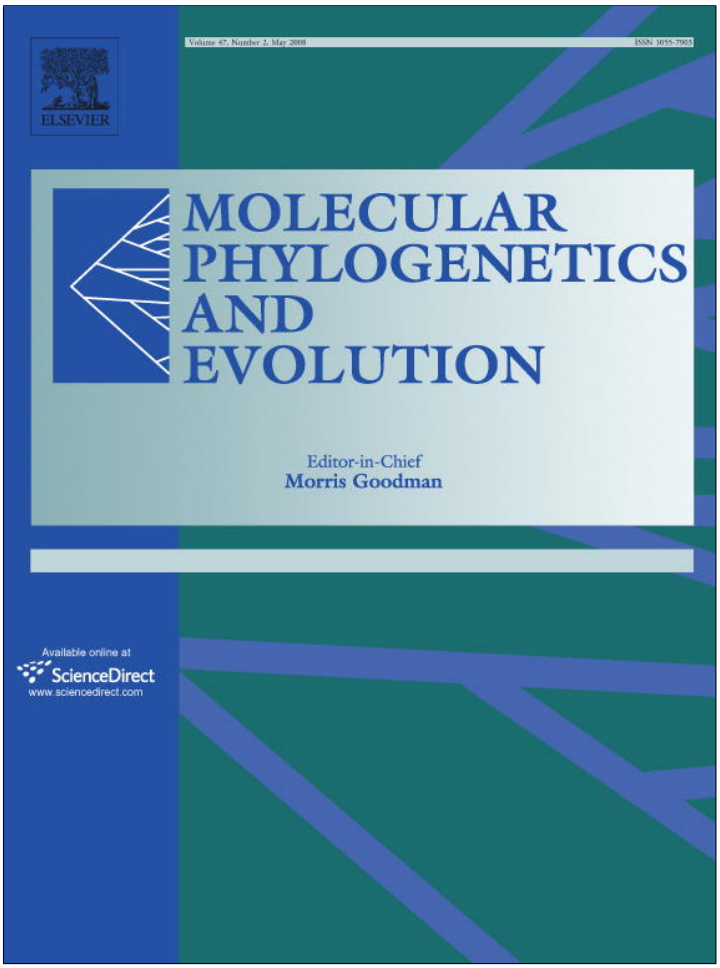

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Geography and host biogeography matter for understanding the phylogeography of a parasite

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Abstract

The co-evolution between hosts and parasites has long been recognized as a fundamental driver of macro-evolutionary patterns of diversification. The effect of co-differentiation on parasite diversification is, however, often confounded by underlying geographic patterns of host distribution. In order to disentangle the confounding effects of allopatric versus host speciation, the mitochondrial cytochrome *b* (*cyt b*) gene was sequenced in seventy individuals of the parasitic nematode genus *Heligmosomoides* sampled in the six *Apodemus* mice species common in the western Palearctic region. The nuclear internal transcribed spacers (ITS) 1 and 2 were also sequenced in fifteen parasites to confirm the mitochondrial data. All lineages differentiated according to a geographic pattern and independently from the sampled host species. This suggests that host speciation did not involve concurrent parasite speciation. However, the geographic distribution range of some parasite lineages mirrors that of *A. sylvaticus* lineages in SW Europe, and that of *A. flavicollis* lineages in the Balkans and in the Middle East. Thus, regional co-differentiation likely occurred between the parasite and the two sister *Apodemus* hosts in different parts of their distribution range. We suggest that differences in regional abundances of *A. sylvaticus* and *A. flavicollis* are responsible for generating this pattern of regional co-differentiation. This study highlights the importance of integrating both geography and biogeographic information from potential hosts to better understand their parasite phylogeography.

Keywords: Biogeography; Comparative phylogeography; Co-differentiation; Common differentiation; Co-speciation; Host; Parasite; *Apodemus*; *Heligmosomoides*; Nematode; Wood mouse; Yellow-necked mouse; Palearctic; Europe; Middle-East

1. Introduction

Interactions among species are a fundamental driver of macro-evolutionary patterns of diversification. One of the most relevant examples of interspecific interactions is the co-evolutionary pattern apparent between hosts and parasites. In host–parasite interactions, co-speciation, i.e., the joint speciation between host and its parasite, can arise if the two organisms share a common evolutionary history, so that the parasite follows the speciation events of its host (Page, 2003). Common history is favoured by traits that strengthen the host–parasite relationship, such as a high level of specificity between the host and its parasite and vertical transmission of parasites among hosts (Page, 2003; Nieberding and Olivieri, 2007). At the phylogenetic level, common history between two taxa remains so far best observed by congruent phylogenetic trees (Huelsenbeck et al., 1997; Charleston and Perkins, 2006).

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Yet, co-speciation is an ongoing process; thus co-differentiation between lineages of a host and its parasite species should be already detectable at the intraspecific level. Since speciation often takes place between allopatric lineages (Schluter, 2001), co-differentiating lineages of a host species and its parasite are expected to be allopatric and the corresponding host and parasite lineages to cover similar geographic areas (Nieberding et al., 2004). In other words, at the intraspecific level, we suggest that a common history is best observed between a host and its parasite whose genetic lineages cover similar allopatric areas (Nieberding et al., 2004; Nieberding and Olivieri, 2007). In this framework, we previously compared the phylogeographic patterns of the wood mouse *Apodemus sylvaticus* Linnaeus 1758 (Muridae, Rodentia), and one of its parasites, the nematode *Heligmosomoides polygyrus* (Dujardin, 1845) (Heligmosomidae, Nematoda) over their continental and insular distribution ranges (Nieberding et al., 2004, 2006). *H. polygyrus* is a specific and direct (i.e., without intermediate host) parasite of rodent hosts of the *Apodemus* Kaup, 1829 genus in the western Palearctic region (Durette-Desset, 1968; N'Zobadila et al., 1996a). *H. polygyrus* reproduces exclusively sexually and its first larval stages are free-living, requiring 4 to 6 days to become infective. Host infection occurs following ingestion of parasitized faeces. Adult parasites live in the intestine and produce eggs in the host faeces about nine days after ingestion, and during up to nine months (Ehrenford, 1954; N'Zobadila et al., 1996b).

H. polygyrus and *A. sylvaticus* were shown to display similar genetic and geographic lineages over south Western Europe (Spain, France and Belgium), Italy and North Africa. Moreover, both species display correlated values of genetic diversity and divergence within and between lineages that share the same geographic range (Nieberding et al., 2004). These shared traits showed that *H. polygyrus* and *A. sylvaticus* displayed a common evolutionary history in the south western Palearctic region over the last 2 millions years, i.e., co-differentiated. We suggested that the common history between the two species might lead to co-speciation over a longer time period and/or larger distribution ranges (Nieberding et al., 2004, 2005b, 2006).

The aim of the present study is to determine whether common differentiation between *H. polygyrus* and *A. sylvaticus* could be observed between parasites from the *Heligmosomoides* genus and other *Apodemus* hosts, and test whether parasite differentiation could be attributed mostly to host or to geography. Our parasite sampling was extended to five additional *Apodemus* species, which are commonly sympatric with *A. sylvaticus* in the western Palearctic region. We expect that, if there is co-speciation at a higher taxonomic level between the two taxa, the genetic lineages of *Heligmosomoides* parasites will be specific to different *Apodemus* host species and thus segregate accordingly to the *Apodemus* species on which they were sampled. In other words, each *Heligmosomoides* lineage would be associated to a particular host species, whatever the geographic origin of the parasites. Alternatively, if co-speciation did not occur at a higher taxonomic level, the genetic lineages of *Heligmosomoides* will rather segregate according to the different geographic regions sampled (allopatric divergence). In other words, each *Heligmosomoides* lineage will be linked to a particular geographic region, whatever the sampled host species might be.

Several pieces of information indicate that the scenario of strict co-speciation between *Heligmosomoides* and *Apodemus* is not the most likely. First, the parasites sampled in the Balkans (former Yugoslavia, Slovenia, Greece, Romania,...) and in Turkey segregated in two distinct genetic lineages (not forming sister taxa) while the corresponding populations of *A. sylvaticus* remained undifferentiated from the Italian ones (Michaux et al., 2003; Nieberding et al., 2004, 2006). This suggests that the history of formation of the parasite lineages might not be tightly linked to *A. sylvaticus* hosts in the eastern part of their distribution range. This would prevent the two taxa from showing a strict co-speciation pattern over a longer time period and a larger distribution range. Second, common differentiation between the parasite and *A. sylvaticus* in south Western Europe was explained by the high level of past and current specificity of the parasite on *A. sylvaticus* (Nieberding et al., 2004). However, field data as well as cumulative bibliographic information highlighted that host specificity of *H. polygyrus* might be less strong than previously supposed (e.g., Durette-Desset, 1968; N'Zobadila et al., 1996a,b; this study). Horizontal transmission of *Heligmosomoides* parasites between different sympatric *Apodemus* host species (see Fig. 1) could prevent the emergence of a co-speciation pattern over longer time periods. Third, parasite lineages from the Balkans and the Middle East rather mirror the geographic differentiation of *A. flavicollis* or of *A. mystacinus* lineages (Michaux et al., 2004, 2005a; Nieberding et al., 2006). The other *Apodemus* species present in the western Palearctic are less likely to have acted upon *Heligmosomoides* differentiation. Indeed, *A. alpicola* and *A. uralensis* display a limited past and present distribution range (Fig. 1). Moreover, fossil records and genetic analysis reveal that *A. agrarius* reached the western Palearctic within the last 10,000 years from the Eastern Palearctic (Kowalski, 2001), which excludes this species as a potential host for *H. polygyrus* in Eastern Europe or in the Middle East during the Quaternary. Thus *A. flavicollis* and *A. mystacinus* will be tested as alternative relevant long-term hosts in the Eastern part of the parasite distribution range.

To discriminate between the alternative hypotheses of co-speciation versus geographic differentiation, we looked for the presence of parasites of the *Heligmosomoides* genus in all *Apodemus* species common in the western Palearctic region: *A. sylvaticus*, *A. flavicollis* Melchior1834, *A. mystacinus* Danford and Alston, 1877, *A. uralensis* (Pallas, 1811) and *A. alpicola* Heinrich, 1952 from the *Sylvaemus* subgenus, as well as *A. agrarius* (Pallas, 1771) from the

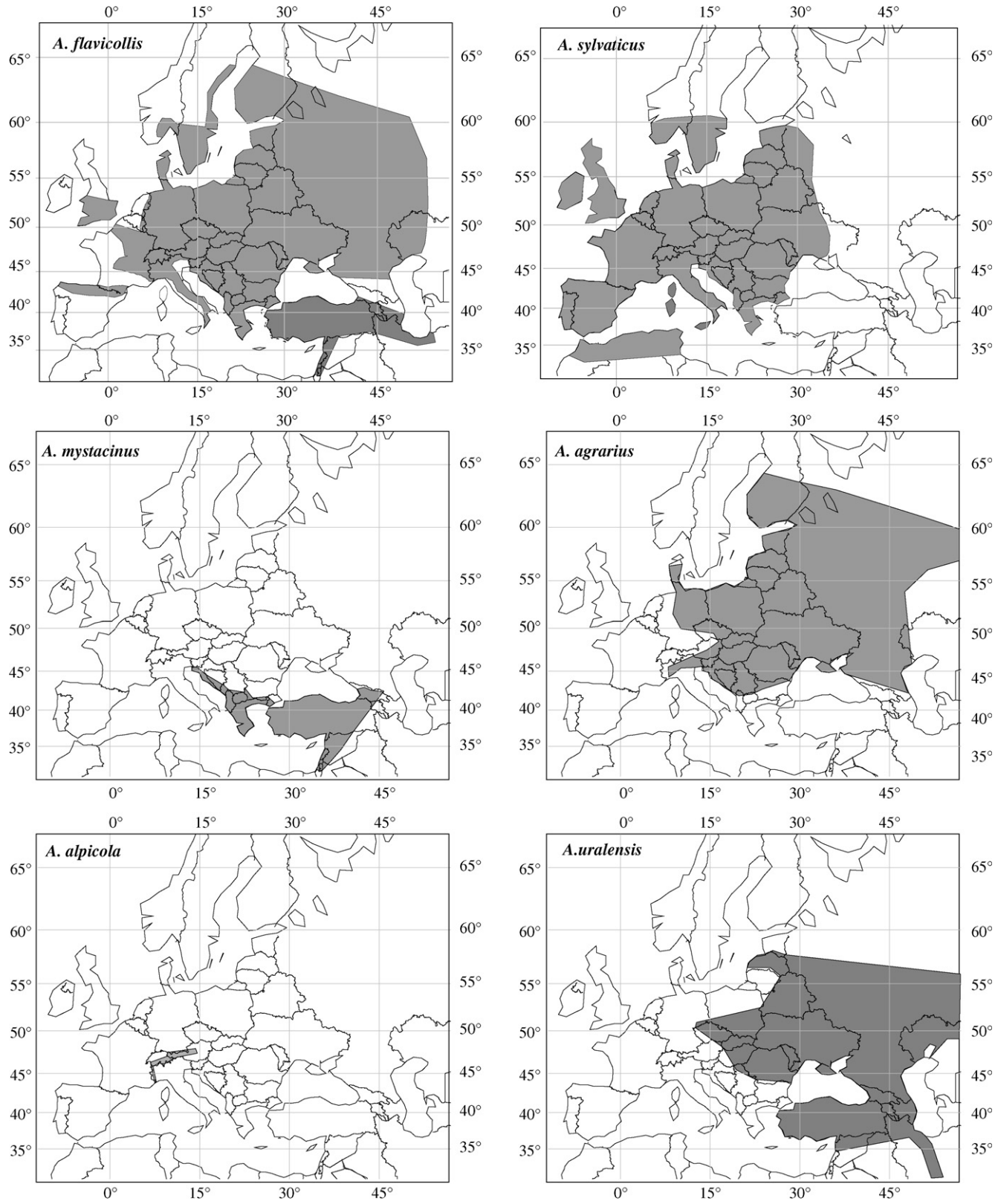

Fig. 1. Distribution range of the six *Apodemus* species common in the south western Palearctic (Europe, Middle East and North Africa), according to updated information found in Panteleyev (1998) and Mitchell-Jones et al. (1999).

Apodemus subgenus (Filippucci et al., 2002; Michaux et al., 2002). Multiple *Heligmosomoides* species were described in *Apodemus* over continental Europe, including *H. skrjarbini* Schulz (1926) and *H. azerbaidjani* Schachnazarova (1949) in *A. sylvaticus*, but they all recently fell into synonymy with *H. polygyrus* (Dujardin, 1845) (see e.g., Tenora

et al., 2003). Four supplementary *Heligmosomoides* species have been described in the western Palearctic region on Arvicolinae (*H. glareoli* Baylis, 1928 in *Clethrionomys glareolus*, *H. laevis* (Dujardin, 1845) Linstow, 1878 in *Arvicola subterraneus*, and *H. tatricus* Erhardova, 1955 in *Microtus nivalis*) and Cricetidae (*H. travassosi* Schulz,1926 in *Cricetus cricetus*) rodent hosts. Yet, the host range of each parasite lineage is not defined, and no genetic data are available, so that the species determinations and their reorganization have been based on morphological data for which diagnostic characters remain limited. Nevertheless, it reveals that *Heligmosomoides* parasites display some level of variation in the studied area. The *Heligmosomoides* individuals found in all *Apodemus* species were sequenced for the mitochondrial cytochrome b (*cyt b*) gene. Because mitochondrial genes do not necessarily convey the evolutionary history of species (Ballard and Whitlock, 2004), we also sequenced the nuclear non coding ITS1 and ITS2 regions of a sub-sample of individuals, in order to confirm the accuracy of the mtDNA-based results. The parasite consensus phylogenetic tree was then compared to the *Apodemus* phylogeny and phylogeographies, to determine whether co-speciation or geographic differentiation was most likely responsible for the observed pattern of parasite divergence. Finally, we dated the divergence times among the parasite lineages.

2. Materials and methods

2.1. Collection of samples

A total of seventy *Heligmosomoides* adult individuals (from twenty-three localities) were sampled on *A. sylvaticus*, *A. flavicollis*, *A. mystacinus*, *A. alpicola*, *A. uralensis* or *A. agrarius*, throughout the south western Palearctic region. Forty of these were obtained in previous studies (Nieberding et al., 2004, 2005b, 2006); the other thirty *Heligmosomoides* individuals sequenced here were sampled from twelve populations in one or more *Apodemus* species (Czech Republic, Slovakia, Romania, Russia, Austria and Poland) (see Table 1 and Fig. 2 for detailed sampling). Our sampling includes representative samples from all previously published genetic lineages for *H. polygyrus*, except the northern European one (present in Ireland and Denmark, see Nieberding et al., 2005b): south western European (Spain, France and Belgium), Moroccan, Italian, Sicilian, Balkan (Austria, Czech Republic, Greece, Slovakia, Slovenia and Romania) and Turkish lineages. This sampling includes *Heligmosomoides* individuals from all common *Apodemus* species in the south western Palearctic, whose distribution ranges partially overlap (Fig. 1). Parasites from each genetic group were identified at the genus level by morphological determination; all hosts were identified at the species level either by morphological determination or by using species-specific markers of *A. sylvaticus*, *A. flavicollis* and *A. alpicola* (Michaux et al., 2001).

2.2. Sequence data

Heligmosomoides DNA was extracted from single individuals using the NaOH digestion protocol of Blaxter et al. (1998). Each nematode was incubated overnight in 20 μl of 0.25 M NaOH. The solution was then heated three min at 99 °C. After the solution had cooled to room temperature, 4 μl of 1 M of HCl, 10 μl of 0.5 M of Tris–HCl (pH 8.0) and 5 μl of 2% of Triton X-100 were added to the solution. The solution was mixed and finally heated for three more min at 99 °C. Six hundred ninety (690) bp of *cyt b* gene were amplified by the polymerase chain reaction (PCR) using the specific primers cytb.1Fc (5′-GRAA TTTTGGTAGTATRTTRG-3′) and cytb.1R (5′-AGMA CGYAAAATWGYAWAAGC-3′) in all new individuals, except Czech and Russian ones, for which the primer cytb.1F (5′-KCWGTRGTTATYACTAG-3′) was used with the primer cytb.1R to amplify the last 400 bp of the 690 bp of the *cyt b* gene. The total length of the nuclear non coding internal transcribed spacers 1 and 2 (ITS1 and ITS2, 500 and 498 bp, respectively) were amplified by PCR using the specific primers ITS1.1F (5′-ACAGT TCAATCGCAATGGC-3′) and ITS2.1R (5′-TTAGTTT CTTTTCCTCCGCT-3′) designed for this study. Amplification reactions of *cyt b* and ITS sequences were carried out in 25 μL including 0.175 μL of 100 μM of the forward and reverse primers, 2 μL of DMSO, 2 μL of 1 mM of dNTP, 2.5 μL of 10× reaction buffer, 0.2 μL of Qiagen *Taq* DNA polymerase, 16.95 μL of ultrapure water and 1 μL of DNA solution. Amplifications were performed in a Labover PTC100 Thermal Cycler using 40 cycles (1 min at 94 °C, 1 min at 48 °C and 1 min 30 s at 72 °C for the *cyt b* amplification; 1 min at 94 °C, 1 min at 53 °C and 3 min at 72 °C for the ITS amplification) with a final extension cycle of 10 min at 72 °C. Four PCRs were performed for each individual. PCR products were concentrated, then purified using the Ultrafree DA Amicon Kit (Millipore) and directly sequenced. Both strands were sequenced using a BigDye Terminator sequencing kit on an ABI 310 automated sequencer (Applied Biosystems) and the primers used for amplification reactions.

2.3. Phylogenetic analyses

The *cyt b* gene data matrix of *Heligmosomoides* comprised 70 *Heligmosomoides* sequences. *Heligmosomoides kurilensis kobayashi* Asakawa and Ohbayashi, 1986 sampled on *Apodemus speciosus* in Japan, and *Heligmonoides sp* sampled on *Mus domesticus* in Syria were used as outgroups. *Heligmosomoides* et *Heligmonoides* belong to the same superfamily (Heligmosomoidea) but to two different families, Heligmosomidae in the case of *Heligmosomoides* and Heligmonellidae in the case of *Heligmonoides*. The sequences from the ITS1 and ITS2 were combined and comprised 15 *Heligmosomoides* sequences; *Heligmosomoides kurilensis kobayashi* was used as outgroup.

Table 1
Geographical distribution, tissue collection codes, host species, and EMBL Accession Numbers of *Heligmosomoides* individuals (690 bp of *cyt b* gene and 988 bp of the ITS regions)

Host	Country	Region	Geo. Code	Samp. Code	EMBL Accession Numbers[a]		
					cytb	ITS1	ITS2
A. sylvaticus	Spain	Catalonia	Sp	Syl.Sp	AJ608806		
			Sp	Syl.Sp	AJ608810	**AM409063**	**AM409079**
	France	Loiret	Fr	Syl.Fr	AJ608848		
			Fr	Syl.Fr	AJ608829		
	Belgium	Liege	Be	Syl.Be	AJ608868		
			Be	Syl.Be	AJ608860		
	Morocco	Haut Atlas	Mo	Syl.Mo	AJ971147	**AM409064**	**AM409080**
			Mo	Syl.Mo	AJ971148	**AM409065**	**AM409081**
			Mo	Syl.Mo	AJ971149		
			Mo	Syl.Mo	AJ971151		
			Mo	Syl.Mo	AJ971152		
	Turkey	Ayder	Tu1	Syl.Tu1	AJ971209		
			Tu1	Syl.Tu1	AJ971210		
		Bozdag	Tu2	Syl.Tu2	AJ971213	**AM409075**	**AM409091**
			Tu2	Syl.Tu2	AJ971211	**AM409076**	**AM409092**
		Ortan	Tu3	Syl.Tu3	AJ971215		
			Tu3	Syl.Tu3	AJ971217		
	Italy	Calabria	It2	Syl.It2	AJ608889	**AM409073**	**AM409089**
			It2	Syl.It2	AJ608884		
			It2	Syl.It2	AJ608885		
		Sicily	It3	Syl.It3	AJ608874		
			It3	Syl.It3	AJ608875		
			It3	Syl.It3	AJ608876		
			It3	Syl.It3	AJ608878		
			It3	Syl.It3	AJ608879		
		Tuscany	It4	Syl.It4	AJ608880		
	Czech Republic	Not known	Cz	**Syl.Cz**	**AM408285**		
	Greece	Crete	Gr1	Syl.Gr1	AJ608914		
			Gr1	Syl.Gr1	AJ608909	**AM409066**	**AM409082**
			Gr1	Syl.Gr1	AJ608913		
		Peloponnese	Gr2	Syl.Gr2	AJ608916		
			Gr2	Syl.Gr2	AJ608917		
A. flavicollis	Spain	Catalonia	Sp	Fla.Sp	**AM408286**	**AM409062**	**AM409078**
			Sp	Fla.Sp	**AM408287**		
	France	Loiret	Fr	Fla.Fr	**AM408288**		
	Turkey	Ayder	Tu1	Fla.Tu1	AJ971219		
	Poland	Lublin	Po	Fla.Po	**AM408290**		
	Italy	Calabria	It2	Fla.It2	**AM408291**		
		Liguria	It1	Fla.It1	**AM408292**		
			It1	Fla.It1	**AM408293**		
			It1	Fla.It1	**AM408294**	**AM409071**	**AM409087**
			It1	Fla.It1	**AM408295**	**AM409072**	**AM409088**
	Greece	Thessaly	Gr4	Fla.Gr4	**AM408296**	**AM409067**	**AM409083**
	Slovakia	Kosicky Kraj	Sa	Fla.Sa	**AM408297**		
			Sa	Fla.Sa	**AM408298**		
			Sa	Fla.Sa	**AM408299**		
			Sa	Fla.Sa	**AM408300**		
	Slovenia	Vipava	Sl1	Fla.Sl1	AJ971193		
			Sl1	Fla.Sl1	AJ971194		
		Pohorje	Sl2	Fla.Sl2	AJ971195		
			Sl2	Fla.Sl2	AJ971196		
	Romania	Baile Herculane	Ro	Fla.Ro	**AM408301**	**AM409070**	**AM409086**
	Czech Republic	Unknown	Cz	Fla.Cz	**AM408302**		
A. uralensis	Russia	Novosibirsk	Ru	Ura.Ru	**AM408303**		
	Slovakia	Kosicky Kraj	Sa	Ura.Sa	**AM408304**	**AM409068**	**AM409084**
			Sa	Ura.Sa	**AM408305**	**AM409069**	**AM409085**
			Sa	Ura.Sa	**AM408306**		
A. agrarius	Poland	Lublin	Po	Agr.Po	**AM408307**		
			Po	Agr.Po	**AM408308**		
			Po	Agr.Po	**AM408309**		

Table 1 (*continued*)

Host	Country	Region	Geo. Code	Samp. Code	EMBL Accession Numbers[a]		
					cytb	ITS1	ITS2
			Po	Agr.Po	**AM408310**		
			Po	Agr.Po	**AM408311**		
			Po	Agr.Po	**AM408312**		
A. mystacinus	Turkey	Ayder	Tu1	Mys.Tu1	AJ971220		
			Tu1	Mys.Tu1	AJ971207	**AM409074**	**AM409090**
		Ortan	Tu3	Mys.Tu3	AJ971222		
			Tu3	Mys.Tu3	AJ971221		
	Greece	Corinthe	Gr3	Mys.Gr3	**AM408313**		
			Gr3	Mys.Gr3	**AM408314**		
A. alpicola	Austria	Tyrol	Au	Alp.Au	**AM408315**		
Outgroups	Japan	*H. kurilensis kobayashi*			AJ971146	**AM409077**	**AM409093**
	Syria	*Heligmonoides sp.*			**AM408289**		

[a] Numbers in bold represent new sequences for the present study.

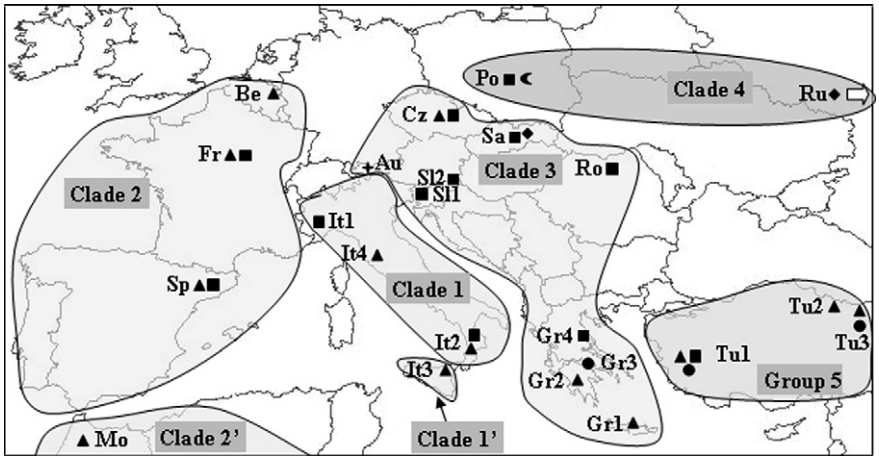

Fig. 2. Geographical distribution of *Heligmosomoides* samples and the extension of their genetic clades. Host species: triangle: *A. sylvaticus*; square: *A. flavicollis*; circle: *A. mystacinus*; cross: *A. alpicola*; diamond: *A. uralensis*; moon: *A. agrarius*. The geographical codes are as in Table 1.

Phylogenetic reconstructions were performed on each of the *cyt b* gene and ITS partitions using a Bayesian procedure as implemented by BayesPhylogenies (Pagel and Meade, 2004). Four chains of 1,000,000 iterations were run and trees were sampled every 10,000 generations to ensure independence of successive trees. The number of generations needed to reach stationnarity in the Markov chain Monte Carlo (MCMC) algorithm was estimated by visual inspection of the plot of the ML score at each sampling point. The trees of the'burn-in' for each run were excluded from the tree set, and the remaining trees from each run were combined to form the full sample of trees assumed to be representative of the posterior probability distribution. An attractive feature of this new MCMC approach is that the performance of increasingly complex substitution models involving several rate matrices can be evaluated without a priori partitioning of the data. We contrasted the performance of increasingly complex models simultaneously employing one to three general time-reversible (GTR) rate matrices, and with or without gamma distributions (with four rate multipliers) to model within-matrix rate heterogeneity. The appropriate model was determined by plotting the log-likelihood values against the standard deviations of the rate matrix parameters. We looked for a cut-off point that corresponds to a slowing in the improvement to the overall log-likelihood and a sharp increase in the error associated with the estimation of the model parameters. In order to test competing hypotheses of host versus geographic differentiation in the parasites, the ITS and *cytb* data matrices were also re-analyzed under the constraint that the clades of the parasite conform to either a host or to a geographic pattern. For that purpose, the Bayesian analysis was re-run under the constraint that the parasites from the same host [e.g., for the ITS data set: ((Syl.Sp, Syl.Mo, Syl.Mo, Syl.It2, Syl.Gr1, Syl.Tu2, Syl.Tu2) (Fla.Sp, Fla.It1, Fla.It1,

Fla.Ro, Fla.Gr4) (Ura.Sa, Ura.Sa) (Mys.Tu1))] or from the same geographic region [e.g., for the ITS data set: ((Fla.Sp, Syl.Sp) (Syl.Mo, Syl.Mo) (Fla.It1, Fla.It1, Syl.It2) (Syl.Gr1, Ura.Sa, Fla.Ro, Fla.Gr4, Ura.Sa) (Syl.Tu2, Syl.Tu2, Mys.Tu1))] are monophyletic. The significance of the difference in average log-likelihood in the constrained and unconstrained runs, respectively, was assessed by computing the Bayes factors. The Bayes factors represent a summary of the evidence provided by the data in favor of a certain model (Kass and Raftery, 1995). A model provides a significantly better representation of the data than another model if twice the difference of the log marginal likelihood returned by the respective models is >5 (Kass and Raftery, 1995; Raftery, 1996; Pagel and Meade, 2004). The marginal likelihoods of the different models are well approximated by the harmonic mean of the likelihoods when the Markov chain is allowed to run for a very large number (millions) of iterations (Kass and Raftery, 1995; Raftery, 1996).

2.4. Co-speciation analyses

TreeMap 1.0b (Page, 1994) was used to test for significant co-differentiation between the parasite and its host. For that purpose, the 50% majority-rule consensus tree of the trees sampled from the posterior probability distribu-

Table 2
Occurrence of the different *Apodemus* species in the Western Palearctic region, gathered from the field missions realized between 1963 and 2004

Country	Year	Number of trapped individuals							References
		Apodemus	*syl*[a]	*fla*[b]	*mys*[c]	*agr*[d]	*ura*[e]	*alp*[f]	
South Eastern Europe									
Czech	2001	69	16	49	0	4	0	0	Vostal and Zakovska (2003)
	2002	67	1	57	0	9	0	0	Vostal and Zakovska (2003)
	1992	11	0	11	0	0	0	0	Zeman and Daniel (1999)
	1992	161	17	144	0	0	0	0	Zeman and Januska (1999)
	1963–1968	537	115	422	0	0	0	0	Prokopic (1972)
	1999–2001	301	78	223	0	0	0	0	Barciová and Macholán (2006)
Greece	2003	13	0	9	4	0	0	0	This study (Nieberding and Deffontaine sampling)
Slovakia	1997–1998	361	0	55	0	306	0	0	Sibold et al. (2001)
	1983–1997	7111	82	3598	0	2538	893	0	Stanko et al. (2002)
	1985–1992	322	21	183	0	109	9	0	Stefancikova et al. (1994)
	2002	22	0	11	0	0	11	0	This study (Morand and Feliu 's teams sampling)
Serbia	1997–1999	2032	0	1524	0	508	0	0	Vukicevic-Radic et al. (2006)
Slovenia	2004	16	1	13	0	2	0	0	This study (Nieberding and Libois sampling)
Total		11023	331	6299	4	3476	913	0	
South Western Europe									
Italy	2001	37	33	4	0	0	0	0	This study (Morand and Feliu's teams sampling)
	2002		2	9	0		0	0	This study (Nieberding and Michaux sampling)
	1992–1993	696	441	255	0	0	0	0	Agnelli and Bellini (1997)
	1991–1992		Dominant	0	0	0	0	0	Canova (1992)
	1984–2001	891	697	194	0	0	0	0	Milazzo (2001)
Belgium	2002	16	16	0	0	0	0	0	This study (Nieberding sampling)
France	1998–1999		Dominant	0	0	0	0	0	de la Pena et al. (2003)
	1995	839	839	0	0	0	0	0	Butet et al. (2006)
	2004–2006	195	60	135	0	0	0	0	J. Deter, N. Charbonnel, JF Cosson, unpubl. data
	2002	24	24	0	0	0	0	0	This study (Nieberding and Goüy sampling)
	2002	34	34	0	0	0	0	0	This study (Morand and Feliu's teams sampling)
Spain	2001–2002	63	43	20	0	0	0	0	Ribas et al. (2004)
	2001–2002	164	150	14	0	0	0	0	Sainz-Elipe et al. (2004)
	1996	29	23	6	0	0	0	0	Arrizabalaga et al. (1999)
	Not known	59	56	3	0	0	0	0	Fernandes et al. (1991)
Switzerland	1993–1994	86	60	26	0	0	0	0	Humair et al. (1995)
	1995–1998	41	8	13	0	0	0	20	Asakawa et al. (1999)
Austria	2002	11	0	0	0	0	0	11	This study (Libois sampling)
Total		3185	2486	679	0	0	0	31	

Northern Europe (including Poland) and the Middle East were not included in the present survey due to the lack of available information. Differences in species abundance was highly significant in each region (proportion tests, $p < 2.2 \times 10^{-16}$).

[a] *A. sylvaticus*.
[b] *A. flavicollis*.
[c] *A. mystacinus*.
[d] *A. agrarius*.
[e] *A. uralensis*.
[f] *A. alpicola*.

tion resulting from the analysis of the *cyt b* gene in *Heligmosomoides* was confronted to the *Apodemus* phylogeny. The latter was inferred from *cyt b* gene sequences for *A. sylvaticus*, *A. flavicollis* and *A. mystacinus* (the three relevant potential host species, see results) that were re-analyzed here in a Bayesian context for consistency (data not shown, available from the authors on request). Because TreeMap requires strictly dichotomous trees, polytomies in the *Heligmosomoides* and *Apodemus* consensus trees were randomly broken by branches of infinitesimal length.

TreeMap computes the fit between the two phylogenies, incorporating a differential cost of the four types of potential events occurring in a host–parasite association: co-differentiation, duplication, sorting and host switching (Page, 2003). Co-speciation tests were performed between partial *cyt b* gene datasets of the parasite and the *cyt b* gene data sets of the hosts sampled in the corresponding geographic locations (see Section 3 for details).

TreeMap includes a testing procedure, by generating random parasite trees and estimating the distribution of the random number of co-speciation events in the host–parasite associations. We generated 10,000 random parasite trees for each host–parasite comparison and the number of co-differentiation events between the 10,000 random parasite trees and the corresponding host tree was calculated. This gives the theoretical distribution of the number of co-differentiation events observed by chance in case the host and the parasite had an independent history. This theoretical distribution was used to estimate whether the number of co-differentiation events observed between the host tree and twenty-one parasite trees sampled within the four hundred trees selected by the Bayesian analysis was significantly higher than by chance alone. Significance was reached if the number of co-differentiation events between a real parasite tree and the host tree was higher than the number of co-differentiation events expected in the 95% confidence interval of the theoretical random distribution. The 95% confidence interval is defined by the mean number of co-differentiation events and two standard deviations of the theoretical distribution. This assessed whether co-speciation in each host–parasite comparison was significantly higher than by chance alone.

2.5. Estimation of divergence time of parasite lineages

It is generally problematic to calibrate the absolute rate of evolution of invertebrates such as nematode parasites, because of the lack of fossil records. Yet, we showed previously, using orthologous DNA coding regions (*cyt b* gene) in both species, that the genetic clades of *H. polygyrus* and *A. sylvaticus* of south western Europe differentiated simultaneously in the past (Nieberding et al., 2004). This allowed us to use the absolute divergence rate between the *cyt b* lineages in *A. sylvaticus*, which was calculated on the basis of rodent paleontological data (Michaux et al., 2003), to estimate the rate of genetic divergence in the corresponding parasite *cyt b* lineages (Nieberding et al., 2004). The level of genetic divergence for *H. polygyrus cyt b* gene was estimated to 3.5–3.7% K_2P (Kimura' s two parameter distance estimator) distance per million years. Genetic divergence was estimated in K_2P distances to allow comparison with most of the published phylogeographic studies (e.g., see Avise et al., 1998), and was corrected for ancestral mtDNA polymorphism (Edwards and Beerli, 2000). This estimation was used in this study to infer the isolation epoch of the new parasite genetic lineages (Polish, Turkish and Moroccan).

3. Results

3.1. Phylogenetic reconstructions of Heligmosomoides

3.1.1. Phylogenetic relationships of cyt b sequences

A total of fifty-two haplotypes were identified among the seventy *Heligmosomoides cyt b* sequences (EMBL Accession Nos. AM408285–AM408314 for the new sequences; Table 1). The matrix provided 690 bp, of which sixty sites were variable. The analysis of the matrix under increasingly complex substitution models simultaneously employing one to three rate matrices, and with or without gamma distributions to model within-matrix rate heterogeneity, suggested that a single-matrix model with a gamma distribution was best appropriate to the data. From that level of complexity indeed, adding more rate matrices did not improve the overall log-likelihood but resulted in a dramatic increase in the standard deviation of the rate parameters (data not shown). The average and standard deviation of the nucleotide proportions, of the rate parameters, and of the shape parameter of the gamma distribution from the posterior probability distribution are provided (additional Table 1 available online).

In the 50% majority-rule consensus of the 394 trees (average $\ln L = -4031.02$) sampled after convergence of the four MCMC's implementing a GTR + Γ substitution model (Fig. 3), all besides the Turkish *Heligmosomoides* sequences fell into the strongly differentiated genetic lineages (Fig. 2). The Italian (clade 1), Sicilian (clade 1′), south-western European (clade 2), Moroccan (clade 2′), the Balkan (clade 3) and the eastern European (Polish and Russian, clade 4) populations form strongly supported lineages (posterior probabilities, noted hereafter p.p., between 0.78 and 1.00). The Turkish populations do not form a clade, but remain clearly differentiated from all other populations (group 5). These clades are also differentiated from the northern European lineage—previously identified in Ireland and Denmark—which was not included in the present study (data not shown; Nieberding, 2005a; Nieberding et al., 2005b).

Constraining the MCMC to sample only trees fitting with the geographic constraint did not result in a significant decrease in log-likelihood (Bayes factor, B. F. = 4.5). By contrast, imposing a structure, wherein parasites from the same host are monophyletic, resulted in a substantial decrease in log-likelihood (B. F. = 707). Thus, *Heligmo-*

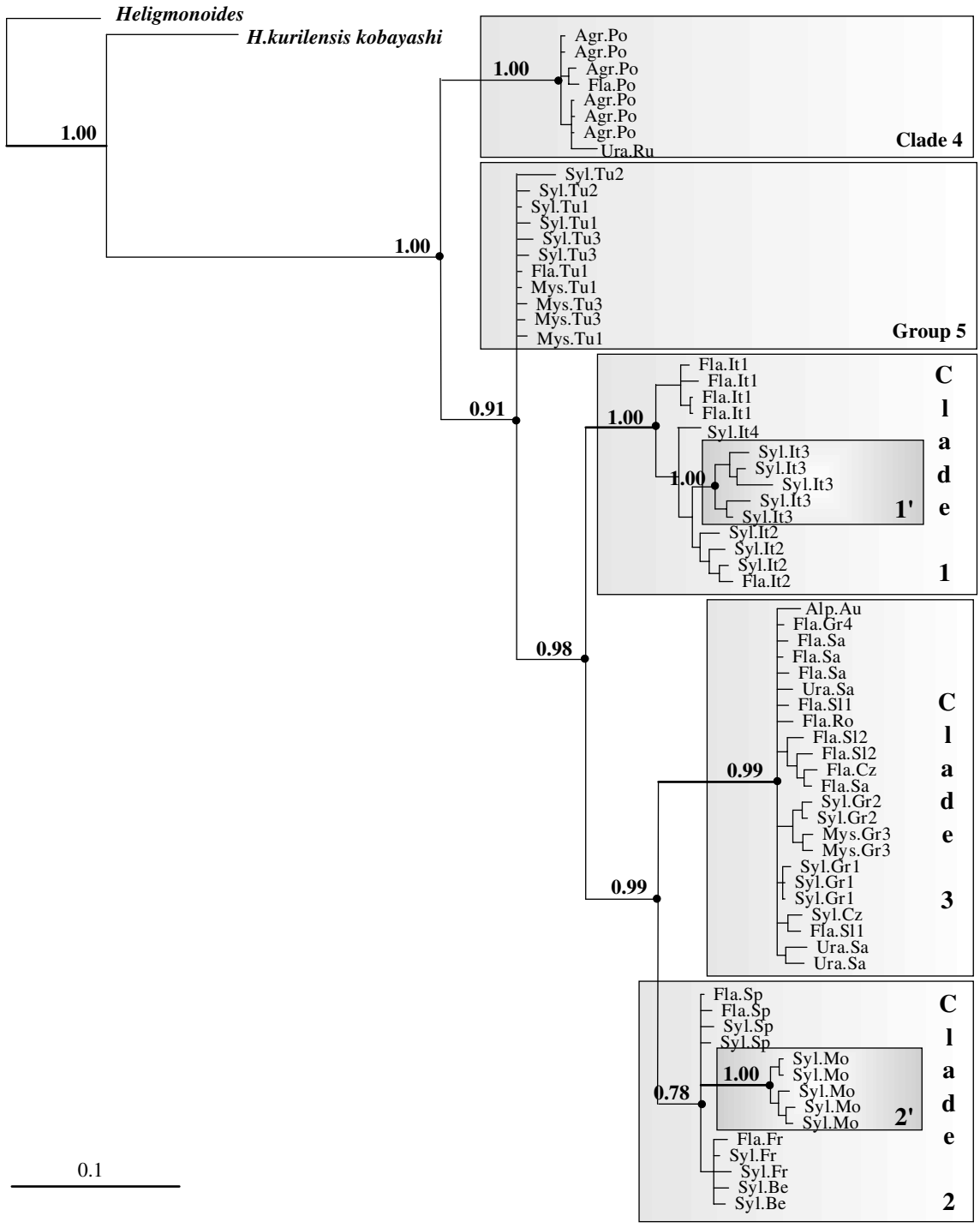

Fig. 3. Fifty percent majority-rule consensus with branch lengths averaged over the 394 trees sampled during the Bayesian analysis of seventy *Heligmosomoides* mitochondrial *cyt b* gene sequences employing four chains and a GTR + Γ substitution matrix. Each accession is represented by its geographical origin and host species name (see Table 1 and Fig. 2). Numbers on branches indicate posterior probabilities >0.75. Bold lines represent nodes that were also supported by posterior probabilities >0.75 in the phylogenetic reconstructions using the ITS data (see Fig. 4).

somoides lineages are not specific to the wood mouse *A. sylvaticus* but can be found in the six most common species of *Apodemus* rodents, namely *A. sylvaticus*, *A. flavicollis*, *A. mystacinus*, *A. alpicola*, *A. uralensis* and *A. agrarius*. Moreover, the phylogenetic reconstructions also reveal that *Heligmosomoides* individuals in the various *Apodemus* hosts did not differentiate according to the host species that they parasitize. Rather, *Heligmosomoides* lineages differentiated in allopatric geographic areas.

3.1.2. Phylogenetic relationships of ITS sequences

ITS1 and ITS2 sequences could be obtained for a few individuals belonging to the clades 1, 2, 2′, 3 and for three Turkish individuals (EMBL Accession Nos. AM409062–

AM409093; Table 1). The matrix provided 988 bp of which thirty sites were variable. The analysis of the matrix under increasingly complex substitution models showed that a single-matrix model was best appropriate to the data (data not shown). The average and standard deviation of the nucleotide proportions and of the rate parameters from the posterior probability distribution are provided (additional Table 1 available online). In the 50% majority-rule consensus of the 396 trees (average ln $L = -2145.50$) sampled after convergence of the four MCMC's implementing a GTR + Γ substitution model (Fig. 4), all *Heligmosomoides* individuals form a monophyletic group (p.p. of 1.00); the existence of the clades 1, 2′ and 3 (p.p. of 1.00, 1.00 and 0.86, respectively) was also confirmed. In contrast to the *cyt b* phylogeny, the south western populations (clade 2 in the *cyt b* phylogenetic tree) do not form a monophyletic lineage. Moreover, the Moroccan subclade is not directly linked to the south western individuals. This could be due to the low level of polymorphism of ITS sequences, which, combined to the small number of sequenced individuals, might limit the resolution of the phylogenetic relationships among the samples. Finally, the Turkish individuals form a monophyletic lineage (clade 5; p.p. of 0.82) distinct from all other populations.

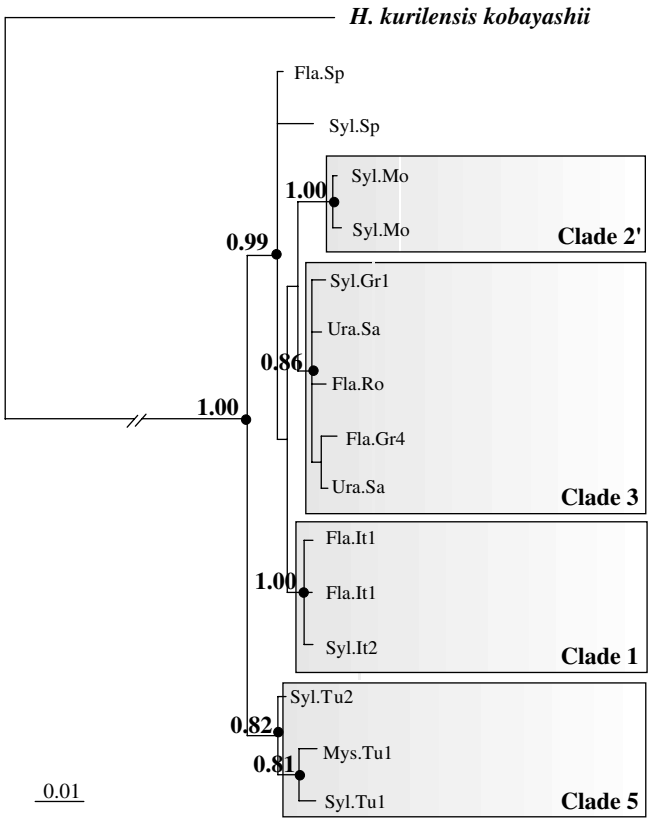

Fig. 4. Fifty percent majority-rule consensus with branch lengths averaged over the 396 trees sampled during the Bayesian analysis of fifteen *Heligmosomoides* ITS sequences employing four chains and a GTR + Γ substitution matrix. Each accession is represented by its geographical origin and host species name (see Table 1 and Fig. 2). Numbers on branches indicate posterior probabilities >0.75.

Both the geographic and host species constraints resulted in significantly lower log-likelihoods. However, while the geographic constraint resulted in a small decrease (B. F. = 13), the species host constraint induced a much bigger decrease in log-likelihood (B. F. = 94). This confirms the results highlighted by the *cyt b* data: parasites group more closely together according to their geographic origin than according to the host species they parasitize.

3.2. Co-differentiation analyses between Heligmosomoides lineages and Apodemus hosts

While strict co-speciation between *Heligmosomoides* parasites and *Apodemus* hosts is not corroborated by the present data, a closer analysis of the geographic subdivision of *Heligmosomoides* lineages highlights some striking similarities with the distribution range of the lineages of some *Apodemus* hosts. Therefore, we next examined whether the parasite lineages showed a level of co-differentiation with either *A. flavicollis* or *A. mystacinus* host species in Eastern Europe and in the Middle East (see Introduction) which was comparable to the level of co-differentiation with *A. sylvaticus* in Western Europe.

First, the level of co-differentiation between *Heligmosomoides* and *A. sylvaticus* in Western Europe, including here the North African populations, was used as a control to interpret the level of co-differentiation observed between *Heligmosomoides* lineages and *A. flavicollis* or *A. mystacinus* in Eastern Europe and in the Middle East. A subset of the *cyt b* gene sequences where hosts and parasites were both sampled was used for phylogenetic reconstructions (Table 3A). The parasite sequences were used here without consideration to the sampled host species, given that it was the hypothesis of geographic differentiation that was tested. The parasite consensus tree, as well as twenty randomly chosen parasite trees sampled from the Bayesian analyses, were generated. The topologies of the host and the twenty-one parasite trees were similar to the phylogenetic trees obtained with the complete datasets (Fig. 5A, left). Using TreeMap, the number of co-differentiation events between each of the 21 parasite trees and the tree of *A. sylvaticus* was calculated. The comparisons of the host and the parasite topologies revealed 15–19 co-differentiation events, depending on which parasite tree was considered in the successive comparisons (Fig. 5C, left). The significance of this result was estimated by generating 10,000 random parasite trees and recalculating the number of co-differentiation events. The results demonstrated that the observed number of co-differentiation events in all of the 21 real parasite trees was significantly higher than what was observed in the 10,000 random associations (Fig. 5B and C, left).

Similarly, we assessed the level of co-differentiation between *A. flavicollis* and *Heligmosomoides* in Eastern Europe and in the Middle East (Table 3B; Fig. 5A, middle). The comparison of the host and the parasite topologies revealed 11–18 co-differentiation events, depending on which of the 21 parasite trees was considered in the succes-

Table 3
Cyt *b* sequences used from *Heligmosomoides* and (A) *A. sylvaticus*, (B) *A. flavicollis* and (C) *A. mystacinus* in the three consecutive TreeMap analyses

Country	Region	Parasite codes	Host	
			Codes	EMBL
A. Heligmosomoides and A. sylvaticus associations (West)				
Spain	Catalonia	Syl.Sp	Sp	AJ511912
		Syl.Sp	Sp	AJ511913
		Fla.Sp	Sp	AJ511880
		Fla.Sp	Sp	AJ511914
France	Loiret	Syl.Fr	Fr	AJ511898
		Syl.Fr	Fr	AJ511899
		Fla.Fr	Fr	AJ511901
Belgium	Liège	Syl.Be	Be	AJ511903
		Syl.Be	Be	AJ511879
Morocco	Haut Atlas	Syl.Mo	Mo	AJ511921
		Syl.Mo	Mo	AJ511920
		Syl.Mo	Mo	AJ511919
		Syl.Mo	Mo	AJ511922
		Syl.Mo	Tn[a]	AJ511917
Italy	Calabria	Syl.It2	It2	AJ511948
		Syl.It2	It2	AJ511949
		Syl.It2	It2	AJ511950
		Fla.It2	It2	AJ511951
	Tuscany	Syl.It4	It4	AJ511929
	Liguria	Fla.It1	It5[b]	AJ511925
		Fla.It1	It5[b]	AJ511926
		Fla.It1	It5[b]	AJ511982
	Sicily	Syl.It3	It3	AJ511962
		Syl.It3	It3	AJ511959
		Syl.It3	It3	AJ511963
		Syl.It3	It3	AJ511960
		Syl.It3	It3	AJ511961
B. Heligmosomoides and A. flavicollis associations (East)				
Turkey	Ayder	Syl.Tu1	Tu1	AJ605681
		Syl.Tu1	Tu1	AJ605680
		Fla.Tu1	Tu1	AJ605668
		Mys.Tu1	Tu1	AJ605667
		Mys.Tu1	Tu1	AJ605670
	Bozdag	Syl.Tu2	Tu2	AJ605677
		Syl.Tu2	Tu2	AJ605679
	Ortan	Syl.Tu3	Tu3	AJ605685
		Syl.Tu3	Tu3	AJ605684
		Mys.Tu3	Tu3	AJ605669
		Mys.Tu3	Tu4	AJ605682
Czeck	Unknown	Syl.Cz	Cz	AJ605607
		Fla.Cz	Cz	AJ605609
Greece	Crete	Syl.Gr1	Gr2[c]	AJ605624
		Syl.Gr1	Gr2[c]	AJ605625
		Syl.Gr1	Gr2[c]	AJ605628
	Peloponnisos	Syl.Gr2	Gr2	AJ605632
		Syl.Gr2	Gr2	AJ605627
	Corinthe	Mys.Gr3	Gr3	AJ605644
		Mys.Gr3	Gr3	AJ605626
	Thessalia	Fla.Gr4	Gr3[d]	AJ605617
Slovaquia	Kosicky Kraj	Fla.Sa	Cz[e]	AJ605605
		Fla.Sa	Cz[e]	AJ605606
		Ura.Sa	Cz[e]	AJ605608
Slovenia	Vipava	Fla.Sl1	Sl	AJ605656
		Fla.Sl2	Sl	AJ605655
	Pohorje	Fla.Sl2	Sl	AJ605657
Romania	Baile Herculane	Fla.Ro	Ro	AJ605646
C. Heligmosomoides and A. mystacinus associations (East)				
Turkey	Ayder	Mys .Tu1	Tu5[f]	AJ748240
	Bozdag	Syl.Tu2	Tu5[f]	AJ748226
		Syl.Tu2	Tu5[f]	AJ748225
	Ortan	Syl.Tu3	Sy[g]	AJ748236
		Syl.Tu3	Sy[g]	AJ748237
		Mys.Tu3	Sy[g]	AJ748238
	Sulema road	Mys.Tu3	Tu5[f]	AJ748239
Greece	Corinthe	Mys.Gr3	Gr1[d]	AJ748234
		Mys.Gr3	Gr2[d]	AJ748230
	Crete	Syl.Gr1	Gr1	AJ748231
		Syl.Gr1	Gr1	AJ748235
		Syl.Gr1	Gr1	AJ748232
	Peloponnese	Syl.Gr2	Gr2	AJ748228
		Syl.Gr2	Gr2	AJ748229
	Thessaly	Fla.Gr4	Gr1[d]	AJ748233
Slovenia	Pohorje	Fla.Sl2	Bu[h]	AJ748241
Romania	Baile Herculane	Fla.Ro	Bu[h]	AJ748227

When the *Apodemus* host sequences and parasite sequences were not available from the same location, we used instead sequences from close regions which belonged to the same genetic clade, as known from the previously published *Apodemus* phylogeographies (see Michaux et al., 2003, 2004, 2005a). Changes are the followings:

[a] *A. sylvaticus* sequence comes from Tunisia (Tn).

[b] *A. sylvaticus* sequences were sampled in the Abruzzi in the centre of Italy (It5).

[c] *A. flavicollis* is absent from Crete. Individuals from the nearby Peloponnese, which colonised Crete (unpublished data), were used instead.

[d] *A. flavicollis* and *A. mystacinus* sequences from different locations in Greece were used according to availability.

[e] *A. flavicollis* sequences come from the nearby Czech Republic.

[f] *A. mystacinus* sequences come from Antalya in Turkey (Tu5).

[g] *A. mystacinus* sequences come from nearby Syria (Sy).

[h] *A. mystacinus* is absent from Romania and from most of Slovenia. Sequences were thus sampled in the nearby Bulgaria (Bu).

sive comparisons. The observed number of co-differentiation events in 11 of the 21 real parasite trees was significantly higher than the ones in the 10,000 random associations (Fig. 5B and C, middle). Finally, the same approach was applied to the comparison between the phylogenetic trees of *Heligmosomoides* and *A. mystacinus* in Eastern Europe and in the Middle East (Fig. 5A, right). When the *Apodemus* host sequences and parasite sequences were not available from the same location, we used instead sequences from close regions which belonged to the same genetic clade (Table 3C). In this case, the comparison of the host and the parasite topologies revealed 4–9 co-differentiation events, and was significantly higher than in the 10,000 random associations in only 7 of the 21 host–parasite comparisons (Fig. 5B and C, right).

To check whether the dissimilarities between host and parasite sampling schemes in the *Heligmosomoides–A. mystacinus* comparison (70% of the host and parasite sequences were not sampled in the very same location, but well in locations belonging to the same genetic clade) were responsible for the lack of co-differentiation, we repeated the co-speciation test between *Heligmosomoides* and *A. sylvaticus*

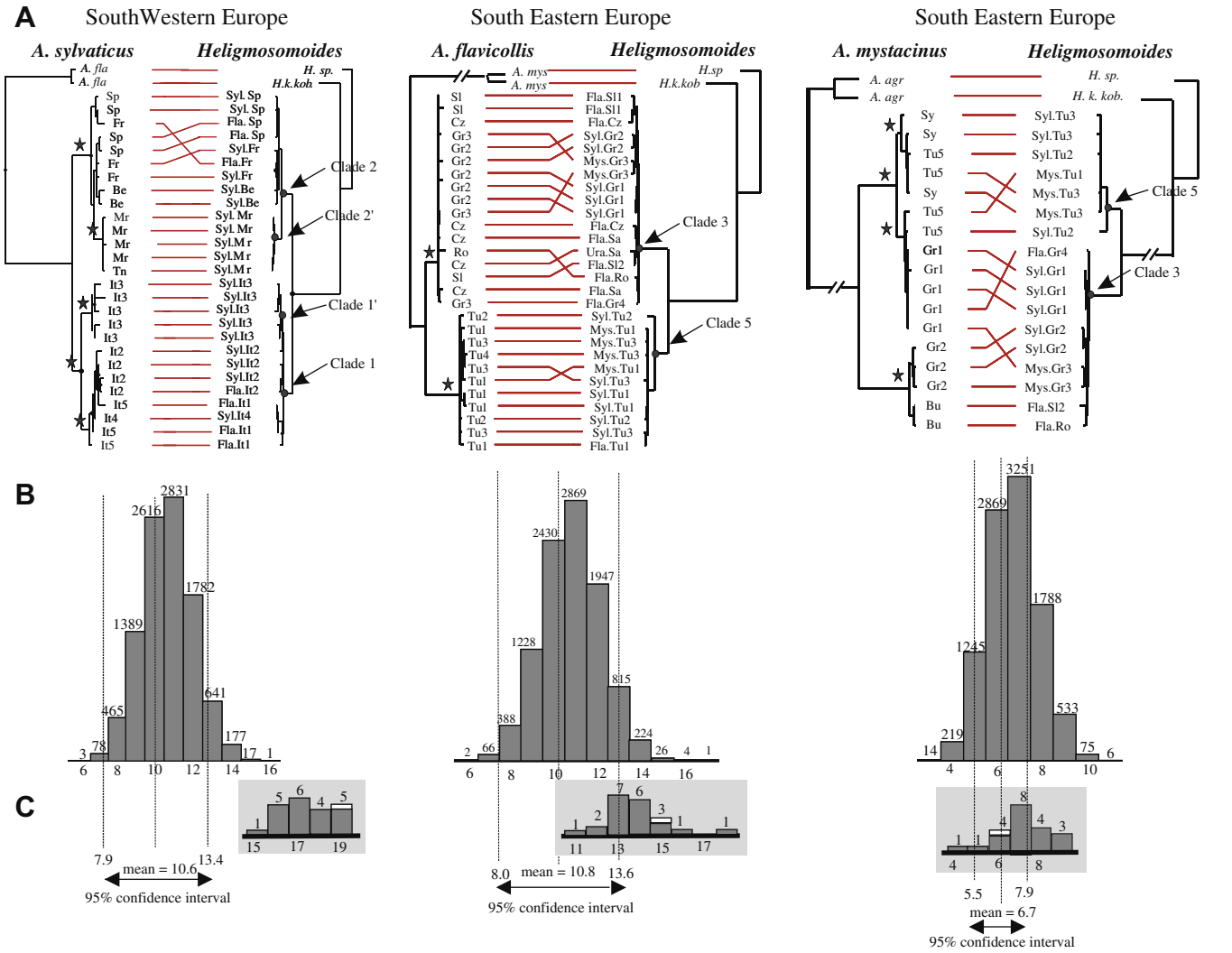

Fig. 5. (A) Pattern of host (*A. sylvaticus* on the left, *A. flavicollis* in the middle and *A. mystacinus* on the right) and parasite associations, using TREEMAP 1.0b (Page, 1994). The host tree and the parasite consensus tree were estimated using Bayesian reconstructions with partial data sets (see M& M). The parasite clades as defined in Figs. 3 and 4 are noted. The host clades that are supported by high bootstrap values when using the whole data sets are mentioned by a star. (B) Histogram showing the distribution of the number of co-differentiation events in associations between the host tree (*A. sylvaticus* on the left, *A. flavicollis* in the middle and *A. mystacinus* on the right) and 10,000 randomly generated parasite trees. The 95% confidence interval of the number of co-differentiation events in these random associations is indicated. (C) Histogram showing the distribution of the number of co-differentiation events in associations between the host tree (*A. sylvaticus* on the left, *A. flavicollis* in the middle and *A. mystacinus* on the right) and 20 real parasite trees (in grey) as well as the parasite consensus tree (in white).

with a similar design using 70% of sequences which had been sampled in different locations but the same clade (data not shown). The number of co-differentiation events (19) observed between the consensus host tree and the parasite consensus tree was identical to the one observed in the original *A. sylvaticus–Heligmosomoides* comparison (see Fig. 5C left). This suggests that the weak host–parasite co-differentiation between *Heligmosomoides* and *A. mystacinus* is probably not due to sampling methodology but is biologically meaningful.

3.3. Estimation of divergence time of parasite lineages

Based on previous estimations (Nieberding et al., 2004), the rate of 3.5–3.7% sequence divergence per Myr in *H. polygyrus cyt b* gene was applied in this study to estimate the differentiation time between the Moroccan, Turkish and Polish lineages of *Heligmosomoides*. The Moroccan lineage was estimated to have differentiated from south Western European populations between 0.54 and 0.57 Million years (Myr) ago. The Turkish lineage differentiated from continental Europe between 1.29 and 1.37 Myr ago, while the Polish lineage differentiated from all other *Heligmosomoides* lineages between 1.81 and 1.91 Myr ago.

4. Discussion

The *cyt b* and ITS phylogenetic reconstructions revealed that *Heligmosomoides* genetic lineages segregate according to the geographic origin of the individuals rather than

according to their host species (Figs. 3 and 4). Yet, the comparison of *Heligmosomoides* regional phylogenetic trees with *A. sylvaticus, A. flavicollis* and *A. mystacinus* phylogenetic trees revealed that co-differentiation was significantly higher than expected by chance between *Heligmosomoides* and *A. sylvaticus* in the West and between the parasite and *A. flavicollis* in the East.

4.1. Regional co-differentiation between Heligmosomoides and A. sylvaticus in the West

Three lines of arguments suggest that *A. sylvaticus* and *Heligmosomoides* co-differentiated in south Western Europe. First, *A. sylvaticus* is the most abundant or unique (in North Africa and in Sicily) potential host species for *Heligmosomoides* in the western part of the parasite continental distribution range. The only other *Apodemus* species present in part of this area is *A. flavicollis* (Fig. 1). Yet, the only European refuge for *A. flavicollis* during the Pleistocene was the Balkan region (Michaux et al., 2004). This is confirmed by paleontological data which show that *A. flavicollis* did not survive up to the last glaciation in western and northern Europe (Aguilar and Michaux, unpubl. data; Cordy, 1984), and attest the presence of *A. flavicollis* in the Iberian peninsula only from the end of the Lower to the beginning of the Upper Pleistocene (Sese, 1994). Because the genetic differentiation of south western *Heligmosomoides* lineages predates that time period by an order of magnitude, *A. flavicollis* cannot be the host on which Iberian and Italian parasites differentiated.

Second, the increasing availability of comparative phylogeographic studies for the western Palearctic region makes it possible to distinguish general phylogeographic trends from specific interactions. Several shared phylogeographic breaks in *Heligmosomoides* and in *A. sylvaticus* in Western Europe are also found in other co-occurring invertebrate or vertebrate species. They include the Pyrenees limiting gene flow between Spain and Northern West Europe and the Alps isolating Italian populations. Although the presence of usual phylogeographic breaks does not exclude co-differentiation between *Heligmosomoides* and its host, co-differentiation might not be the most likely cause of these interruptions. In contrast, other phylogeographic breaks shared by *Heligmosomoides* and *A. sylvaticus* in South Western Europe do not correspond to phylogeographic breaks typically found in many other taxa: i.e., the differentiation of Sicily from continental Italy (even if few studies showed that Sicily is a hotspot of biodiversity in Europe (Randi et al., 2003; Fineschi et al., 2005; Fritz et al., 2005)), and the differentiation of Maghreb populations from Spain. These shared specific phylogeographic breaks are, in contrast to usual phylogeographic fractures, good clues arguing for long-term co-differentiation between the two species (Thompson, 2005). Third, this study confirmed that co-differentiation was significantly more likely than independent history between *A. sylvaticus* and *Heligmosomoides* in south Western Europe (TreeMap analyses, Fig. 5, left).

These three arguments argue in favour of regional co-differentiation between *Heligmosomoides* and *A. sylvaticus* in south-western Europe (Spain, France and Belgium), North Africa, Italy and Sicily. The consequences of co-differentiation for the host and parasite histories have already been discussed elsewhere (Nieberding et al., 2004), except for North Africa. In North Africa, paleontological data attest the presence of the wood mouse since 10,000 years only (Dobson, 1998; Dobson and Wright, 2000). Libois et al. (2001) and Michaux et al. (2003) showed that North African *A. sylvaticus* reached North Africa via the Strait of Gibraltar as a result of anthropogenic introductions during the Holocene. *Heligmosomoides* phylogeographic data in this region confirms this scenario as the Moroccan parasite population clusters within the south western European clade 2. As *A. sylvaticus* is the only possible host species of *Heligmosomoides* in this region, these parasite data strongly suggest that colonization of North Africa by both species likely occurred from Spain.

4.2. Regional co-differentiation between Heligmosomoides and A. flavicollis in the East

In Eastern Europe, the parasite displays differentiated lineages in the Balkans and in Turkey, while the corresponding populations of *A. sylvaticus* remain undifferentiated from the Italian ones (Nieberding et al., 2006). Two relevant alternative host species for *Heligmosomoides* in these regions are *A. flavicollis* and *A. mystacinus*, because both species are abundant in the region and display a deep phylogeographic break between the Balkan and Turkish regions (Michaux et al., 2004, 2005a). Yet, this phylogeographic rupture is usual as the hedgehog *Erinaceus concolor*, the grasshopper *Chorthippus parallelus*, the Black alder *Alnus glutinosa*, the lesser white toothed shrew *Crocidura suaveolens* (reviewed in Hewitt, 1999), and more recently in *Fraxinus angustifolia* and in the brown trout *Salmo trutta* (Bardakci et al., 2006; Heuertz et al., 2006) display it too. Thus, the fracture between Balkan and Turkish populations is not in itself sufficient to argue for co-differentiation between *Heligmosomoides* and either *A. flavicollis* or *A. mystacinus*.

The TreeMap analyses revealed that it was more likely that *Heligmosomoides* co-differentiated in the East with *A. flavicollis* than with *A. mystacinus* (Fig. 5). This result is corroborated by two factors. First, *A. mystacinus* is absent from a large part of the distribution area covered by *Heligmosomoides* Balkan clade (Czech Republic, Slovakia, Slovenia), while in contrast *A. flavicollis* is and has been abundant in the region (Mitchell-Jones et al., 1999). Second, *A. mystacinus* displays two genetic lineages in Turkey, one of which colonized the island of Crete (Michaux et al., 2005a). In contrast, the Turkish populations of *Heligmosomoides* remain undifferentiated, and it was shown

that the parasite colonized Crete from continental Greece (Nieberding et al., 2006).

However, the TreeMap analysis of co-differentiation between *Heligmosomoides* and *A. flavicollis* is less conclusive than between the parasite and *A. sylvaticus* in South Western Europe (Fig. 5). The presence of a second Balkan genetic lineage in *A. flavicollis*, which was not observed in *Heligmosomoides* sampling, might partly explain this result (Michaux et al., 2004). We expect that additional sampling in *Heligmosomoides* eastern distribution range might reveal the additional Balkan lineage in the parasite.

4.3. Relative abundance of host species might matter in parasite co-differentiation

Currently, each *Heligmosomoides* lineage infects all *Apodemus* species present over its distribution range. The situation was likely similar during the Quaternary. During this period, each *Apodemus* species reacted uniquely to the climatic cycles, leading to unique phylogeographic patterns (Michaux et al., 2005b). Therefore, the long-term presence of *Heligmosomoides* in several *Apodemus* species in each regional lineage should have led ultimately to a mixed phylogeographic pattern in the parasite, obscuring the phylogeographic imprint of a particular host species.

Nevertheless, *Heligmosomoides* phylogeographic pattern mirrors regionally the phylogeography of two (and not more) *Apodemus* hosts across its distribution range, *A. sylvaticus* in the west and *A. flavicollis* in the east. How can this have happened? We suggest that regional differences in past and present abundance between *Apodemus* hosts could have led to the observed pattern of regional co-differentiation. In order to estimate the relative present abundances of *Apodemus* rodents in the western Palearctic, the sampling records of available field studies over the last 40 years were compiled (Table 2). This shows that *A. sylvaticus* is the dominant or even unique host species in those regions where *Heligmosomoides* mirrors its phylogeographic pattern, while *A. flavicollis* is predominant in Eastern Europe (proportion tests are highly significant; $p < 2.2e^{-16}$; data from field missions in the Middle East remain too scarce to be included in the present summary). Recent estimations of *A. sylvaticus* and *A. flavicollis* local abundances point in the same direction: *A. sylvaticus* density is maximum in south western Europe (50 individuals per hectare), while *A. flavicollis* is absent from western France and much of the Iberian peninsula, has a limited distribution in the west and south, but has a density of over 100 ind/ha in Eastern Europe (Mitchell-Jones et al., 1999). Similarly, the compilation of *Apodemus* fossil records over Europe highlights that *A. sylvaticus* dominated south Western Europe in the past too (supplementary Table 2). The past predominant abundance of *A. flavicollis* in Eastern Europe appears less straightforward, although the southern part of the Balkans was relatively richer in *A. flavicollis* than the northern Balkans, which is in agreement with the long-term survival of *A. flavicollis* in the south (Michaux et al., 2004). Therefore, past and present predominance of *A. sylvaticus* in south Western Europe and in North Africa might be responsible for the local phylogeographic similarity between its phylogeography and the one of *Heligmosomoides*, despite the parasite presence in other *Apodemus*. The present and possibly past predominance of *A. flavicollis* in Eastern Europe might similarly explain the significant, but less strong, pattern of co-differentiation between its regional phylogeographic structure and the one of *Heligmosomoides*.

4.4. Northern refuge for Heligmosomoides Polish populations?

It is worth mentioning that *Heligmosomoides* displays a distinct and highly differentiated (since around 1.8 Myr ago) clade in Poland and extends into Russia (Fig. 1). Similarly, some species such as the brown bear (*Ursus arctos*) (Taberlet et al., 1998) or different small mammal species [*Clethrionomys glareolus* (Deffontaine et al., 2005); *Sorex minutus* and *S. araneus* (Bilton et al., 1998) and *M. arvalis* (Jaarola and Searle, 2004) and *M. oeconomus* (Brunhoff et al., 2003)] display genetic lineages suggesting that these species also survived in more easterly refuges (the Caucasus or the southern Urals) and in Central Europe (Kotlık et al., 2006). Moreover, the examination of the fossil record of European temperate species during the last ice age (26000–13000 years ago) reveals that environmental conditions were not severe enough in these regions to prevent the survival of forest-dependent species, notably in Poland (Sommer and Nadachowski, 2006). Thus the model of exclusively southern glacial refuges in Europe becomes more and more limited as phylogeographic studies accumulate in north Eastern Europe.

5. Conclusion

Here we tested whether host specificity or geography played a role in structuring the parasite phylogeography. Thus, the parasite shows no specificity (i.e., has no lineages that segregate among host species), and geography, which is the usual cause of genetic structuring for most organisms, explains most of the parasite structuring. Yet, in areas where there is one dominant host species for long evolutionary periods, the parasite shows congruence with host phylogeography. This indicates likely co-differentiation with the host. Therefore, both geography and host phylogeography are important in determining *Heligmosomoides* phylogeographic structure.

Co-differentiation is expected to lead to co-speciation over a longer time period. Yet, this study suggests that co-speciation might not be observed at a higher phylogenetic scale (i.e., over a longer time period) if host specificity is not limited to a single host species. Indeed, regional co-differentiation with different closely related host species might rather ultimately mix the co-differentiation signal if the different host species overlap partially in their distribu-

tion ranges and if the parasite life history traits, such as in *Heligmosomoides*, authorize host switching events between syntopic host species (Weckstein, 2004). In addition, the signal of regional co-differentiation, if generated by differences in the regional abundance of the different hosts, might also be lost if the relative regional host abundance changes over evolutionary time. This is likely to occur when considering the amplitude of climatic changes typical of the last two million years (VanAndel and Tzedakis, 1996; Hewitt, 1999). Third, invasion of new hosts may also disrupt co-differentiation patterns. Therefore, we expect that the high number of strictly co-evolving host–parasite interactions described at the population level (for a review, see Whiteman and Parker, 2005; Wirth et al., 2005; Nieberding and Olivieri, 2007) might not lead to co-speciation between these host and parasite groups over a longer time period. In agreement with this idea, studies showing actual host–parasite co-speciation patterns remain rare among the many studies that compared host and parasite phylogenies (Page, 2003; Charleston and Perkins, 2006).

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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.2008.01.028.

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