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Phylogenetic relationships of haemosporidian parasites in New World Columbiformes, with emphasis on the endemic Galapagos dove ☆

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ABSTRACT

DNA-sequence analyses of avian haemosporidian parasites, primarily of passerine birds, have described the phylogenetic relationships of major groups of these parasites, which are in general agreement with morphological taxonomy. However, less attention has been paid to haemosporidian parasites of non-passerine birds despite morphological and DNA-sequence evidence for unique clades of parasites in these birds. Detection of haemosporidian parasites in the Galapagos archipelago has raised conservation concerns and prompted us to characterise the origins and diversity of these parasites in the Galapagos dove (*Zenaida galapagoensis*). We used partial mitochondrial cytochrome *b* (cyt *b*) and apicoplast caseinolytic protease C (ClpC) genes to develop a phylogenetic hypothesis of relationships of haemosporidian parasites infecting New World Columbiformes, paying special attention to those parasites infecting the endemic Galapagos dove. We identified a well-supported and diverse monophyletic clade of haemosporidian parasites unique to Columbiformes, which belong to the sub-genus *Haemoproteus* (*Haemoproteus*). This is a sister clade to all the *Haemoproteus* (*Parahaemoproteus*) and *Plasmodium* parasites so far identified from birds as well as the *Plasmodium* parasites of mammals and reptiles. Our data suggest that the diverse *Haemoproteus* parasites observed in Galapagos doves are not endemic to the archipelago and likely represent multiple recent introductions.

1. Introduction

Haemosporidian parasites are vector-born parasites in the order Haemosporida (Phylum Apicomplexa) that are commonly found in reptiles, birds and mammals (Valkiūnas, 2005). Avian haemosporidian parasites have a cosmopolitan distribution and are divided into four genera: *Plasmodium*, *Haemoproteus*, *Fallisia* and *Leucocytozoon* (Atkinson and van Riper, 1991; Atkinson, 1991; Valkiūnas, 2005). Only recently have evolutionary biologists and ecologists applied molecular approaches to study this group of parasites in free-ranging hosts (e.g., see Schall and Marghoob, 1995; Schall and Pearson, 2000; Salkeld and Schwarzkopf, 2005 for lizards; and Bensch et al., 2000; Ricklefs and Fallon, 2002; Fallon et al., 2003, 2004, 2005; Pérez-Tris and Bensch, 2005; Valkiūnas, 2005; Hellgren et al., 2007a for birds). These studies have revealed that the diversity of parasite DNA lineages greatly surpasses the number of named species based on morphological characters (Ricklefs and Fallon, 2002; Križanauskienė et al., 2006; Martinsen et al., 2006; Hellgren et al., 2007a).

Phylogenetic studies of avian haemosporidian parasites, mostly based on mitochondrial (mtDNA) cytochrome *b* (cyt *b*), have identified many parasite lineages of the genera *Plasmodium* and *Haemoproteus* infecting passerine birds (Perkins and Schall, 2002; Ricklefs and Fallon, 2002; Martinsen et al., 2008); however, few studies have specifically addressed the haemosporidian parasites of non-passerine birds. Traditional taxonomy based on morphological characters places *Haemoproteus* parasites that infect Columbiformes in the sub-genus *Haemoproteus*, whereas all other *Haemoproteus* parasites belong to the sub-genus *Parahaemoproteus* (Valkiūnas, 2005). Sequence (mtDNA cyt *b* and cytochrome *c* oxidase subunit I (COI), apicoplast caseinolytic protease C (ClpC) and nuclear adenylosuccinate lyase (asl), genes) have verified the phylogenetic position of the sub-genus *Haemoproteus* (*Haemoproteus*) clade as sister to the sub-genus *Haemoproteus* (*Parahaemoproteus*) and the genus *Plasmodium* (Martinsen et al., 2008); however, that study only included three samples from domestic pigeons (*Columba livia*), which represent one morphological species of parasite (*Haemoproteus columbae*). Thus, sampling of haemosporidian parasites from pigeons and doves, as well as from other non-passerine birds, has been rather limited in phylogenetic analyses. Wide geographical sampling of blood parasites infecting non-passerine birds

☆ *Note:* Nucleotide sequence data reported in this paper are available in the GenBank™ database under the accession numbers FJ462649–FJ462685 and FJ467560–FJ467608.

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will certainly provide a refinement of the taxonomy and systematics of the order Haemosporida.

Traditional taxonomy recognises 19 morphological species of haemosporidian parasites infecting Columbiformes (Bennett and Peirce, 1990; Valkiūnas, 2005). Of these, six belong to the genus *Haemoproteus*, 11 to *Plasmodium*, one to *Fallisia* and one to *Leucocytozoon* (Valkiūnas, 2005). *H. columbae*, *H. sacharovi*, *H. palumbis*, *H. turtur*, *Plasmodium gabaldoni*, *P. columbae*, *Fallisia neotropicalis* and *Leucocytozoon marchouxi* have been described from bird species of the family Columbidae (Valkiūnas, 2005). The other two *Haemoproteus* parasites (*H. krylovi* and *H. pteroclis*) that infect pigeons and doves have been described from the bird family Pteroclididae. The remaining parasites infecting pigeons and doves have been described from birds belonging to the families Passeridae, Turdidae, Hirundinidae, Phasianidae, Mimidae and Psittacidae (Valkiūnas, 2005). The vertebrate hosts of the present study belong to the family Columbidae, sub-family Columbinae, with approximately 60 species recognised across the American continent (Baptista et al., 1997). The endemic Galapagos dove (*Zenaida galapagoensis*) belongs to a genus comprising seven species, within which its sister species is the widespread South American eared dove (*Z. auriculata*) (Johnson and Clayton, 2000).

The study of avian haemosporidian parasites has contributed to understanding emerging infectious diseases in novel hosts (e.g., Mackenzie et al., 2004; Kilpatrick et al., 2006; Jourdain et al., 2007). Because millions of migratory birds travel enormous distances, they potentially can transmit parasites between distant geographical locations, even between continents (e.g., Waldeström et al., 2002; Ricklefs et al., 2005; Pérez-Tris and Bensch, 2005; Fallon et al., 2006; Svensson et al., 2007; but see Hellgren et al., 2007b). In addition, many avian haemosporidian parasites are able to infect species from different bird families (Ricklefs and Fallon, 2002; Ricklefs et al., 2004; Križanauskienė et al., 2006; but see Iezhova et al., 2005).

Introduced avian diseases are a concern for the conservation of endemic species in the Galapagos archipelago (Padilla et al., 2004; Parker et al., 2006). Recently, we have detected high prevalence (>80%) and infection intensities of *Haemoproteus* spp. in endemic Galápagos doves from eight different islands of the archipelago (Padilla et al., 2004; Santiago-Alarcon et al., 2008). We suspected that these haemosporidian parasites might have arrived in the Galapagos recently, perhaps via introduced domestic pigeons, which were repeatedly brought to the islands during the last century (Harmon et al., 1987; Padilla et al., 2004). An extermination program removed the last remaining domestic pigeons from the Galapagos in 2002, leaving the endemic Galapagos dove as the only columbiform species inhabiting the archipelago.

Until now, no attempt has been made to characterise the diversity of columbiform haemosporidian parasites using molecular methods. In this study, we provide a comprehensive phylogenetic analysis of columbiform haemosporidian parasites using partial mtDNA cyt *b* and ClpC genes. We also characterise the haplotype diversity of haemosporidian parasites of Galapagos doves in relation to parasite lineages obtained from doves across Latin America.

2. Materials and methods

2.1. General field and laboratory methods

Pigeons and doves were captured using mist nets and hand nets. Our sample comprised 439 blood samples from doves and pigeons of North and South America and the West Indies. We collected 166 blood samples from Galapagos doves on eight islands (Santiago, Santa Cruz, Santa Fe, Española, San Cristobal, Genovesa, Darwin and Wolf) between 2002 and 2005. In 2002, we also obtained samples from 14 domestic pigeons on San Cristobal Island. We obtained blood samples from New World Columbiformes (17 species from seven genera) in the United States (two samples), Mexico (seven), Caribbean islands (10), Venezuela (126), Peru (29), Uruguay (two), Ecuador (73) and Guatemala (10). Samples from Ecuador (Galapagos and mainland), Peru, USA and Caribbean Islands were collected by the authors. Samples from other localities were provided to us by colleagues. To develop a broader phylogenetic perspective, we also used published DNA sequences available in GenBank from Martinsen et al. (2008).

We collected ~50 μl of blood from each captured bird via puncture of the wing vein. Samples were preserved in lysis buffer (Longmire et al., 1988). DNA was extracted by phenol–chloroform followed by dialysis in 1× TNE_2 (Sambrook and Russell, 2001). Infection was determined by visual examination of blood smears and by PCR amplification of parasite gene sequences (Fallon et al., 2003) in endemic Galapagos doves, and by PCR only in all other columbiform samples. We prepared two thin blood smears from each sampled Galapagos dove. Smears were air dried, fixed in methyl alcohol and stained with Giemsa's stain. We scanned from 100 to 200 fields at 100× magnification to visually identify infected individuals (Valkiūnas et al., 2006). We sequenced a fragment of ~600 bp of mtDNA cyt *b* and ~550 bp of the apicoplast gene ClpC using published primers that are specific for haemosporidian parasites (Perkins and Schall, 2002; Martinsen et al., 2008). PCR was conducted in 50 μl reactions containing 5 μl 10× Ex Taq buffer, 4 μl of 25 mM $MgCl_2$, 4 μl of dNTP mixture (2.5 mM each), 0.25 U of Takara Ex Taq polymerase, 1 μl of 10 mM primers, 1 μl of stock DNA, and 1 μl of BSA only for mtDNA cyt *b* reactions.

We conducted nested PCRs. For mtDNA cyt *b* we used primer pairs DW2–DW4 for the outer reaction and DW1–HaemoR for the nested reaction (Perkins and Schall, 2002) and for ClpC we used primer pairs ClpC/outer and ClpC/nested (Martinsen et al., 2008). When several bands were present in the amplified products, we optimised the PCR to suppress most or all unspecific product. Then, we purified the targeted fragment by using a QIAquick gel extraction kit (QIAGEN). When a single band was obtained, we directly cleaned the PCR product using Antarctic phosphatase and Exonuclease I (# M0289S and # M0293S, respectively, New England Bio Labs, Inc.). We used sequence chromatograms to determine samples with possible mixed infections. When we detected double peaks in sequence chromatograms, we sequenced the sample again and adjusted conditions to verify that it was not an artefact of the sequencing reaction. If we again obtained a chromatogram with double peaks we removed the sample from analyses. We either used an ABI 3100 microcapillary genetic analyzer to sequence DNA products or sent the samples for sequencing at Macrogen, Inc. (Korea). DNA sequences were compared with sequences of the GenBank database by using the BLAST algorithm to verify the amplification of the correct gene. All sequences have been deposited in GenBank (Accession Nos.: FJ462649–FJ462685 and FJ467560–FJ467608).

2.2. Phylogenetic analyses

Sequences were edited in SeqManII version 4 (1989–1999, DNASTAR, Inc.) and aligned by eye in Se-Al version 2.0a11 (1996–2002, http://tree.bio.ed.ac.uk/software/seal/). We constructed phylogenetic hypotheses using maximum parsimony, maximum likelihood and Bayesian methods. Phylogenetic analyses were performed in PAUP* version 4.0b10 (Swofford, D., 2002. Paup*version 4.0b10. Massachusetts: Sunderland.) and Mr. Bayes 2.01 (Huelsenbeck and Ronquist, 2001), and the best-fit model of DNA evolution was determined using MODELTEST (Posada and Crandall, 1998). All analyses were executed in the Beowulf computing cluster of the University of Missouri – St. Louis, USA

(http://www.umsl.edu/services/kellogg/Beowulf/index.html). For each Bayesian analysis we did four independent runs, with four chains in each run for a total of 25 million generations, sampling every 1000 generations. We discarded as burn-in the first 10,000 trees, and used a total of 10,000 trees from each run to build our majority-rule consensus tree. We conducted 100 bootstrap pseudoreplicates for the maximum likelihood and maximum parsimony analyses. For the model-based analyses we used a GTR+I+Γ model of molecular evolution with shape parameter $\alpha = 0.74$ and proportion of invariable sites Pinvar = 0.35 as calculated with MODELTEST. To build our majority-rule consensus from the maximum parsimony analysis we used 50,000 trees. After building our majority-rule consensus tree from cyt *b*, we forced the topology to match our ClpC dataset to test for incongruence among the two datasets. mtDNA cyt *b* and ClpC datasets were incongruent (Templeton test, $z = -10.6$, $P < 0.0001$), thus we did not concatenate those and instead conducted separate analyses. After we obtained our consensus phylogeny of columbiform haemosporidian parasites using the ClpC dataset, we applied RELL S–H tests (Shimodaira and Hasegawa, 1999; Felsenstein, 2004) to alternative tree topologies (hypotheses). There is no consensus on how to delimit haemosporidian species based on mtDNA cyt *b* (e.g., Perkins, 2000; Valkiūnas et al., 2007). Thus, we used haplotypes as our units of diversity and avoided any attempt to define species based on these data; we defined a haplotype as a DNA sequence that differs by one or more bp from other DNA sequences obtained for the same molecular marker.

3. Results

We recovered 160 sequences of the mtDNA cyt *b* gene and 107 sequences of the apicoplast ClpC gene. We identified 30 cyt *b* haplotypes and 46 ClpC haplotypes of the sub-genus *H.* (*Haemoproteus*), six cyt *b* and one ClpC haplotypes of the genus *Plasmodium*, and one cyt *b* and two ClpC haplotypes of the sub-genus *H.* (*Parahaemoproteus*) (Supplementary Table S1). The maximum sequence divergence between two haplotypes of the *H.* (*Haemoproteus*) subgenus was 5.5% for the mtDNA cyt *b* and 8% for the ClpC genes. The maximum sequence divergence between haplotypes of the subgenus *H.* (*Haemoproteus*) infecting endemic Galapagos doves was 3.1% as estimated with the cyt *b* dataset and 7.5% as estimated with the ClpC dataset.

Our phylogenetic hypotheses based on cyt *b* sequences (Fig. 1) placed most of the dove parasites in a sister clade (*H.* (*Haemoproteus*)) to parasite haplotypes of the sub-genus *H.* (*Parahaemoproteus*) and the genus *Plasmodium*. This clade was highly supported by our three reconstruction methods, and was supported by the ClpC dataset (Supplementary Fig. S1). The cyt *b* phylogeny shows a well-supported clade that contains four haplotypes in the genus *Plasmodium* recovered from columbiform hosts (Fig. 1). That same clade contains a parasite haplotype identified as *Plasmodium* (*Huffia*) sp. recovered in a passerine bird in the study of Martinsen et al. (2008); however, species from the sub-genus *Huffia* can also infect Columbiformes. The ClpC and cyt *b* datasets conflict with respect to the placement of certain columbiform haplotypes: these lineages, identified with cyt *b* as columbiform *Plasmodium*, are nested within the *H.* (*Haemoproteus*) clade in the ClpC phylogeny (Figs. 1 and 2). The two datasets also disagreed with respect to the relationships among haplotypes within the sub-genus *H.* (*Haemoproteus*); for example, haplotypes CB4Ecu and CB8Ecu are identical in the cyt *b* tree, but belong to different clades in the ClpC tree. Another example is that of haplotype LV13Ecu, which is identified as *H.* (*Haemoproteus*) in the cyt *b* phylogeny and as *H.* (*Parahaemoproteus*) in the ClpC phylogeny (see Supplementary Table S1 and Figs. 1, 2).

The cyt *b* data did not resolve relationships among haplotypes of the sub-genus *H.* (*Haemoproteus*). In contrast, the ClpC data revealed four well-supported clades containing lineages infecting endemic Galapagos doves (Fig. 2). These exhibited no geographic structure and most were widely distributed among dove populations throughout the archipelago (Fig. 2 and Supplementary Table S1). None of the four recovered ClpC clades was specific to endemic doves; instead, they included haplotypes from parasites infecting continental pigeons and doves (Fig. 2). This is supported by the cyt *b* data, where all haplotypes infecting Galapagos doves are included in a polytomy that contains parasite haplotypes from across the American continent.

To test whether clades A, B, C and D (Fig. 2), recovered with ClpC, might, with similar support, represent an unresolved clade (polytomy), we built alternative topologies (hexagons 1 and 2 in Fig. 2). We first collapsed all nodes with support values lower than 80, and then constructed two alternative topologies. First, we converted clade D (hexagon 1 in Fig. 2) into a polytomy. The new topology received significantly less support than the topology presented in Fig. 2 (S–H test, $P = 0.015$), indicating that clade D is well supported. A second topology (hexagon 2 in Fig. 2) tested the hypothesis that clades A, B and C comprise a single unresolved clade. This topology also received significantly less support than the topology presented in Fig. 2 (S–H test, $P < 0.001$), strengthening the phylogenetic hypothesis shown in Fig. 2.

4. Discussion

Our study based on partial sequences of the mtDNA cyt *b* and ClpC apicoplast genes confirmed the existence of a diverse clade of haemosporidian parasites (sub-genus *H.* (*Haemoproteus*)) unique to Columbiformes. Searches using the BLAST algorithm of the GenBank data base failed to find related haplotypes infecting hosts other than Columbiformes. Our results are in agreement with the morphological taxonomic placement of these parasites in the sub-genus *H.* (*Haemoproteus*), which is sister to the sub-genus *H.* (*Parahaemoproteus*) and the genus *Plasmodium* that infect passerine birds, reptiles and mammals (Fig. 1 and Supplementary Fig. S1). As reflected in the phylogenetic trees, *H.* (*Haemoproteus*) haplotypes found in well-supported clades present no geographical structure. Rather, they are widely distributed across the American continent. Furthermore, most of the *H.* (*Haemoproteus*) haplotypes were not restricted to a single host species (Supplementary Table S1). Indeed, haplotypes recovered from Galapagos doves were not unique to the archipelago, but were distributed widely in continental dove populations, from which they were likely brought to the Galapagos Islands.

Valkiūnas (2005) identified six *Haemoproteus* parasites infecting Columbiformes. From these, two (the widely distributed *H. columbae* and *H. sacharovi*) are the only described species recorded in New World pigeons and doves. The other four species have been recorded only in the Palearctic region (Valkiūnas, 2005). The maximum sequence divergences between two haplotypes of the *H.* (*Haemoproteus*) sub-genus were 5.5% for the mtDNA cyt *b* and 8% for the ClpC genes. Morphologically described species with accompanying mtDNA sequence differ by 3–5% sequence divergence (Perkins, 2000; Valkiūnas et al., 2007), and often much less (Beadell et al., 2006); therefore parasite lineages recovered from Galapagos doves likely represent at least two species. However, this should be corroborated with morphological identification of parasites in blood smears.

From the *H.* (*Haemoproteus*) parasite species, *H. turtur* was first described from *Streptopelia turtur* (Columbidae). It also infects other columbiform hosts and as such is considered to belong to the sub-genus *H.* (*Haemoproteus*) (Valkiūnas, 2005). In our analy-

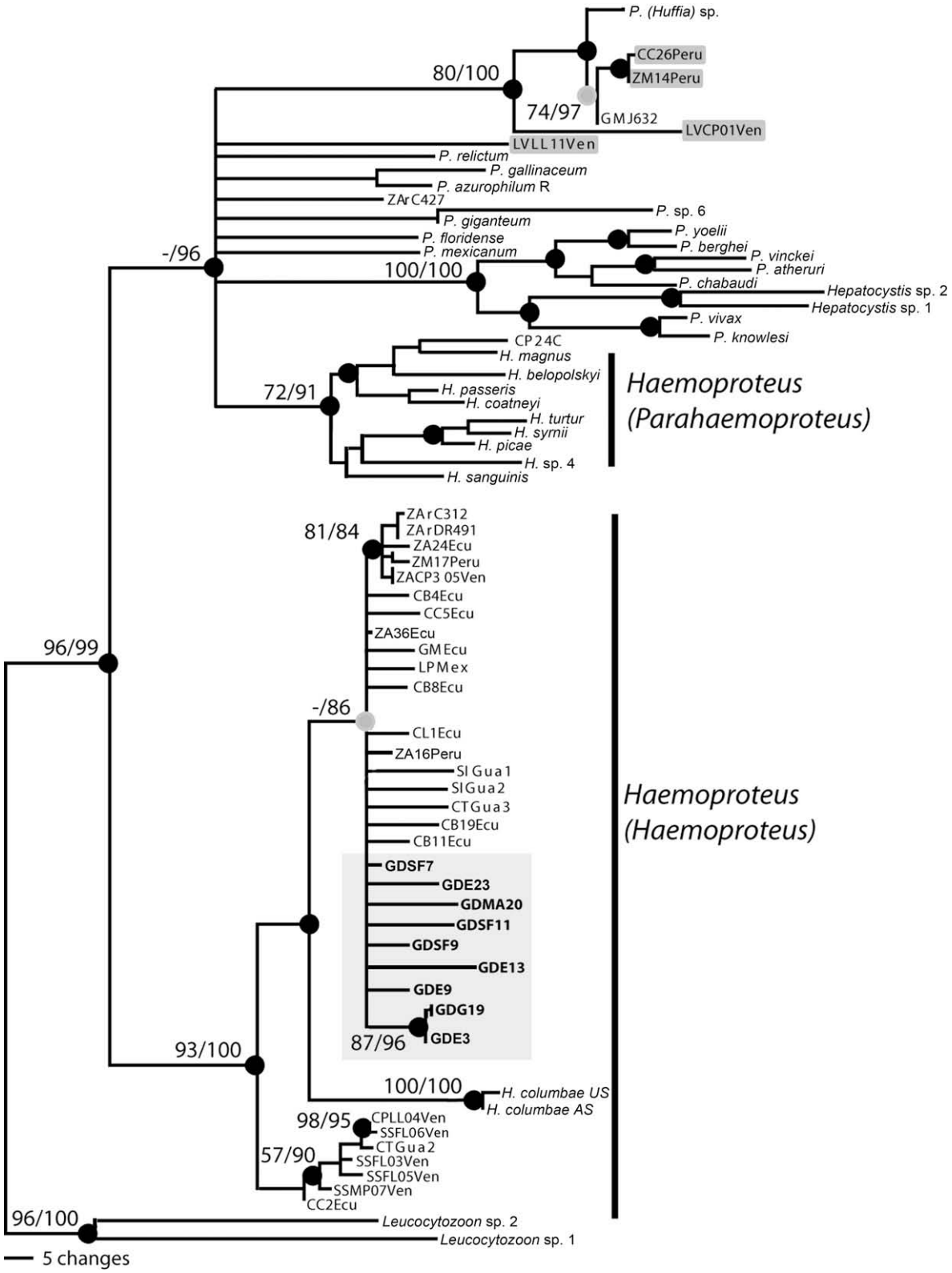

Fig. 1. Majority-rule consensus phylogram of 600 bp of mtDNA cyt *b* showing relationships among haemosporidian parasites. Black dots represent nodes with ⩾99% posterior probability and grey dots represent nodes with ⩾90% posterior probability. Numbers above each node represent bootstrap support values from maximum likelihood and maximum parsimony analyses, respectively. Parasite haplotypes recovered from endemic Galapagos doves are in bold face and are enclosed by a light grey box. Geographic locations of haplotypes are as follows: GD = Galapagos Islands, Ecu = Ecuador, Ven = Venezuela, Peru = Peru, Mex = Mexico, US = United States, Gua = Guatemala, AS = Southeast Asia. The rest of the haplotypes from the sub-genus *Haemoproteus* (*Haemoproteus*) were obtained from the Caribbean Islands. In grey boxes are the haplotypes that were identified as belonging to the sub-genus *H.* (*Haemoproteus*) in the apicoplast caseinolytic protease C (ClpC) tree. Haplotypes identified by species name were obtained from Martinsen et al. (2008). For more detailed information on parasite haplotypes, see Supplementary Table S1.

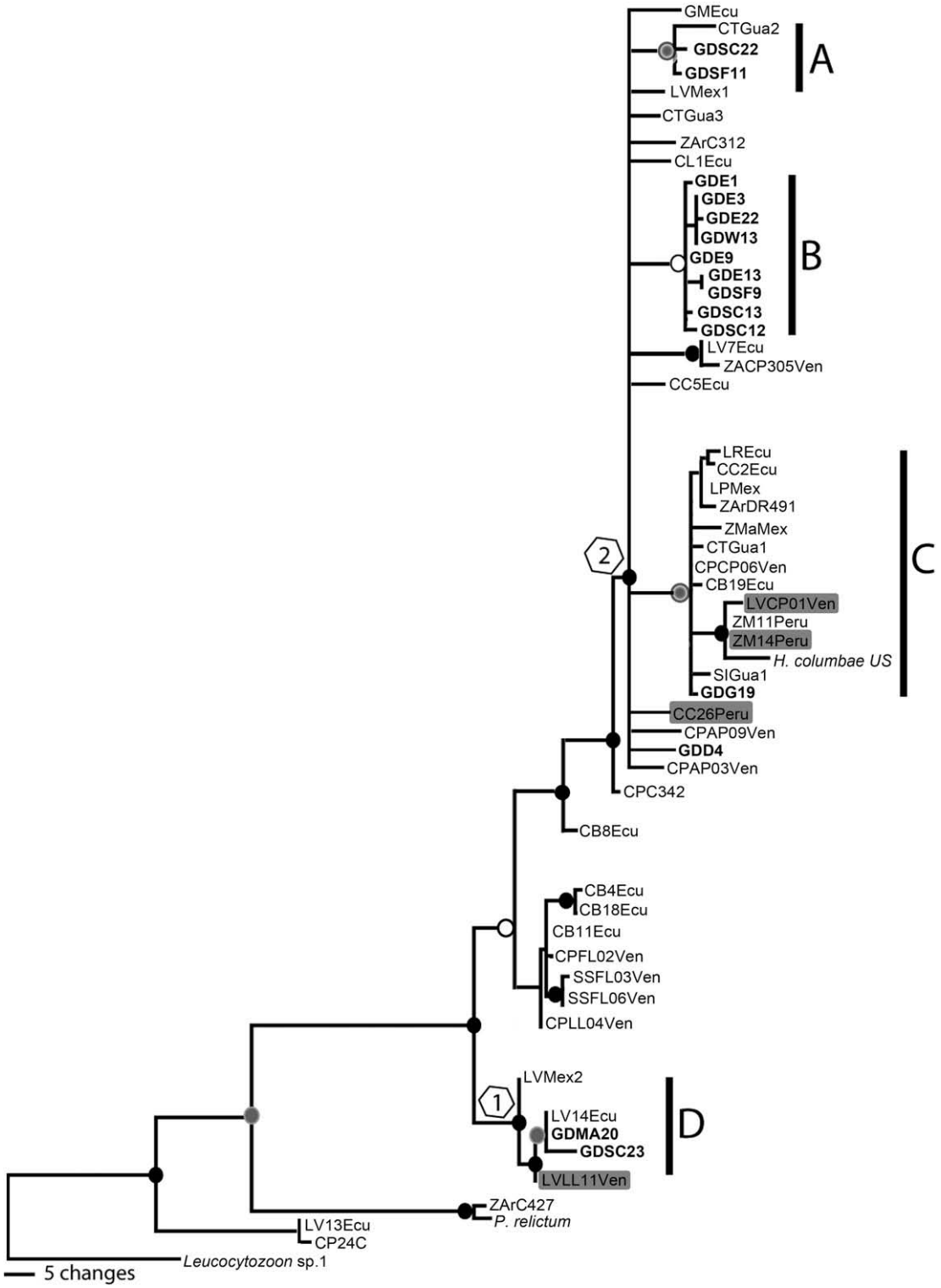

Fig. 2. Majority-rule consensus Bayesian phylogram of 550 bp of apicoplast caseinolytic protease C (ClpC) showing relationships among *Haemoproteus* (*Haemoproteus*) parasites. Black dots represent nodes with ⩾99% posterior probability, grey dots represent nodes with ⩾90% posterior probability, and white dots represent nodes with ⩾85% posterior probability. Parasite haplotypes recovered from endemic Galapagos doves are in bold face. The four groups shown (A, B, C and D), represent well-supported clades that include haplotypes recovered from endemic Galapagos doves. Notice that none of those four groups is unique to the Galapagos archipelago; a mainland sample matches each haplotype obtained from Galapagos doves (see also Supplementary Table S1). Sequences in grey boxes correspond to the same samples identified in the cyt *b* tree as belonging to the genus *Plasmodium* in Columbiformes. Haplotype GMJ632 (it belongs to the genus *Plasmodium* in the cyt *b* tree) is not shown because we were unable to obtain a high quality sequence. Some samples belonging to the same haplotype are presented in the phylogeny for comparative purposes with the cyt *b* phylogeny. For more detailed information on parasite haplotypes, see Fig. 1 and Supplementary Table S1.

ses, we included one parasite haplotype from the study of Martinsen et al. (2008) that was identified as *H. turtur* based on morphology. However, this parasite haplotype falls with parasite haplotypes from the sub-genus *H.* (*Parahaemoproteus*) in both our

analyses and those of Martinsen et al. (2008). This problem was not discussed in the work of Martinsen et al. (2008), and we suggest two possible explanations for this situation. First, it may be due to a mistake in the taxonomic and/or sequence assignment of the sample (Valkiūnas et al., 2008a). Such a mistake may have arisen from an undetected mixed infection. We recommend that this haplotype be checked against original sources (blood smear(s) and DNA sample(s)). Second, the molecular study of columbiform haemosporidian parasites is not extensive (as shown in this study) and we do not know the range of vertebrate hosts that *H. turtur* can infect (Valkiūnas, 2005). Furthermore, many records of *H. turtur* are misidentifications recorded as *H. columbae* and *H. palumbis* (Valkiūnas, 2005). Thus, the general lack of knowledge at the molecular level of *H. turtur* and how its morphology varies according to the host it infects, may be possible reasons why the lineage of Martinsen et al. (2008) falls within *H.* (*Parahaemoproteus*). This sort of problem can be addressed as we continue to attach molecular DNA lineages to morphologically identified haemosporidian parasites, which will allow us to determine the extent of genetic diversity within different morphological species (e.g., Valkiūnas et al., 2007).

Despite incongruence between mtDNA cyt *b* and ClpC, both genes strongly support the placement of the sub-genus *H.* (*Haemoproteus*) as a separate clade restricted to Columbiformes. The disagreement between the two genes relates to the relationships among lineages of the sub-genus *H.* (*Haemoproteus*). Whereas cyt *b* identifies four haplotypes (CC26Peru, ZM14Peru, LVCP01Ven and LVLL11Ven) in the genus *Plasmodium*, ClpC includes those same haplotypes in the sub-genus *H.* (*Haemoproteus*) (Figs. 1 and 2). The ClpC gene distinguishes four well-supported clades that include parasite lineages infecting the Galapagos dove; however, none of the clades is unique to the Galapagos archipelago and each clade includes lineages from mainland samples.

We considered three hypotheses to explain the disagreement between datasets, which may not be mutually exclusive. First, the two genes used in this study belong to different intracellular organelles (cyt *b* to the mitochondrion and ClpC to the apicoplast; Wilson et al., 1996; Wilson and Williamson, 1997). Because both organelles are strictly inherited through the female gametes in very close physical proximity (Wilson et al., 1996; Wilson and Williamson, 1997), they may behave non-independently as if linked. Although both are presumed to be maternally inherited in close proximity, the organelles have different evolutionary origins and may be under different selective regimes (e.g., homologues of ClpC in bacteria have been associated with virulence and are changing rapidly; Wilson et al., 1996; Wilson and Williamson, 1997). These factors may affect, at the very least, the amount of phylogenetic signal within each gene, and consequently the tree structure may reflect these different evolutionary patterns. However, this would not explain the shift of haemosporidian cyt *b* haplotypes associated with the genus *Plasmodium* to the sub-genus *H.* (*Haemoproteus*) based on ClpC. Second, the primers used for both genes in this study may have biases in amplifying parasites from different genera. This could explain the incongruence if some samples contained undetected mixed infections, where one parasite lineage will be preferentially amplified over the other(s) (see Valkiūnas et al., 2006). Experimental mixed infections of domestic pigeons might provide information concerning the possibility for primer bias. Third, parasite lineages might hybridise or exchange genetic material during the sexual stage in the insect vector (Valkiūnas et al., 2008b). This assumes that a single vector is able to transmit both parasite genera, which remains to be determined.

With the resolution and haplotype structure provided by the ClpC marker, it is difficult to define the exact number of colonisation events of the Galapagos Islands. One interpretation of the ClpC data is that haemosporidian parasites have arrived in the islands with hosts more than once. Alternatively, if the observed parasite ClpC haplotype diversity was already present in the ancestor of the Galapagos dove, which colonised the archipelago about 2 million years ago (Johnson and Clayton, 2000), the Galapagos haplotypes should be more closely related to each other than to any continental haplotypes. However, this is not the case. The weak differentiation between haplotypes recovered from island and continental populations suggests that parasites infecting endemic doves represent recent arrivals. Thus, the parasite diversity observed in the Galapagos doves appears to have been generated in continental dove populations.

Continental species of doves have been recorded in the Galapagos Islands, particularly the eared dove (Curry and Stoleson, 1988; H. Vargas, personal communication), which is widely distributed in South America. It is interesting to note that parasite haplotypes obtained from *Z. auriculata* are closely related to haplotypes recovered from endemic Galapagos doves (Fig. 2 and Supplementary Table S1). In addition to natural arrivals from continental doves, domestic pigeons were repeatedly introduced into the archipelago during the last 200 years, which suggests that this exotic bird might have been the source of the observed parasite diversity in the archipelago.

In recent years, conservation concerns have been raised in the Galapagos Islands due to the discovery of many pathogens that can cause problems in populations of endemic birds (Padilla et al., 2004; Parker et al., 2006; Soos et al., 2008). Harmon et al. (1987) suggested that domestic pigeons were responsible for introducing *Trichomonas gallinae* into populations of the endemic Galapagos dove. We also identified *T. gallinae* in domestic pigeons in 2002, pathogens including *Chlamydophila psittaci*, and high prevalence ($\geqslant$80%) and high infection intensities (up to 12%) of *H.* (*Haemoproteus*) sp. in endemic Galapagos doves (Padilla et al., 2004; Santiago-Alarcon et al., 2008). None of the haemosporidian lineages infecting endemic doves belonged to the genus *Plasmodium*, which includes species that have contributed to population declines of Hawaiian endemic birds (van Riper et al., 1986). However, our cyt *b* data identified several lineages of *Plasmodium* spp. in samples collected from continental pigeons and doves, and lineages of *Plasmodium* spp. have been isolated from Galapagos penguins on five different islands (Levin et al., 2009). Furthermore, a competent vector (*Culex quinquefaciatus*) of avian *Plasmodium* malaria has established populations on the islands (Whiteman et al., 2005; Bataille et al., 2009). Thus, avian malaria (*P. relictum*) might arrive in the Galapagos Islands via vagrant continental birds carrying these parasites. Unfortunately, it is logistically impossible to monitor birds that arrive at the archipelago by their own means. Moreover, the endemic dove is highly mobile across the islands, showing no genetic structure (Santiago-Alarcon et al., 2006), which is also reflected in the lack of geographical genetic structure of the parasites infecting this endemic bird. Hence, if a parasite able to infect endemic doves arrives at the archipelago, this pathogen will likely spread throughout the islands.

In summary, this study shows the existence of a diverse clade of haemosporidian parasites belonging to the sub-genus *H.* (*Haemoproteus*) that is unique to Columbiformes. This clade is sister to parasite lineages from the sub-genus *H.* (*Parahaemoproteus*) and the genus *Plasmodium*. Our results are in agreement with morphological taxonomy. Parasite lineages of the sub-genus *H.* (*Haemoproteus*) are widely distributed across the American continent. Haemosporidian parasites infecting endemic Galapagos doves are not unique to the archipelago and are widely distributed across the islands, suggesting multiple colonisations of parasite lineages existing on the continent. None of the parasite lineages infecting endemic Galapagos doves belong to the genus *Plasmodium*.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijpara.2009.10.003.

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