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A parasite reveals cryptic phylogeographic history of its host

C. Nieberding[1,†2*] S. Morand S[3], R. Libois[1] and J. R. Michaux[1,2]

[1]*Unité de Recherches Zoogéographiques; Institut de Zoologie; Quai Van Beneden, 22, 4020 Liège, Belgium*
[2]*Laboratoire de Paléontologie—cc064, Institut des Sciences de l'Evolution (UMR 5554-CNRS), Université Montpellier II, Place E. Bataillon, 34095 Montpellier, France*
[3]*Centre de Biologie et de Gestion des Populations, Campus international Agropolis de Baillarguet CS 30016, 34988 Montferrier-sur-Lez, France*

This study compares the continental phylogeographic patterns of two wild European species linked by a host–parasite relationship: the field mouse *Apodemus sylvaticus* and one of its specific parasites, the nematode *Heligmosomoides polygyrus*. A total of 740 base pairs (bp) of the mitochondrial cytochrome *b* (cyt b●●1●●) gene were sequenced in 122 specimens of *H. polygyrus* and compared to 94 cyt b gene sequences (974 bp) previously acquired for *A. sylvaticus*. The results reveal partial spatial and temporal congruences in the differentiation of both species' lineages: the parasite and its host present three similar genetic and geographical lineages, i.e. western European, Italian and Sicilian, and both species recolonized northwestern Europe from the Iberian refuge at the end of the Pleistocene. However, *H. polygyrus* presents three particular differentiation events. The relative rate of molecular evolution of the cyt b gene was estimated to be 1.5-fold higher in the parasite than in its host. Therefore, the use of *H. polygyrus* as a biological magnifying glass is discussed as this parasite may highlight previously undetected historical events of its host. The results show how incorporating phylogeographic information of an obligate associate can help to better understand the phylogeographic pattern of its host.

Keywords: *Heligmosomoides polygyrus*; *Apodemus sylvaticus*; comparative phylogeography; host–parasite relationships; mitochondrial cyt b gene; relative molecular rate

1. INTRODUCTION

Phylogeography is a field of research which studies the processes determining the geographical distribution of genetic lineages at the intraspecific or congeneric levels, and is useful for detecting processes such as population subdivision, speciation events or ecological adaptation and migration routes associated with past climatic changes (Avise 2000). For several years, comparative phylogeography has been developed on sympatric species that are taxonomically and ecologically distant, in order to determine whether they exhibit congruent phylogeographic patterns. Congruence would indicate that the species differentiated in response to similar, possibly the same, geological or environmental events (concerted versus independent response of co-distributed species in reaction to past climatic fluctuations) (Sullivan *et al.* 2000; Lyons 2003). Concordant phylogeographic patterns among distant taxa have been reported in the southeastern USA (Avise 2000) and in the Baja California desert (Riddle *et al.* 2000), as well as among rodent species in central America (Sullivan *et al.* 2000), various bat, rodent and marsupial species in southern America (i.e. da Silva & Patton 1993, 1998; Ditchfield 2000), in Australian amphibians, reptiles (Schneider *et al.* 1998) and snails (Hugall *et al.* 2002). However, other studies have shown that such a concordance is not a general trend as many incongruences appear in the phylogeographic structure of different plants and animals, such as in northwestern North America (Brunsfeld *et al.* 2001; for a review, see Arbogast & Kenagy 2001). This situation is particularly evident in Europe: indeed, although some general trends can be recognized concerning refuge regions, postglacial recolonization routes or localization of contact zones, concordant geographical distribution among genetic lineages of various taxa is rare, suggesting that taxa reacted independently from each other to Quaternary ice ages (Taberlet *et al.* 1998; Hewitt 2001).

In this context, the comparative phylogeography of phylogenetically or ecologically linked species provides an interesting tool to identify and evaluate the roles of historical, stochastic and ecological factors on phylogeographic patterns. When sympatric sister species have been compared, divergences in phylogeographic patterns have been postulated as resulting from recently derived 'life history' or ecological traits. Such divergences are frequent, and of the same order of magnitude, as those found when comparing distant taxa (i.e. Evans *et al.* 1997; Bermingham & Martin 1998; Avise *et al.* 2000; Dawson *et al.* 2002; Rocha *et al.* 2002; Pastorini *et al.* 2003; Michaux *et al.* 2004). In this respect, studies on the comparative phylogeny of taxa strongly linked by an ecological factor, such as parasitism, have shown that the degree of phylogenetic congruence increases with the obligate character of the host–parasite relationship. This has been demonstrated for rodents and

* Author for correspondence (caroline.nieberding@ulg.ac.be).
† Present address: Centre de Biologie et de Gestion des Populations, Campus international Agropolis de Baillarguet CS 30016, 34988 Montferrier-sur-Lez, France

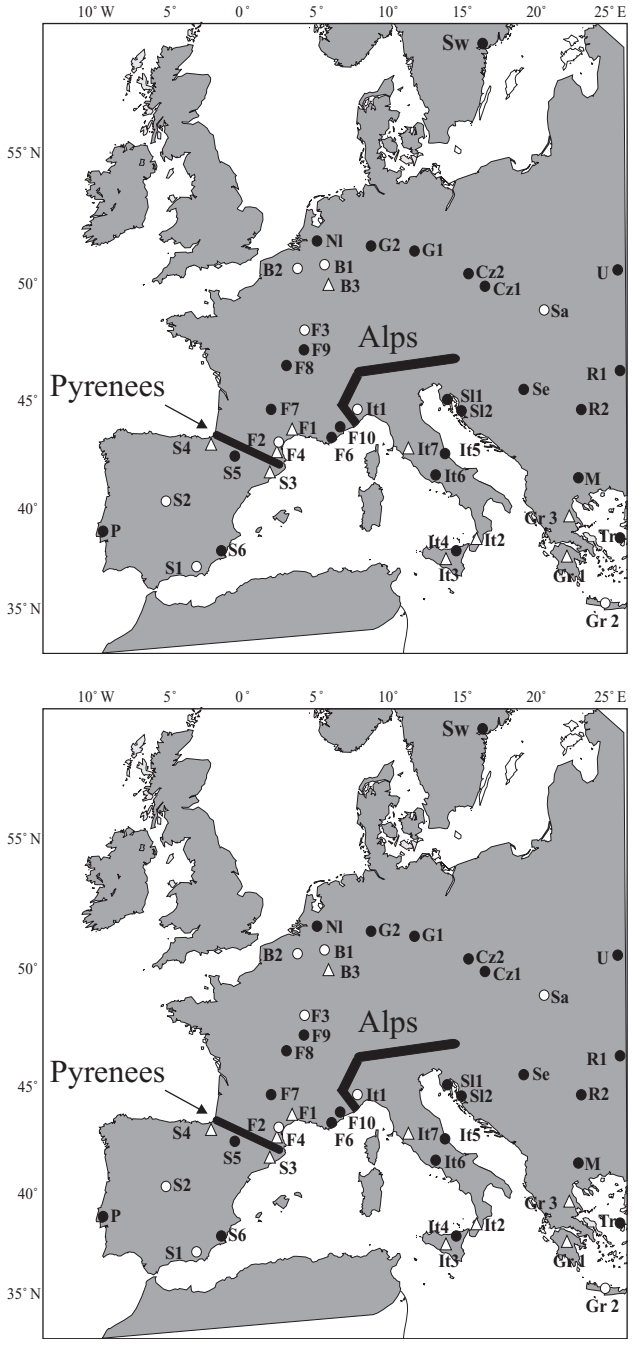

Figure 1. Geographical distribution of *A. sylvaticus* and *H. polygyrus* samples. The shaded zone corresponds to the species distribution area. Sampling localities of *H. polygyrus*: open circles; *A. sylvaticus*: filled circles; both species: triangles. Samples are designated by the countries in which they were collected: B, Belgium; Cz, Czech republic; F, France; G, Germany; Gr, Greece; It, Italy; M, Macedonia; Nl, Netherlands; P, Portugal; R, Romania; S, Spain; Sa, Slovaquia; Se, Serbia; Sl, Slovenia; Sw, Sweden; Tr, Turkey; U, Ukrainia. The thick lines correspond to the main European biogeographic barriers: the Pyrenees (west) and the Alps (east).

their specific ectoparasites at the species or at higher taxonomic levels (Hafner *et al.* 2003). Therefore, at an intraspecific level, it can be assumed that the phylogeographic patterns observed between species linked by a parasitic relationship are likely to be congruent in time as well as space, provided the parasite is specific and obligate (Price 1980).

The aim of the present study was to compare the phylogeographic patterns of a 'host–parasite' pair, the woodmouse *Apodemus sylvaticus* (Rodentia, Muridae), and one of its parasites, *Heligmosomoides polygyrus* (Dujardin, 1875; Nematoda, Heligmosomoidea). These species were chosen because *H. polygyrus* is a direct (without intermediate host) and specific endoparasite of *A. sylvaticus*, and reproduces on an exclusively sexual mode. Its first larval stages are free and require 4–6 days to become infective. Host contamination occurs after ingestion of parasitized faeces; adult parasites live in the intestine and produce eggs in the host faeces *ca.* 9 days after ingestion, and continues for up to nine months (Ehrenford 1954). *Heligmosomoides polygyrus*'prevalence—i.e. percentage of hosts infected and abundance—i.e. average number of parasites per host on *A. sylvaticus*, are high, respectively 50% and 11%, and do not vary among populations from different geographical origins (Goüy de Bellocq *et al.* 2002, 2003). The variation in helminth density is cyclic and seasonal and follows the population dynamics of *A. sylvaticus* (Montgomery & Montgomery 1988●●2●●). Importantly, *A. sylvaticus* is a forest dweller, present throughout Europe since at least 3 Myr ago (Michaux *et al.* 2003), and the two species are common and geographically widespread in the Western Palaearctic region.

The phylogeographic pattern of these two species were compared by sequencing the cytochrome *b* mitochondrial gene (cyt b●●1●●). This allowed us to: (i) estimate the degree of spatial and temporal congruences of their phylogeographic histories; (ii) estimate the relative rate of evolution of their homologous cyt b gene; (iii) identify the ecological factors possibly responsible for the observed phylogeographic patterns.

2. MATERIAL AND METHODS

(a) *Samples collection and DNA sequencing*

A total of 122 adult *H. polygyrus* specimens sampled in 81 *A. sylvaticus* in 19 localities distributed throughout its European range were analysed (figure 1), and compared with 94 previously described *A. sylvaticus* specimens trapped in 35 localities (Michaux *et al.* 2003). Detailed locality data and sampling are available from the authors on request. All animals were identified at the species level, by morphological or species-specific PCR analyses (Michaux *et al.* 2001). Tissues of both species are preserved in the collection of J. Michaux and C. Nieberding housed at the Centre de Biologie et de Gestion des Populations, Montferriez-sur-Lez (France).

Since the genetic distances (Kimura's 2 parameters distance estimator (K_2P)) calculated among *H. polygyrus* present in a single host are of the same order of magnitude as those between parasites living in different individuals of the population (data not shown), the parasites found in a single host can be considered as independent samples.

DNA from *H. polygyrus* was extracted as reported in Goüy de Bellocq *et al.* (2001). A total of 740 base pairs (bp) of the cyt b gene were amplified by the PCR specific primers 1F (5′-GRAATTTTGGTAGTATRTTRG-3′) and 1R (5′-AGMACGYAAAATWGYAWAAGC-3′) for the western European and Balkan clades, and 4F (5′-TTCAGATTGTYACYGGYAC-3′) and 4R (5′-ACGGTAAAATTGTATAAGC-3′) for the Italian clade. DNA extraction, amplification and sequencing of 974 bp of cyt b of *A. sylvaticus* samples were carried out as described in Michaux *et al.* (2003).

(b) *Data analysis*

i) *Phylogenetic analyses*

Cyt b sequences were aligned using the MUST package (Philippe 1993). The complete *H. polygyrus* data matrix comprises 114 haplotypes as well as *Heligmosomoides kurilensis kobayashii* Asakawa and Ohbayashi, 1986, used as an outgroup (EMBL accession numbers AJ608805 to AJ608917). The matrix includes 740 bp of which 228 sites are variable and 157 parsimony informative. The mean transition to transversion ratio is 2.18 and the nucleotide frequencies are 25.08%, 5.96%, 19.46% and 49.49% for A, C, G and T respectively (see Michaux *et al.* (2003) for *A. sylvaticus* data information). Both cyt b datasets were analysed for saturation by comparing, in scatterplots, the ratio transition–transversion at each codon position. No saturation was observed for any type of substitution and at any codon position for both species datasets.

Heligmosomoides polygyrus data were analysed by distance (neighbour joining, NJ) using PAUP 4.0b8 (Swofford 1998), and maximum likelihood using the PhyML package (Guindon & Gascuel 2003●●2●●). Modeltest version 3.06 (Posada & Crandall 1998) was used to determine the best-fit substitution model for the parasite data, which was GTR + I + G ($-\ln L = 4775.73$, I = 0.46, Gamma distribution shape parameter = 0.52). A Bayesian approach to phylogeny reconstruction was also implemented using MrBayes 3.0 (Huelsenbeck & Ronquist 2001). The robustness of inferences was assessed by bootstrap resampling (BP) (100 random repetitions for the Bayesian method, Douady *et al.* (2003); 10000 random repetitions for NJ and PhyML analyses).

Apodemus sylvaticus data were analysed by distance (NJ) and maximum parsimony (MP) methods, using PAUP 4.0b8 (TBR branch swapping option, Maxtrees = 1000; Swofford (1998)). The best substitution model for the host data was GTR + I + G. The robustness of inferences was assessed by BP (10 000 random repetitions for NJ and MP analyses).

TreeMap 1.0b (Page 1994) was used to test for significant co-differentiation of host and parasite phylogenetic trees (heuristic search); it computes the fit between the two phylogenies, incorporating a differential cost of the four types of potential events occurring in a host–parasite association (cospeciation, duplication, sorting and host switching, see Page (1994)). TreeMap includes a testing procedure, by generating random host and/or parasite trees and comparing the random number of cospeciation events in the association to the observed number to assess whether it is significantly higher than by chance alone. This analysis was based on partial host and parasite datasets: 43 haplotypes of the 10 populations shared by the two species, i. e. B3, F1, F4, S3, S4, Gr1, Gr3, It2, It3, It7 (see figure 1). The partial consensus trees of both species were obtained by distance (NJ) using PAUP 4.0b8 (TBR branch swapping option, Maxtrees = 1000). The substitution model for the parasite and host data was GTR+I+G.

ii) *Phylogeographic analyses*

A minimum spanning network was constructed using the MINSPNET algorithm available in the Arlequin 2.0 program (Schneider *et al.* 2000), using the Tamura–Nei and Gamma options of substitution model of evolution. Population genetic structure was determined by analysing the molecular variance (AMOVA) and calculating ϕ_{st}, available in the Arlequin 2.0 program. Genetic distances between the groups of samples were obtained using a distance analysis (K_2P). Nucleotide (π) and haplotype (h) diversities were estimated using the DnaSP 4.0 program (Rozas & Rozas 1997). A 'mismatch distribution' of substitutional differences between pairs of haplotypes was calculated within the main genetic lineages and compared with a fit to the Poisson model using DnaSP 4.0.

(c) *Estimation of time differentiation*

On the basis of paleontological data of the genus *Apodemus* sp., an approximate timing of divergence between the observed mtDNA lineages was calculated for the cyt b in *A. sylvaticus* (Michaux *et al.* 2003); the rate of 2.6–2.85% K_2P distance per million years, taking into account the correction for ancestral mtDNA polymorphism (Edwards & Beerli 2000), allowed to provide an absolute molecular dating to be determined for the different dichotomies observed within *A. sylvaticus*.

It is generally problematic to calibrate the absolute rate of evolution of invertebrate parasites, because of the lack of fossil records. However, the use of orthologous DNA coding regions (cyt b) in both species allowed us to test for simultaneous differentiation of *H. polygyrus* and *A. sylvaticus* genetic clades. The principle, as proposed by Hafner & Nadler (2003)●●3●●, is to plot the genetic distances of pairs of hosts (*X*-axis) against corresponding pairs of cospeciating parasites (*Y*-axis). Although it is not statistically valid to fit a line to these points, because of the dependence among the elements within each taxa, the resulting plot provides an assessment of the relationship between the two distance data (Hafner & Nadler 1990); the relative rate of evolution of both species is given by the slope, and the *y*-intercept reveals information about the relative timing of divergence events in the two taxa.

Two different methods were tested. The first method (test A) is based on the comparison of the genetic distance between five groups defined in both species co-differentiated datasets according to the geographical and genetic structures of *H. polygyrus* in Western Europe and in Italy: two groups were defined in the western European group (2No, 2SW), and three in the Italian group (1a, 1b, 3) (see figure 2 and table 1 for the definition of the groups), allowing a total of 10 comparison points in each species. The population average pairwise distance between each pair of groups in both species were calculated using Arlequin 2.0 (Schneider *et al.* 2000), using the Tamura–Nei and Gamma options of substitution model of evolution. The correlation coefficient, the confidence interval at 95%, and the standard error associated to the variation of the slope within the confidence interval, and to the *y*-intercept, were calculated with Statistica 6.1 (Statsoft France). As these points are non independent, the significance of the correlation coefficient was tested by the estimation of the linear correlation between the two distance matrices by a permutative procedure implemented using Permute 3.4 (Casgrain 1994).

Second, we used TreeMap 1.0b (Page 1994), which compares branch lengths of host haplotypes and co-differentiating parasites in a bivariate plot and displays the correlation coefficient between the branch lengths in the two trees (test B). Moreover, TreeMap includes a testing procedure, by generating random parasite trees and comparing the random correlation coefficient in the association to the observed value to assess whether it is significantly higher than by chance alone. This analysis was based on partial host and parasite datasets (eight co-differentiating populations shared by the two species, i.e. B3, F1, F4, S3, S4, It2, It3, It7).

As the divergence time calculated for the separation of the *A. sylvaticus* lineages was viewed as equivalent to the one separating the corresponding clades of *H. polygyrus*, the estimate of mean K_2P distances between these parasite lineages, corrected for ancestral mtDNA polymorphism, gives the mean K_2P distance

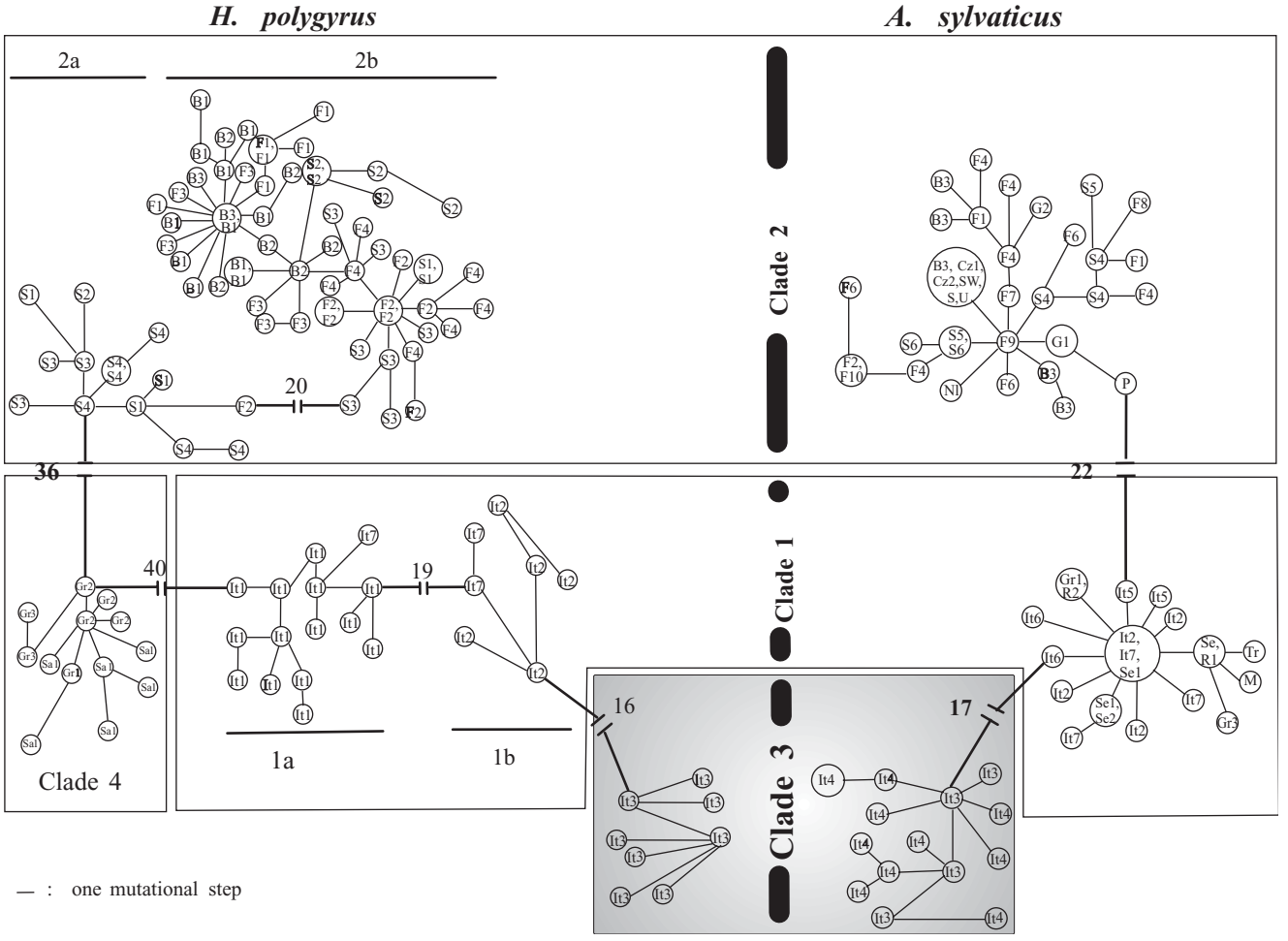

Figure 2. Minimum-spanning networks constructed using mitochondrial cyt b gene sequences. The *H. polygyrus* network is shown on the left and the *A. sylvaticus* network is on the right. Branches length corresponds to the mutational steps separating haplotypes; for simplicity, haplotypes separated by only one mutational step have been associated. Geographical origins are described in figure 1. Clade 2 corresponds to the western European populations, clade 3 to the Sicilian populations, clade 4 to the Balkan populations. Clade 1 corresponds to the Italian populations in *H. polygyrus* network, and to the Italo-Balkan ones in *A. sylvaticus*. In *H. polygyrus*, clade 1 is further divided in sub-clades 1a (northern Italian populations) and 1b (southern Italian populations); clade 2 is divided in two partially syntopic groups, 2a and 2b.

per million years for *H. polygyrus*, which was then used to date the isolation time of the own clades of the parasite.

3. RESULTS

(a) *Spatial comparison of host and parasite phylogeographic patterns*

The minimum spanning networks of 114 haplotypes of *H. polygyrus* and 94 haplotypes of *A. sylvaticus* show partial genetic and geographical congruences (figure 2): in both species, a western European (clade 2), an Italian (clade 1) and a Sicilian clade (clade 3) appear. The Sicilian *H. polygyrus* and *A. sylvaticus*' clades 3 are linked to their clade 1 populations by 16 and 17 mutational steps, respectively. However, the parasite's genetic structure appears more diverse and complex. In *A. sylvaticus*, the Italian clade also includes Balkan populations, and is separated from the western European clade by 22 mutational steps. By contrast, *H. polygyrus* Italian (clade 1) and Balkan (clade 4) populations form two distinct clades and are separated from the western European clade by 36 mutational steps. Moreover, *H. polygyrus*' clades 1 and 2 are further divided into several sub-clades: the Italian clade 1 is itself formed by 2 geographical and genetic lineages, 1a (northern Italy) and 1b (southern Italy), separated by 19 mutational steps; and clade 2 forms two genetic groups (sub-clades 2a and 2b), separated by 20 mutational steps. By contrast, the corresponding populations of *A. sylvaticus* cannot be distinguished. Moreover, the mean number of mutational steps separating haplotypes is higher in the parasite than in its host (6.0 versus 3.7).

The host clade 1 and parasite sub-clade 2b show a star-like topology, suggesting that these populations are the result of a recent expansion from a small number of animals. The other clades of both species appear more heterogeneous.

The consensus phylogenetic trees of 114 haplotypes of *H. polygyrus* and 94 haplotypes of *A. sylvaticus* globally show the same genetic structure as their corresponding networks (data not shown): clade 1 of *A. sylvaticus* (96 and 81% BP in NJ and MP) is formed by two highly differentiated clades in *H. polygyrus*, clade 1 (98, 100 and 53% BP in NJ, PhyML and Bayesian methods respectively) and clade 4 (100, 100 and 98% BP respectively). Similarly, *A. sylvaticus*' clade 2 (86 and 77% BP) is formed in *H. polygyrus* by sub-clades 2a (94, 78 and 51% BP) and 2b

Table 1. Genetic variability observed within the five main genetic lineages of *H. polygyrus* (*H. pol*; clades 1, 2a, 2SW, 2No, 3 and 4) and the corresponding genetic lineages of *A. sylvaticus* (*A. syl*; clades 1, 2SW, 2No and 3). (*Apodemus sylvaticus* clade 2 and *H. polygyrus* clade 2b were divided into Iberian populations (group 2SW; populations S1-S6 and P) and all northern European populations (group 2No; populations B1-3, Cz1-2, F1-F10, G1-G2, Nl, R1-R2, Sw and U). The genetic divergence observed within each of these groups is calculated according to the distance method K_2P (%).)

groups	sample size		number of haplotypes		genetic divergence(%) ± s.d.		nucleotide diversity (π) ± s.d.		haplotype diversity (h) ± s.d.	
	H. pol	*A. syl*	*H. pol*	*A. syl*	*H. pol*	*A. syl*	*H. pol*	*A. syl*	*H. pol*	*A. syl*
group 2No	46	26	41	20	1.46±0.059	1.09±0.006	0.012±0.001	0.005±0.001	0.997±0.012	0.965±0.028
goup 2SW	12	27	11	23	2.50±0.017	1.41±0.004	0.023±0.005	0.009±0.001	0.997±0.034	0.981±0.023
clade 2a	14	—	12	—	1.99±0.018	—	0.018±0.006	—	0.995±0.034	—
clade 1	23	26	21	25	2.70±0.015	1.22±0.004	0.026±0.003	0.006±0.001	0.992±0.015	0.968±0.014
clade 4	14		13		1.32±0.060		0.012±0.002		0.981±0.031	
cade 3	12	15	10	15	2.78±0.011	1.33±0.005	0.021±0.002	0.013±0.001	1.000±0.045	1.000±0.024

(100, 94 and <50% BP). Sicilian populations of both species form a distinct clade 3 (100 and 98% BP for *A. sylvaticus* and 51, 85 and 100% BP in its parasite). Moreover, *H. polygyrus* clade 1 is further subdivided in two lineages, 1a (97, 93 and <50% BP) and 1b (67, 81 and 78% BP respectively). However, in contrast to the network analysis, the parasite Sicilian populations (clade 3) are not the sister clade of continental Italy (clade 1), but form a distinct sub-clade within sub-clade 1b. *Heligmosomoides polygyrus* clade 4 is not directly connected to the clade 1 but is the sister clade of clade 2, but this association is not well supported by BP (73%, <50%, and <50% BP).

To test for significant co-differentiation between host and parasite phylogenetic trees, the distance trees based on the 10 populations where hosts and parasites were both sampled (see § 2), were realized for both species and compared using TreeMap. The distance trees showed identical topologies as the phylogenetic trees obtained with the whole data ets (figure 3). The reconciliation of both topologies called upon 21 cospeciation, 19 duplication, 1 host switch and 146 sorting events. The fit between both trees was estimated by generating 10 000 random parasite trees and recalculating the number of cospeciation events. The results demonstrated that the observed number of cospeciation events is significantly higher than the ones in the 10 000 random associations (mean number of cospeciation events: 16.38 ± 3.58).

The estimate of nucleotide (π) and haplotype (h) diversities, and mean genetic divergence (K_2P distance), allowed inference of the general population dynamic trends of the two species. Analyses were performed on clades 1 and 3 in *A. sylvaticus* and on clades 1, 2a, 3 and 4 in *H. polygyrus*. Moreover, to assess whether nucleotide diversity was higher within potential refuge regions compared with northern populations, *A. sylvaticus*' clade 2 and *H. polygyrus*' clade 2b were divided into two subgroups: the first corresponding to the Iberian populations (group 2SW) and the second to all the northern European populations (group 2No) (table 1). Within the five main groups, the 2SW group is characterized by a significantly higher π ($p < 0.001$, Student t-test) and mean genetic divergence ($p < 0.001$, Kruskal–Wallis test) for both species. Both parasite clade 4 and host clade 1 show very low levels of genetic divergence, π and h, which reflect genetic bottlenecks and recent population expansion from a small number of founder individuals. In clade 3, both species present high levels of π and h, typical of stable populations with large long-term effective population numbers. Finally, h, π and mean genetic divergence values are globally two to three times higher in *H. polygyrus* than in *A. sylvaticus*, confirming the more diverse genetic structure of the parasite as compared to that of its host.

(b) *Temporal comparison of host and parasite phylogeographic patterns*

AMOVA analyses were performed on both parasite and host sequences in order to estimate the part of genetic variability distributed either among the major clades, among populations within the major clades or within populations. In both species, most of this variability (62.8% for *H. polygyrus* and 76.2% for *A. sylvaticus*) is distributed among the previously defined major genetic clades. Moreover, ϕ_{st} values of both species, 0.83 for

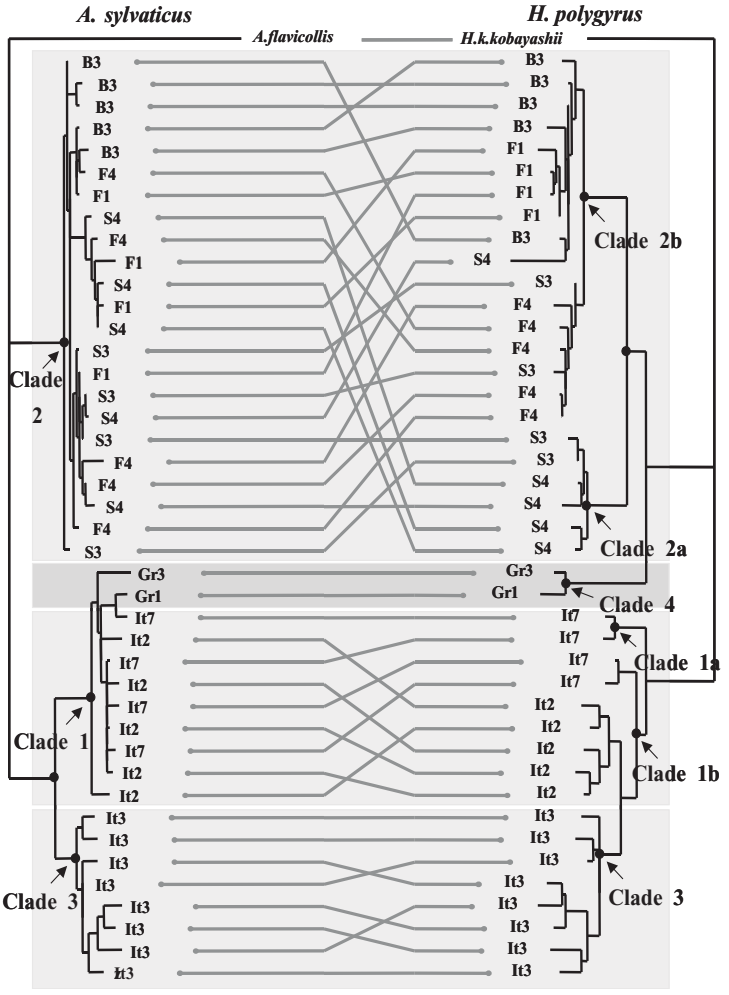

Figure 3. Pattern of host and parasite associations, using TREEMAP 1.0b (Page 1994). Host and parasites trees were estimated by NJ on partial datasets (see § 2); both trees showed similar topologies to the phylogenetic trees obtained with the whole datasets.

H. polygyrus and 0.79 for its host, are similar and important. Therefore, both in the host and in the parasite, the majority of mutations distinguish the main clades, and have accumulated since their isolation. The remaining genetic variability in the AMOVA analysis (37.2% for *H. polygyrus* and 24.8% for its host) is distributed in populations within clades and within populations.

Second, to estimate more precisely the relative rates of evolution and the timing of divergence events in both species, we plotted the genetic distances of pairs of hosts (*X*-axis) against the corresponding pairs of co-differentiating parasites (*Y*-axis). The Balkan populations (Gr1, Gr2, Gr3) were therefore excluded from this analysis as the minimum spanning network and the phylogenetic analyses disagree about its connection with either the Italian or western European groups, in *H. polygyrus*. The *H. polygyrus* Spanish endemic haplotypes (clade 2a) were also excluded, as they did not correspond to any *A. sylvaticus* co-differentiating populations.

The first method (test A in § 2) consisted in calculating the population average pairwise distance between five co-differentiating geographical groups in both species (figure 4*a*). The determination coefficient obtained ($r^2 = 0.82$) is highly significant ($p = 0.002$) and the *y*-intercept of the linear relationship ($y = 1.40x + 3.29$) is not significantly different from zero (confidence interval at 95%). The range of variation of the slope within the confidence interval at 95% is 1.40 ± 0.23 (s.e., $p < 0.001$).

TreeMap compared the branch lengths of partial host and parasite datasets (test B in § 2). The correlation coefficient of the linear relationship ($y = 1.57x - 2.82$) between the branch lengths in the two trees, r^2, reached 0.79 (figure 4*b*) and the *y*-intercept is not significantly different from zero (confidence interval at 95%). The range of variation of the slope within the confidence interval at 95% is 1.57 ± 0.11 (s.e., $p < 0.001$). The randomization of parasite tree and the comparison of the random correlation coefficient in the association (mean correlation coefficient value $= 0.23 \pm 0.12$) to the observed value showed that this correlation is significantly higher ($p = 0.0001$) than by chance alone.

(c) *Evolutionary molecular rates of cyt b gene*

The hypothesis of temporal congruence—or synchronous co-differentiation—between host and parasite genetic clades, and the use of orthologous DNA coding regions (cyt b gene), allowed us to examine the relative rates of evolution in the two groups by comparing the degree of evolutionary change that each species underwent during the period of parallel phylogenesis (Hafner *et al.* 2003). Figure 4 shows that this relative rate is 1.5 higher for *H. polygyrus* than for its

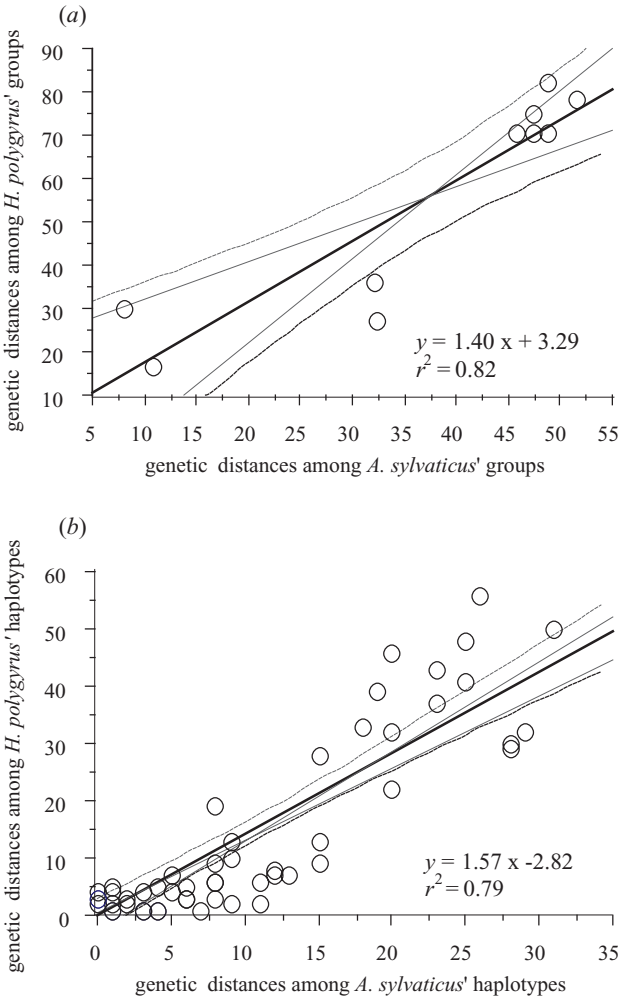

Figure 4. Genetic divergence between correspondent pairs of genetic groups (Test A in § 2; (*a*)) or haplotypes (Test B in § 2; (*b*)) of *H. polygyrus* and *A. sylvaticus*. The coefficient of determination (r^2) and the equation of the correlation line are given. The relative rate of molecular evolution of cyt b between both species is given by the slope (bold line). The variation of the slope of the correlation line within the confidence interval at 95% is represented (normal lines). The *y*-intercept informs about the relative timing of divergence events in the two species; note that in both graphs the *y*-intercept is not significantly different from 0 (confidence interval at 95%, dashed lines).

host. Moreover, since *H. polygyrus* may have co-differentiated synchronously with *A. sylvaticus*, the calibration points derived from palaeontological data of the genus *Apodemus* sp. (Michaux *et al.* 2002) can be used to date the internal nodes of the parasite (Page *et al.* 1998). We previously estimated the molecular evolution rate of the *A. sylvaticus* cyt b gene at 2.6–2.85% K_2P distance per million years, and dated the separation between the western European and the Italo-Balkan clades at 1.5–1.6 Myr ago (Michaux *et al.* 2003). Assuming the separation time of the two correspondent clades (western European and Italian) of *H. polygyrus* is similar, and taking into account a correction factor for ancestral mtDNA polymorphism, the molecular evolution rate of the parasite can be estimated at 5.58% K_2P

distance, i.e. 3.5–3.7% K_2P distance per million years, a value nearly 1.5-fold higher than that of the corresponding *A. sylvaticus* lineages. Therefore, the separation time between sub-clades 2a and 2b, 1 and 3, and 1a and 1b, can be estimated at 0.98–1.04, 0.53–0.56 and 0.60–0.64 Myr, respectively. Divergence time for the Balkan clade was not assessed due to a lack of concordance between the phylogenetic trees and the network.

4. DISCUSSION

(a) *Comparison of parasite and host phylogeographic patterns*

i) *Similarities between parasite and host phylogeographic patterns*

The present study shows that *H. polygyrus* and *A. sylvaticus* partially display the same phylogeographic history. Indeed, the parasite and host continental European populations both differentiated into three main geographical and genetic lineages: the first is widespread from southern Spain in the South to Sweden in the North and Central Europe in the East (clades 2b and 2 respectively), the second is located in Italy (clade 1) and the third one in Sicily (clade 3). This structure could be explained by the isolation of three groups of hosts and parasites into three different refuges (Iberian Peninsula, Italy and Sicily) during previous ice ages. This isolation was probably reinforced by the presence of the Alps, which acted, and probably continue to act, as a biogeographic barrier for both species. Based on our calculations, these two factors probably prevented genetic exchanges between the two groups in both species for at least 1.5–1.6 Myr ago●●4●●. At the end of the last Ice Age (*ca.* 10 000 years ago), both species expanded northward within the Western Palaearctic region exclusively from the Iberian refuge, as shown by the distribution of the clades (parasite sub-clade 2b and host clade 2) and by the significantly higher genetic diversity for both species in southwestern Europe compared with northern populations (table 1). This scenario of expansion is corroborated by a bell-shaped distribution in the mismatch distribution analyses performed for both species (data not shown). By contrast, Italian populations of both species did not expand to the North, while Sicily constitutes another major refuge for both species.

ii) *Differences between parasite and host phylogeographic patterns*

However, these major phylogeographic similarities in the parasite and host patterns are counterbalanced by the presence, in *H. polygyrus*, of particular clades that do not exist in its host, which highlights that the parasite genetic structure is more diverse that the one of its host. Indeed, within *H. polygyrus*:A second clade (sub-clade 2a) is found in the Iberian Peninsula and in southern France, but it did not contribute to northern recolonization of Europe, in contrast to syntopic sub-clade 2b. They differentiated *ca.* 1 Myr ago.

Within clade 1, two allopatric lineages exist, one located in northern Italy (1a), and the other in southern Italy (1b). They may have differentiated *ca.* 1.3 Myr ago. These subdivisions can be explained by independent parasite differentiation inside the common ancestral host lineage. This ancient isolation of host populations in the Iberian Peninsula and in Italy may have been associated with the fragmentation of the Mediterranean forests during the Quaternary ice ages (Blondel 1995).

The parasite Sicilian populations (clade 3) are differentiated within the southern Italian ones (clade 1b), while in *A. sylvaticus*, the Sicilian populations are well differentiated from the continental Italian ones (clade 1). This result highlights the southern Italian origin of Sicilian populations of both species.

The phylogeographic history of the two species appears more complex in the Balkan region. Indeed, *A. sylvaticus* populations from Italy and the Balkans form a single genetic group, while the parasite Balkan populations (clade 4) are genetically well differentiated from the Italian ones. Further, considering both the network or phylogenetic analyses, the relationship of the parasite Balkan populations remains unclear (figures 2 and 3). The analysis of the genetic diversity in Balkan populations shows that both species are characterized by a very low genetic diversity (genetic divergence, π and h values; table 1). This is somewhat surprising because palaeontological and palaeoclimatological data (Reille & de Beaulieu 1995; Tzedakis *et al.* 1997) attest that the Balkans were a refuge region for *A. sylvaticus* during the Quaternary glaciations. This low genetic variability could be explained by the appearance of a genetic bottleneck during one of the last Ice Ages. This hypothesis is supported by our data: (i) the star-like topology of the minimum spanning network suggesting a rapid expansion from a small number of founder animals, and (ii) the slope of the mismatch analysis fitting a Poisson distribution typical from populations in expansion (data not shown; Avise 2000). Therefore, one scenario could be proposed: the bottleneck that severely affected parasite and host populations in the Balkans allowed Balkan parasite populations to rapidly differentiate from the Italian ones and accumulate mutations due to low population effective size. *Apodemus sylvaticus* residual Balkan populations may also have differentiated, more slowly, from the Italian ones, but they were replaced by the latter during expansion at the end of the last Ice Age, after transmission of the Balkan parasite populations. This scenario may explain the unclear origin and low diversity values of parasite Balkan populations.

iii) *Biological 'magnifying glass': Inferring host phylogeographic history from its parasite*

The present study showed partial co-differentiation between *H. polygyrus* and *A. sylvaticus* in Western Europe (comparison of network, phylogenetic, TreeMap and diversity analyses). In a second step, we tested for simultaneous differentiation time of the major clades detected in both species. AMOVA and ϕ_{st} suggested ancient and concomitant genetic differentiation in both species. The hypothesis of temporal congruence was further tested by comparing the genetic distances co-differentiating parasites and hosts. Both tests (A and B) showed a good correlation between parasite and host data and estimated that the y-intercept of the linear relationship is not significantly different from zero, suggesting that corresponding genetic groups or haplotypes in both species differentiated simultaneously.

Therefore, we feel confident to consider that the parasite and its host differentiated in response to the same major

environmental and ecological events, and that the differentiation pattern of one species influenced that of the other.

In consequence, the evolutionary pressure that generated the parasite genetic structure may logically also act on that of its host, even if the current genetic marker does not reveal it in the latter. Indeed, the cyt b molecular rate relatively higher in the parasite may be one factor explaining this lack of host phylogeographic signal (see below●●5●●).

In this context, *H. polygyrus* highlights undetected historical events of its host. The presence of two ancient lineages in the *H. polygyrus* western European group, and in continental Italy, suggests the existence of at least four disjunctive glacial refuge areas within the Iberian Peninsula and in Italy during the Quaternary ice ages both for the parasite and its host. The isolation of these refuges was probably not sufficient to involve a genetic differentiation among *A. sylvaticus* populations. In Spain, this hypothesis is supported by other studies suggesting the existence of several distinct refuge areas in the Iberian Peninsula during the past 2 Myr in the European rabbit *Oryctolagus cunniculus* (Queney *et al.* 2001) and the rodent *Microtus agrestis* (Jaarola & Searle 2004).

Within the Western European group (clade 2) of the parasite, nucleotide diversity is higher in the Iberian Peninsula than in southern France (table 1). This result strongly suggests that the French side of the Pyrenees was recolonized recently from the Iberian region and was not a refuge for the wood mouse or for its parasite.

Although the Sicilian populations of both species are differentiated from the Italian continental ones (figure 2), the isolation between continental and insular populations of *H. polygyrus* seems to have occurred *ca*. 0.55 Myr ago, while the Sicilian host populations appear to have separated from each other 0.8–0.9 Myr ago (Michaux *et al.* 2003). Therefore, contacts between Sicilian and southern continental *A. sylvaticus* populations seem to have persisted until 0.5 Myr ago according to the genetic proximity of parasites in both areas and indicates the existence of several connections between Sicily and southern Italy during the last million years (Thaler 1973).

iv) *Ecological and molecular mechanisms for parasite speciation*

The partial co-differentiation pattern of *H. polygyrus* and *A. sylvaticus* can be explained by the life-history traits of both species: (i) host–parasite specificity, (ii) direct cycle of the parasite, (iii) short survival time of larvae during the free stage, and (iv) limited dispersal abilities of the parasites on their own. Therefore, parasite gene flow is strictly dependent on movements and contacts between individual hosts (Blouin *et al.* 1995). *Heligmosomoides polygyrus* abundance and prevalence on *A. sylvaticus* are high, respectively 11 and 50% (Goüy de Bellocq *et al.* 2003). As such, there is a reduced risk of extinction and of 'missing the boat' respectively (Clayton *et al.* 2003), and this explains how the parasite was able to follow its host in its response to Pleistocene climatic changes.

However, the population genetic differentiation is more pronounced and the genetic diversity in *H. polygyrus* twice that in its host (table 1). According to Frankham *et al.* (2002), the genetic diversity and differentiation in a population depends on five main factors: the breeding system, the selection, the migration (gene flow), the current and historical effective size *Ne* (demographic events), and the mutation rate μ. First, both the parasite and its host display sexual reproduction and a sex ratio of approximately 1 : 1; therefore, this factor cannot explain the difference in genetic diversity and differentiation between them. Second, the selection on the cyt b gene can be considered equivalent in both species. Third, *H. polygyrus* gene flow, determined primarily by the movement of its host (Blouin *et al.* 1995), involves parasite differentiation because successful reproduction between wood mice does not guarantee successful *H. polygyrus* populations genetic mixing in their intestines (Nadler & Hafner 1990); only 50% of *A. sylvaticus* are infected, transmission by infected migrants might not be successful and nematodes might be locally adapted to different environments. As such, gene flow between parasite populations lags behind that of their hosts; during Quaternary ice ages, sporadic gene flow between host refuges were probably not transmitted to its parasite. This kind of barrier promotes parasite self-differentiation (such as in the Western European or Italian lineages), and, from a macroevolutionary perspective, reduces congruence (Blouin *et al.* 1995; Clayton *et al.* 2003). Fourth, phylogeographic datasets are not appropriate to estimate *Ne* because they cannot be considered as a single population from an extended temporal perspective (large, high gene flow and historically non-sundered population; Avise (2000)). In a phylogeographic context, *Ne* does not depend on the current intrinsic diversity but rather on the species demographic history in the evolutionary time (Avise 2000). However, the mtDNA diversity in parasitic nematodes depends on the abundance and prevalence of the parasite in its host, two factors that directly affect *Ne* (Blouin *et al.* 1998). We may therefore propose the following scheme: the abundance and prevalence of *H. polygyrus* on *A. sylvaticus* are quite high, involving a rapid accumulation of mutations, and the mode of infestation of *H. polygyrus* involving continual founding events may strengthen the persistence and transmission of distantly related copies of mtDNA (Page *et al.* 1998), thus increasing the parasite genetic diversity and differentiation in comparison with the one of its host. Fifth, important sequence diversity can also result from an accelerated rate of nucleotide substitution (Blouin *et al.* 1995). Indeed, as shown above, the rate of evolution of cyt b gene is 1.5-fold higher in *H. polygyrus* than in *A. sylvaticus*. This result is consistent with phylogenetic studies highlighting a faster molecular evolutionary rate of the mitochondrial DNA of nematodes, compared with all other taxa (Blouin *et al.* 1998; Anderson *et al.* 1998; Denver *et al.* 2000). Four similar studies have suggested more rapid rates of substitutions (2.5–5.5-fold) in the parasitic taxa (lice of the genus *Dennyus*, *Collodennyus* and *Halipeurus*; Hafner *et al.* (1994); Page *et al.* (1998); Paterson & Banks (2001)) and bacteria of the genus *Buchneria* (Moran *et al.* 1995) relative to their hosts. At the molecular level, a range of mechanisms were proposed to account for these differences, including metabolic rate, base composition and population size (Page *et al.* 1998), but none have been conclusively demonstrated.

5. CONCLUSION AND PERSPECTIVES

These results argue that comparative phylogeography can be used to evaluate phylogeographic patterns as well as evolutionary processes (Bernatchez & Wilson 1998). The

temporal congruence in genetic differentiation among corresponding lineages in *H. polygyrus* and *A. sylvaticus* shows the relevance of this model for the comparison of the evolution of phylogenetically distant species over the same time-period. In particular, such an approach provides estimates of relative rates of evolution of a homologous gene, cyt b, in two distantly related taxa.

The accuracy of concerted versus independent response of co-distributed species in response to past climatic fluctuations (Zink 1996; Sullivan *et al.* 2000) seems to depend mainly on the strength of the ecological link among species. The present results show that partial congruent phylogeographic patterns in Western Europe, in Italy and in Sicily can be observed between species linked by a strong ecological trait, such as specific and direct-cycle endoparasitism. In these regions, the parasite may act as a biological magnifying glass as it may reveal previously undetected phylogeographic information in the host, such as distinct allopatric refuges in the Italian and Iberian peninsulas, and historically persistent gene flow between Sicily and southern Italy. By contrast, this hypothesis cannot be supported or refuted in the Balkan region, for which the parasite phylogenetic affinities with Italy remain unclear. Therefore, incorporating phylogeographic information of an obligate associate, i.e. a parasite, may allow a better understanding of the phylogeographic pattern of its host, as parasites can be treated as biological markers of their hosts (Hafner *et al.* 2003).

6. UNCITED REFERENCE

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