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Phylogeography analysis and molecular evolution patterns of the nematode parasite *Heligmosomum mixtum* based on mitochondrial DNA sequences

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Abstract

Mitochondrial DNA was explored to study phylogeography of the nematode parasite *Heligmosomum mixtum* and elucidate molecular evolution pattern of cytochrome b gene. The size of cyt b gene ranged from 511 bp to 591 bp and the average of GC contents was 28.9%. The overall transition/transversion ratio R was 5.773 indicating that the transitions are more frequent than transversion. The aligned sequences allowed identifying 54 mtDNA haplotypes among the 119 examined individuals. The genetic divergence registered among the populations of *H. mixtum* was low (0.3% to 1.5%). Neighbor–joining and maximum Likelihood trees evidenced a huge polytomy and unstructured phylogeographic pattern among the studied populations. The demographic analyses tend to evidence a recent and rapid expansion of *H. mixtum.* Our results imply a positive selection and the genetic hitchhiking effect is unlikely. Parameters performed supported scenario of sweep selection and recent expansion of *H.mixtum* populations. Both positive selection and demographic histories have jointly contributed to the observed patterns of nucleotide diversity and haplotypes structure. The comparison of the phylogeographical pattern of *H. mixtum* with the one of its most common rodent host *M. glareolus,* confirmed a strong incongruence between the two species. These results strongly suggest that the parasite would not be specific to *M. glareolus* and that it would switch easily from one rodent species to another. The mitochondrial diversity seems to be unstructured with any biogeographic repartition of the variability and that the genetic structure of *H. mixtum* is probably associated with weak host specificity.

Keywords

Mitochondrial DNA, Cytochrome b gene, Phylogeography, *Myodes glareolus*, Nematode parasite, *Heligmosomum mixtum*

Introduction

Phylogeography is a field of research that studies the processes determining the geographical distribution of genetic lineages at the intra-specific or congeneric levels and is useful for detecting processes such as population subdivision, speciation events and ecological adaptation, and migration routes associated with past climatic changes (Avise 2000). However, the phylogeography of invertebrate taxa, particularly parasite species, was still poorly studied until recently (Wickström *et al*. 2003; Nieberding *et al.* 2004, 2005, 2008). Indeed, over the last few years, there has been increasing interest in investigating the dispersal abilities of invertebrates and micro-organisms (Nieberding *et al*. 2005). In this context, phylogeographical studies have been performed on parasites like nematodes and they enabled to understand whether and how the Pleistocene climatic fluctuations may have affected the genetic structure of parasite species. *H. mixtum* has a direct life-cycle (no intermediate host), it reproduces sexually with three free-living larval stages and a 4th, parasitic larval stage dwelling in the intestinal wall (Asakawa 1987). The micro-habitat of the adult is the lumen of the anterior small intestine (duodenum) (Haukisalmi and Henttonen 1993). The mating habits of *H. mixtum* are unknown, but polygamy is thought to prevail in nematodes (Haukisalmi *et al.* 1996). Its first larval stages are free and require 3 days to become infective. Host contamination occurs after ingestion of contaminated faeces. Adult parasites live in the intes-

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tine and produce eggs in the host faeces about 12 days after ingestion. Individuals in the free-living stage have no dispersal activity, so it seems that the gene flow in this species is determined by host movements (Goüy de Bellocq *et al*. 2002, 2003). The prevalence of *H. mixtum* (percentage of host infected) and its abundance (average number of parasites per host) on *Myodes glareolus*, are high, 45% and 12 correspondingly (N'Zobadila 1994).

Myodes glareolus (Arvicolinae, Rodentia) is a forest dweller present throughout Europe since at least the middle Pleistocene (1.2 Ma) (Bauchau and Chaline 1987). The phylogeography and the post glacial colonization history of the bank vole have been extensively studied in Europe showing a complex phylogeographical structure (Deffontaine *et al*. 2005, 2009). The results revealed the presence of four Mediterranean (Basque, Spanish, Italian and Balkan) and three continental (western, eastern and 'Ural') phylogroups.The host and its parasite are common and geographically widespread in all over the western Palearctic region except in the Mediterranean biome (Spitzenberger 1999).

Comparative phylogeographical approaches have also been developed in several studies (Wickström *et al*. 2003; Nieberding *et al*. 2005; Biek *et al*. 2006; Whiteman *et al*. 2007). Parasites have notably been used to resolve the evolutionary and ecological history of their host (Nieberding and Olivieri 2007). It have been found to have often more structured populations than their host, suggesting lower rates of gene flow, but the reverse has also been found in some species (McCoy *et al*. 2005). The extent to which the spatial structure of parasite populations mirrors patterns seen in host populations seems to depend on the degree to which they are dependent on the host (Barrett *et al*. 2008). Indeed, host vagility should be a major determinant of parasite gene flow because many parasites have no free-living stages or have low dispersal capability in their free-living stages (McCoy *et al*. 2003; Criscione *et al*. 2005). Consequently, gene flow in a parasite with multiple host species will be controlled by the more mobile host (Criscione *et al.* 2005). Divergent patterns between host and parasite may imply that additional host species play a role in dispersing the parasite (Jones and Britten 2010). However, the links between the host specificity of a parasite and a congruent signal between their phylogeographic patterns are still poorly studied.

Mitochondrial DNA was used for phylogeographic studies. It is a molecule that has specific characteristics different from those of the nuclear DNA, which made it very attractive for the researchers (Ballard and Whitlock 2004). Characteristics as its abundance, its simple genetic structure (haploid) due to its uniparental transmission (maternal inheritance generally), its small size and the absence of introns and recombination facilitate its amplification and its sequencing. The arrangement of its genes was very stable between various taxonomic classes. However, phylogeographic studies using genetic marker such as the gene coding for the cytochrome b of the mitochondrial DNA (mtDNA) have been developed in order to examine the phylogeographic patterns of vertebrate and invertebrate taxa. For this purpose, we studied the phylogeographical structure of the nematode parasite *(Heligmosomum mixtum*) (Heligmosomidae) and we compared it to those of its most commonly host, the bank vole (*Myodes glareolus*). However, this nematode seems to have also the ability to infect other rodent hosts such *as Clethrionomys rutilus,* the vole *Microtus arvalis, Microtus agrestis* and *Microtus socialis* and the gerbill *Meriones persicus* (Haukisalmi *et al*. 1996; Mezeika *et al.* 2003; Grikieniennë 2005; Kia *et al*. 2010; Zhigileva 2011). In contrast to its related species, the nematode *Heligmosomoides polygyrus*, which is characterized by an important congruent phylogeographic pattern as compared to its specific host, the wood mouse (*Apodemus sylvaticus*), we therefore hypothesize that *H. mixtum* would be characterized by a weaker phylogeographic structure, which would be divergent to those of its major host, the bank vole. In this scope, the aims of the present study are: (i) to verify, by sequencing the mitochondrial DNA cytochrome b gene (*cyt* b), the hypothesis of a weak congruence between the phylogeographic structure of *H. mixtum* and *M. glareolus*; and (ii) to discuss the particularities of *H. mixtum* phylogeographical pattern as compared with those of other invertebrate taxa, in the light of the knowledge on the population genetic structure and the genetic diversity of nematode parasite species.

Materials and Methods

Sample collection and sequencing

A set of 119 adult *Heligmosomum mixtum* from 10 localities in Europe were analyzed (Table I, Fig. 1). All samples were identified at the species level. Tissues are held in the collection of Michaux J., Nieberding C., Deffontaine V., Libois R., Henttonen H., Niemima J.

DNA from *H. mixtum* was extracted as reported by Goüy de Bellocq *et al*. (2001). A total of 591 bp for the *cyt* b gene were amplified by the polymerase chain reaction (PCR). Specific primers are used 3F (5'-CTGCTGATGGYTCAA TAGCTT-3') and 3R (5'-GGGTCACCCAACCTAAAAGG-3'. These primers were defined using Primer3 software (version 4.0.0) (Koressaar and Remm 2007). Amplifications were carried out in 50µL volumes including 0.35 µL of each 100 µM primer, 4 µL of 1 mM dNTP, 10 µL of 5Xreaction buffer, 29 µL of purified water, 4µL DMSO and 0.5 µL of 5U/µL Go TAQ DNA polymerase. PCR amplification used 2 µL of DNA extract. Amplifications were performed at 94°C for 4 min, followed by 40 cycles (45 s at 94°C, 45 s at 54°C and 1 min 30 s at 72°C) with final extension cycle of 10 min at 72°C with a Labover PTC100 Thermal Cycler. PCR products were purified using the Ultrafree DA Amicon kit and directly sequenced. Both strands were sequenced by Macrogen Society (Seoul, South Korea).

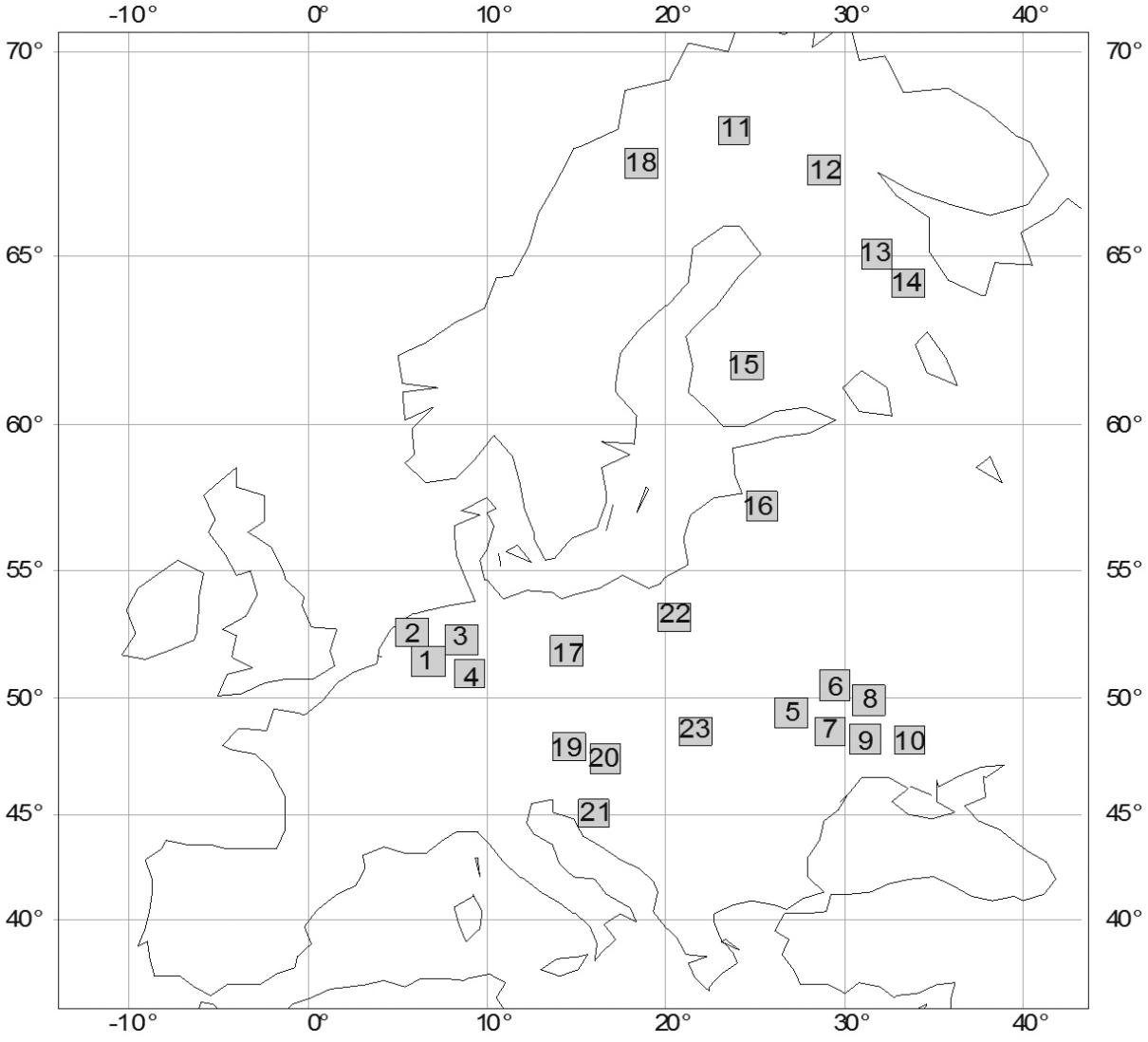

Fig. 1. Geographic distribution of *H. mixtum* in the Palearctic region: **1** – Virelles; **2** – Colonster; **3** – Marcourt; **4** – Havelanj; **5** – Transylvanie Tirgu; **6** – Transylvanie Zarnesti; **7** – Crisana Ineu; **8** – Banat Baile; **9** – Brasov; **10** – Timisoara; **11** – Pallasjärvi; **12** – Sodankyla; **13** – Transect, Rautjärvi; **14** – Transect, Joutseno; **15** – Finland South; **16** – Lithuania; **17** – Germany; **18** – Sweden **19** – Austria Ginzling; **20** – Austria Tirol ventetal; **21** – Croatia; **22** – Poland; **23** – Hungary

Data analysis

Sequences of haplotypes were submitted to NCBI GenBank (Table I) (Accessions numbers: HG966618 to HG966671 for the 54 haplotypes of *H. mixtum*).

The sequences were aligned using the ClustalW package in the BIOEDIT program, version 7.0.0 (Hall 1999) and analyzed with MEGA program version 5 (Tamura *et al*. 2011). Aligned sequences were analyzed with DnaSP software version 5.10.01 (Librado and Rozas 2009) to estimate polymorphisms indices. Indices of haplotypes diversity (Hd) (Nei and Tajima 1983) and pairwise estimates of nucleotide diversity (Pi) (Jukes and Cantor 1969) were used to appreciate genetic diversity among the studied populations. The mean of genetic diversity was calculated for each population and for overall *H. mixtum* animals.

Phylogenetic reconstruction

The alignment was manually checked and pairwise sequence divergence between individuals of *H. mixtum* was calculated according to the Maximum Composite Likelihood (MCL) (Tamura *et al*. 2004). Phylogeny reconstruction was performed using Neighbor-Joining (NJ) (Saitou and Nei 1987), Maximum parsimony (MP) methods applying MEGA Version 5 software (Tamura *et al*. 2011) and Maximum Likelihood approaches, with the PHYML package (ML) (Guindon and Gascuel 2003). PHYML trees were constructed with 1000 bootstrap replicates. Phylogenetic trees were rooted with *cyt* b sequences from two *Heligmosomoides polygyrus*, a related species. The Akaike Information Criterion in Model test version 3.06 (Posada and

Table I. Geographical locations, sample abbreviations and Genbank accession numbers of *H. mixtum* haplotypes used in this study

Countries	Total number of samples	Localities	Number of animals (number of haplotypes)	Abbreviations	Sequences code	Genbank accession numbers (for haplotypes only)
Belgium	12	Virelles	5 (3)	Be1	Be1.1	HG966618
					Be1.2	HG966619
					Be1.3	HG966620
		Colonster	3 (1)	Be2	Be2	HG966621
		Marcourt	3 (1)	Be3	Be 3	HG966622
		Havelanj	1 (1)	Be4	Be4	HG966623
Romania	29	Transylvanie, Tirgu Mures, Sovata	7 (5)	Ro1	Ro1.1	HG966624
					Ro1.2	HG966625
					Ro1.3	HG966626
					Ro1.4	HG966627
					Ro1.5	HG966628
		Transylvanie, Zarnesti, Plaiul Foii	6 (3)	Ro2	Ro2.1	HG966629
					Ro2.2	HG966630
					Ro2.3	HG966631
		Crisana, Ineu, Moneasa	4 (2)	Ro3	Ro3.1	HG966632
					Ro3.2	HG966633
		Banat, Baile, Herculane, Vallée de la Cerna	5	Ro4		
		Brasov	5 (3)	Ro5	Ro5.1	HG966634
					Ro5.2	HG966635
					Ro5.3	HG966636
		Timisoara	1	Ro6		
Finland	23	Pallasjärvi: kittila	6 (2)	PJ	PJ1	HG966637
					PJ2	HG966638
		Sodankyla	3 (2)	So	So1	HG966639
					So2	HG966640
		Transect: Finland SE (Rautjärvi)	5 (3)	T1	T1.1	HG966641
					T1.2	HG966642
					T1.3	HG966643
		Transect: Finland SE (Joutseno)	4 (1)	T2	T2	HG966644
		Finland South	5 (4)	Fin	Fin1	HG966645
					Fin2	HG966646
					Fin3	HG966647
					Fin5	HG966648
Lithuania	2	SE, Alytus	2 (2)	Li	Li1	HG966649
					Li2	HG966650
Germany	14	Gera	14 (5)	Ger	Ger1	HG966651
					Ger2	HG966652
					Ger3	HG966653
					Ger4	HG966654
					Ger5	HG966655
Sweden	10	Norrbotten-northeasternmost Sweden	10 (3)	Sw	Sw1	HG966656
					Sw2	HG966657
					Sw3	HG966658
Austria	10	Ginzling, Tirol, Zemmtal	5 (3)	Au1	Au1.1	HG966659

Countries	Total number of samples	Localities	Number of animals (number of haplotypes)	Abbrevia-tions	Sequences code	Genbank accession numbers (for haplotypes only)
					Au1.3	HG966661
		Tirol, Ventetal	5 (2)	Au2	Au2.1	HG966662
					Au2.2	HG966663
Croatia	12	Croatia	12 (5)	Cr	Cr1	HG966664
					Cr2	HG966665
					Cr3	HG966666
					Cr4	HG966667
					Cr5	HG966668
Poland	8	O Lublin: Pulawi PO45	8 (3)	Po	Po1	HG966669
					Po2	HG966670
					Po3	HG966671
Hungary	1	Zala	1	Hun		
H. polygyrus	2					AJ608890; AJ608902

Crandall 1998) was used to select the best substitution model for the parasite data in the ML reconstructions which was GTR plus gamma.

The genetic relationship of the haplotypes was also graphically displayed using the algorithm MINSPNET available in the program Arlequin 3.5 (Excoffier *et al*. 2005). The Minimum Spanning Network (MSN) computes a Minimum Spanning Tree (MST) and Network (MSN) among haplotypes. F_{st} (Hudson *et al.* 1992), G_{st} (Nei 1973) and N_{st} (Lynch and Crease 1990) were analyzed with DnaSP, version 5.10.01 (Librado and Rozas 2009). F_{st} index is widely used to estimate the degree of subdivision between populations and G_{st} depends only on the frequencies of the haplotypes. N_{st} is influenced by both haplotype frequencies and genetic distances between haplotypes. Moreover, the strengths of gene flow (N_m) and random drift were accessed from F_{st}, G_{st} and N_{st} parameters.

Phylogeographical and genetic structure analyses

Selection neutrality for the detected mutations was tested by both Tajima's D (Tajima 1989) and Fu and Li's D* and F* methods (Fu and Li 1993). Demographic parameters were assessed using the distribution of pairwise sequence differences (mismatch distribution) of Rogers and Harpending (1992) and site-frequency spectra (distribution of the allelic frequency at a site) of Tajima (1989) using the program DnaSP software version 5.10.01 (Librado and Rozas 2009). This analysis provided an estimate of the population dynamics either in recent expansion or rather stable in time in the different lineages. The smoothness of the observed distribution was quantified by the raggedness statistic, r (Harpending 1994) and Ramos-Onsins statistic, R2 (Ramos-Onsins *et al*. 2002). The confidence intervals were provided by computer simulations using the coalescent algorithm in DnaSP software. This powerful population expansion test takes into account haplotype frequencies under neutrality, stationarity and panmixis, as described by Ewens (1972), but is sensitive to background selection. Fu's Fs test (Fu and Li 1993) was used to access historical changes in population size. Fu's Fs is sensitive to demographic expansion and can be used to estimate exponential population growth or decline (Lessa *et al*. 2003).

On the basis of the percentage of genetic divergence (GD) obtained with a distance analysis (K2P distance), and was corrected for ancestral mtDNA polymorphism, as proposed by Avise (2000) using the formula: $P_{net} = P_{AB} – 0.5 (P_A + P_B)$. Where P_{net} is the corrected distance between the isolated lineages A and B, P_{AB} is the mean genetic distance in pairwise comparisons of individuals A vs. B, and P_A and P_B are mean genetic distance among individuals within these lineages. However, it is generally problematic to calibrate the absolute rate of evolution of invertebrate parasites because of lacking fossil records.

Results

Sequence variation in the cytochrome b gene

The length of the *cyt*b mt DNA ranged from 511 bp to 591 bp with an average of 596 bp among the 119 sequences of *H. mixtum* analysed. The average GC contents of the *cyt*b mt DNA was 28.9%. The transition/transversion rate ratios were: K1 = 21.104 (purines) and K2 = 10.067 (pyrimidines). The overall transition/transversion bias (R) was 5.773. This result shows that the transitions are more frequent than transversions at the cytb mtDNA (Table II). The different substitutions detected are given in table II and shows that the A→G and T→C transitions are more frequent than G→A and C→T transitions in the *cyt* b gene.

Table II. Maximum Composite Likelihood Estimate of the Pattern of Nucleotide Substitution in cytochrome b gene

	A	T	C	G
A	–	2.78	0.45	**26.17**
T	1.4	–	**4.54**	1.24
C	1.4	**28**	–	1.24
G	**29.55**	2.78	0.45	–

Rates of different transitional substitutions are shown in bold and those of transversionsal substitutions are shown in italics. Evolutionary analyses were conducted in MEGA5 (Tamura *et al*. 2011)

Table III. Summary of genetic polymorphisms observed in *H. mixtum* animals used in this study

H. mixtum	**All individuals**
Number of sequences	119
Alignment length (bp)	487
Monomorphic characters	422
Variable characters	65
Parsimony informative characters	36
Singleton variable sites	29
Total number of mutations	66
Number of polymorphic sites (S)	65
Number of haplotypes (H)	54
Haplotype diversity (Hd) ± SD	0.968 ± 0.007
Variance of haplotype diversity	0.00004
Nucleotide diversity (Pi) ± SD	0.00851 ± 0.00048
Theta (per site) from Eta	0.02532
Average of pairwise differences (K)	4.144
Minimum number of recombination events (Rm)	8
%GD genetic diversity (K2P)	0.009
Tajima's D	–2.10850 (*P<0.05)
Fu and Li's D*	–3.30214 (**P<0.02)
Fu and Li's F*	–3.35691 (**P<0.02)
Fu's Fs statistic	–52.426 (P=0.000)

SD: Standard deviation, Tajima's D, Fu and Li's D* and Fu and Li's F*: neutrality tests

Table IV. Genetic variability and mean genetic diversity observed within the different populations of *Heligmosomum mixtum*

Heligmosomum mixtum	**Number of samples**	**% GD genetic diversity (K2P)**	**Number of haplotypes**	**Hd ± SD**	**Pi ± SD**
Belgium	12	1	9	0.939 ± 0.058	0.00905 ± 0.00086
Romania	28	0.5	13	0.854 ± 0.055	0.00481 ± 0.00091
Lithuania	2	1.5	2	1 ± 0.5	0.01197 ± 0.00598
Austria	10	0.5	6	0.844 ± 0.103	0.00437 ± 0.00058
Hungary	1	n/c	n<2		
Poland	8	0.9	3	0.607 ± 0.164	0.00768 ± 0.00192
Croatia	12	0.3	6	0.818 ± 0.096	0.00433 ± 0.0014
Germany	14	0.5	5	0.769 ± 0.083	0.00539 ± 0.00118
Sweden	11	0.3	5	0.618 ± 0.164	0.00316 ± 0.00125
Finland	21	0.6	15	0.938 ± 0.04	0.00636 ± 0.00052

SD: Standard deviation; Hd: haplotypic diveristy; Pi: nucleotide diversity; GD: genetic diversity

Table V. Genetic divergences (K2P distances) between the populations of *Heligmosomum mixtum*

H. mixtum	Romania	Belgium	Lithuania	Finland	Sweden	Germany	Croatia	Austria	Poland	Hungary
Romania										
Belgium	0.010/ 0.003									
Lithuania	0.012/ 0.002	0.015/0.003								
Finland	0.008/0.003	0.011/0.003	0.012/0.002							
Sweden	0.008/0.004	0.011/0.005	0.013/0.004	0.005/0.000						
Germany	0.008/0.003	0.011/0.003	0.012/0.002	0.009/0.003	0.009/0.005					
Croatia	0.007/0.003	0.010/0.003	0.010/0.001	0.007/0.003	0.008/0.005	0.008/0.003				
Austria	0.008/0.003	0.010/0.003	0.011/0.001	0.008/0.002	0.008/0.004	0.007/0.002	0.007/0.003			
Poland	0.014/0.007	0.016/0.007	0.017/0.006	0.013/0.005	0.013/0.007	0.012/0.005	0.014/0.008	0.013/0.006		
Hungary	0.003/0.000	0.008/0.003	0.010/0.003	0.006/0.003	0.007/0.005	0.007/0.004	0.005/0.004	0.006/0.003	0.012/0.008	

The two values correspond to the Pmean and P distances

Sequence alignment resulted in a matrix of 591 bp characters. Of the 65 variable sites, 36 were parsimony-informative and 29 were singleton variable sites (Table II). The nucleotide frequencies were 23.84%, 47.36%, 21.12% and 7.6% for A, T, C and G respectively.

Genetic variability and differentiation

The Aligned Sequences allowed identifying 54 mtDNA haplotypes among 119 individuals examined. Within the whole dataset, the haplotype diversity (Hd) was estimated to 0.968 and the nucleotide diversity (pi) to 0.00851 (Table III). The mean of genetic diversity was estimated to 0.9% for the studied *H. mixtum* animals (Table III). The Théta (per site) from Eta and the average number of pairwise differences (K) are 0.02532, 4.144 respectively for overall data set (Table III).

The recombination rate is also an important parameter affecting patterns of DNA polymorphism. In *H. mixtum*, 8 recombination events were detected at whole data sequences. Sites touched by recombination events were (99,243); (267,285); (285,303); (324,327); (390,396); (396,417); (438,447) and (447,477). Value of Rm per informative sites was 0.222 for the overall data set sequences (Table III).

The nucleotide diversity was calculated for each population of *H. mixtum*. The results of these analyses are summarized in table IV and indicate that the Belgium and Poland animals have the highest level of nucleotide diversity 0.00905 and 0.00768, respectively. Moreover, the Finnish animals showed a relatively high level of nucleotide diversity (0.00636). The other populations have an intermediate value of nucleotide diversity. Animals from Lithuania showed a high value of nucleotide diversity. However, this result must be taken with caution because the sampling for this region was very low (N = 2) (Table IV).

The genetic variability registered between the populations of *H. mixtum* was low and ranged from 0.3 to 1.5% (maximum 1.5% K2P genetic diversity is observed in Lithuania animals). However, these data must be interpreted with caution because the sampling for this region was very low (N = 2). Populations of *H. mixtum* from Belgium and Poland showed a relatively high level of genetic diversity, 1% and 0.9% respectively (Table IV). The K2P genetic distances observed among the animals from the different regions were high (between 0.2 to 1.7% of K2P distance; Table V). F_{st}, G_{st} and N_{st} parameters were estimated. G_{st} was calculated based on haplotype frequencies, whereas N_{st} takes into account the genetic relation among haplotypes. The level of differentiation in allele frequencies between groups was measured by F_{st} (Wright 1951). When N_{st} value is higher than the G_{st} estimated, it indicates the presence of a phylogeographical structure as noted by Petit *et al*. (2005). The F_{st} values in the range of 0.07–0.59 were registered between the different populations of *H. mixtum*.The highest value of F_{st} (0.59) is registered between populations from Croatia and Sweden. Parasites from Lithuania

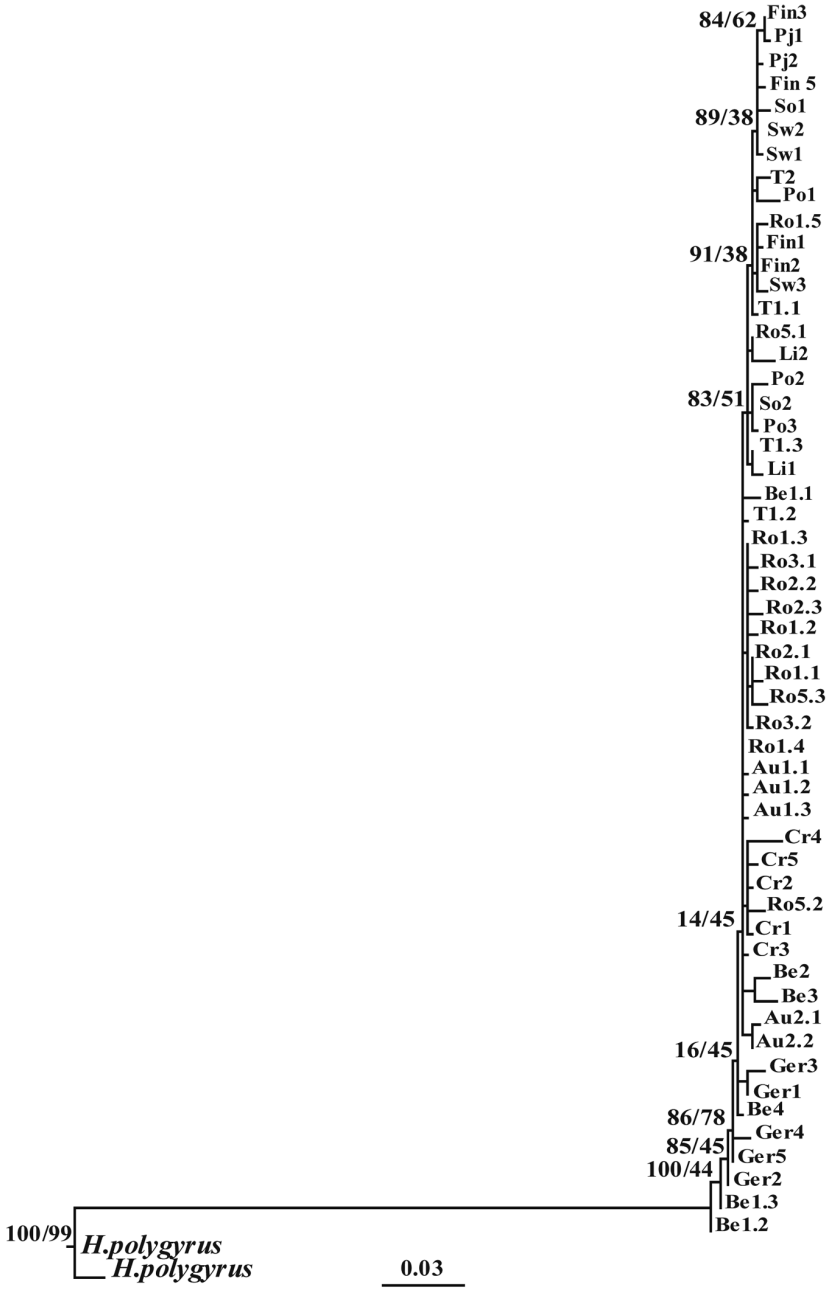

Fig. 2. Most likely tree of the PHYML reconstruction for the 54 mt DNA haplotypes of *Heligmosomum mixtum* and the two *Heligmosomoides polygyrus* outgroups. Numbers on branches indicate, from left to right (a) bootstrap support in the PHYML analysis, (b) bootstrap support obtained in the NJ reconstruction

and Poland have a low value of F_{st} (0.08) suggesting a frequent gene flow between these regions. However, F_{st} is also low between the Finnish animals and those from Sweden (0.07) suggesting recent contacts between these regions (Table VI).

The estimated F_{st} (0.365) and G_{st} (0.175) are low and agree with assumption that a balancing selection activates at the *cyt*b mtDNA. Here, the N_{st} (0.366) and the G_{st} (0.175) ($P < 0.05$) suggested the presence of gene flow between the different populations of *H. mixtum* as demonstrated by values of N_m (0.43; 1.17 and 0.43 estimated on the basis of F_{st}, G_{st} and N_{st} indices, respectively).

Genetic relationships based on cytochrome b

Phylogenetic trees were reconstructed using Maximum Parsimony (MP), ML and neighbor-joining (NJ) methods. Parsimony analysis showed a low homoplasy. Indeed, the consistency index (CI), the retention index (RI) and the homplasy index (HI) were 0.7364, of 0.7434 and 0.2566, respectively. The Maximum

Table VI. Genetic differentiation (F_{st} values) between the studied populations of *Heligmosomum mixtum*

H. mixtum	Belgium	Romania	Lithuania	Austria	Poland	Croatia	Germany	Sweden	Finland
Belgium									
Romania	0.27956								
Lituania	0.16898	0.18981							
Austria	0.25613	0.39139	0.11111						
Poland	0.42483	0.50611	0.33049	0.47913					
Croatia	0.31074	0.44707	0.08442	0.40783	0.55694				
Germany	0.28303	0.41351	0.19741	0.28790	0.43574	0.45320			
Sweden	0.41209	0.52410	0.30376	0.52823	0.53416	0.59753	0.55248		
Finland	0.26256	0.34902	0.13418	0.32218	0.42130	0.39158	0.36957	0.07121	

Likelihood (ML) tree is illustrated in Fig. 2. This tree shows a huge polytomy among the studied animals and suggests the existence of a great genetic homogeneity within this dataset. The topology of ML tree is made independently of the geographic origin of parasites. Indeed, the NJ tree showed similar topology than obtained by ML. The bootstrap values of this last analysis were added to the ML tree (Fig. 2).

Demographic analysis

We employed different statistical methods to determine if the patterns of diversity observed in *H. mixtum* species in the *cytochrome* b of mtDNA deviated significantly from an equilibrium neutral model. Selective neutrality tests show that Tajima's as well and Fu and Li's tests were negative and significant for the animals studied (Table III). The observed variation patterns provide evidence that *H. mixtum* have been undergoing rapid expansion. The Fu's Fs statistic results demonstrate that the genetic effect of hitchhiking was ruled out by Fu's test. The calculated values were highly negative and significant (Fu's Fs = –52.426; P = 0.000 for the all data set sequences) (Table III and Fig. 3a). This result confirms a signal of population expansion of *H. mixtum* and positive selection without a hitchhiking effect (negative D-value) as described by Eswaran *et al*. (2005). This was also supported by significant values of Fu and Li's statistical tests (F*:–3.30214; **P<0.02; D*: –3.35691; **P<0.02) for all data set sequences. A signature of population growth (unimodal pattern) was also clearly evident in the distribution of pairwise nucleotide distribution within the total animals (n = 119) (Fig. 3b). These results are corroborated by Harpending's raggedness index and Ramos-Onsins statistic (r = 0.08921, R2 = 0.16021) in *H. mixtum* cytb DNA sequences.

Haplotype distribution

To improve the genealogical relationships assessment between haplotypes in *H. mixtum*, a minimum spanning network was

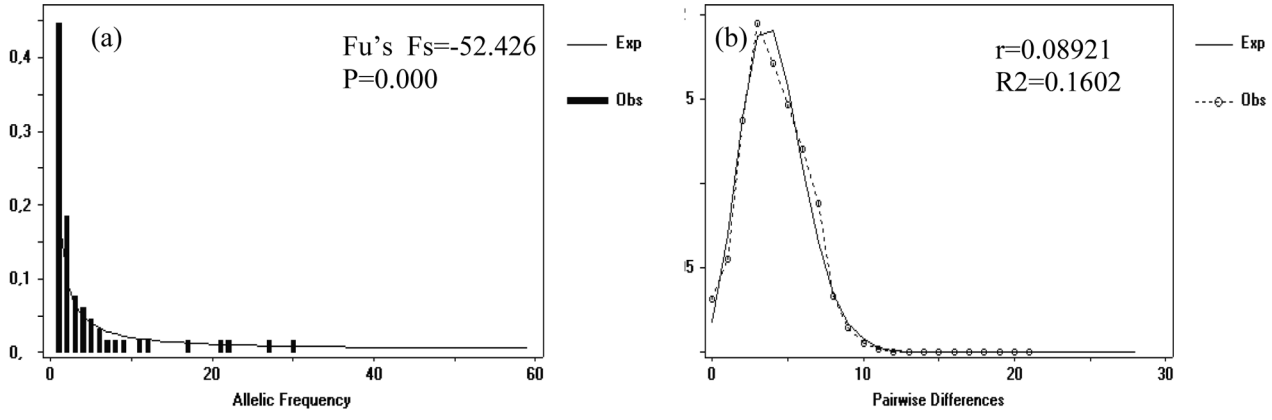

Fig. 3. Site frequency spectra and mismatch distribution of the cytochrome b sequences of *H. mixtum* based on pairwise nucleotide differences in the overall data set sequences. (**a**) Solid lines in the site-frequency spectra indicate the expected distributions under neutrality and at equilibrium. Fu's Fs statistics and corresponding P- values are given; (**b**) Solid lines in the curves indicate the expected distribution under expansion and dotted lines indicate the observed distribution under population expansion. The ranggedness and Ramos-Onsins and Rozas statistics, corresponding r and R2 statistic values are given.

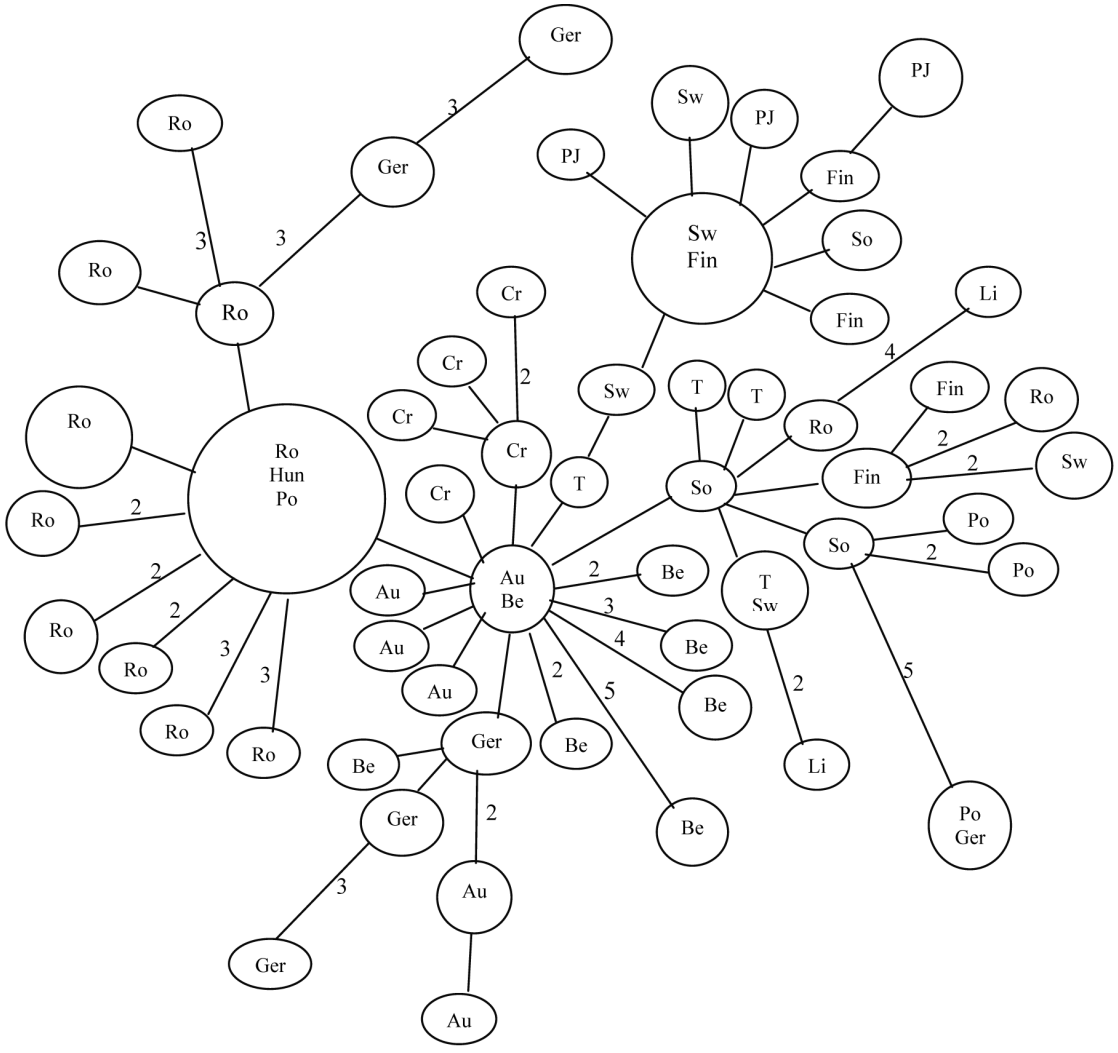

Fig. 4. Minimum spanning network constructed using mitochondrial cytochrome b gene sequences. Numbers correspond to the mutational steps observed between the inferred haplotypes of *H. mixtum*: **Ro** – Romania; **Cr** – Croatia; **Be** – Belgium; **So** – Sodankyla; **Ger** – Germany, **Au** – Austria **T** – Transect; **Hun** – Hungary; **Po** – Poland; **Li** – Lithuania; **Fin** – Finland, **PJ** – Pallas jarvi, **Su** – Sweden

constructed by linking sequences in a hierarchical manner based on mutational changes between them. Fig. 4 shows the genetic network of the detected haplotypes using the cytb mt DNA. Because most genetic variants arose recently, the haplotype patterns should reflect a signature of relative recent expansion *of H. mixtum*. Indeed, populations of *H. mixtum* are linked to three main haplotypes. The first one is composed of animals from Romania, Hungary and Poland; the second one is represented by Sweden and Finnish animals and finally the third one is composed of animals from Belgium and Austria which display the highest number of connections (Fig. 4). The other individuals are dispersed among the described haplotypes. The number of mutational steps which separated haplotypes ranged from one to five suggesting close relationships between the inferred haplotypes. Fig. 4 revealed the same result as the phylogenetic tree. Indeed, the distribution of haplotypes showed a star-like topology without any geographic structure.

Discussion

Genetic variability in cytochrome b for *H. mixtum*

The mean length of the *cyt* b mtDNA in *H. mixtum* is 596 bp and the nucleotide frequencies are 23.84%, 47.36%, 21.12% and 7.6% for A, T, C and G respectively. Similar results have been reported in *H. polygyrus* (687 bp), specific parasite of *A. sylvaticus,* where the base composition was 25.68%, 48.97% 5.73% and 19.62% for A, T, C and G, respectively (Nieberding *et al.* 2005). The level of transition/transversion which registered in *H. Mixtum* (5.773) was higher than those observed in *H. polygyrus* (ti/tv = 2.18) (Nieberding *et al*. 2005).

The mean *cytochrome* b level of genetic diversity of *Heligmosomum mixtum* (0.9% in the whole data set) was low in comparison with its related species *Heligmosomides polygyrus* (6.7%) (Nieberding *et al*. 2005). This result would be partly

explained by abundance and prevalence of *H. polygyrus* on *A. sylvaticus* which is quite high, involving a rapid accumulation of mutations and ensuring the parasite genetic diversity (Nieberding *et al*. 2005). The level of genetic diversity observed in *H. mixtum* also appears very low as compared to other invertebrate taxa like the cestode *Paranoplocephala arctica* paraziting collared lemmings (4.5%) (Wickström *et al*. 2003) and other species like the snails *Biomphalaria glabrata* (5.9%); the insects (e.g. *Maoricicada campbelli* (2%); *Peltoperla tarteri* (4%); *Tarphius canariensis* (5.8%)) (Emerson *et al*. 2000; Buckley *et al*. 2001; Trewick and Wallis 2001; Mavarez *et al*. 2002; Schulthesis *et al*. 2002). The nucleotide and haplotype diversities observed in *H. mixtum* appear also very low as compared to the values observed in other parasite or invertebrate species. Such results could be explained by three scenarios:

– (1) A historical one: the parasite may have undergone frequent bottlenecks during the quaternary glaciations which may have induced a reduction of the genetic diversity of this parasite.
– (2) The eradication of the diversity within populations of the parasite may also be related to its biology and the reproductive cycle. Indeed, cold conditions may affect the living cycle of *H. mixtum* and the development of the free-living larval stages to infective L3s is almost certainly slowed and or suspended during cold winter (Haukisalmi *et al*. 1988).
– (3) The low genetic variation in *H. mixtum* is explained by the fact that *H. mixtum* is a host generalist. It seems that host specialist parasites have more genetic structure.

Lack of phylogeographic structure in *H. mixtum*

The different phylogenetic trees constructed with the inferred haplotypes evidenced a great genetic homogeneity among the studied *H. mixtum* (Fig. 2) as compared to the complex phylogeographic structure of its main host, the bank vole (*M. glareolus*) (see Deffontaine *et al*. 2005, 2009). This strong incongruence between the host and parasite phylogeographic patterns could be explained by the ability of *H. mixtum* to switch on different other host species like the fieldmouse *Apodemus flavicollis*, the vole *Microtus arvalis and Microtus socialis* and even the gerbill *Meriones persicus* (Mezeika *et al*. 2003; Grikieniennë 2005; Kia *et al*. 2010). Indeed, the high vagility of *H. mixtum* and its host generalist behavior probably influenced its degree of genetic differentiation and its phylogeographic structure. Switching on different host species, its dispersal habits could be totally different as compared to those of its main host, *M. glareolus.* This would have facilitated a genetic homogenisation of its populations throughout all Europe, which lead to the present weak phylogeographic structure. Similar data have been found since unstructured genetic variability have been registered using other molecular markers (Dybdahl and Lively 1996; Nieberding *et al*. 2008).

This host switching hypothesis was also proposed to explain some incongruences between the phylogeographic structure of *H. polygyrus* and *A. sylvaticus* (Nieberding *et al*. 2008). Indeed, this parasite species can also switch sometimes on other *Apodemus* species (eg *A. flavicollis*, *A. uralensis*, *etc.*). This probably led to particular phylogeographic patterns observed in some regions for the parasite, as compared to the one of its host (Nieberding *et al.* 2008).

The same signal of a weak geographic structure was also observed on the cestode *Paranoplocephala artica*. For this latter species, an indeterminate intermediate host might be responsible of such pattern (Wickström *et al*. 2003). Another example concerns the snail *Potamopyrgus antipodarum*, which forms a collection of highly structured populations; whereas its trematode parasite *Microphallus* exhibits weak population structure because of the dispersal abilities of its final bird host species (Dybdahl and Lively 1996).

Our results and these last examples tend to confirm that the phylogeographic congruence between parasites and their host should be rather rare and that this phenomenon would be only observed when there is a high specificity between the parasite and its host (e.g. Nadler and Hafner 1990; Mulvey *et al*. 1991; Parker and Spoerke 1998).

In contrast, when some particular conditions exist, like a (i) strong host–parasite specificity, (ii) a direct cycle of the parasite, (iii) a short survival time of larvae during the free stage, and (iv) a limited dispersal abilities of the parasites on their own, consequently the genetic structure of the parasite could be higher. This was particularly illustrated on the nematode *Heligmosomoides polygyrus* (Heligmosomoidea) which was more strongly structured than its host (*Apodemus sylvaticus*). This result suggests lower rates of gene flow, the parasite playing the role of a biological "evolutionary print" (Nieberding *et al*. 2004, 2005).

Population expansion

Population size changes give particular footprints that may eventually be detected in DNA sequence data (Tajima 1989; Slatkin and Hudson 1991; Rogers and Harpending 1992). The haplotypic relationships portrayed in the network (Fig. 4) showed clear the structuring independently of geographical origin, and the presence of a star-like topology, which suggest a recent population expansion of *H. mixtum*. Further expansion was provided by the smooth mismatch distributions of mtDNA haplotypes observed in the whole sampled animals. Simulations based on coalescent process provided statistical support for the smoothness of the observed distributions (as quantified by the raggedness indices). At demographic equilibrium, mismatch distribution is usually multi-modal, but unimodal in a population having passed through a recent demographic expansion (Slatkin and Hudson 1991; Rogers and Harpending 1992). Although, the mismatch distribution of *H. mixtum* is unimodal for the overall dataset suggesting that the parasite has undergone rapid population expansion. This result is corroborated by Fu's Fs statistic (Fu's Fs = –52.426; P = 0.000 for the all data set sequences) which con-

firmed these inferences about the population dynamics of *H. mixtum*. Moreover, negative and significant Fs statistical values in the total sample gave strong evidence of past population expansion and the positive selection undergone the cytb of mt DNA (F*:–3.30214; **P<0.02; D*:–3.35691; **P<0.02) for all data set sequences.

Although it is difficult to date accurately the expansion time from mtDNA data, we may suggest that the recent expansion in *H. mixtum* population reported here could be facilitated by the Pleistocene or glaciation's overflow. Phylogeography studies on many mammals and plants have revealed that their genetic structure were influenced by geological and climatic events of Pleistocene, which result in population isolation or extinction, and subsequent expansion of the surviving types when environmental conditions became favourable. Pleistocene violent climatic cycles were occurring and geological evidence suggested considerable geological activity occurred in middle Pleistocene, affecting climate and hydrology of this region (Cordy 1991). Indeed, the expansion signal in *H. mixtum* populations would be associated to past bottlenecks associated to the last glacial maximum, which would have been followed by rapid population expansions at the beginning of the present interglacial period. The host generalist behavior of *H. mixtum*, would have favoured its postglacial recolonisation, switching from one rodent species to another and leading to its particular genetic patterns: weak phylogeographic structure, low level of genetic diversity and a rapid population expansion signal.

Conclusion

This study represents the first attempt to use DNA sequence data in a study of intraspecific relationships in *H. mixtum.* This study has shown that intraspecific variations occur in the mt DNA of *H. mixtum* parasites which indicates that there has been a recent and rapid expansion in their evolutionary history. According to Price (1980), parasites populations will tend to be more strongly structured than their hosts suggesting lower rates of gene flow (Burban and Petit 2003; Galbreath *et al.* 2012) and in a few cases host and parasites showed similar degrees of population structure (Nieberding *et al*. 2005). Our results showed that *H. mixtum* is less structured than its main host *M. glareolus*. Indeed, this nematode is not specific to one host species and can authorize host switching events between other syntopic species (Nieberding *et al*. 2008). The cytoplasmic diversity seems to be unstructured with any biogeographic repartition of the variability. The general conclusion emerging from the present study is that the genetic structure of *H. mixtum* is probably associated to weak host specificity.

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