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SHORT COMMUNICATION

Genetic characterization of avian malaria (Protozoa) in the endangered lesser kestrel, *Falco naumanni*

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Abstract We genetically analyzed avian malaria (Protozoa) isolated from lesser kestrels (*Falco naumanni*) breeding in La Mancha, Central Spain. A total of 586 adult individuals were screened for blood parasites using a very efficient polymerase chain reaction approach that amplifies a partial segment (498 bp) of the cytochrome *b* gene of avian malaria of the genera *Haemoproteus* and *Plasmodium*. The prevalence of *Plasmodium* was 8.2%, and the prevalence of *Haemoproteus* was 4.1%. Sequence analyses revealed six unique lineages of avian malaria, three *Plasmodium* (LK5, LK6, RTSR1) and three *Haemoproteus* (LK2, LK3, LK4). According to sequence divergence, these lineages seem to correspond to at least three different species, although all recovered lineages could be independent evolutionary units. The third most common lineage (RTSR1) has been previously retrieved from two other avian host species, including a resident African bird species and a trans-Saharan migrant passerine, suggesting that lesser kestrels could acquire this *Plasmodium* lineage at their winter quarters in Africa.

Introduction

Avian malaria parasites are a well-known group of parasites that are increasingly being studied due to the interesting implications of this host–parasite system for both ecology and evolution and their importance in conservation research. In recent years, the advent and application of molecular methods have revealed that the number of avian malaria species is much higher than previously thought and that host switching is extensive (Bensch et al. 2000; Waldenström et al. 2002; Bensch et al. 2004). On this sense, species described based on morphological criteria have been found to be constituted by a number of independent evolutionary units, indicating that the traditional view that host taxon is a valid character to distinguishing malaria parasite species is controversial (Bensch et al. 2000, 2004).

The lesser kestrel (*Falco naumanni*) is a small-sized colonial bird of prey. This species was once one of the most abundant birds of prey in Europe, but it suffered a sharp population decline in its Western Paleartic breeding range in the second half of the twentieth century that led to a complete extinction in several countries and to strong declines in others (Biber 1990). Thus, of great conservation interest is the knowledge of the factors, such as avian malaria parasites, which could potentially affect the maintenance of their populations. Based on morphological data, *Haemoproteus tinnunculi* is the only avian malaria parasitising lesser kestrels (Tella et al. 1996, 1999). However, the fact that the lesser kestrel is a trans-Saharan migrant makes intuitive that it is likely to be infected by a wider array of avian malaria parasites from both Europe and Africa rather than by a single species (Waldenström et al. 2002). As has been found for other malaria parasites, *H. tinnunculi* could be constituted by a complex of genetically differentiated lineages or even different good species with different and potentially pathological effects on host individuals (e.g. Bensch et al. 2004). On the other hand, malaria parasites of the genera *Plasmodium* could have gone undetected because they generally occur at low

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intensity and are hard to detect in blood smears using the traditional light microscopy (Waldenström et al. 2004).

In this paper, we genetically characterize avian malaria parasites in a lesser kestrel population from central Spain. For this purpose, we screened 586 individuals using a highly efficient nested polymerase chain reaction (PCR) method that allowed us in identifying malaria lineages infecting lesser kestrels and establishing their phylogenetic relationships on the basis of sequences of a partial segment (498 bp) of the cytochrome *b* gene of avian malaria.

Materials and methods

The study was conducted in La Mancha, central Spain (39° 20′N, 3°15′W; 600–800 m above sea level), in an area covering approximately 1,000 km^2 where we study 29 lesser kestrel colonies. The climate is meso-Mediterranean, with mean temperatures ranging from 24–26°C in July to 4–6°C in January and 300–400 mm of rainfall mainly concentrated in spring and autumn (see Ortego et al. 2007). During the 2001–2006 breeding seasons, adult lesser kestrels were trapped with a noose carpet or by hand during incubation, measured and individually marked with metallic and coloured plastic rings for further identification. Blood samples (100 μl) were obtained by venipuncture of the brachial vein and preserved in ~1,200 μl ethanol 96% at −20°C.

We used QIAamp DNA blood mini kits (QIAGEN®) to extract and purify total DNA from blood samples. A total of 586 individuals were screened for malaria infection using a highly efficient nested 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 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 we bidirectionally sequenced the fragments on an ABI 310 genetic analyser (Applied Biosystems®).

Sequences were edited and aligned using the program BioEdit (Hall 1999). A sequence divergence of at least one nucleotide was used to define lineages (Waldenström et al. 2002). The sequences determined from our isolates were aligned against homologous sequences of other avian malaria parasites registered in the GenBank by Clustal W multiple alignment program (Thompson et al. 1994). Then, a phylogenetic tree was constructed in the program PAUP 4.0 using a neighbour-joining method with a Kimura 2-parameter distance matrix (Swofford 1993). The tree was rooted with sequences from three primate malaria species (GenBank accession numbers: AF069619, AF069624, AF069609).

For a subset of the lesser kestrels captured during 2004–2006 (n=288), we compared the estimates of prevalences based on the PCR approach with ocular examinations of blood smears. For this purpose, we smeared a drop of blood on two individually marked microscope slides. Each smear was 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 per 2,000 erythrocytes (Ortego and Espada 2007). Determination of prevalence and intensity of infection was carried out by the same person (L. Zapero) who had no information about the individual birds except ring number.

Results and discussion

We found six unique lineages of avian malaria parasitising lesser kestrels (Fig. 1). The sequences have been deposited in the GenBank international nucleotide sequence database with accession numbers EF564175–EF564179 (Fig. 1). The sequence divergence between the lineages of *Haemoproteus* sp. varied from 0.2% (corresponding to 1-bp substitution between LK3/LK4) to 0.6% (between LK2/LK3). The corresponding values among *Plasmodium* lineages ranged from 0.6% (LK5/LK6) to 7.1% (LK5/RTSR1). According to the obtained sequence divergences, these lineages seem to correspond to at least three well-differentiated species (Bensch et al. 2004). However, some avian malaria cyt *b* lineages with less than 0.5% sequence divergence seem to be reproductively isolated entities (Bensch et al. 2004), suggesting that all six lineages could be independent evolutionary units.

We compared the sequences obtained from our samples with homologous sequences of other avian malaria lineages registered in the GenBank. Interestingly, we found that one of the most frequent lineages of *Plasmodium* (RTSR1) isolated in lesser kestrels has been previously retrieved in great reed warblers *Acrocephalus arundinaceus*, a trans-Saharan migrant passerine, sampled in their breeding grounds in Sweden (Bensch et al. 2007), and in rufous scrub robins *Cercotrichas galactotes*, a resident African bird species, sampled in Nigeria (Waldenström et al. 2002). This points to the possibility that this lineage is transmitted

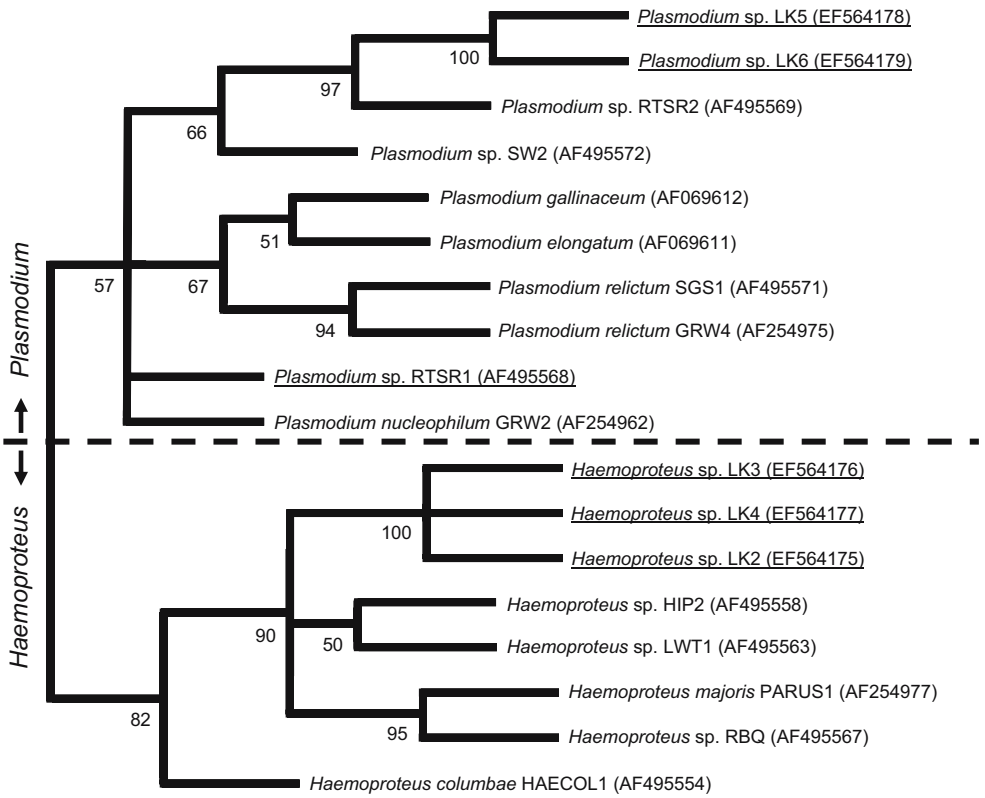

Fig. 1 Neighbour-joining tree (Kimura two-parameter distance) based on 478-bp partial cytochrome *b* sequences of six malaria parasites infecting lesser kestrels sampled in Spain (*underlined*) and 12 published avian *Plasmodium* and *Haemoproteus* lineages as references. Bootstrap values are based on 1,000 replicates and are shown when larger than 50. The GenBank accession numbers of each isolate are shown in *parentheses*

to lesser kestrels at their winter quarters in Africa (Waldenström et al. 2002), suggesting a potential cost of migration due to increased risk of encounter parasite faunas from vastly separated areas (Waldenström et al. 2002).

The mean prevalence of all lineages of *Plasmodium* combined was 8.2%, and the prevalence of *Haemoproteus* was 4.1%. Prevalence of the three most common lineages was 3.4% (RTSR1), 4.1% (LK2) and 4.6% (LK6). Two lineages (LK3 and LK5) were only detected in a single individual (0.2%), and another lineage was only recovered from four individuals (LK4; 0.7%). We found no bird infected with more than one parasite lineage. The fact that a half of the recovered lineages has only been detected in a few individuals suggests that they may represent sporadic infections by malaria lineages that mainly depend on other hosts species (Bensch et al. 2007). As found in previous studies, the ability of microscope examinations to detect haemosporidian infections was low compared with the nested PCR approach (Waldenström et al. 2004). All visually detected infections gave positive PCR amplifications. No positive amplification for the lineages RTSR1 (n=18), LK3 (n=1), LK5 (n=1) and LK6 (n=22) was detected in blood smears using light microscopy. However, 77% of positive amplifications of LK2 (n=13) and two out of three positive amplifications of LK4 were also detected by ocular methods, which, most likely, was a result of higher levels of parasitemia in individuals infected with these lineages (LK2: mean±SE=10.3±8.2 parasites per 2,000 erythrocytes; range=1–22; LK4: mean±SE=6.5±0.7 parasites per 2,000 erythrocytes; range=6–7). Morphological examinations of LK2 and LK4 infections tentatively identified them as *H. tinnunculi* (Apanius and Kirkpatrick 1988). Prevalence obtained using light microscopy was 3.1%, similar to 3.2% reported in another Spanish population of lesser kestrels (Tella et al. 1996). Overall, this prevalence was low when compared with other raptorial species (Tella et al. 1999). This may be a consequence of the scarcity of suitable vectors in open and arid areas occupied by lesser kestrels, which could reduce transmission rates in comparison with species inhabiting habitats more suitable for vector proliferation (Tella et al. 1999; Ortego and Espada 2007). Another possibility is that a high virulence of the studied parasites causes high mortality rates among infected individuals during the acute phase, thus, reducing the observed prevalences as a result of only live birds being sampled (Bensch et al. 2007).

Overall, we obtained that lesser kestrels are parasitised by six lineages of malaria parasites that may have evolved different adaptations and affect host differentially. These lineages seem to correspond to at least three different species, and a more detailed genetic analysis might allow to differentiate whether closely related isolates are good species or cases of intraspecific mitochondrial genetic polymorphism.

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