doi:10.1111/j.1420-9101.2009.01863.x

Genetic diversity, population structure and sex-biased dispersal in three co-evolving species

S. FOITZIK, S. BAUER, S. LAURENT & P. S. PENNINGS

Department of Biology, Ludwig Maximilians Universität Munich, Planegg-Martinsried, Germany

Keywords:

ants;
coevolution;
host–parasite interaction;
phylogeography;
population genetics;
population structure;
sex-biased dispersal.

Abstract

Genetic diversity and spatial structure of populations are important for antagonistic coevolution. We investigated genetic variation and population structure of three closely related European ant species: the social parasite *Harpagoxenus sublaevis* and its two host species *Leptothorax acervorum* and *Leptothorax muscorum*. We sampled populations in 12 countries and analysed eight microsatellite loci and an mtDNA sequence. We found high levels of genetic variation in all three species, only slightly less variation in the host *L. muscorum*. Using a newly introduced measure of differentiation (Jost's D_{EST}), we detected strong population structuring in all species and less male-biased dispersal than previously thought. We found no phylogeographic patterns that could give information on post-glacial colonization routes – northern populations are as variable as more southern populations. We conclude that conditions for Thompson's geographic mosaic of coevolution are ideal in this system: all three species show ample genetic variation and strong population structure.

Introduction

To understand the course of evolution of a species or a group of interacting species, we need to measure not only the selective forces that work on them, but also their adaptive potential (Gandon & Nuismer, 2009). It is hard to study the evolutionary potential of natural populations directly as we would need to know the genes currently under selection and variation at these loci. Although we do not know how strongly heritable variation in relevant traits is correlated to neutral variation, both parameters are equally influenced by the demographic history of populations, effective population size, standing genetic variation, population structure and patterns of gene flow (Bos *et al.*, 2008). Thus neutral genetic markers can help us to understand the adaptive potential of populations. The same data can shed light on the recent history of species, for example the impact of the last ice age (Hewitt, 2004). Finally, by studying both biparentally inherited nuclear microsatellite loci and mitochondrial markers, which are only maternally transmitted, we can indirectly examine male and female dispersal (e.g. Holzer *et al.*, 2009).

Species that are engaged in a coevolutionary arms race, a continuing process of reciprocal adaptation, are required to adapt as fast as they can to keep up with their opponent (Dybdahl & Storfer, 2003). For these species it is important that their adaptive potential is higher than that of their antagonists. In most parasite–host interactions, parasites such as bacteria and viruses have a much higher evolutionary potential than their hosts, because of their larger population sizes and shorter generation times. An exception to this rule is found in parasites that are phylogenetically more closely related to their hosts, such as avian brood parasites (Martinez *et al.*, 1999) and the social parasites of ants, bees and wasps (Brandt *et al.*, 2005). The latter take advantage of the social system of heterospecific insect societies, in a fashion analogous to avian brood parasites that exploit the brood care behaviour of other species. Social parasites are very closely related to their hosts (Emery, 1909), and the antagonists in these systems are therefore characterized by similar generation times, population sizes, and mutation and recombination rates.

Correspondence: Susanne Foitzik, Biologie II, Ludwig Maximilians Universität München, Grosshaderner Str. 2, D-82152 Planegg-Martinsried, Germany. Tel.: 49 89 2180 74 209; fax: 49 89 2180 74 221; e-mail: foitzik@biologie.uni-muenchen.de

In this paper, we analyse eight nuclear microsatellite loci and mtDNA sequences to investigate neutral genetic variation and population structure of three ecologically and behaviourally well studied ant species, which engage in coevolutionary arms races (Foitzik *et al.,* 2003; Fischer & Foitzik, 2004; Fischer-Blass *et al.,* 2006; Bauer *et al.,* in press a,b). *Harpagoxenus sublaevis* (Nylander 1852) is an obligate social parasite, which needs enslaved hosts to take care of its brood. *Leptothorax muscorum* is the preferred host species of this parasite. *Leptothorax acervorum*, the second host species, is slightly larger, more common and more widespread than *L. muscorum*. The slavemaker *H. sublaevis* completely depends on its hosts throughout its life cycle. To found a new colony it kills or drives away all adult ants and takes over a host nest. The brood that is left will grow up to be the first generation of slaves. Workers of *H. sublaevis* are incapable of carrying out routine colony tasks such as brood care, foraging, and nest maintenance (Buschinger, 1974; Buschinger *et al.,* 1980). Slavemaker brood is raised by enslaved hosts. Slavemaker workers regularly conduct slave raids on neighboring host colonies and steal their brood to replenish their labor force (Buschinger, 1974; Buschinger *et al.,* 1980). Colonies can survive for 10–15 years. Because of its parasitic life style, *H. sublaevis* has an approximately 10-fold lower density (0.06–0.1 nests m^{-2}) than the host species (Fischer-Blass *et al.,* 2006). It also has a smaller range, likely because some regions have too low host densities to sustain parasite populations. Its reproductive strategy also makes it a relatively slow disperser: the vast majority of *H. sublaevis* queens are wingless and attract winged males by emitting pheromones (female calling) (Buschinger, 1966, 1971; Heinze, 1993; Hölldobler & Wilson, 1990). The mated queens attack host colonies in walking distance from their mother nest to establish new colonies.

Both host species, *Leptothorax acervorum* (Fabricius 1793) and *Leptothorax muscorum* (Nylander 1846), inhabit the leaf litter layer of pine forests of the boreal regions of Eurasia (Collingwood, 1971; Ratschenko *et al.,* 1999), where they nest in pine twigs and logs. *Leptothorax acervorum* can also be found in deciduous forests. A typical colony consists of 10–200 workers and one to several queens. Life expectancy for queens is estimated to be around 10 years. Slave raids impose severe selection on host populations lowering the mean life expectancy of host colonies considerably (Fischer-Blass *et al.,* 2006). Ecological field data indicate that the slavemaker *H. sublaevis* shows a slight preference for the smaller host species *L. muscorum*, and consequently has a stronger impact upon it (Fischer-Blass *et al.,* 2006; Bauer *et al.,* in press a,b). In many sites the social parasite co-occurs with the two host species and mixed parasite nests containing slaves of both species are frequently found. *Leptothorax muscorum* has higher densities than the parasite, but slightly lower than the other host (*L. muscorum* up to 0.8 nests m^{-2}, *L. acervorum* 1 nest m^{-2}). The reproductive biology of *L. muscorum* is similar to that of its parasite, only that both sexes are winged. Virgin queens attract mates by emitting pheromones (female calling), and there are no mating flights in this species. After mating, *L. muscorum* queens can return into the mother nest or start a colony in the vicinity on their own. Of the three species, *L. acervorum* shows the highest nest densities and the widest distribution (Ratschenko *et al.,* 1999; Fischer-Blass *et al.,* 2006). This species has winged queens and winged males that mate in large mating swarms. It is therefore expected to be most dispersive.

A recent population genetic study, based mainly on mitochondrial gene sequences revealed higher genetic variation in the larger host species *L. acervorum* than in the parasite and the smaller host *L. muscorum*. In the same study, it was found that *L. acervorum*, which is the species that performs mating flights, showed much lower Φst values than both *H. sublaevis* and *L. muscorum* (Brandt *et al.,* 2007). Both findings qualitatively fit to what we know about nest densities and reproductive strategies in these species. However, mtDNA is inherited through the female line only and stochastic effects can have a huge effect on sequence variation found in an mtDNA locus. For example, a recent selective sweep in one of the species on the mtDNA could have removed a large part of the genetic variation—making it impossible to determine whether differences in genetic variation or population structuring reflect the real situation for most of the genome. For this reason, Brandt *et al.* (2007) studied also a small number of microsatellites in the three species.

In this study, we considerably extend the microsatellite study of Brandt *et al.* (2007) to more populations and eight microsatellite loci for each species and each population. With this larger dataset, we will focus on three questions. First, we are interested in the amount of genetic variation each of the three species harbour, because this is relevant for the adaptive potential of the species.

Second, we want to know whether there is population structuring and whether there is evidence for isolation by distance. For this we used the program Structure (Pritchard *et al.,* 2000) and a newly introduced measure of differentiation (D_{EST}), which is not sensitive to levels of heterozygosity (Jost, 2008). We are also interested in the effects of the last ice age on the distribution of genetic variation and test whether there is any effect of latitude or longitude on the amount of genetic variation. Third, we will compare male and female migration patterns. This is possible, because we have data for maternally inherited mtDNA, and biparentally inherited nuclear microsatellites. This comparison however, is not straightforward, mainly because of the large differences in population wide mutation rates (N*mu) and the resulting differences in heterozygosities which greatly influence Fst measures (Hedrick, 2005; Jost, 2008). Therefore we again used the measure of differentiation, D_{EST}, which is independent of heterozygosity (Jost, 2008).

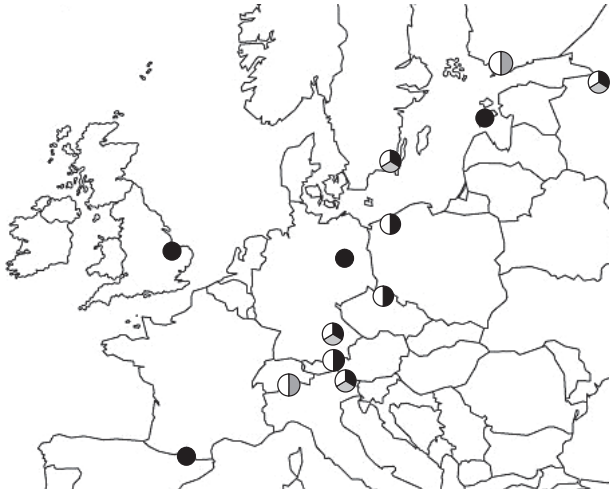

Fig. 1 Collection sites. The geographic position of these study sites are: Germany R (Regensburg: N 48°48′59.62′; E 11°50′45.40′), Germany B (Berlin: N 52°17′39.72′; E 13°37′31.18′), Russia (N 59°56′20.54′; E 30°18′47.55′) Sweden (N 56°39′41.23′; E 16° 21′45.78′), Austria (N 47°19′56.19′; E 11°11′03.13′), Italy (N 46°43′33.66′; E 12°17′46.88′), Poland (N 54°03′36.48′; E 14°56′14.99′), Spain (N 42°42′16.81′; E 0°47′31.37′), Switzerland (N 46°22′03.55′; E 8°10′54.54′), England (N 52°27′31.32′; E 0°13′22.26′). Circles symbolise species found at the different sites; in black: *L. acervorum*, in white: *L. muscorum*, in grey: *H. sublaevis.*

Materials and methods

Sampling sites

Colonies of the parasite *H. sublaevis* and its hosts *L. muscorum* and *L. acervorum* were collected in pine forests throughout Europe, where they were found mainly in logs and twigs on the forest floor. In the summers of 2004–2008, ant colonies were collected in twelve countries (Fig. 1; Table 1).

DNA extraction for microsatellite analyses and mtDNA sequencing

For microsatellite analysis, DNA was extracted from a total of 78 *H. sublaevis*, 284 *L. acervorum* and 221 *L. muscorum* workers. Each worker came from a different colony, for sample sizes per location, see Table 1. DNA was extracted using Puregene DNA extraction kit (Gentra Systems, Minneapolis, MN, USA). In total, the same eight highly variable microsatellite loci were amplified with PCR using the primers LXA GA1 (Bourke *et al.,* 1997), L-18 (Foitzik *et al.,* 1997), LX GT 223 (Hamaguchi *et al.,* 1993) LXA GA2 (Bourke *et al.,* 1997), LXA GT2 (Bourke *et al.,* 1997), LXA GT1 (Bourke *et al.,* 1997), LX GT 218 (Hamaguchi *et al.,* 1993) and Myrt 3 (Evans, 1993). These polymerase chain reactions were performed in a 20 μL volume containing 2.0 μL 10× *Taq* polymerase buffer (*Taq* Core Kit 10; MP Biomedical Europe, Illkirch, France), between 2.0 and 2.2 mM, depending on loci $MgCl_2$ (*Taq* Core Kit 10, MP Biomedical), 4 mM of each dNTP, 0.5 μM of labelled forward primer, 0.5 μM of reverse primer and 1 U of *Taq* DNA polymerase (*Taq* Core Kit 10, MP Biomedical). The following amplification conditions were used in a thermocycler (Thermal Cycler PxE 0.2; Fisher Scientific, Schwerte, Germany) LXA GA1, L-18, LX GT 223: one cycle of 94 °C for 1 min 30 s, 54 °C for 45 s and 72 °C for 30 s. 28 cycles 92 °C for 45 s, 54 °C for 45 s, 72 °C for 30 s and followed by a final extension at 72 °C for 7 min and hold at 4 °C. Annealing temperature varied LXA GA2, LXA GT2: 48 °C, for Myrt 3: 45 °C, for LXA GT1: 42 °C and for LX GT 218: 44 °C. For control we transferred part of our product on a TBE agarose gel (1.5%). Fragments were then analysed in a 96 capillary sequencer (Megabace, Amersham Biosciences Europe, Freiburg, Germany) and evaluated using the software Fragment Profiler (Amersham Biosciences).

For the mtDNA analysis, we used the sequences from Brandt *et al.* (2007), and added new sequences to enlarge

Table 1 Number of colonies samples per species at each of the study sites.

	L. acervorum		*L. muscorum*		*H. sublaevis*	
Sampling Location	Microsat.	mtDNA	Microsat.	mtDNA	Microsat.	mtDNA
Germany	57	12 (10)	56	20 (10)	33	19 (11)
Italy	84	15 (10)	86	14 (10)	30	11 (8)
Russia	30	14 (10)	21	15 (10)	10	12 (8)
Sweden	25	12 (2)	30	12	5	0
Poland	10	4	7	6	0	1
Czech Republic	25	15	11	7	0	0
Austria	6	5	10	5	0	0
Switzerland	14	13 (8)	0	0	0	2 (2)
Finland	0	8 (7)	0	0	0	6 (5)
England	30	10 (5)	0	0	0	0
Spain	3	1	0	0	0	0
Estonia	0	4 (3)	0	0	0	0

In brackets are the number of individuals that were also used in Brandt *et al.* (2007).

the dataset. DNA extraction and sequencing was done as described in Brandt *et al.* (2007) for a total of 51 (34) *H. sublaevis*, 113 (55) *L. acervorum* and 79 (30) *L. muscorum* individuals (in brackets are the number of individuals that were already used in Brandt *et al.* (2007)). For the sample sizes per location, see Table 1.

Data analysis

Summary statistics for the microsatellite data were calculated using Microsatellite-Analyzer 4.05, and GENEPOP 3.4 (web version). The data were tested for heterozygote deficiency (test for H-W equilibrium, using a *U* test (Rousset and Raymond, 1995)) and the program GENEPOP, option 1 sub-option 4. Expected heterozygosities (H_{exp}) and observed heterozygosities (H_{obs}) were calculated for each locus, in each population for each species using MSA. The data were tested for linkage disequilibrium between loci (Fisher exact test for each pair of loci across all populations) using GENEPOP (option 2 sub-option 1).

To compare levels of genetic variation between species, H_{exp} was used, because it is less sensitive to null-alleles than H_{obs}. We fitted a linear model with H_{exp} as response variable and population, species and locus as explanatory variables using R (version 2.5.1, {http://www.R-project.org}). We did not allow for interactions. For the linear model we had to make the assumption that populations of a species are independent, which is not strictly the case as they are related by genealogy.

We are interested in the effects of the last ice age—with large glaciers over northern Europe and the alpine regions—on the distribution of genetic variation in the species we study. For example, we are interested whether more northern populations, which were resettled after the ice ages, are genetically less diverse compared to areas, which could have served as refuges. We therefore repeated the same analysis as described in the previous paragraph with explanatory variables latitude and longitude instead of population name.

For the mtDNA sequences, summary statistics were calculated using DNASP (Rozas *et al.*, 2003). With the mtDNA data, we repeated the same analysis (linear model in R) as with the microsatellite data. We used a web version of Phylip dnapars (v3.66, http://bioweb2.pasteur.fr/docs/phylip/doc/dnapars.html, Felsenstein 2004) to find the most parsimonious tree and used this to make a haplotype network by hand.

Global and pairwise G_{ST} values for the microsatellite data and the mtDNA sequences were calculated with R, following Nei & Chesser (1983). It was noted by several authors that standard *F* statistics and their relatives, such as G_{ST}, are not good measures of differentiation (Hedrick, 2005; Jost, 2008). Specifically, F_{ST} and relatives greatly underestimate differentiation when heterozygosity is high as it is commonly found with microsatellite markers (Hedrick, 2005; Jost, 2008). When heterozygosity is high, G_{ST} is automatically low, even when populations carry completely distinct sets of alleles. We therefore also calculated the *D* statistic, which was proposed by Jost (2008). For comparison:

$$G_{ST} = \frac{Ht - Hs}{Ht}, D_{EST} = \frac{n}{n-1} * \frac{Ht - Hs}{1 - Hs};$$

(*n* is the number of populations that were sampled, *Ht* is heterozygosity over all populations, *Hs* is mean heterozygosity within the populations). We calculated D_{EST} according to formula 12 in Jost (2008).

In addition to the G_{ST} analysis, we analysed our data with the programs Structure 2.3.1 (Pritchard *et al.*, 2000, Falush *et al.* 2003, 2007). This program uses a Bayesian MCMC approach and multi-locus genotype data to infer the presence of distinct clusters or subpopulations, without using information on where individuals were sampled. For Structure, we did the standard analysis with admixture, burn in period of 50 000 steps, then 100 000 steps. *K* values (number of clusters) ranged from 1 to 4 for *H. sublaevis*, 1 to 7 for *L. muscorum* and 1 to 12 for *L. acervorum* and we did four repeats per *K* value.

To test for isolation by distance a Mantel test was performed over the whole data set using IBDWS (Jensen *et al.*, 2005) for both microsatellites and mtDNA, using both pairwise G_{ST} values and pairwise D_{EST} values.

Results

Microsatellites

The eight microsatellite loci were highly variable for all three species with between 14 and 76 (mean 31) alleles for *L. acervorum*, between 9 and 65 (mean 26) alleles for *L. muscorum* and between 9 and 36 (mean 21) alleles per locus for *H. sublaevis*. H-W equilibrium over all populations and loci could be rejected due to heterozygote deficiency for all three species ($P < 0.0001$). The difference between expected heterozygosity (Nei) and observed heterozygosity indicates how ''far'' a population is from H-W equilibrium. In *H. sublaevis* the difference was largest (H_{obs} 0.70 and H_{exp} 0.80), in *L. acervorum* smaller (H_{obs} 0.77 and H_{exp} 0.82) and in *L. muscorum* smallest (H_{obs} 0.73 and H_{exp} 0.72) (averages over loci and populations). The underlying reason could be the presence of null alleles and/or inbreeding. No significant linkage disequilibrium could be detected in any of the species (in *L. acervorum* two locus pairs out of 28 had *P*-values <0.05, *L. muscorum*: two significant locus pairs out of 28 and *H. sublaevis*: two significant locus pairs out of 28).

Microsatellites genetic variability

We tested whether the amount of genetic variation, as measured by Nei's expected heterozygosity, depends on location, species, and locus. There was a significant effect of species (*L. muscorum* populations had 10%

lower heterozygosity than the other two species, $P < 0.005$; Fig. 2a) and of locus ($P < 0.0001$), but there was no effect of location ($P = 0.83$). We repeated the same ANOVA using geographic location (coordinates) instead of the name of the location. There was no significant effect of latitude or longitude ($P = 0.68$) on the amount of genetic variation as measured by microsatellites. There was a clear effect of locus: Ga2 and GT1 were more variable than the other loci ($P < 0.0001$ in both cases).

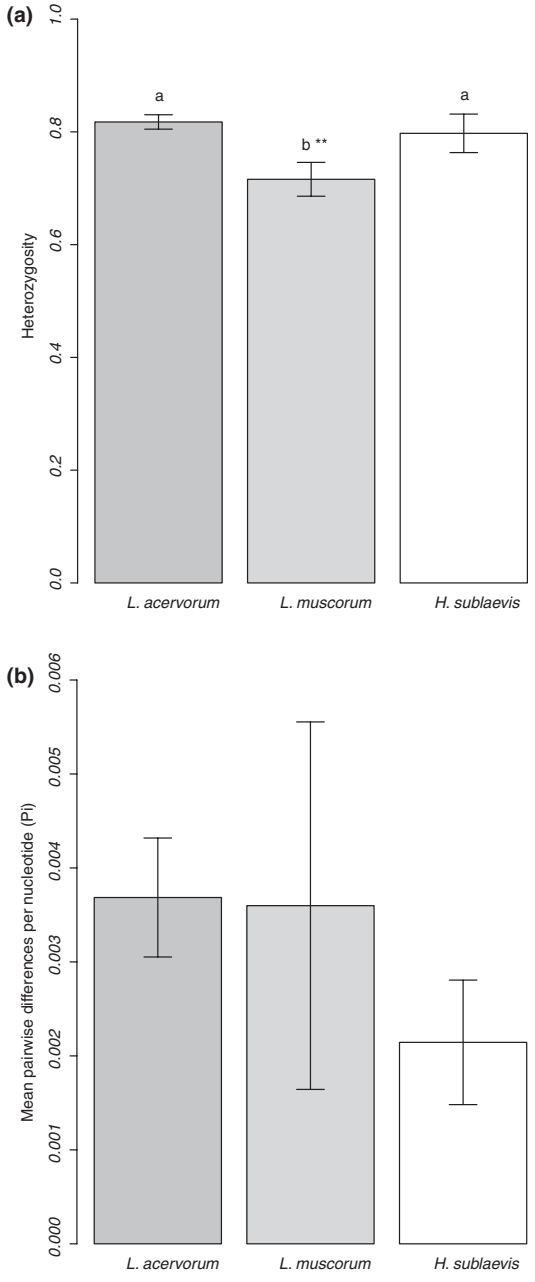

Fig. 2 (a) Heterozygosity (microsatellites) averaged over populations and loci. (b) Mean pairwise differences per nucleotide, calculated within each population and averaged over all populations.

mtDNA genetic variability

Genetic variability, calculated as mean pairwise differences (Pi) within populations and then averaged over populations was (mean ± SE): 0.0037 ± 0.00063 for *L. acervorum*, 0.0036 ± 0.00196 for *L. muscorum* and 0.0021 ± 0.00066 for *H. sublaevis* (Fig. 2b). Genetic variability within populations, was not significantly different in the different species ($P = 0.66$) nor in the different locations ($P = 0.78$). However, this could be due to the fact that we have only one data point per species and population (for the microsatellites, we have eight data points per species and population).

Haplotype networks

For *L. acervorum* and *H. sublaevis*, the haplotype network of the mtDNA sequences looked very similar to the ones published in Brandt *et al.* (2007) (not shown). However, we found a large discrepancy for *L. muscorum* between our current results (Fig. 3) and previous results. In the Brandt *et al.* (2007) study, only three populations were analysed for *L. muscorum*: Germany, Italy and Russia. These three populations formed clear clusters in the haplotype network and we found a correspondingly high G_{ST} value. In this study four more populations were added. Two of these populations, Austria and Czech Republic, have haplotypes that do not cluster like the ones from Italy, Russia and Germany. Austrian individuals cluster with both Germany and Italy. Czech individuals cluster with both Russia and Germany. In addition, both Austria and Czech Republic carry haplotypes that are not in the clusters originally found. Individuals from Sweden and Poland cluster with the German haplotypes.

Genetic differentiation

Global G_{ST} values (from microsatellites) were small (estimates ± standard errors: *L. acervorum* 0.030 ± 0.007, *L. muscorum* 0.066 ± 0.012, *H. sublaevis* 0.043 ± 0.015. However, G_{ST} values are greatly influenced by the amount of heterozygosity in the populations (Hedrick, 2005; Jost, 2008). We therefore also calculated Jost's *D* statistic (Jost, 2008), which is independent of the level of heterozygostity. D_{EST} values are much higher than the G_{ST} values: *L. acervorum* 0.23 ± 0.073, *L. muscorum* 0.28 ± 0.085, *H. sublaevis* 0.35 ± 0.073 (see Fig. 4). D_{EST} values are not significantly different between species ($P > 0.05$).

We also calculated G_{ST} values for mtDNA and found 0.16 for *L. acervorum*, 0.34 for *L. muscorum* and 0.19 for

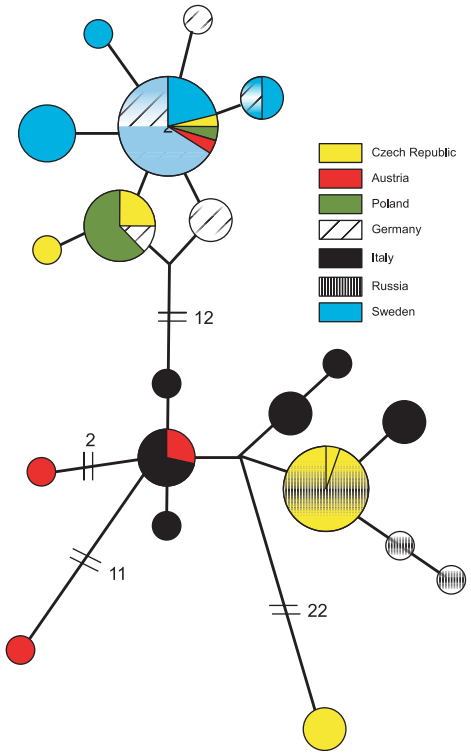

Fig. 3 Haplotype network for *L. muscorum* (the numbers along the branches are distances of more than one mutation).

H. sublaevis (based on a single locus, so no standard errors can be given). Also for mtDNA data it is possible to calculate Jost's *D* statistic, which allows a direct comparison of the mtDNA data and the microsatellite data. D_{EST} values were 0.50 for *L. acervorum*, 0.82 for *L. muscorum* and 0.86 for *H. sublaevis* (see Fig. 4).

We found no evidence for isolation by distance (IBDWS) for either mtDNA or microsatellites using G_{ST} values (*P*-values microsatellites: *L. acervorum* 0.10, *L. muscorum* 0.13, *H. sublaevis* 0.36; *P*-values mtDNA: *L. acervorum* 0.62, *L. muscorum* 0.10, *H. sublaevis* 0.54). When using D_{EST} values, we found a significant effect of isolation by distance in *L. muscorum* when using mtDNA sequence data. This was no longer significant after a Bonferroni correction (*P*-values microsatellites: *L. acervorum* 0.22, *L. muscorum* 0.27, *H. sublaevis* 0.54; *P*-values mtDNA *L. acervorum* 0.14, *L. muscorum* 0.02, *H. sublaevis* 0.14).

Evidence for population substructure was found using the software Structure (3.2.1) for two of the three species. Structure calculates the likelihood, by which the dataset could be separated in a given number of clusters, *K*. When there is detectable population structure, the likelihood of the data goes up when the number of clusters is increased. In the case of *L. acervorum*, the likelihood was highest for $K = 5$, but this value was only slightly higher than for $K = 1$ (Table 3). When we look at the barplot graph of Structure for $K = 5$ (see Fig. 5a), it becomes apparent that no meaningful structure was found by the program. No individual can convincingly be assigned to one of the five inferred clusters. The picture looks different for the other two species. For the smaller host *L. muscorum*, the highest likelihood was found for $K = 5$, but this value was not much higher than for $K = 4$ (Table 3). The barplot for $K = 4$ shows clear differences between the geographic locations (Fig. 5b). Individuals from the Czech Republic, for example, are consistently assigned to the green cluster, while the Swedish and to a lesser extent the German ants were assigned to the blue cluster. In Italy, we sampled in two different years in South Tyrol at two samples sites, which were 1 km apart. It was surprising to find that these two subsamples were clearly separated by the Structure analysis. For the social parasite *H. subleavis*, the highest likelihood was found for $K = 3$ clusters (Table 3). From the barplot it becomes clear that the samples from Sweden and Russia are clustered together, as well as the samples from Germany and Italy (Fig. 5b).

Discussion

In this study, we analysed genetic variation and population structure of three European ants: two host species and an obligate social parasite. Compared to our previous study (Brandt *et al.*, 2007) we have looked at eight microsatellite loci (compared to three in the previous study) and mtDNA sequences in more individuals and in more populations per species. In addition, for the analysis of population structure we have used a newly introduced measure of genetic differentiation, which allows a better comparison between nuclear and mtDNA data.

When looking at the microsatellite data, we find that the three species each harbor a lot of genetic variation. The smaller host species *L. muscorum*, which has lower nest densities and a more restricted range (Ratschenko *et al.*, 1999; Fischer-Blass *et al.*, 2006) exhibits less genetic variability than the larger host, *L. acervorum*, and the slavemaker, *H. sublaevis*. It is surprising that the parasite *H. sublaevis* has similar high levels of genetic variation as *L. acervorum*, given that populations of this species have approximately 10-fold lower nest densities and therefore a much lower census population size. A previous study (Brandt *et al.*, 2007) found that *H. sublaevis* had higher genetic variation than *L. acervorum*, but with our larger dataset we could not confirm this. Our results show again that census population size does not necessarily correlate with the amount of neutral genetic variation even among closely related species (Bazin *et al.*, 2006). It is currently unclear why this is the case, but we suppose that populations of these two species are so large that genetic variability at these highly variable microsatellite loci is not limited by population size. A similar result was found in a comparison of the Bonobo with its low population

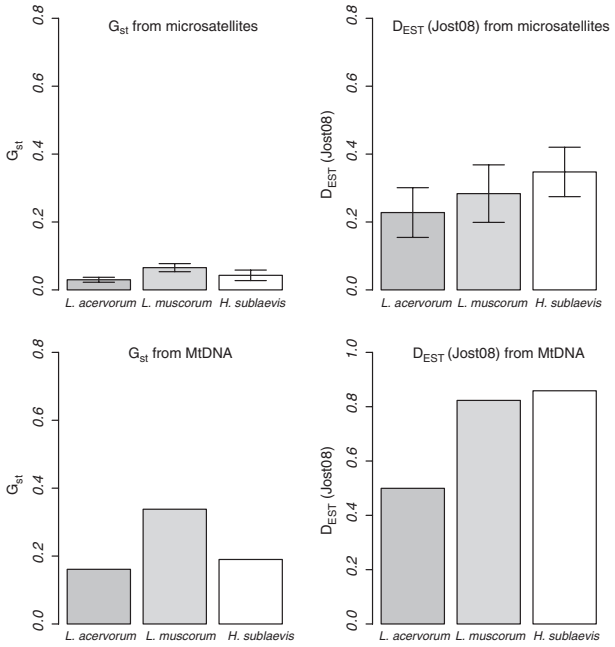

Fig. 4 Different measures of genetic differentiation for microsatellites (upper two panels) and mtDNA (lower two panels).

sizes to its closest relative, the common chimpanzee, whose larger populations were found to harbor not much more genetic variation at neutral microsatellite loci (Reinhartz *et al.*, 2000). On the other hand, numerous studies, which investigated endangered species with shrinking populations, uncovered the expected low genetic variation also at microsatellite loci (e.g. Ellis *et al.*, 2006; Aguilar *et al.*, 2008; Pastor *et al.*, 2007).

In all three ant species we find high levels of genetic diversity in the mtDNA sequences. However, the social parasite *H. sublaevis* exhibits only 60% of the pairwise differences on the mitochondrial Cytochrom Oxidase I and II genes, while it shows similar or even higher levels of heterozygosities on the microsatellite loci. Genetic studies comparing variation in mtDNA sequences and microsatellites frequently uncover much higher levels of variation on microsatellite markers (e.g. Lundy *et al.*, 2000; Dalebout *et al.*, 2006). These differences are generally thought to be due to higher mutation rates in microsatellites or selective sweeps in the mtDNA. The interesting aspect in our study is that we compare very closely related species, where we would not expect vastly different mutation rates. However, analyses of mtDNA have the disadvantage that this marker behaves like a single locus. mtDNA sequences are linked by a single genealogical tree, because there is no recombination. Large differences may therefore also occur by chance.

We found low G_{ST} values for the microsatellite data, which is the typical pattern found in many ant species. However, G_{ST} is strongly influenced by levels of heterozygosity and if heterozygosity is high as it is typical for microsatellite loci, G_{ST} is invariably low. Our low G_{ST} values may therefore simply reflect the high values of within population heterozygosity. We therefore also looked at the D statistic, which was recently proposed by Jost (2008) and which is independent of levels of heterozygosity and therefore allows a direct comparison of different marker systems. D_{EST} values calculated from microsatellites are relatively similar for all species and are four to eight times larger than the G_{ST} values (*L. acervorum* G_{ST}: 0.03, D_{EST}: 0.23, *L. muscorum* G_{ST}: 0.07, D_{EST}: 0.28, *H. sublaevis* G_{ST}: 0.04, D_{EST}: 0.35). The D_{EST} values show that our populations are much more differentiated than would have been suggested by the G_{ST} values. The fact that *L. acervorum* has the lowest value of D_{EST} (even though the difference is not significant) fits our expectations, because it is the only species of the three, which has large scale mating flights so it is likely to be the most dispersive.

We also calculated G_{ST} and D_{EST} values from the mtDNA data (*L. acervorum* G_{ST}: 0.16 D_{EST}: 0.50, *L. muscorum* G_{ST}: 0.34, D_{EST}: 0.82 and *H. sublaevis* G_{ST}: 0.19, D_{EST}: 0.86) and found that the D_{EST} values for mtDNA were between 2.1 and 2.9 times larger than D_{EST} values based on microsatellites. This can be explained by male biased dispersal, which was commonly found in ants. Especially in *L. muscorum* and *H. sublaevis*, where females do not participate in mating flights, male biased dispersal is expected. Females of *L. acervorum* however do fly, so the twofold difference between D_{EST} from microsatellites and mtDNA is somewhat surprising in this species. We think it can be explained by facultative polygyny. Some of the young queens return to the mother nest after mating and are readopted. Polygynous

Table 2 Main summary statistics for each of the species.

	Microsatellites				mtDNA		
	Number of alleles per locus (min, max)	H_{exp} averaged over loci and populations ± SE	G_{ST} ± SE	D_{EST} ± SE (Jost, 2008)	Mean pairwise differences (Pi) ± SE	G_{ST}	D_{EST} (Jost, 2008)
L. acervorum	31 (14–76)	0.82 ± 0.013	0.030 ± 0.007	0.23 ± 0.073	0.0037 ± 0.00063	0.16	0.50
L. muscorum	26 (9–65)	0.72 ± 0.030	0.066 ± 0.012	0.28 ± 0.085	0.0036 ± 0.00196	0.34	0.82
H. sublaevis	21 (9–36)	0.80 ± 0.034	0.043 ± 0.015	0.35 ± 0.073	0.0021 ± 0.00066	0.19	0.86

Table 3 Results of the structure (version 2.3.1) analysis. The Log likelihood of the data given K (the number of subpopulations), averaged over four independent runs is given. For the settings of the program, see text.

K (N of subpopulations)	*L. acervorum*	*L. muscorum*	*H. sublaevis*
1	−10216	−6687	−2577
2	−10389	−6723	−2565
3	−10770	−6562	−2539
4	−10252	−6333	−2813
5	−10185	−6317	
6	−10458	−6342	
7	−10720	−6842	
8	−11049		
9	−11245		
10	−10944		
11	−11623		
12	−11215		

colonies and readoption of daughter queens can also occur in *L. muscorum*, but are completely absent in *H. sublaevis*, where queens mainly disperse on foot and start new colony by invading host nests in the vicinity.

G_{ST} values from mtDNA were much higher (roughly fivefold) than G_{ST} values from microsatellites. Most of this difference disappears when we convert to D_{EST} values. Many studies on ant population genetics compare the G_{ST} values directly and find up to 20 times higher values in the mtDNA based calculations (for example, Doums *et al.*, 2002; Clemencet *et al.*, 2005; Brandt *et al.* 2007; Goropashnaya *et al.*, 2007). They conclude that male dispersal is much more pronounced in ants than female dispersal. We think that this pattern may at least partly be caused by the differences in variability, rather than a real difference in differentiation. It is good that there are now other statistics available (G_{ST}' by Hedrick, 2005 and D_{EST} by Jost, 2008), which do not have this

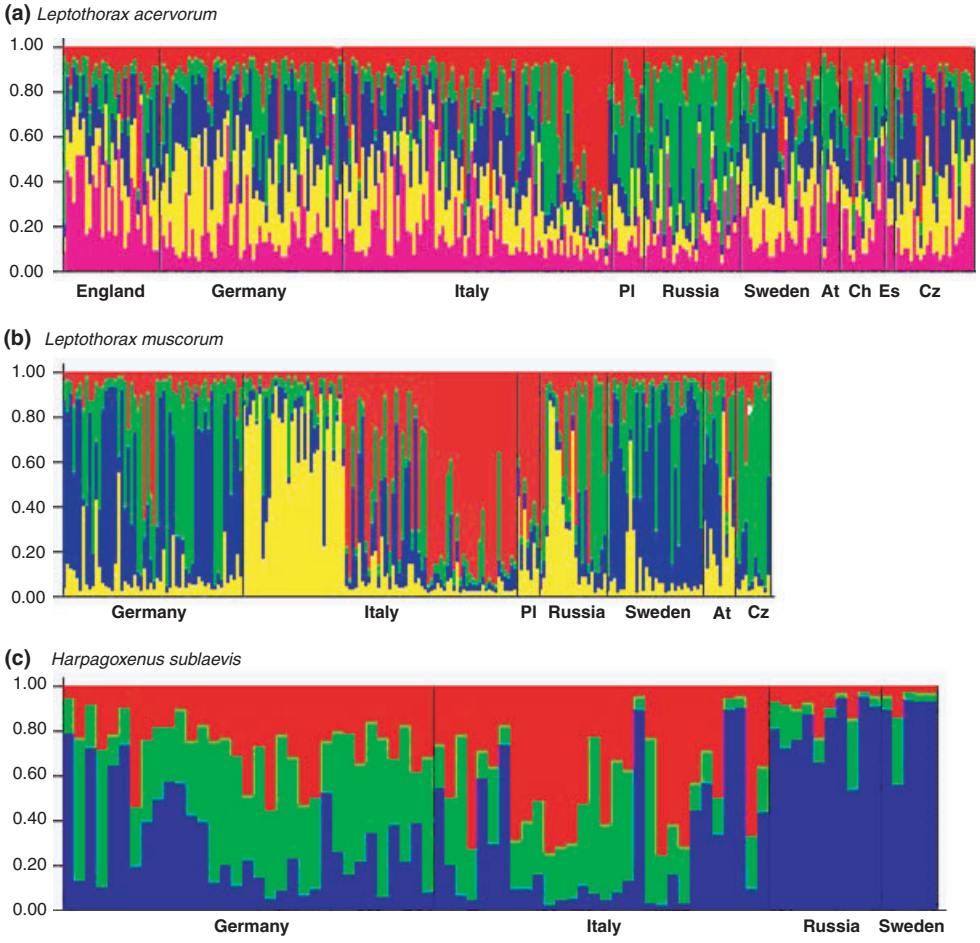

Fig. 5 Plots of the estimates of Q (estimated membership coefficient for each individual) for each cluster (K). The most probable number of genetic populations present in the data is K = 5 for *L. acervorum* (a), K = 4 for *L. muscorum* (b) and K = 3 for *H. sublaevis* (c). The vertical lines are broken into colored segments (greyscale in print publication) showing the proportion of each individual assigned to each of the inferred K. Names or abbreviations at the bottom of the graph correspond to the country of the various sample sites (Pl = Poland; At = Austria; CH = Switzerland; ES = Spain; CZ = Czech Republic).

bias. In addition, it would be very useful if nuclear sequences would become available for ants so that they can be compared to the other two marker systems. This would give us a much better insight in male and female dispersal in ants.

However, this problem is not restricted to the population genetics of ants alone. Studies on other organisms also concluded male-biased dispersal based on a comparison of G_{ST} values in nuclear microsatellites and mitochondrial sequences (e.g. Escorza-Trevino & Dizon, 2000, van Hooft *et al.*, 2003). Also in these systems the application of novel measures of differentiation (G_{ST}' by Hedrick, 2005 and D_{EST} by Jost, 2008) might be helpful to determine how strongly sex-biased dispersal really is.

We found evidence for population structure with *Structure* analysis for the smaller host species *L. muscorum* and the social parasite *H. sublaevis*. In *L. muscorum* *Structure* revealed no larger geographic pattern, but clearly differentiated between local sub sites in Italy, which were only a km apart. In *H. sublaevis* the northern populations in Russia and Sweden clustered together as well as the Southern populations in Germany and Italy. *Structure* revealed no evidence for population structure in the larger host *L. acervorum*. This could be due to rare alleles and the relatively small sample sizes, the combination of which means that alleles were often only observed in one or few populations. Rare alleles contribute to differentiation (when D_{EST} is used to estimate differentiation), but do not have a large influence on the likelihood estimates for *Structure*, which are based on H-W proportions of genotypes. For both marker systems, we find no clear evidence for isolation by distance, nor do we find that northern populations have lower genetic variation. This is different in some other ant species (e.g. Viginier, 2004; Clémencet *et al.*, 2005; Schlüns *et al.*, 2009). The three ant species we study depend largely on pine forests. Pine (*Pinus sylvestris*) probably occupied several refugia in Central and Eastern Europe in addition to the ''standard'' ones in southern Europe (Cheddadi *et al.*, 2006). Even a small forest can harbour a large population of ants. We therefore expect that also relatively large ant populations survived the last ice age in several refugia in Europe. As the forest moved back up North, the ants would have moved along. Ants can move much faster than trees, but because they depend on the trees they would have moved at exactly the same speed as the trees. For the ants, this meant a rather slow dispersal, allowing for a lot of individuals to contribute to the dispersal. We therefore expect that there were large (effective) population sizes all along and not much genetic variability was lost during migration north or in the high alpine sites. Large population sizes in the past and now, combined with relatively slow migration could explain why the species have high variability even in the northern populations.

Hosts and the social parasite in our study system exert strong selection pressure on each other, leading to antagonistic coevolution (Fischer-Blass *et al.* 2006). Thompson (2005) predicts in its geographic mosaic model of coevolution that the strength of selection in such interactions will vary from one geographic region to the other, depending, for example, on local population sizes, the community composition and the resulting parasite pressure. Where selection is strong, the coevolutionary arms race may go faster (coevolutionary hotspots) and where selection is weaker it will be slow (coldspots). This population genetic study indicates that we should find evidence for Thompson's geographic mosaic of coevolution (Thompson, 2005) in this host–parasite system because (a) there is abundant genetic variation and population sizes are large in all three species. This probably means that adaptation should not be hindered by lack of variation. In addition, (b) the populations of all three species are strongly structured. Coevolutionary processes in one community are therefore somewhat independent of other communities and we would expect parasite and host to adapt to their local opponent. This was indeed found in behavioral studies, which investigated the crucial host–parasite encounter—the slave raids (Fischer & Foitzik, 2004).

Acknowledgments

We thank Annette Leingärtner, Yvonne Cämmerer and Bettina Rinjes for their support with the laboratory work. We are grateful to Tobias Pamminger, Aurelien Tellier, Dirk Metzler and Matthias Birkner for fruitful discussions. This work was supported by the DFG (Fo 298/7).

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Received 1 July 2009; revised 16 September 2009; accepted 25 September 2009