Parasitol Res (2009) 104:467–470
DOI 10.1007/s00436-008-1207-x

SHORT COMMUNICATION

# PCR-based detection and genotyping of haematozoa (Protozoa) parasitizing eagle owls, *Bubo bubo*

**Joaquín Ortego · Pedro J. Cordero**

Received: 26 May 2008 / Accepted: 12 September 2008 / Published online: 26 September 2008
© Springer-Verlag 2008

**Abstract** We genetically analysed haematozoa parasites (Protozoa) isolated from nestling eagle owls (*Bubo bubo*) in Toledo province, Central Spain. A total of 206 nestlings from 74 nests were screened for parasites of the genera *Leucocytozoon*, *Plasmodium*, and *Haemoproteus* using a very efficient polymerase chain reaction (PCR) approach that amplifies a partial segment of the mitochondrial cytochrome *b* gene of these parasites. PCR-based detection and sequence analyses revealed a unique lineage of *Leucocytozoon* (EO1) parasitizing nestling eagle owls. Ocular examination of blood smears identified these parasites as *Leucocytozoon ziemanni*, the only species deemed valid in owls based on morphology. Preliminary phylogenetic analyses using homologous published sequences isolated from other owl species suggest that *L. ziemanni* constitutes a monophyletic clade that may be composed by a complex of genetically differentiated cryptic species.

## Introduction

Avian haematozoa of the genera *Plasmodium*, *Haemoproteus*, and *Leucocytozoon* comprise a group of vector-borne transmitted parasites that have extensively been used as model organisms to study several aspects of host–parasite ecology and evolution (Bensch et al. 2000, 2004; Hellgren et al. 2008). In recent years, DNA sequencing has greatly contributed to understand several aspects of this host–parasite system, including relevant information on their vectors and their interactions with hosts (Hellgren et al. 2008). Some studies have revealed that the number of avian haematozoa species is much higher than previously thought (Bensch et al. 2000, 2004; Waldenström et al. 2002) and several species described based on morphology or host range have been found to be constituted by a number of independent evolutionary units with frequent host switching (Bensch et al. 2000; Bensch et al. 2004). However, most research has focused on the genera *Plasmodium* and *Haemoproteus* and lower attention has been paid on the sister genera *Leucocytozoon* (Hellgren et al. 2004, 2007, 2008; Hellgren 2005; Sehgal et al. 2006). The knowledge of the genetic characteristics of these parasites is of great interest to understand parasite virulence and distribution, reservoirs and transmission routes, and potential pathological and differential consequences of cryptic lineages (Ortego et al. 2008).

In eagle owls (*Bubo bubo*) and all other nocturnal raptors, *Leucocytozoon ziemanni* is the only species of the genus deemed valid based on morphological criteria (Remple et al. 2004). Although some aspects on the ecology of this host–parasite system have been recently studied (Ortego and Espada 2007; Ortego et al. 2007a; Penteriani et al. 2007), no information is available on the genetic characteristics of this parasite. On the light of recent evidence for cryptic speciation in several avian blood parasites, leucocytozoids parasitizing eagle owls could be constituted by a complex of genetically differentiated lineages or even different good species that may be

J. Ortego (✉) · P. J. Cordero
Grupo de Investigación de la Biodiversidad Genética y Cultural, Instituto de Investigación en Recursos Cinegéticos-IREC (CSIC, UCLM, JCCM),
Ronda de Toledo s/n,
E-13005 Ciudad Real, Spain
e-mail: joaquin.ortego@mncn.csic.es

J. Ortego
Departamento de Ecología Evolutiva,
Museo Nacional de Ciencias Naturales (CSIC),
C/ José Gutiérrez Abascal 2,
E-28006 Madrid, Spain

confined to a single host species or shared with other Strigiformes (e.g. Bensch et al. 2004; Sehgal et al. 2006). On the other hand, haematozoa of the genera *Plasmodium* and *Haemoproteus* have been previously recorded infecting adult eagle owls (Martinho and Canavilhas 2002) but, to the best of our knowledge, they have never been found parasitizing nestlings (Ortego and Espada 2007). Thus, it may be that infections by these parasites are only acquired later on life or they could have gone undetected in nestlings if they occur at intensities of infection below the limit of detection of traditional visual blood smear analyses (Hellgren et al. 2004; Waldenström et al. 2004).

Here, we genetically characterise haematozoa parasitizing an eagle owl population from central Spain. For this purpose, we screened 203 nestlings for parasites of the genera *Leucocytozoon*, *Plasmodium*, and *Haemoproteus* using a very efficient polymerase chain reaction (PCR) approach that amplifies a partial segment of the mitochondrial cytochrome *b* gene of these parasites. Sequence analyses allowed us identifying blood parasite lineages infecting eagle owls and establishing the phylogenetic relationships with isolates previously recovered from other bird species.

## Materials and methods

The study was conducted in Toledo province, central Spain (39°47′N, 4°04′W; 400–900 m above sea level), in an area covering 2,400 km$^2$ where we study a highly dense eagle owl population (Ortego and Espada 2007). 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. During 2003–2005 breeding seasons we obtained blood samples from 206 nestlings in 74 eagle owl nests. Nestlings were individually marked with metallic rings for further identification and when they were 20–50 days old we obtained blood samples (100 µl) by venipuncture of the brachial vein. Blood was preserved in ~1,200 µl ethanol 96% at −20°C.

We used NucleoSpin Tissue Kits (MACHEREY-NAGEL®) to extract and purify total DNA from blood samples. All nestlings were screened for infections using a highly efficient nested PCR that amplifies a partial segment of the mitochondrial cytochrome *b* of parasites of the genera *Leucocytozoon*, *Plasmodium*, and *Haemoproteus* (Hellgren et al. 2004). We used negative controls (i.e. samples with ddH$_2$O instead of genomic DNA as template) and positive controls (i.e. DNA from individuals with known infections) to ascertain that the outcome of each PCR run was not affected by contamination (Hellgren et al. 2004). Further, negative infections were confirmed by repeated PCR. Positive or negative infections 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). The sequences determined from our isolates were aligned against homologous sequences of other haematozoa 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 maximum parsimony method (Swofford 1993). The tree was rooted with a sequence from a primate malaria species (GenBank accession number: AF069619).

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 (Ortego and Espada 2007). Determination of prevalence was carried out by the same person (F. Espada), who had no information about the individual birds except ring number.

## Results and discussion

Nestling eagle owls were only infected by leucocytozoids and no infection by parasites of the genera *Plasmodium* and *Haemoproteus* was detected. Among the individual nestlings from all the nests we found a prevalence of 52.71% and 68.92% of the nests analysed had at least one infected nestling, similar to previously described in our study population (Ortego et al. 2007a). As found in previous studies, the ability of microscope examinations to detect infections by leucocytozoids was lower compared with the nested PCR approach (Hellgren et al. 2004). On this respect, all visually detected infections gave positive PCR amplifications whereas only 83.17% of positive amplifications were also detected by ocular methods. The fact that no individual was positive for *Plasmodium*/*Haemoproteus* parasites may have resulted if the high intensities of parasitemia by *Leucocytozoon* (up to 109 infected gametocytes/2,000 erythrocytes; Ortego et al. 2007a) take over the PCR and this prevents amplification of the other genera that may occur at lower intensities (Bensch et al. 2007). To avoid this potential methodological problem, we also screened all individuals for *Plasmodium*/*Haemoproteus* parasites using another nested PCR approach that amplifies

the same segment of the cytochrome *b* gene only in these genera (Waldenström et al. 2004; see Ortego et al. 2007b for detailed description), but again we found no single individual infected. Thus, although *Plasmodium*/*Haemoproteus* parasites have been previously described infecting adult eagle owls (Martinho and Canavilhas 2002), it may occur that adequate vectors for parasite transmission are not available during the chick development period and, thus, infections would be only acquired latter on life. Another possibility is that chicks become parasitised during the nesting period but infections are not patent before fledgling and, thus, these parasites may go undetected even using a very efficient nested PCR approach.

Among 107 nestlings that were positive for *Leucocytozoon* infections, we sequenced PCR products of the mitochondrial cytochrome *b* gene of the parasite from 48 individuals sampled in 31 different nests. We identified a unique lineage of *Leucocytozoon* (EO1) parasitizing nestling eagle owls. The sequence has been deposited in the GenBank International Nucleotide Sequence Database with accession number EU624137 (Fig. 1). Morphological examinations of infections tentatively identified them as *L. ziemanni*, the only species of the genus deemed valid in owls based on morphological criteria. We compared the sequence obtained from our samples with homologous sequences of other *Leucocytozoon* lineages registered in the GenBank, with particular attention to three lineages previously isolated from other species of the family Strigidae (Fig. 1). The sequence divergence between the lineage isolated from eagle owls (EO1) and those recovered from other Strigidae ranged from 0.4% (corresponding to 2-bp substitutions with an isolate from tawny owls, *Strix aluco*, EF607285) to 4.18% (between EO1/EF607286) and the corresponding value between the most divergent isolates infecting Strigiformes was 4.39% (EF607285/EF607286). Our preliminary phylogenetic analysis together with information on sequence divergence suggests that *L. ziemanni* constitutes a monophyletic clade that may be composed by a complex of genetically differentiated cryptic species (Fig. 1; Bensch et al. 2004; Hellgren et al. 2004; Sehgal et al. 2006).

Overall, we have found that nestling eagle owls are parasitised by a single lineage of *Leucocytozoon*, a figure much smaller than previously found for other bird species (e.g. Hellgren et al. 2004, 2007; Hellgren 2005; Sehgal et al. 2006). The presence of a single blood parasite lineage is likely to have reduced within-host competition between genetically diverse parasites (de Roode et al. 2005) and this may help to explain the scarce virulence of leucocytozoids infecting nestling eagle owls (Ortego and Espada 2007; Ortego et al. 2007a). Further, the lineage described is phylogenetically close to isolates previously recovered from other owl species and more detailed genetic analysis of leucocytozoids parasitizing owls, together with additional sequence information from nuclear genes, will help to resolve their taxonomic status and give valuable information on the evolution and host specificity of these parasites.

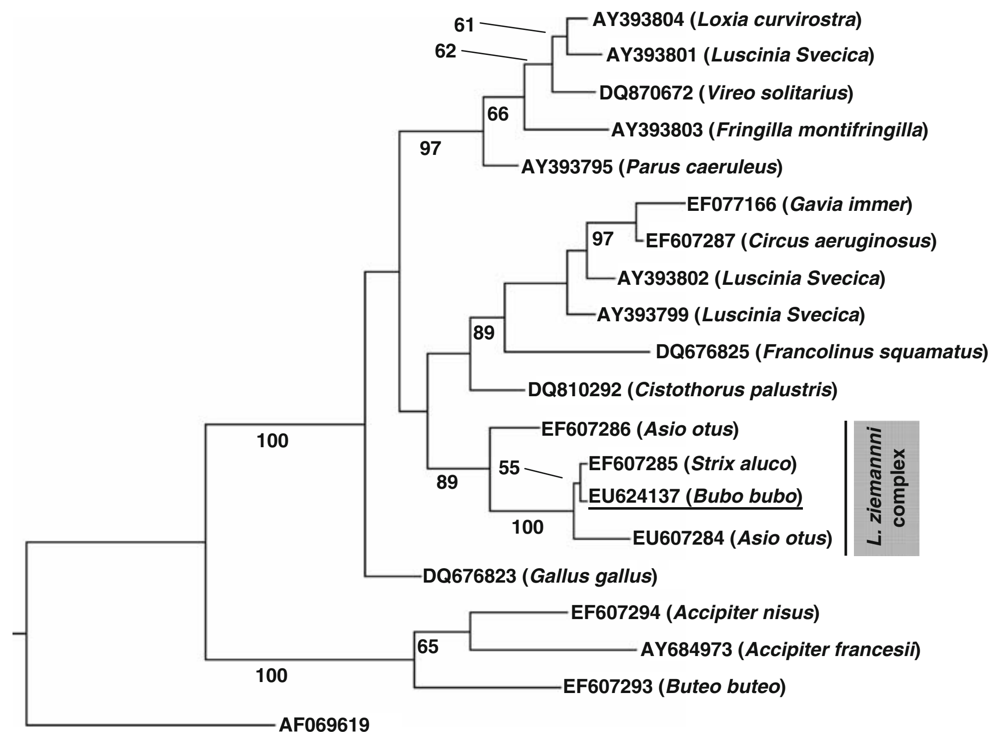


**Fig. 1** Maximum parsimony tree based on 478 bp partial cytochrome *b* sequences of the *Leucocytozoon* parasite infecting eagle owls (*underlined*) and 18 published *Leucocytozoon* lineages as references. The tree was rooted with a cytochrome *b* gene sequence from one primate malaria species (AF069619). Bootstrap values are based on 1,000 replicates and are shown when larger than 50. GenBank accession numbers and initial host species for each isolate (*in parentheses*) are shown

**Acknowledgments** We wish to thank E. Espada for determining blood parasite prevalence in the blood smears. R. Bonal assisted us with the program PAUP. We manipulated and banded nestling eagle owls under license from the Spanish institutional authorities (Environmental Agency of the Community of Castilla–La Mancha and the Ringing Office of the Ministry of Environment) and we followed general ethical guidelines for animal welfare and nature conservation. This work received financial support from the projects: CGL2005-05611-C02-02/BOS (MEC), PAI05-053 (JCCM), and PCI08-0130-3954 (JCCM). During this work J.O was supported by a pre-doctoral fellowship from the Junta de Comunidades de Castilla–La Mancha/ European Social Fund and a post-doctoral JAE-Doc (CSIC) contract. We performed all the laboratory work at the Laboratory of Genetics of the IREC and sequencing was performed by the Centro de Investigaciones Biológicas (CSIC) of Madrid.

## References

Bensch S, Stjernman M, Hasselquist D, Östman Ö, Hansson B, Westerdahl H, Torres-Pinheiro R (2000) Host specificity in avian blood parasites: a study of *Plasmodium* and *Haemoproteus* mitochondrial DNA amplified from birds. Proc R Soc B 267:1583–1589

Bensch S, Pérez-Tris J, Waldenström J, Hellgren O (2004) Linkage between nuclear and mitochondrial DNA sequences in avian malaria parasites—multiple cases of cryptic speciation? Evolution 58:1617–1621

Bensch S, Waldenström J, Jozén N, Westerdahl H, Hansson B, Sejberg D, Hasselquist D (2007) Temporal dynamics and diversity of avian malaria parasites in a single host species. J Anim Ecol 76:112–122

de Roode JC, Pansini R, Cheesman SJ, Helinski MEH, Huijben S, Wargo AR, Bell AS, Chan BHK, Walliker D, Read AF (2005) Virulence and competitive ability in genetically diverse malaria infections. Proc Natl Acad Sci USA 102:7624–7628

Hall TA (1999) BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser 41:95–98

Hellgren O (2005) The occurrence of haemosporidian parasites in the Fennoscandian bluethroat (*Luscinia svecica*) population. J Ornithol 146:55–60

Hellgren O, Waldenstrom J, Bensch S (2004) A new PCR assay for simultaneous studies of *Leucocytozoon*, *Plasmodium*, and *Haemoproteus* from avian blood. J Parasitol 90:797–802

Hellgren O, Waldenstrom J, Perez-Tris J, Szollosi E, Hasselquist D, Krizanauskiene A, Ottosson U, Bensch S (2007) Detecting shifts of transmission areas in avian blood parasites - a phylogenetic approach. Mol Ecol 16:1281–1290

Hellgren O, Bensch S, Malmqvist B (2008) Bird hosts, blood parasites and their vectors—associations uncovered by molecular analyses of blackfly blood meals. Mol Ecol 17:1605–1613

Martinho F, Canavilhas MP (2002) Prevalence of haematozoa in wild birds admitted for rehabilitation at Centro de Recuperação de Animais Selvagens do Parque Florestal de Monsanto (C.R.A.S.P. E.M.). Proceedings of the Veterinary Sciences Congress. Sociedade. Portuguesa de Cîencias Veterinarias, Oeiras, pp 427–436

Ortego J, Espada F (2007) Ecological factors influencing disease risk in eagle owls *Bubo bubo*. Ibis 149:386–395

Ortego J, Espada F, Baquero RA (2007a) Ecology of parasitism of nestling Eurasian eagle-owls (*Bubo bubo*) by *Leucocytozoon ziemanni*. J Raptor Res 41:247–251

Ortego J, Calabuig G, Cordero PJ, Aparicio JM (2007b) Genetic characterization of avian malaria (Protozoa) in the endangered lesser kestrel, *Falco naumanni*. Parasitol Res 101:1153–1156

Ortego J, Cordero PJ, Aparicio JM, Calabuig G (2008) Consequences of chronic infections with three different avian malaria lineages on reproductive performance of lesser kestrels (*Falco naumanni*). J Ornithol 149:337–343

Penteriani V, Delgado MD, Alonso-Alvarez C, Pina NV, Sergio F, Bartolommei P, Thompson LJ (2007) The importance of visual cues for nocturnal species: eagle owl fledglings signal with white mouth feathers. Ethology 113:934–943

Remple JD (2004) Intracellular hematozoa of raptors: a review and update. J Avian Med Surg 18:75–88

Sehgal RNM, Hull AC, Anderson NL, Valkiunas G, Markovets MJ, Kawamura S, Tell LA (2006) Evidence for cryptic speciation of *Leucocytozoon* spp. (Haemosporida, Leucocytozoidae) in diurnal raptors. J Parasitol 92:375–379

Swofford DL (1993) PAUP: Phylogenetic Analysis Using Parsimony. Mac version 3.1.1 (Computer program and manual). Illinois Natural History Survey, Champaign

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680

Waldenström J, Bensch S, Kiboi S, Hasselquist D, Ottosson U (2002) Cross-species infection of blood parasites between resident and migratory songbirds in Africa. Mol Ecol 11:1545–1554

Waldenström J, Bensch S, Hasselquist D, Östman Ö (2004) A new nested PCR method very efficient in detecting *Plasmodium* and *Haemoproteus* infections from avian blood. J Parasitol 90:191–194