A restriction site to differentiate *Plasmodium* and *Haemoproteus* infections in birds: on the inefficiency of general primers for detection of mixed infections

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SUMMARY

Avian *Plasmodium* and *Haemoproteus* parasites are easily detected by DNA analyses of infected samples but only correctly assigned to each genus by sequencing and use of a phylogenetic approach. Here, we present a restriction site to differentiate between both parasite genera avoiding the use of those analyses. Alignments of 820 sequences currently listed in GenBank encoding a particular cytochrome B region of avian *Plasmodium* and *Haemoproteus* show a shared restriction site for both genera using the endonuclease *Hpy* CH4III. An additional restriction site is present in *Plasmodium* sequences that would initially allow differentiation of both genera by differential migration of digested products on gels. Overall 9 out of 326 sequences containing both potential restriction sites do not fit to the general rule. We used this differentiation of parasite genera based on *Hpy* CH4III restriction sites to evaluate the efficacy of 2 sets of general primers in detecting mixed infections. To do so, we used samples from hosts infected by parasites of both genera. The use of general primers was only able to detect 25% or less of the mixed infections. Therefore, parasite DNA amplification using general primers to determine the species composition of haemosporidian infections in individual hosts is not recommended. Specific primers for each species and study area should be designed until a new method can efficiently discriminate both parasites.

Key words: avian malaria, endonuclease *Hpy* CH4III, haemoparasites, Haemosporidia, host-parasite interactions, mixed infections, molecular detection, PCR.

INTRODUCTION

Studies of avian malaria have increased in recent years because avian malaria provides an excellent system for investigating several aspects of the parasite-host interaction: sexual selection (Hamilton and Zuk, 1982), immunocompetence (Nordling *et al.* 1998; Tomás *et al.* 2007), costs of host reproduction (Norris *et al.* 1994; Merino *et al.* 2000; Marzal *et al.* 2005), stress (Merino *et al.* 2002; Tomás *et al.* 2005), host specificity (Bensch *et al.* 2000), host switching and evolutionary relationships (Ricklefs *et al.* 2004) or latitudinal distribution of diseases (Merino *et al.* 2008).

The use of the sensitive polymerase chain reaction (PCR) has become a routine technique for detecting these parasites (e.g., Feldman and Freed, 1995; Bensch *et al.* 2000; Perkins and Schall, 2002; Ricklefs *et al.* 2005; Durrant *et al.* 2006; Merino *et al.* 2008) and several studies have been published contributing to the improvement of the method of detection (Richard *et al.* 2002; Fallon *et al.* 2003; Waldenström *et al.* 2004; Freed and Cann, 2006). However, due to the tight phylogenetic proximity between parasites of the genera *Plasmodium* and *Haemoproteus* (7·7% average genetic divergence calculated in a variable region; Beadell and Fleischer, 2005) it is very difficult to design a universal specific pair of primers to amplify only one of them and, therefore, they are frequently amplified indiscriminately (Perkins and Schall, 2002; Pérez-Tris *et al.* 2005). In spite of this, several authors have developed specific primers designed for *Haemoproteus* or *Plasmodium* but they are generally useful for parasite species in a particular geographical region and not in others (Bentz *et al.* 2006; Merino *et al.* 2008). Alternatively, Beadell and Fleischer (2005) described a restriction enzyme-based assay to distinguish

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between avian haemosporidians. This study specifically permitted differentiation of *Plasmodium* from *Haemoproteus* in 38 lineages of both genera by using a conserved fragment of mitochondrial DNA encoding an *Xmn*I restriction site unique to *Haemoproteus*. The selection of the specific restriction site in that study was based on only 5 sequences (2 *Haemoproteus* and 3 *Plasmodium*). The ability of the assay to distinguish between the two genera was then tested using samples from 33 avian host species with known infections to sum up the 38 tested samples. However, these authors did not perform an exhaustive study on the power of the assay to detect mixed infections. Of course it is very useful to have a restriction site to differentiate both parasite genera at the conserved region of the mitochondrial DNA but the lack of sequences deposited in GenBank containing this mitochondrial region prevents a firm conclusion being reached on the universality of the assay.

On the other hand, the efficacy of general primers to detect mixed infections has been recently discussed (Pérez-Tris and Bensch, 2005; Valkiūnas *et al*. 2006). The main problem to detect these infections using general primers could be the different affinity shown for each lineage or the low intensity of infection by one of these parasite lineages providing low DNA concentrations and poor amplification. Although mixed infections could be important from an ecological and evolutionary perspective (Marzal *et al*. 2008; Merino *et al*. 2008), no studies to date have examined the efficacy of general primers in detecting mixed infections of haemosporidian species based on samples from multiple hosts and parasite lineages (Valkiūnas *et al*. 2006).

Here, we report another restriction enzyme-based assay using a nucleotide fragment of the cytochrome B encoding an *Hpy* CH4III restriction site that allows differentiation of *Haemoproteus* from *Plasmodium*. In addition, we tested the efficacy of 2 sets of general primers to detect mixed infections of *Plasmodium* and *Haemoproteus* in wild birds.

MATERIALS AND METHODS

Initially, we searched all cytochrome B sequences encoding for a particular region of avian *Plasmodium* (432), *Haemoproteus* (388) and *Leucocytozoon* (204) currently listed in GenBank (Table 1). Recently, it has been suggested that several *Haemoproteus* species be transferred to the genus *Parahaemoproteus* (Martinsen *et al*. 2008). However, to date, there are no sequences assigned to this genus in GenBank. Thus, we use the nomenclature present in GenBank.

Subsequently, we achieved multiple restriction maps for groups of sequences using a tool available in http://insilico.ehu.es/restriction/main. All restriction maps were analysed in detail reporting an *Hpy* CH4III restriction site unique to *Plasmodium* with the exception of 9 non-conforming sequences. To check preliminary data obtained from restriction maps, all sequences were aligned using CulstalW program (Thompson *et al*. 1994), edited with the software BioEdit (Hall, 1999).

In order to clarify the phylogenetic placement of non-conforming sequences listed in GenBank we performed a phylogenetic analysis in the following way. DNA sequences were aligned using the ClustalW program. The BioEdit program was used to edit the sequences. The MEGA4 (Tamura *et al*. 2007) software package was used in phylogram construction/drawing. The computer programs were set at their default parameters in all analyses. Phylogenetic analyses were carried out using the Neighbour-Joining method (Kimura substitution model). Nodal support was estimated by bootstrap analysis with 1000 replications. Phylogenetic analysis was carried out using sequences with a length of 304 bp after removing columns containing gaps or missing data. The *Plasmodium* lineage LIN34 was included in another tree due to the low overlap with the other non-conforming sequences, 256 bp being the length of the sequences. Several GenBank sequences from parasites recovered from birds were included in the phylogenetic trees to clarify the taxonomic position of these non-conforming sequences.

Overall 168 samples from Spanish blue-tits captured in the spring of 2007 were used (see Martínez-de la Puente *et al*. 2007 for details on areas of study and blood sampling) to select individuals infected with both parasites, *Plasmodium* and *Haemoproteus*, by using specific primers to detect each genus. In addition, we also selected 15 blood samples from Chilean birds that were previously analysed for molecular detection of parasites and showed mixed infections (see Merino *et al*. 2008). Bird blood samples were stored in FTA classic cards (Whatman International Ltd, UK) and DNA was extracted to form a soluble solution before polymerase chain reaction (PCR) using the following protocol: cored samples were transferred to collection vials with 250 μl of SET buffer (0·15 M NaCl, 0·05 M Tris, 0·001 M EDTA, pH8) at 4 °C for 6 h. Then 7 μl of 20% SDS and 50 μg proteinase K were added to the vials and incubated at 55 °C overnight. After incubation, 250 μl of 4 M ammonium acetate were added to the vials at room temperature for 30 min. Subsequently, vials were centrifuged at 13 000 ***g*** for 15 min. After removing the pellet, DNA was precipitated with ethanol and re-suspended in sterile water.

We designed 2 sets of primers to specifically detect the lineages of *Plasmodium* or *Haemoproteus* present in blue-tits (*Cyanistes caeruleus*) from our study area in Spain (see Merino *et al*. 2000). In particular, primers HML (5′-GCT ACT GGT GCT ACA TTT GT-3′) and HMR (5′-CCT AAA GGA TTA GAG CTA CC-3′) were designed for *Haemoproteus*

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(367 bp; see Merino *et al*. 2008) and Plas-F (5′-GTA ACA GCT TTT ATG GGT TAC-3′) and 4292Rw (5′-TGG AAC AAT ATG TAR AGG AGT-3′) for *Plasmodium* (422 bp). The primer 4292Rw was previously published (Beadell *et al*. 2004; Durrant *et al*. 2006). The genus specificity of the two sets of primers is due to HML and Plas-F primers for *Haemoproteus* and *Plasmodium*, respectively. The design of these primers was carried out using an alignment file with several sequences from both parasite genera. Later, the specificity of the primers was checked by performing a NCBI BLAST. The identity of the HML and Plas-F primers is not complete with any *Plasmodium* and *Haemoproteus* lineages respectively. However, the HML primer might hybridize with 2 *Plasmodium* sequences (DQ368386 and DQ241534) and the primer Plas-F with 7 *Haemoproteus* sequences (AF495574, AY714192, FJ462661, FJ462662, FJ462663, FJ462664 and FJ462657) because they have only 1–3 defective positions. Thus, the use of these primers avoids the amplification of parasites of the undesirable genus with relative confidence. PCR reactions consisted of 25 μl reaction volumes containing 20 ng template DNA, 50 mM KCl, 10 mM Tris-HCl, 1·5 $MgCl_2$, 0·2 mM of each dNTP, 0·5 μM of each primer, and 1·25 U of AmpliTaq Gold (Applied Biosystems, Foster City, California). The reactions were cycled at the following parameters using a thermal cycler (MasterCycler Personal, Eppendorf): 94 °C for 10 min (polymerase activation), 40 cycles at 95 °C for 40 sec, 58 °C for primers HML/HMR or 60 °C for Plas-F/4292Rw for 1 min, 72 °C for 1 min, and a final extension at 72 °C for 10 min. The specificity of the genus-specific primers designed was tested by sequencing using an ABI 3130 (Applied Biosystems) automated sequencer. Sequences of amplicons obtained from 10 samples of Spanish blue-tits using primers HML/HMR showed only 1 lineage of *Haemoproteus* (corresponding to *H. majoris* GenBank Accession number AY099045). Likewise, the sequentiation of 5 amplicons obtained with primers Plas-F/4292Rw also revealed only 1 lineage of *Plasmodium* in the blue-tits (GenBank Accession number FJ494966). The parasite lineages present in the Chilean samples were previously identified (see Merino *et al*. 2008).

We also designed a pair of general primers Palu-F (5′-GGG TCA AAT GAG TTT CTG G-3′) and Palu-R (5′-DGG AAC AAT ATG TAR AGG AGT-3′) selecting a conserved region of the cytochrome B on the basis of the alignment file that showed the specific restriction site for *Plasmodium*. A total of 59 Spanish blue-tits and 15 Chilean birds with mixed infections were analysed using this pair of primers. In this case, we used the same PCR conditions described above except that the annealing temperature was 56 °C. The size of the obtained amplicons was 390 bp. In order to check the effectiveness of the primers amplifying both parasites, the amplicons obtained after PCR were digested with the endonuclease *Hpy* CH4III (New England Biolabs, Beverly, Massachusetts). To carry out the digestion, 3 μl of PCR product was mixed with 1 μl (1 U/μl) of enzyme plus 5 μl of distilled water and 1 μl of 10× NEBuffer 4. The mix was incubated for 3 h at 37 °C. The digestion products were resolved on 5% polyacrylamide gels (Mini-protean III, Bio-Rad) at 200 V for 35 min. After digestion with the endonuclease *Hpy* CH4III, amplicons from *Plasmodium* were cut into 3 fragments of 27, 36 and 327 bp and *Haemoproteus* in 2 fragments of 27 and 363 bp. The smaller fragments of 27 and 36 bp were indistinguishable in electrophoresis from those produced by the primers.

In order to compare the efficacy of the assay between different sets of general primers in detecting double infections, we also analysed the same 59 blue-tit samples using previously published general primers (Beadell *et al*. 2004; Durrant *et al*. 2006). Unfortunately, we only obtained sufficient DNA from 9 Chilean samples to conduct this second analysis. The primers were 3760F (5′-GAG TGG ATG GTG TTT TAG AT-3′) and 4292Rw (see above), amplifying a fragment of 565 bp from parasite cytochrome B. PCR reactions were conducted using the same conditions described above except that the annealing temperature was 52 °C. The endonuclease *Hpy* CH4III cut in 2 sites on sequences from *Plasmodium* yielding 3 fragments of 327, 206 and 37 bp. However, it only cut at 1 site on sequences from *Haemoproteus* yielding 2 fragments of 206 and 363 bp. Fragments of 37 bp were indistinguishable from those produced by the primers (see Figs 1 and 2). Under-digestion of PCR products obtained by using both sets of primers was not a serious problem because in both genera there is at least 1 restriction site. Nevertheless, under-digestion was never detected using the conditions reported above.

RESULTS

Overall 326 sequences of *Plasmodium* (185) and *Haemoproteus* (141) present enough length to include the regions where both hypothetical restriction sites are encoding. The first restriction site is present in all sequences listed on Genbank encoding for at least the portion for that particular cytochrome B region of avian *Plasmodium* (307 sequences) and *Haemoproteus* (299 sequences) except one that cannot be clearly assigned to any of that genera (see Discussion section). However, among the sequences including the fragment where the endonuclease could produce the second cut (310 *Plasmodium* and 230 *Haemoproteus* sequences), 9 sequences do not conform to the expected cutting pattern; 3 *Haemoproteus* sequences show the second *Hpy* CH4III restriction site and 6 *Plasmodium* sequences do not show it. The alignment

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Table 1. GenBank Accession numbers of sequences encoding a particular cytochrome B region from avian *Plasmodium* and *Haemoproteus* currently listed

Accession numbers (*Haemoproteus*)											
AF069613	AF495547	AY455658	AY640150	AY714166	AY817754	DQ241542	DQ451420	DQ847190	EF380167	EF380201	FJ462666
AF465562	AF495550	AY455659	AY640151	AY714167	AY817755	DQ241543	DQ451421	DQ847191	EF380168	EF380202	FJ462667
AF465563	AF495551	AY540196	AY640152	AY714168	AY831750	DQ241544	DQ451422	DQ847192	EF380169	EF380203	FJ462668
AF465564	AF495552	AY540198	AY714134	AY714169	AY831751	DQ241545	DQ451423	DQ847193	EF380170	EF380205	FJ462670
AF465565	AF495553	AY540199	AY714135	AY714170	AY831752	DQ241546	DQ451424	DQ847194	EF380171	EF380206	FJ462671
AF465566	AF495554	AY540201	AY714136	AY714171	AY831753	DQ241547	DQ451425	DQ847195	EF380172	EF380207	FJ462672
AF465567	AF495555	AY540202	AY714137	AY714172	AY831754	DQ241548	DQ451426	DQ847196	EF380173	EF380208	FJ462673
AF465568	AF495556	AY540203	AY714138	AY714173	AY831755	DQ241549	DQ451427	DQ847197	EF380174	EF380209	FJ462674
AF465569	AF495557	AY540204	AY714139	AY714174	AY831756	DQ241550	DQ451428	DQ847198	EF380175	EF564175	FJ462675
AF465570	AF495558	AY540205	AY714140	AY714175	AY831757	DQ241551	DQ451429	DQ847200	EF380176	EF564176	FJ462676
AF465571	AF495559	AY540209	AY714141	AY714176	AY831758	DQ241552	DQ451430	DQ847201	EF380177	EF564177	FJ462677
AF465572	AF495560	AY540212	AY714142	AY714177	AY831759	DQ241553	DQ451431	DQ847202	EF380178	EF607289	FJ462678
AF465573	AF495561	AY540213	AY714143	AY714178	AY831760	DQ241554	DQ630004	DQ847203	EF380179	EF607290	FJ462679
AF465574	AF495562	AY540214	AY714144	AY714179	AY831761	DQ241555	DQ630005	DQ847204	EF380180	EU254548	FJ462682
AF465575	AF495563	AY540215	AY714145	AY714180	AY831762	DQ241556	DQ630006	DQ884876	EF380181	EU254549	
AF465576	AF495565	AY540216	AY714146	AY714181	AY831763	DQ241557	DQ630007	DQ991077	EF380182	EU254553	
AF465577	AF495567	AY540224	AY714147	AY714182	AY831764	DQ241558	DQ630008	DQ991078	EF380183	EU676187	
AF465578	AF495570	AY640124	AY714148	AY714183	AY831765	DQ241559	DQ630009	DQ991079	EF380184	EU676188	
AF465579	AF495573	AY640125	AY714149	AY714184	AY831766	DQ278434	DQ630010	DQ991080	EF380185	EU676189	
AF465580	AF495574	AY640126	AY714150	AY714185	AY831767	DQ278435	DQ630011	EF032811	EF380186	EU676190	
AF465581	AF495575	AY640127	AY714151	AY714186	AY831768	DQ278436	DQ630012	EF032812	EF380187	FJ025895	
AF465582	AF495579	AY640129	AY714152	AY714187	AY831769	DQ278437	DQ630013	EF032813	EF380188	FJ025896	
AF465583	AF495580	AY640131	AY714153	AY714188	AY840997	DQ451408	DQ630014	EF032871	EF380189	FJ462649	
AF465584	AY167239	AY640133	AY714154	AY714189	AY840998	DQ451409	DQ659592	EF153646	EF380190	FJ462650	
AF465585	AY167240	AY640138	AY714155	AY714190	AY840999	DQ451410	DQ847180	EF153647	EF380191	FJ462651	
AF465586	AY167241	AY640139	AY714156	AY714191	DQ000320	DQ451411	DQ847181	EF153648	EF380192	FJ462652	
AF465587	AY167242	AY640140	AY714157	AY714192	DQ000321	DQ451412	DQ847182	EF153649	EF380193	FJ462653	
AF465588	AY167243	AY640141	AY714158	AY714193	DQ000322	DQ451413	DQ847183	EF153650	EF380194	FJ462654	
AF465589	AY167244	AY640142	AY714160	AY817748	DQ000323	DQ451414	DQ847184	EF153652	EF380195	FJ462655	
AF465590	AY167245	AY640144	AY714161	AY817749	DQ000324	DQ451415	DQ847185	EF153653	EF380196	FJ462657	
AF465591	AY167246	AY640146	AY714162	AY817750	DQ000325	DQ451416	DQ847186	EF153654	EF380197	FJ462658	
AF465592	AY172842	AY640147	AY714163	AY817751	DQ241539	DQ451417	DQ847187	EF380164	EF380198	FJ462659	
AF465593	AY455656	AY640148	AY714164	AY817752	DQ241540	DQ451418	DQ847188	EF380165	EF380199	FJ462660	
AF465594	AY455657	AY640149	AY714165	AY817753	DQ241541	DQ451419	DQ847189	EF380166	EF380200	FJ462665	
Accession numbers (*Plasmodium*)											
AB30289	AY540208	AY831748	DQ508376	DQ659567	DQ839002	DQ839045	DQ839085	EF011173	EF380116	EF380156	
AF465547	AY540210	AY831749	DQ508396	DQ659568	DQ839003	DQ839046	DQ839086	EF011174	EF380117	EF380157	
AF465548	AY540211	AY841000	DQ508397	DQ659569	DQ839004	DQ839047	DQ839087	EF011175	EF380118	EF380158	

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AF465549	AY540217	AY841001	DQ508398	DQ659570	DQ839005	DQ839048	DQ839088	EF011176	EF380119	EF380159
AF465550	AY540218	DQ241508	DQ508399	DQ659571	DQ839006	DQ839049	DQ839089	EF011177	EF380120	EF380160
AF465551	AY540219	DQ241509	DQ508400	DQ659572	DQ839007	DQ839050	DQ839090	EF011178	EF380121	EF380161
AF465552	AY540220	DQ241510	DQ508401	DQ659573	DQ839008	DQ839051	DQ839091	EF011179	EF380122	EF380162
AF465553	AY540221	DQ241511	DQ508402	DQ659574	DQ839009	DQ839052	DQ839092	EF011180	EF380123	EF380163
AF465554	AY540222	DQ241512	DQ508403	DQ659575	DQ839010	DQ839053	DQ839093	EF011181	EF380124	EF564178
AF465555	AY540223	DQ241513	DQ508404	DQ659576	DQ839011	DQ839054	DQ847258	EF011182	EF380125	EF564179
AF465556	AY640128	DQ241514	DQ508405	DQ659577	DQ839012	DQ839055	DQ847259	EF011183	EF380126	EF607288
AF465557	AY640130	DQ241515	DQ659538	DQ659578	DQ839013	DQ839056	DQ847260	EF011184	EF380127	EF607291
AF465558	AY640132	DQ241516	DQ659539	DQ659579	DQ839014	DQ839057	DQ847261	EF011185	EF380128	EU600217
AF465559	AY640134	DQ241517	DQ659540	DQ659580	DQ839015	DQ839058	DQ847262	EF011186	EF380129	EU600218
AF465560	AY640135	DQ241518	DQ659541	DQ659581	DQ839016	DQ839059	DQ847263	EF011187	EF380130	EU600219
AF465561	AY640136	DQ241519	DQ659542	DQ659582	DQ839019	DQ839060	DQ847264	EF011188	EF380131	EU600220
AF495548	AY640137	DQ241520	DQ659543	DQ659583	DQ839020	DQ839061	DQ847265	EF011189	EF380132	EU600221
AF495549	AY640143	DQ241521	DQ659544	DQ659584	DQ839021	DQ839062	DQ847266	EF011190	EF380133	EU600222
AF495564	AY640145	DQ241522	DQ659545	DQ659585	DQ839022	DQ839063	DQ847267	EF011191	EF380134	EU600223
AF495566	AY714194	DQ241523	DQ659546	DQ659586	DQ839023	DQ839064	DQ847268	EF011192	EF380135	EU600224
AF495568	AY714195	DQ241524	DQ659547	DQ659587	DQ839024	DQ839065	DQ847269	EF011193	EF380136	EU600225
AF495569	AY714196	DQ241525	DQ659548	DQ659588	DQ839025	DQ839066	DQ847270	EF011194	EF380137	EU600226
AF495571	AY714197	DQ241526	DQ659549	DQ659589	DQ839026	DQ839067	DQ847271	EF011195	EF380138	EU600227
AF495572	AY714198	DQ241527	DQ659550	DQ659590	DQ839027	DQ839068	DQ884877	EF011196	EF380139	EU600228
AF495576	AY714199	DQ241528	DQ659551	DQ659591	DQ839028	DQ839069	DQ991068	EF011197	EF380140	EU600229
AF495577	AY714200	DQ241529	DQ659552	DQ838987	DQ839029	DQ839070	DQ991069	EF011198	EF380141	EU600230
AF495578	AY714201	DQ241530	DQ659553	DQ838988	DQ839030	DQ839071	DQ991070	EF032870	EF380142	EU600231
AY167247	AY714202	DQ241531	DQ659554	DQ838989	DQ839031	DQ839072	DQ991071	EF153638	EF380143	EU600232
AY167248	AY714203	DQ241532	DQ659555	DQ838990	DQ839032	DQ839073	DQ991072	EF153639	EF380144	EU676191
AY167249	AY714204	DQ241533	DQ659556	DQ838991	DQ839033	DQ839074	DQ991073	EF153640	EF380145	EU708328
AY167250	AY714205	DQ241534	DQ659557	DQ838992	DQ839034	DQ839075	DQ991074	EF153641	EF380146	EU883534
AY455660	AY714206	DQ241535	DQ659558	DQ838993	DQ839035	DQ839076	DQ991075	EF153642	EF380147	EU883535
AY455661	AY714207	DQ241536	DQ659559	DQ838994	DQ839036	DQ839077	DQ991076	EF153643	EF380148	
AY455662	AY714208	DQ241537	DQ659560	DQ838995	DQ839037	DQ839078	EF011166	EF153644	EF380149	
AY455663	AY714209	DQ241538	DQ659561	DQ838996	DQ839039	DQ839079	EF011167	EF153645	EF380150	
AY540195	AY714210	DQ451403	DQ659562	DQ838997	DQ839040	DQ839080	EF011168	EF380111	EF380151	
AY540197	AY714211	DQ451404	DQ659563	DQ838998	DQ839041	DQ839081	EF011169	EF380112	EF380152	
AY540200	AY817747	DQ451405	DQ659564	DQ838999	DQ839042	DQ839082	EF011170	EF380113	EF380153	
AY540206	AY817756	DQ451406	DQ659565	DQ839000	DQ839043	DQ839083	EF011171	EF380114	EF380154	
AY540207	AY831747	DQ451407	DQ659566	DQ839001	DQ839044	DQ839084	EF011172	EF380115	EF380155	

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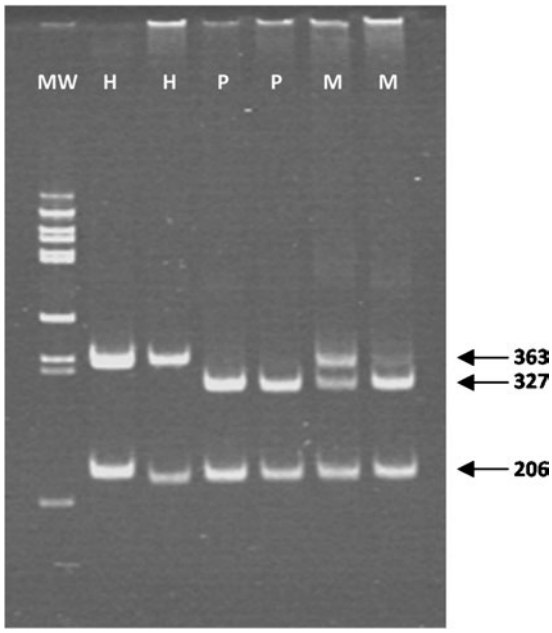

Fig. 1. Restriction fragments electrophoretically resolved on 5% polyacrylamide gel using primers 3760F/4292R. Two lineages of *Haemoproteus* from Chilean birds (H), 2 lineages of *Plasmodium* from Chilean birds (P) and 2 mixed infections (M) can be distinguished on the gel. Arrows show the length of fragments in base pairs. MW (molecular weight, 100 bp ladder).

of these 9 sequences showing the DNA region containing the second restriction site is presented in Fig. 3. Another 6 problematic sequences (AF069613, DQ241534, DQ241553, EF380131, EF380157 and EF380163) were incorrectly assigned at the genus level as shown by phylogenetic analysis, probably due to errors during the GenBank submission (Fig. 4).

The alignment of *Leucocytozoon* sequences including the fragment where the endonuclease could produce the first cut (204 sequences) showed that all of them present it. However, 50% of the 84 sequences including the fragment where *Hpy* CH4III nuclease could produce the second cut showed this restriction site. Accordingly, in the hypothetical case that *Leucocytozoon* would be amplified using the primers specifically selected for *Plasmodium* and *Haemoproteus* species, in one half of the cases the electrophoretic restriction maps would be identical to *Plasmodium* and in the other half to *Haemoproteus*.

The average genetic divergence calculated on a nucleotide fragment of 332 bp (from first to second *Hpy* CH4III restriction site, see Fig. 2) using Kimura-2 model in MEGA 4.0 showed a divergence within groups of 4·1%, 5·1% and 12·2% for *Plasmodium* (185 sequences), *Haemoproteus* (141 sequences) and *Leucocytozoon* (83 sequences), respectively. Otherwise, genetic divergence between groups was 8·3%, 15·8% and 15·5% for *Plasmodium*/*Haemoproteus*, *Plasmodium*/*Leucocytozoon* and *Haemoproteus*/*Leucocytozoon*, respectively.

In order to test the efficacy of this assay to detect mixed infections, we first selected birds infected with both parasites from 168 blue-tit samples using the specific primers for *Haemoproteus* (HML/HMR) and *Plasmodium* (Plas-F/4292Rw). The results showed 87·5% (147) and 44% (74) of samples infected with *Haemoproteus* and *Plasmodium*, respectively. In total, 59 samples found infected by both parasite genera using specific primers were then amplified using general primers 3760F/4292Rw and Palu-F/Palu-R. The efficacy in detecting mixed infections by both pairs of primers in the two study regions was tested by one-tailed Fisher exact test comparing the expected versus observed number of mixed to single infections. The primers Palu F/R detected fewer mixed infections than expected in both Spanish and Chilean bird groups ($\chi^2_1=69{\cdot}57$, $P<0{\cdot}0001$ and $\chi^2_1=22{\cdot}18$, $P<0{\cdot}0001$, respectively). Similar results were obtained for the primers 3760F/4292Rw both, for Spanish ($\chi^2_1=117$, $P<0{\cdot}0001$), and Chilean birds ($\chi^2_1=10{\cdot}82$, $P=0{\cdot}001$). However, the primers Palu F/R detected more mixed infections than the primers 3760F/4292Rw in the Spanish bird group (Fisher exact test, two tailed $\chi^2_1=13{\cdot}38$, $P=0{\cdot}0001$) although the difference was not significant for the Chilean group (Fisher exact test, two tailed $\chi^2_1=0{\cdot}22$, $P=1{\cdot}00$). In addition, the primers Palu F/R detected more *Plasmodium* infections than the primers 3760F/4292Rw in the Spanish bird group (Fisher exact test, two tailed $\chi^2_1=12{\cdot}53$, $P=0{\cdot}0003$) but there was no significant difference between both sets of primers detecting *Plasmodium* or *Haemoproteus* infections in the Chilean bird group (Fisher exact test, two tailed $\chi^2_1=0{\cdot}25$, $P=0{\cdot}76$ and $\chi^2_1=0{\cdot}40$, $P=0{\cdot}68$, respectively) or *Haemoproteus* infection in the Spanish group (Fisher exact test, two tailed $\chi^2_1=0{\cdot}04$, $P=0{\cdot}90$). A summary of the results is shown in the Table 2.

DISCUSSION

This restriction enzyme-based assay is valid to discriminate between avian parasites of the genera *Plasmodium* and *Haemoproteus* in the vast majority of cases, but it has a possible error rate of about 3%. Next, we discuss the non-conforming sequences found in GenBank. (i) The sequence with Accession number AF069613 (*Haemoproteus columbae* from Venezuela) has the second *Hpy* CH4III restriction site typical of the genus *Plasmodium* while the sequences EF380157 (*Plasmodium* spp. LIN29D) and EF380163 (*Plasmodium* spp. LIN34) do not show this site. However, a phylogenetic analysis including these sequences confirms that they are incorrectly assigned at the genus level because (see Valkiūnas *et al*. 2008 for a similar conclusion for *H. columbae*) those assigned to *Plasmodium* are in fact *Haemoproteus* and *vice versa*. This result strongly suggests that a revision of nomenclature for these three

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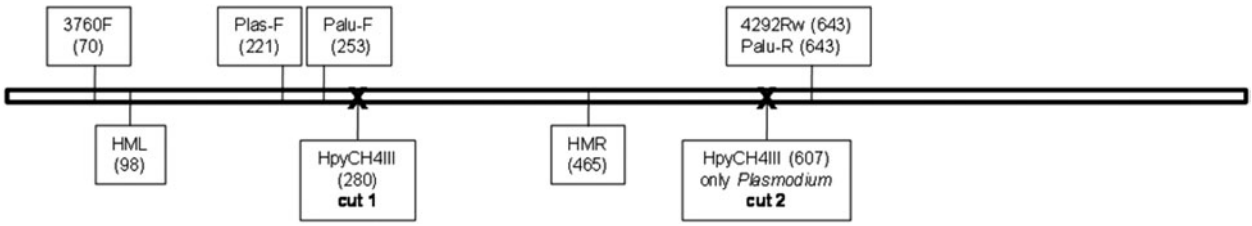

Fig. 2. Schematic representation of the cytochrome B sequence showing the *Hpy* CH4III restriction sites and the regions recognized by the different sets of primers used in this study (based on AF465554 sequence of *Plasmodium*).

***Hpy* CH4III**		5′ **AC NGT** 3′
***Haemoproteus* sp.**	**(EF380167)**	CATCCTGATA ATGCAATT.. ...AGATAGA TATGCA.end
***Haemoproteus* sp.**	**(EF380194)**	CATCCTGATA ATGCAATT.. ...AGATAGA TATGCA.end
***Haemoproteus* sp.**	**(EF380198)**	CATCCTGATA ATGCAATT.. ...AGATAGA TATGCA.end
***Plasmodium* sp.**	**(EF380142)**	CATCCAGATA ATGCAATA.T ...AGATAGA TATGCT.end
***Plasmodium* sp.**	**(EF380143)**	CATCCAGATN ATGCAATA.T ...AGATAGA TATGCT.end
***Plasmodium* sp.**	**(DQ241515)**	CATCCAGATA ACGCAATT.T ...AGATAGA TATGCT.end
***Plasmodium* sp.**	**(DQ659567)**	CATCCAGATA ATGCTATT.T ...AGATAGA TATGCTACTC CTTTACATAT→
***Plasmodium* sp.**	**(DQ659576)**	CATCCAGATA ATGCTATT.. .A.AGATAGA TATGCTACTC CTTTACATAT→
***Plasmodium* sp.**	**(AY540221)**	CATCCTGATA ATGCTATTG. ...AGATAGA TATGCTACTC CTTTACATAT→

Fig. 3. Alignment of the *Plasmodium* and *Haemoproteus* cytochrome B sequences that cannot be differentiated using the *Hpy* CH4III restriction site-based assay. Only the fragment corresponding to the second restriction site is shown.

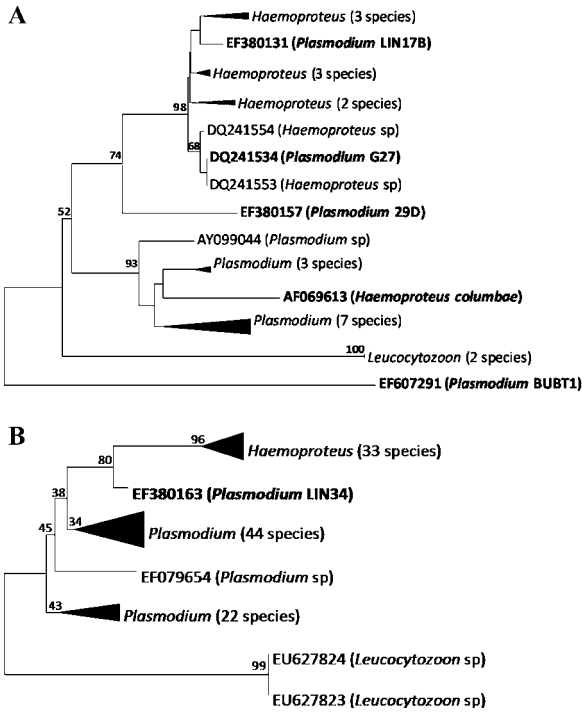

Fig. 4. Phylogenetic tree based on a cytochrome B fragment of 304 bp (A) or 256 bp (B) using the Neighbour-Joining method (Kimura substitution model). The misidentified sequences are marked in bold.

lineages is needed. (ii) The sequence EF607291 (*Plasmodium* spp. BUBT1) is the only parasite lineage without the first restriction site, but it is far from clear that this sequence was really a *Plasmodium* or even an *Haemoproteus* as it is not clearly grouped within these parasites (identity with other lineages only reach 85%; see also Krone *et al*. 2008). (iii) Sequences DQ241534 (*Plasmodium* sp. G27) and EF380131 (*Plasmodium* sp. LIN17B) are clearly errors because sequences DQ241553 (*Haemoproteus* sp. G46) and EF380174 (*Haemoproteus* sp. LIN11) respectively are completely identical. These sequences do not present the specific restriction site for *Plasmodium* and phylogenetic analysis grouped it within *Haemoproteus*. (iv) Only the sequences DQ659576 (*Plasmodium* sp. P35), DQ241515 (*Plasmodium* sp. U8), DQ659567 (*Plasmodium* sp. P26), EF380142 (*Plasmodium* sp. LIN24A), EF380143 (*Plasmodium* sp. LIN24B), AY540221 (*Plasmodium* sp. OZ42), EF380167 (*Haemoproteus* sp. LIN5), EF380194 (*Haemoproteus* sp. LIN29) and EF380198 (*Haemoproteus* sp. LIN33) are not correctly assigned to their genera using the assay described here because the *Plasmodium* sequences do have not the second restriction site for *Hpy* CH4III endonuclease whereas *Haemoproteus* sequences have it. Therefore, all sequences from both parasite genera show the first

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Table 2. Efficiency of two sets of general primers amplifying *Plasmodium* and *Haemoproteus* from birds infected with both parasites and tested applying the *Hpy* CH4III restriction assay

(Sample size is shown in parentheses.)

Primers	Birds (*N*)	*Haemoproteus*	*Plasmodium*	Mixed infection
3760F/4292Rw	Spanish birds (59)	97% (57)	3% (2)	0%
	Chilean birds (9)	22·2% (2)	100% (9)	22% (2)
Palu-F/Palu-R	Spanish birds (59)	91·5% (54)	33·9% (20)	25·4% (15)
	Chilean birds (15)	40% (6)	73·3% (11)	13·3% (2)

restriction site and only 9 do not follow the rule of the second restriction site. Among these 9, chromatograms for DQ659576 and DQ659567 were re-inspected and did not contain any obvious errors; in addition, unpublished sequence data appear to confirm the validity of AY540221 (J. Beadell, personal communication) Although the other 6 non-conforming sequences may also be exempt from any errors, we can venture several reasons to explain the origin of hypothetical errors. (i) The hypothetical errors could be generated during the GenBank submission process. (ii) In some sequences, the second restriction site is very close to the extreme 3′, a very sensitive zone where wrong readings are often observed. (iii) In some of them, the nucleotide change that knocks out the second restriction site consists of a thymine appearing instead of a cytosine and in another there is a guanine instead of an adenine. This points to the existence of a possible mistake in sequence readings due to the presence of a low DNA quantity of *Haemoproteus* in those samples because 96% and 94% of the *Haemoproteus* sequences present thymine and guanine in the second and the first position, respectively, within the second restriction site. This possibility cannot be ruled out due to the fact that general primers were used to amplify the parasite DNA and *Haemoproteus* presence may be unnoticed. In any case, we have evidence that at least 3 sequences, as mentioned above, do not contain errors and, therefore, strictly speaking the proposed test will be useful only after first surveying the lineage diversity in the host or geographical area under study.

The possibility that the general primers used in the present study could amplify *Leucocytozoon* species is remote, as indicated by performing a normal NCBI BLAST with the primers used in this study. The analysis BLAST showed that the primers HML, 4292Rw and Palu-R are unlikely to hybridize efficiently to the gene encoding the cytochrome B of *Leucocytozoon* species, whereas the other primers will do so. In addition, the high average genetic divergence between the *Leucocytozoon* group and *Plasmodium* or *Haemoproteus* groups (higher than 15%) also indicates that the amplification of *Leucocytozoon* is unlikely. Moreover, we have never amplified this parasite genus using the mentioned primers on samples with well-known *Leucocytozoon* infection and, to our knowledge, nobody has communicated it. Thus, we can state with relative confidence that the presence of *Leucocytozoon* does not affect the specificity of the test presented. Nonetheless, this should be tested using DNA from other *Leucocytozoon* isolates. It is also important to note that, as previously mentioned, the genera *Haemoproteus* and *Parahaemoproteus* cannot be discriminated using this assay.

The effectiveness of the assay in detecting double infections from samples of Spanish or Chilean birds was less than expected. The primer sets behaved very differently in the two groups of birds. While both primer sets detected over 90% of *Haemoproteus* infections in Spain, the case was just the opposite in the Chilean birds where both sets of primers detected a relatively high proportion of *Plasmodium* infections but failed to amplify a similar high proportion of *Haemoproteus* infections. In addition, primers Palu-F/Palu-R detected significantly more *Plasmodium* infections that primers 3760F/4292Rw in blue-tits. These facts could be due to a different affinity of the primers for the parasite lineages present in both areas or a lower intensity of *Haemoproteus* or *Plasmodium* infection in the Chilean or Spanish population, respectively. In this respect, previous data from the Spanish population of blue-tits indicated that only about 10% of samples were infected with *Plasmodium* using microscopy (authors' unpublished data; see also Merino *et al*. 2000), implying that *Plasmodium* DNA is probably in lower concentration as compared to *Haemoproteus* DNA in our blue-tit samples. In fact, detection of *Plasmodium* increased up to 70% by PCR using specific primers Plas-F/4292Rw (J. Martínez, unpublished data).

As mixed infections were constituted by different lineages, we can suspect that detection is completely dependent on the parasitic lineage implied and on the DNA quantity available as it was previously suggested by Beadell and Fleischer (2005) and Pérez-Tris and Bensch (2005). These authors have tried to develop molecular methods to solve this problem but their efficiency is still very dependent on parasitic

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lineages and the intensity of infections. The most efficient method described to date to detect mixed infections is the highly expensive and time-consuming method of sequencing and TA cloning described by Pérez-Tris and Bensch (2005). As the assay presented here has practically 100% efficacy to differentiate both genera in samples with single infections, it can be used in combination with TA cloning to identify the parasite genus present in each clone but without sequencing, thus reducing the economic costs of detection of mixed infections by cloning. Unfortunately, the amplicons obtained with the primers used by Pérez-Tris and Bensch (2005) do not contain the specific restriction site for *Plasmodium* as described here, thus, another set of primers should be used before applying the assay of the *Hpy* CH4III restriction site.

The conclusions that we can extract from the present study are the following. (i) The general primers tested yielded a poor efficiency in detecting known mixed infections, although they were successful in detecting at least 1 of the 2 genera, thus they could be used with single-infected samples. (ii) This method may be a cost-effective way to discriminate *Plasmodium* and *Haemoproteus* infections when lineage-specific information and the exact number of mixed infections are not needed, or to reduce the costs of sequencing when using cloning to detect mixed infections. However, (iii) as the detection of mixed infections is completely dependent on the parasitic lineages and/or parasite intensity, we highly recommend the use of different pairs of general primers in the initial screening of samples, the genetic characterization of the amplicons obtained and the development of specific primers for the characterized lineages if the prevalence of each lineage is needed.

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