

# High degree of population subdivision in a widespread amphibian

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## Abstract

In general, amphibians are known to exhibit a higher degree of population subdivision than any other major animal taxa, but large-scale population genetic surveys of widely distributed species are still scarce, especially in the Eurasian continent. Using microsatellite markers and mitochondrial DNA sequences, we investigated the large-scale population genetic structure of the common frog (*Rana temporaria*) – one of the most widespread amphibians of the Palearctic region. Analyses of cytochrome *b* sequences revealed evidence for two distinct lineages inhabiting western and eastern parts of Europe. The separation of these lineages *c.* 700 000 years ago may have been induced by the onset of the Middle Pleistocene continental glaciations. Analyses of the variability of microsatellite loci within each of the clades revealed evidence for evolution of a high degree of population subdivision ( $F_{ST} \sim 0.23$ ) even in northern Fennoscandia, colonized less than 10 000 years ago. The high level of substructuring is puzzling in the face of an apparently high dispersal capacity, as evidenced by the rather rapid recolonization of northern Europe. This suggests that processes other than restricted dispersal capacity need to be explored as explanations for the high degree of population subdivision in amphibians. The colonization of northern Europe has been accompanied by loss of genetic variability as evidenced by decreasing levels of intrapopulational genetic variability in microsatellite loci from south to north across Europe.

**Keywords:** amphibians, cytochrome *b*, microsatellite, phylogeography, *Rana temporaria*, Scandinavia

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## Introduction

The continental glacial advances during the Middle and Late Pleistocene, starting *c.* 0.9 million years ago (Ma) constitute one of the most important factors that have shaped the North European ecosystems. With approximately 0.1 million year (Myr) intervals, the advancing ice sheets have destroyed boreal terrestrial habitats and restricted species distributions to hospitable areas south of the ice (Hewitt 1996). During the interglacials, organisms surviving in these refugia recolonized the previously glaciated areas. These invasions, although limited by the organism's

specific environmental requirements and dispersal abilities, have at times been remarkably rapid (Taberlet *et al.* 1998; Hewitt 2000). Several European refugial regions have now been identified in the Iberian and Apennine peninsulae, as well as in the Balkans and southwestern Russia (Hewitt 2001).

The colonization of the Fennoscandian region by the present flora and fauna was not possible until the withdrawal of the ice sheet *c.* 10 000 years ago; as a result the ecosystems are young and less complex than their southern counterparts. These dynamics should result in a low level of genetic differentiation between populations, especially in long-lived species (e.g. Ibrahim *et al.* 1996). During and after the deglaciation, two principal routes were open for the recolonization of Fennoscandia: from the south

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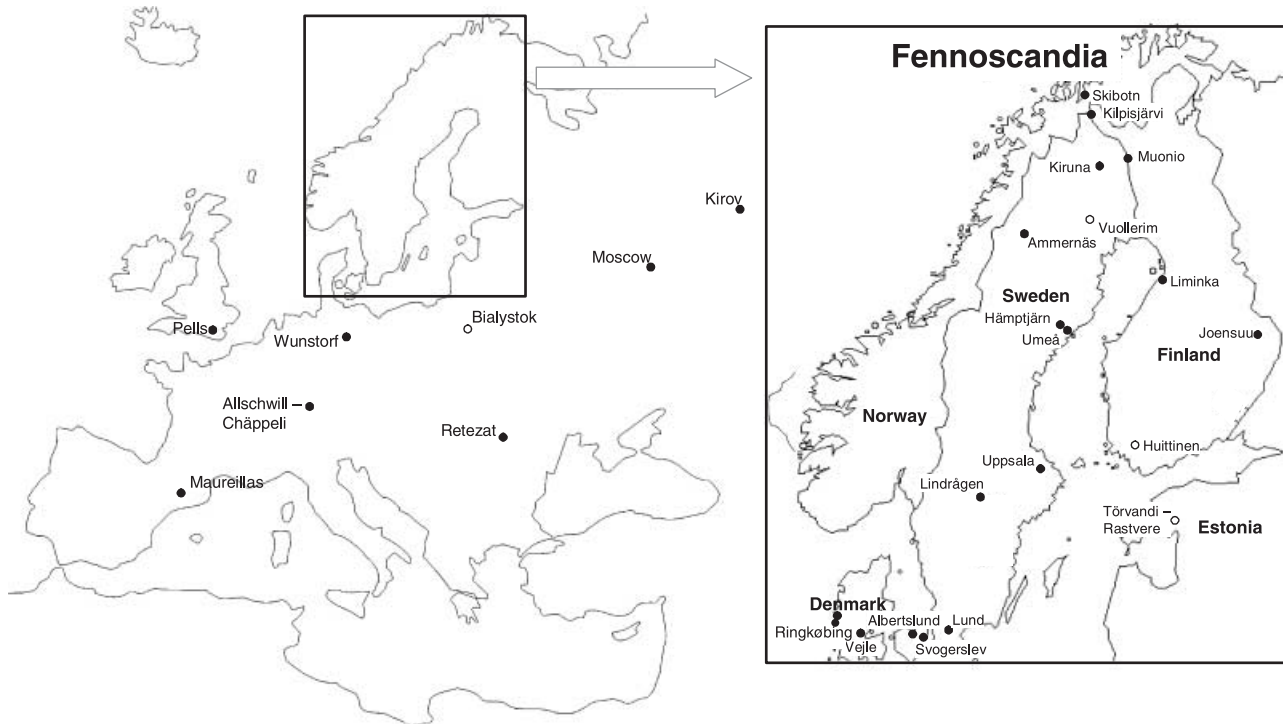


Fig. 1 A map showing the locations of study populations. Open circles designate populations for which only *Cytb* data was available.

through Denmark and from the southeast through Finland (cf. Fig. 1). Consequently, genetic studies in several species have revealed two phylogenetic lineages that meet in Fennoscandia (reviewed in Taberlet *et al.* 1998; Hewitt 2000), originating from distinct western and eastern glacial refugia. Notably, the estimated age of most of these lineages, *c.* 0.3 Myr, suggests that the intraspecific divergence took place prior to the most recent glacial cycle (Taberlet *et al.* 1998; Hewitt 2000).

In the postglacial recolonization context, the number of species studied to date is still limited, and taxonomically biased towards mammals (e.g. Taberlet & Bouvet 1994; Jaarola & Tegelström 1995; Fedorov *et al.* 1999; Petit *et al.* 1999; Davison *et al.* 2001; Seddon *et al.* 2001), fishes (Hansen *et al.* 1999; Bernatchez & Wilson 1998; Koljonen *et al.* 1999; Nesbø *et al.* 1999; Kontula & Väinölä 2001; Nilsson *et al.* 2001; Koskinen *et al.* 2002), and plants (e.g. Sinclair *et al.* 1999; Soranzo *et al.* 2000; Comps *et al.* 2001; Gugerli *et al.* 2001; Matyas & Sperisen 2001; Kremer *et al.* 2002). These studies have revealed somewhat differing phylogeographical patterns in terrestrial and aquatic organisms. For instance, the refugial lineages of terrestrial organisms frequently meet in central Sweden (e.g. Jaarola *et al.* 1999), where ice persisted for the longest time (Andersen & Borns 1997), whereas with aquatic organisms the primary contact zone appears to be located in the northern parts of Finland and Sweden (e.g. Kontula & Väinölä 2001; Koskinen *et al.* 2002;

Vainio & Väinölä 2003). However, the phylogeographical pattern of North European amphibians is largely unknown at present. Amphibians are apparently poorer dispersers than most other animal taxa as indicated by their very high degree of population subdivision (Ward *et al.* 1992).

The common frog (*Rana temporaria*) is the most widespread amphibian in Europe, ranging from the Iberian Peninsula to northern Norway in the west and to western Siberia in the east (Gasc *et al.* 1997; Kuzmin 1999). To date, only local surveys of genetic structuring have been performed (e.g. Reh & Seitz 1990), but these have discovered quite extensive population divisions, hinting at a possible high degree of genetic differentiation at larger scales. For instance, in a comparative study of population differentiation in Swedish common frogs, Palo *et al.* (2003) found a relatively high degree of population subdivision in both microsatellite loci ( $F_{ST} = 0.25$ ) and genes coding for quantitative traits ( $Q_{ST} = 0.81$ ). Several allozyme analyses of the population substructure of *R. temporaria* report  $F_{ST}$  ranging from 0.109 (Veith *et al.* 2002) up to 0.388 (Hitchings & Beebee 1997). However, lower  $F_{ST}$  was reported for island populations from southern Finland (Seppä & Laurila 1999).

The aim of this study was to investigate the phylogeography and genetic population structure of common frogs on a Europe-wide scale with special emphasis on Fennoscandia. In particular, we aimed to address the following questions. (i) Is there evidence for one or several genetic

lineages of common frogs in Northern Europe, and if for several, was Fennoscandia colonized by more than one of these lineages? (ii) Given that the colonization of previously glaciated areas has often been associated with loss of genetic diversity (Hewitt 2000), is there any evidence for latitudinal trends in the levels of within population genetic variability? (iii) What is the level of interpopulation differentiation in the postglacially established amphibian populations? We approached these questions by examining genetic variation in mitochondrial cytochrome *b* (*Cytb*) and nuclear microsatellite marker loci in *R. temporaria* populations spanning a region of about 2.5 million km<sup>2</sup>.

## Materials and methods

### *The study species and populations*

The common frog is a medium sized (c. 30 g) brown frog reaching maturity at an age of 2–3 years in southern

latitudes, and at 4–6 years in northern latitudes and at high altitudes (Miaud *et al.* 1999; ter Schure *et al.* 2002). Female common frogs lay 700–3000 eggs in single egg clutches (Ryser 1988). The species is a communal breeder preferring shallow ponds (Glandt 1986), at which they may arrive while ice still covers a large part of the pond (personal observation). Adults are site tenacious (Savage 1961; Haapanen 1970), but migrate between winter, breeding, and summer habitats (Savage 1961; Tramontano 1998). There is some variation in migration distances between individuals (Blab 1978; Tramontano 1998), but nothing is known about juvenile dispersal.

In total, 740 *Rana temporaria* individuals from 29 different locations were sampled for the analyses. These individuals originated from 18 Fennoscandian locations, and from 11 more southerly reference locations in Estonia (EE), France (FR), Germany (DE), Poland (PL), Romania (RO), Russia (RU), Spain (ES), Switzerland (CH) and the United Kingdom (UK) (Fig. 1, Table 1). Most of the samples were

**Table 1** The study populations, samples and basic descriptive statistics

Population	Country	Coordinates	$N_{Cytb}$	$N_{ms}$	$H$	$A$	$Ar$	$F_{IS}$
Pells*	United Kingdom – UK	50°45′ N, 00°03′ E	3	27	0.775	8.13	4.91	0.117
Allschwili-Chäppeli	Switzerland – CH	47°32′ N, 07°32′ E	2	28	0.751	10.25	5.13	0.040
Kirovt†	Russia – RU	58°40′ N, 49°36′ E	2	30	0.602	6.25	3.57	0.055
Moscow†	Russia – RU	55°50′ N, 37°28′ E	2	32	0.661	7.63	4.11	0.063
Retezat	Romania – RO	44°25′ N, 27°58′ E	2	22	0.805	10.88	5.61	0.184
Maureillas	Spain – ES	42°40′ N, 01°19′ E	1	28	0.700	7.25	4.17	–0.018
Kilpisjärvi	Finland – FI	69°03′ N, 20°47′ E		42	0.602	6.00	3.41	–0.055
Liminka	Finland – FI	64°53′ N, 25°33′ E	1	31	0.676	5.25	3.64	–0.051
Muonio	Finland – FI	67°55′ N, 24°04′ E		36	0.680	6.88	4.07	0.014
Joensuu	Finland – FI	62°43′ N, 29°42′ E		16	0.628	4.13	3.42	0.032
Lund	Sweden – SE	55°42′ N, 13°26′ E	2	36	0.683	9.38	4.49	0.095
Umeå	Sweden – SE	63°49′ N, 20°14′ E	2	36	0.594	5.88	3.61	–0.050
Ammarnäs	Sweden – SE	65°54′ N, 16°18′ E		36	0.515	4.63	2.99	–0.134
Hampjärn	Sweden – SE	63°52′ N, 20°13′ E		33	0.546	5.88	3.22	–0.013
Lindrågen	Sweden – SE	59°27′ N, 13°34′ E		30	0.710	7.63	4.30	0.033
Häggedal	Sweden – SE	59°51′ N, 17°14′ E		42	0.575	5.88	3.41	0.005
Kiruna	Sweden – SE	67°51′ N, 21°20′ E		36	0.658	5.50	3.61	–0.011
Roskilde–Svogerslev†	Denmark – DK	55°40′ N, 12°01′ E	2	25	0.404	4.38	2.73	0.127
Roskilde–Albertslund†	Denmark – DK	55°40′ N, 12°22′ E	2	30	0.515	5.50	3.30	0.133
Vejlet	Denmark – DK	55°43′ N, 09°34′ E	2	22	0.719	8.13	4.59	0.123
Ringkøbing†	Denmark – DK	56°09′ N, 08°14′ E	2	19	0.714	7.50	4.59	0.062
Wunstorf	Germany – DE	52°29′ N, 09°20′ E	4	21	0.584	4.88	3.52	0.063
Skibotn	Norway – NO	69°23′ N, 20°18′ E		27	0.633	5.25	4.23	–0.169
Huittinen*	Finland – FI	61°51′ N, 22°52′ E	2	29				
Törvandi + Rastvere	Estonia – EE	58°33′ N, 24°13′ E	2	22				
Vuollerim	Sweden – SE	66°26′ N, 20°37′ E	2					
Bialystok	Poland – PL	53°03′ N, 22°55′ E	2					
Overall			37	736	0.640	6.65	3.91	0.029

$N_{Cytb}$  = number of individuals sequenced for *Cytb*;  $N_{ms}$  = number of individuals genotyped for microsatellites;  $H$  = gene diversity;  $A$  = average allele number per locus;  $Ar$  = average allelic richness per locus. The Huittinen and Törvandi-Rastvere populations were not included in the actual analysis because of deviations from Hardy–Weinberg equilibrium.

\*Independent tadpole samples.

†Independent embryo samples, all other samples consist of toe clips from adult individuals.

originally collected as breeding adults, and a minority as independent tadpoles or embryos (one embryo per spawn clump).

### Molecular genetic methods

Genetic variation was assessed in partial mitochondrial *Cytb* sequences and at eight nuclear microsatellite loci. The template DNA was extracted using standard sodium dodecyl sulphate–proteinase K digestion treatment followed by NaCl purification and isopropanol precipitation (e.g. Bruford *et al.* 1992).

*Cytb* sequence data was obtained for one to four individuals from 18 locations (Table 1). A 605-base-pair (bp) *Cytb* segment was amplified using primers L14850 (5'-TCTCATCTGATGAACTTTGGCTC-3'; Tanaka *et al.* 1994) and H15410 (5'-GTCTTTGTAGGAGAAGTATGG-3'; Tanaka *et al.* 1996). Polymerase chain reaction (PCR) amplifications were performed in a total volume of 20 µL using approximately 20 ng of genomic frog DNA, 250 µM of each dNTP, 1 × PCR buffer (10 mM Tris–HCl pH 8.3, 10 mM KCl, 1.5 mM MgCl<sub>2</sub>; PE Biosystems), 0.5 U AmpliTaq DNA polymerase (PE Biosystems) and 100 nM of each primer. The cycling profile consisted of 94 °C for 3 min, followed by 35 cycles of 94 °C, 53 °C and 72 °C for 30 s each, and a final elongation step of 5 min at 72 °C.

*Cytb* fragments were sequenced in both directions using the PCR primers. Unincorporated nucleotides and primers were removed using the GFX DNA purification kit (Amersham Pharmacia Biotech). The fluorescent Big Dye-terminator sequencing kit (Applied Biosystems) was used for the sequencing reactions. Unincorporated dye terminators were removed with Centri-Sep gel filtering columns (Princeton Separations, Inc.). Sequences were resolved on an ABI Prism 377 sequencer (Applied Biosystems). The analyses were based on 440 bp of manually aligned sequences.

The two main observed *Cytb* lineages (see Results) can be separated by the presence/absence of a diagnostic site at position 277 that is cut by the *StyI* restriction enzyme. Therefore, a random fragment length polymorphism (RFLP) method was applied to scan a subset of populations for presence of the two main *Cytb* lineages. Restriction digests were performed overnight at 37 °C using 10 µL of the PCR reaction, 1 U of *StyI* enzyme (Promega) and 1 × restriction buffer F (Promega). The digests were resolved in 1.5% agarose gels and scored under UV-light.

Microsatellite analyses were conducted on 736 individuals from 25 locations (Table 1) using the following eight loci: Rt2Ca2–22, Rt2Ca25 (Trent Garner unpublished), RRD590 (Vos *et al.* 2001), RtµH (Pidancier *et al.* 2002), RtSB03 (Berlin *et al.* 2000), Rtempµ4, Rtempµ5 and Rtempµ7 (Rowe & Beebe 2001). PCR amplifications were performed in a total volume of 10 µL, with conditions as described in Palo

*et al.* (2003). PCR products were resolved by denaturing polyacrylamide gel electrophoresis (5% LongRanger gel, BMA, Rockland, USA) using an ABI 377 DNA sequencer; data collection and allele scoring were performed using the GENESCAN 3.1 and GENOTYPER 2.5 softwares.

### Data analyses

Phylogenetic analyses based on the *Cytb* sequences included heuristic searches under maximum likelihood criterion (heuristic search with TBR branch swapping and 100 random stepwise additions of taxa) using PAUP\*4b10 (Swofford 2003). Confidence values for the nodes were obtained by bootstrapping (100 replicates). The choice of the mutation model assumed in the maximum likelihood analyses were based on a hierarchical log likelihood test implemented in MODELTEST 3.04 (Posada & Crandall 1998) using a 1% significance cut-off level. The suggested model for the 440 bp data set, including *Rana arvalis* *Cytb* sequence (AY156954; Palo & Merilä 2003) as an outgroup, was HKY85 (Hasegawa *et al.* 1985) with nucleotide frequencies A: 0.226, C: 0.288, G: 0.166, T: 0.320 and a transition/transversion ratio  $ti/tv = 7.547$ . Haplotype and nucleotide diversities both within the observed clades (see below) and for the total data set were estimated using DNASP 3.53 (Rozas & Rozas 1999).

To assess the age and the position of the eastern and western clades within *R. temporaria*, *Cytb* data from six *R. temporaria* morphotypes from Spain, France and Germany deposited in the GenBank (*R. t. parvipalmata*, *R. t. temporaria*, *R. t. honnorati*, *R. t. canigonensis*, *R. t. 'gasseri'* and *R. t. 'aragonensis'*, accession numbers AY147951–AY147956; Veith *et al.* 2003) were aligned with our haplotype data. For this 356-bp data set, the suggested model of sequence evolution was HKY85 (A: 0.234, C: 0.257, G: 0.176, T: 0.333,  $ti/tv = 5.367$ ). The constancy of evolutionary rates between lineages was tested by comparing the log likelihood scores of tree topologies found with and without enforcing the molecular clock during the maximum likelihood search.

Population genetic analyses based on microsatellite data were performed using FSTAT v.2.9.3 software (Goudet 2001) unless otherwise indicated. Allele frequencies were determined within each of the 25 populations. Microsatellite diversity was estimated by calculating allelic richness (El Mousadik & Petit 1996) and unbiased estimates of gene diversity (Nei 1987). Deviations from Hardy–Weinberg equilibrium within populations were assessed by calculating the  $F_{IS}$  at each locus as well as over all loci and populations; the statistical significance of the  $F_{IS}$  values were determined by 10 000 randomization steps. The significance of association between genotypes (linkage disequilibrium) between pairs of loci was assessed using the randomization method implemented in FSTAT.



The difference in average intrapopulation allelic richness and gene diversity was compared between the populations from nonglaciated (RO, RU, DE, CH, UK, ES) and glaciated (Fennoscandia) areas using the one-sided test and randomization procedure to assess the statistical significance implemented in the *FSTAT* (1000 randomization steps). Latitudinal trends in genetic diversity estimates were examined by regressing the intrapopulation allelic richness and gene diversity estimates against the latitude.

The degree of differentiation in allele frequencies between each pair of populations was quantified using the standardized  $F_{ST}$  (i.e.  $\theta$ ; Weir & Cockerham 1984), and statistical significance was tested by bootstrapping (10 000 replicates). The allelic differentiation between each population pair was tested using Fisher's exact test, implemented in *GENEPOP* 3.4 software (Raymond & Rousset 1995). To examine interpopulation relationships, a neighbour-joining tree was constructed based on microsatellite  $D_A$  distances (Nei *et al.* 1983; see also Takezaki & Nei 1996) and the reliability of the tree was evaluated by bootstrapping (over loci, 10 000 replicates) using the program *POPULATIONS* 1.2.28 (Olivier Langella 1999, unpublished, <http://www.pge.cnrs-gif.fr/bioinfo/populations/index.php>). In addition, multidimensional scaling was used to visualize the clustering of populations based on microsatellite  $D_A$  distances.

The association between the estimates of interpopulation genetic distances ( $D_A$ ) and land-based geographical distances were assessed using the Mantel test, implemented in the *IBD* 1.2 – software (Bohonak 2002). For the  $F_{ST}$ , the association between  $F_{ST}/(1 - F_{ST})$  and the natural logarithm of the geographical distances was examined, as suggested by Rousset & Raymond (1997). The statistical significance of the values was obtained by 10 000 randomization steps.

To examine further the origin of the Fennoscandian populations, individual assignment tests, implemented in the software package *GENECLASS* 2.0 (Cornuet *et al.* 1999; Piry *et al.* submitted for publication), were employed. Using the Bayesian module, individuals from Fennoscandian populations were assigned either to the eastern (Retezat – RO, Moscow – RU, and Kirov – RU) or western (Pells – UK, Allschwill-Chäppeli – CH, Maureillas – ES, Wunstorf – DE) reference population from which their respective multilocus genotypes were most likely to have occurred, i.e. assignment to the population with the lowest log(Likelihood) value (Rannala & Mountain 1997). This differs from a standard assignment test as Fennoscandian individuals could not be assigned to their 'correct' population, but only to one of the eastern or western reference populations (see above). The proportion of each Fennoscandian populations' individuals assigned to each of the clades therefore gives an indication of the respective genetic contribution of each clade.

## Results

### Mitochondrial DNA differentiation

Within the analysed 440-bp *Cytb* segment, 16 polymorphic sites defining seven haplotypes were observed among the 37 individuals sequenced (GenBank accession numbers AY619558–AY619564; Table 2). Fifteen sites contained only transitional and one site transversional changes. The majority of the substitutions occurred in the third codon position; only the two Romanian samples contained a nonsynonymous substitution in the first codon position (base 74, leading to A → T amino acid replacement). No *Cytb* variation was observed among the Fennoscandian *R. temporaria* individuals sequenced.

In the maximum likelihood tree (Fig. 2a), the haplotypes observed in our sample clustered into two major clades: an eastern clade containing all the Fennoscandian and east European samples, and a western clade comprising the samples from DE, ES, CH and UK. The exclusive presence of eastern or western clade *Cytb* haplotypes in the Danish, Norwegian and German samples was confirmed using the RFLP method. The mean interlineage divergence between haplotypes in the two clades was 3.2% (HKY85 corrected distances). Within the clades, there was very little variation (Table 2).

The *Cytb* haplotypes representing five *R. temporaria* morphotypes from Spain and France (see Veith *et al.* 2003) clustered within the western clade together with our samples from Germany, UK and Switzerland (Fig. 2b). The *R. t. parvipalmata* lineage has separated earlier and appears

**Table 2** Cytochrome *b* haplotypes and their frequencies found in *Rana temporaria*

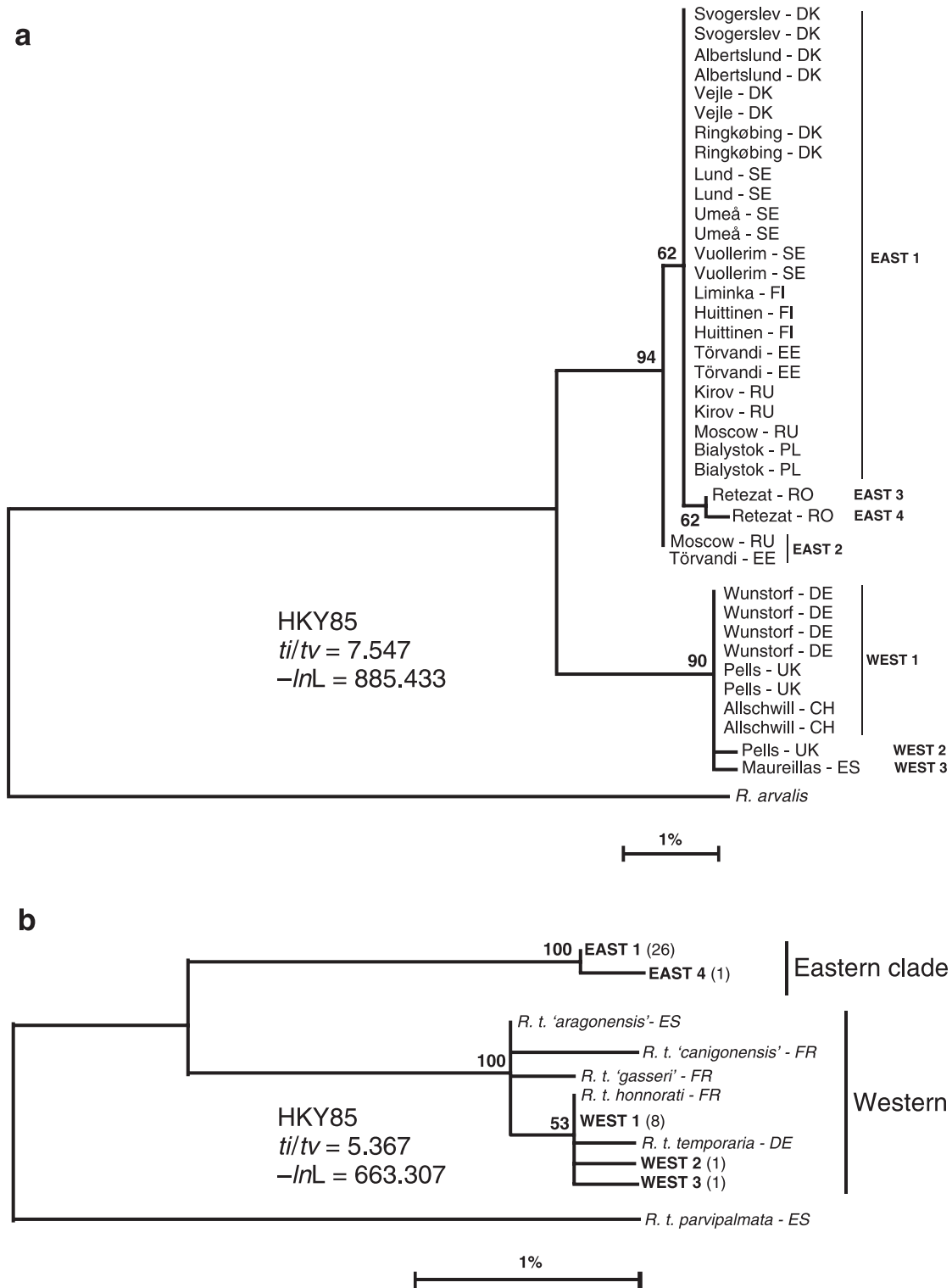
Haplotype code	Site position		N
	11112222	233334	
	7703564678	904792	
	4960164870	513341	
East 1	GTACCCGCGA	TTTATC	23
East 2	.C.....	.....	2
East 3	A.....	.....	1
East 4	A.....	.....T	1
West 1	.CGTTTATAG	C.CGAT	8
West 2	.CGTTTATAG	C.CGAT	1
West 3	.CGTTTATAG	.CCGAT	1

Only variable positions are shown.  $N$  = number of observed haplotypes,  $H$  = haplotype diversity,  $\pi$  = nucleotide diversity within the clades and in the total data set.

East,  $N = 27$ ,  $H = 0.276$ ,  $\pi = 0.0008$ .

West,  $N = 10$ ,  $H = 0.378$ ,  $\pi = 0.0009$ .

All,  $N = 37$ ,  $H = 0.577$ ,  $\pi = 0.0130$ .



**Fig. 2** Maximum likelihood trees based on (a) the 440-bp (our sample) and (b) the 356-bp *Cytb* data sets. The latter includes published *Cytb* sequences for six west European *Rana temporaria* morphotypes. For both data sets, the HKY85 model was assumed; bootstrap support values > 50 are indicated by the nodes. All Fennoscandian populations harboured the same eastern *Cytb* haplotype.

equidistant to the eastern and the western clade haplotypes (average HKY85 distances 5.40% and 5.43%, respectively) in this data set. The basal position of *R. t. parvipalmata* was confirmed by rooting the tree with *R. arvalis* sequence (not shown). Constancy of the evolutionary rates was confirmed by the likelihood ratio test ( $-\ln L = 663.307$  vs.  $-\ln L = 666.423$ , d.f. = 9,  $P = 0.716$ ).

Assuming a 'standard' mtDNA divergence rate of 2%/Myr (Brown *et al.* 1979), the split between the eastern and western clade haplotypes (average divergence 3.4%) would be dated to *c.* 1.7 Ma. However, using a molecular clock calibration derived from allozyme data on a multigene data, Veith *et al.* (2003) dated the divergence of the *R. t. parvipalmata* lineage to 1.12 (0.84–1.50) Ma. Assuming equal rates of sequence evolution between the lineages, the eastern and western clade divergence (3.4%) comprises 63% of the divergence between *R. t. parvipalmata* and the western clade. Taking this calibration, the split between the eastern and western European clades of *R. temporaria* could be roughly dated to *c.*  $1.12 \times 0.63 = 0.71$  (0.53–0.95) Ma.

#### Microsatellite variability

The eight microsatellite loci were highly polymorphic; the total allele number per locus varied from nine to 34 (average 24.8). The average intrapopulation allelic richness per locus was 3.9, ranging from 3.5 (Svogerslev, DK) to 7.3 (Allschwill-Chäppeli, CH). These populations also demonstrated the greatest range in gene diversity:  $0.35 \pm 0.04$  and  $0.72 \pm 0.03$ , respectively.

The Estonian Tõrvandi-Rastvere and Finnish Huittinen populations showed significant heterozygote deficit, mainly at locus SB03 ( $F_{IS} = 0.579$  and  $F_{IS} = 0.524$ ) and were excluded from further population genetic analyses. In the remaining 23 populations, the overall  $F_{IS} = 0.021$  did not deviate significantly from zero (95% C.I.  $-0.006$ – $0.050$ ), nor did the genotype frequencies deviate from the Hardy–Weinberg expectations in any of the populations after correction for multiple tests (Rice 1989). Linkage disequilibrium between four pairs of loci (Rt2Ca25  $\times$  Rtemp $\mu$ 7, Rtemp $\mu$ 4  $\times$  Rtemp $\mu$ 5, Rtemp $\mu$ 4  $\times$  RtSB03 and Rtemp $\mu$ 5  $\times$  RtSB03) bordered on the level of significance following correction for multiple tests. These cases were caused by strong linkage disequilibrium in one or two populations, and are therefore unlikely to indicate physical linkage.

There was a significant difference between populations originating from previously nonglaciated regions and postglacially established populations both in the average allelic richness ( $AR_{NON-GLACIATED} = 5.98$  vs.  $AR_{GLACIATED} = 4.71$ ,  $P = 0.015$ ) and gene diversity ( $H_{NON-GLACIATED} = 0.694$  vs.  $H_{GLACIATED} = 0.614$ ;  $P = 0.020$ ). Across Europe, there was a significant negative correlation between the genetic diversity indices and the latitude (Fig. 3).

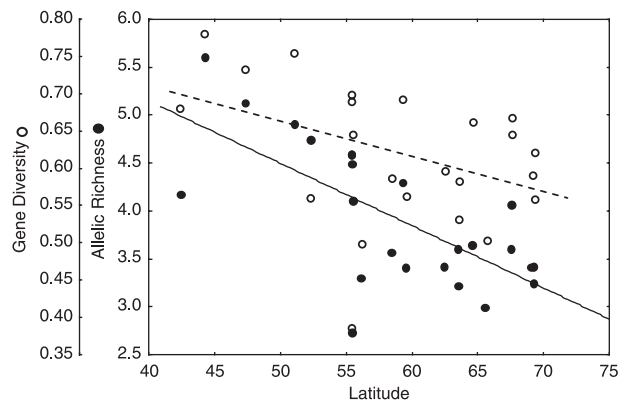


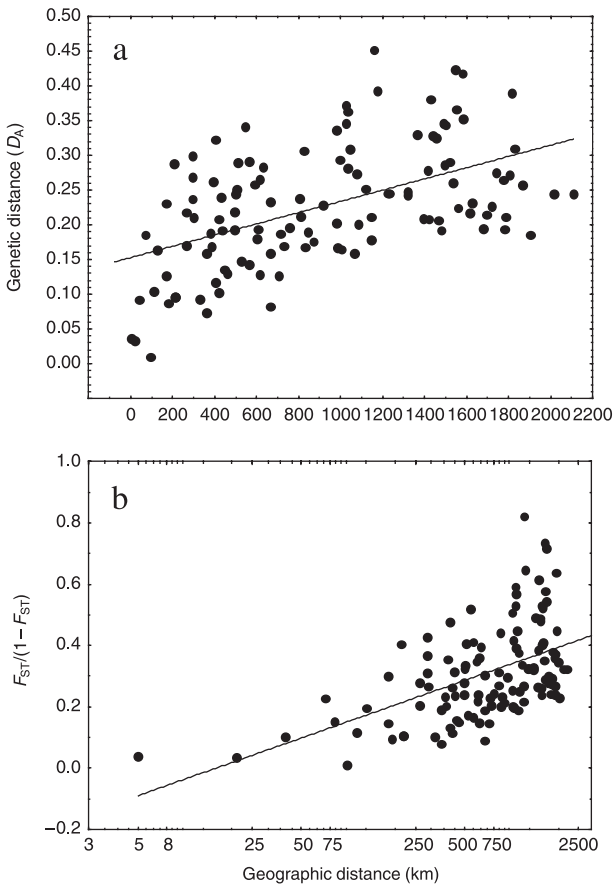
Fig. 3 Latitudinal trends in *Rana temporaria* genetic diversity. Allelic richness and latitude ( $\bullet$   $r_{21} = -0.686$ ,  $P < 0.001$ ), gene diversity and latitude ( $\circ$   $r_{21} = -0.450$ ,  $P = 0.031$ ).

#### Population structure and differentiation

All the studied populations were significantly differentiated from each other (Fisher's exact test,  $P < 0.0004$ ). The overall  $F_{ST}$  estimate from microsatellite data across all populations was 0.238 (95% C.I. 0.187–0.297). Differentiation among the postglacially established Fennoscandian populations was of the same magnitude  $F_{ST} = 0.229$  (95% C.I. 0.182–0.283), ranging from 0.010 (Vejle–Ringkøbing) to 0.451 (Svogerslev–Ammarnäs; Appendix I). Among the Fennoscandian populations, there was a highly significant isolation-by-distance pattern both in the  $D_A$  genetic distances and in the  $F_{ST}$  (Fig. 4).

As for the *Cytb* data, the microsatellites clustered the German, British and Swiss samples into one distinct clade in the neighbour-joining tree (Fig. 5a); the Spanish population appeared basal to all other populations. Of the 168 alleles observed in the eastern (RU, RO) and western (ES, UK, CH, DE) reference populations, 72 were observed in one clade only. Surprisingly, the mainland Danish populations Vejle and Ringkøbing grouped together with the western reference populations (Fig. 5a), despite their eastern mtDNA haplotype. The rest of the Fennoscandian populations and east European populations formed a diverse and poorly supported clade with Retezat (Romania) as the basal population. In the multidimensional scaling (Fig. 5b) this pattern manifests as three main groups in Fennoscandia. One group consists of the Finnish, Norwegian, northern Swedish and Russian populations. The second group includes only the southern Swedish and the Danish populations from the Sjælland Island. As the third group, the central Swedish populations Ammarnäs, Umeå and Hamptjärn cluster closely together, but appear intermediate between the groups of northern/eastern and southern Fennoscandia.

The assignment tests gave further support to this pattern (Fig. 6). No individuals in the Vejle and Ringkøbing



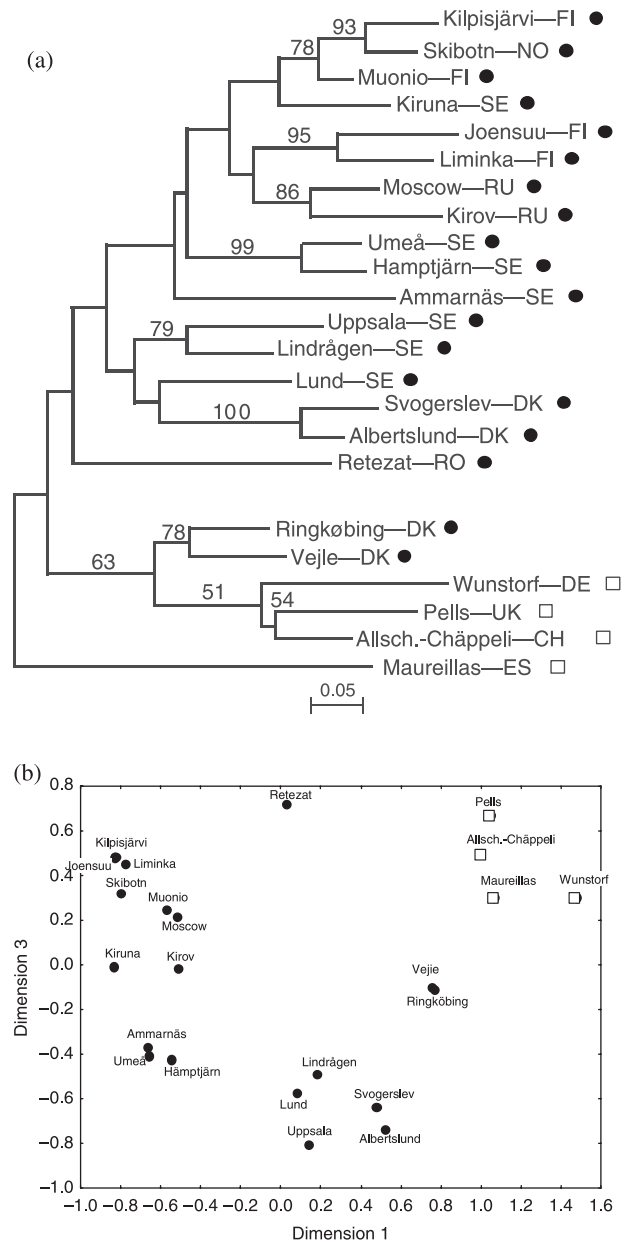
**Fig. 4** Isolation by distance in the Fennoscandian *Rana temporaria* populations (Mantel-test) using (a) interpopulation genetic distances ( $D_A$  distance;  $r_{118} = 0.800$ ,  $P < 0.001$ ) and (b) the transformed  $F_{ST}$  ( $r_{118} = 0.523$ ,  $P < 0.001$ ).

populations were assigned to the eastern group (defined based on the alleles observed in the Russian and Romanian sample). In the southern Swedish populations Lund, Häggedal and Lindrängen more than 30% of the individuals were assigned to the western group. In all other populations, the proportion of the eastern type individuals exceeded 75% (close to 100% in most).

## Discussion

### *A deep phylogenetic split between eastern and western European Rana temporaria populations*

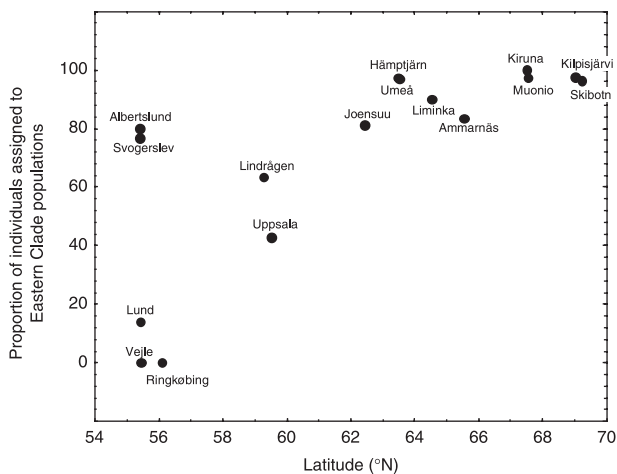
The seven haplotypes observed in the *Cytb* data were clustered in two clearly distinct and geographically separate clades. The eastern clade haplotypes were observed in all Fennoscandian and east European populations, whereas the individuals from the Spanish, Swiss, British and German populations carried the western clade mtDNA. As with many organisms studied before (e.g. Hewitt 2000),



**Fig. 5** (a) Neighbour-joining tree (support values  $> 0\%$  from 1000 bootstrap replicates are indicated above the branch) and (b) the results of multidimensional scaling illustrating the interpopulation relationships based on the microsatellite data ( $\bar{D}$ :  $\Phi = 3.58$ , stress = 0.08). ● and □ indicate populations harbouring eastern and western clade *Cytb* haplotypes, respectively.

the age of these main clades clearly exceeds the onset of the last glaciation phase. Using the previously dated *R. t. parvipalmata* divergence (1.12 Ma) as a calibration point places the east–west divergence to the Middle Pleistocene c. 0.71 (0.53–0.95) Ma. This calibration would denote a divergence rate of 4.8% per million years between sequence pairs, which is several times higher than rates commonly





**Fig. 6** Assignment test results depicting the proportion of individuals from each Fennoscandian population assigned to the eastern (Romanian and Russian) reference populations based on microsatellite data.

proposed for anurans (1.3%; Macey *et al.* 2001; Mønsen & Blouin 2003 and references therein) or generally for mtDNA (2%/Myr; Brown *et al.* 1979). Taking the latter calibration, the divergence of eastern and western clades would be dated to the Late Pliocene *c.* 2.8 Ma, which appears surprisingly ancient for an intraspecific lineage. Nevertheless, these deep phylogenetic divergences are plausibly explained by allopatric separations that may have been caused by the increasingly dramatic continental glaciations in the early Middle Pleistocene (e.g. Andersen & Borns 1997).

At present, *R. temporaria* is one of the most widely distributed anurans in Eurasia. It appears to cope well with cold climate — subfossil remains show that even during the glacial maxima it inhabited areas close to the southern limits of the continental ice sheets (Holman 1998). Veith *et al.* (2003) concluded that the (west European) *R. temporaria* is genetically and morphologically the most diverse of the Palearctic frog species (see also Veith *et al.* 2002) and suggested that it may have survived the glacial maxima in a number of 'cryptic' refugia in central Europe. In the light of this, the uniformity of the east European *R. temporaria* populations (from a much broader geographical range) appears surprising, suggesting survival in one main glacial refugium. As *R. temporaria* shows a preference for moist habitats, this difference could plausibly stem from a more arid continental climate in the eastern parts of Europe, especially during the glacial times. Alternatively, the mtDNA variation may have been affected by selection (selective sweep). No evidence for selection can be inferred from the *Cytb* sequences, though, as the eastern and western clades were defined uniquely by nonsynonymous substitutions. However, as the mtDNA genes are linked, we cannot reliably exclude this possibility.

### Geographical trends in the microsatellite variability

The recurrent reductions in population sizes (bottlenecks) associated with the colonization events are expected to lead to reduced genetic diversity, often decreasing with increasing latitude (i.e. increasing distance from the refugium, e.g. Sage & Wolff 1986; Hewitt 1996; Merilä *et al.* 1997). The recurrent bottlenecks are expected to have more acute impact on the allele number than on the gene diversity, because rare alleles are readily eliminated during times of increased genetic drift (Nei *et al.* 1975).

In agreement with these expectations, allelic richness and gene diversity were significantly lower in the Fennoscandian *R. temporaria* populations than in the populations inhabiting non-glaciated parts of Europe, where common frogs survived during the glacial maxima (cf. Holman 1998). Overall, there was a negative correlation between the latitude and the two different diversity measures (Fig. 3), but surprisingly, the lowest allele numbers and very low gene diversities were encountered in the Danish populations Svogerslev and Albertslund. These two populations, situated in the Danish island Sjælland, are clearly distinct from the two other Danish populations from the Jylland peninsula (Vejle and Ringkøbing) and may have been subjected to stronger genetic drift than most of the other populations (see below).

### Population subdivision

In general, amphibians are considered to be philopatric, showing a high degree of population subdivision over relatively short geographical distances (e.g. Ward *et al.* 1992; Hitchings & Beebe 1997; Veith *et al.* 2002). This is indeed supported by the present study: the overall  $F_{ST}$  was estimated to be 0.23. Significant differentiation in the allele frequencies was observed even over a small geographical scale, and the  $F_{ST}$  between the Umeå and Hämtjärn populations, separated by only *c.* 3.5 km, was 0.036. The lowest level of differentiation was observed in the Jylland populations Vejle and Ringkøbing ( $F_{ST} = 0.01$ ), separated by *c.* 100 km. Furthermore, the two populations from the Danish Sjælland Island were highly differentiated from the rest (average  $F_{ST} = 0.31$ ), but showed rather limited interpopulation divergence ( $F_{ST} = 0.03$ ).

The high population subdivision observed in the Fennoscandian common frog populations appears surprising given the relatively recent and rapid recolonization. The divergence estimates are also high when contrasted with the divergence observed in several *R. temporaria* morphotypes from Spain and France ( $F_{ST} \sim 0.1$  from allozymes, Veith *et al.* 2002).

Several possible explanations for the accumulation of interpopulation divergence in a species with a potential of rapid colonization could be proposed: (i) truly poor dispersal

ability as suggested by capture–recapture studies of contemporary populations, (ii) poor dispersal ability in the present day fragmented landscapes, (iii) establishment of local populations accompanied with rapid adaptation to local conditions leading to the exclusion of migrant gene combinations, (iv) smaller effective population sizes in northern Europe, and (v) rapid colonization and subsequent reduced migration rates for ecological reasons (e.g. breeding system).

The first hypothesis would entail a slow recolonization of Fennoscandia during the Holocene. This appears unlikely, as there is evidence for the early Holocene occurrence of ranids in Scandinavia (Aaris-Sørensen 1988). Also hypothesis (ii), poor dispersal ability at present, seems unlikely as there was no evidence for higher level of population substructuring in the more fragmented (human-influenced) southern habitats of Denmark and southern Sweden than in the more pristine northern Fennoscandia. Furthermore, fragmentation is a relatively recent phenomenon – it takes several dozens or hundreds of generations for the differentiation to build up, depending on the effective population sizes. The last three scenarios are more likely explanations.  $Q_{ST}$  values from several life history characteristics (age and weight at metamorphosis, growth rate) show that there is significant diversifying selection acting along a latitudinal gradient through Fennoscandia (Palo *et al.* 2003). Hence, local adaptations might indeed buffer populations against invasion of migrating (foreign) alleles, leading to increased fluctuations of deme sizes through random individual reproductive success allowing weakly selected genes to affect neutral diversity (Charlesworth *et al.* 2003; see also Reusch *et al.* 2001). Additionally, populations tend to be smaller towards northern Fennoscandia, further exhibiting bias in the sex ratio (Alho 2004) and reproductive success (unpublished data). Together with the founder effects during the colonization, the lower effective population sizes may explain the high degree of genetic substructure in Fennoscandian populations. However, this difference may be partly narrowed by the increased longevity of *R. temporaria* towards high latitudes (ter Schure *et al.* 2002).

The factor contributing the most to the level of interpopulation divergence might, however, be explanation (v) different dynamics of migration to free habitats and between established populations (cf. leading edge expansion; Hewitt 2000, 2001). Once the habitats are colonized, there is a significant reduction in gene flow between populations, leading to higher levels of differentiation accumulated during the Holocene than would be expected by the species' colonization ability alone (cf. Hewitt 2000).

Assuming that the microsatellites evolve under stepwise (SMM), two-phase (TPM), generalized (GSM), or K-allele (KAM) mutation models (see Estoup *et al.* 2002), size homoplasy would lead to underestimation of interpopulation

differences and mask the phylogenetic signal at microsatellite loci. However, as noted by Estoup & Angers (1998), assuming a 'standard' mutation rate of  $5 \times 10^{-4}$  the effect of size homoplasy is substantial if the population divergence times are  $> 2000$  generations. As individuals in the northern Fennoscandian frog populations reach maturity at an age of 4–6 years (see above), we can assume that the generation interval is above 6 years. This would mean that the divergence times of Fennoscandian populations are below 10 000 per six to 1670 generations, i.e. under the limit where homoplasy would have a significant effect on estimates of population divergence. The highly significant isolation-by-distance pattern observed in the Fennoscandian populations can be taken as additional evidence for the accuracy of the markers in estimating interpopulation differentiation. The data may, however, underestimate the differentiation in the more southern (i.e. older) reference populations.

#### *Post-glacial colonization of Fennoscandia*

Based on the *Cytb* data it appears that west Europe was colonized by common frogs surviving in different, possibly multiple, areas as compared to those colonizing the north and east of Europe. Unfortunately, the slowly evolving *Cytb* does not allow detailed inferences of the origin of the east European and Fennoscandian frog populations. The low mtDNA diversity suggests that these originate from one refugial area, which may have been in any of the three traditionally identified refugia in the Appenines, Balkans and/or Russia (e.g. Taberlet *et al.* 1998). However, the microsatellite data cluster the east European and eastern/northern Fennoscandian populations into a distinct group, separate from the southern Swedish/Danish populations (Fig. 5a,b), indicating postglacial recolonization of Fennoscandia proceeding via two routes after the deglaciation: from the south through Denmark and from the southeast through Finland and northern Sweden. Assuming that the genetic diversity is adversely affected by the recurrent bottlenecks occurring along the colonization route, the distal populations of the colonization front should show lowest diversity. Indeed, the Swedish populations of Ammarnäs, Umeå and Hamptjärn show lower allele number and heterozygosity than either the southern Swedish, or the northernmost populations. The biogeographical pattern of two colonizing lineages meeting in Central Sweden has been observed in a number of terrestrial and aquatic organisms (reviews by Taberlet *et al.* 1998; Jaarola *et al.* 1999; Koljonen *et al.* 1999; Kontula & Väinölä 2001). In contrast to these studies, however, our assignment tests suggest that the southern colonization route was used by common frogs representing both the eastern and western lineage (see below).

The Danish *R. temporaria* populations are of particular interest. No *Cytb* variation was observed; all four populations

harbour uniquely eastern mtDNA type. However, there was a clear distinction in the microsatellite diversity, despite their geographical proximity. The island populations Svogerslev and Albertslund harbour eastern-type microsatellite variation and are genetically depauperated. In contrast, the mainland Danish populations Vejle and Ringkøbing are significantly more diverse genetically and carry microsatellite alleles found only in the west European populations having the western mtDNA type.

This contradictory pattern most likely denotes a contact zone between the western and eastern *R. temporaria* clades. During the deglaciation, until c. 8500 years ago, the Danish straits area was largely dry land and could have been colonized by *R. temporaria*. This landbridge also opened a temporary route for the colonization of southern Sweden. The Danish straits became submerged again c. 8500 years ago as a result of the eustatic rise of the northern oceans, isolating the Danish islands and separating the Scandinavian peninsula from mainland Europe (e.g. Andersen & Borns 1997). This plausibly explains the isolation of the Sjælland populations, as evidenced by their low microsatellite variability, indicating that the large water bodies form an effective barrier limiting *R. temporaria* migration. In contrast, the Danish mainland (Jylland) has remained physically connected to mainland Europe. The discordance between the nuclear and the mitochondrial markers in the Denmark area could be caused by range shifts over a narrow contact zone between the eastern and western *R. temporaria* clades. Introgression and subsequent fixation of eastern mtDNA, rather than biparental microsatellites, into the Jylland area is more likely in *R. temporaria*, in which migration appears to be female biased (Palo *et al.* in press).

The distinctiveness of the Sjælland and southern Sweden populations (Fig. 5) may denote that they originate from a separate (eastern) refugial area from common frogs of eastern and northern Fennoscandia. However, the *Cytb* data do not support this. Alternatively, the observed pattern would fit a scenario where the eastern and western *R. temporaria* clades have formed a narrow primary contact zone in the Jylland area during the Late Pleistocene or early Holocene. After formation of the transient landbridge between southern Sweden and Denmark c. 10 500 years ago, individuals carrying microsatellite alleles distinctive to both lineages could disperse northwards. This hypothesis would also plausibly explain the high microsatellite diversity observed in the Lund population, as well as the high proportion of individuals assigned to the western clade in the three southern Swedish populations. As the clades harbour a substantial number of private alleles, the signatures of the two clades are retained even in areas where the individuals representing both clades are interbreeding.

## Conclusions

In agreement with theoretical expectations, the postglacially established Fennoscandian *R. temporaria* populations harbour lower levels of genetic variability than the reference populations from western and eastern Europe. All studied Fennoscandian individuals had an eastern type mtDNA, suggesting colonization from the east. However, microsatellite data indicated that this postglacial colonization might have proceeded via two routes: from the south through Denmark and from the southeast through Finland and northern Scandinavia. These two colonization fronts may currently meet in central Sweden, but resolution of the applied markers is too low to ascertain this. It also appears that a second contact zone between west and east European *R. temporaria* clades has formed in Denmark as assignment tests suggested that individuals representing both the western and eastern *R. temporaria* lineages have invaded southern Sweden, most probably during the transient Ancylus-phase of the Baltic Sea development c. 10 500 years ago. Hence, the results suggest a complex colonization history of Fennoscandia by *R. temporaria*. A denser sampling scheme and additional markers are required to clarify the precise location and extent of the suspected contact zones in the central Sweden and Denmark regions.

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This study is the result of a collaboration between researchers interested in utilizing molecular methods to study population history and microevolutionary processes in wild vertebrates.

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## Appendix I

Pairwise  $D_A$  (upper diagonal) and  $F_{ST}$ -estimates (lower diagonal) of the investigated populations

	Allsch.-																						
	Kilpisjärvi	Kiruna	Umeå	Ammaarnäs	Uppsala	Lund	Hampfjärn	Muonio	Joensuu	Liminka	Maureillas	Chäppeli	Pellham	Retezat	Lindrägen	Moscow	Kirov	Albertslund	Svogerslev	Vejle	Ringköping	Wunstorf	Skibotn
Kilpisjärvi	0.260	0.419	0.436	0.652	0.506	0.435	0.133	0.352	0.307	0.812	0.669	0.673	0.462	0.541	0.345	0.437	0.759	0.710	0.636	0.604	0.777	0.777	0.120
Kiruna	0.164	0.277	0.349	0.470	0.488	0.259	0.179	0.413	0.310	0.738	0.694	0.742	0.495	0.448	0.353	0.435	0.704	0.691	0.589	0.571	0.740	0.740	0.255
Umeå	0.258	0.130	0.340	0.426	0.379	0.123	0.326	0.475	0.393	0.754	0.643	0.723	0.532	0.463	0.460	0.512	0.562	0.532	0.562	0.547	0.759	0.759	0.356
Ammaarnäs	0.322	0.210	0.237	0.386	0.443	0.435	0.392	0.542	0.556	0.711	0.729	0.744	0.563	0.373	0.509	0.612	0.677	0.630	0.603	0.634	0.717	0.717	0.307
Uppsala	0.363	0.229	0.244	0.363	0.298	0.421	0.497	0.694	0.643	0.671	0.608	0.677	0.574	0.218	0.551	0.564	0.440	0.416	0.436	0.432	0.618	0.618	0.522
Lund	0.260	0.208	0.167	0.159	0.410	0.211	0.437	0.550	0.509	0.599	0.601	0.654	0.514	0.304	0.462	0.466	0.348	0.303	0.459	0.397	0.652	0.652	0.527
Hampfjärn	0.283	0.135	0.036	0.251	0.203	0.300	0.343	0.443	0.412	0.741	0.643	0.669	0.522	0.434	0.486	0.534	0.526	0.531	0.509	0.498	0.740	0.740	0.409
Muonio	0.087	0.104	0.168	0.278	0.186	0.188	0.082	0.291	0.226	0.736	0.590	0.635	0.391	0.416	0.248	0.353	0.663	0.607	0.540	0.513	0.709	0.709	0.148
Joensuu	0.168	0.187	0.212	0.336	0.206	0.238	0.082	0.082	0.216	0.745	0.678	0.747	0.469	0.599	0.283	0.384	0.661	0.595	0.642	0.630	0.823	0.823	0.377
Liminka	0.147	0.117	0.193	0.294	0.192	0.218	0.073	0.093	0.808	0.808	0.642	0.706	0.451	0.589	0.309	0.396	0.601	0.584	0.625	0.607	0.803	0.803	0.327
Maureillas	0.331	0.273	0.292	0.281	0.211	0.320	0.267	0.270	0.273	0.273	0.584	0.727	0.660	0.620	0.695	0.706	0.655	0.662	0.697	0.708	0.638	0.638	0.779
Allsch.-	0.278	0.243	0.283	0.257	0.232	0.299	0.201	0.251	0.210	0.212	0.213	0.515	0.537	0.515	0.611	0.664	0.591	0.557	0.331	0.286	0.249	0.249	0.698
Chäppeli	0.264	0.239	0.291	0.242	0.206	0.299	0.203	0.248	0.212	0.230	0.050	0.584	0.584	0.625	0.716	0.764	0.673	0.651	0.369	0.350	0.353	0.353	0.684
Pellham	0.192	0.170	0.224	0.239	0.181	0.236	0.111	0.136	0.129	0.202	0.124	0.128	0.477	0.477	0.344	0.472	0.551	0.516	0.562	0.553	0.631	0.631	0.487
Retezat	0.252	0.165	0.180	0.096	0.102	0.194	0.159	0.209	0.201	0.207	0.189	0.179	0.147	0.147	0.477	0.547	0.367	0.354	0.346	0.356	0.603	0.603	0.449
Lindrägen	0.195	0.166	0.220	0.298	0.204	0.257	0.119	0.130	0.133	0.272	0.221	0.235	0.132	0.186	0.085	0.196	0.529	0.460	0.601	0.579	0.735	0.735	0.337
Moscow	0.233	0.200	0.250	0.329	0.221	0.284	0.178	0.216	0.191	0.298	0.270	0.280	0.190	0.242	0.085	0.322	0.453	0.442	0.639	0.642	0.820	0.820	0.481
Kirov	0.233	0.200	0.250	0.329	0.221	0.284	0.178	0.216	0.191	0.298	0.270	0.280	0.190	0.242	0.085	0.322	0.453	0.442	0.639	0.642	0.820	0.820	0.481
Albertslund	0.424	0.381	0.346	0.342	0.185	0.372	0.346	0.389	0.343	0.360	0.340	0.318	0.314	0.259	0.293	0.322	0.453	0.120	0.470	0.422	0.686	0.686	0.764
Svogerslev	0.366	0.328	0.281	0.291	0.131	0.309	0.287	0.310	0.290	0.310	0.292	0.271	0.256	0.192	0.238	0.265	0.034	0.230	0.438	0.387	0.649	0.649	0.699
Vejle	0.275	0.231	0.245	0.196	0.170	0.245	0.195	0.244	0.214	0.246	0.076	0.081	0.151	0.129	0.222	0.267	0.288	0.218	0.173	0.173	0.432	0.432	0.652
Ringköping	0.272	0.226	0.243	0.189	0.159	0.247	0.193	0.244	0.212	0.243	0.067	0.081	0.157	0.127	0.222	0.274	0.269	0.218	0.010	0.176	0.402	0.402	0.622
Wunstorf	0.376	0.328	0.386	0.333	0.307	0.404	0.303	0.368	0.313	0.277	0.087	0.134	0.212	0.265	0.318	0.372	0.443	0.384	0.163	0.176	0.402	0.402	0.797
Skibotn	0.092	0.127	0.217	0.273	0.224	0.265	0.096	0.176	0.143	0.283	0.267	0.244	0.181	0.178	0.179	0.236	0.418	0.352	0.265	0.257	0.365	0.365	0.797