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Additional data for nuclear DNA give new insights into the phylogenetic position of *Sorex granarius* within the *Sorex araneus* group

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ABSTRACT

Many species contain genetic lineages that are phylogenetically intermixed with those of other species. In the *Sorex araneus* group, previous results based on mtDNA and Y chromosome sequence data showed an incongruent position of *Sorex granarius* within this group. In this study, we explored the relationship between species within the *S. araneus* group, aiming to resolve the particular position of *S. granarius*. In this context, we sequenced a total of 2447 base pairs (bp) of X-linked and nuclear genes from 47 individuals of the *S. araneus* group. The same taxa were also analyzed within a Bayesian framework with nine autosomal microsatellites. These analyses revealed that all markers apart from mtDNA showed similar patterns, suggesting that the problematic position of *S. granarius* is best explained by an incongruent behavior by mtDNA. Given their close phylogenetic relationship and their close geographic distribution, the most likely explanation for this pattern is past mtDNA introgression from *S. araneus* race Carlit to *S. granarius*.

1. Introduction

The climatic oscillations that characterized the Pleistocene imposed important range shifts on Palaearctic biota and helped shape their demographic history and genetic diversity (Avise et al., 1998). Climatic cooling forced temperate species to retract into fragmented Southern refugia within the northern hemisphere, creating high levels of diversity and endemism in these areas (Bilton et al., 1998; Hewitt, 1996). In Europe, the Balkans, as well as the Italian and Iberian Peninsulas represent the major ice age refugia (Taberlet et al., 1998). For many species, the warming of the climate could have represented periods of range expansion while the cooler stage may be a time of range contraction (see Hewitt, 2001). Consequently, some regions must have been occupied by an alternation of species as the climate oscillated. This probably set the conditions for dynamic overlaps of species' ranges, interspecific competition, and potentially hybridization.

The phylogenetic relationships and phylogeographic patterns in shrew species of the *Sorex araneus* group in southwestern Europe represent an illustrative example of the interplay between closely related species potentially influenced by climatic oscillations. Among the four species occurring in this region (Fig. 1), *S. granarius* is restricted to the northwest of the Iberian Peninsula, whereas *S. antinorii* is present on the Italian Peninsula as well as in the French and Swiss Alps. By contrast, *S. araneus* and *S. coronatus* have much larger distributions that cover most of western and central Europe. Although the distribution of these species occasionally overlaps, few cases of natural hybridization have been reported. A notable exception is the introgressive hybridization observed between the common shrew *S. araneus* and the Valais shrew *S. antinorii* in two hybrid zones located in the Swiss and French Alps (Basset et al., 2006a; Yannic et al., 2008a, 2009). Interestingly, these studies have determined that the level of introgression may vary according to the portion of the genome investigated. This suggests that some parts of the genome remain porous long after speciation is completed and highlights the risks of misinterpretations of single gene genealogies (Chan and Levin, 2005; Mallet, 2005). The challenge in molecular taxonomy is to distinguish species that have low levels of interspecific genetic divergence, either because speciation is recent or because the species continue to exchange genes (Petit and Excoffier, 2009).

Therefore, investigation of genomic regions with different inheritance patterns, coalescence time and mutation rates is warranted in order to obtain an accurate picture of the species' evolutionary history (e.g., Leaché, 2010) (but see the recent debate on the use of mtDNA versus nuclear DNA in avian phylogeography; Barrowclough and Zink, 2009; Edwards and Bensch, 2009; Zink and Barrowclough, 2008).

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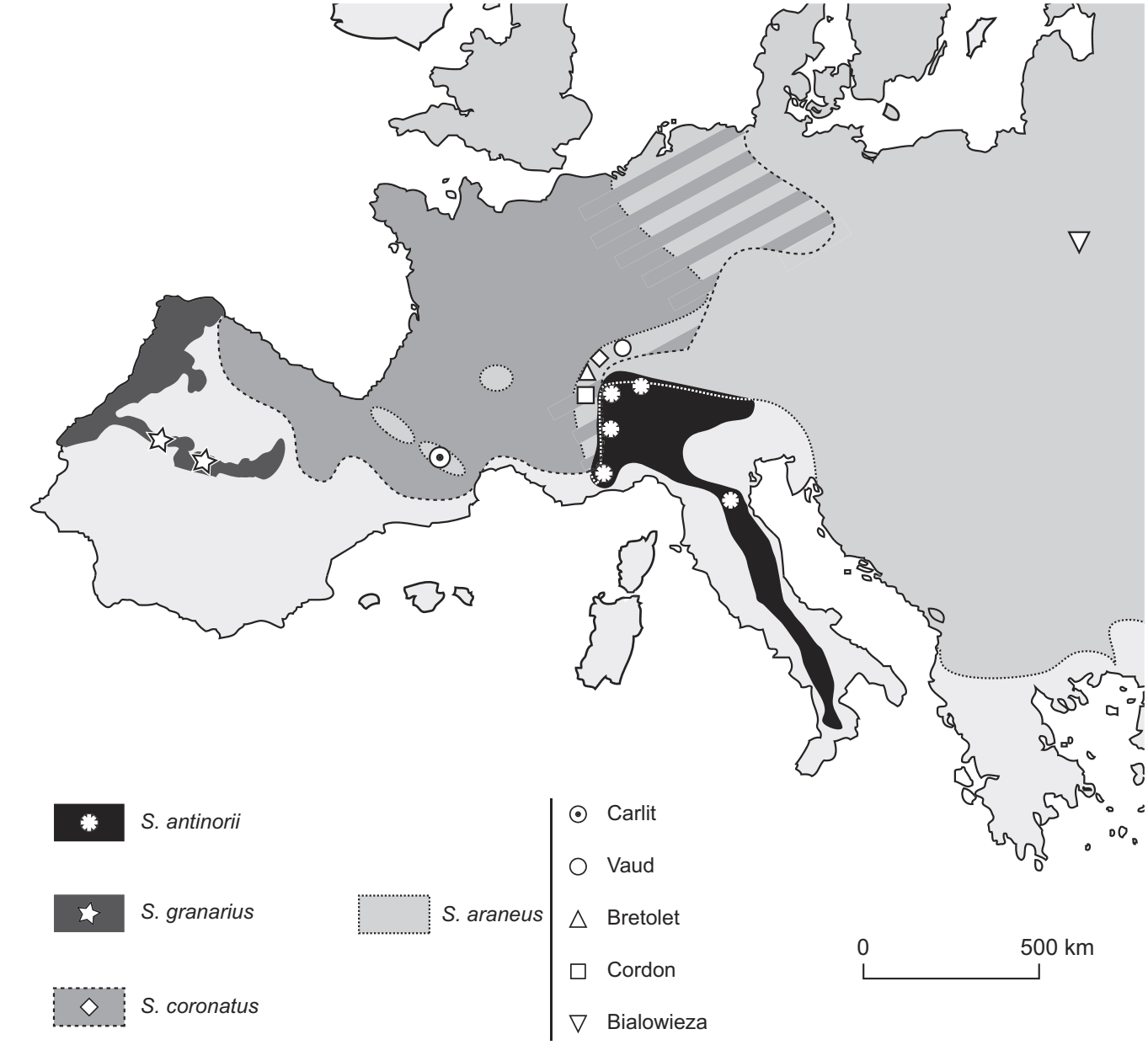

Fig. 1. Approximate locations of samples and current known distribution of the different species and races of the *S. araneus* group used in this study, according to Taberlet et al. (1994) and Mitchell-Jones et al. (1999). The zones of overlap between *S. araneus* and *S. coronatus* (dashed) are depicted only schematically.

The shrews of the *S. araneus* group are morphologically very similar but considerable chromosomal variation has been observed between and even within species (Yannic et al., 2008b). Several researchers, studying karyotype (for a review, see Zima et al., 1998), proteins (Catzeflis et al., 1982; Ruedi, 1998), mtDNA (Taberlet et al., 1994; Yannic et al., 2008b), and Y chromosome DNA (Yannic et al., 2008b) have proposed scenarios to explain the radiation of the *S. araneus* group in southwestern Europe (see Fig. 2). Despite general consensus, these studies disagree about the position of *S. granarius* (Fig. 2). *S. granarius* was generally considered to retain the ancestral karyotype of the group, thus representing an early divergence of the European lineage (Volobouev and Catzeflis, 1989; Volobouev and Dutrillaux, 1991; Wójcik and Searle, 1988). However, the strong association of *S. granarius* with several chromosomal races of *S. araneus* inferred from some markers (mtDNA, allozymes) and with *S. coronatus* from others (Y-chromosome) (Fig. 2) highlights that the forces shaping the phylogenies of these shrews still need to be clarified.

As previously shown (Yannic et al., 2008b), in some situations, no single-locus topology (gene tree) will reconstruct the real relationships of species (species tree) with complete accuracy. By definition, closely related species have recent evolutionary divergences and consequently low degrees of differentiation, making them particularly susceptible to introgression (when sympatric or parapatric) or differential lineage sorting (Tosi et al., 2003). The pervasive web of genetic connections resulting from these processes is becoming better appreciated as more and more comprehensive molecular data sets are analyzed (Arnold, 2006). Adding differently inherited kinds of markers with different mutation rates will bring new information and lead to a more accurate picture of the species relationships in the *S. araneus* group.

In this study, we used two autosomal nuclear genes, nine autosomal nuclear microsatellites and three X-linked markers to resolve this problem and compare our results to a previous study based on mtDNA and Y chromosome sequence data (Yannic et al., 2008b). Our aims were to: (i) determine the precise

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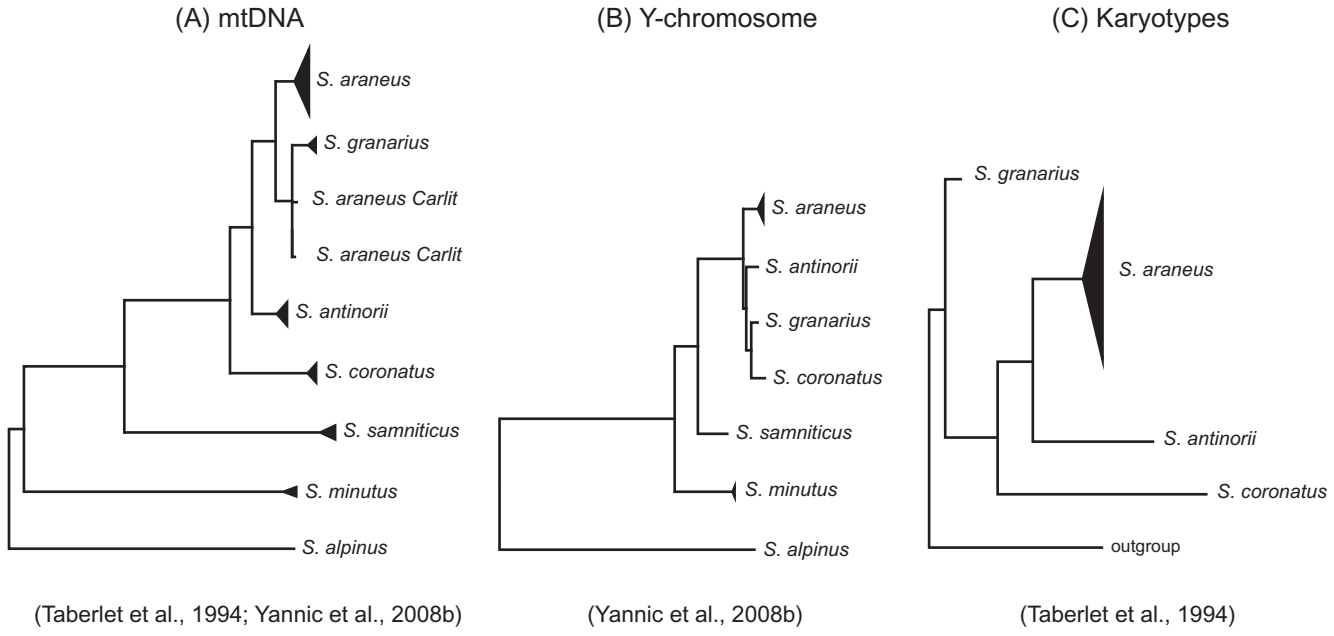

Fig. 2. Previous molecular studies of west-eastern *S. araneus* group species evolution. (A) Mitochondrial DNA tree of Taberlet et al. (1994) and Yannic et al. (2008b). (B) Y-chromosomal DNA tree of Yannic et al. (2008b). (C) Karyotype relationships reconstructed by Taberlet et al. (1994). The different data sets disagreed on a main point: the position of *S. granarius* relative to other taxa of the *S. araneus* group. In the mtDNA tree, *S. granarius* is closely related to the *S. araneus* races. In the Y-chromosome tree, *S. granarius* is linked to *S. coronatus*. Based on karyotypic data, the fully acrocentric karyotype of *S. granarius* is in a basal position and is most reasonably considered as the common ancestor of all species within the *S. araneus* group (see also White et al., 2010).

phylogenetic position of *S. granarius* within the *S. araneus* group and (ii) assess whether this position results from incomplete lineage sorting or introgressive hybridization.

2. Material and methods

2.1. Sampling, DNA extraction and locus information

A total of 47 individuals from the *S. araneus* group subdivided into four species (*S. antinorii*, *S. araneus* sensu stricto, *S. coronatus* and *S. granarius*) and five chromosomal races of *S. araneus* s.s. (Cordon, Bretolet, Carlit, Vaud and Bialowieza) were analyzed (Table 1; Fig. 1). All these races (expect Bialowieza) belong to the Western European Karyotypic Group (WEKG), characterized by metacentrics *gm, hi* (Searle, 1984). Species and chromosome race identification of individuals followed karyotype analysis but in a few unambiguous cases, it was deduced from sampling localities. Individuals of a sister species of this group, *Sorex samniticus*, were also analyzed as well as individuals of *S. minutus* and *S. alpinus*, which are more distantly related (Fumagalli et al., 1999; Taberlet et al., 1994; Yannic et al., 2008b). All individuals were collected between 1977 and 2002 from localities in various parts of the distribution of each species (Table 1). Tissue samples (liver or toe clips) were stored at −80 °C or in absolute ethanol and total DNA was extracted using the DNeasy Extraction Kit (QIAgen). Tissues preserved in ethanol were washed with sterile distilled water before extraction.

2.2. Gene amplification

Amplification of the Breast cancer susceptibility 1 (BRCA1) and Apolipoprotein B (ApoB) nuclear genes (exons) was performed using the primer pairs B1f/B1r (Dubey et al., 2006) and ApoBf/ApoBr (Dubey et al., 2007). Amplification of the Amelogenine (AMELX intron) and Zinc Finger Protein (ZFX part 1 intron and ZFX part 2 intron 6 and partial cds) X-chromosome-linked genes were performed with primer pairs AMELXf (5′-TTTAAGGTGCTTACTCCTCTGAAG-3′)/AMELXr (5′-GGAACATCGGAGGCAGAG-3′), ZFX-1f (5′-AGAGAAAGAAAGGAGCATGA-3′)/FX-1r (5′-GTTAAAGATGGGGCTGGA-3′) and ZFX-2f (5′-TCAGTGACAATCTCTGCTTC-3′)/ZFX-2r (5′-CAGTTTCCTGGTAGAAAGTCA-3′), respectively, specifically developed for this study.

PCRs generally contained 50–100 ng DNA, 0.2 μM of each primer, 0.2 mM dNTPs, 1× PCR buffer, 1.5 mM $MgCl_2$, and 1.25 units *Taq* polymerase (QIAgen) in a total volume of 25 μl. PCRs were performed in a PE9700 (Applied Biosystems) thermal cycler with cycling conditions as follows: initial denaturation at 95 °C for 5 min, followed by 35–40 cycles of 95 °C for 45 s, annealing (T_n, PCRs were performed in a PE9700 (Applied Biosystems) thermal cycler with cycling conditions as follows: initial denaturation at 95 °C for 5 min, followed by 35–40 cycles of 95 °C for 45 s, annealing T_n (BRCA1: 52 °C; ApoB: 50 °C; AMELX: 55 °C; ZFX-1: 60 °C; ZFX-2: 55 °C) for 60 and 90 s extension at 72 °C, and a final extension of 72 °C PCR products were electrophoresed on a 1% agarose gel and visualized with ethidium bromide staining to verify PCR quality.) for 60 s and 90 s extension at 72 °C, and a final extension of 72 °C PCR products were electrophoresed on a 1% agarose gel and visualized with ethidium bromide staining to verify PCR quality. Products were then purified by centrifugal dialysis using the QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's instructions. Cycle sequencing was performed in 10 μl total volume containing 1–3 μl of amplified DNA, 1 μl of 10 μM primer, 2 μl of ABI PRISM BigDye Terminator vs 3.1 (Applied Biosystems). Sequence reactions were visualized on an ABI 3100 genetic analyzer (Applied Biosystems).

When direct sequencing of purified PCR amplicons of autosomal and X-linked loci revealed more than one heterozygous site within a sequence, we resolved haplotypes probabilistically using phase vs 2.1 (Stephens and Donnelly, 2003; Stephens et al., 2001).

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Table 1
Species and specimens used in the present study; specimen identification code for each individual (ID), geographic origin of the samples, alleles of analyzed samples for the different genes and samples used for microsatellites analysis. Asterisks (∗) refer to samples already present in the data set developed by Basset et al. (2006b) (<http://www.unil.ch/dee/page6759_fr.html>).

N	ID Code[a]	Species	Race	Locality	Country	Coordinates		BRCA1		ApoB		AMELX	ZFX 1	HapX	ZFX 2	microsat
1	IZEA 632	*S. granarius*		Rascafria	Spain	40°54'13.73"N	3°52'45.83"W	A4		B		AMELX4	ZFX1.2	HX1	ZFX2.1	new
2	IZEA 634	*S. granarius*		Rascafria	Spain	40°54'13.73"N	3°52'45.83"W	A4		B		AMELX4	ZFX1.1	HX2	ZFX2.1	new
3	IZEA 635	*S. granarius*		Rascafria	Spain	40°54'13.73"N	3°52'45.83"W	A4	A3	B		AMELX4	ZFX1.1	HX2	ZFX2.1	new
4	IZEA 636	*S. granarius*		Candelario	Spain	40°22'4.96"N	5°44'40.23"W	A1	A2	B	G	AMELX4	ZFX1.1	HX2	ZFX2.1	new
5	IZEA 637	*S. granarius*		Piedrahita	Spain	40°27'48.23"N	5°19'41.50"W	A4		B		AMELX4	ZFX1.1	HX2	ZFX2.1	new
6	IZEA 639	*S. granarius*		Rascafria	Spain	40°54'13.73"N	3°52'45.83"W	A4	A3	B		AMELX4	ZFX1.1	HX2	ZFX2.1	new
7	IZEA 683	*S. granarius*		Candelario	Spain	40°22'4.96"N	5°44'40.23"W	A4		B		AMELX4	ZFX1.1	HX2	ZFX2.1	new
8	IZEA 5940	*S. granarius*		Rascafria	Spain	40°54'13.73"N	3°52'45.83"W	A4		G	G	AMELX4	ZFX1.1	HX2	ZFX2.1	new
9	IZEA 6151	*S. granarius*		Piedrahita	Spain	40°27'48.23"N	5°19'41.50"W	A4		A	B	AMELX4	ZFX1.1	HX2	ZFX2.1	new
10	IZEA 4356	*S. araneus*	Carlit	Les Bouillouses	France	42°39'42.63"N	1°29'17.44"E	A6		A		AMELX1	ZFX1.4	HX4	ZFX2.1	new
11	IZEA 4359	*S. araneus*	Carlit	Les Bouillouses	France	42°39'5.72"N	1°4'48.55"E	A9		A		AMELX1	ZFX1.3	HX3	ZFX2.1	new
12	IZEA 5309	*S. araneus*	Carlit	Sorpe Levide	Spain	43°6'58.33"N	1°41'21.79"E	A5		C		AMELX1	ZFX1.4	HX4	ZFX2.1	new
13	IZEA 4354	*S. araneus*	Carlit	Etang de Balcère	France	42°39'42.63"N	1°29'17.44"E	A9		C	A	AMELX1	ZFX1.4	HX4	ZFX2.1	new
14	IZEA 4357	*S. araneus*	Carlit	Les Bouillouses	France	42°39'42.63"N	1°29'17.44"E	A9		C		AMELX1	ZFX1.4	HX4	ZFX2.1	new
15	IZEA 4358	*S. araneus*	Carlit	Les Bouillouses	France	42°39'5.72"N	1°4'48.55"E	A5		L		AMELX1	ZFX1.3	HX3	ZFX2.1	new
16	IZEA 4355	*S. araneus*	Carlit	Etang de Balcère	France	42°39'42.63"N	1°29'17.44"E	A9		Z		AMELX1	ZFX1.4	HX4	ZFX2.1	new
17	IZEA 5310	*S. araneus*	Carlit	Sorpe Levide	Spain	43°6'58.33"N	1°41'21.79"E	A5		A		AMELX1	ZFX1.4	HX4	ZFX2.1	new
18	IZEA 2980	*S. araneus*	Vaud	Champ-Pittet	Switzerland	46°46'54.48"N	6°39'42.78"E	A7		A		AMELX6	ZFX1.4	HX6	ZFX2.1	*
19	IZEA 2981	*S. araneus*	Vaud	Champ-Pittet	Switzerland	46°46'54.48"N	6°39'42.78"E	A9		A		AMELX6	ZFX1.4	HX6	ZFX2.1	*
20	IZEA 2683	*S. araneus*	Bretolet	Morzine	France	46°3'34.26"N	6°39'47.63"E	A7	A9	D		AMELX6	ZFX1.3	HX5	ZFX2.1	*
21	DEE C4.3	*S. araneus*	Cordon	Cordon	France	45°55'21.23"N	6°36'41.64"E	A8		M	A	AMELX6	ZFX1.3	HX5	ZFX2.1	*
22	DEE C3.1	*S. araneus*	Cordon	Cordon	France	45°55'21.23"N	6°36'41.64"E	A7		J		AMELX6	ZFX1.3	HX5	ZFX2.1	*
23	B11-B 95.17	*S. araneus*	Bialowieza	Jurowce	Poland	53°11'47.81"N	23°9'10.32"E	A9		J		AMELX6	ZFX1.4	HX6	ZFX2.1	/
24	B4-B 93.399	*S. araneus*	Bialowieza	Jurowce	Poland	53°11'47.81"N	23°9'10.32"E	A9		B		AMELX6	ZFX1.4	HX6	ZFX2.1	/
25	IZEA 5325	*S. antinorii*		Gran Sasso	Italy	42°30'32.47"N	13°30'45.99"E	A12		B		AMELX3	ZFX1.8	HX7	ZFX2.1	*
26	DEE P2	*S. antinorii*		Pralognan-La-Vanoise	France	45°22'52.02"N	6°43'17.96"E	A12	A9	B		AMELX3	ZFX1.5	HX8	ZFX2.1	/
27	FP 14	*S. antinorii*		Tournoux - St Paul	France	44°29'17.35 " N	6°44'22.05" E	A12		B	H	AMELX3	ZFX1.5	HX8	ZFX2.1	/
28	DEE Si-92.15	*S. antinorii*		Undre-Rothwald, Ried-Brig	Switzerland	46°16'30.70" N	8°02'20.52" E	A12		B		AMELX3	ZFX1.5	HX8	ZFX2.1	*
29	IZEA 5326	*S. antinorii*		Gran Sasso	Italy	42°30'32.47" N	13°30'45.99" E	A12		B		AMELX3	ZFX1.8	HX7	ZFX2.1	*
30	IZEA 5664	*S. antinorii*		Serramazzoni	Italy	44°25'34.00" N	10°47'15.00" E	A12		H		AMELX3	ZFX1.5	HX8	ZFX2.1	*
31	IZEA 7515	*S. antinorii*		San Nicolo Piacenza	Italy	45°3'21.97"N	9°36'28.86"E	A12		H	B	AMELX3	ZFX1.5	HX8	ZFX2.1	*
32	DEE V2	*S. antinorii*		Les Allues	France	45°21'20.40"N	6°35'39.10"E	A12	A9	H		AMELX3	ZFX1.5	HX8	ZFX2.1	/
33	FP 16	*S. antinorii*		Tournoux - St Paul	France	44°29'17.35 " N	6°44'22.05" E	A12		H		AMELX3	ZFX1.5	HX8	ZFX2.1	/
34	IZEA 5315	*S. antinorii*		Fivizzano Toscane	Italy	44°14'21.80" N	10°07'37.33" E	A12		I		AMELX3	ZFX1.5	HX8	ZFX2.1	*
35	DEE Sb-82.15	*S. antinorii*		Saint-Rhemy-En-Bosses	Italy	45°50'09.64" N	7°11'01.22" E	A12	A9	/		AMELX3	ZFX1.5	HX8	ZFX2.1	*
36	IZEA 5319	*S. antinorii*		Passo del Cerreto, Toscane	Italy	44°14'21.80" N	10°07'37.33" E	A12		B		AMELX3	ZFX1.5	HX8	ZFX2.1	*
37	IZEA 3376	*S. coronatus*		Jorat	Switzerland	46°36'23.53"N	6°44'32.06"E	A10	A12	B		AMELX4	ZFX1.5	HX10	ZFX2.1	*
38	IZEA 5391	*S. coronatus*		Bassin	Switzerland	46°27'48.18"N	6°14'0.62"E	A11		B		AMELX4	ZFX1.5	HX10	ZFX2.1	*
39	IZEA 2984	*S. coronatus*		Champ-Pittet	Switzerland	46°46'54.48"N	6°39'42.78"E	A12		B		AMELX4	ZFX1.5	HX10	ZFX2.1	*
40	IZEA 3018	*S. coronatus*		Champ-Pittet	Switzerland	46°46'54.48"N	6°39'42.78"E	A12		B		AMELX4	ZFX1.6	HX9	ZFX2.1	*
41	IZEA 3223	*S. coronatus*		Jorat	Switzerland	46°36'23.53"N	6°44'32.06"E	A12	A13	B		AMELX4	ZFX1.5	HX10	ZFX2.1	*
42	IZEA 3242	*S. coronatus*		Jorat	Switzerland	46°39'23.64"N	6°38'31.55"E	A10		B		AMELX4	ZFX1.5	HX10	ZFX2.1	*
43	IZEA 3245	*S. coronatus*		Le Mont-sur-Lausanne	Switzerland	46°33'35.16"N	6°38'8.99"E	A12	A13	F		AMELX4	ZFX1.5	HX10	ZFX2.1	*
44	IZEA 2341	*S. coronatus*		Verolliey	Switzerland	46°12'19.81"N	7°0'8.44"E	A12	A10	F		AMELX4	ZFX1.5	HX10	ZFX2.1	*
45	IZEA 2342	*S. coronatus*		Verolliey	Switzerland	46°12'19.81"N	7°0'8.44"E	A10		F		AMELX4	ZFX1.5	HX10	ZFX2.1	*
46	IZEA 2982	*S. coronatus*		Champ-Pittet	Switzerland	46°46'54.48"N	6°39'42.78"E	A12		U		AMELX4	ZFX1.5	HX10	ZFX2.1	*
47	IZEA 5703	*S. coronatus*		Roscoff	France	48°43'36.44"N	3°59'10.51"W	A10		N		AMELX4	ZFX1.5	HX10	ZFX2.1	*
48	IZEA 4739	*S. samniticus*		Fivizzano	Italy	44°14'21.80"N	10°07'37.33"E	A14		N		/	ZFX1.9	/	ZFX2.2	/
49	IZEA 5332	*S. samniticus*		Pescasseroli	Italy	41°48'29.69"N	13°47'22.49"E	A14		E		/	ZFX1.9	/	ZFX2.2	/
50	IZEA 5943	*S. minutus*		Bretolet	Switzerland	46°10'34.06"N	6°52'2.74"E	A16		O		AMELX5	ZFX1.7	HX11	ZFX2.3	/
51	IZEA 4746	*S. minutus*		Val d'Illiez	Switzerland	46°12'16.28"N	6°53'33.60"E	A16		O		AMELX5	ZFX1.7	HX11	ZFX2.3	/
52	IZEA 5337	*S. minutus*		Opi	Italy	41°47'3.84"N	13°49'46.58"E	A15		P		/	ZFX1.7		ZFX2.3	/
53	IZEA 4709	*S. alpinus*		Mase	Switzerland	46°11'38.39"N	7°26'3.77"E	A17		/		AMELX2	ZFX1.10	HX12	ZFX2.4	/
54	IZEA 3423	*S. alpinus*		Haslital	Switzerland	46°42'3.51"N	8°13'53.93"E	A17				/	/	/	/	/

[a] ID codes: « IZEA » refers to samples deposited in the Museum of Zoology collection in Lausanne, whereas those designed « DEE » are maintained in the Department of Ecology and Evolution, Lausanne. Other samples were kindly provided by Agatha Banaszek, Mirek Ratkiewicz and Françoise Poitevin (see Acknowledgments).

2.3. Phylogenetic analyses

The sequences were aligned using the multiple alignment algorithm implemented in clustalX (Thompson et al., 1997) and further checked by eye. Three methods of phylogenetic analyses were carried out for the different genes. Maximum parsimony analyses on the complete data set were performed using paup*4.0b10 (Swofford, 1998) with 10,000 random addition sequence followed by TBR branch swapping, keeping at most 100 trees at each replicate. Support values were estimated using 1000 bootstrap replicates using the same heuristic settings. For maximum likelihood (ML) and Bayesian analyses (BA), the models of DNA substitution were selected for each DNA region using modeltest v 3.1 (Posada and Crandall, 1998). For BRCA1, the HKY model was selected with base frequencies ($A = 0.2996$, $C = 0.2348$, $G = 0.1484$, $T = 0.3172$) estimated from the data, an unequal distribution of rates at variable sites ($\alpha = 0$), and different transition/transversion ratios for purines (8.761) and pyrimdines (3.184). For ApoB, the TrN model was selected with a proportion of invariable sites ($\gamma = 0$), and base frequencies (0.3828, 0.1724, 0.2070, and 0.2092) and three different substitution types (rate [A–C] = [A–T] = [A–T] = [C–G] = 1.000, rate [A–G] = 9.9203, rate [C–T] = 3.5933) estimated from the data. For AmelX, the F81+I+G model was selected with a proportion of invariable sites ($\gamma = 0.9794$), and base frequencies (0.2730, 0.3588, 0.1677, and 0.2004) estimated from the data set and an unequal distribution of rates at variable sites ($\alpha = 0.2193$). For ZFX-1, the F81 model was selected with base frequencies (0.3240, 0.2192, 0.1748, and 0.2820) estimated from the data set. Because of the lack of polymorphism, ZFX-2 was excluded from the phylogenetic reconstruction (see Section 3 section). Partitioned ML heuristic searches and bootstrap analyses (1000 replicates) were performed with the online program phyml v 3.0 (Guindon and Gascuel, 2003), using the inferred nucleotide substitution models as indicated above. Bayesian analyses were performed with partition-specific models as indicated above, using MrBayes v 3.0 b4 (Huelsenbeck and Ronquist, 2001). Two independent runs were performed, each consisting of four parallel MCMC chains of 3 million generations. Trees were sampled every 1000 generations. We selected an appropriate burn-in based on examination of the trends and distributions of log-likelihoods and parameter values using TRACER 1.4 (Rambaut and Drummond, 2007). To assess convergence among MCMC runs, we also examined the correlations of split frequencies among runs in the program AWTY (Nylander et al., 2008). Samples showed patterns consistent with stationarity and convergence after 600,000 generations for all runs and all data sets; hence the first 20% of samples were discarded as burn-in for all analyses. The remaining trees were used to construct a 50% majority rule consensus tree.

2.4. Microsatellite amplification and population genetic analyses

S. granarius ($n = 9$) and *S. araneus* race Carlit ($n = 8$) samples (Table 1) were genotyped at nine microsatellite loci (B10, B15, B3, B5, C5, L13, L67, L9 and L99) as described in Basset et al. (2006b) and added to the dataset developed by the same authors (available at http://www.unil.ch/dee/page7010.html#5). The dataset contains three distinct species of the *S. araneus* group: *S. araneus* (races Vaud, $n = 41$; Bretolet, $n = 25$; Cordon, $n = 30$), *S. antinorii* ($n = 83$) and *S. coronatus* ($n = 33$). We explored the genetic relationship among taxa using Bayesian analyses implemented in structure 2.2 (Falush et al., 2007; Pritchard et al., 2000). This model is particularly suitable when studying a potentially hybridizing group of species as it considers that an individual could originate from more than one population. It was designed to identify the K genetic clusters (or populations/species) of origin of individuals, and simultaneously probabilistically assign individuals to one cluster or several clusters if they are genetically admixed as a result of hybridization. structure was run with the 'admixture model', and ten repetitions of 100,000 iterations following a burn-in period of 20,000 iterations. Individuals with a proportion of membership to each cluster $q_{ind} < 0.90$ (admixed individual) were assigned to more than one cluster whereas individuals with $q_{ind} \geqslant 0.90$ were assigned to only one cluster. A neighbor-joining tree based on Cavalli-Sforza genetic distance (Cavalli-Sforza and Edwards, 1967) was computed with the program populations (Langella, 1999).

3. Results

Forty-seven individuals representing eight shrew taxa (four species and five races) of the *S. araneus* group and the three outgroup species were sequenced for 1322 bp from two unlinked nuclear genes (790 bp of BRAC1 and 532 bp of ApoB) and 1125 bp from three X-linked genes (535 bp of AMELX intron, 210 bp of ZFX-1 and 380 bp of ZFX-2). GenBank Accession numbers are as follows: GU473723-GU473787 (BRCA1), GU473788-GU473846 (ApoB), GU473953-GU473996 (AMELX), GU473847-GU473899 (ZFX-1) and GU473900-GU473952 (ZFX-2). Sequence alignments are available as supplementary online material (Supplementary files S1 to S5). The haplotypic diversity was particularly low for X-linked genes in the *S. araneus* group since only 17 sites were polymorphic (5 of which were parsimony-informative) among the 1125 base pairs sequenced. No variation was found among taxa for ZFX-2 within the *S. araneus* group. This gene was therefore excluded from further analyses. Similar results were obtained for the two autosomal genes: 15 sites were polymorphic (6 parsimony-informative) among the 532 bp analyzed in ApoB and 10 sites were polymorphic (5 parsimony-informative) among the 709 bp of BRAC1. The name and number of different alleles found for each locus is detailed in Table 1. Within the *S. araneus* group, 13 different alleles were found for BRCA1, 13 for ApoB, 4 for AMELX, 7 for ZFX-1 and only 1 for ZFX-2. The three phylogenetic methods gave identical arrangements of the main branches. Therefore, the relationship between alleles is given only for the ML analysis (Fig. 3 and 4). The topology of terminal branches within the *S. araneus* group was poorly supported for both the autosomal and the X-linked genes. Consequently, we obtained polytomous trees in all three phylogenetic reconstruction methods.

The analysis of autosomal genes (ApoB and BRCA1) showed that numerous alleles are shared among the different species within the *S. araneus* group (Fig. 3 and 4). For instance, allele 9 of BRCA1 is common to *S. antinorii* and some *S. araneus s.s.* races (Carlit, Bretolet and Bialowieza); allele B of ApoB is shared between *S. granarius* and *S. antinorii* (see Fig. 3 and 4). *S. granarius* individuals form a monophyletic group for BRCA1, but with poor support (66% bootstrap, 65% bootstrap and 0.85 posterior probabilities for ML, MP and BA analyses, respectively). *S. araneus* races form a monophyletic group for ApoB, but also with weak support (52%, 60% and 0.81).

Analyses of X-linked genes yielded similar results: *S. araneus* races form a monophyletic group with weak support (57%, 62% and 1.0). This probably results from the few numbers of parsimony-informative sites among species within this group. The relationships are concordant among the three analyses for the three outgroups (*S. samniticus*, *S. minutus* and *S. alpinus*).

3.1. Microsatellites

Bayesian analyses using the software structure indicate that the data set most likely included three distinct groups ($K = 3$; inferred with the 'Log probability of data' (Pritchard et al., 2000) and the statistic ΔK (Evanno et al., 2005). The average proportions of membership (q_{group}) over 10 runs showed that *S. granarius* individuals

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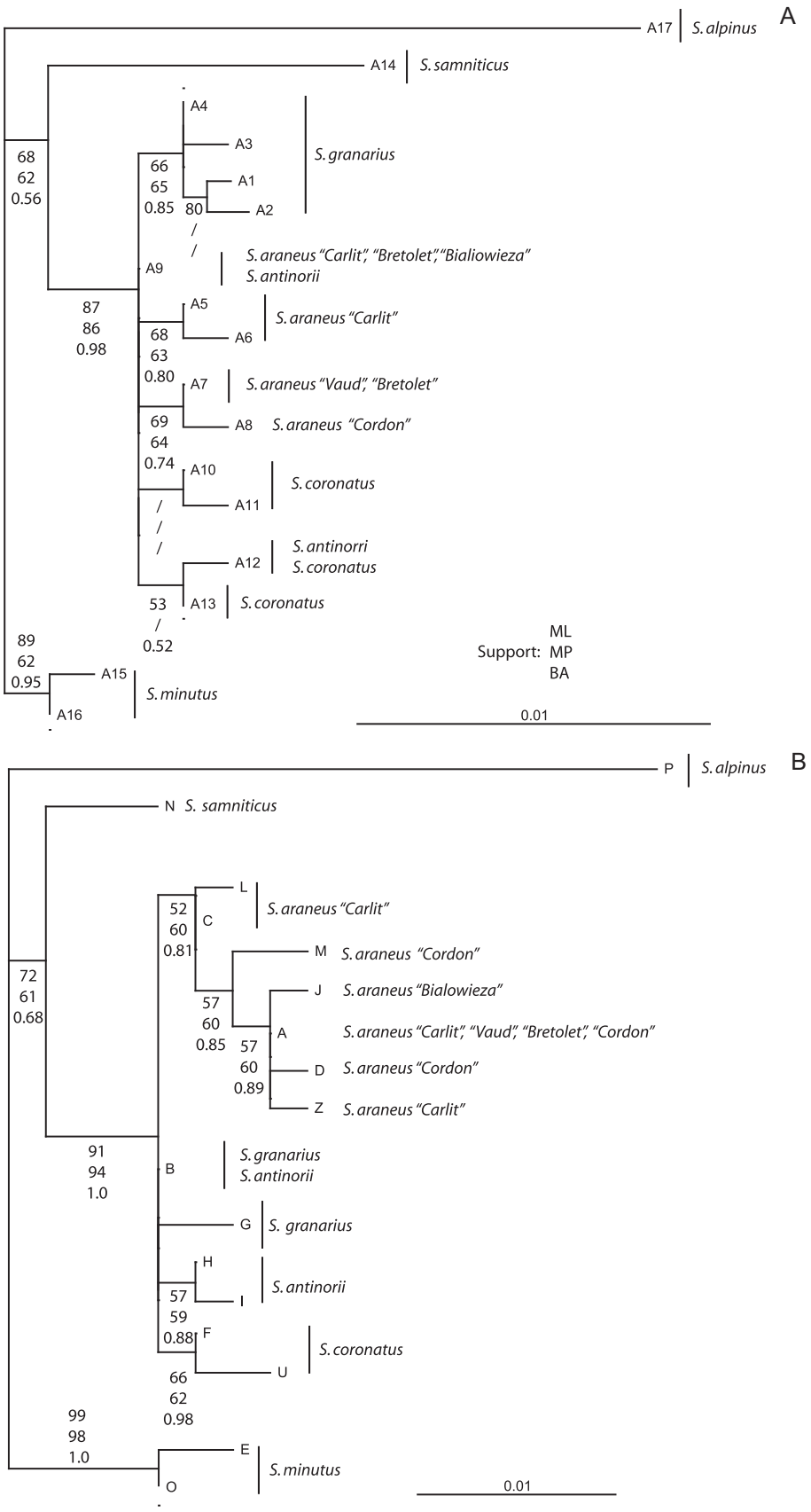

Fig. 3. Phylogenies of (A) the 790 bp *BRCA1* fragment and (B) the 532 bp *ApoB* fragment analyzed with a maximum likelihood (ML) procedure and using the GTR model and TrN model of substitution, respectively. Values in branches are indices of support for the major branches for Bayesian (BA), ML and maximum parsimony (MP) analyses (percentage of 1000 replications for ML and MP, posterior probabilities based on 3,000,000 generations for BA).

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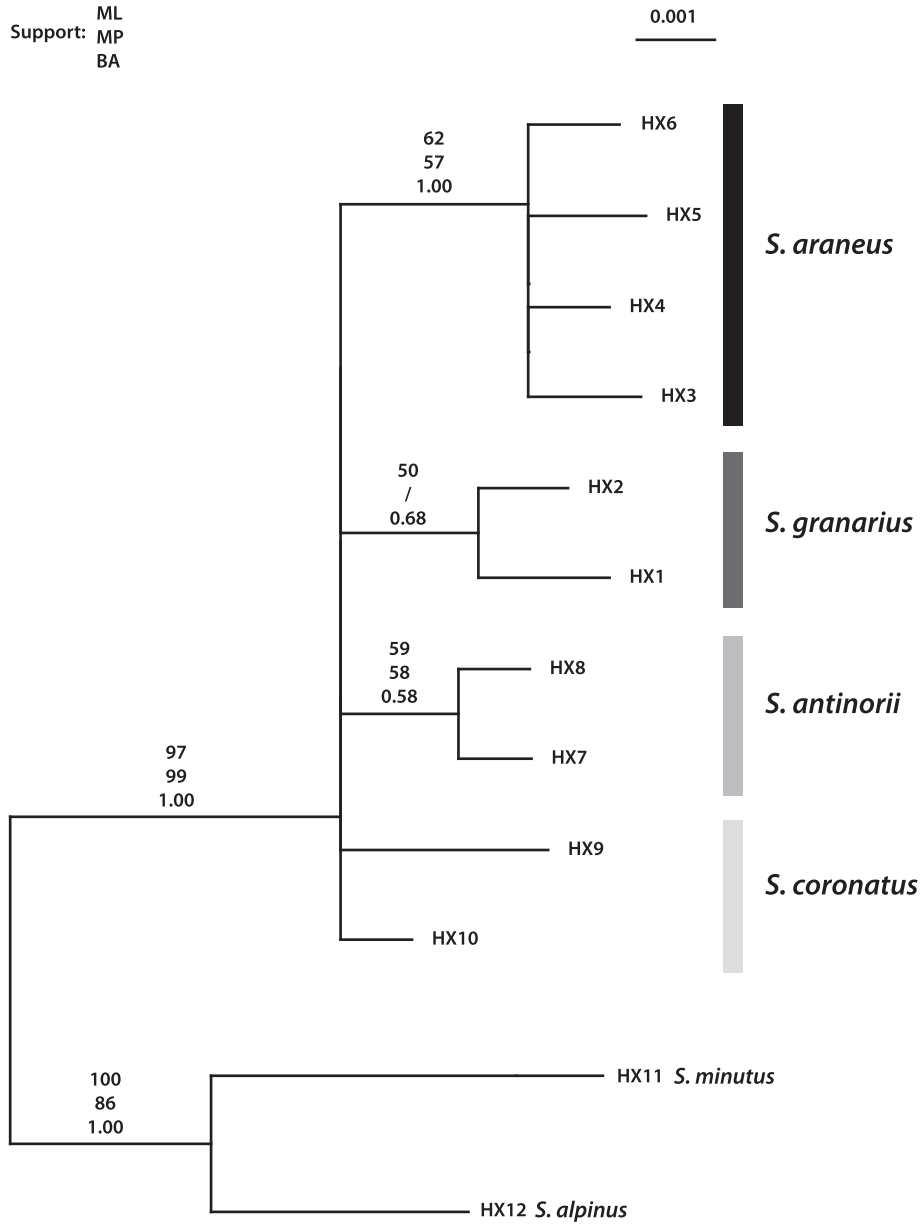

Fig. 4. Phylogeny of the concatenated 745 bp X-linked fragment analyzed with a maximum likelihood (ML) procedure and using the F81 model of substitution. Values in branches are indices of support for the major branches for BA, maximum likelihood (ML) and maximum parsimony (MP) analyses (percentage of 1000 replications for ML and MP, posterior probabilities based on 3,000,000 generations for BA).

grouped mostly with *S. coronatus* (q_{group} = 0.89, *SD* = 0.17; Fig. 5A) with some admixture with the *S. antinorii* cluster. The individuals of *S. araneus* Carlit grouped with the other *S. araneus* races (q_{group} = 0.96, *SD* = 0.02). Higher values of *K* (*K* = 4) suggested a split of *S. antinorii* into two clusters, as previously shown by Basset et al. (2006b), while *S. granarius* remained mainly associated with *S. coronatus*. Finally, at *K* = 5, *S. granarius* individuals composed a genetically independent cluster. The unrooted neighbor-joining tree presented in Fig. 5B depicts the relationships among species. All *S. araneus* races formed a monophyletic group, whereas the position of *S. granarius* is intermediate between *S. coronatus* and *S. antinorii*.

4. Discussion

The main goal of the present study was to elucidate the phylogenetic position of *S. granarius* within the *S. araneus* group. According to previous studies, *S. granarius* is closely related to *S. araneus* sensu stricto according to mtDNA and to *S. coronatus* according to Y-linked markers (Taberlet et al., 1994; Yannic et al., 2008b). Our results on autosomal (DNA sequence and microsatellites) and X-linked markers suggest a close phylogenetic affinity between *S. granarius* and both *S. coronatus* and *S. antinorii*. By contrast, we did not find genetic similarities between *S. granarius* and *S. araneus*. All the markers except mtDNA revealed a similar pattern; therefore, the problematic position of *S. granarius* within the *S. araneus* group is best explained by incongruous results with mtDNA relative to nuclear markers.

The phylogenetic incongruence between the mtDNA and the nuclear DNA markers can be explained by two major phenomena that are not mutually exclusive: introgression and/or incomplete lineage sorting (reviewed by Funk and Omland, 2003). Introgression occurs when interspecific hybridization results in DNA crossing species boundaries. Incomplete lineage sorting occurs if species divergence was too recent for ancestral polymorphisms to have

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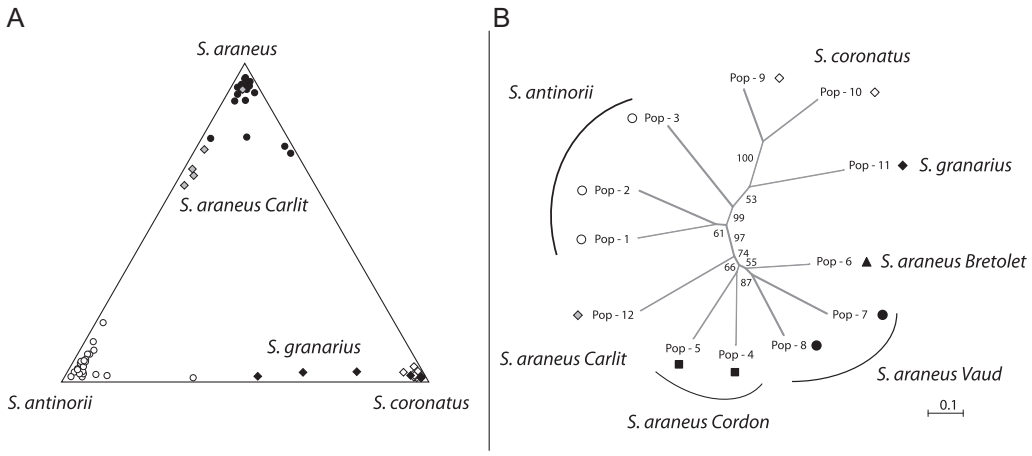

Fig. 5. Microsatellite analyses: (A) STRUCTURE triangle plot revealing the patterns of clustering of *S. granarius* individuals mostly with *S. coronatus*. (B) Neighbor-joining tree based on Cavalli-Sforza genetic distance (Cavalli-Sforza and Edwards, 1967) computed with the program populations (Langella, 1999). Numbers on nodes indicate bootstrap percentages (1000 replicates on loci). Population numbers refer to the data set of Basset et al. (2006b, http://www.unil.ch/dee/page7010_en.html#1).

sorted to reciprocal monophyly. Distinguishing between these two hypotheses is often difficult because both produce similar topologies in gene trees (Holder et al., 2001). Extensive sharing of nuclear DNA polymorphisms is often interpreted as evidence for recent divergence and retention of ancestral polymorphisms (Sefc et al., 2005; Wilson et al., 2000). In our study, the absence of shared nuclear polymorphisms between *S. araneus* and *S. granarius* (i.e. differences are fixed between the two species) can thus be interpreted as evidence of a deeper species divergence and suggests mtDNA introgression.

Additionally, the introgression hypothesis predicts that allele sharing should be more common where the two species are sympatric since there are opportunities for hybridization (e.g., Berthier et al., 2006; Donnelly et al., 2004; Dubey et al., 2008; Sefc et al., 2005). By contrast, incomplete lineage sorting does not make any predictions regarding the geographic distribution of lineages. Repeated range variations during the glacial oscillations set the conditions for recurring secondary contact between species. The exact distribution of the species of the *S. araneus* group during the last glacial period is poorly known, but past contact between *S. araneus* and *S. granarius* populations were likely in the northern part of Spain and in the Pyrenees. Interestingly, the race Carlit of *S. araneus* occurs in this region and is closely related to *S. granarius* for mtDNA. These results therefore suggest an introgression of mtDNA from *S. araneus* Carlit to *S. granarius* without Y chromosome and autosomal DNA introgression.

These conclusions implicitly assume that mtDNA has higher potential for moving across species boundaries and that autosomal DNA and Y chromosome better reflect phylogenetic history (Brumfield et al., 2001). Other studies have described a contrast between the pervasiveness of mtDNA introgression and the limited or undetectable introgression of nuclear genes (e.g., Bachtrog et al., 2006; Berthier et al., 2006; Roca et al., 2005). Several sources of sex-biased asymmetry in reproductive and demographic processes may contribute to such patterns. For example, higher mtDNA introgression could be explained by higher female dispersal. However, this pattern is unlikely in our situation since male-biased dispersal has been shown in this group (G. Yannic, P. Basset, L. Büchi, J. Hausser and T. Broquet, unpublished data) and male-biased dispersal is a general pattern in mammals (see Lawson Handley and Perrin, 2007 for a review).

This reasoning implies a positive correlation between intraspecific and interspecific gene flow although this view has recently been challenged by Petit and Excoffier (2009). According to these authors, the level of interspecific gene flow at a specific marker would negatively correlate with the level of intraspecific gene flow at the same marker. If a species characterized by male-biased dispersal expands its range and meets a closely related species with which reproductive barriers are still incomplete, asymmetric introgression of mtDNA markers will take place from the local species to the colonizing species. If there is sufficient intraspecific gene flow among populations of the colonizing species, genetic drift for the Y chromosome markers will be reduced and mtDNA introgressed haplotypes will be less likely to increase in frequency by chance (Currat et al., 2008). The relevance of this prediction is well supported in the hybrid zone between *S. antinorii* and *S. araneus* Cordon, where several studies suggested that *S. antinorii* genotypes have recently advanced into the *S. araneus* substrate (Basset et al., 2007; Brünner et al., 2002; Hausser, 1994; Lugon-Moulin et al., 1999). Interestingly, in this zone, relative rates of mtDNA introgression are high, while those of the Y chromosome are absent (Balloux et al., 2000). However, while it is difficult to extend the pattern observed in a given context to a rule for the entire *S. araneus* group, such a process could explain the difference of introgression between mtDNA and Y chromosome and autosomal DNA between *S. araneus* and *S. granarius*.

Alternatively, several lines of evidence indicate that sex chromosomes carry more genes involved in low hybrid fertility and/or viability than other parts of the genomes (Muller, 1942; Orr, 1997; Turelli and Orr, 1995). The primary explanation for this pattern is Haldane's rule (Haldane, 1922), the idea that inviability or sterility is first acquired by the heterogametic sex in animals with sex-determining chromosomes (Coyne and Orr, 2004). As a consequence, in hybrid zones between mammals, genes located on the paternally transmitted Y chromosome are expected to introgress less than autosomal and mitochondrial genes. This hypothesis cannot be ruled out as alternative explanation for mtDNA introgression into *S. granarius*.

Finally, selection has repeatedly been invoked to account for massive mtDNA introgression (e.g. Bernatchez et al., 1995; Rognon and Guyomard, 2003) and could have facilitated the introgression of the *S. araneus* mtDNA into *S. granarius*. Even though mitochondrial DNA has long been considered to be an 'innocent bystander' of population history for the convenience of phylogeographical reconstruction, evidence is accumulating that it is subject to various selective pressures (reviewed in Ballard and Whitlock, 2004; Galtier et al., 2009). Adaptive introgression of mtDNA in response to particular environmental pressures has been suggested in charrs (Doiron et al., 2002) and hares (Alves et al., 2008). In humans, there are strong arguments that temperature shapes the mtDNA

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variation in humans (Balloux et al., 2009) and in shrews as well (Fontanillas et al., 2005). Although this hypothesis still needs to be addressed, adaptive advantages of the *S. araneus* mtDNA in cold climate could explain its evolutionary success in all the species present in the colder northern part of the Iberian Peninsula. Occasional hybridizations followed by multiple mtDNA selective sweeps could explain the observed situation whatever the histories of the Iberian species. Interestingly, the same arguments have been invoked to account for the past introgression of mtDNA from a relict arctic hare species, *Lepus timidus*, to the Iberian hare, *L. granatensis*, in the north of the Iberian Peninsula (Alves et al., 2008; Melo-Ferreira et al., 2005), roughly the same region considered here between *S. granarius* and *S. araneus*. Examples of introgressive events with partial or complete replacement of some part of the genome (especially mtDNA) are not rare in the literature and could be the most likely explanation of the situation observed between these species of the *S. araneus* group.

5. Conclusion

In this study, we used a comparative approach to understand the incongruence between the mtDNA and Y chromosome phylogenies within the *S. araneus* group (Yannic et al., 2008b). As expected, a single molecular topology can, in some case, reconstruct with difficulty the real relationships of species with complete accuracy. Our results suggest a case of mtDNA introgression between *S. araneus* and *S. granarius*. We observed a fixation of mtDNA haplotype of *S. araneus* within *S. granarius*, suggesting a complete mitochondrial replacement, although this study is based on only a few individuals (often from the same locality) per species or race. Additional samples from the global distribution of *S. granarius* are necessary to estimate its genetic diversity and to appreciate the extent of the introgression. Finally, this study emphasizes the benefit of multilocus approaches to resolve genetic relationships among species and indicates that divergence with gene flow certainly occurred in this group.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2010.09.015.

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