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ORIGINAL PAPER

Molecular characterization of avian malaria parasites in three Mediterranean blue tit (*Cyanistes caeruleus*) populations

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Abstract We genetically analysed malaria parasites (Protozoa) in three Mediterranean blue tit (*Cyanistes caeruleus*) populations from central Spain. A total of 853 breeding individuals were screened for parasites of the genera *Plasmodium* and *Haemoproteus* using a very efficient polymerase chain reaction approach that amplifies a partial segment of the mitochondrial cytochrome *b* gene of these parasites. We have found six lineages of *Plasmodium* (SGS1, GRW11, COLL1, DELURB4, GRW04 and BLUTI10) parasitizing the studied populations but we did not detect any infection by *Haemoproteus*. One of the detected lineages (BLUTI10) has not been previously described in any bird species and this is the first study recording lineages DELURB4 and GRW04 in blue tits. SGS1 (belonging to the morphospecies *Plasmodium relictum*) was the most frequent lineage (overall prevalence, 24 %), whereas the other lineages showed a much lower prevalence (<4 %). Only a small proportion (12.2 %) of positive amplifications of the most common lineage (SGS1) was detected in blood smears using light microscopy and infection intensities were very low (mean ±SE, 2.0±1.4 parasites/2,000 erythrocytes). We have also found strong inter-population variability in prevalence patterns (12–41 % for lineage SGS1), suggesting important differences in parasite transmission rates among the geographically close studied localities.

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Introduction

Avian malaria is a mosquito-borne disease caused by parasites in the genus *Plasmodium* and *Haemoproteus* (*sensu* Pérez-Tris et al. 2005). These parasites have been widely used as study system to understand the dynamics of infectious diseases (e.g. Wood et al. 2007; Knowles et al. 2011; Lachish et al. 2011a; Shurulinkow and Chakarov 2006; Shurulinkow and Ilieva 2009) and their consequences in wild populations (e.g. Ortego et al. 2008; Martínez-de la Puente et al. 2010; Lachish et al. 2011b; Shurulinkow et al. 2012). The application of molecular methods to the study of these parasites has opened up the possibility of detecting genetically different lineages, further increasing the interest for this host-parasite system in recent years (Bensch et al. 2000; Pérez-Tris et al. 2005; Howe et al. 2012). For instance, molecular tools have revealed that the number of avian malaria species is much higher than previously thought and that host switching is extensive (e.g. Bensch et al. 2000; Ortego et al. 2007; Illera et al. 2008; Valkiunas et al. 2009).

The blue tit (*Cyanistes caeruleus*) is a model species that has been extensively studied in the fields of ecology, behaviour and evolution. Genetic characterization of avian malaria in this species has revealed the presence of several lineages of the genus *Plasmodium* and *Haemoproteus* (Bensch et al. 2009; Szollosi et al. 2011; Fig. 1). Some studies have also found that the two most widespread lineages (the *Plasmodium* SGS1 and the *Haemoproteus* PARUS1) have negative fitness consequences in this species (Martínez-de la Puente et al. 2010; Lachish et al. 2011a). The factors determining malaria prevalence and infection dynamics at a local spatial scale have been also recently studied, suggesting complex patterns that depend on both habitat and host characteristics (Shurulinkow and Chakarov 2006; Wood et al. 2007; Knowles et al. 2011; Lachish et al. 2011a). A recent study analysing nine blue tit populations across

Europe has reported strong differences among populations in parasite lineage composition, indicating that their transmission success is site specific (Szollosi et al. 2011).

In spite of the ecological and genetic distinctiveness of blue tit populations from southern Europe (Blondel et al. 2006; Illera et al. 2011; García-Navas and Sanz 2011), avian malaria parasites have been only genetically characterised in a single population located at these latitudes and no information is available for any genuine Mediterranean population (Szöllösi et al. 2011). In the present study, we characterise avian malaria in three Mediterranean blue tit populations from Montes de Toledo (central Spain), an area located close to the southern edge of the species distribution range (Illera et al. 2011). In the Mediterranean region, habitat fragmentation due to both natural and anthropogenic processes has resulted in mosaic landscapes (Blondel and Aronson 1999). In Montes de Toledo, prevailing climatic conditions have favoured the predominance of vegetation with low water requirements, while the deciduous oak woodlands preferred by blue tits are restricted to the most humid environments such as valleys and riverbanks (Blanco et al. 1997). This has probably contributed to increase population fragmentation and reduce dispersal and gene flow in comparison with other European blue tit populations (Ortego et al. 2011). For this reason, we predict that spatial heterogeneity in malaria transmission rates results in strong differences in parasite prevalence among the studied populations, a pattern which can be compared with that reported for populations studied across a much larger geographical scale (Szöllösi et al. 2011).

Materials and methods

The study was conducted in three localities of central Spain, Quintos de Mora (Toledo province: 39° 25′N, 4° 04′W; 2008–2011 breeding seasons), San Pablo de los Montes (Toledo province: 39° 31′N, 4° 21′W; 2011 breeding season), and Cabañeros National Park (Ciudad Real province: 39° 24′N, 3° 35′W; 2008 breeding season; Fig. 1). The study area comprises deciduous forests dominated by Pyrenean oak *Quercus pyrenaica* and Mediterranean scrublands. Adult birds were captured when feeding 8-day-old chicks by means of a spring trap shutting the entrance hole as the bird entered the nest. All birds were individually marked with aluminium rings for further identification. Blood samples (≤25 μl) for genetic analyses were obtained by brachial venipuncture and stored in ethanol 96 %.

We used NucleoSpin Tissue Kits (Macherey-Nagel) to extract and purify genomic DNA from the blood samples. A total of 853 adult individuals were screened for malaria infection using a highly efficient nested polymerase chain reaction (PCR) that amplifies 480 bp of the cytochrome *b* of both *Plasmodium* and *Haemoproteous* parasites (Waldenström et al. 2004). We used negative controls (i.e. samples with ddH_2O instead of genomic DNA as template) and positive controls (i.e. DNA from individuals with known malarial infections) to ascertain that the outcome of each PCR run was not affected by contamination (Waldenström et al. 2004). Further, negative infections were confirmed by repeated PCR. Positive or negative infections (i.e. birds having or not gametocytes or

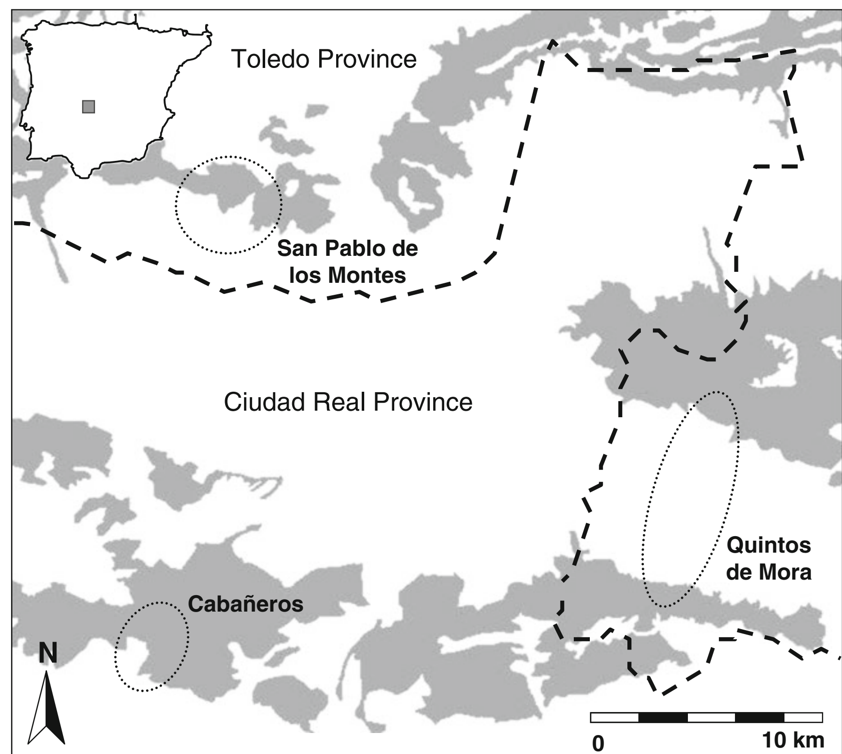

Fig. 1 Map of the study area showing main woodlands (*shaded areas*) and the spatial location of the studied blue tit populations (*dotted areas*)

merozoites in their blood stream) were scored separating PCR products on 2 % agarose gels. PCR products from positive samples were purified using NucleoSpin Extract II (Macherey-Nagel) kits and sequenced on an ABI 310 Genetic Analyser (Applied Biosystems).

Sequences were edited and aligned using the program SEQUENCHER 5.0 (GeneCodes Corporation). A sequence divergence of at least one nucleotide was used to define lineages (Waldenström et al. 2004). Chromatograms were examined for conspicuous overlapping peaks indicative of co-infection (e.g. Wood et al. 2007). The obtained sequences were compared to the MalAvi database (Bensch et al. 2009) and by the National Center for Biotechnology Information Basic Local Alignment Search Tool to those other published sequences available from GenBank. Then, a phylogenetic tree was constructed in the program MEGA 3.1 using a neighbour-joining method with a Kimura two-parameter distance matrix (Kumar et al. 2001). Node support was tested using 1,000 bootstrap replications. For comparison, we also included in this phylogenetic analysis all the avian malaria lineages (*Plasmodium* and *Haemoproteus*) previously isolated from blue tits according to the MalAvi database (Bensch et al. 2009) and other recent published studies (Szollosi et al. 2011; Lachish et al. 2011a). The tree was rooted using a sequence of *Leucocytozoon* sp. also isolated in blue tits (lineage Parus4; GenBank accession number: AY393795).

For a subset of the blue tits captured in 2011 breeding season (San Pablo de los Montes, 280 individuals; Quintos de Mora, 37 individuals), we compared the estimates of prevalence based on the PCR approach with ocular examinations of blood smears. For this purpose, we smeared a drop of blood on an individually marked microscope slide. Blood smears were rapidly air dried, fixed with absolute ethanol and later stained in the laboratory with Giemsa's solution (1:10) for 45 min. At least 10,000 erythrocytes per slide were examined at 1,000× magnification under oil immersion to determine presence of blood parasites. In positive smears, we estimated infection intensity as number of parasites/2,000 erythrocytes (Ortego and Espada 2007). Determination of prevalence and intensity of infection was carried out by the same person (E.S. Ferrer), who had no information about the origin of the samples except ring number of analysed birds.

Results and discussion

We have identified six lineages of avian malaria infecting blue tits in the studied populations (Fig. 2). Two of these

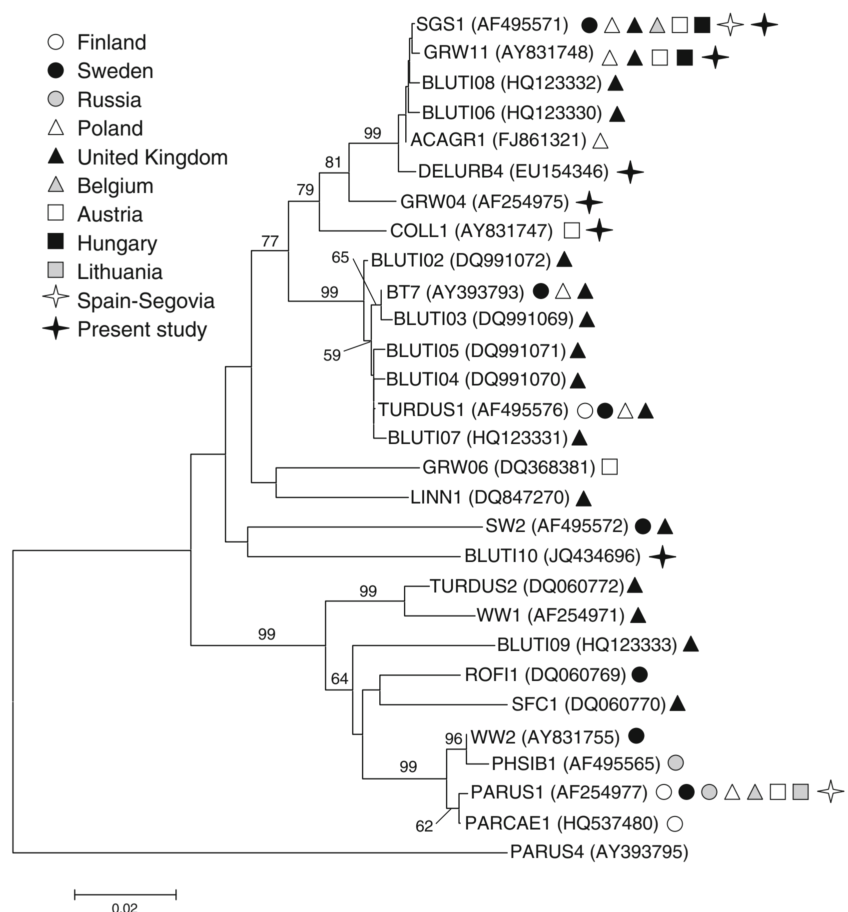

Fig. 2 Neighbour joining tree (Kimura two-parameter distance) of avian malaria lineages infecting blue tits based on partial sequences of the cytochrome *b* gene. Occurrence of these lineages in blue tit populations from different European countries is indicated. We used a sequence from *Leucocytozoon* sp. isolated from a blue tit (lineage PARUS4) as an outgroup to root the tree. Bootstrap values are based on 1,000 replicates and are shown when larger than 50. GenBank accession numbers of each isolate are shown in parentheses

lineages (SGS1 and GRW11) belong to the morphospecies *Plasmodium relictum* (Lachish et al. 2011a) and have been previously found parasitizing several other blue tit populations (Fig. 2) and more than 50 bird species from 16 families worldwide distributed (MalAvi database). COLL1 is a less frequent lineage that has been recently found parasitizing another blue tit population from Austria (Szöllösi et al. 2011). Lineages DELURB4 and GRW04 have not been previously recorded parasitizing blue tits but they have been detected in several other passerines (MalAvi database). Finally, lineage BLUTI10 has not been hitherto isolated from any bird species. The sequence for this lineage has been deposited in the GenBank International Nucleotide Sequence Database with accession number JQ434696. Based on the occurrence of conspicuous double peaks in electropherograms, we detected three individuals carrying mixed infections that always involved the lineages SGS1 and COLL1 (Table 1). It is noticeable the absence of infections by *Haemoproteus*, despite lineage PARUS1 within this genus reaches 100 % prevalence in a close Iberian population (Szöllösi et al. 2011). The blue tit is a sedentary species and shows very low dispersal rates in the study area (Ortego et al. 2011) indicating that all the detected lineages must be locally transmitted.

Sequence divergence between DELURB4 and SGS1-GRW11 is very low (<0.4 %, corresponding to 2 bp synonymous substitutions), suggesting that DELURB4 may also belong to *P. relictum* (Bensch et al. 2004). Phylogenetic analyses placed GRW04 and COLL1 in the main clade including *P. relictum* (Fig. 2) (see also Zehtindjiev et al. 2008). These lineages have sequence divergences with SGS1-GRW11 (2–3 %) similar to those previously reported among reproductively isolated avian malaria parasites, suggesting that they could be independent evolutionary units (Bensch et al. 2004). A previous study has also reported that lineages SGS1 and GRW11 occur at much lower parasitemia than GRW04 within the same host species, indicating its ecological distinctiveness (Zehtindjiev et al. 2008). Finally, phylogenetic analyses placed lineage BLUTI10 in a different clade (Fig. 2). Lineage BLUTI10 probably belongs to a different species according to the sequence divergences (6–8 %) with the other lineages detected in the studied populations (Bensch et al. 2004).

As found in previous studies, the ability of microscope examinations to detect haemosporidian infections was very low compared with the nested PCR approach (Waldenström et al. 2004; Ortego et al. 2007). No positive amplification for the lineages COLL1 and GRW04 was detected in blood smears using light microscopy (Table 1). Only 12.2 % of positive amplifications of lineage SGS1 were also positive by microscopic examination of blood smears and infection intensities were very low (mean±SE=2.0±1.4 parasites/2,000 erythrocytes; n=16). Two individuals infected with the lineages GRW11 (16.7 %; mean±SE=2.3±1.1 parasites/2,000 erythrocytes) and DELURB4 (33 %; mean±SE=1.0±0.0 parasites/2,000 erythrocytes) also showed infections detectable by traditional microscopic methods. The single individual infected with lineage BLUETI10 that was also examined at blood smears had detectable infections by microscopic examination (5.0 parasites/2,000 erythrocytes).

Prevalence strongly differed among the detected lineages (Table 1). SGS1 was the most frequent lineage, whereas other lineages showed a much lower prevalence (<4 %; Table 1). Considering that lineages GRW11-DELURB4 probably belong to the same species that SGS1, the lower frequency of the former could just reflect genetic bottlenecks or other demographic phenomena (Vardo and Schall 2007). The fact that the other recovered lineages (COLL1, BLUTI10 and GRW04) have been only detected in a few individuals suggests that they may represent sporadic infections by malaria lineages that mainly depend on other hosts species (e.g. Szöllösi et al. 2011). Another possibility is that

Table 1 Prevalence (percent) of the different malaria lineages (the percentage of infected individuals in relation to all screened individuals in a population) detected in the three study populations

	Quintos de Mora					Cabañeros	San Pablo de los Montes
Year	2008 (n=121)	2009 (n=149)	2010 (n=99)	2011 (n=93)	Years combined	2008 (n=111)	2011 (n=280)
SGS1	19.8 (24)	16.1 (24)	8.1 (8)	20.4 (19)	16.2 (75)	11.7 (13)	41.4 (116)
GRW11	1.7 (2)	0.0 (0)	0.0 (0)	1.1 (1)	0.6 (3)	2.7 (3)	3.9 (11)
COLL1	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.4 (1)
DELURB4	0.8 (1)	0.0 (0)	0.0 (0)	2.2 (2)	0.6 (3)	0.0 (0)	2.1 (6)
GRW04	0.8 (1)	0.0 (0)	0.0 (0)	0.0 (0)	0.2 (1)	0.0 (0)	0.0 (0)
BLUTI10	0.8 (1)	0.0 (0)	0.0 (0)	0.0 (0)	0.2 (1)	0.9 (1)	0.4 (1)
SGS1+COLL1[a]	0.8 (1)	0.0 (0)	0.0 (0)	0.0 (0)	0.2 (1)	0.9 (1)	0.4 (1)

Absolute number of infected individuals with each lineage is shown in parentheses

[a] Chromatograms showing conspicuous overlapping peaks indicating co-infection with the lineages SGS1 and COLL1

a high virulence of these lineages causes high mortality rates among infected individuals during the acute phase, thus, reducing the observed prevalence as a result of only live birds are sampled (Ortego et al. 2008).

Probability of parasitism combining all the studied lineages strongly differed among study populations (general linear model; $F_{1,\ 850}$=13.38, P<0.001) and years ($F_{1,\ 850}$= 3.86, P=0.009). A similar pattern was found only considering individuals infected with the most frequent lineage SGS1 (area, $F_{1,\ 850}$=11.12, P<0.001; year, $F_{2,\ 850}$=2.55; P=0.055). Post hoc Tukey tests showed that probability of parasitism was significantly different between San Pablo de Los Montes and the two other studied localities both considering all the lineages combined and only SGS1 (all P values<0.001). However, we found no significant differences between Cabañeros and Quintos de Mora (P values >0.5). This inter-population variability could be due to different landscape features and/or climate among the studied localities that may strongly influence vector abundance (Surulinkow and Chakarov 2006; Ortego et al. 2007; Shurulinkow and Ilieva 2009). These differences may be also maintained by the low dispersal and high isolation of the studied blue tit populations (Ortego et al. 2011; see also Illera et al. 2008). Given that Cabañeros and San Pablo de los Montes were only sampled during one breeding season (2008 and 2011, respectively), we repeated the analyses only comparing these populations with Quintos de Mora within the same study year. We found significant differences between Quintos de Mora and San Pablo de los Montes (all lineages, $F_{1,\ 371}$=15.17; P<0.001; lineage SGS1, $F_{1,\ 371}$= 18.91; P<0.001), but not between Quintos de Mora and Cabañeros (all lineages, $F_{1,\ 230}$=2.93; P=0.088; lineage SGS1, $F_{1,\ 230}$=2.99; P=0.085). We also found inter-annual differences in probability of parasitism within Quintos de Mora (all lineages, $F_{1,\ 459}$=4.77; P=0.003; lineage SGS1, $F_{1,\ 459}$=3.20; P=0.023). Post hoc Tukey tests showed that this pattern was driven by the lower probability of parasitism in 2010 in comparison with 2008 and 2011 breeding seasons (Table 1). Inter-annual variation of avian malaria prevalence has been observed in other studies and may be consequence of temporal fluctuation of vector abundance or due to the different origin of immigrant individuals in different years (see Surulinkow and Ilieva 2009 and references therein).

Overall, we have detected six lineages of avian malaria infecting the studied blue tit populations. All these lineages showed very low levels of parasitemia that cannot be efficiently detected using traditional microscopic examination of blood smears. We have also found strong spatial variability in infection patterns among geographically close localities, indicating that these fragmented populations constitute an ideal study system to analyse the factors determining the patterns of avian malaria transmission at the landscape scale.

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