

# Postglacial Recolonization of Continental Europe by the Pygmy Shrew (*Sorex minutus*) Inferred From Mitochondrial and Y Chromosomal DNA Sequences

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**Abstract** The postglacial recolonization of the pygmy shrew (*Sorex minutus*,  $n=68$ ) across continental Europe was examined using control region sequences (342 bp) and concatenated sequences (2,939 bp) of four paternally inherited introns, specific for the Y chromosome. Phylogenetic analysis (NJ, MP, ML, and Bayesian inference) of the control region retrieved five major lineages (Northern, Western, Italian, Balkan, and Spanish) in Europe, in agreement with the previous cytochrome *b* data. Our study identifies a new refugium for the pygmy shrew located in France during the Pleistocene glacial cycles and the most important refugium with regards to the recolonization of Eurasia was probably located in the Carpathians. In contrast, Y chromosome intron sequences retrieved four major lineages (Northern, Western, Italian, and Balkan). This may indicate contact between Western and Spanish mtDNA lineages after expansion from their respective refugia post-LGM. Our results also revealed differential introgression of mtDNA and Y chromosome markers at a local level when populations from different glacial refugia come into contact. These results highlight the importance of adopting a multiple marker approach when inferring phylogeographic structure and colonization history of species.

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## 1 Introduction

The Pleistocene glaciations and intervening warming periods have undoubtedly played a major role in the formation of distinct genetic lineages across the geographic distributions of species (Hewitt 2000). The Last Glacial Maximum (LGM) occurred approximately from 23,000 to 19,000 calendar years before present (y BP; Mix et al. 2001). During this period, extensive areas of Europe were covered by ice and permafrost. Many species could only survive in relatively sheltered areas and would, therefore, have retreated to refugia with more suitable environmental conditions. Phylogeographic studies of European mammals have revealed several recolonization patterns from these refugia during the Pleistocene climatic fluctuations (Taberlet et al. 1998; Hewitt 1999). Glacial refugia that have been identified from a wide variety of studies of mammalian fauna include the Iberian Peninsula, Italy, and the Balkans (see Seddon et al. 2001; Taberlet et al. 1998; Mascheretti et al. 2003; Michaux et al. 2003). However the Mediterranean regions would have been more important as hotspots for endemism rather than a source of Northward recolonization for the rest of Europe (Bilton et al. 1998). The importance of refugia other than the Mediterranean peninsulas has become apparent through studies of a variety of mammals (Bilton et al. 1998; Stewart and Lister 2001). These include the Ural (Jaarola and Searle 2002; Brunhoff et al. 2003; Deffontaine et al. 2005) and Carpathian (Pazonyi 2004; Kotlík et al. 2006; Sommer and Nadachowski 2006; Saarma et al. 2007) mountain ranges. Some species of small mammals (e.g., bank vole *Myodes glareolus*) tolerated the climate conditions present during the LGM (Kotlík et al. 2006) to survive and recolonize Europe from these more Northern latitudes. Glacial refugia may also have been present further North in Scandinavia. For instance, populations of root voles, *Microtus oeconomus*, may have persisted through the LGM as far North as Andøya, an island situated off Northern Norway (Brunhoff et al. 2006).

Since the conception of phylogeographical studies, the most popular molecular markers have been mitochondrial DNA (mtDNA) sequences (Avise 2000). There are many reasons for this; mtDNA has many favorable properties, such as haploidy, a lack of recombination, rapid evolution compared to nuclear DNA (Brown et al. 1979), and is predominantly maternally-inherited (Nishimura et al. 2006). But perhaps the main reason for its widespread application is its relative ease of use (Petit et al. 2002) since universal primers were developed to amplify across a wide range of mammalian families (Kocher et al. 1989; Irwin et al. 1991). Despite the many obvious benefits, the sole use of mtDNA data can potentially lead to the construction of an incorrect species history because only the maternally-inherited mitochondrial lineage is being investigated (Ballard and Whitlock 2004). This is particularly important when it is evident that males and females do not play ‘symmetrical roles’ in establishing and maintaining population structure (Greenwood 1980).

Recently, the human genome sequencing project provided Y-specific genetic markers for humans (Petit et al. 2002). This has allowed numerous studies on the colonization history of humans (Underhill et al. 2000; Hammer et al. 2003) based

on male-inherited Y chromosome genes and the results have been consistent with the ‘Out of Africa’ hypothesis of modern humans based on mitochondrial, X-linked, and autosomal regions of DNA (Templeton 2002). However, there have been few phylogeographic studies in non-human mammalian species using Y chromosome markers. This may be due to the low levels of nucleotide diversity found in Y chromosome regions (Hellborg and Ellegren 2004). Recently, there have been studies on hybrid zones using Y chromosome structure (Jaarola et al. 1997) and Y-linked microsatellites (Balloux et al. 2000). Furthermore, combined datasets of mitochondrial, X and Y chromosomal genes have detected ‘cryptic species’ in the African elephant, *Loxodonta africana* (Roca et al. 2005), field vole, *Microtus agrestis* (Hellborg et al. 2005), and ground squirrels, *Spermophilus* (Gündüz et al. 2007). Brändli et al. (2005) used a similar set of markers to clarify the evolutionary history of the greater white-toothed shrew, *Crocidura russula* and its subspecies. Most of these studies have shown concordant tree topologies of mitochondrial, X and Y chromosomal DNA sequences when inferring evolutionary history, at the species- or subspecies-level. Therefore, the main finding of previous studies has been the reproductive isolation of genetic lineages. Little attention has been focused on sex-biased gene admixture when inferring the colonization history of a particular species.

The purpose of the present study is to investigate the female- and male-biased historical gene flow of the pygmy shrew, *Sorex minutus*. This study utilized a female-only inherited sequence (segment of the mitochondrial control region) and male-inherited sequences (four Y chromosome introns), and was centred on continental Europe and integrated in the context of the postglacial colonization history of the species. The pygmy shrew is the most widely distributed shrew species found in Europe (Mitchell-Jones et al. 1999) and it is also the oldest, with a fossil record dating back to the Pliocene, ca. 2 million years ago (Rzebik-Kowalska 1998). Its range extends from Western Iberia and Ireland to Siberia in the East, but is absent from Southern Iberia and most of the Mediterranean coasts and islands (Mitchell-Jones et al. 1999).

The pygmy shrew is an ideal choice for such a study because of its widespread distribution and fossil evidence of possible pygmy shrew survival in two refugia; with dates of approximately 17,000 y BP in Northern Spain (Pokines 2000) and 12–15,000 y BP in the Carpathian Basin (Pazonyi 2004). The pygmy shrew has also been the subject of previous postglacial colonization studies. Bilton et al. (1998) sequenced a 447 basepair (bp) segment of the cytochrome *b* gene from 14 specimens to reveal the importance of an unidentified glacial refugia located further North than the classic Mediterranean refugia. A similar result was found by Mascheretti et al. (2003) using a much larger dataset and a near-complete sequence (1,110 bp) of the cytochrome *b* gene when investigating the colonization history of Ireland. Glacial refugia identified thus far are the Iberian Peninsula, Italy, the Balkans, and a refugium located further North. Here we extend these findings using a combination of sex-linked and mitochondrial markers in an attempt to provide a more complete understanding of the pygmy shrew’s colonization history.

## 2 Materials and Methods

### 2.1 Sampling and DNA Extraction

A total of 68 samples were analyzed in this study. Forty eight of these samples were obtained from collections in the Universities of York, United Kingdom, and Lausanne, Switzerland. Live pygmy shrews were also sampled in France ( $n = 5$ ), including the island of Belle Île ( $n = 10$ ), using Trip Traps (Procter Bros. Ltd.). Details of sample locations are given in Table 1. DNA was extracted from ethanol-preserved tissue from tail tips, toe clippings or liver using the DNeasy Blood & Tissue Kit (Qiagen Ltd.) according to the manufacturer's protocol.

### 2.2 Mitochondrial DNA

The left domain of the control region was amplified using primers tRNA<sup>Pro</sup> (Stewart and Baker 1994a) and PS-CD (5' - GGCGAGATTAAATGTTAGCTGG - 3'). PCR reactions consisted of 2  $\mu$ l of DNA (40–200 ng/ $\mu$ l), 1.5  $\mu$ l (0.375  $\mu$ M; Sigma Aldrich) of forward and reverse primer and 45  $\mu$ l of MegaMix~Blue (Microzone Ltd.). PCR amplification was carried out in a Peltier Thermal Cycler (MJ Research) using an initial denaturation of 95°C for 3 min followed by 30 cycles of 95°C for 45 s, annealing temperature of 60°C for 1 min, and an extension of 72°C for 90 s. This was followed by a final extension step of 10 min. Fragment sizes ranged from approximately 500–800 bp, depending on the number of tandem repeats as previously reported in other *Sorex* species (Stewart and Baker 1994a; Fumagalli et al. 1996).

### 2.3 Y Chromosome Introns

Five primer sets were chosen from Y chromosome introns which amplified in the common shrew, *Sorex araneus* (Hellborg and Ellegren 2003). The introns chosen were DBY1, DBY3, DBY7, UTY11, and ZFY4 generating products totaling approximately 3.2 kbp. Two other introns (DBY8, DBY14), which amplified in *S. araneus* (Hellborg and Ellegren 2003) were not chosen due to a lack of polymorphism in several races of the common shrew (G. Yannic, unpublished data). Amplification of the Y chromosome introns was based on the protocol used by Hellborg and Ellegren (2003). Briefly, PCR reactions consisted of 3  $\mu$ l DNA (60–300 ng/ $\mu$ l), 5.0  $\mu$ l 10 $\times$ Buffer (Invitrogen), 2.5  $\mu$ l of MgCl<sub>2</sub> (2.5 mM; Invitrogen), 1.0  $\mu$ l dNTPs (0.2 mM; Invitrogen), 1.0  $\mu$ l (0.25  $\mu$ M; Sigma Aldrich) of forward and reverse primer for each locus and 0.3  $\mu$ l of Platinum Taq Polymerase (5 U/ $\mu$ l; Invitrogen) to make up a 50  $\mu$ l reaction.

PCR amplification was carried out using an initial denaturation at 95°C for 10 min followed by 20 cycles of 95°C for 30 s, a touchdown annealing temperature

**Table 1** List of samples and haplotypes (both mtDNA and concatenated Y chromosome introns) present in each locality. The number of individuals per locality is represented by *n*. The coordinates of each sampling locality (numbered from 1 to 38) is plotted in Figs. 3 and 4

Location	Country	Coordinates	Map		mtDNA	
			No.	<i>n</i>	haplotype	Y haplotype
Rascafria	Spain	40°54' N 03°53' W	1	3	Spa1,2	Spa1,2
Encamp	Andorra	42°35' N 01°35' E	2	2	And1,2	Fra3
Le Mas-d' Artige	France	45°42' N 02°08' E	3	1	Fra1	–
Nexon	France	45°45' N 01°15' E	4	1	Fra2	Fra1
Belle Île	France	47°20' N 03°11' W	5	10	BI1,2,3,4,5	BI1
Fresseneville	France	50°05' N 01°34' E	6	1	Fra3	–
Broualan	France	48°28' N 01°37' W	7	2	Fra4,5	Fra2,3
Plancoet	France	48°30' N 02°15' W	8	1	Fra6	Fra4
Morlaix	France	48°35' N 03°50' W	9	2	Fra6	Fra3
Boxtel	Belgium	51°36' N 05°20' E	10	1	Bel1	–
Wageningen	Holland	51°58' N 05°40' E	11	1	Hol1	–
Hartz Mountains	Germany	51°45' N 10°40' E	12	2	Ger1	Ger1,2
Eberswalde	Germany	52°10' N 13°45' E	13	1	Ger2	Ger3
Kobbero	Denmark	56°43' N 08°20' E	14	1	Den1	North1
Askland	Norway	58°40' N 08°35' E	15	3	Nor1,2,3	Nor1
Klockkarvik	Norway	60°07' N 05°00' E	16	2	Nor4	–
Steinkjer	Norway	64°00' N 13°53' E	17	1	Nor5	North1
Tromso	Norway	68°50' N 19°00' E	18	1	–	North1
Karlskrona	Sweden	56°09' N 15°15' E	19	1	Swe1	Swe1
Vehkalahti	Finland	60°15' N 27°00' E	20	1	Fin1	–
Val d' Illiez	Switzerland	46°16' N 06°43' E	21	1	Swz1	Swz1
Bretolet	Switzerland	46°13' N 06°40' E	22	1	Swz2	Swz1
Bassin	Switzerland	46°32' N 06°39' E	23	1	Swz3	–
Trento	Italy	46°15' N 11°50' E	24	2	Ita1,2	–
Abruzzo	Italy	42°00' N 14°00' E	25	4	Ita3,4,5,6	Ita1
Calabre	Italy	37°07' N 16°38' E	26	1	Ita7	Ita2
Reggio	Italy	44°32' N 11°21' E	27	1	Ita8	Ita3
Cesky Jiretin	Czech Republic	50°41' N 13°34' E	28	2	Cze1,2	North1, Cze1
Donnerskirchen	Austria	47°56' N 16°40' E	29	1	Aut1	Aut1
Bratislava	Slovakia	48°07' N 17°00' E	30	5	Svk1,2,3,4	North1, Svk1
Blizocin	Poland	51°36' N 22°16' E	31	2	Pol1,Pol2	North1,Pol1
Vilnius	Lithuania	54°40' N 25°19' E	32	2	Lit1	North1
Kanev	Ukraine	49°41' N 32°00' E	33	1	Ukr1	North1
Brjansk	Russia	52°20' N 34°00' E	34	1	–	North1
Novosibirsk	Siberia	54°49' N 83°06' E	35	1	Sib1	North1
Lake Baikal	Siberia	53°40' N 108°00' E	36	1	–	Sib1
Pelister Mountain	Macedonia	41°00' N 21°10' E	37	1	Mac1	–
Strandzha Mountains	Turkey	41°45' N 27°41' E	38	2	Tur1,2	Tur1

from either 60–50°C (DBY1, DBY7) or 55–45°C (DBY3, UTY11) for 1 min, decreasing by 0.5°C per cycle and then an extension of 72°C for 90 s. This was followed by 20 cycles of 95°C for 30 s, 50°C (DBY1, DBY7) or 45°C (DBY3, UTY11) for 1 min, and 72°C for 90 s and a final extension step at 72°C for 10 min. All PCR reactions included a known female sample as a negative control. Amplification of a fragment in the female sample was used as evidence that the gene fragment was not specific to the Y chromosome. For this reason, intron ZFY4 was disregarded after amplification in several female samples.

## 2.4 Sequencing

PCR products were purified using Novagen® Spinprep™ PCR Clean-up Kit (Merck Biosciences) according to the manufacturer's protocol. Control region fragments were sequenced using the forward (tRNA<sup>Pro</sup>) and reverse primers (PS-CD). Y chromosome introns, DBY3 and DBY7, were sequenced using forward primers only while introns, DBY1 and UTY11, were sequenced using forward and reverse primers. Sequencing was carried out by Macrogen Inc. (Seoul, Korea). Control region fragment and Y chromosomal intron sequences were deposited in GenBank (Accession nos. for control region fragments: EU564340–EU564393; DBY1 intron: EU564394–EU564407 and EF636561–EF636562; DBY3 intron: EU564408–EU564414 and EF636573; DBY7 intron: EU564415–EU564422 and EF636584; UTY11 intron: EU564423–EU564434 and EF636606. Concatenated Y chromosomal intron sequences are available upon request).

## 2.5 Data Analysis

Chromatogram contiguous sequences were assembled in Sequencer 4.5 (GeneCodes) and sequences were aligned manually using Se-Al 2.0 (Rambaut 1996). The 5' end of the *Sorex* control region contains a tandem repeat region (Stewart and Baker 1994a,b; Fumagalli et al. 1996), with repeat motifs ranging from 78 to 80 bp in length. The number of tandem repeat copies ranged from 3 to 7 per individual and length heteroplasmy due to repeat numbers within and between individuals was present. All tandem repeat copies within an individual were screened for mutations between repeats (Rhymer et al. 2004). For analyses, in accordance with previous phylogenetic studies on *Sorex* species (Stewart and Baker 1994a,b, 1997; Rhymer et al. 2004) only the last tandem repeat and the unique flanking regions at the 5' end containing the hypervariable region were used, in addition to the 3' end of the repeat region (including part of the conserved Central Domain). This resulted in 342 bp (including indels) being used in the analyses. Chromatogram contigs for the four Y introns were assembled and

analyzed individually before being combined in Se-AL 2.0 (Rambaut 1996) to form a concatenated dataset (2,939 bp including indels).

Control region and concatenated Y intron haplotypes were identified using DAMBE 4.5.50 (Xia and Xie 2001). Nucleotide diversity ( $\pi$ ) was calculated using DnaSp 4.10.9 (Rozas et al. 2003). Net divergence ( $D_a$ ) was calculated in MEGA v. 3.1 (Kumar et al. 2004) using Kimura's 2-parameter distances (Kimura 1980). Standard errors (SE) were calculated by bootstrapping with 10,000 replicates.

Neighbor-joining (NJ), Maximum Parsimony (MP) and Maximum Likelihood (ML) phylogenetic trees derived from control region haplotypes were constructed in PAUP\* 4.0b10 (Swofford 1998). ML and NJ trees were constructed using the HKY85+I+G (Hasegawa et al. 1985) model ( $I=0.39$ ;  $\alpha=0.7831$ ), selected by the Akaike Information Criterion (AIC) in ModelTest 3.06 (Posada and Crandall 1998). Indels were treated as missing data when constructing trees. Of the 342 bp that were sequenced, 72 characters were parsimony informative. Bootstrap support was calculated from 1,000 NJ, MP, and ML parametric replicates, respectively. Bayesian posterior probabilities were estimated in MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003) from 1,000,000 generations sampled every 1,000th generation excluding a burn-in of 100,000 steps. *Sorex volnuchini* was used as the outgroup species, in accordance with previous phylogeographic studies on *S. minutus* (Bilton et al. 1998; Mascheretti et al. 2003).

The amount of genetic variation among and within phylogenetic lineages was determined by an analysis of molecular variance (AMOVA) implemented in Arlequin version 3.11 (Excoffier et al. 2005).

Recent population expansion from small founder populations for control region data was tested using mismatch distributions and Fu's (1997)  $F_s$ . Mismatch distributions of pairwise nucleotide differences of each lineage were calculated and compared with expected values for an expanding population (Rogers and Harpending 1992). Tests for goodness-of-fit statistics based on the sum of square deviations (SSD) for a model of sudden expansion were determined in Arlequin version 3.11 (Excoffier et al. 2005). Fu's  $F_s$  tests were performed to detect an excess of low frequency mutations in each lineage conforming to a model of sudden expansion. Tests for goodness-of-fit and Fu's  $F_s$  were generated in Arlequin v. 3.11 using parametric bootstrapping with 10,000 replicates.

Because sequence regions of the control region evolve at different rates (Stewart and Baker 1994a; Pesole et al. 1999), expansion times for lineages conforming to the sudden population expansion model were estimated using the unique sequence region at the 5' end from the mismatch distribution ( $\tau$ ) of each lineage, using the formula  $\tau = 2 \mu t$  (where  $\tau$  is the time since expansion in units of  $1/2 \mu$  generations), using a generalized least squares approach adapted from Rogers (1995), as described in Schneider and Excoffier (1999). Generation time was assumed to be 1 year (Churchfield and Searle 2008). An average evolutionary rate of 23% (15–31%) per million years ( $\text{My}^{-1}$ ) was used based on the rate derived from other *Sorex* species for the unique sequence region at the 5' end (Stewart and Baker 1994a). The divergence time ( $T$ ) between lineages for control region data was estimated by  $T = D_a / 2 \mu$ , where  $2 \mu$  is the divergence rate of 23%  $\text{My}^{-1}$  for the unique sequence



region at the 5' end with 95% confidence intervals (CI) estimated according to Jaarola and Searle (2004).

A median-joining network of Y chromosomal introns was constructed using Network 4.2 (Bandelt et al. 1999) to illustrate relationships among haplotypes. Indels were included as informative characters (Hellborg et al. 2005). Bootstrap support for lineages was calculated separately in PAUP\* 4.0b10 from 1,000 NJ and MP replicates, respectively. NJ analysis was performed using Kimura's 2-parameter distances (Kimura 1980) due to differences in the substitution patterns of the four introns (Hellborg et al. 2005).

### 3 Results

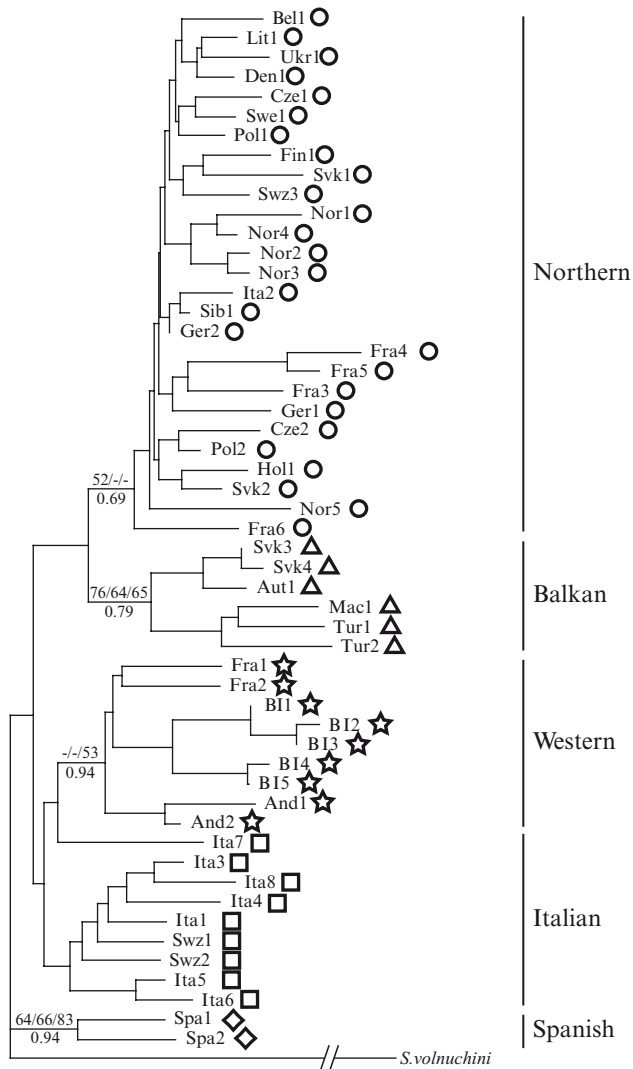
#### 3.1 Mitochondrial DNA Data

We examined 63 pygmy shrews from continental Europe for 342 bp of the mitochondrial control region. Of the 342 nucleotides, 108 were polymorphic (including indels). Fifty one (47.2%) polymorphic sites were found in the 5' unique flanking region. Thirty (27.8%) polymorphic sites were found in the last tandem repeat which was 78–80 bp in size and 27 (25%) polymorphic sites were found in the 3' end and part of the Central Domain. Fifty three unique haplotypes were identified (see Table 1). Only one haplotype was shared between localities: Fra6 between two localities in Northern France. NJ, MP, ML, and Bayesian analysis of the 53 haplotypes revealed concordant topologies resulting in five major lineages (Fig. 1), with poor to relatively good statistical support. The Western lineage included individuals from Andorra, central France, and the French island Belle Île. The Northern lineage included individuals from Northern France and Norway in the West to Siberia in the East. The Spanish lineage was confined to Spain. The Balkan lineage was present as far North as Austria and Slovakia, and the Italian lineage was present in Italy and the Alps in Switzerland and France. One outlier haplotype, Ita7, was identified from this region (Fig. 1). The Northern lineage is separated from both Western and Italian lineages by two indels, 3 bp and 4 bp in size. The Spanish lineage is separated from the Northern lineage by an 8 bp indel, and the Spanish and Balkan lineages are separated by a 2 bp indel. This corroborates the lineages identified by Mascheretti et al. (2003) using cytochrome *b* but with certain lineages more widespread because of better sampling coverage. An AMOVA showed that the majority of control region variation (62.8%) was distributed among lineages with a little variation (10.13%) found among populations within lineages.

The nucleotide diversity ( $\pi$ ) for lineages ranged from 0.01786 to 0.0331 (Table 2). Net divergence ( $Da \pm SE\%$ ) between lineages and estimated divergence in 1,000 s of calendar years before present (ky BP) are given in Table 3. Mismatch distributions for four out of the five lineages significantly conformed to the sudden population expansion model (Table 4). The Spanish lineage was excluded because of the small sample size ( $n=3$ ). Fu's  $F_s$  values and estimates for time since expansion for the four lineages based on Rogers (1995)  $\tau$  are given in Table 4.



— 0.005 substitutions/site



**Fig. 1** Neighbour-joining tree using the HKY85+I+G model of substitution of 53 control region haplotypes (342 bp) showing the 5 lineages found in European *S. minutus*. See Table 1 for haplotype designations. Bootstrap values obtained by NJ, MP, ML (>50%) are shown above nodes and Bayesian probabilities (>0.50) are shown below nodes for each lineage

### 3.2 Y Chromosome Intron Data

We examined 39 pygmy shrews for 2,939 bp (including indels) of concatenated Y introns (DBY1: 1098 bp, DBY3: 596 bp, DBY7: 441 bp, UTY11: 804 bp), which resulted in 23 unique haplotypes (see Table 1 and Fig. 2). Sixty polymorphic sites

**Table 2** Nucleotide diversity ( $\pi \pm \text{SD}$ ) of and number of individuals ( $n$ ) in each mtDNA and concatenated Y chromosomal intron lineage

Lineage	$n$	mtDNA	Y chromosome introns	
		$\pi \pm \text{SD}$	$n$	$\pi \pm \text{SD}$
Northern	30	0.02706 $\pm$ 0.00176	23	0.00033 $\pm$ 0.00009
Western	14	0.03193 $\pm$ 0.00334	10	0.00084 $\pm$ 0.00017
Italian	9	0.02746 $\pm$ 0.00276	5	0.00136 $\pm$ 0.00048
Balkan	7	0.02863 $\pm$ 0.00516	1	–
Spanish	3	0.01786 $\pm$ 0.00842	–	–
–	–	–	–	–
Total	63	0.04406 $\pm$ 0.00181	39	0.00384 $\pm$ 0.00034

**Table 3** Estimates of percentage divergence ( $Da \pm 95\%$  CI) between control region lineages (upper diagonal) and estimates of divergence times [ky BP ( $\pm 95\%$  CI)] (lower diagonal) using an evolutionary rate of 23% My<sup>-1</sup>

ky/ $Da \pm \text{SE}\%$	Northern	Western	Italian	Balkan	Spanish
Northern	–	6.06 $\pm$ 2.13%	5.09 $\pm$ 1.97%	5.53 $\pm$ 2.21%	5.45 $\pm$ 2.14%
Western	263 (171–356)	–	3.08 $\pm$ 1.45%	8.22 $\pm$ 2.73%	3.62 $\pm$ 1.74%
Italian	221 (136–307)	134 (71–197)	–	6.93 $\pm$ 2.58%	3.03 $\pm$ 1.73%
Balkan	240 (144–337)	357 (239–476)	301 (189–413)	–	6.49 $\pm$ 2.26%
Spanish	237 (144–330)	157 (82–233)	132 (57–207)	282 (184–380)	–

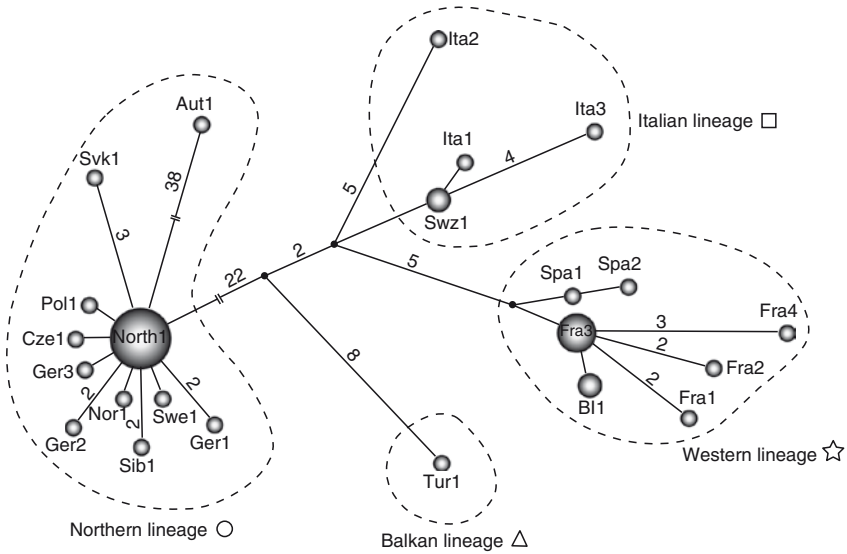
**Table 4** Estimates of SSD,  $F_s$ ,  $\tau$  and time since expansion (TSE) in y BP for each lineage

mtDNA lineage	SSD	$F_s$	$\tau$ (95% CI)	TSE (95% CI)
Northern	0.00375	–15.945*	4.088 (2.801–5.260)	72,844 (49,911–93,728)
Western	0.07546	0.680	4.578 (0.420–8.410)	87,300 (8,009–160,374)
Italian	0.00367	–2.661	3.363 (1.408–6.920)	64,698 (27,087–133,128)
Balkan	0.03880	0.083	3.234 (0.025–6.068)	54,499 (421–102,258)

\*Indicates significance ( $p < 0.05$ )

(including indels) were found in the concatenated Y chromosome dataset. Twenty-two (36.7%) polymorphic sites were found in DBY1; 8 (13.3%) in DBY3; 8 (13.3%) in DBY7, and 22 (36.7%) in UTY11. A 4 bp indel separated the Northern lineage from all other samples in DBY1 and the Northern lineage also possessed an extra repeat motif (CAAA) in the UTY11 intron.

Four major lineages were identified from the concatenated dataset by the median-joining network (Fig. 2). The Western Y lineage consisted of individuals from the Western and Spanish mtDNA lineages plus individuals from the Northern mtDNA lineage found in Northern France (Fig. 2). The Northern Y lineage included all individuals found in the Northern mtDNA lineage with the exception of those from Northern France and also included individuals from Austria and Slovakia, which belonged to the Balkan mtDNA lineage. The Italian Y lineage contained the same individuals as the Italian mtDNA lineage. Bootstrap support was high for



**Fig. 2** Median-joining network of 23 concatenated Y chromosomal intron haplotypes (2,939 bp). Numbers on branches indicate more than one mutation. Inferred haplotypes are represented by black dots. Bootstrap support for the Northern Y lineage is 100% and support for the Western Y lineage is 96% using both NJ and MP methods, respectively. See Table 1 for haplotype designations

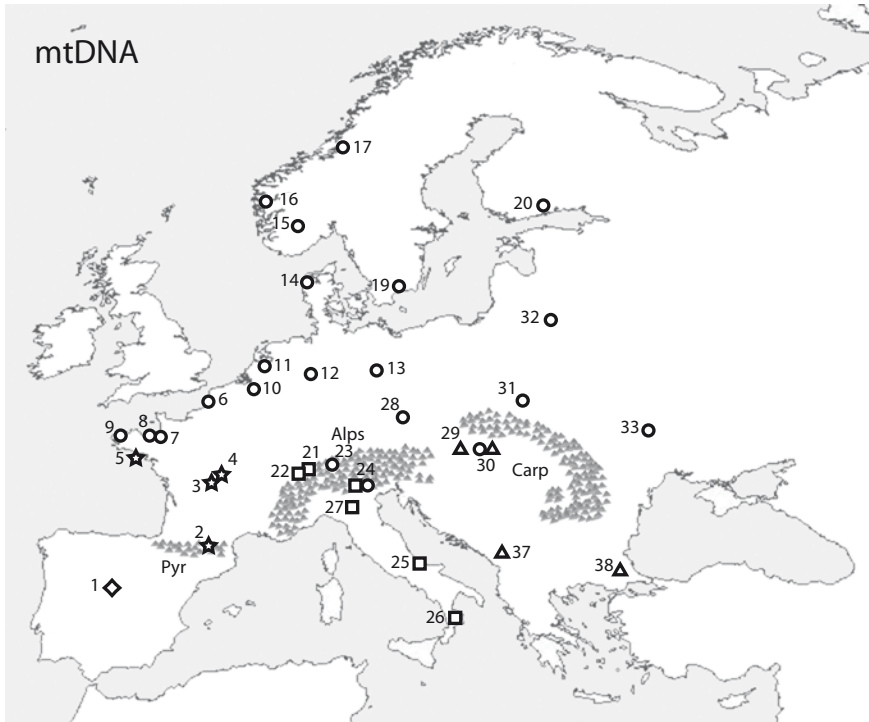
Western (NJ/MP: 96/96%) and Northern (NJ/MP: 100/100%) Y lineages, with weak support for the Italian Y lineage. Only one individual belonged to the Balkan Y lineage. These lineages were also identified from each Y intron individually, with the exception of DBY3 which failed to separate the Western and Italian lineages (data not shown). The nucleotide diversity ( $\pi$ ) of each lineage is given in Table 2.

Eleven haplotypes were found in 23 individuals in the Northern Y lineage (see Table 1 and Fig. 2), with Y haplotype North1 found in 10 localities spread from Norway to Siberia. The Y haplotype Aut1 had several large indels. Seven haplotypes were found in the Western Y lineage and four in the Italian Y lineage. However, the Y haplotype Ita2 was quite different from other Italian individuals (Fig. 2). This was also the case for this individual in the control region analysis (haplotype Ita7; Fig. 1).

## 4 Discussion

### 4.1 Postglacial Colonization History Inferred From mtDNA

The five lineages identified from control region data agrees with previously published data for the cytochrome *b* gene (Mascheretti et al. 2003). However, statistical support for all but two (Balkan and Spanish lineages) of these control region lineages were



**Fig. 3** Distribution of control region lineages of continental Europe (see Fig. 1 for symbol designation of lineages). Samples 34–36 belonging to the Northern mtDNA lineage are not shown. Clustered grey triangles represent the major mountain ranges; Pyr=Pyrenees and Carp=Carpathians

generally very low (<75%, Fig. 1), and this may be due to the large amount of variation and indels present in the *Sorex* control region. Although the Mediterranean peninsulas were important refugia for the pygmy shrew, they contributed relatively little to the spread of the pygmy shrew throughout Europe (Bilton et al. 1998).

From Fig. 3, the Spanish lineage appears to be confined to Spain which could indicate that the Pyrenees separated the glacial refugia for the Spanish lineage from that of the Western lineage in Southern France. The Andorran samples belong to the Western lineage as reported previously (Mascheretti et al. 2003) and could represent a Southern colonization after the LGM. Likewise, the Alps could have acted as a barrier between the Italian and other lineages at the LGM, with a subsequent spread of the Italian lineage into the mountains from an Italian refugium after the LGM.

With regards to the possible glacial refugium for the pygmy shrew Western lineage in Southern France, this is a new refugial location not previously suggested for the pygmy shrew. The common vole, *Microtus arvalis*, is proposed to have persisted North of the Pyrenees (Heckel et al. 2005) and fossil finds of the common shrew (*S. araneus*) or Millet's shrew (*S. coronatus*) indicate that it, along with other

temperate species, survived in the Dordogne in South-Western France during the LGM (Sommer and Nadachowski 2006). From such a refugium, the Western lineage could have easily spread further North to the small French island, Belle Île, which only became separated from the French mainland approximately 8,000 years ago (A. Tresset, pers. comm.). This accounts for the relatively high nucleotide diversity (data not shown) present on the island compared to large islands, such as Ireland (McDevitt et al. 2009) which was probably colonized by a small number of individuals introduced by humans (Mascheretti et al. 2003; McDevitt et al. 2009). The Balkan lineage is present as far North as Slovakia and Austria to which it presumably spread from a Balkan refugium. Five individuals from the same locality in Slovakia include individuals representing two separate lineages, the Northern and Balkan. This mtDNA contact zone is located near the Carpathians which is also likely to have been a glacial refugium during the LGM for the pygmy shrew (Pazonyi 2004) as well as other mammalian species (Kotlík et al. 2006; Saarma et al. 2007). We propose that it was the Northern lineage that occupied this refugium. However, further sampling with mtDNA is needed to provide more support for this and other refugial locations.

The most widespread mtDNA lineage of the pygmy shrew found in Europe is the Northern lineage which occurs from Northern France in the West to Siberia in the East to the Scandinavian countries in the North. Mismatch distribution and a negative and significant  $F_u$ 's  $F_s$  value indicate that this lineage originated from a single refugium and spread rapidly. The extensive distribution of this lineage may be due to a selective advantage of this mtDNA type in North temperate and arctic environments. Fontanillas et al. (2005) showed that certain mtDNA haplotypes in *C. russula* may be better adapted to colder environments than others, and this was particularly evident in females. A similar selective advantage has also been shown in humans (Mishmar et al. 2003) and been advocated in hares (Melo-Ferreira et al. 2005). Pygmy shrews inhabiting regions, such as the Carpathians during glacial cycles may have been better adapted to the temperature regimes found in North temperate and arctic regions than those present in refugia in the Mediterranean peninsulas and Southern France.

The approximate divergence times between the mtDNA lineages of the pygmy shrew indicate that separation may have occurred several glacial cycles ago (Renssen and Vandenberghe 2003). This is also the case with other small mammals in Europe (Jaarola and Searle 2004; Deffontaine et al. 2005). Larger, more mobile mammals show a lack of phylogeographical structuring pre-LGM (Hofreiter et al. 2004) but small mammals are more limited in their dispersal abilities and would, therefore, have remained isolated between glacial cycles, promoting divergence in mtDNA sequences. On the assumption of the molecular clock used, the Balkan and Northern lineages diverged from all other lineages approximately 240–357,000 and 221–263,000 y BP, respectively. The Western, Italian, and Spanish lineages diverged more recently (approximately 132–157,000 y BP).

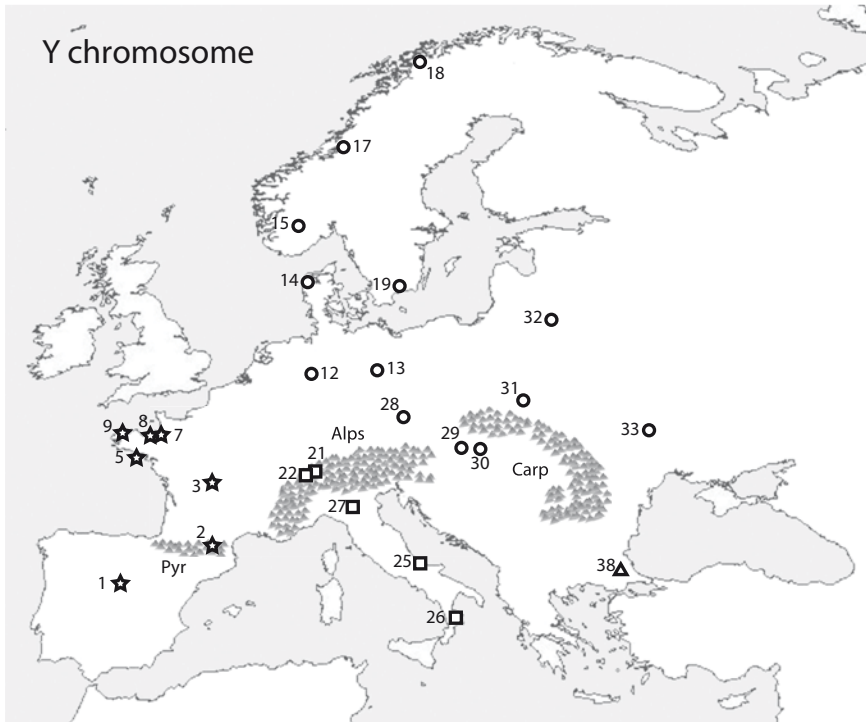
Estimates of time since population expansion vary among lineages. The Northern and Italian lineages expanded 72,844 and 64,498 y BP, respectively, with lower 95% CI values (49,911 and 27,087 y BP, respectively) before the LGM

(19–23,000 y BP). Estimates for the other two lineages were somewhat similar. The estimate for the Western lineage is 87,300 y BP with a lower 95% CI value (8,009 y BP) which stretches into the Holocene. This was affected by the large sample size from Belle Île as the estimate was lower when excluding individuals from the island (57,952 y BP; 95% CI: 11,575–108,009 y BP). The Balkan lineage has an estimate of 54,499 y BP, but with a large 95% CI range (421 years BP–102,258 y BP). These large 95% CI ranges (approximately 50–100,000 y) are obviously affected by the low sample size used for all lineages apart from the Northern lineage. It is also worth noting that only the Northern lineage had a negative and significant  $F_s$  value. The data here support the view that lineages might have expanded during or after the Early Pleniglacial (74–59,000 y BP; Renssen and Vandenberghe 2003) but for all except the Northern and Italian lineages a later date for the expansion, after the LGM, cannot be excluded.

However, the results presented here should be treated with caution as the evolutionary rate varies greatly among different regions of the *Sorex* control region (Stewart and Baker 1994a). A divergence rate of 15–20% My<sup>-1</sup> has been used previously for *S. araneus* (Andersson et al. 2005) based on an error in the abstract of the original paper (D. Stewart, *pers. comm.*). The true divergence rate quoted in the discussion section of Stewart and Baker (1994a) is 8.3–14.3% My<sup>-1</sup> for the unique sequence and repeat regions combined. The value used here (23% My<sup>-1</sup>) for the 5' end of the unique flanking sequence may in fact underestimate the divergence between lineages and their expansion times. Evolutionary rates as high as 60.4% My<sup>-1</sup> for the control region have been reported in *M. arvalis* (Heckel et al. 2005). The use of this value places the expansion of lineages as follows; Northern: 27,738 y BP; Western: 33,243 y BP (22,067 y BP without individuals from Belle Île); Italian: 24,636 y BP, and Balkan: 20,753 y BP with lower 95% CI values occurring at the end or after the LGM (160 y BP–19,006 y BP). This interpretation fits much better with the expectation of small refugial populations at the LGM and extensive expansion thereafter.

## 4.2 Postglacial Colonization History Inferred From Y Chromosomal Introns

Four major Y chromosome lineages were identified throughout Europe as opposed to five identified using mtDNA (Fig. 2). One of the major differences between female-mediated (mtDNA) and male-mediated (Y introns) lineages was the strong grouping of all French and Spanish shrews to form a Western Y lineage (Fig. 4). Pygmy shrews from Northern France belong to the Western Y lineage as opposed to the Northern lineage for mtDNA. The well supported Western Y lineage is possibly caused by the slower mutation rate in Y chromosome genes compared to faster evolving mitochondrial genes (Hellborg and Ellegren 2004). According to the mtDNA data, the Western, Spanish, and Italian mtDNA lineages diverged more recently from each other than the Northern and Balkan mtDNA lineages (Table 3),



**Fig. 4** Distribution of concatenated Y chromosomal intron lineages of continental Europe (see Fig. 2 for symbol designation of lineages). Samples 34–36 belonging to the Northern Y lineage are not shown. Clustered grey triangles represent the major mountain ranges; Pyr=Pyrenees and Carp=Carpathians

and the grouping of French and Spanish Y haplotypes in the same Y chromosome lineage may reflect a more ancient association. However, it could be expected that French, Spanish, and Italian individuals would group together in a Y lineage if this was the case, and although the Italian Y lineage is poorly supported, it is clearly distinct from the Western Y lineage (Fig. 2). Therefore, it is possible that there was gene flow between Spanish and French refugia more recently than the mtDNA data suggests, perhaps after they came into contact after the range expansion following the LGM.

The Y chromosome data supports the existence of a separate glacial refugium in Italy for the pygmy shrew, as already suggested by the mtDNA data. Interestingly, the individual from Southern Italy (Y haplotype Ita2, mtDNA haplotype Ita7; Figs. 3 and 4) did not group with the other Italian haplotypes for both Y chromosome introns and mtDNA. There may have been multiple refugia within Italy during the Pleistocene glaciations, causing isolation of certain populations from others. Further samples are needed to fully resolve this. Genetic differentiation has been



reported within the Italian peninsula for *Sorex antinorii* because of putative post-glacial recolonization routes (Basset et al. 2006).

The Northern Y lineage is typified by low nucleotide diversity, in comparison to the other lineages (with the exception of the Balkan Y lineage where only one individual was sequenced). Only 11 haplotypes were found in 23 individuals (Fig. 3), with the North1 Y haplotype found in 13 individuals from 10 locations ranging from Norway in the Northwest to Siberia in the East. This supports the contention that expansion of the mtDNA Northern lineage over much of Northern Eurasia occurred from a single refugium. Individuals from the Balkan mtDNA lineage in Slovakia and Austria belonged to the Northern Y lineage. As is the case for Northern France, this indicates differential introgression of mtDNA and Y chromosome markers at a local level when populations from different glacial refugia come into contact.

## 5 Conclusions

Analyses of multiple molecular markers with different means of inheritance has demonstrated contrasting patterns of recolonization for the pygmy shrew in continental Europe. Five major lineages were retrieved from mtDNA data, in accordance with Mascheretti et al. (2003). In contrast, four major lineages were retrieved from Y chromosome data. By using mtDNA alone, contact between French and Spanish refugia would not have been identified and this study has highlighted the importance of adopting a multiple marker approach in phylogeographic studies.

The pygmy shrew is the oldest shrew found in Europe (based on fossil data; Rzebik-Kowalska 1998) and is relatively rare on the European mainland (often restricted to high altitudes) in comparison to other shrew species because of competition with species which occupy similar niches and as a result of habitat fragmentation. The genetic lineages described in this study diverged several glacial cycles ago and therefore each has an ancient evolutionary history (Jaarola and Searle 2004). Some of these lineages are relatively rare on the European continent in comparison to others. For example, the Northern lineage is spread throughout Northern and Eastern parts of Europe while the other lineages are relatively restricted in their distribution, often confined to the Southern refugial regions. Fully identifying refugial locations and protecting reservoirs of genetic diversity is vital for the long-term persistence and conservation of pygmy shrew populations (Bhagwat and Willis 2008).

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