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How a haemosporidian parasite of bats gets around: the genetic structure of a parasite, vector and host compared

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ABSTRACT

Parasite population structure is often thought to be largely shaped by that of its host. In the case of a parasite with a complex life cycle, two host species, each with their own patterns of demography and migration, spread the parasite. However, the population structure of the parasite is predicted to resemble only that of the most vagile host species. In this study we tested this prediction in the context of a vector-transmitted parasite. We sampled the haemosporidian parasite *Polychromophilus melanipherus* across its European range, together with its bat fly vector *Nycteribia schmidlii* and its host, the bent-winged bat *Miniopterus schreibersii*. Based on microsatellite analyses, the wingless vector, and not the bat host, was identified as the least structured population and should therefore be considered the most vagile host. Genetic distance matrices were compared for all three species based on a mitochondrial DNA fragment. Both host and vector populations followed an isolation-by-distance pattern across the Mediterranean, but not the parasite. Mantel tests found no correlation between the parasite and either the host or vector populations. We therefore found no support for our hypothesis; the parasite population structure matched neither vector nor host. Instead, we propose a model where the parasite's gene flow is represented by the added effects of host and vector dispersal patterns.

INTRODUCTION

Parasites are more and more being used as genetic tags of their hosts, which can give additional demographic information when a host population shows low genetic variability (Bruyndonckx *et al.* 2010; McCoy *et al.* 2005a). Moreover, parasites can reveal non-reproductive contact between different

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host populations or even different host species (Bruyndonckx *et al.* 2009). Naturally, not all parasite species are reliable tags: their particular mating systems, life-history traits or demographic characteristics can make them less convenient or positively misleading proxies for a host's genealogy (Nieberding & Olivieri 2007).

Parasites with complex life cycles are parasites which need a minimum of two hosts to complete their life cycle. Such heteroxenous parasites are potentially unsuitable tags since any genetic signal should be a mix of that of the two host species. How exactly the population structure of heteroxenous parasites is shaped by both hosts remains unclear. Jarne and Théron (2001) proposed that, a priori, the population structure of a heteroxenous parasite should closely mimic that of its least structured host. The reasoning being that for its overall gene-flow, the parasite is dependent on the host with highest dispersal. Any potential signal of the more strongly isolated species would be erased by a more motile host with more frequent dispersal events (Louhi *et al.* 2010).

The strength of such correlations between parasite and host population structures are, however, also dependent on their respective life-history traits. For parasites, the duration of the free living stage in particular could influence the tightness of this correlation. The longer the free-living stage of a parasite lasts, the more incongruence would be expected between parasite and host. Spores and eggs have the potential to be distributed over a large range, yet their effect on the parasites gene-flow has been studied only minimally (Jarne & Theron 2001). Important host traits that could alter the expected relation between parasite and host gene flow include the host's timing of reproduction and dispersal.

Many socially and economically important diseases, like malignant malaria, chagas and Lyme disease, are caused by vector-transmitted parasites. These can be considered a special class of heteroxenous parasites, for several reasons. First of all, the two host species required for completion of their life cycle are from much diverged taxa, different phyla in general. Moreover, these parasites are obligate, permanent parasites; they cannot live outside the host and have no free-living stages. As a consequence, for the parasite to complete its life-cycle, multiple encounters between the host and

vector species are needed. A last peculiarity of many vectored parasites is that one of the two hosts, ‘the vector’, is itself dependent on the other host for survival and can be considered a parasite itself.

Despite the peculiarities of the system, surprisingly few studies have looked into how vector and host populations interact in shaping the genetic structure of the parasite. Levin and Parker (2013) found a complete absence of differentiation in an island population of the haemosporidian parasite *Haemoproteus iwo*. The hippoboscid vector *Olfersia spinifera* had much lower population differentiation than the avian host *Fregata minor*, which might explain the lack of differentiation in *H. iwo*. However, the authors could not compare patterns of gene-flow due to the absence of polymorphism in the parasite *cytb* marker used. Gomez-Diaz *et al.* (2011) detected large genetic variability in populations of *Borrelia garinii* across an entire oceanic region but found it to be unstructured despite the presence of host-races within the tick vector across this region (McCoy *et al.* 2005b). In both studies, the parasite was a generalist, and both vectors, the tick and hippoboscid fly, may feed on several species of bird. Dispersal patterns of parasite and hosts are therefore not expected to compare one-to-one; dispersal opportunities provided by unsampled host species could cause unexplainable discrepancies in the patterns of gene-flow.

The goal of the current study is to test the hypothesis that heteroxenous parasite dispersal patterns mimic those of its most vagile host (Jarne & Theron 2001) by comparing and quantifying the patterns of gene-flow of a specialist parasite, its vector and its vertebrate host. To this end, we use a haemosporidian parasite, *Polychromophilus melanipherus* (Apicomplexa: Haemosporida), a malaria-like blood parasite of bats. In its European range, it mainly parasitizes *Miniopterus schreibersii* (Chiroptera: Miniopteridae), but is known to infect other members of the Miniopteridae in Africa (Duval *et al.* 2012; Garnham 1966; Lanza 1999). *Nycteribia schmidlii* (Diptera: Nycteribiidae) is a small wingless bat fly that is a host-specific ectoparasite of *M. schreibersii* (Theodor 1957), though can occasionally be found on other bat species sharing the same cave (Lanza 1999). Although it has never been formally demonstrated, it is the suspected vector of *P. melanipherus*, as it is the most common bat fly of *M. schreibersii* (Estrada-Peña & Serra-Cobo 1991), occurring throughout its

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European range (Theodor 1967) and is closely related to *N. kolenatii*, the confirmed vector of *P. murinus* (Gardner *et al.* 1987). Using microsatellites, we first tested which of the two host species is more vagile, the bat *M. schreibersii* or the bat fly *N. schmidlii*. We then compared both the dispersal patterns of host and vector with that of the parasite *P. melanipherus* using mitochondrial markers, identifying whether the dispersal patterns mimic more those of its vector or its host.

METHODS

Sampling

Sampling was done in May 2011 (Croatia, Portugal, Spain, Switzerland) and August/September 2012 (Italy, Slovakia, France, Switzerland) on 23 different sites across Southern and Central Europe (Table 1, Figure 1A). *Miniopterus schreibersii* were captured at their roosts either upon emergence using mist nets and harp traps at dusk, or by entering the roosts at day time and collecting them while they were resting. Young-of-the-year, born in June/July, were not sampled. Bat flies were looked for by blowing through the fur of the bat, collected using soft forceps and immediately stored on 96% ethanol.

A wing biopsy was taken using a 1 mm gauge punch (Stiefel) and stored in 96% ethanol. Approximately 15 μ L of blood was taken by puncturing the uropatagial vein with a 0.5 mm gauge needle (Neolus, Terumo). Blood beads forming on the patagium were captured by pipetting or using a heparinized glass microcapillary tubes (Marcel Blanc & Cie) and ejected on white blotting paper (3MM, Whatman) which was left to dry and later stored in separate envelopes. Wounds were treated with haemostatic cotton until bleeding had stopped after which bats were released at the site of capture. All captures were done in accordance with local regulations.

DNA extraction

DNA was extracted from the blood-soaked blotting paper using the DNeasy Blood and Tissue kit (Qiagen) according to the manufacturer's tissue protocol with the following exceptions. A fragment of paper of ~10mm in diameter was cut in smaller pieces using sterile scissors, added to a 2 mL tube (Eppendorf) containing 360 μ L of ATL buffer and incubated at 90°C for 15 min. After cooling down,

40 μ L of Proteinase K was added to each sample and left for overnight digestion at 56°C. At the final step 55 μ L elution buffer was added to the filter and samples were incubated at 37°C for 15 min. before centrifuging. This step was repeated once to maximize recuperation. For a subset of individuals, extra DNA was extracted from the wing biopsy following the same tissue protocol with the addition of an initial rinsing phase where ethanol was removed from the tissue by soaking the biopsy in 1 mL of pure MilliQ water (Millipore).

For all collected bat flies, species was determined following the key by Theodor (1967). All specimens identified as *Nycteribia schmidlii* were rinsed of their ethanol by soaking in 1mL of pure MilliQ water (Millipore) at room temperature for 2-3 hrs. Because females most often carry a developing larva, their abdomens were removed to prevent paternal contamination. Afterwards individual flies were triturated using sterile pestles. After an overnight digestion the standard Biosprint 96 tissue protocol (Qiagen) was followed.

Marker amplification

To determine the genetic structure of *M. schreibersii* in Europe, all individuals were genotyped at 14 polymorphic microsatellite loci (Supp. Table S1; Miller-Butterworth *et al.* 2002; Wood *et al.* 2011). Primers, PCR thermal profiles and reagent concentrations can be found in Supplementary Tables S1-S3. PCR products were controlled on an agarose gel 2% under UV light. Four post-PCR multiplexes were realized after different tests by adjusting the ratio of each reaction in a final volume of 3 μ l (Supp. Table S1). PCR products were typed in an ABI Prism 3100 sequencer (Applied Biosystems).

For *N. schmidlii* 10 new microsatellite markers were developed commercially (Ecogenics, Zurich-Schlieren, Switzerland). Size selected fragments from genomic DNA were enriched for microsatellite content by using magnetic streptavidin beads and biotin-labelled CT and GT repeat oligonucleotides. The microsatellite-enriched library was analysed on a Roche 454 platform using the GS FLX titanium reagents. Reads were selected for tetra- or trinucleotide repeats of at least 6 units long or a dinucleotide repeat of at least 10 units long. Of these reads 1173 were suitable for primer design, which resulted in primers for 10 new markers (Supp. Table S4).

The 10 new microsatellite markers for *N. schmidlii* were combined in 2 multiplexes, each reaction combining 5 loci each. See Supplementary Tables S2, S3 for the thermal profiles and reagent concentrations respectively. Post-PCR reaction products were diluted 6x of which 3 μ L was finally genotyped on an ABI prism 3100 sequencer.

The detection of *P. melanipherus* infection in the vertebrate host's blood was done by amplifying a 705 bp cytochrome *b* fragment (*cytb*) of the parasite following a nested PCR protocol. Primers, reagents and PCR temperature profile were taken from Megali et al. (2011; Supp. Tables S2, S3). Bands were run on a 1% agarose gel stained with ethidium bromide and were visualized under UV light. Each sample was tested in duplicate. If, after retesting, the sample continued to give ambiguous results, we considered it an infection of very low intensity and therefore included it in further analysis. Samples that, after retesting gave no bands at all, were presumably contaminated during the previous run, considered negative, and left out of further analyses. Positive samples were either purified with the Wizard PCR clean-up system (Promega) and sequenced locally on a ABI Prism 3100 genetic analyser (Applied Biosystems), or sent to a commercial agent for sequencing (Microsynth, Switzerland).

To compare the molecular variability of the parasite with that of the vector and host, also a mitochondrial gene fragment of vector and host was amplified. Based on preliminary tests with several candidate markers and 10 samples, we selected for each species a marker which produced a workable level of diversity. For *N. schmidlii*, 408 bp of the 16S ribosomal subunit (16S) was amplified using the primers fw: 5'-CGC CTG TTT AAC AAA AAC AT-3' and rv: 5'-TGA ACT CAG ATC ATG TAA GAA A-3' (Petersen *et al.* 2007). For *M. schreibersii* 310 bp of the mitochondrial control region (CR, often referred to as 'd-loop') was amplified using the forward primer 5'-CAT CTG GTT CTT ACT TCA GG- 3' (Fumagalli *et al.* 1996) and a newly designed reverse primer 5'-GTG CAC AGT CGT AAT CTC-3'. Conditions and reagents for both amplifications can be found in Supp. Tables S2, S3. Purification and sequencing using the forward primers was performed by a commercial agent (Microsynth, Switzerland).

Microsatellite analyses

Microsatellite allele sizes were scored and sized by hand with the aid of the software GeneMapper (Applied Biosystems). Micro-Checker v2.2.3 (Van Oosterhout *et al.* 2004) was used to investigate large allelic drop out, stuttering and null alleles. The software FSTAT v2.9.3.2 (Goudet 2001) was used to test for linkage disequilibrium between loci. Deviation from Hardy-Weinberg equilibrium across all markers and populations was tested for with the X^2 -test from the 'genetics' package in R v3.0.1 (R Core Team 2012). Using the R-package hierfstat v. 0.04-10 (Goudet 2005) the observed heterozygosity, within population and overall gene diversity, allelic richness and within population heterozygote deficiency F_{IS} were calculated for each species.

For both the bats and the bat flies, a hierarchical population structure was assumed where sampling sites were clustered in regions (Table 1). To partition the components of genetic variation among regions and sites, a hierarchical analysis of variance (ANOVA) was performed using the hierfstat function varcomp.glob. Significance of the variance components was tested using likelihood ratio G-statistic permutation tests from the same package. To make the fixation indices comparable between species, F^* -values, corrected for maximum polymorphism, were calculated by dividing each F-value by its theoretical maximum (Jost 2008).

A pattern of isolation-by-distance between sampling sites was examined by looking at the relation between the pairwise F_{ST} 's, $x / (1-x)$ transformed, and the natural logarithm of the overland geographical distances (Rousset 1997). The correlations between the genetic and geographical distance matrices were tested using a mantel test (1000 permutations) using the mantel function from the R-package ade4 v1.5-2 (Dray & Dufour 2007).

For both the bats and bat flies a principle component analysis (PCA) was performed on the population allele frequencies, weighted by population size to prevent small populations from having disproportional effects. The eigenvectors were extracted using the function dudi.pca from the ade4 package. Significance of the inertia of the first two components was calculated by permuting the

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population assignment of each individual and recalculating the inertia 1000 times using a custom script in R.

The most likely number of clusters in each species' European population was estimated within the Bayesian framework of Structure v2.3.4 (Pritchard *et al.* 2000). A model with admixture and correlated alleles (Falush *et al.* 2003) was used to assign individuals to any of the predestined number of clusters, ranging from 1 to 8, based on the number of regions sampled. The sample location was not used as a prior. Each simulation had a burn-in of 50'000 generations and data collection for 100'000 generations and 10 replicates were performed for each parameter setting. The most likely number of clusters was determined by looking at the raw log likelihood, as well as ΔK , the ratio of the absolute rate of change of the log likelihood and its standard deviation (Evanno *et al.* 2005), as implemented in Structure Harvester (Earl & Vonholdt 2012).

Mitochondrial sequence analysis

The chromatographs of all mtDNA sequences were manually checked, edited and aligned with the software Mega 5.03 (Tamura *et al.* 2011). Identification and characterization of haplotypes and the sequence diversity of the sampling sites were done with the aid of the pegas v0.4-5 package in R (Paradis 2010). A minimum-spanning network was created of the haplotypes for each of the three species in TCS v1.21 (Clement *et al.* 2000). Neutral evolution of each fragment was assessed by calculating Tajima's D and tested for significant deviation from zero using the function `tajima.test` from pegas, customized to take into account insertion-deletions (indels).

As with the microsatellite analyses, the components of genetic variation were partitioned hierarchically, though not only based on the local haplotype frequencies, but also on their molecular distances, in an analysis of molecular variance (AMOVA; Excoffier *et al.* 1992). To calculate the molecular distance between sequences two different models of sequence evolution were used: 1) the Kimura 2 parameter model of nucleotide evolution, ignoring indels (Kimura 1980) or 2) the absolute number pairwise differences, where the presence of different sized indels were considered a single difference, irrespective of their size difference in base pairs. Besides the AMOVA, a standard

ANOVA (disregarding molecular distances among haplotypes) was performed allowing the identification of the effects of the evolutionary model, and again the corrected F²-values were calculated as well. The components of variation of each distance model were tested using the AMOVA randomisation test from the ade4 package.

Arlequin v3.1 (Excoffier *et al.* 2005) was used to produce pairwise Φ_{ST} distance matrices for each species. These population distance matrices were based on both the frequency and sequence data of the mitochondrial fragments and were used as measures of genetic distance between each sampling site for each species. These were used to test for isolation-by-distance between sites after transformation as described above for the microsatellites. Moreover, the bat and bat fly matrices were compared with their microsatellite counterpart using the mantel test (1000 permutations) from the ade4 package. Lastly, the Φ_{ST} distance matrix of *P. melanipherus* was compared with both nuclear and mitochondrial distance matrices of both *M. schreibersii* and *N. schmidlii*. To test for different dispersal patterns based on infection, the mantel test was repeated for *M. schreibersii*, including only individuals of the host population infected with *P. melanipherus*.

RESULTS

Miniopterus schreibersii and *N. schmidlii*: comparison of microsatellites

Three microsatellite loci of *M. schreibersii*, MM01, MM29 and MM34, had only a single allele and were removed from any further analyses, leaving the total number of markers at 11. No signs of stuttering, allelic drop out or null alleles were found in the bat microsatellite dataset and no loci showed signs of linkage. Eight population-locus combinations were not in Hardy-Weinberg equilibrium after Bonferroni correction. However, these were scattered randomly across the total 253 comparisons and were therefore left in the data set.

In the *N. schmidlii* data all markers were polymorphic. However, two loci, Ns134 and Ns719, produced null alleles consistently across populations. These loci were removed from any further analyses. No marker pair showed linkage and all markers were in Hardy-Weinberg equilibrium for each population.

The bat fly markers tended to be more diverse than those of the bats. Both the absolute number of alleles and gene diversities (H_S , H_T) seemed higher in the flies (Supp. Table S5). Bat flies showed slightly higher values of F_{IS} , both across markers and populations (Supp. Tables S5, S6). At higher hierarchical levels, F_{CT} and F_{ST} were 8-10 times higher in bats than bat flies (Table 2). Corrected for the lower gene diversity the bats still show a 6-fold higher level of isolation (Table 2: F' -values). Within region, the differentiation between sampling sites was only significantly different from zero in the bats, not in the bat flies. In both species, the differentiation among sites followed an isolation-by-distance pattern (mantel test; *M. schreibersii*: $r=0.396$, $p=0.001$; *N. schmidlii*: $r=0.275$, $p=0.002$; Supp. Figure S1A-B) but had twice the explanatory power in the bat ($r^2=0.157$) than in the bat fly ($r^2=0.076$). In contrast, the population isolation patterns of each species did not correlate with each other (mantel test; *M. schreibersii* vs. *N. schmidlii*: $r=0.020$, $p=0.434$; Supp. Figure S1C).

For both *M. schreibersii* and *N. schmidlii*, the first principle component (PC1) explained a significant amount of the allelic variation observed in the European populations (permutation tests: *M. schreibersii*: inertia=0.148; $p=0.001$; *N. schmidlii*: inertia= 0.114; $p=0.006$). In both species, this axis demonstrated an east-west pattern, except Portugal remarkably taking up a somewhat central position (Figure 1B-C). Inertia of PC2 was significant for *M. schreibersii*, but not *N. schmidlii* (permutation tests: *M. schreibersii*: inertia = 0.105, $p=0.001$; *N. schmidlii*: inertia = 0.081; $p=0.884$). PC2 separates the more Western and Southern Italian sites (together with the Sicilian site), from the other *M. schreibersii* samples (Figure 1B). Overall, the *M. schreibersii* samples were more clumped by region whereas the *N. schmidlii* sites were more mixed among regions.

Following the method of Evanno (2005), $K=2$ was the most likely number of clusters in the European population of *M. schreibersii* ($\Delta K_2 = 31.01$, mean $\ln L = -8210.01$, Supp. Figure S2). According to the structure plot, the Spanish, French and Swiss sites formed one cluster and all sites more east a second. The Portuguese samples were identified as an admixture of the two clusters. In contrast with the bat host, the most likely number of clusters for the *N. schmidlii* data was $K=1$.

Simulations with a single cluster produced the highest absolute log-probability (mean lnL = -9068.26), which decreased monotonically with an increasing number of suggested clusters.

Host, vector, parasite: mtDNA sequence description

Of the 310 bats examined, 231 proved PCR-positive for *Polychromophilus melanipherus*, resulting in a cross-European infection rate of 74.5% (Table 1, Supp. Figure S3). After sequencing, 32 of the 231 infections (14%) had multiple ambiguous base calls, indicating these hosts were infected with multiple strains of *P. melanipherus* at the time of sampling. Because haplotypes could not be called unambiguously in these cases, the multiple infections were disregarded in the consecutive analyses.

Sequence analysis of the *P. melanipherus* *cytb* fragment revealed 8 haplotypes in the European population, based on 22 segregating sites. The 310 bp CR fragment of *M. schreibersii* had 51 segregating sites and 103 haplotypes. The 455 bp 16S fragment of *N. schmidlii* had 32 haplotypes, based on 15 segregating sites plus 3 indels: a single adenine indel, an adenine-thymine indel and an indel consisting of 0-5 repeated thymines. Independent reamplification and resequencing confirmed these indels. For both *M. schreibersii* (51 out of 103) and *N. schmidlii* (15 out of 32) approximately half of the haplotypes were singletons, found in only 1 individual. All unique haplotype sequences of *P. melanipherus* (acc. nb. KJ131270 – KJ131277), *M. schreibersii* (acc. nb. KJ131278 – KJ131380) and *N. schmidlii* (acc. nb. KJ131381 – KJ131412) were deposited at the publicly accessible online database GenBank (<http://www.ncbi.nlm.nih.gov/genbank/>).

Both haplotypic diversity and nucleotidic diversity were of the same order of magnitude for each species (Supp. Table S7). Despite having the shortest fragment, *M. schreibersii* had haplotypic diversity close to unity, whereas the vector and parasite had very similar, slightly lower values. The reverse trend had been observed in the microsatellites, where heterozygosity levels were higher in the vector than in the host. The vector *N. schmidlii*'s gene fragment had the lowest nucleotide diversity of the three species. However, this is partly an artefact caused by the indels in the sequence, which are ignored when calculating nucleotide diversity.

The minimum spanning network of *P. melanipherus* consisted of the eight haplotypes loosely connected by branches varying in length from 1-12 mutational steps with no alternative paths between haplotypes (Figure 2D). The most numerous haplotypes were also common across all regions, whereas a few rare haplotypes were specific to certain locations. Both the bat and bat fly minimum spanning networks formed large strongly interconnected nets. Most haplotypes were separated by only 1-3 mutational steps, and many alternative paths existed between haplotypes. Because of the many haplotypes and many connections, no clear minimum spanning network figures could be produced, however Figures 2A-C show the frequency and distribution of haplotypes across the different regions. None of the bats' CR haplotypes were shared among all sites; most of them were unique to a specific region. In contrast, the bat flies' 16S fragment had one extremely prevalent haplotype, followed by a few considerably less prevalent haplotypes, but all except the rarest were shared among regions (Figure 2B).

Selection might be in part responsible for the marked differences in minimum spanning networks. Indeed, the mtDNA fragment of *M. schreibersii* and *N. schmidlii* showed no signs of selection (*M. schreibersii*: $D = -1.115$, $p = 0.265$; *N. schmidlii*: $D = -0.370$, $p = 0.712$). In contrast, the *cytb* fragment of *P. melanipherus* had a high segregating-sites-to-haplotypes ratio ($D = 2.178$, $p = 0.029$), a sign of balancing selection or population contraction.

When not correcting for mutations, the host *M. schreibersii* showed the highest levels of population differentiation (F' -values, Table 3: 'Equidistant'), compared to vector and parasite. When mutations are considered, the contrast between the species' population differentiation values becomes less pronounced (Table 3: 'Pairwise differences', 'Kimura 2p'). The vector *N. schmidlii* had the lowest (and non-significant) within-region differentiation (Φ_{SC}), but slightly higher levels of isolation among sites compared to parasite and host (Φ_{ST} , Φ_{CT}). The host *M. schreibersii* has the highest, significant, levels of within-region isolation (Φ_{SC}), whereas the parasite *P. melanipherus* is overall the least differentiated among sites. The two modes of nucleotide evolution differed very little in their effect on the Φ -statistics of parasite and host (Table 3). Notable exception was seen with the vector, where a

clear difference is shown between the Kimura model (ignoring indels) and pairwise differences (including indels in the distance matrix).

The pairwise Φ_{ST} matrices of *N. schmidlii* and *M. schreibersii* were produced using pairwise number of differences as the mutation model. The pairwise genetic distance matrix of *P. melanipherus* was calculated using no mutation model, as the differences in F-values between the mutation models were minimal (Table 3). The patterns of population differentiation based on the mtDNA fragment agreed with the patterns based on microsatellites in both carrier species (mantel test, microsatellite F_{ST} – mtDNA Φ_{ST} ; *M. schreibersii*: $r=0.333$, $p=0.004$; *N. schmidlii*: $r=0.294$, $p=0.003$). As with the microsatellites, the mitochondrial fragments of both the bat (mantel test; $r=0.182$, $p=0.005$) and the bat fly (mantel test; $r=0.475$, $p=0.001$) demonstrated an isolation-by-distance pattern. Visual inspection further indicates that this pattern is mainly present on the long, interregional distances; the left of the graph, representing sites separated by short distances, suggests no relation between the genetic and geographic distances within region (Supp. Figure S4A-B). The malaria parasite, however, showed no such geographical pattern (mantel test; $r=0.072$, $p=0.191$; Supp. Figure S4C).

Comparisons between parasite and vector or host genetics

The parasite's genetic distance matrix did not correlate with either that of the host or the vector; neither when the distances were based on microsatellites, nor on mtDNA (Table 4, Figure 3, Supp. Tables S8-S10). This was also true when only actual 'hosts' were used, that is, when only the bat host genotypes were included in the distance table that were actually infected with the parasite (Table 4). In contrast to their microsatellites, the host and vector mtDNA distance matrices did correlate significantly with each other (mantel test; $r=0.529$, $p=0.001$, $r^2=0.280$), though this correlation disappeared when corrected for geographic distance (mantel test; $r=0.077$, $p=0.164$).

DISCUSSION

Parasite gene flow is dependent on both vector and host dispersal behaviour. In this study, we determined the population genetic structure of the haemosporidian parasite *P. melanipherus* and tested

the hypothesis that its structure matches that of its most vagile host species. We demonstrated that it is the wingless vector *N. schmidlii* that is most vagile and not the vertebrate host *M. schreibersii*. However, the pattern of genetic differentiation of *P. melanipherus* resembled neither that of the vector nor that of the host.

Which is the most vagile species?

When comparing the microsatellite data of the host *M. schreibersii* and the vector *N. schmidlii*, a clear pattern emerges. Though the F_{ST} was significant in both species, the detected levels of isolation were 10 times higher in the bat than in the bat fly, whose level of subdivision was close to zero. Even after correcting for the slightly higher levels of gene diversity in the bat fly, differentiation among bat populations remained higher. In contrast, at the mitochondrial marker the regional structuring was relatively similar for both species, with the exception that only the bat flies showed no significant structuring within regions. The clustering analysis and PCA confirm this pattern of stronger regional clustering in the bat hosts. Lastly, both species showed a pattern of isolation-by-distance but this relation was much stronger in the bat. All these findings indicate that more genetic exchange takes place between populations of bat flies and less between the populations of their bat hosts. The *N. schmidlii* bat flies should therefore be considered the more vagile species in Europe.

However, the lower level of differentiation observed between bat fly sites might also be due to a possibly larger population size, reducing drift. Indeed most *M. schreibersii* would be carrying multiple *N. schmidlii* in summer making the bat fly population size correspondingly larger compared to the bat. Yet when hosts were sampled in spring (Croatia) and autumn (Switzerland) lower numbers of bat flies were found, averaging less than one fly per host (Table 1). Though bat fly sampling was not exhaustive in this study, and geography might be a confounding factor, seasonal bottlenecks have been observed in *N. schmidlii* (Estrada-Peña & Serra-Cobo 1991) as well as other temperate *Nycteribia* species (Witsenburg *et al.* 2014). These severe bottlenecks should decrease the effective population size N_e and thereby increase relative levels of differentiation between bat fly populations. The shorter

life cycle of temperate bat fly species (estimated at two generations per year; Reckardt & Kerth 2006) should also increase relative population differentiation compared to the more slowly reproducing bat host. Despite these two possible factors, *N. schmidlii* had a lower fixation values, strongly suggesting more migration between vector populations compared to the host.

Despite their extensive seasonal migrations between roosts, actual dispersal events of marked *M. schreibersii* to a different population have very rarely been observed (Rodrigues *et al.* 2010). Both males and females are philopatric, in this case meaning faithful to their natal group, rather than to any geographic location, though females will always return to the same maternity roost (Rodrigues & Palmeirim 2008; Rodrigues *et al.* 2010). Despite the philopatry of both sexes, the Φ_{ST} of the maternally inherited CR fragment was much higher than the expected fourfold increase compared to the nuclear data. This implies that females disperse less than males (Ramos Pereira *et al.* 2009), which would contradict the behavioural studies. However, though males do not disperse to other groups, the seasonally migrating *M. schreibersii* do meet other local populations and mating at these meetings have been observed (Rodrigues *et al.* 2010), allowing for the transfer of nuclear, but not mitochondrial, genes to the other population. Moreover, since the mitochondrial control region is the fastest evolving sequence of the mammalian genome (Fumagalli *et al.* 1996), the mutation model we used might not have been able to correctly capture all of the observed variation, artificially inflating the mtDNA fixation indices.

The exchange of bat flies appears much stronger than that of the bats' own genes. Though peak reproduction of bat flies is synchronized to that of the bat, *N. schmidlii* can reproduce throughout the season (Lourenço & Palmeirim 2008). Moreover, hibernacula, shared among multiple subpopulations of *M. schreibersii* (Rodrigues & Palmeirim 2008), could facilitate bat fly dispersal to other groups. But contact of bat subpopulations needs not be direct for *N. schmidlii* dispersal to occur. Roosting in the same cave at different time points would already allow for the exchange of bat flies, which leave their pupae to develop on the cave walls.

The *N. schmidlii* microsatellites and mitochondrial sequences agree that within regions all sites are strongly connected, with regular exchanges. But the between-region mitochondrial levels of

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fixation are 80 times the nuclear values. Again, our mutation model might not have effectively captured the mutational processes going on at the ribosomal RNA fragment. Yet, this large discrepancy also suggests a strongly reduced N_e , caused by processes such as skewed sex ratios in the bat flies or strong female fly philopatry. Skewed sex ratios in favour of males are known to occur in bat flies (Dick & Patterson 2008), but in this study, more female flies were found (but no exhaustive sampling was performed). Female bat flies have to leave their host frequently to deposit a pupa on the roost wall (Marshall 1970). This risky behaviour could lower the survival of females. On the other hand, this behaviour should cause the females to change hosts more often, increasing their dispersal likelihood. Sex differences in survival have not been observed in bat flies (Marshall 1970; Witsenburg *et al.* 2014). However, the performed experiments concerned short term off-host survival, yet these flies can live up to six months at least (Ryberg 1947 cited in Marshall 1970); sex differences in long-term survival might therefore very well be possible, and have been observed with regard to haemosporidian infections in hippoboscids flies (Waite *et al.* 2012). Females might also be less likely to survive the winter season, causing bottlenecks in the mitochondrial genome. Bat fly female philopatry could also have caused the high level of differentiation of the mitochondrial gene, but to our knowledge, no study has looked into bat fly dispersal behaviour.

Population structure of Polychromophilus melanipherus

The *cytb* fragment of *P. melanipherus* was the only fragment that showed signs of past selection. It was also the only protein-coding gene in this study, which might explain the relatively few haplotypes separated by relatively long branches. The balancing selection indicates negative frequency-dependent selection, suggesting a coevolutionary alternation of defences between the parasite and either the vector or host. However, how the mitochondrial cytochrome *b*, involved in the trans-membrane transport of electrons and ultimately in ATP production, could be a target for host immune responses is not clear.

The four major haplotypes were shared among the different regions, with the other four being more geographically restricted. The bat fly also had its more frequent haplotypes shared among

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regions yet still had an isolation-by-distance pattern. The malaria parasite showed no such geographical pattern. Despite a relatively low expected N_e (infection rates < 1 , few multi-clone infections, high expected inbreeding rate), and therefore high expected levels of differentiation among the parasite populations, the relative contribution of Φ_{CT} to the parasites' structuring was low compared with those of vector and host. This suggests that the cross-region exchange of parasites is more frequent than either that of vector and host.

In avian malaria research, all cytb haplotypes are considered distinct non-recombining genetic 'lineages', since they often have non-overlapping host distributions (Bensch *et al.* 2000) and show linkage with nuclear haplotypes (Bensch *et al.* 2004). Though host distributions clearly overlapped in the case of *P. melanipherus*, we have no data on linkage with nuclear haplotypes. If our haplotypes actually represented cryptic species, gene diversity for each species should actually be higher, resulting in lower fixation values and therefore even more panmictic parasites. However, if a species were defined by its genetic haplotype, it will always be panmictic, as it would be genetically identical across its range by definition. The host distribution of the *P. melanipherus* haplotypes suggests they are a single species, but nuclear markers are required to determine whether they are truly recombining.

Does the parasite mimic the vector's dispersal patterns?

As stated by Wright (1951) F_{ST} is inversely related to the estimated number of migrants Nm . With its lower F_{ST} , *N. schmidlii* provides a higher number of migrants between demes compared to *M. schreibersii*. Even if a majority of these migrants was male, as suggested by the mtDNA, both male and female Nycteribiidae take blood meals and can therefore transmit *P. melanipherus*. The bat fly *N. schmidlii* should therefore be the main agent transporting the parasite between demes, but only if infection rates among bats and bat flies are equal. Although we did not measure the infection status of *N. schmidlii* here, the infection rate of its sister species *N. kolenatii* with the *P. murinus* parasite was 17%, four times lower than of its host *M. daubentonii* (Witsenburg *et al.* 2014). If similar rates were to apply to this system, the effective number of parasite dispersal events caused by vector or host might approach each other. However, the parasite's population structure neither resembled that of the bat fly

vector, nor that of the bat host. And since the dispersal patterns of host and vector were correlated at the mitochondrial level, it is unlikely that the parasite's pattern would lie somewhere 'in the middle' of its two hosts, as an average of their patterns of gene flow.

An alternative hypothesis to the idea that a parasite's population structure should resemble that of its most vagile host, is a simple additive model where the parasite's gene flow is represented by the added effects of host and vector dispersal patterns. Indeed, there is no a priori argument for ignoring any effect which an intermediate host might have on the parasite's dispersal. If one of the two hosts is a stationary, highly structured species, this hypothesis should produce the same results as Jarne and Théron's hypothesis of mimicking the most vagile species. However, if both host species show a certain degree of genetic exchange, the results of the two models should radically differ. Whereas the original hypothesis would expect the parasite's gene flow pattern still to be similar to that of the most vagile host, our proposed alternative states the parasite's gene flow should be higher than that of either hosts.

The fixation indices of *P. melanipherus* were lower than that of either host, but due to different expected mtDNA mutation rates, we should be cautious of direct comparisons. However, the absence of an isolation-by-distance pattern in the blood parasite, in contrast with either host, indicates that *P. melanipherus* does have higher levels of gene flow than either host. This lack of differentiation between parasite populations due to higher gene flow could also have caused the apparent absence of correlation between parasite and host populations.

A possible cause for the lack of correlation between the population structures of the parasite and its host and vector might also be the presence of a second vector. *Penicilidia conspicua* is a large bat fly species that can also be found on *M. schreibersii* (Theodor 1967). However, we found this species at lower intensities or not at all and it was therefore considered a less likely candidate-vector. Moreover, the dissection of 26 individuals revealed no oocysts. If *P. conspicua* would also transmit *P. melanipherus*, we would expect an overall increase in gene flow for the parasite relative to the vector, which might be the case in our study.

Dispersal rate heterogeneity within species, where infected individuals of a species show different dispersal behaviours than their uninfected counterparts, could have caused the discrepancy between host and parasite gene-flow. However, *Nycteribia kolenatii* bat flies carrying *P. murinus* have lower survival than their uninfected counterparts (Witsenburg *et al.* 2014), which should reduce dispersal rate of infected flies, increasing the parasite population differentiation. No clear effects of a *Polychromophilus* infection on its bat host have been found as of yet, but an increase in migration behaviour of infected bats, by e.g. being expelled from the current colony as a form of infection-avoidance, could cause the parasite to be a more effective disperser than its host population (Curtis 2014).

We cannot say how much the discrepancy between hosts and parasite genetic structure is caused by the specific gene histories. As seen with *N. schmidlii*, the message from different types of markers can be markedly different. A comparison between parasite and hosts with microsatellites should give a higher resolution picture of gene flow patterns, but despite multiple efforts, we did not manage to develop microsatellite markers for *P. melanipherus*. Only once have microsatellites been used to compare dispersal patterns of a parasite with its two hosts (Prugnolle *et al.* 2005). Yet, as in our study, the authors found no correlation between their parasite (the trematode *Schistosoma mansoni*) and its little dispersing intermediate mollusc host *Biomphalaria glabrata*, nor with its definite host the rat *Rattus rattus*. Though undeniably dispersed by the rat, no apparent correlation existed between parasite and host gene flow. Only the genetic distance between the trematodes' infrapopulations showed a positive relationship with the shared allelic distance of each rat. Instead of a shared dispersal patterns, this might rather suggest a gene-by-gene model of coevolution between parasite and host (Prugnolle *et al.* 2005).

To our knowledge, the Prugnolle *et al.* (2005) study is the only other study where a quantitative comparison has been made between parasite and both hosts. Blasco-Costa and Poulin (2013) found in a meta-analysis of gene flow of trematode species that the most important factor influencing parasite gene flow was final host's mode of dispersal (aquatic or terrestrial/by air), though no traits of intermediate hosts were tested. A more quantitative comparison was made between the malaria

parasite *Plasmodium falciparum* and its mosquito host *Anopheles gambiae* in Kenya. In concordance with the additive effects hypothesis, the authors found that the parasite *P. falciparum* had no structure at all whereas the vector *A. gambiae* still showed some structuring between villages (Prugnolle *et al.* 2008). But this study also lacked data on the other (human) host. Yet these type of studies will be required more to discriminate between the hypotheses of resembling the most vagile host and the additive effects of both hosts.

In conclusion

The parasite's genetic structure was clearly not a mere copy of that of one of its hosts, nor an intermediate of the two. We thus found no support for the hypothesis that a parasite's population structure resembles that of its most vagile host species. On the other hand, the observations would be in concordance with a model where the parasites population structure is determined by the additive effects of both vector and host. Our study system, with a specialized parasite and easily traceable vector is the ideal candidate to find such a pattern and discriminate between these different hypotheses. The next important step would be to develop nuclear (microsatellite) markers for *P. melanipherus*. These would allow us to have a more quantifiable and comparable measure of population differentiation in the parasite, as well as enable us the detection of any cryptic species. In parallel, mathematical models need to be developed which can create quantifiable predictions on how host and vector dispersals combined create the parasites pattern of gene flow.

The genetics of *N. schmidlii* indicated that ample non-reproductive contact exists between European *M. schreibersii* subpopulations, which demonstrates the utility of using (ecto)parasites as an ecological 'tag' for host behaviour. In contrast, even though *P. melanipherus* is unquestionably 'tagged' onto both of its hosts, its complex message is much harder to read than any single-host parasite system. A future disentanglement of these effects should, however, prove to be some of the more valuable insights into epidemiology of any vector-transmitted disease.

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DATA ACCESSIBILITY

- DNA sequences of the new *N. schmidlii* microsatellites: GenBank accessions KJ189108 - KJ189117

- Mitochondrial haplotypes of *P. melanipherus*, *M. schreibersii* and *N. schmidlii*: GenBank accessions KJ131270 – KJ131277, KJ131278 – KJ131380 and KJ131381 – KJ131412 resp.

- All DNA sequence alignments: Dryad doi: 10.5061/dryad.2m1p0

- Microsatellite genotypes, sex, host id. and sampling locations of *M. schreibersii* and *N. schmidlii*:
Dryad doi: 10.5061/dryad.2m1p0

AUTHOR CONTRIBUTIONS

FW, NS and PC conceived and designed the study. LC, ALB, JP, IP, DS and MS organised local permissions, material and sampling schemes. FW, LC, ALB, JP, IP, DS, MS and LD collected the samples. LC and FW tested and designed molecular tools, performed molecular analyses, scored alleles and haplotypes and performed a preliminary data analysis. FW performed the final data analysis and wrote the manuscript. JG and PC contributed to the data analysis, interpretation of the results and writing of the manuscript.

TABLES

Table 1 Overview of sampling effort and infection rates.

	Name	Region	N	N inf.	N nyct.
Pt1	Soïdos	Portugal	11	10 (10)	5
Pt2	Preguiça	Portugal	11	11 (11)	8
Pt3	Cercal	Portugal	15	12 (11)	6
Pt4	Nabão	Portugal	15	15 (11)	22
Es1	Llaberia	Spain	15	15 (12)	24
Es2	Collserola	Spain	15	14 (11)	14
Es3	Montnegre	Spain	14	13 (10)	21
Es4	Savassona	Spain	15	11 (8)	9
Es5	Les Gavarres	Spain	14	14 (10)	7
Fr1	St-Médard	France	15	7 (7)	3
Ch1	Baulmes	Switzerland	16	9 (8)	0
It1	Monte Catini	Italy	15	6 (4)	43
It2	Riolo Terme	Italy	15	9 (8)	17
It3	San Marino	Italy	15	12 (12)	29
It4	Velo Veronese	Italy	3	1 (1)	9
Si1	Marzamemi	Sicily	16	11 (11)	0
Hr1	Karin Gornji	Croatia	6	6 (6)	2

Hr2	Kijevo	Croatia	15	13 (11)	5
Hr3	Drnis	Croatia	15	12 (11)	9
Hr4	Rumin	Croatia	9	8 (5)	2
Sk1	Chvalovská jaskyňa	Slovakia	15	10 (9)	17
Sk2	Nandraž Bradlo	Slovakia	15	4 (4)	16
Sk3	Drienovecká jaskyňa	Slovakia	15	8 (8)	10

N: number of *Miniopterus schreibersii* hosts sampled, N inf.: number of bats infected with the parasite *Polychromophilus melanipherus* (number of single clone infections), N nyct.: number of *Nycteribia schmidlii* bat fly vectors collected.

Table 2 Hierarchical analysis of the variance components of genetic diversity of the host *Miniopterus schreibersii* and the vector *Nycteribia schmidlii*. Sampling site was the lowest hierarchical level, the middle level consisted of the regions, loosely following country borders (see table 1).

		F-value	CI	p	F'
the host	F _{ST}	0.0629	0.0482 - 0.0798	0.001	0.1391
<i>M. schreibersii</i>	F _{SC}	0.0203	0.0111 - 0.0311	0.001	0.0449
	F _{CT}	0.0434	0.0332 - 0.0534	0.001	0.0986
the vector	F _{ST}	0.0053	0.0012 - 0.0108	0.014	0.0240
<i>N. schmidlii</i>	F _{SC}	-0.0010	-0.0040 - 0.0015	0.497	-0.0046
	F _{CT}	0.0063	0.0019 - 0.0137	0.002	0.0284

N.B. CI: 95% confidence interval of the F-value; p: associated p-value based on 1000 permutations

Table 3. Analysis of molecular variance of the mtDNA fragments, for three mutation models. Significant values are in bold.

		Equidistant			Pairwise differences			Kimura 2p			
		F-value	%	p-value	F'	Φ-value	%	p-value	Φ-value	%	p-value
the host	F _S	0.191	80.8	0.000	1	0.324	67.5	0.000	0.323	67.6	0.000
	T	4	6	1		5	5	1	1	9	1
<i>M. schreibersii</i>	F _S	0.096		0.000	0.506	0.108		0.000	0.108		0.000
	C	1	8.6	1	7	9	8.26	1	4	8.23	1
	F _C	0.105	10.5	0.000	1	0.241	24.1	0.000	0.240	24.0	0.000
	T	4	4	1		9	9	1	7	7	1
the vector	F _S	0.088	91.1	0.000	0.331	0.406	59.3	0.000			0.000
	T	5	5	1	8	8	2	1	0.502	49.8	1
<i>N. schmidlii</i>	F _S	-			-						
	C	0.000		0.667	0.002	0.026		0.217	0.033		0.228
		7	-0.07	9	8	5	1.61	8	8	1.74	3
	F _C	0.089		0.000	0.333	0.390	39.0	0.000	0.484	48.4	0.000
	T	1	8.91	7	6	6	6	2	6	6	1
the parasite	F _S			0.000	0.494	0.159	84.0	0.000	0.159	84.0	0.000
	T	0.163	83.7	1	8	2	8	1	2	8	2
<i>P. melanipherus</i>	F _S				0.145						
	C	0.053		0.047	6	0.052		0.075	0.052		0.075
		1	4.69	5	7	7	4.68	5	6	4.67	2
	F _C	0.116	11.6	0.000	0.408	0.112	11.2	0.001	0.112	11.2	0.002
	T	1	1	1	7	5	5	9	5	5	7

Table 4 Mantel test results correlating the parasite's *Polychromophilus melanipherus* pairwise genetic distances with those of its host *Miniopterus schreibersii* and its vector *Nycteribia schmidlii*.

Compared dataset	r	p-value ¹
<i>M. schreibersii</i>		
microsatellites	0.0427	0.364
mtDNA	-0.0604	0.600
<i>only infected bats</i>		
microsatellites	0.0959	0.215
mtDNA	0.0462	0.431
<i>N. schmidlii</i>		
microsatellites	-0.0950	0.665
mtDNA	0.0740	0.182

¹based on 1000 permutations

FIGURES

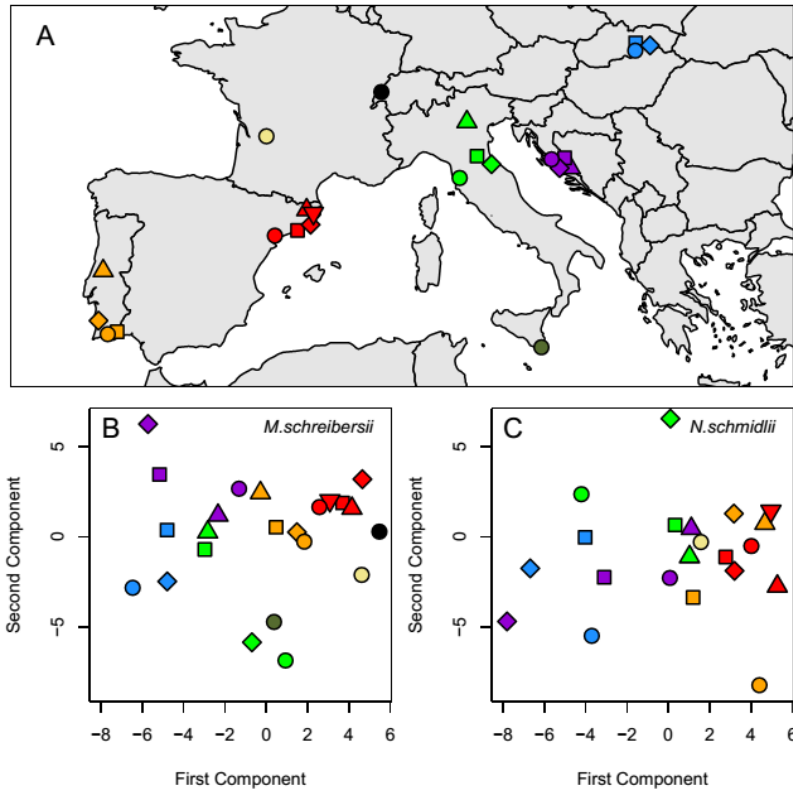


Figure 1 The geographic distribution of sampling sites and PCA results. *A.* Map of Southern Europe with sampling sites; *B* and *C.* Scatterplots of populations according to the first two axes of the PCA based on the microsatellite allele frequencies. *B.* The host *Miniopterus schreibersii*; *C.* The vector *Nycteribia schmidlii*. No vectors were collected in Sicily and Switzerland. PC1 was significant for both species, PC2 only for *M. schreibersii*. Symbols coloured by region and shaped according to population abbreviation (Table 1): 1 = circles, 2 = squares, 3 = diamonds, 4 = upward-pointing triangles, 5 = downward-pointing triangles.

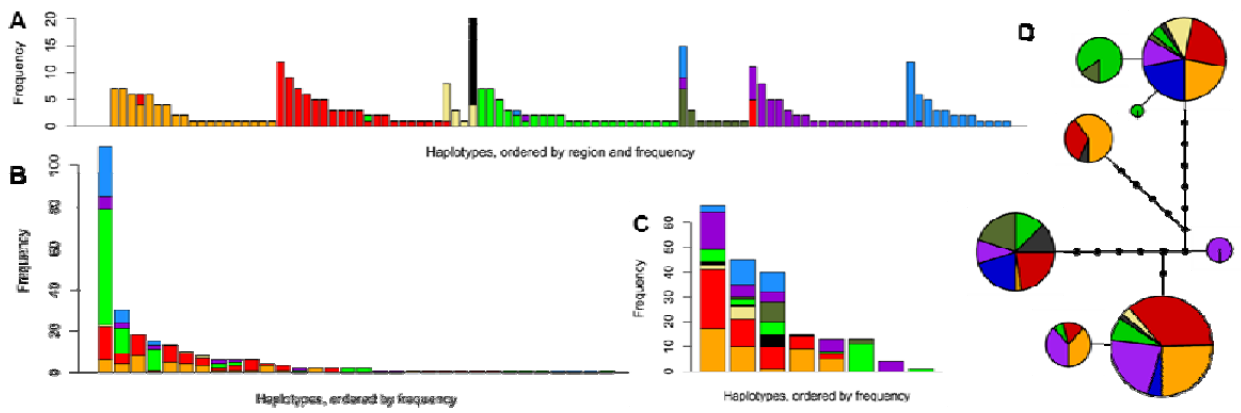


Figure 2 Frequency of each mitochondrial haplotype and distribution among the different regions sampled. Colour-coding according to region (see also Figure 1). *A.* 103 haplotypes of the host *Miniopterus schreibersii*; *B.* 32 haplotypes of the vector *Nycteribia schmidlii*; *C.* 8 haplotypes of the parasite *Polchromophilus melanipherus*. *D.* A minimum spanning network of the 8 *cytb* haplotypes of *P. melanipherus* in Europe. Haplotypes are represented by pie charts, the colour represent the regions where this haplotype was found and the size of the pie chart is relative to the total frequency of the haplotype. Each line represents a single nucleotide mutation, black dots represent unobserved hypothetical haplotypes.

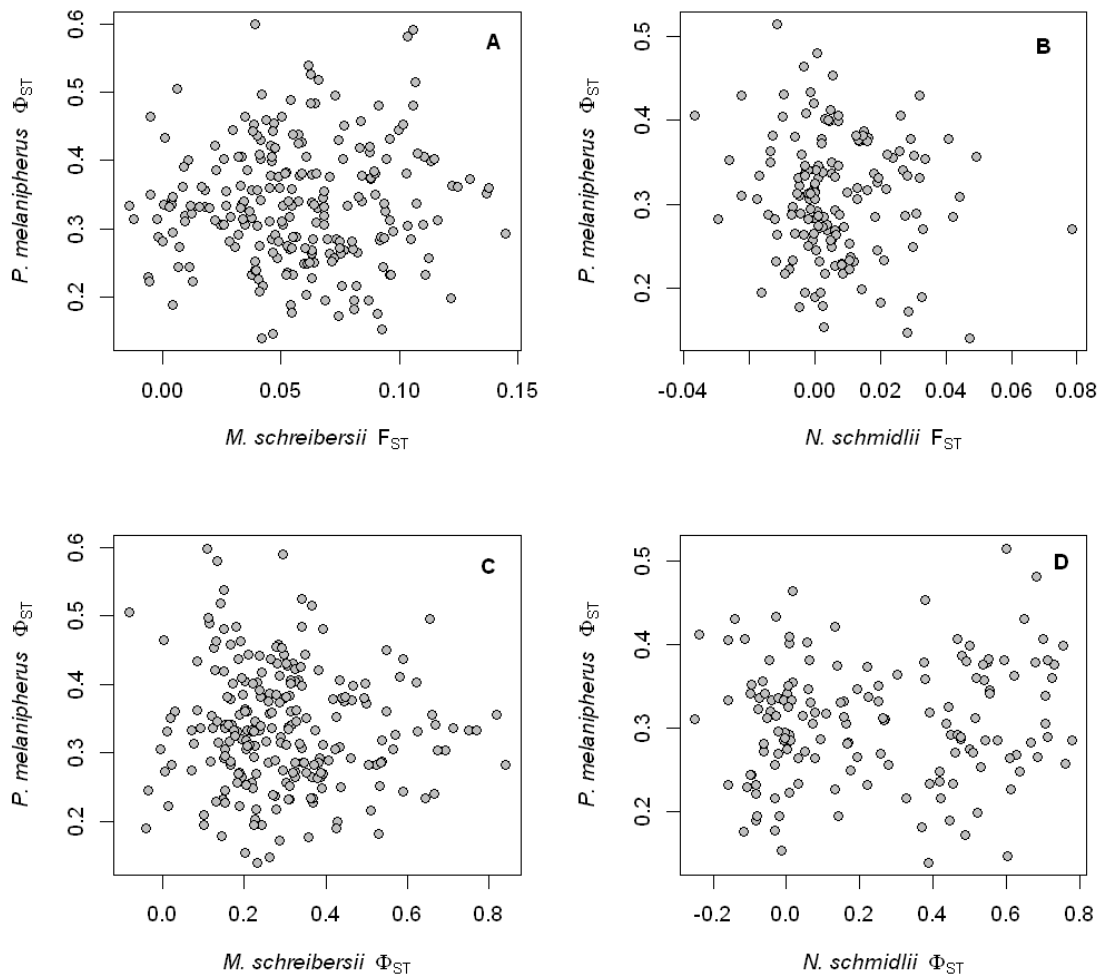


Figure 3 Population differentiation patterns do not correlate between either host species and the malaria parasite. Microsatellite (F_{ST} , top row A, B) and mitochondrial sequence (Φ_{ST} , bottom row C, D) based pairwise population genetic distances for the host *Miniopterus schreibersii* (left column A, C) and the vector *Nycteribia schmidlii* (right column B, D) compared with the pairwise population genetic distances for the parasite *Polychromophilus melanipherus*. See Supp. Tables S8, S9, S10 for the raw pairwise distance tables.