ver. 10; ESRI 2011). Two general atmospheric circulation models were used to generate Last Glacial Maximum (LGM) climate scenarios: the Community Climate System Model (CCSM; http://www.ccsm.ucar.edu) and the Model for Interdisciplinary Research on Climate (MIROC; http://www.ccsr.u-tokyo.ac.jp/~hasumi/MIROC/).

Following methodology from Hijmans et al. (2005) and Waltari et al. (2007) and to avoid highly correlated and redundant climatic variables (which can cause over-parameterization and loss of predictive power), correlation between pairs of variables was assessed using the Pearson correlation coefficient by ENMTools. Two variables sharing correlation coefficients of 0.7 or higher were considered highly correlated. Previous knowledge about the biology and requirements of the studied species is crucial for optimal modeling (Sardà-Palomera and Vieites 2011); we therefore selected climatic variables based on known preferences of *P. fuscus*.

Models were generated by Maxent (ver. 3.3.3k; Phillips et al. 2006; Phillips and Dudík 2008), an algorithm that uses environmental parameters in combination with geographical coordinates that produce high-quality predictions of species distribution, often more reliable when evaluated and compared with other predictive models (Hernandez et al. 2006; Jiménez-Valverde et al. 2008; Giovanelli et al. 2010). This model minimizes the relative entropy between two probability densities (one from presence data and one from landscape configuration) defined in covariate space (Elith et al. 2010a, b), and the model output displays relative occurrence probability of a species within grid cells of the study area. Maxent was used with default settings: convergence threshold=0.00001, maximum number of iterations=500 and β_j=1. Averages of ten pseudo-replicated models with randomly selected test samples were used to produce potential niche models plotted in logistic format.

Final models were reclassified in ArcGIS into binary presence-absence maps. Following the assumption that ten percent of records were either incorrectly identified or georeferenced (Raes et al. 2009), the average ten percentile threshold was used, and 10 % of model outputs with the lowest predicted probabilities fall into the 'absence' region of the thresholded model, and 'presence' regions include 90 % of distribution records with the highest model values.

All models were tested with receiver-operating characteristics (ROC) curve plots, which plot the true-positive rate against the false-positive rate. The average area under the curve (AUC) of the ROC plot measures the degree to which a species is restricted to a range of variation of the predictive variables, and its values range between 0.5 (highly unsuitable) and 1.0 (highly suitable). Models with test AUC values above 0.75 are considered useful, and values above 0.90 are very good (Swets 1988; Elith 2002).

Niche overlap between *P. f. fuscus* and *P. f. vespertinus* was estimated using Schoener's (1968) *D* and modified Hellinger distance I_{mod} (see Rödder and Engler 2011) in ENMTools with niche similarity quantified statistically from 0 (no overlap) to 1 (identical niche models) based on potential niche models of both subspecies.

Results

Allozyme variation and differentiation

Four (*Gtdh-1*, *Gtdhp-2*, *Mdh-1* and *Sod-2*) of the 23 analyzed loci were monomorphic in all the samples. Allele frequencies at variable loci are shown in Appendix S1. No deviation from the expected Hardy-Weinberg and linkage equilibria was observed. Estimates of population genetic variability are provided in Table 1. The values of observed heterozygosity and the average number of alleles per locus in *P. f. fuscus* were slightly higher than those in *P. f. vespertinus*, i.e., 3.48 (0.4–7.8) vs. 3.01 (0–7.2) and 1.13 (1.0–1.3) vs. 1.09 (1.0–1.2), but these differences were statistically not significant (U-tests; Z=-0.584, *P*=0.559 and Z=-1.536, *P*=0.125). The loci studied had 1–3 alleles in *P. f. fuscus* and 1–2 alleles in *P. f. vespertinus*. In both subspecies, no significant correlation between geographic location and genetic diversity was observed.

Four loci (*Aat-1*, *G6pdh-1*, *Gtdhp-1* and *Ldh-1*) proved to be diagnostic for *P. f. fuscus* and *P. f. vespertinus*: 10 loci (*Est-2*, *Est-P, G3pdh, Hem-1, Hem-2, Ldh-1, Ldh-2, Pgm-1, Prot-2* and *Sod-1*) for *P. cultripes* and *P. varaldii*; 13 loci (*Aat-1*, *Aat-2*, *Est-2*, *Est-P*, *G3pdh-1*, *Hem-1*, *Hem-2*, *Ldh-1*, *Ldh-2*, *Pgm-1*, *Prot-1*, *Prot-2* and *Sod-1*) for *P. fuscus* and for *P. cultripes* with *P. varaldii*, whereas 8 loci (*Est-2*, *Est-P*, *Hem-1*, *Iddh-2*, *Ldh-2*, *Mdh-2*, *Pgdh-1* and *Sod-1*) for *P. syriacus* and other *Pelobates* species. The syntopic populations of *P. fuscus* and *P. syriacus* (Jagodina, Banatska Palanka and Gurbuki) possessed only their own diagnostic alleles.

Table 2 and Appendix S2 provide genetic distances (D_{Nei}) among samples. Mean distances between species ranged from 0.544 to 1.024, whereas the average difference between *P. f. fuscus* and *P. f. vespertinus* was 0.337 and groups of populations of *P. f. fuscus* from Western and Eastern Europe, respectively, was 0.228. Italian *P. f. fuscus* were very similar (0.061) to West European *P. f. fuscus*.

In the UPGMA analysis (Fig. 2a), the eastern spadefoot toad (*P. syriacus*) was the most distant cluster. Three main groups of populations can be identified: (1) *P. f. vespertinus*, (2) *P. f. fuscus* from Eastern Europe and (3) *P. f. fuscus* from Western Europe (no differentiation observed between *P. f. fuscus* and the sample from northern Italy), while the NJ

Table 2 Genetic differences (allozymes; D_{Nei}) between populations of the analyzed taxa

Taxon	*n*	Mean±SD	Range
Within groups			
P. f. fuscus (EE)	54	0.009±0.008	0–0.034
P. f. fuscus (WE)	21	0.048±0.032	0.007–0.118
Within subspecies			
P. f. fuscus	153	0.124±0.110	0–0.310
P. f. vespertinus	210	0.012±0.012	0–0.062
P. s. balcanicus	3	0.010±0.007	0.004–0.017
Within species			
P. fuscus	741	0.208±0.180	0–0.639
P. cultripes	1	0.040	–
P. syriacus	6	0.183±0.190	0.004–0.368
Between groups			
P. f. fuscus (EE) and *P. f. fuscus* (WE)	77	0.228±0.042	0.142–0.310
Between subspecies			
P. f. fuscus and *P. f. vespertinus*	357	0.337±0.114	0.194–0.639
P. s. syriacus and *P. s. balcanicus*	3	0.356±0.019	0.334–0.368
Between species			
P. fuscus and *P. cultripes*	78	0.980±0.157	0.797–1.333
P. fuscus and *P. varaldii*	39	0.887±0.177	0.712–1.318
P. fuscus and *P. syriacus*	156	1.024±0.173	0.804–1.513
P. cultripes and *P. varaldii*	2	0.544±0.018	0.531–0.556
P. cultripes and *P. syriacus*	8	0.993±0.059	0.911–1.083
P. varaldii and *P. syriacus*	4	0.994±0.078	0.890–1.086

n, number of comparisons; EE, Eastern Europe; WE, Western Europe

analysis (Fig. 2b) revealed only two main groups of populations: *P. f. fuscus* and *P. f. vespertinus*.

The first two coordinate dimensions from the MCA analysis (Fig. 2c) explained 50 % and 21 % (respectively) of the total genetic variance in *P. fuscus*. As in the UPGMA phenogram, along this coordinate dimension, three main groups of populations can be identified. The first group included populations of *P. f. vespertinus*, the second populations of *P. f. fuscus* from Eastern Europe and the third *P. f. fuscus* from Western Europe. According to MCA analysis, most samples from Kursk Province of Russia (nos. 22–32) were intermediate between East European *P. f. fuscus* and *P. f. vespertinus*.

In the Structure analysis (Fig. 2e), the model assuming two ancestral gene pools (*P. f. fuscus* and *P. f. vespertinus*) was preferred (Appendix S3).

The hierarchical AMOVA analysis revealed that the largest portion of observed genetic variation (96.3 %) within *P. fuscus* is observed among groups (two groups of *P. f. fuscus* and *P. f. vespertinus*). The remaining variation is almost entirely explained by the within-population level of variation (2.6 %), whereas a minimal part of genetic variation is explained by the among-population within-group level (1.1 %). All the variance components and the associated fixation indices (F_{IS}=0.083, F_{SC}=0.289, F_{CT}=0.963 and F_{IT}=0.976) were statistically significant ($P<0.05$).

Mitochondrial DNA variation and differentiation

The phylogenetic analyses (Fig. 3a) identified two well-supported clusters that correspond to *P. f. fuscus* and *P. f. vespertinus*. All *P. fuscus* sequences from Italy group among samples of *P. f. fuscus*; therefore, Italian haplotypes are unequivocally attributed to the nominal subspecies.

When comparing new data with previously published cyt *b* data (Crottini et al. 2007), the analysis of the samples belonging to *P. f. vespertinus* identified a single new haplotype (E13), found at Stupino (locality no. 2). This haplotype has only one mutational step difference with the previously published and widespread haplotype E8 (Fig. 3b). The analyses of other samples of *P. f. vespertinus* confirm the subspecies attribution of these samples, and new samples bear haplotypes E1, E3 and E8 (see Table 1 for more details).

All analyzed samples of the East European *P. f. fuscus* bear the haplotype W14 (Fig. 3b), the most widespread haplotype in Central Europe, while the samples of *P. f. fuscus* from Italy have the haplotype (W19), one of the most common haplotypes found in the Po Plain.

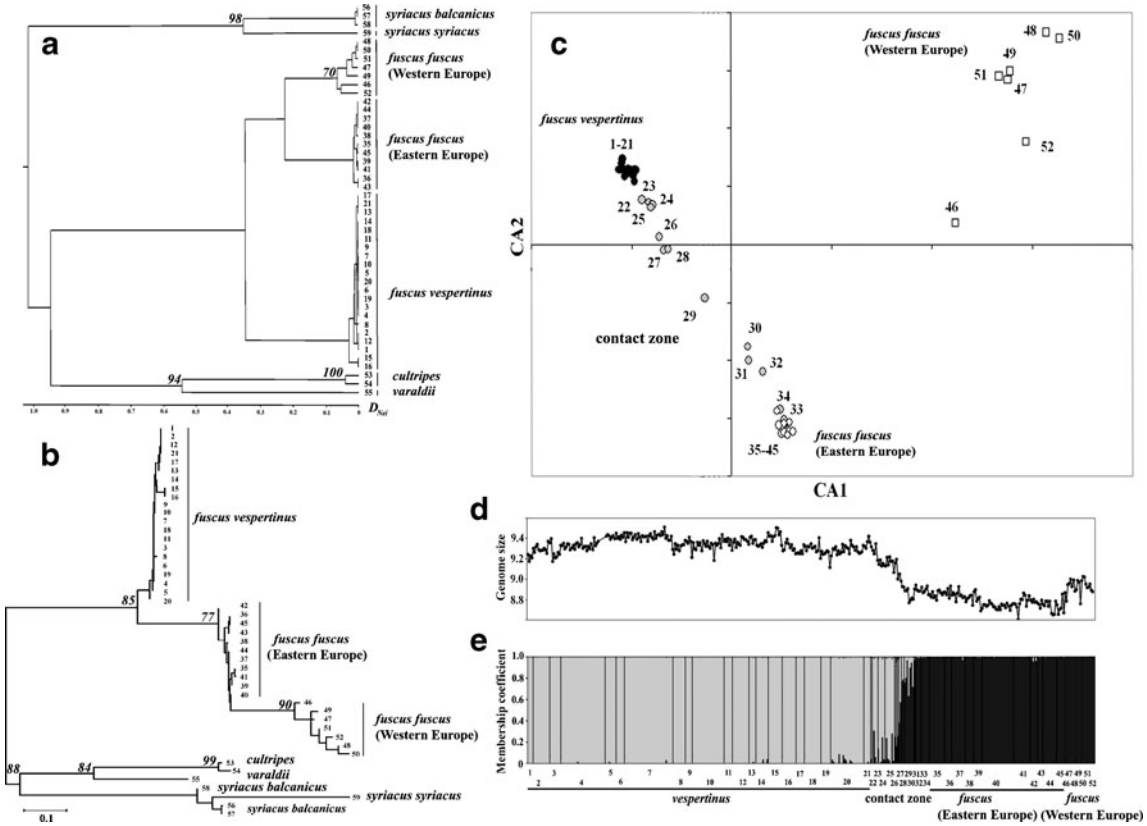

Fig. 2 Unweighted pair group method with arithmetic mean phenogram (**a**) and neighbor-joining tree (**b**) showing genetic (allozyme) relationship among the *Pelobates* species populations sampled based on Nei's (1978) unbiased genetic distance (D_{Nei}); bootstrap values≥ 70 %. Correspondence analysis of allele frequencies among the studied samples of *P. fuscus* (**c**), where *dark circles* represent *P. f. vespertinus* samples, *gray circles* are samples from the contact zone from Kursk Province of Russia, *open circles* are *P. f. fuscus* samples from Eastern Europe, and *small open squares* are *P. f. fuscus* samples from Western Europe. Genome size (in pg) variation for *P. fuscus* (**d**; each individual is represented as a point). Bayesian clustering results of the Structure analysis (with $K=2$) based on allozyme data (**e**), where the vertical line is partitioned into K colored segments, whose length is proportional to the individual's estimated membership coefficient (**e**; *black lines* separate different populations). Localities are numbered as in Table 1

The most interesting results come from the analysis of the samples originating from the contact zone between *P. f. fuscus* and *P. f. vespertinus* in Kursk Province of Russia. The analysis of these samples identified one new haplotype (W30), found at Peschanoe (locality no. 31), Guevo (locality no. 32) and Gaponovo (locality no. 33). This haplotype has only one mutational step difference with the previously published and widespread haplotypes W24 and W25 (Fig. 3b) and corresponds to a previously unsampled putative haplotype as forecasted by the previously published haplotype network reconstruction (Crottini et al. 2007). Haplotypes belonging to *P. f. vespertinus* were found in the localities 22, 23, 24, 25, 29, 31, 33 and 34, while haplotypes belonging to *P. f. fuscus* were found in the localities 28, 31, 32 and 33. In two localities, Peschanoe (locality no. 31) and Gaponovo (locality no. 33), individuals with haplotypes of both subspecies were found together (see Tables 1 and 3 for more details). In the contact zone, we could find only four haplotypes (W25, W30, E1 and E8), and the newly identified haplotype (W30) was found together with haplotypes of *P. f. vespertinus* in both the above-mentioned localities.

Contact zone between *Pelobates f. fuscus* and *P. f. vespertinus*

By use of allozyme, genome size and mitochondrial cyt *b* gene data, we localized a contact zone between *P. f. fuscus* and *P. f. vespertinus* in Kursk Province of Russia (Tables 1 and 3; Figs. 3b and 4). This zone can be qualified as a hybrid zone with interbreeding between the separate lineages leading to patterns of mixed ancestry in the center.

Due to the moderate sampling, cline analyses are best carried out on a linear array of populations. The southernmost populations from Kursk Province were arranged into a one-dimensional transect at latitudinal direction to estimate the width of the hybrid zone. The clines for all four diagnostic allozyme loci (*Aat-1*, *G6pdh-1*, *Gtdhp-1* and *Ldh-1*) for *P. f. fuscus* and *P. f. vespertinus* had congruent shapes and centers (Fig. 5). Population 22, located about 44 km

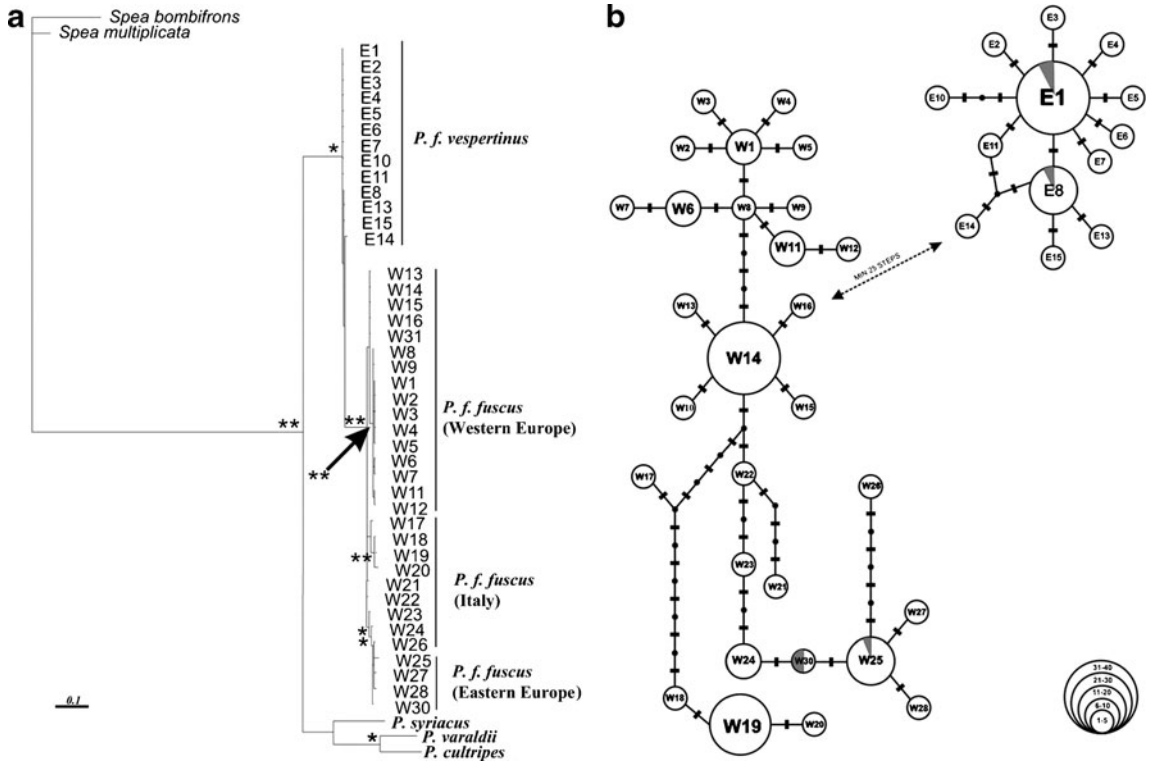

Fig. 3 **a** Bayesian phylogenetic analysis based on a 571-bp fragment of the mitochondrial cyt *b* gene. Only haplotype sequences have been used. *Spea bombifrons* was used as outgroup. Asterisks denote Bayesian posterior probabilities values: *95–98 %; **99–100 %. **b** Haplotype network reconstruction of 29 haplotypes of *Pelobates fuscus fuscus* (W) and of 13 haplotypes of *P. f. vespertinus* (E), based on the analysis of a 571-bp fragment of the mitochondrial cytochrome *b* gene. Size of circles is proportional to the number of individuals sharing a given haplotype. The frequency of each haplotype has been computed based on published data (Crottini et al. 2007) and on new sequences obtained in this study. In gray is the proportion of a given haplotype identified in samples originating from the contact zone. Each *black bar* represents a single nucleotide substitution. Using the option of the fix connection limit at 30 steps, provided by TCS (Clement et al. 2000), we defined the minimum number (inferior to 30) of mutational steps required to connect the two networks (*dashed line connections*) as identified using the method of Templeton et al. (1992). *Small black circles* correspond to hypothetical inferred haplotypes (not found or extinct). The names of haplotypes follow Crottini et al. (2007)

from the center of the hybrid zone and at the eastern limit of the studied contact area, was fixed for diagnostic loci for *P. f. vespertinus* and was characterized by (1) typical *P. f. vespertinus* allozyme loci, (2) typical *P. f. vespertinus* genome size and (3) typical *P. f. vespertinus* mtDNA haplotypes (Tables 1 and 3; Figs. 2d, 3, 4, and 5). Population 24 (about 15 km away from the center of the hybrid zone) had *P. f. fuscus* allozymes in the frequency of 30 % for *G6pdh-1* and 40 % for *Gtdhp-1* in an otherwise *P. f. vespertinus* genetic background. Samples from this locality had *P. f. vespertinus* mtDNA haplotypes and genome size data intermediate between *P. f. vespertinus* and *P. f. fuscus* (Table 1 and Figs. 2d, 3b, 4, and 5). Population 25 (about 7 km away from the center of the hybrid zone) possessed predominantly *P. f. vespertinus* allozymes, with *P. f. fuscus* alleles in the frequency of 14 % for *G6pdh-1* and 21 % for both *Aat-1* and *Ldh-1*. This population had only *P. f. vespertinus* mtDNA haplotypes and genome size data intermediate between the two subspecies (Figs. 2d, 3b, 4, and 5). Populations 27 and 28, located in the center of the hybrid zone, consist of single individuals. The sample from population 27 was heterozygote at *Aat-1* and *Ldh-1* in otherwise *P. f. vespertinus* alleles for the other two diagnostic loci. The sample from population 28 was homozygous for *P. f. vespertinus* allozymes at *Aat-1*, *G6pdh-1* and *Gtdhp-1* and homozygous for *P. f. fuscus* allozymes at *Ldh-1* locus. Both samples had intermediate genome size values and population 28 had a *P. f. fuscus* mtDNA haplotype. In population 29 (two individuals analyzed), located near the center of the hybrid zone one individual was heterozygote at *Aat-1* and *Ldh-1* for *P. f. vespertinus* and for *P. f. fuscus* allozymes and was homozygote for *P. f. vespertinus* at *G6pdh-1* and homozygote for *P. f. fuscus* at *Gtdhp-1*. The second individual was heterozygote at *G6pdh-1* for *P. f. vespertinus* and *P. f. fuscus* allozymes and was homozygote for *P. f. vespertinus* allozymes at *Aat-1* and for *P. f. fuscus* allozymes at *Gtdhp-1* and *Ldh-1*. Both specimens had intermediate genome size values for *P. f. vespertinus* and *P. f. fuscus*, and the single specimen that was analyzed for the mtDNA screen had a *P. f. vespertinus* haplotype. Population 31 (about 4 km away from the center

Table 3 Genetic data of samples from the contact zone between *P. f. fuscus* and *P. f. vespertinus* in Kursk Province of Russia

Locality		Allozymes						Cyt *b*
		n	%	Vesp	Fusc	Hybrids	Intermediate	
22	Bobryshevo	1	91	100(100)	0(0)	0	0(0)	V(1)
23	Selihovi Dvori	4	88	75(75)	0(0)	25	0(25)	V(2)
24	Bushmenovo	5	88	60(60)	0(0)	0	40(40)	V(3)
25	Bobrava	7	87	71(71)	0(0)	14	14(29)	V(5)
26	Vanina	1	72	0(0)	0(0)	0	100(100)	–
27	Ivanovsky	1	80	0(0)	0(0)	0	100(100)	–
28	Loshakovka	1	71	0(0)	0(0)	0	100(100)	F(1)
29	Dolgiy Kolodez	2	51	0(0)	0(0)	100	0(100)	V(1)
30	Pervyi Zyabkin	2	25	0(0)	0(0)	0	100(100)	–
31	Peschanoe	7	21	0(0)	29(43)	29	42(67)	V(3)+F(2)
32	Guevo	1	4	0(0)	100(100)	0	0(0)	F(1)
33	Gaponovo	6	1	0(0)	100(100)	0	0(0)	V(1)+F(1)
34	Zabolotovka	4	1	0(0)	100(100)	0	0(0)	V(2)

n, number of analyzed specimens; %, proportion of membership of the *P. f. vespertinus* cluster according to results of the Structure analysis. Vesp and Fusc, percentage of specimens attributed to *P. f. vespertinus* and *P. f. fuscus,* respectively, according to the results of New Hybrids and Structure (in parentheses) analyses. Hybrids, percentage of specimens attributed to hybrids, according to the results of New Hybrids analysis. Intermediate, percentage of specimens not attributed to hybrid and parental classes, according to the results of New Hybrids and Structure (in parentheses) analyses. Cyt *b*, haplotypes (number of specimens is given in parentheses) belonging to *P. f. fuscus* (F) and *P. f. vespertinus* (V)

of the hybrid zone) possessed predominantly *P. f. fuscus* allozymes with *P. f. vespertinus* allozymes at a frequency of 21 % for *Aat-1* and 14 % for both *G6pdh-1* and *Ldh-1*. This population had *P. f. fuscus* genome sizes and possessed two individuals with *P. f. fuscus* mtDNA haplotypes and three individuals with *P. f. vespertinus* mtDNA haplotype (see Tables 1 and 3). Populations 32–34 (from 30 to 100 km away from the center of the hybrid zone), at the western limit of the study area, were fixed for allozymes diagnostic for *P. f. fuscus* and were characterized by typical genome size valued for the nominal subspecies. Population 32 had the *P. f. fuscus* mtDNA haplotype; population 33 possessed one individual with *P. f. fuscus* mtDNA haplotype and one individual with *P. f. vespertinus* mtDNA haplotype; population 34 had *P. f. vespertinus* mtDNA haplotype (Table 3).

The width of the multilocus cline was estimated at about 12.5 km (Fig. 5). The widest separation of populations characterized by intermediate values of genome sizes (nos. 24 and 29) is 15 km. We found no evidence of allozyme introgression beyond the hybrid zone. The introgression of *P. f. fuscus* mtDNA was lacking, but *P. f. vespertinus* mtDNA was identified more than 100 km westward to the center of hybrid zone.

According to the Structure analysis results, all samples of *P. f. vespertinus* and *P. f. fuscus* collected beyond the contact zone were characterized by dominance (96–100 %) of membership of the own subspecies. In the contact zone (locality nos. 22–34), the easternmost seven samples (nos. 22–28) had 71–91 % of membership of *P. f. vespertinus*, whereas the five westernmost ones (nos. 30–34) had 75–99 % of membership of *P. f. fuscus*. Only one sample (no. 29) was characterized by nearly half of the membership of both subspecies (Table 3). According to both Structure and New Hybrids results based on allozyme data (see Appendix S4), the easternmost (locality no. 22) and westernmost (localities nos. 33–34) samples from the contact zone were characterized by specimens identified as pure parental *P. f. vespertinus* or *P. f. fuscus*, respectively (Table 3). Samples located in the center of the contact zone (nos. 26–30) consisted of only hybrid (6 individuals) or "intermediate" individuals. Other populations (nos. 23–25 and 31) were represented by pure parental (29–75 %) and hybrid (or "intermediate") individuals (25–71 %).

Ecological niche modeling

After correcting for correlation among data layers, nine bioclimatic variables were retained: Bio1 (annual mean temperature; °C), Bio2 (mean diurnal range; °C), Bio3 (isothermality), Bio5 (maximum temperature of warmest month; °C), Bio7 (temperature annual range; °C), Bio8 (mean temperature of wettest quarter; °C), Bio12 (annual precipitation; mm), Bio15 (precipitation seasonality) and Bio18 (precipitation of warmest quarter; mm).

The ENMs of both subspecies had high mean test AUC values (0.901±0.003 for *P. f. fuscus* and 0.943±0.003 for *P. f. vespertinus*) and showed significance for all binomial omission tests, indicating a good performance of the

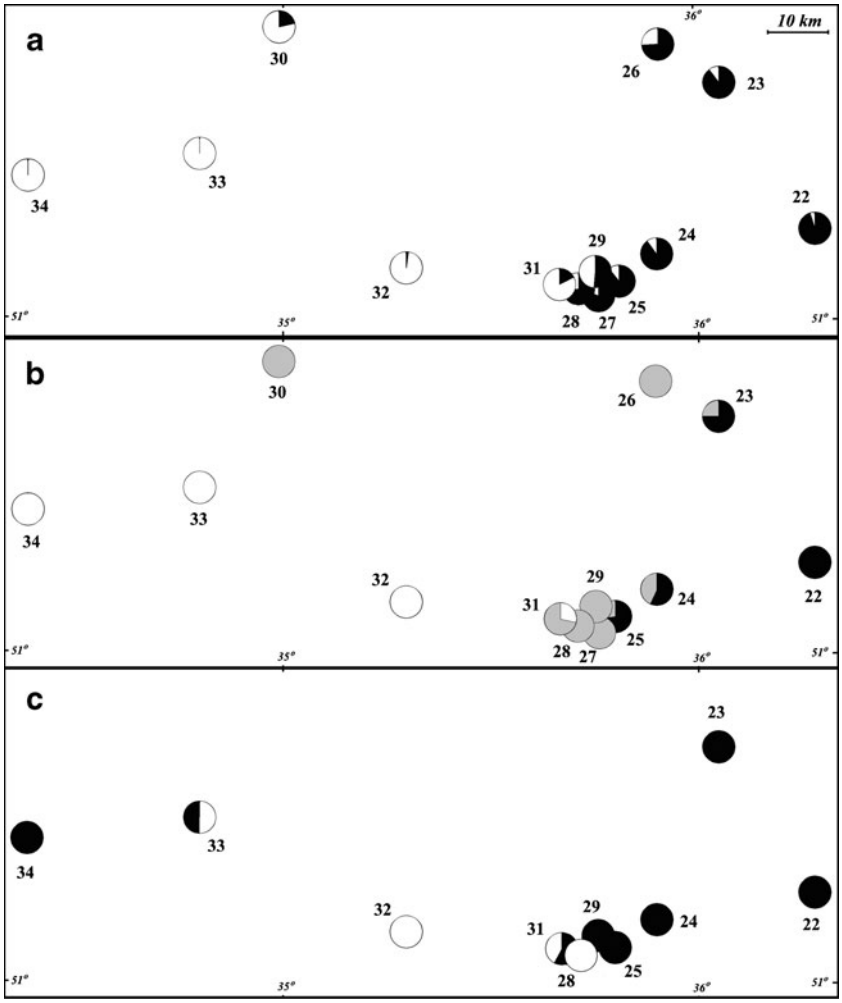

Fig. 4 Geographic location of populations sampled in the contact zone between *Pelobates fuscus fuscus* and *P. f. vespertinus* in Kursk Province of Russia. **a** *Light and dark sectors* show the proportion of membership of each population in clusters of *P. f. fuscus* and *P. f. vespertinus*, respectively, according to results of the Structure analysis based on allozyme data; **b** sectors show proportion of individuals determined as pure parental species (*P. f. fuscus* in *light* and *P. f. vespertinus* in *dark colors*) and hybrid plus "intermediate" individuals (*gray color*) in each population, according to results of the New Hybrids analysis based on allozyme data; **c** *light and dark sectors* show the proportion of membership of each population in clusters of *P. f. fuscus* and *P. f. vespertinus*, respectively, according to cyt *b* data. Localities are numbered as in Table 1 and Fig. 1

models. The predicted potential niche models under the current climate conditions are shown in Fig. 6. Despite the

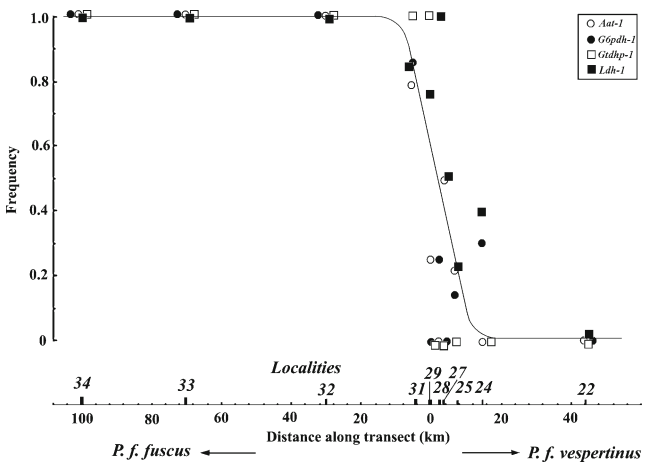

Fig. 5 A multilocus cline at four diagnostic allozyme loci along transect in the contact zone of *Pelobates fuscus fuscus* and *P. f. vespertinus*. The vertical axis shows the frequency of genetic variants diagnostic for *P. f. fuscus* (variation diagnostic for *P. f. vespertinus* is the inverse)

partial overlap between predictions, the variables with the greatest contribution to the models differed for each subspecies. For *P. f. fuscus*, two variables (Bio7 and Bio8) accounted for 83 % of the predicted range, whereas in *P. f. vespertinus* four variables (Bio3, Bio5, Bio 7 and Bio 8) accounted for 80 % of the predicted range. The ecological niche overlap between *P. f. fuscus* and *P. f. vespertinus* was very small (D=0.096 and I=0.217), meaning that the subspecies predominantly inhabit different environmental niches.

The projected potential niche models for the LGM indicate a substantial southward retraction of the ranges for both subspecies (Fig. 7). The extent of range shrinkage is, however, variable depending on the Global Circulation Model. According to the MIROC model, the suitable environmental conditions for *P. f. fuscus* were widely distributed in Western Europe, both south and north of the Alps, in the western part of the Pannonian Plain and in the northwestern part of the Black Sea area. Only the latter region showed a suitable climate according to the CCSM model. Both models identified Last Glacial refugia for *P. f. vespertinus* in

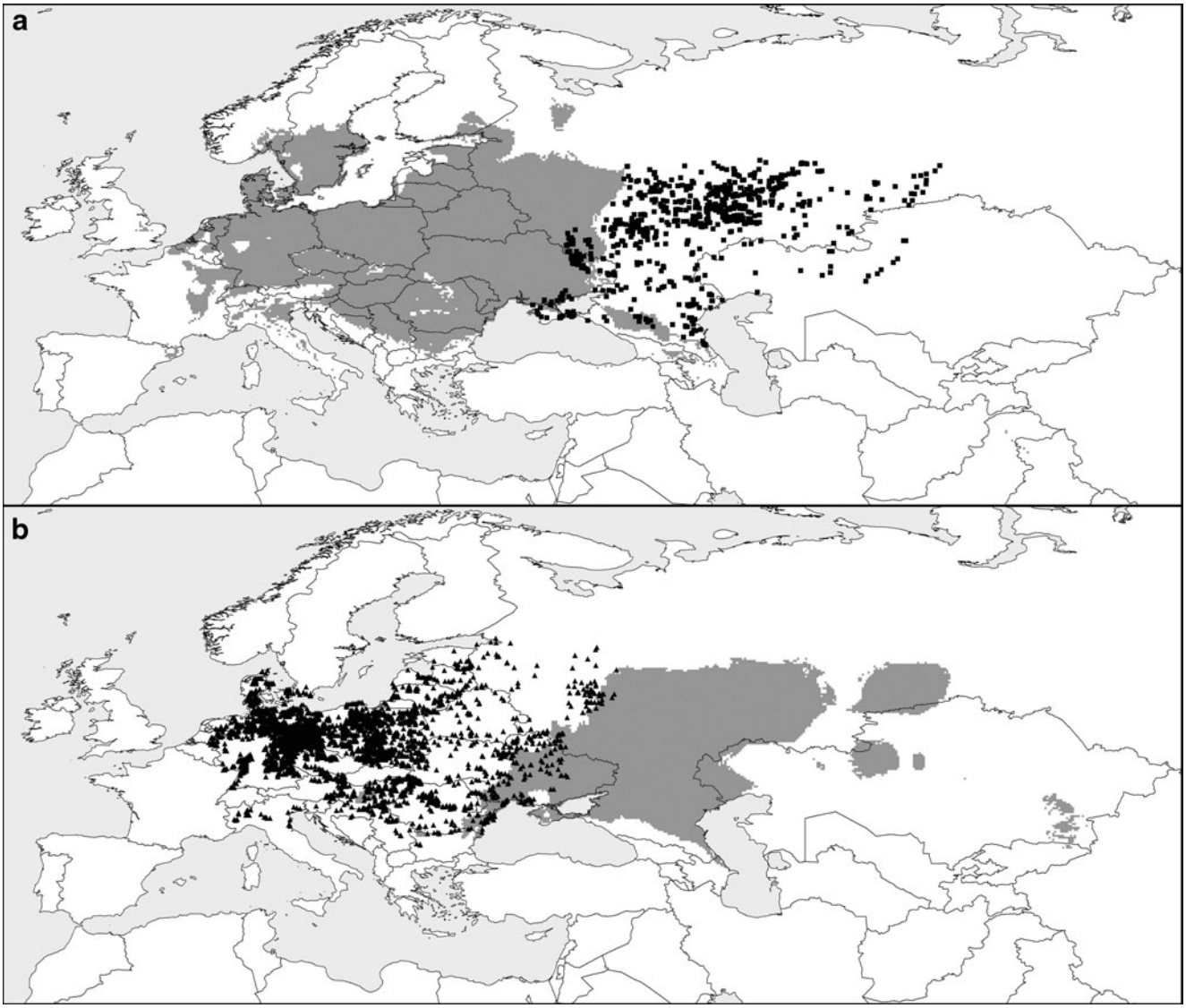

Fig. 6 Potential niche models (*dark gray area*) of *Pelobates f. fuscus* (**a**) and *P. f. vespertinus* (**b**) based on Maxent. Localities of *P. f. fuscus* and *P. f. vespertinus* are designated as *triangles* (**b**) and *squares* (**a**), respectively. Models are above the average 10-percentile training threshold

the territory between the Azov and Caspian Seas and in some parts of the Volga River drainage (Fig. 7).

Discussion

Allozyme and mtDNA analyses identified the existence of two main groups within *P. fuscus* (*P. f. fuscus* and *P. f. vespertinus*), initially revealed by the genome size variation studies of Borkin et al. (2001, 2002, 2003). Crottini et al. (2007) estimated the time of divergence between these groups at the early Pleistocene (2.42±1.40 Ma, lower and upper 95 % credibility interval is 0.81–6.15 Ma). Based on allozyme data, the divergence time between *P. f. fuscus* and *P. f. vespertinus* is the Pliocene (3.30 Ma). According to paleontological data (Appendix S5), the late Miocene and Pliocene ancestors of *P. fuscus* inhabited a territory from France in the west to eastern Kazakhstan in the east. In the middle Pliocene (3.3–4.0 Ma), the rapid cooling that occurred in Eastern Europe (Chepalyga 1987) probably induced a southward retraction of the range and initiated geographic isolation of *P. f. fuscus* and *P. f. vespertinus*. The Pliocene paleontological records of presumed ancestors of *P. f. fuscus* are known from northern Italy, Slovakia, Hungary, Moldova, western Ukraine, southern regions of Poland and Germany, and of *P. f. vespertinus* from Don River in the Voronezh Province of Russia (Appendix S5). Several other cladogenetic events in amphibians have been reported in this part of Europe within the same temporal frame: *Bombina bombina*/*B. variegata* (Fromhage et al.

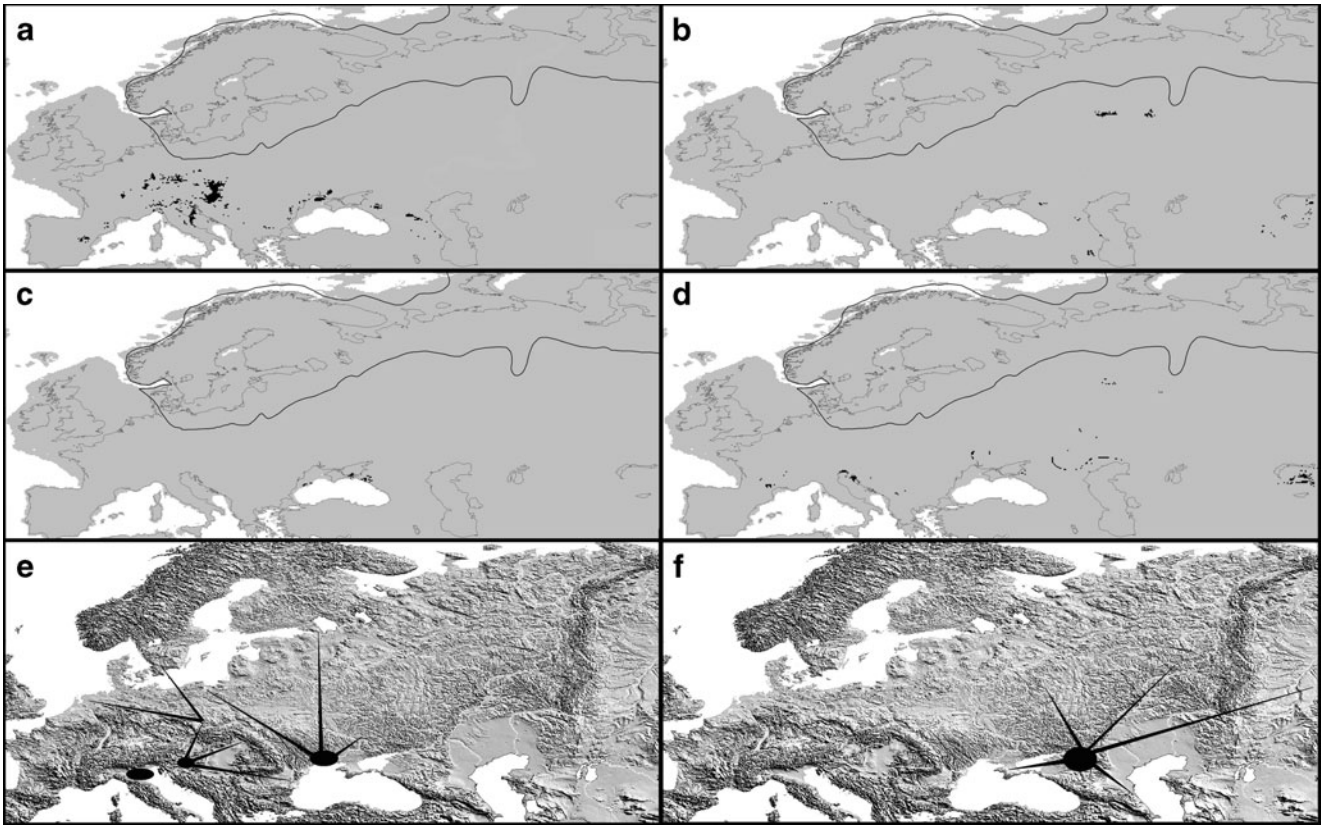

Fig. 7 Predictive potential niche models (*black areas*) of *Pelobates f. fuscus* (**a** and **c**) and *P. f. vespertinus* (**b** and **d**) for Last Glacial Maximum based on the MIROC (**a** and **b**) and CCSM (**c** and **d**) models. Models are above the average 10-percentile training threshold. The *solid black line* (in **a**–**d**) designates the position of the Fennoscandian glacier shield edge. Hypothesized postglacial range expansion routes from Last Glacial refugia (*black ovals*) are shown for *Pelobates f. fuscus* (**e**) and *P. f.vespertinus* (**f**)

2004; Hofman et al. 2007), *Hyla arborea/H. orientalis* (Stöck et al. 2008, 2012), *Pelophylax ridibundus*/*P.* cf. *bedriagae* (Akın et al. 2010), and *Bufo viridis*/*B. variabilis/B. balearicus* (Stöck et al. 2006).

Two genetic sublineages within *P. f. fuscus* were revealed in the haplotype network of the mtDNA analysis of Crottini et al. (2007). One of them included West European populations, and the second one shared two geographically distant East European and Italian populations (Fig. 3). The time of divergence between West European and East European lineages was estimated at the middle Pleistocene (0.55± 0.46 Ma, lower and upper 95 % credibility interval is 0.11–1.69 Ma). Our UPGMA and MCA analyses also revealed a West European and East European group within *P. f. fuscus* (the time of divergence is the early Pleistocene, 2.27 Ma). The central part of the Carpathian Mountains seem to form the range border between these groups: the West European group occurs exclusively to the west of this part of mountain range, but the East European group solely to the east. Unlike the mtDNA analysis results of Crottini et al. (2007), according to our allozyme data, the Italian population of *P. f. fuscus* corresponds to the West European clade of *P. f. fuscus*. Therefore, we assume that the Pleistocene climatic oscillations with strong southward expansions of glacial shields might have affected the isolation and genetic differentiation of East European and West European groups of *P. f. fuscus* (see also Eggert et al. 2006). Among western Palearctic amphibians, the same processes initiated genetic differentiation between *Rana arvalis arvalis* and *R. a. wolterstorffi* (Babik et al. 2004) and among eastern Mediterranean brown and green frogs (Veith et al. 2003; Akın et al. 2010).

According to our allozyme, ecological niche modeling and mtDNA analysis results, we suggest the existence of four different Last Glacial refugia of *P. fuscus*: the first in the area between the Caspian and Azov Seas as the origin for the expansion of *P. f. vespertinus*; the second in the northwestern part of the Black Sea area for the East European group of *P. f. fuscus*; the third in the southwestern part of the Pannonian Plain (the Sava River drainage) for the part of the West European group of *P. f. fuscus*, characterized by the "own" West European mtDNA; and the fourth in the Po Plain (with adjacent foothills of the Alps) for the part of the West European group of *P. f. fuscus*, which also bear East European mtDNA. All these refugia are located to the south of the southern limit of permafrost (Taberlet et al. 2008;

Hewitt 1999; Provan and Benett 2008), which is a very important environmental factor for terrestrially hibernating amphibians with no special adaptations to freezing (our data). According to the MIROC model, the suitable environmental conditions for *P. f. fuscus* were distributed in the northeastern part of the Iberian Peninsula. This territory is inhabited by *P. cultripes*. We have no paleontological evidence that this region was inhabited in the Pleistocene by *P. fuscus* (Appendix S5).

Among amphibians, the Po Plain and the southern foothills of the Alps could have been Last Glacial refugia for *Ichthyosaura alpestris* (Sotiropoulos et al. 2007), *Salamandra atra* (Bonato and Steinfartz 2005), *Triturus carnifex* (Canestrelli et al. 2012), *Hyla intermedia* (Canestrelli et al. 2007), *Pelophylax lessonae* (Snell et al. 2005; Zeisset and Beebee 2007; Canestrelli and Nascetti 2008) and *Rana temporaria* (Palo et al. 2004; Teacher et al. 2009). Fossil records of *P. fuscus* from the last glaciation in the late Pleistocene (0.027 Ma) are known from the adjacent territory in the Istrian Peninsula (Appendix S5), where the species recently became extinct (Džukić et al. 2008). The southernmost part of the Pannonian Plain (the Sava River drainage) likely acted as a refugium for *Triturus dobrogicus* during the late Pliocene (Litvinchuk and Borkin 2009; Vörös and Arntzen 2010). The Danube, Dnestr, Bug and Dnepr River deltas in the northwestern part of the Black Sea region were refugia for *Triturus dobrogicus* (Litvinchuk 2005; Litvinchuk and Borkin 2009) and *Bombina bombina* (Hofman et al. 2007; Fijarczyk et al. 2011). The southern foothills of the Moldavian Highland and the Carpathians could have been a refugium for *Triturus cristatus* (Litvinchuk and Borkin 2009). Stöck et al. (2006) proposed that the southern Ukrainian steppe may have been a refugium for *Bufo viridis* as well. The area between the Caspian and Azov Seas (southern European Russia and eastern Ukraine) could have been refugia for such cold-tolerant species as *Rana arvalis*, *Rana temporaria* and *Pelophylax lessonae* (Babik et al. 2004; Palo et al. 2004; Mezhzherin et al. 2010). Hence, Pliocene and Pleistocene climatic oscillations left their imprint in the evolution of *P. fuscus* characterized by successive vicariance and dispersal events.

The previous mtDNA analysis of Crottini et al. (2007) indicated that most populations went through bottlenecks during postglacial range expansions. The analysis of mismatch distributions for mtDNA lineages showed that all *P. f. vespertinus* and some populations of *P. f. fuscus* (the Pannonian Plain) exhibited signatures of postglacial range expansions. Riverbanks with alluvial sand deposits are the most suitable habitats for *P. fuscus* (Meissner 1970; our data) and could have been corridors for rapid postglacial range expansions. After postglacial warming, in the Dryas (12.9–11.5 years bp) and/or the early Holocene, the range expansion of *P. f. vespertinus* probably took place in valleys along the Don, Volga and Ural rivers and their tributaries (Fig. 7f). An early Holocene fossil record of *P. fuscus* is known from the southern Ural (Yakovleva and Yakovlev 2009). This area is currently inhabited by *P. f. vespertinus*. The East European group of *P. f. fuscus* could have restored the range along the Dnestr, Bug and Dnepr Rivers (Fig. 7e). The fossil remains of presumptive representatives of the group were found in northwestern Ukraine (Ratnikov 2009). The West European group of *P. f. fuscus* could have penetrated from the Pannonian Plain in the lower part of Danube River (Fig. 7e). The Moravian Gate, a natural depression between the Sudetes and the Carpathians, could have been used by the group for penetration from the Pannonian Plain in more northern and western parts of Western Europe (Fig. 7e). The early Holocene fossils of potential members of the group were recorded in northern Germany (Böhme 1983). The Moravian Gate, as routes of postglacial colonization, was presumably used by *Bombina bombina* and numerous other species of animals and plants (Hofman et al. 2007; Schmitt 2007; Hewitt 2011b).

The current study examined a contact zone between *P. f. vespertinus* and *P. f. fuscus* with the goal of determining whether they are maintaining their evolutionary independence. The results of this study indicate that the secondary contact between these subspecies results in a hybrid zone centered on Loshakovka and Dolgiy Kolodez villages (nos. 28 and 29) in Kursk Province of Russia (Figs. 1, 4, and 5). Here the subspecies interbreed, including admixture in the center of the hybrid zone (Fig. 4). Very low abundance of *P. fuscus* in the center of the hybrid zone (five year observation) did not allow us to estimate linkage disequilibrium suggesting selection against hybrid genotypes. However, taking into account dispersal capabilities of *P. fuscus* (up to 73 m per night; Eggert 2002), the hybrid zone width estimated in this study (about 12.5 km) is narrow. Given that contact between *P. f. vespertinus* and *P. f. fuscus* must have been possible since the Dryas/early Holocene, it is reasonable to assume that the hybrid zone formed 2,000–3,000 generations ago and is now at dispersal/selection equilibrium. All diagnostic allozyme loci had congruent cline centers (Fig. 5). The cline in genome size was similar to the allozyme clines (Fig. 2d, e). There is evidence of selection against hybrid genotypes in the center of the hybrid zone. Therefore, we suggest this may be a tension zone, though more sampling is necessary to make this determination with more confidence.

We found discordance in the extent of introgression into the tails of the clines by allozymes and mtDNA. Some populations (nos. 31, 33 and 34) with predominance of *P. f. fuscus* alleles had *P. f. vespertinus* haplotypes (Table 3). It is not uncommon for mtDNA to show discordant patterns relative to nuclear markers (e.g., Moritz et al. 1992; Litvinchuk et al. 1999; Jockusch and Wake 2002; Ballard and Whitlock 2004; Currat

et al. 2008; Veith et al. 2012). In most cases, the mito-nuclear discordance is attributed to adaptive introgression of mtDNA and sex-biased demographic asymmetries (Toews and Brelsford 2012).

In the light of the newly presented data, despite hybridization, the two lineages appear to maintain their independence as distinct evolutionary species (de Queiroz 2005): *P. fuscus* (common spadefoot toad) and *P. vespertinus* (Pallas's spadefoot toad). At the same time, our allozyme, genome content and mtDNA data on genetic divergence between *P. f. fuscus* and *P. f. insubricus* do not support the validity of this subspecies. We therefore propose to synonymize *P. f. insubricus* with *P. f. fuscus*.

The type locality of *P. fuscus* is "habitat in paludibus" (Laurenti 1768: 28), which later was restricted to "Wien," Austria (Mertens and Müller 1928: 18). The type locality of *P. vespertinus* is "Bach Sarbai," i.e., Zarbai Brook, the tributary of the Kinel' River, near a bridge 8.534 km ("8 Werste") from Ilmen' Village, Samara Province, Russia (Pallas 1771: 202). The taxonomic status of West European and East European populations of *P. fuscus* (sensu strictu) remains unclear and needs further investigation.

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