Development of a PCR for detection and species identification of avian *Plasmodium* infection in UK penguins

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Abstract

Avian malaria is a significant cause of mortality in captive penguins in the UK, resulting from infection with apicomplexan protozoa of the genus *Plasmodium* spread by *Culex spp.* mosquitos. Detection of disease in penguins is challenging, peracute mortality precedes development of parasitaemia and therefore blood smears are frequently non-diagnostic, whilst post-mortem histopathological diagnosis requires specialist operator knowledge. A test capable of identification and speciation of avian malaria in penguins would allow zoological collections to screen for or confirm infection, and ultimately may guide treatment and prevention strategies. This project aimed to develop a PCR and probe(s) capable of identifying infective *Plasmodium* species using bioinformatic techniques, and apply these to frozen and formalin fixed paraffin embedded (FFPE) tissues from 25 penguins from the UK with known or suspected avian malaria infection. The novel PCR targeting the Cytochrome B gene demonstrated *Plasmodium* DNA in both frozen and FFPE penguin tissues. Sanger sequencing of the PCR product indicated 100% (25/25) birds were infected with *Plasmodium relictum* whilst 4.0% (1/25) birds was coinfected with *Plasmodium matutinum*. These findings demonstrate potential utility of this PCR in confirming avian malaria infection in penguins, although further validation, including testing on known *Plasmodium*-negative penguin tissue, is essential. Development of a *Plasmodium relictum* specific probe targeting the amplicon generated using this protocol was unsuccessful, owing to insufficient interspecies genetic diversity in the target region. In addition, incomplete characterisation of avian infective *Plasmodium*, geographical diversity and epidemiology of avian malaria parasites compound the challenges inherent in malaria diagnostics.

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Preface

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Author’s declaration

I declare this thesis to be my own work, and that all source material has been acknowledged. I declare that I performed work described herein almost exclusively; any collaborative contributions have been indicated and acknowledged. The contents of this thesis have not been published elsewhere, nor submitted for any other degree.

List of abbreviations

°C	Degrees centigrade
A	Adenine
bp	Base pairs
C	Cytosine
COI	Cytochrome C Oxidase subunit 1 gene
cyt B	Cytochrome B gene
DHFR-TS	Dihydrofolate reductase-thymidylate synthase
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
FFPE	Formalin fixed paraffin embedded
G	Guanine
m	Minutes
ml	Millilitres
PCR	Polymerase chain reaction
pmol	Picomoles
RNA	Ribonucleic acid
rpm	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
s	Seconds
T	Thymine
TAE	Tris-Acetate-Ethylenediaminetetraacetic acid
µl	Microlitres

1. Introduction

1.1 Introduction

Avian malaria is caused by infection with apicomplexan protozoa of the genus *Plasmodium* (Plasmodiidae). These intracellular protozoa belong to the order Haemosporidia, and are amongst its most widely studied genera, along with *Leukocytozoon* (Leukocytozoidae) and *Haemoproteus* (Haemoprotidae). Historically, little distinction between members of the Haemosporidia was implied by the term “avian malaria”, however in recent years use of the term “avian malaria” has evolved to describe disease due to infection with *Plasmodium spp*. exclusively (Valkiūnas et al., 2005; Atkinson, 2008; Vanstreels, Braga and Catão-Dias, 2016; Stidworthy and Denk, 2018).
A large number of *Plasmodium* species are able to infect birds; at present over 50 avian-infective morphospecies have been identified and this is not considered an exhaustive list (Valkiūnas and Iezhova, 2018). Currently, avian malaria parasites are divided into 5 subgenera: *Haemamoeba, Giovannolaia, Novyella, Bennettinia* and *Huffia*. These parasites demonstrate variable host fidelity and some individual *Plasmodium* species are highly generalist; for example, *P. relictum* can infect members of over 70 avian families. The majority of avian orders assessed for *Plasmodium* infection have been found to harbour the parasite, although just under half of extant avian species have yet to be examined for infection (Atkinson, 2008). The outcome of infection depends on a number of host, and well as parasite factors. This is well demonstrated by *P. relictum*; infection of native European passerines is typically associated with minimal reductions in activity or fitness, whilst infection of non-native captive penguins typically results in high mortality (Fantham and Porter, 1944; Atkinson, 2008; Vanstreels, Braga and Catão-Dias, 2016; Stidworthy and Denk, 2018; Valkiūnas and Iezhova, 2018).

1.2 Avian malaria in penguins

Most avian hosts of *Plasmodium* do not suffer significant morbidity or mortality, and infection causes mild reductions in fitness, or subclinical disease. However, the *Sphenicidae* (penguins) are amongst a minority of avian families which are

acutely sensitive to infection (Fantham and Porter, 1944; Valkiūnas et al., 2005; Vanstreels et al., 2015; Vanstreels, Braga and Catão-Dias, 2016; Stidworthy and Denk, 2018); outbreaks of avian malaria in captive penguins typically result in mortality between 50-80% (Stidworthy and Denk, 2018). Infection with *P. relictum* is the most commonly reported cause of avian malaria in penguins in Europe, whilst *P. relictum* and *P. elongatum* are most common in North America; this reflects the parasitic populations present in the local native avifauna (Bensch, Hellgren and Pérez-Tris, 2009; Vanstreels et al., 2015; Stidworthy and Denk, 2018; Hernandez-Colina et al., 2021). Avian malaria in penguins due to other members of the genus is also reported, including infection with *P. cathemerium*, *P. juxtanucleare*, *P. tejerai*, *P. nucleuphilum*, *P. matutinum* and *P. unalis* (Stidworthy and Denk, 2018; Iurescia et al., 2021). Additionally, a wide range of dipteran hosts from multiple genera are capable vectors of avian malaria parasites, and *Plasmodium spp.* vector fidelity remains poorly understood (Valkiūnas et al., 2005; Garamszegi, 2011; Valkiūnas and Iezhova, 2018; Fecchio et al., 2020).

Many penguin species are susceptible to avian malaria but reports of disease are most common in Humbolt (*Spheniscus humboldti*) and African (*Spheniscus demersus*) penguins; this likely reflects their popularity in zoological collections, as well as their native geographic range. *Plasmodium* infections are also reported in Chinstrap (*Pygoscelis antarcticus*), Gentoo (*Pygocelis papua*), Fjordland (*Eudyptes pachyrhynchus*), Galapagos (*Spheniscus mendiculus*), King (*Aptenodytes patagonicus*), Little (*Eudyptula minor*), Macaroni (*Eudyptes chrysolophus*), Magellanic (*Spheniscus magellanicus*), Northern Rockhopper (*Eudyptes moseleyi*), Snares (*Eudyptes robustus*), Southern Rockhopper (*Eudyptes chrysocome*) and Yellow eyed (*Megadyptes antipodes*) penguins (Fantham and Porter, 1944; Brossy, 1992; Levin et al., 2009; Jansen van Rensburg, 2010; Quillfeldt et al., 2011; Sallaberry-Pincheira et al., 2015; Vanstreels, Braga and Catão-Dias, 2016; Sijbranda et al., 2017; Hernandez-Colina et al., 2021). Penguin species in which infection with *Plasmodium spp.* is not reported are typically less studied, native to Antarctica and/or are less commonly kept in zoological collections. In these species, absence of malaria is speculated to be due to lack of exposure and/or diagnosis rather than disease resistance (Vanstreels, Braga and Catão-Dias, 2016).

Avian malaria is much less commonly reported in wild penguins than their captive or rehabilitant counterparts (Table 1), this is thought to reflect both a lower rate of disease and less surveillance in this population. The lower disease incidence in wild birds is attributable to habitat and bird factors. Wild birds often live in geographic regions which are inhospitable to the mosquito vector, typically due to cold temperatures, desiccation and/or wind. Wild birds also have far greater freedom of movement than captive birds, enabling reduced contact with vectors, wild birds are also postulated to experience lower rates of corticosteroid–induced immunosuppression compared to their captive counterparts, as the latter experience a variety of husbandry factors including handling and movement (Fantham and Porter, 1944; Hernandez-Colina et al., 2021). In line with the above, environmental stressors have been found to be implicated in disease in wild birds; one study examining seropositivity for *Plasmodium* infection in African penguins detected an increase from 31% to 55% following an oil spill (Brossy, 1992).

Rates of exposure to avian malaria vary markedly between wild penguin populations; seropositivity rates of 0% are reported for Antarctic Adelie penguins, 100% from penguins sampled in New Zealand, and birds from a range of other geographic locations demonstrated rates of seropositivity between 33-92% (Graczyk, Cranfield, et al., 1995). This is thought to reflect exposure to the parasite rather than differences in susceptibility to disease. Other surveillance studies have employed polymerase chain reaction (PCR) -based methods (Table 1), one such study assessed 800 penguins from across South America which were negative for *Plasmodium spp.* in all cases. It ought to be noted that PCR testing relies on presence of the pathogen in the tissue sampled, and negative results therefore reflect an absence of detectable parasites at the time of sampling rather than providing definitive evidence of lack of exposure.

Table 1 Avian malaria in wild penguins. Adapted from Ings and Denk, 2022. An overview of published cases of avian malaria infection in wild penguins outlining host and parasite species, location and diagnostic method.

Penguin Species	Location	Diagnostic method	Plasmodium spp. implicated	Reference
African	South Africa	Blood smear	*P. relictum*	(Brossy, 1992)
	South Africa	Serology	Not specified	(Graczyk, Cranfield, et al., 1995)
Yellow Eyed	New Zealand	Blood smear	*P. relictum*	(Fantham and Porter, 1944)
Snares	New Zealand	Blood smear	*P. relictum*	(Fantham and Porter, 1944)
Northern Rock Hopper	Gough Islands	Blood smear	*P. relictum*	(Fantham and Porter, 1944)
Galapagos	Galapagos Islands	PCR	Not specified	(Levin et al., 2009)
Little	New Zealand	PCR	Not specified	(Jansen van Rensburg, 2010)

1.3 Clinical disease

Avian malaria in captive penguins frequently presents as sudden death. Less common acute symptoms include behavioural separation, dullness, anorexia, vomiting, dyspnoea, and pallor (Stidworthy and Denk, 2018). Neurological signs, including ataxia, paralysis and seizures are occasionally observed, they occur due to the presence of intra-endothelial meronts within the capillaries of the central nervous system, which cause both endothelial necrosis and endothelial expansion leading to vascular occlusion. The ensuing hypoperfusion in both cases leads to necrosis of the neuropil and resultant clinical signs (Stidworthy and Denk, 2018; Valkiūnas and Iezhova, 2018).

The cause of death in avian malaria is typically circulatory failure secondary to release of large numbers of cytokines ('cytokine storm'), cardiac tamponade following development of a substantial hydropericardium, or respiratory failure (Delhaye et al., 2018; Stidworthy and Denk, 2018). Haematocrit is commonly used to monitor mammalian malarial infections, and in infected birds a reduction of packed cell volume of up to 50% may be seen. However this approach has less utility in the context of penguin malaria as death typically occurs prior to erythrocyte lysis (Atkinson, 2008). Indeed, the acute nature of the disease in penguins provides a significant diagnostic challenge.

Chronic infection with avian malaria is rarely recognised in penguins, however extrapolation from disease manifestation in other avian species suggests that, should penguins survive the initial infection, there is a possibility of chronic latent or subclinical disease. Chronic infection causes reduced longevity of the host, and reductions in fitness, primarily due to reduced clutch size and fledging success (Knowles, Palinauskas and Sheldon, 2010; Asghar et al., 2015; Delhaye et al., 2018). The primary physiological driver of these findings is likely to be increased metabolic demand, both due to demands exerted by the parasite and those required to mount a suppressive immune response. Exposure to a novel pathogen can trigger recrudescence of avian malaria, suggesting that the requirement for the immune system for suppression of *Plasmodium* is ongoing, and that additional infection pressure leads to decreased suppression of avian malaria (Knowles, Palinauskas and Sheldon, 2010; Asghar et al., 2015; Delhaye et al., 2018). It is plausible that the metabolic demands exerted by suppression of chronic *Plasmodium* infection also underlie the initially counterintuitive observation that, in contrast with the studies referenced above, in some cases birds with higher brood sizes have higher *Plasmodium* burdens (Sheldon and Verhulst, 1996; Knowles, Palinauskas and Sheldon, 2010). These findings provide interesting context within which to speculate on the implications of chronic avian malaria in penguins, however such infections are far less commonly identified than acute disease and their impact on clinical disease and population health is considered minimal.

1.4 Epidemiology

Variation in the life cycle of *Plasmodium spp*. occurs with species, host, vector, geography and climatic conditions. Despite this, a common life cycle proposed in the early 20th century by Huff and colleagues remains the basis for our understanding (Figure 1) (Sheldon and Verhulst, 1996; Atkinson, 2008; Lapointe, Atkinson and Samuel, 2012; Liao et al., 2017; Ishtiaq, 2021). The lifecycle can be summarised as follows: when an infected vector takes a blood meal from an avian host, vast numbers of sporozoites enter the host tissue at the site of the bite and form cryptozoites within local fibroblasts and inflammatory cells. From these, merozoites are released, infect macrophages within viscera, where they undergo merogony. The resultant merozoites can form phanerozoites within the viscera, exoerythrocytic meronts within endothelial cells (except for members of the subgenus *Huffia)*, or they may infect erythrocytes. Extraerythrocytic parasites are not able to undergo sexual reproduction but can undergo further cycles of merogony, whilst erythrocytes support both merogony and gametocytogenesis. In the latter case, the resultant gametocytes are infective to the mosquito vector, within which they produce micro- and macrogametes within the midgut. These combine and form an ookinete which is capable to penetrating the gut wall and becoming an oocyst. Sporozoites form within the oocyst, rupture the cell and are released into the haemocoel of the mosquito, from where they migrate to the salivary glands and infect a new avian host following a mosquito bite (Figure 1). Greater understanding of interspecific variations of the life cycle of *Plasmodium* may facilitate development of improved diagnostics and inform prevention and treatment protocols in penguins.

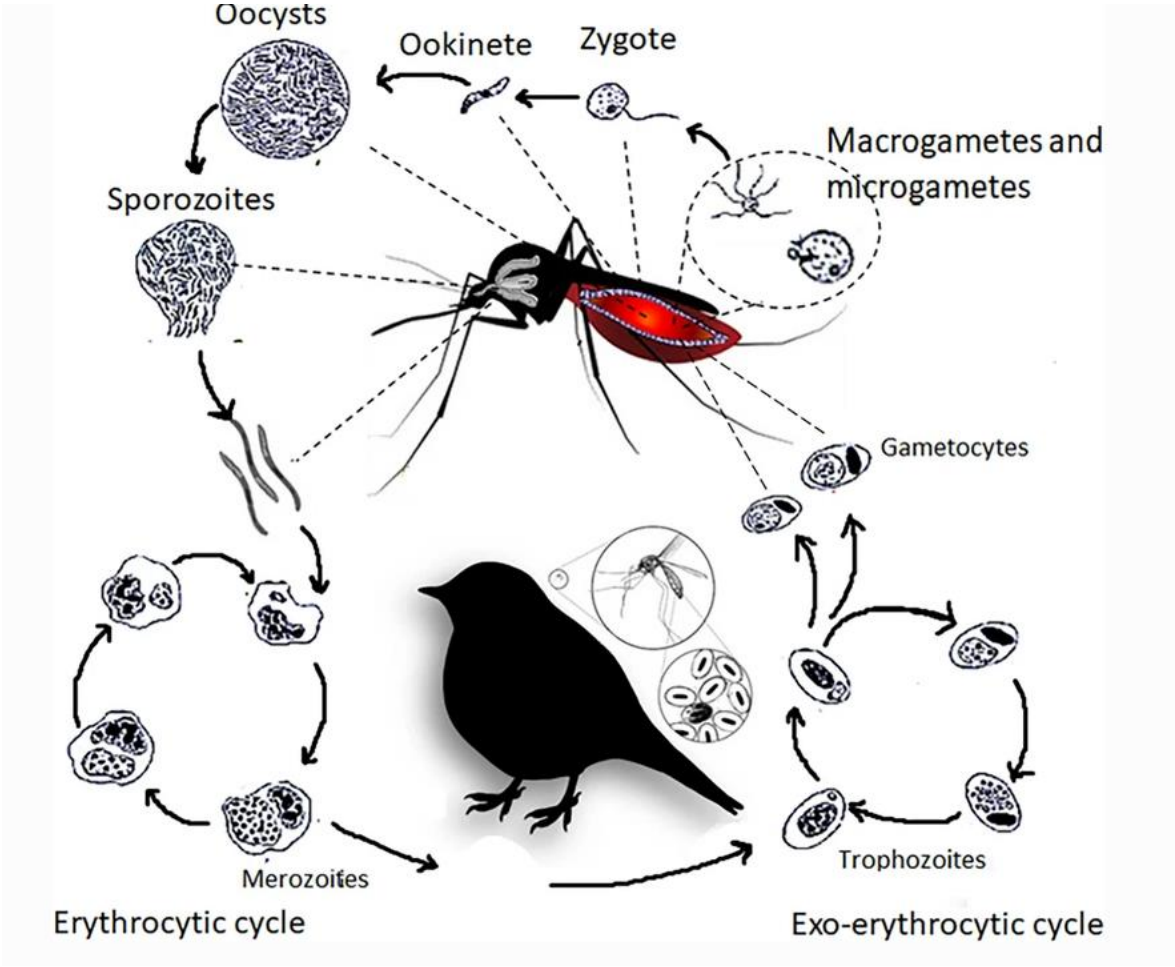

Figure 1 Life cycle of avian malaria parasites. From Ishtiaq, F. Ecology and Evolution of Avian Malaria: Implications of Land Use Changes and Climate Change on Disease Dynamics. J Indian Inst Sci 101, 213–225 (2021). https://doi.org/10.1007/s41745-021-0

Avian malaria in penguins captive in Europe typically results in death prior to detectable parasitaemia (*i.e.* during tissue infection stages). Therefore penguins are unlikely to be sources for onward transmission of disease and resident avifauna are considered the reservoir for infection (Atkinson, 2008; Stidworthy and Denk, 2018). Infection of native birds results in an initial spike in blood-parasite levels, followed by a steady decline in the face of a mounting immune response (Atkinson, 2008). Despite this, *Plasmodium* parasites may persist and the host can remain infective for considerable periods; indeed, some evidence suggests lifelong persistence of the parasite (Lapointe, Atkinson and Samuel, 2012; Liao et al., 2017). Avian malaria is therefore very difficult to eradicate from wild birds. Furthermore, chronic, low-level infections, such as those seen in native European birds, have been implicated in the evolution of more virulent parasitic strains (Lapointe, Atkinson and Samuel, 2012; Liao et al., 2017). Avian malaria is typically seasonal in Europe, increasing in spring owing to increased vector activity, avian immunosuppression (due to metabolic demands of

reproduction), and increase in total bird numbers as migratory species arrive (Fantham and Porter, 1944; Knowles, Palinauskas and Sheldon, 2010).

The reason for the exquisite susceptibility of penguins to avian malaria is not entirely understood, however, the fact that a number of penguin species evolved in regions free from avian malaria and therefore were not subject to the selective pressure the parasite exerts may partially explain their lack of resistance (Fantham and Porter, 1944; Levin et al., 2009; Stidworthy and Denk, 2018). As would be anticipated, immunologically naïve penguins are more susceptible to *Plasmodium* infection, so recently imported/acquired and juvenile penguins are at particular risk (Stidworthy and Denk, 2018). In addition, as briefly discussed above, several physiological and environmental factors may cause glucocorticoid-induced immunosuppression. Examples include concurrent disease (these may be considered primary or secondary in relation to malaria), inappropriate management practices, reproduction, and moult.

1.5 Avian malaria and climate change

Increasing global temperatures are anticipated to increase the activity, reproductive rate, abundance and range of the dipteran (mosquito) vectors of avian malaria, with a resultant increase in *Plasmodium* infections, particularly at the northerly and southerly extremes of the current mosquito range (Garamszegi, 2011; Lapointe, Atkinson and Samuel, 2012; Loiseau et al., 2013; Liao et al., 2017; Fecchio et al., 2020). Introduction of novel vector species to previously naïve areas is also a likely consequence; indeed Culex *quinquefaciatus* has recently been recorded in both New Zealand and the Galapagos Islands, in both cases the effects on native avifauna are under close observation. The notion of expansion of malaria-endemic regions is of particular concern in malaria-adjacent regions inhabited by penguin species categorised as either endangered or at risk, here introduction of *Plasmodium spp.* could reasonably be anticipated to have devastating effects. For example, should vector ranges reach the southerly extremes, the impact on Antarctic penguin species could potentially reach catastrophic dimensions (‘BirdLife International. *Eudyptes moseleyi*. The IUCN Red List of Threatened Species.’, 2020; ‘BirdLife International. *Spheniscus mendiculus*. The IUCN Red List of Threatened Species.’, 2020; ‘BirdLife International. *Spheniscus demersus*. The IUCN Red List

of Threatened Species.', 2020; 'BirdLife International. *Megadyptes antipodes*. The IUCN Red List of Threatened Species', 2020; 'BirdLife International. *Eudyptes chrysolophus*. The IUCN Red List of Threatened Species', 2020; 'BirdLife International. 2020. *Eudyptes chrysocome*. The IUCN Red List of Threatened Species', 2020; 'BirdLife International. *Aptenodytes patagonicus*. The IUCN Red List of Threatened Species', 2020; 'BirdLife International. *Eudyptula minor*. The IUCN Red List of Threatened Species', 2020; 'BirdLife International. *Pygoscelis papua*. The IUCN Red List of Threatened Species', 2020; 'BirdLife International. *Pygoscelis adeliae*. The IUCN Red List of Threatened Species', 2020; 'BirdLife International. *Spheniscus magellanicus*. The IUCN Red List of Threatened Species', 2020). In addition, meteorological changes associated with climate change may affect the epidemiology of *Plasmodium spp*. themselves, for example Fecchio et al demonstrated increased host fidelity in association with increased seasonality of rainfall. The underlying reasons for such findings are likely complex, and therefore speculating on the likely impact of individual findings on avian malaria rates is challenging (Fecchio et al., 2020). Despite this, overall rates of avian malaria have been demonstrated to have increased over the past 20 years, and this is predicted to continue (Garamszegi, 2011; Lapointe, Atkinson and Samuel, 2012; Loiseau et al., 2013; Liao et al., 2017; Fecchio et al., 2020).

1.6 Monitoring and prevention of avian malaria in captive penguins

Monitoring and prevention are the keystones of managing avian malaria in captive penguins. Chiefly, reducing or preventing access to birds by vector-competent mosquitoes is required. Approaches to this have included introduction of infertile or malaria-resistant mosquito strains, manipulation of the environment to make it inhospitable to development of *Culicoides spp.* larvae and implementation of mosquito-proof enclosures (Graczyk et al., 1994; Graczyk, Cranfield, et al., 1995; Valkiūnas et al., 2005). Additionally, prophylactic use of antimalarial medication is undertaken in some zoological collections (Hernandez-Colina et al., 2021). Development of protective malaria vaccines is challenging; considerable efforts have been made in human malaria vaccinology and a DNA vaccine is currently in use which is reduces clinal disease

in children, however frequent dosing is required to maintain efficacy (Grim et al., 2004; Duffy and Patrick Gorres, 2020). Similar vaccines for avian malaria are not currently commercially available and development of such vaccines is challenging, both due to lower availability of resources and greater abundance of causative agents and hosts in avian malaria compared with human disease. Nevertheless, DNA vaccines for birds have been developed and have demonstrated effective protection for the first year in penguins and canaries. However, likely because the vaccinal response was weaker than that generated by true infection, vaccinated birds were significantly more susceptible to avian malaria the subsequent year (Grim et al., 2004; Atkinson, 2008). Overall, no one of the approaches outlined above is effective in managing *Plasmodium spp*. infection in captive penguins, and many are expensive, time consuming and challenging to impose. However, some reduction in risk can be achieved by strict adherence to a combination of the approaches outlined above.

1.7 Current diagnostics

At present several diagnostic methods are used to identify avian malaria in penguins. The gold-standard for diagnosis is a combination of light microscopic and molecular methods. In the context of acute, pauciparasitaemic disease such as that seen in penguins, the majority of diagnoses occur post-mortem.

1.7.1 Light microscopy

1.7.1.1 Examination of blood smears

Plasmodium spp. parasites can be identified by the presence of intraerythrocytic meronts on Giemsa-stained blood smears. Historically this has been the most common method of diagnosing infection with *Plasmodium*, and early characterisation of species was based on the morphologic appearance of the parasite on light microscopy (Fantham and Porter, 1944; Valkiūnas and Iezhova, 2018). Consistent with this, an updated diagnostic key to avian-infective *Plasmodium spp*. was published in 2018, which describes 55 morphospecies and also identifies additional putative species which require further evaluation (Valkiūnas and Iezhova, 2018). Unfortunately, mortality outbreaks in captive penguins are typically associated with rapid death prior to development of

significant parasitaemia, making this method unreliable. Furthermore, pauciparasitaemia typical of chronic infection in a range of avian species makes diagnosis of chronic infection by blood smear unreliable. In those cases where parasitaemia could be sufficient to make a diagnosis of avian malaria, this method relies on operator skill and may be hampered by the absence of one or more life stages, poor blood sampling or inexpert blood smear preparation (Valkiūnas and Iezhova, 2018). Despite these considerations, blood smear evaluation remains one of the mainstays of diagnosis of avian malaria, is one of few validated methods suitable for application in live patients and facilitates identification of mixed infections (where more than one *Plasmodium spp*. is present).

1.7.1.2 Histopathology

Gross post-mortem examination findings can be highly suggestive of avian malaria and allows directed sampling for impression smears and histopathology. Impression smears may be sufficient to identify intraerythrocytic meronts, and therefore can be diagnostic (Stidworthy and Denk, 2018). Histopathology may identify additional supportive changes including granulocytic myocarditis, pneumonia, hepatitis, and splenitis, in association with intralesional meronts and/or hemozoin pigment (the iron-containing product of *Plasmodium* haemoglobin breakdown) (Stidworthy and Denk, 2018). In either case, absence of the parasite in the tissue sections/smear examined may lead to false negative/inconclusive results.

Speciation may be possible using light microscopy (with the caveats mentioned in section 1.7.1.1), providing required parasitic life stages are present, and that the operator is sufficiently skilled and versed in identification (Valkiūnas and Iezhova, 2018). Often however, histological speciation of *Plasmodium* parasites is challenging or impossible as it frequently relies on meront-morphology alone (Stidworthy and Denk, 2018; Valkiūnas and Iezhova, 2018). Furthermore, poor tissue preservation may reduce diagnostic sensitivity. In such cases in-situ hybridisation may be valuable; a probe targeting *Plasmodium spp*. 18S small subunit ribosomal RNA gene (18S SSU rRNA gene) has been published, however, it is not currently commercially available (Dinhopl et al., 2011; Weissenböck. H. (University of Veterinary Medicine, Vienna), 2022).

1.7.2 Enzyme-linked immunoassay (ELISA)

ELISA testing has been widely used to assess levels of antibody to *Plasmodium spp.* in both wild and captive penguins (Graczyk et al., 1994; Graczyk, Brossy, et al., 1995; Dinhopl et al., 2011; Atkinson et al., 2013). Such studies are excellent at demonstrating exposure at a population level and illuminate geographic and temporal distribution of infected birds. However, the nature of humoral responses creates an inevitable lag between infection and seropositivity, and in cases of acute mortality antibodies are typically absent. This renders such tests unsuitable for diagnosis of individual cases of malaria, especially when considering acute disease in penguins. Use of ELISA testing as a prognostic tool had been considered, however the degree of antibody response does not predict disease outcome or degree of parasitaemia, and is again unlikely to be of clinical utility in the context of the acute mortality seen in typical penguin infections (Graczyk, Cranfield, et al., 1995). ELISA testing therefore has application in epidemiological work on avian malaria but is not a useful diagnostic or prognostic tool in acute disease.

1.7.3 Polymerase chain reaction

PCR testing is a mainstay of avian malaria diagnostics, and is particularly valued for its sensitivity and, in combination with Sanger sequencing, ability to identify infective *Plasmodium* species (Jarvi, Schultz and Atkinson, 2002; Fallon et al., 2003). Many PCR protocols have been published to identify avian *Plasmodium* infection. Early work focused primarily on targeting the 18S SSU rRNA gene; more recent (nested) protocols target the Cytochrome B gene (cyt B), and have become the most popular approach (Richard et al., 2002; Fallon et al., 2003; Freed, 2006; Clark, Clegg and Lima, 2014; Videvall, 2019). Whilst the 18S SSU rRNA and cyt B genes are by far the most widely published approaches, a range of other molecular targets have also been considered for avian malaria diagnosis, including large subunit rRNA, dihydrofolate reductase-thymidylate synthase (DHFR-TS) and cytochrome oxidase 3 (Richard et al., 2002; Fallon et al., 2003; Freed, 2006; Murray et al., 2008; Clark, Clegg and Lima, 2014; Videvall, 2019). Nevertheless, despite the ubiquity of PCR testing in avian malaria research, diagnostic PCR testing is not widely commercially available.

1.7.3.1 PCRs targeting the 18S small subunit ribosomal RNA gene

The 18S SSU rRNA gene (or 16S in the case of prokaryotes) has been a widely used target for PCR in a wide range of species since its first publication in 1977 (Fox et al., 1977). It is particularly suitable because of its high copy number, evolutionary preservation, and low rate of polymorphism within hosts. It also has a mutation rate sufficient to generate interspecies but not intraspecies variation in many cases (Fox et al., 1977; Field et al., 1988; Li et al., 1995). Additionally, reports of 'genus-conserved' regions of the 18S SSU rRNA gene in *Plasmodium spp.* enhance to its suitability and explain its popular adoption as a target for PCR testing for malaria diagnosis (Fox et al., 1977; Field et al., 1988; Feldman, Freed and Cann, 1995a; Li et al., 1995; Grim et al., 2004). Despite these advantages, primers optimised on samples derived solely from one host type or geographic region may not be globally applicable due to variation in parasitic genome with host and location (Richard et al., 2002). Additionally, has been suggested that specificity for parasite rather than host sequences may be unreliable with 18S SSU gene rRNA targeting primers, and studies comparing the two approaches indicate improved specificity with primers targeting cyt B (Richard et al., 2002).

1.7.3.2 PCRs targeting the Cytochrome B gene

The cytochrome B gene is currently the most published target for avian malaria PCR (Richard et al., 2002; Fallon et al., 2003; Clark, Clegg and Lima, 2014; Videvall, 2019). PCR testing based on this target forms the basis for the MalAvi database, a collection of haemosporidian reference lineages classified by parasite species, host species, and location, which has contributed significantly to reducing ambiguity in both speciation and naming of the avian haemosporidia (Bensch, Hellgren and Pérez-Tris, 2009). The cyt B gene shares a number of the traits outlined above which make 18S SSU rRNA gene suitable as a PCR target including high copy number, evolutionary preservation, and low rate of intrahost polymorphism, with the additional benefits of greater specificity (Richard et al., 2002). Again, it is conceivable that primers optimised on samples derived from one host type or geographic region may not be applicable in other regions, although this phenomenon is less clearly demonstrated in the case of cyt B

targeting PCR when compared with 18S SSU rRNA gene targeting PCR (Richard et al., 2002).

1.8 Project outline

1.8.1 Study objective

The aim of the study was to develop a method to identify causative *Plasmodium* species in avian malaria infection. A combination of PCR and probe(s) was considered most suitable. Given the diversity of avian infective *Plasmodium spp.* already discovered and the anticipated additional species, designing a probe-based test to identify all possible causative species would be highly challenging. Instead, the study aims were modified to focus initially on development of a probe specific to *P. relictum*, as it is the most commonly identified aetiological agent of malaria in penguins in Europe. The same methodology could then be used to develop additional probes targeting other *Plasmodium* species.

1.8.2 Assay type

PCR is considered the most sensitive diagnostic method currently available and, in combination with Sanger sequencing, facilitates speciation of *Plasmodium* parasites. Additionally, PCR testing can be performed on a variety of tissue types subject to a range of preservation methods, can be performed on a small amount of tissue, and is relatively rapid. PCR – based testing was therefore selected as the initial method for developing a diagnostic test for avian malaria. The apparent ubiquity and suitability of 18S SSU rRNA gene and cyt B as targets for avian malaria diagnosis in the literature informed the decision to trial these as targets when developing a novel diagnostic tool.

PCR can be utilised in a variety of ways to enable testing for diagnostic purposes. Nested PCR is an example of this in which two sequential rounds of PCR are utilised, the amplicon of the first PCR test is used as the substrate for the second PCR test. This approach increases both sensitivity and specificity compared with a single round, and nested PCR protocols for detection of *Plasmodium spp.* infection were reported for use in humans 20 years ago (Snounou et al., 1993). This method does require increased time, labour and

reagent costs compared with a single round, however this can be justified in the case of malaria infection by the fact that the relative proportion of parasite to host DNA is small, particularly given the propensity of avian malaria parasites to cause latent infection (in many avian species), and therefore highly sensitive and specific approaches are required. Nested PCR tests now form the basis for most avian *Plasmodium spp.* PCR based diagnostics, and is the approach adopted in this study (Richard et al., 2002; Snounou and Singh, 2002; Videvall, 2019).

1.8.3 Probe

DNA probes are stretches of nucleic acids which are specifically labelled with a detectable compound and directly bind target sequences in the template DNA [60,61]. Probes allow rapid detection of pathogens, and batteries of individual probes can be used to test for the presence of multiple pathogens simultaneously. The nature of the labelling compound dictates the methods used for detection and may also affect test sensitivity; in particular colorimetric labels are associated with lower specificity when compared to fluorescence or bioluminescent labels (Richards, 1991). The sensitivity of probes can be increased by combining them with PCR testing, as the amplicon generated by the PCR is typically a smaller, more predictable sequence (or group of sequences).

This study aimed to develop a PCR/probe combination for detection of *Plasmodium* by species, beginning with *P. relictum* and with the ultimate aim of developing a battery of *Plasmodium* species-specific probes (Pfaller, 1991; Richards, 1991; de Muro, 2005).

1.8.4 Challenges

There are several inherent challenges to the development of molecular methods for the diagnosis of avian malaria.

Firstly, the genomes of the avian malaria parasites contain uniquely high levels of adenine (A) and thymine (T), which results in relative homogeneity, genomic instability and reduced avidity for primers. Additionally, this feature also necessitates the development of primers with a higher A/T ratio which have a

higher incidence of non-specific binding (Maddocks and Jenkins, 2017; Videvall, 2018, 2019).

Secondly, the avian-infective haemosporidia are closely related and the resultant lack of genetic diversity makes development of genus-specific primers challenging. Whilst species-specific PCR protocols exist for the four human malaria parasites, no such protocols are available for avian malaria parasites (Snounou et al., 1993; Cosgrove, Day and Sheldon, 2006; Bensch, Hellgren and Pérez-Tris, 2009; Clark, Clegg and Lima, 2014; Ciloglu et al., 2018). Sanger sequencing of PCR products is currently required for speciation of avian infective *Plasmodium spp*. Furthermore, some PCR protocols have resulted in misidentification of the haemosporidia at a genus level (Cosgrove, Day and Sheldon, 2006).

Thirdly, whilst species-specific protocols for mixed infections have been combined in a multiplex PCR in human malaria, no such test is available for use in birds; developing such a test would be highly challenging owing to the vast number of causative species and the incomplete characterisation of avian malarial genomes (McNamara et al., 2004). Most published avian malaria PCR protocols are not able to identify mixed *Plasmodium spp*. infections; instead one of the species present will have a greater affinity for the primers and will be amplified at a far greater rate due to the exponential nature of PCR testing. However, a very promising protocol has recently been published which is capable of identifying both single and mixed avian haemosporidia infection (Ciloglu et al., 2018).

Finally, the increasing use of molecular methods has identified large numbers of novel avian infective *Plasmodium* species, and disagreement between morphospecies and molecular species is an increasingly common phenomenon. The current lack of clarity regarding characterisation of species of avian-infective *Plasmodium* is a significant barrier to the development of species-specific diagnostic tools.

1.9 Summary of project aims

The aim of this study is to develop a sensitive and specific diagnostic PCR and probe combination to diagnose penguins infected with *Plasmodium spp.*, beginning with *Plasmodium relictum*. The study will utilise diagnostic material from previously identified cases of avian malaria in penguins resident in the UK to trial novel primers/probes targeting *Plasmodium* mitochondrial DNA.

2. Materials and Methods

This project was approved by the University of Glasgow School of Veterinary Medicine Research Ethics Committee (Application EA31/21).

2.1 Positive controls

Positive control material was generously supplied by Dr Francesco Baldini (Institute of Biodiversity Animal Health & Comparative Medicine, University of Glasgow, Scotland) who provided extracted DNA from *Plasmodium relictum* (Sample ID: FBPR) and *Plasmodium falciparum* (Sample ID: FBPF) and Dr Merit Gonzales-Olvera (Department of Infection Biology, University of Liverpool, England), who provided extracted DNA from *Plasmodium relictum* (Sample ID: MUNCH), *Plasmodium vaughani* (Sample ID: C11-33) and *Plasmodium matutinum* (Sample ID: C7WBL). *Plasmodium falciparum* causes malaria in humans and this material is intended for use as a non-avian infective positive *Plasmodium spp.* control. Control DNA extracted from horse blood was kindly supplied by Dr Robert Coultous (Institute of Biodiversity Animal Health & Comparative Medicine, University of Glasgow, Scotland).

2.2 Sample acquisition and storage

Sample material comprised embedded formalin-fixed paraffin tissue (FFPE) and fresh frozen tissues acquired from cases submitted to a commercial pathology lab based in the United Kingdom. The samples comprised 8 sets of frozen tissue, and 16 sets of FFPE tissues (Table 2), derived from captive penguins identified as (or strongly suspected of being) infected with *Plasmodium spp.,* and two sets of frozen tissue (P17 and P19) from penguins not anticipated to be infected with *Plasmodium.* Diagnosis prior to submission was based on microscopic evaluation of direct impression smears of parenchyma, or histopathological evaluation of tissues. Diagnosis had been made to genus but not species level.

Table 2 Summary of penguin samples used in this study. Description of sample material used in this project, including the species, sex and age of the birds (where available), the tissue type(s) available from each bird, and the *Plasmodium* infection status. A dash indicates that the information is not available. Confirmed cases are those in which the parasite has been demonstrated, suspected are those with histopathological lesions and history consistent with avian malaria, but without direct confirmation of the presence of the parasite.

Penguin ID	Penguin species	Sex	Age (years)	Frozen tissue	FFPE tissue	*Plasmodium* infection status
P1	Macaroni	F	13	No	Yes	Confirmed
P2	-	-	-	No	Yes	Confirmed
P3	-	M	-	No	Yes	Confirmed
P4	Macaroni	F	14	No	Yes	Confirmed
P5	Macaroni	F	8	No	Yes	Confirmed
P6	Macaroni	M	Adult	No	Yes	Suspected
P7	Humboldt	F	25	No	Yes	Confirmed
P8	Macaroni	F	6	No	Yes	Confirmed
P9	Macaroni	M	4	No	Yes	Confirmed
P10	African	M	2	Yes	Yes	Confirmed
P12	-	F	-	No	Yes	Confirmed
P13	-	F	3	No	Yes	Confirmed
P14	-	-	3	No	Yes	Confirmed
P15	-	-	21	Yes	No	Suspected
P17	-	-	-	Yes	No	Negative
P18	-	-	-	Yes	No	Suspected
P19	-	-	-	Yes	No	Negative
P20	Magellanic	F	-	Yes	No	Suspected
P21	Magellanic	F	8	Yes	No	Suspected
P22	Humboldt	F	19	Yes	No	Suspected
P23	Humboldt	F	7	Yes	No	Confirmed
P24	Magellanic	F	-	Yes	No	Suspected
H5	Humboldt	-	-	No	Yes	Confirmed
H6	Humboldt	-	-	No	Yes	Confirmed
H7	Humboldt	-	-	No	Yes	Confirmed

Frozen tissue samples that had previously been stored at -80 ^{0}C were shipped by same-day courier on dry ice and stored at -80 ^{0}C on arrival. FFPE tissues that had been stored at room temperature, were shipped at room temperature by same day courier, and were stored at room temperature on arrival.

2.3 DNA extraction and handling

2.3.1 DNA extraction from FFPE tissues

DNA extraction from FFPE tissues was performed using the QIAamp DNA FFPE Advanced Kit (50) (Qiagen, Germany) and the recommended protocol. All reagents were stored at room temperature, and two or three paraffin scrolls were used per sample. The quantity of scrolls used was based on a subjective assessment of the amount of tissue in each block; two scrolls were used from the more abundant blocks and three from the less abundant blocks. The centrifuge used was an Eppendorf 5415R Microcentrifuge, the heat bock was a Star Labs Dry Bath System, and the vortexer was a Scientific Industries ST™ Vortex-Genie™ 2, used at maximum speed (10).

For each sample, the recommended QIAamp DNA FFPE Tissue Kit protocol was applied (*QIAamp DNA FFPE Tissue Kit*). The resultant samples were each labelled with their unique sample identifier and stored at -20ºC until further use.

2.3.2 DNA extraction from fresh frozen tissues

Extraction of DNA from fresh chicken liver, chicken spleen and lamb liver, and from frozen penguin tissues was performed using a QIAamp DNA Mini Kit (Cat. No. 51304 and 51306, Qiagen, Germany) and application of the recommended protocol ('QIAamp DNA Mini Blood Mini Handbook - EN', 2016). The resultant extract from each sample was frozen at -20ºC until further use.

2.3.3 Assessing success of DNA extraction

2.3.3.1 Assessing success of DNA extraction by PCR

PCR targeting avian DNA was performed to assess success of DNA extraction from avian tissues. Polymerase chain reaction using published avian primers BirdF1 (5' TTCTCCAACCACAAAGACATTGGCAC 3') and COIbirdR2 (5' ACGTGGGAGATAATTCCAAATCCTGG 3') (summarised in Table 3) was performed on DNA extracted from commercially available chicken and lamb liver, chicken spleen and horse blood (prepared using the method described above).

This primer pair had been trialled using the published protocol which can be briefly summarised as follows; denaturation at 94ºC for 60 s, five cycles at 94ºC for 60 s, at 45ºC for 40 s and at 72ºC for 60 s, followed by 35 cycles at 90ºC for 60 s, at 51ºC for 40 s, and at 72ºC for 60 s, with a final elongation step at 72ºC for 5 m (Hebert et al., 2004; Lijtmaer et al., 2012). The anticipated product length was 746 base pairs.

Table 3 Published avian primers targeting the Cytochrome C Oxidase gene which were used to assess success of DNA extraction. The name, sequence and expected product length of primers published by Lijtmaer et al, 2012 are outlined.

Forward Primer		Reverse Primer		Product Length
Name	Sequence (5' to 3')	Name	Sequence (5'-3')	
BirdF1	TTCTCCAACCACAAAGACATTGGCAC	COIbirdR2	ACGTGGGAGATAATTCCAAATCCTGG	746

2.3.3.2 Quantification of DNA extracted

Quantification of DNA in extracted samples was undertaking using a Fluorometer (Qubit 4 Fluorometer, Thermo Fisher Scientific, Massachusetts, USA), and repeated using a NanoDrop (Thermo Fisher, Massachusetts, USA) for comparison, both according to manufacturer guidelines. Fluorometry is considered a more accurate means of quantifying DNA than spectrophotometry, and therefore the results of fluoroscopy were considered more reliable (Paul et al., 2021).

2.3.4 Gel electrophoresis

The final products of all PCRs were visualised using gel electrophoresis on a 1% agarose gel. The gel was made by heating 1 g of UltraPure™ agarose (Thermo Fisher, Massachusetts, USA) with 100 ml of 1 x Tris-Acetate-EDTA (TAE) in a microwave (Samsung, Suwon-si, South Korea) at 1000 W for 90 s in a conical flask. The flask was then transferred to a fume cupboard, and 5 µl of ethidium bromide (Sigma-Aldrich, Missouri, USA), added and thoroughly mixed (Note: a number of safer alternatives are available, and would be recommended). The

hot gel is then poured into a gel casting tray and rested until set (approximately 30-45 m).

Once set, 10 µl of 100 bp DNA ladder (Promega, Wisconsin, USA) was added to the left-most well, and 8 µl of each PCR product was combined with approximately 2 µl of QIAgen GelPilot Loading Dye (Qiagen, Germany), and then added to each well. The gel was then submerged in 1x TAE within a Gibco BRL Model H5 Horizontal Gel Electrophoresis Apparatus (Thermo Fisher, Massachusetts, USA), and a voltage of 72 V applied for approximately 45-60 m (depending on the rate of migration of the product).
The resultant migration was then visualised using a FluorChem 5500 (Alpha Innotech, Cambridge Scientific, Cambridge, UK) and AlphaEase FC software (Alpha Innotech, Cambridge Scientific, Cambridge, UK).

2.4 Development of a novel PCR for *Plasmodium spp.*

2.4.1 Trial of previously published primers

The positive control material was initially tested using published PCR protocols which target the 18S SSU rRNA gene of *Plasmodium spp*. (Table 4 and Table 5)(Feldman, Freed and Cann, 1995a; Fallon et al., 2003). The resultant product was submitted for Sanger sequencing (Eurofins Genomics, Germany).

Table 4 PCR protocol targeting the 18S SSU rRNA gene published by Feldman et al, 1995, outlining primer names, sequences, protocol and expected amplicon length.

Forward Primer	Reverse Primer	Protocol	Expected length
Primer 90 5'GCATGGCCGTTTTTAGTTCGTGAAT3'	**Primer 89** 5'TATCTTTCAATCGGTAGGAGCGACG3'	30 cycles at 94ºC for 40 s, at 48ºC for 2 m and at 72ºC for 45 s, then 10 m at 72ºC	580 bp

Table 5 PCR protocol targeting the 18S SSU rRNA gene published by Fallon et al, 2003, outlining primer names, sequences, protocol and expected amplicon length.

Outer forward	Outer reverse	Outer conditions	Inner forward	Inner reverse	Inner conditions	Expected size
>292F 5'CGGTAGATAGG	**>631R** 5'GCGAGAAGG	2 m at 94ºC, 35 cycles of 60 s at 94ºC, 60 s at 52-	**>343F** 5'GCTCACGCAT	**>496R** 5'GACCGGTCAT	2 m at 94ºC, 35 cycles of 60 s at 94ºC, 60 s at 57ºC,	154 bp

GAACAA ACTGC3'	GAAGT GTGTTT C3'	42ºC, 70 s at 72ºC, then 72ºC for 3 m	CGCTTC T3'	TTTCTT TG3'	and 60 s 10 s at 72ºC, then 72ºC for 3 m	

The positive control material also was initially tested using a published PCR protocol which targets cyt B of *Plasmodium spp*. (Table 6) (Snounou and Singh, 2002). The resultant product was submitted for Sanger sequencing (Eurofins Genomics, Germany) using the inner forward primer (>rPLU3).

Table 6 PCR protocol targeting the Cytochrome B gene published by Snounou and Singh, 2002, outlining primer names, sequences, protocol and expected amplicon length.

Outer Forward Primer	Outer reverse Primer	PCR conditions for outer primers	Inner forward Primers	Inner reverse Primers	PCR conditions for inner primers	Expected amplicon size
>rPLU1 TC5'AA AGATTA AGCCAT GCAAGT GA3'	**>rPLU5** 5'CCTG TTGTTG CCTTAA ACTTC3'	95°C for 5 m. 25 cycles at 58°C for 2 m, at 72°C for 2 m, at 94°C for 60 s. Then 58°C for 2 m and 72°C for 5 m.	**>rPLU3** TTTTTA TAAGGA TAACTA CGGAAA AGCTGT	**>rPLU4** TACCCG TCATAG CCATGT TAGGCC AATACC	95°C for 5 m. 30 cycles of at 64°C for 2 m, at 72°C for 2 m, at 94°C for 60 s. Then 64°C for 2 m and 72°C for 5 m.	235 bp

2.4.2 Acquisition and alignment of previously published sequences

2.4.2.1 Acquisition of Plasmodium 18S SSU rRNA gene sequences

The 18S SSU rRNA gene sequences were acquired from the non-redundant NCBI GenBank database (https://www.ncbi.nlm.nih.gov/genbank/), using the search terms 'plasmodium 18S' and 'plasmodium small subunit' which generated 1009 sequences. These sequences were downloaded for analysis and incomplete, duplicated, or misidentified sequences were deleted. The resultant 183 sequences are detailed in the supplementary material (Table 11).

2.4.2.2 Acquisition of Plasmodium Cytochrome B gene sequences

The same process was employed using the search terms 'plasmodium' and 'cytochrome B', which generated 2509 sequences, from which 40 sequences were selected based on genus (*Plasmodium*) and completeness (Table 12).

2.4.2.3 Alignment of selected sequences

Geneious Prime (Auckland, New Zealand) was used to perform alignments, employing the 'Multiple Align…' function, and the 'MUSCLE Alignment' and the settings outlined in Table 7. In addition, AliView software was employed for generation of a consensus sequence of *Plasmodium spp*. cyt B sequences (Larsson, 2014).

Table 7 MUSCLE alignment settings used to create alignments of *Plasmodium* Cytochrome B gene sequences sourced from the non-redundant NCBI GenBank database.

Max. iterations	3	
Number of trees to build	1	
Distance measure	Iteration 1: kmer4_6	Subsequent: pctid_kimura
Clustering method	Iteration 1&2: UPGMB	Subsequent: UPGMB
Tree Rooting method	Iteration 1&2: pseudo	Subsequent: pseudo
Sequence weighting scheme	Iteration 1&2: CLUSTALW	Subsequent: CLUSTALW
Objective score	spm	
Anchor spacing	32	
Diagonals	Min length: 24	Margin: 5
Hydrophobicity	Multiplier: 1.2	Window size: 5
Maximum memory (MB)	500	

2.4.3 Novel primer design

2.4.3.1 Principles of primer design

Possible novel primer sites were selected to flank hypervariable regions of the consensus sequence (in order to facilitate subsequent probe development) adhering as closely as possible to the following principles (Maddocks and Jenkins, 2017):

- G/C ratio of approximately 50-55%
- Length of 18-25 nucleotides

- Melting temperature of 60-64ºC and <2ºC variance in melting temperature within a pair
- No more than 3 consecutive bases should be the same (to reduce the risk of formation of secondary structures)
- No stretches of >3 base pairs which are complementary, either within the primer or between pairs (to reduce the risk of self-binding or primer dimer formation)

However, as detailed in the introduction, the *Plasmodium* genome is one of the most adenine (A) and thymine (T) rich eukaryotic genomes ever sequenced (Videvall, 2018), which means that in addition to exhibiting a reduced heterogeneity, these genomes are less stable than more variable sequences and therefore exhibit lower affinity during primer annealing (Videvall, 2018, 2019). This means that not only do *Plasmodium* genomes offer fewer suitable sites for primers to bind, they are also likely to force selection of binding sites with higher AT composition and longer consecutive runs of a single nucleotide than would be considered optimal; this held true for the primers in this study. In addition, AT-rich primers require lower annealing and extension temperatures, which increases the risk of non-specific binding (Dhatterwal, Mehrotra and Mehrotra, 2017). Suitable primer sites in conserved regions were identified using Geneious Prime software (Auckland, New Zealand), followed by manual optimisation of the primers to best satisfy the criteria outlined above.

2.4.3.2 Primers targeting the 18S SSU rRNA gene

Based on the consensus sequence generated and the principles outlined previously, two sets of nested primers were developed (Table 8). The anticipated product lengths are 1227 bp for the outer primers for set 1, and 739 for the inner primers of set 1. For set 2 the anticipated product length from the outer primers is 905 bp, and from inner primers is 298 bp.

Table 8 Overview of primers designed to target the 18S SSU rRNA gene of avian infective *Plasmodium spp.*, including primer names, sequences, length, melting temperature (Tm) in ºC and guanine and cytosine content (%GC). The expected amplicon lengths are 1227 bp for the outer primers for set 1, and 739 bp for the inner primers of set 1. For set 2 the anticipated product length from the outer primers is 905 bp, and from inner primers is 298 bp.

	Set 1				Set 2			
	Name and Sequence	Seq. length	Tm	%GC	Name and Sequence	Seq. length	Tm	%GC
Inner	**>370_Inner_F** AGGCGCGTAAATTACCCAATTCTAAA	26	58.5	33.3	**>1457_Inner_F** TGCATGGCCGTTTTTAGTTCG	21	60.1	47.6
	>1273_Inner_R GCCCCAGAACCCAAAGACTT	20	60.2	55	**>1930_Inner_R** TAGCACGCGTGCAGCCTA	20	60.2	55
Outer	**>16_Outer_F** TTATAAGGATAACTACGGAAAAGCTGT	27	58.5	55	**>1273_Outer_F** GCCCCAGAACCCAAAGACTT	20	60.2	55
	>1456_Outer_R GAACTAAAAACGGCCATGCA	20	57.3	45	**>2199_Outer_R** TTTCAATCGGTAGGAGCGACG	21	60.5	52.4

2.4.3.3 Primers targeting the Cytochrome B gene

Based on the Cytochrome B gene consensus sequence and the principles outlined previously, using the 'Design New Primers…' function in Genious Prime (settings were as follows; Task: Generic, Product size 50-479 base pairs), twenty potential primer sites were identified. Suitable primer sites were annotated into the consensus sequence. From these, two sets of nested primers which best satisfied the criteria set out for primer design in section 2.4.3.2 were selected and manually optimised. Of these, four primers were developed with the intention of using these in various combinations as three sets of inner primers (Table 9) together with already established and published outer primers HaemN'I (5'-CATATATTAAGAGAGITATGGAG-3') and HeamR'L (5'-ATAGAAAGATAAGAAATACCA'TC-3') (Hellgren et al., 2015).

These 3 nested primer sets were trialled using positive control material (FBPR and FBPF) and the published protocol for outer primers which can be summarised as follows: denaturation at 94ºC for 180 s, then 35 cycles at 94ºC for

30 s, at 50ºC for 30 s and at 72ºC for 45 s, with a final extension at 72ºC for 10 m (Hellgren et al., 2015). Each set of inner primers was then trialled using the following protocol: denaturation at 94ºC for 180 s, then 35 cycles at 94ºC for 30s, over a temperature gradient of 8ºC and a central temperature of 54ºC for 30 s and at 72ºC for 45 s, with a final extension at 72ºC for 10 m. Resultant product was submitted for Sanger sequencing (Eurofins Genomics, Germany) using the relevant inner forward primer.

Table 9 Overview of novel inner primers used in PCR targeting Cytochrome B gene, including primer names, sequences, sequence length in base pairs, melting temperature (Tm) in ºC, guanine and cytosine content (%GC) and expected amplicon length in base pairs.

	Forward Primer				Reverse Primer				Amplicon length
	Name and Sequence	Seq. length	Tm	%GC	Name and Sequence	Seq. length	Tm	%GC	
Set A	**>P_1_F** TCTATAGTAACAGCTTTTATGGG	23	58.5	35	**>P_ 3_R** TTGGGTCACTTACAAGATATCC	22	60.2	41	98
Set B	**>P_ 1_F** TCTATAGTAACAGCTTTTATGGG	23	58.5	35	**>P_ 4_R** CCCTTCTATCCAAATCTATTAAGTC	25	60.1	36	251
Set C	**>P_ 2_F** GGATATCTTGTAAGTGACCCAA	22	61.6	41	**>P_ 4_R** CCCTTCTATCCAAATCTATTAAGTC	25	60.1	36	132

2.4.4 Working solution for primers

Any primers produced commercially (Eurofins Genomics, Germany) were reconstituted from the lyophilised form with sterile molecular grade water to achieve a concentration of 100 pmol/µl according to the manufacturer's instructions. The reconstituted primers were then stored frozen at -20ºC. A working solution of 10 pmol/µl was made by diluting 20 µl of 100 pmol/µl in 180 µl DNA free water.

2.5 Design of a novel *Plasmodium relictum* specific probe

Following development of a set of primers capable of identifying *Plasmodium spp*. DNA, approaches to speciation were considered. Approaches using either additional primer sets or oligonucleotide probes were considered. Given the nested design of the current PCR, the anticipated minimal genetic diversity within the cyt B gene of avian malaria parasites, and the ultimate aim of multiplex testing a probe-based approach was considered most suitable.

2.5.1 Principles of probe design

There are a number of considerations when designing a probe (de Muro, 2005). The most pertinent of these include:

- Length, 18-50 base pairs is most suitable
- Base composition, 40-60% G/C ratio is most suitable
- No more than 4 identical consecutive bases
- Absence of complementary regions within the sequence (as they would predispose to formation of hairpin bends).

2.5.2 Identification of suitable probe target sites

Consensus sequences were generated from 5 *Plasmodium relictum* cyt B sequences and 35 non-relictum *Plasmodium* cyt B sequences, respectively. The anticipated product of the novel primers >P_1_F and >P_3_R was extracted from each consensus sequence and compared, identifying 6 non-matching base pairs (Figure 2).

Figure 2 A comparison between sequence of novel PCR product derived from non- relictum *Plasmodium (top)* and *P. relictum* (bottom) consensus sequences, demonstrating 6 non-matching base pairs and the location of the candidate probe. BLAST analysis identified only the *P. relictum* genome as containing the probe sequence. No other *Plasmodium spp.* genomes were identified as containing the probe sequence following BLAST analysis.

Using 'Geneious Prime' software (Auckland, New Zealand), the same *Plasmodium spp*. cyt B gene sequences as used for the initial primer design were

examined, all *P. relictum* genomes were removed and the 'MUSCLE Align' function was used to generate a consensus sequence. A separate consensus sequence of the *Plasmodium relictum* sequences was also generated. The 101 base pair region targeted by the inner primers >P_1_F and >P_3_R was selected from both these consensus sequences. These regions were compared using the 'Pairwise Align' function, which demonstrated 6 base pair variances between them. Of these 6 base pair variances, three were clustered in the region of positions 47-53. This was considered the most variable region and therefore most suitable for probe targeting.

2.5.3 Testing the novel P. relictum specific probe in a nested PCR

The putative probe sequence ('P_Prob)' was synthesised as a primer in order to assess its specificity for *P. relictum*. It was used as an inner primer in a nested PCR, and positive control material FBPR and FBPF were used in initial trials.

The PCR was performed as follows; outer primers HaemNFI (5'-CATATATTAAGAGAGITATGGAG-3') and HaemR2L (5'-ATAGAAAGATAAGAAATACCATTC-3') were used in the initial PCR as previously described (see section 2.4.3.3). The second rounds utilised the probe sequence as a forward primer and either >P_3_R or >P_4_R as the inner reverse primer (Table 10), and was conducted under the following conditions: denaturation at 94ºC for 180 s, then 35 cycles at 94ºC for 30 s, at 57ºC for 30 s and at 72ºC for 45 s, with a final extension at 72ºC for 10 m.

Table 10 Overview of primer pairs targeting the Cytochrome B gene trialled to assess novel probe 'P_Prob', outlining primer names, sequences and expected amplicon lengths.

	Forward primer	Sequence	Reverse primer	Sequence	Expected amplicon length (bp)
Pair 1	P_Prob	GGGTGCTACCG TAATAACTAAT	>P _3_R	TTGGGTCACTTAC AAGATATCC	38
Pair 2	P_Prob	GGGTGCTACCG TAATAACTAAT	>P _4_R	CCCTTCTATCCAAA TCTATTAAGTC	129

The resulting PCR product underwent gel electrophoresis as previously described (see Section 2.3.3.3), and suitable products were extracted using the QIAquick Gel Extraction Kit (Qiagen, Germany) ('QIAquick® Spin Handbook', 2020), and submitted for sequencing (Eurofins Genomics, Germany).

Since the resultant DNA fragment was short (approximately 125 base pairs) and therefore challenging to identify on gel electrophoresis, 'P_Prob' was incorporated into a heminested PCR, using the outer primers HaemNFI (5'-CATATATTAAGAGAGITATGGAG-3') and HeamR2L (5'-ATAGAAAGATAAGAAATACCATTC-3'), followed by 'P_Prob' and HeamR2L as inner primers, which generated a product of approximately 300 base pairs.

2.6 Application of novel PCR in recent UK outbreak of avian malaria

2.6.1 Outbreak summary

An outbreak of avian malaria in captive penguin colony of 69 birds resident in the UK resulted in mortality of over 70% in January 2022 (*BBC News*, 2022). FFPE tissue from three affected birds (H5, H6, H7) was donated.

2.6.2 Application of novel PCR

DNA was extracted from the FFPE samples as described previously (Section 2.3.1). The extracted DNA was screened using the novel PCR protocol which incorporates >P_1_F and >P_3_R and is outlined in section 2.4.3.3.

Supplementary Material

Table 11 Accession numbers of 18S SSU rRNA gene sequences used in this study.

MK650560	MK650548	MK650551
MK650558	MK650555	MK650559
MK650553	MK650557	MK650572
MK650542	MK650543	MK650538
MK650539	MK650541	MK650537
MK650563	MK650566	MK650544
MK650536	MK650540	XR_002273095
KU510230	XR_002966679	XR_001974520
XR_003699314	XR_003699303	XR_003699387
XR_606809	XR_001678646	XR_002696555
XR_004611896	XR_002688202	XR_004618857
XR_004618869	AF180727	XR_002696559
AJ243513	XR_002688199	PFARGDC
XR_004611894	KF696370	KF696374
KF696372	KF696373	KF696369
KF696371	KF018657	KF696361
KF219559	KF018654	KF219560
KF696365	KF696364	KF696360
KF696359	KF219558	KF219561
KF018656	KF018658	KF018655
XR_003001217	JQ627156	JQ627157
JQ627158	XR_003001206	XR_003001225
JQ627154	JQ627155	JQ627153
HM032051	XR_005506366	XR_005506386
XR_002198253	XR_002198261	XR_003001211
PVU93234	DQ660817	XR_002198256
XR_001111607	XR_005506393	XR_002273101
XR_003699327	MK650535	MK650590
MK650589	MK650582	MK650608
MK650606	MK650600	MK650609
MK650601	MK650610	MK650611
MK650607	MK650593	MK650586
MK650588	MK650592	MK650594
MK650597	MK650569	MK650554
MK650561	MK650546	MK650547
MK650556	MK650552	MK650545
MK650565	MK650550	MK650549
MK650564	MK650567	MK650570
MK650571	MK650562	MK650568

XR_003699198	MK650508	MK650505
MK650506	MK650516	MK650534
MK650507	MK650526	MK650522
MK650519	MK650517	MK650531
MK650533	MK650521	MK650515
MK650518	MK650523	MK650613
MK650625	MK650619	MK650612
MK650621	MK650622	MK650630
MK650617	MK650627	MK650628
MK650618	MK650615	MK650623
MK650624	MK650631	MK650626
MK650614	MK650620	AY625607
MK650476	MK650477	MK650499
MK650492	MK650498	MK650484
MK650503	MK650502	MK650487
MK650494	MK650478	MK650490
MK650474	MK650479	MK650480
MK650481	MK650485	MK650491
MK650504	MK650489	MK650483
MK650482	MK650475	MK650473
MK650497	MK650488	MK650493
MK650486	MK650500	MK650495

Table 12 Accession number, lineage and species of Cytochrome B gene sequences used in this study

Accession Number	Lineage	Species
FJ389155	P_ALEDIA02	*Plasmodium parahexamerium*
EU770151	P_ANLAT01	*Plasmodium globularis*
FJ389157	P_ANLAT07	*Plasmodium multivacuolaris*
KY653784	P_COLL4	*Plasmodium homocircumflexum*
KU529943	P_COLL6	*Plasmodium delichoni*
FJ389156	P_CYAOLI09	*Plasmodium lucens*
MF817774	P_DENPET03	*Plasmodium nucleophilum*
KF537305	P_DIGCYA08	*Plasmodium lutzi*
MH457423	P_DIGLAF01	*Plasmodium lutzi*
KM211353	P_DIGLAF02	*Plasmodium lutzi*
MW175901	P_FANTAIL01	*Plasmodium collidatum*
MT724388	P_GALLUS01	*Plasmodium gallinaceum*

KC142195	P_GALLUS02	*Plasmodium juxtanucleare*
KM361483	P_GRW02	*Plasmodium ashfordi*
MT925877	P_GRW04	*Plasmodium relictum*
JX029877	P_GRW06	*Plasmodium elongatum*
KR049255	P_GRW11	*Plasmodium relictum*
MK443241	P_LINN1	*Plasmodium matutinum*
KC342644	P_LZFUS01	*Plasmodium relictum*
MK018109	P_PADOM02	*Plasmodium cathemerium*
MT724397	P_PADOM09	*Plasmodium cathemerium*
JX196865	P_PADOM16	*Plasmodium rouxi*
MG724747	P_PHCOL01	*Plasmodium relictum*
MG598399	P_PYCJOC01	*Plasmodium juxtanucleare*
DQ847268	P_PYSUN1	*Plasmodium spp.*
MF817783	P_SEIAUR01	*Plasmodium cathemerium*
KC787080	P_SGS1	*Plasmodium relictum*
JX272844	P_SPMAG01	*Plasmodium tejerai*
HQ591361	P_SPMAG02	*Plasmodium tejerai*
MT281527	P_SW2	*Plasmodium homonucleophilum*
MK929544	P_SW5	*Plasmodium circumflexum*
MF817773	P_SYAT05	*Plasmodium vaughani*
KC138226	P_TFUS05	*Plasmodium lutzi*
KF537293	P_TFUS06	*Plasmodium unalis*
MT724476	P_TUMIG03	*Plasmodium unalis*
KM361481	P_TURDUS1	*Plasmodium circumflexum*
KU573817	P_TURUF03	*Plasmodium unalis*
KU573815	P_TURUF04	*Plasmodium unalis*
AY178904	P_UNKNOWN02	*Plasmodium rouxi*

3. Results

3.1 DNA Extraction

Proving the presence of extracted DNA is essential in order the evaluate the utility of the novel primer and probe, as failure to yield sufficient template material would preclude primer/probe binding. Successful extraction of DNA from fresh, frozen and FFPE tissues was demonstrated using combinations of established avian primers and protocols and DNA quantification using fluoroscopy.

3.1.1 Quantification of DNA extracted from fresh control material

The primers BirdF1 and COIBIRD R2 target avian Cytochrome C Oxidase subunit 1 (COI) gene and are widely used in genetic 'barcoding' of avian species. The DNA extracted from both chicken liver and spleen subject to the established PCR yielded product of the anticipated length (749 bp) following gel electrophoresis, however DNA extracted from lamb liver also yielded product of the anticipated length (Figure 3). When the PCR was repeated on DNA extracted from equine blood, no detectable product was present.

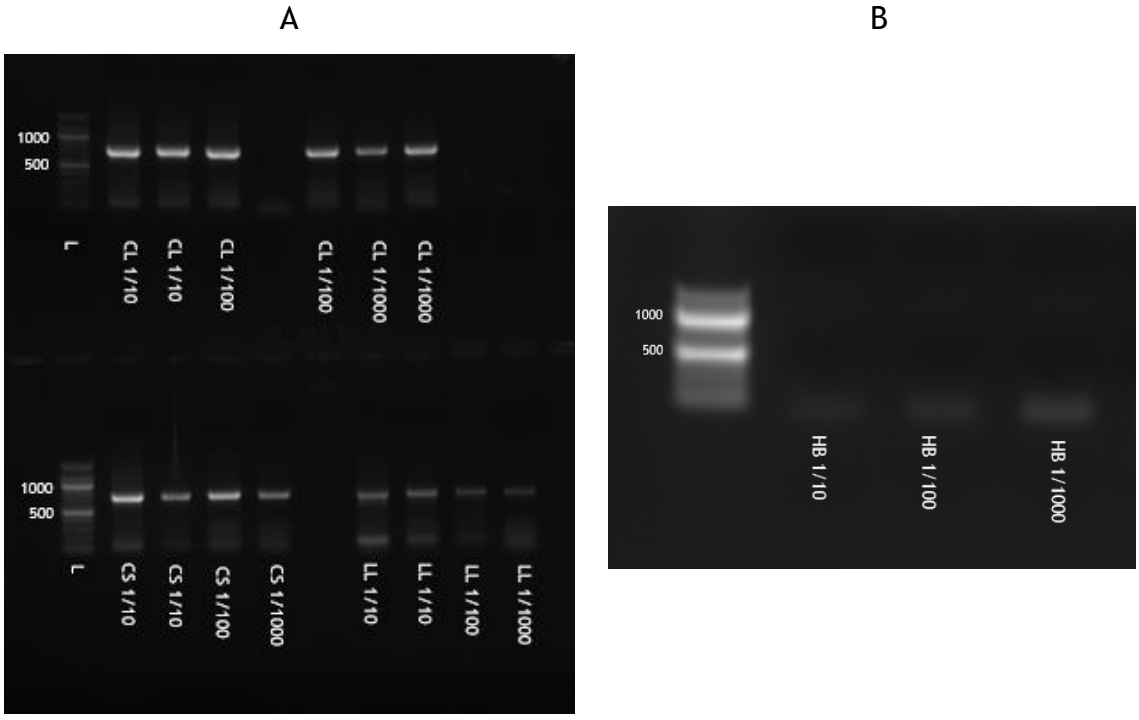

Figure 3 Assessment of the quality of DNA extracted from fresh tissues. A) An electrophoresis gel showing the final product from the PCR protocol published by Lijtmaer et al, targeting the Cytochrome C Oxidase 1 gene, applied to DNA extracted from fresh chicken liver (CL), chicken spleen (CS) and lamb liver (LL) with a 100bp ladder (L). B) An electrophoresis gel showing the final PCR product from DNA extracted from horse blood (HB) with a 100bp ladder. In all cases the expected amplicon length is 749 base pairs.

3.1.2 Quantification of DNA extracted from FFPE

The PCR using established avian primers BirdF1 and COIBIRD R2 on DNA extracted from FFPE penguin tissue did not produce a detectable product. The same sample of extracted DNA was quantified using a fluorometer demonstrated successful extraction DNA at concentrations between 2.38 – 30.9 ng/L (Table 13).

Table 13 Quantification of DNA extracted from FFPE tissues from cases P1-P10 by high-sensitivity fluoroscopy, showing concentration of DNA in each sample in ng/uL.

Sample Name	Concentration (ng/uL)
P1	10.7
P2	16.7
P3	7.47

P4	2.38
P5	15.9
P6	3.12
P7	30.9
P8	26.0
P9	17.7
P10	20.1

3.1.3 Quantification of DNA extracted from frozen tissues

Quantification of extracted DNA from 7 frozen samples using a fluorometer indicated successful extraction of DNA of concentrations of between 14.1-57.0 ng/L (Table 14).

Table 14 Quantification of DNA extracted from frozen tissue samples by high-sensitivity fluoroscopy, showing concentration of DNA in each sample in ng/uL.

Sample Name	Concentration (ng/uL)
P22	51.0
P23	29.2
P24	57.0
P20	53.0
P21	57.0
P15	14.1
P10	44.4

3.2 Development of a novel PCR for *Plasmodium spp.*

3.2.1 Trial of previously published primers

Trial of published primers targeting either the 18S SSU rRNA gene or cyt B on known positive *P. relictum* (FBPR) and *P. falciparum* (FBPF) control material demonstrated product sequences as outlined in Table 15. The PCR protocol targeting cyt B published by Snounou and Singh (Snounou and Singh, 2002) identified parasite DNA from the 1/10 (most concentrated) *P. falciparum* sample, which demonstrated 100% identity with *P. ovale* (Acession No. MW426207.1), and 99.5% identity to *P. falciparum*. This protocol failed to detect the less concentrated *P. falciparum* or *P. relictum* samples.

Primers targeting the 18S SSU rRNA gene published by Feldman et al (Feldman, Freed and Cann, 1995a) successfully identified the two most concentrated *P. relictum* samples, but also amplified vector (*Anopheles gambiae*) DNA from the *P. falciparum* sample (Table 15).

Finally, the protocol published by Fallon et al (Fallon et al., 2003) amplified the expected target (18S SSU rRNA gene) sequence from all the positive control material (Figure 4, Table 15), demonstrating that the positive control material is suitable for our purposes.

Table 15 Results of published PCR protocols trialled on positive control material, indicating the amplicon sequence and the BLAST identity for each positive sample derived using published PCR protocols targeting the Cytochrome B gene (Snounou and Singh, 2002) and the 18S SSU rRNA gene (Feldman et al, 1995 and Fallon et al, 2003).

Authors	Positive sample	Sequence	BLAST identity
Snounou and Singh, 2002	PF 1/10	CGCGGCCTTATACTTGCTTTATTATCCTTTGATTTTTATCTTTGGATAAGTATTTGTTAGGCCTTATAAGAAAAAAGTTATTAACTTAAGGAATTATAACAAAGAAGTAACACGTAATAAATTTATTTTATTTAGTGTGTATCAATCGAGTTTCTGACCTATCAGCTTTTGATGTTAGGGTATTGGCCTAACATGGCTATGACGGGTA	*P. falciparum*
Feldman et al, 1995	PR 1/10	TCTGAAAATATATACAAAAGGTGTAATTTGTCCTCCACTGAAAAGTGTAGGTAATCTTTATCAATATATATCGTGATGGGGATAGATTATTGCGATTATTAATCTTGAACGAGGAATGCCTAGTAAGCATGATTCATCACATTGTGCTGACTACGTCCCTGCCCTTTGTACACACCGCCCGTCGCTCCTACCGATTGAGAGATAAA	*P. relictum*
	PR 1/100	AAATAACAAATAAGAGAAAATATTAGGATGTTATTATATATAATATCCTTTTCCCTTTTCTTCTTATTATGTTTTTTTATTCTATTTCTTTTTTTACTTAAGAATGTATTTACTTGATTGTAAAGCTTCTTAGAGGAACATTGTGTGTCTAACACAAGGAAGTTTAAGGCAACAACAGGTCTGTGATGTCCTTAGATGAACTAGGCTGCACGCGTGCTACACTGATATATATAACGAGTTATTAAAAATATATCTTTGTATATATGTATTTTATATATATATACAAAAGGTATAATTTTCCTCCACTGAAAAGTGTAGGTAATCTTTATCAATATATATCGTGATGGGGATAGATTATTGCAATTATTAATCTTGAACGAGGAATGCCTAGTAAGCATGATTCATCACATTGTGCTGACTACGTCCCTGCCCTTTGTACACACCGCCCGTCGCTCCTACCGATTGAAAGAT	*P. relictum*
	PR1/1000	No meaningful sequence	No meaningful sequence
	PF 1/10	ACTTAGAACGCTTGTCAGTAGTGTGCCTCCGGGCGCACCTGACGTTAGGTAGTGGCGGGTGTCCTCACGGGTGCCCGTCACTTAGTTTGCCCTGCTTAGCGGGACAACTTGTGTTTAGCAAGATGAGATTGAGCGATAACAGGTCCGTGATGCCCTTAGATGTTCTGGGCTGCACGCGTGCTACAATGTGAGCAGCAGCGTGTTCTCGCCTTATGGCGCCCCCATTCCGAGAGGAACGGGAAATCACCCAAATGCTCATTTAGTAGGGATTGGGGACTGCAATGGTCCCCATGAACCTGGAATTTCTAGTAAGTGCTAGTCATTAGCTAGCGCTGATTACGTCCCTGCCCTTTGTACACACCGCCCGTCGCTCCTACCGATTGAAAGATA	*A. gambiae*
	PF 1/100	TTCGATACGAACGCGACTCAGTCAAGCTAACTAGAACGCTGTCAGTAGTGTGCCTCCGGGCGCACCTGACGTTAGGAGTGGCGGGTGTCCTCACGGGTGCCCGTCACTTAGTTTGCCCTGCTTAGCGGGACAACTTGTGTTTAGCAAGATGAGATTGAGCGATAACAGGTCCGTGATGCCCTTAGATGTTCTGGGCTGCACGCGTGCTACAATGTGAGCAGCAGCGTGTTCTCGCCTTATGGCGCCCCCATTCCGAGAGGAACGGGAAATCACCCAAATGCTCATTTAGTAGGGATTGGGGACTGCAAGGG	*A. gambiae*

	PF 1/1000	GACCGTCACTTAGTTTGCCCTGCTTAGCGGGACAACTTGTGTTTAGCAAGATGAGATTGAGCGATAACAGGTCCGTGATGCCCTTAGATGTTCTGGGC TGCACGCGTGCTACAATGTGAGCAGCAGC	*A. gambiae*
Fallon et al, 2003	PF 1/10	CAGTTCAATAGATTGGTAAGGTATAGTGTTTACTATCAAATGAAACAATGTGTTCCACCGCTAGTGTTTGCTTCTAACATTCCACTTGCTTATAACTGTA TGGACGTAACCTCCAGGCAAAGAAAATGACCGGTCAGAT	*P. falciparum*
	PF 1/100	CATGGACTTGTTCAGTTCAATAGATTGGTAAGGTATAGTGTTTACTATCAAATGAAACAATGTGTTCCACCGCTAGTGTTTGCTTCTAACATTCCACTTG CTTATAACTGTATGGACGTAACCTCCAGGCAAAGAAAATGACCGGTCAATTTTA	*P. falciparum*
	PF 1/1000	CATGGACTTGTTCAGTTCAATAGATTGGTAAGGTATAGTGTTTACTATCAAATGAAACAATGTGTTCCACCGCTAGTGTTTGCTTCTAACATTCCACTTG CTTATAACTGTATGGACGTAACCTCCAGGCAAAGAAAATGACCGGTCAATTTTA	*P. falciparum*
	PR 1/10	TAATGGGAACCTTGTTTCAAGTTCAAATAGATTGGGTAAGGTATAGCGTTTACTATCGAATGAAACAATGTGTTCCACCGCTAGTGTTTGCTTCTAACAT TCCATTTGCTTATAACTGTATGGACGTAACCTCCAGGCAAAGAAAATGACCGGTCAAAACAG	*Plasmodium spp.*
	PR 1/100	TGGACTTGTTCAGTTCAATAGATTGGTAAGGTATAGCGTTTACTATCGAATGAAACAATGTGTTCCACCGCTAGTGTTTGCTTCTAACATTCCATTTGCT TATAACTGTATGGACGTAACCTCCAGGCAAAGAAAATGACCGGTCAAAAACGGA	*Plasmodium spp.*
	PR 1/1000	AAACAATGTGTTCCACCGCTAGTGTTTGCTTCTAACATTCCATTTGCTTATAACTGTATGGACGTAACCTCCAGGCAAAGAAAATGACCGGTCAGAT	*Plasmodium spp.*

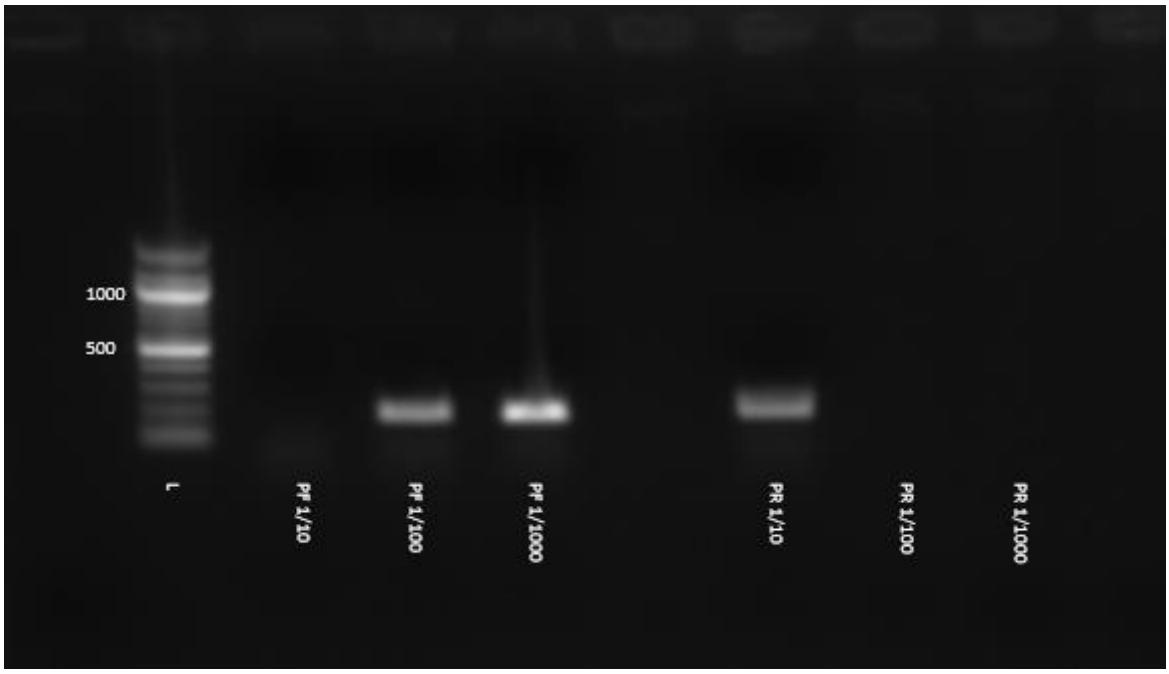

Figure 4 An electrophoresis gel demonstrating the final PCR product of positive control *Plasmodium falciparum* (PF) and *Plasmodium relictum* (PR) DNA at dilutions of 1/10, 1/100 and 1/1000 subject to primers targeting the 18S SSU rRNA published by Fallon et al (2003), alongside a 100bp ladder (L). The expected amplicon length is 286 base pairs.

3.2.2 Design of novel primers targeting the 18S Small Subunit rRNA gene

3.2.2.1 Alignment of 18S SSU rRNA gene Sequences

Alignment of 183 *Plasmodium* 18S SSU rRNA gene sequences demonstrated several highly conserved regions, interspersed with variable regions (Figure 5). A *Plasmodium spp.* consensus sequence was generated and used as the template for primer design (Figure 6).

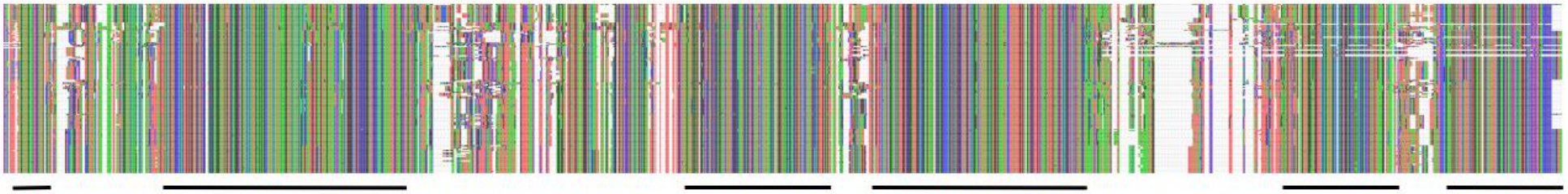

Figure 5 Alignment of Plasmodium spp. 18S SSU rRNA gene. Each sequence is represented by a horizontal line, base pairs are identified by colour, adenine is green, thymine is red, guanine is grey, and cytosine is blue. Conserved regions are indicated by black lines.

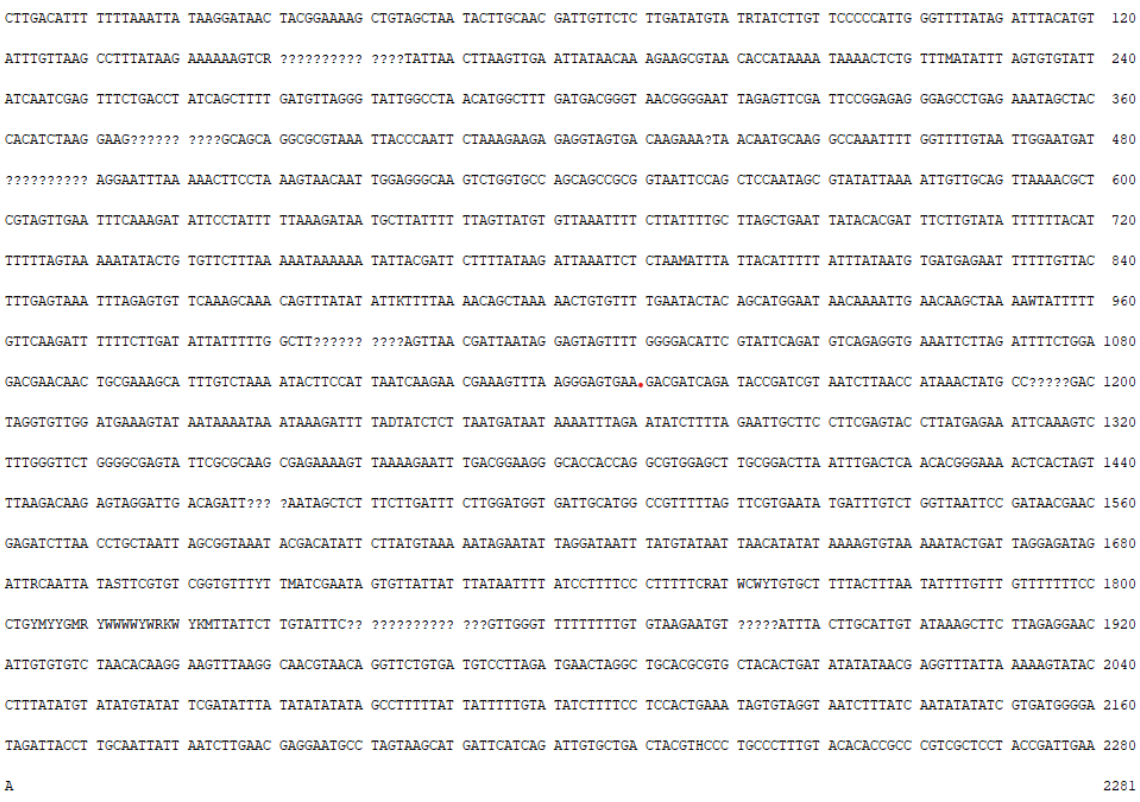

Figure 6 Consensus sequence generated for *Plasmodium spp.* 18S SSU rRNA gene sequences derived from the non-redundant NCBI GenBank database.

3.2.2.2 Development of candidate primers targeting the 18S rRNA gene

An initial visual assessment of suitable primer sites which would flank conserved regions in the *Plasmodium* consensus sequence identified candidate regions. These were selected on the basis that primer pairs flanking variable regions would be more useful in subsequent speciation efforts. Two sets of nested primers which best satisfied the criteria for primer design discussed previously (section 2.4.3.1) were selected and manually optimised, the resultant primers are shown in Figure 7.

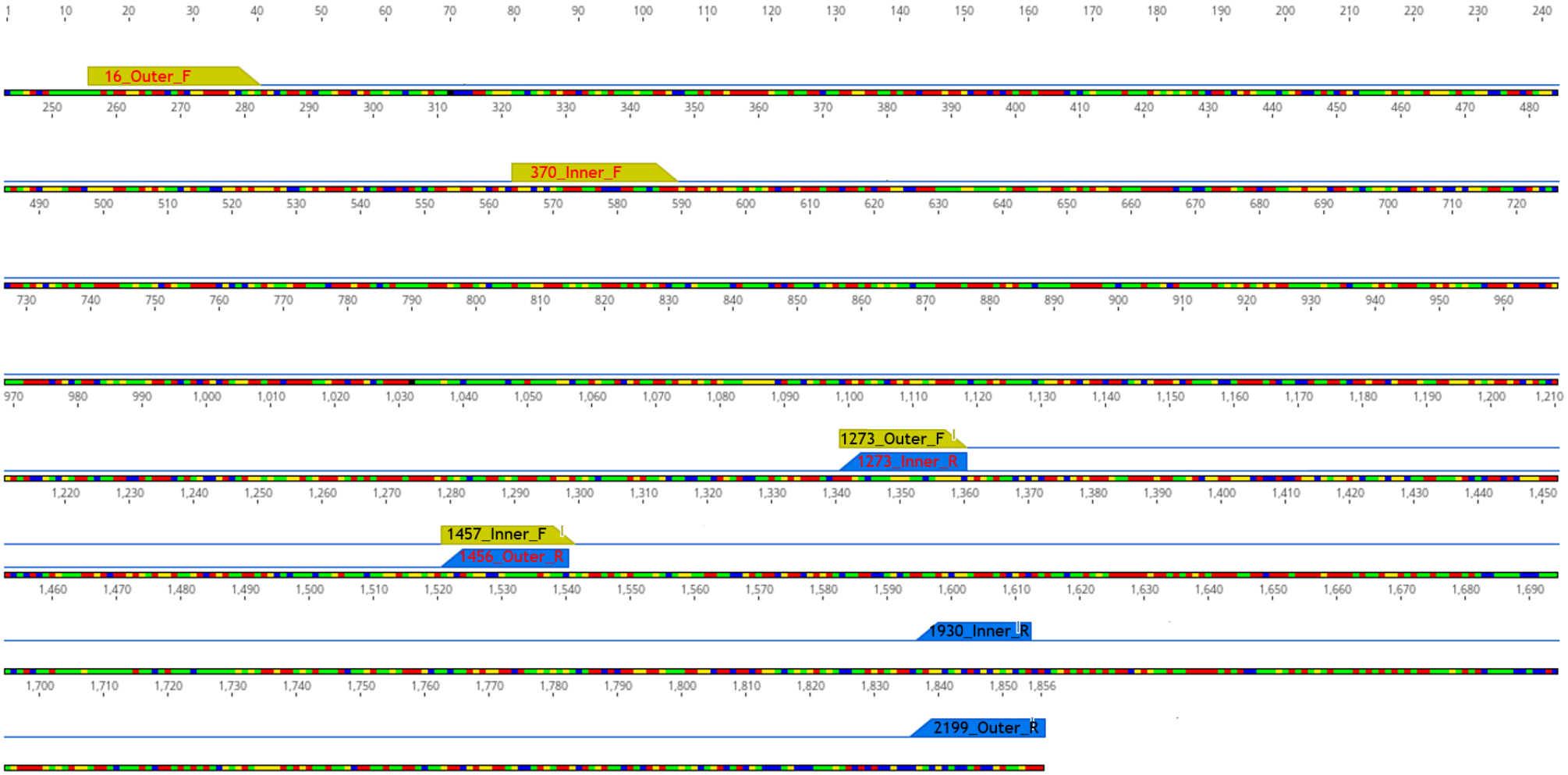

Figure 7 The binding sites of two sets of nested primers (Set 1 in red text and Set 2 in black text) on the consensus 18S SSU rRNA sequence of avian infective *Plasmodium spp.*.

3.2.2.3 Trial of novel primers targeting 18S SSU rRNA gene

Set 1

A PCR using inner primers >370_Inner_F and >1273_Inner_R did not yield detectable product from positive control tissues FBPR and FBPF on gel electrophoresis. A nested PCR ('Set 1') using >16_Outer_F and >1456_Outer_R as outer primers, followed by >370_Inner_F and >1273_Inner_R as inner primers also failed to yield detectable product. Therefore, this nested PCR was considered insufficiently sensitive to *Plasmodium spp*. DNA and was abandoned.

Set 2

A second nested PCR ('Set 2') using >1273_Outer_F and >2199_Outer_R as outer primers, followed by >1930_Inner_R and >1457_Inner_F as inner primers also failed to detect *P. relictum* positive control material, however it yielded product from the *Plasmodium falciparum* positive control material at dilutions of 1/10 (PF_1/10_S2 and 1/100 (PF_1/100_S2). The DNA sequences of the products are summarised in Table 16.

Table 16 Sequences generated from extracted product acquired by PCR targeting the 18S SSU rRNA gene using primers >1930_Inner_R and >1457_Inner_F, indicating the product sequence, expected length, actual length and BLAST identity. The BLAST identity of sample >PF_1/10_S2 was not investigated as the sequence was significantly shorter than expected and of poor quality.

Sample Name	Sequence	Expected Length (bp)	Actual length (bp)	BLAST identity
>PF_1/10_S2	TTCGGTCCATCTCCGTCGATTTGGCCGCT GAAATCTCTGACAATTTCGCT	296	50	N/A
>PF_1/100_S 2	TTGGGGTTGCTGGTAATTCCGATAACGAA CGCGACTCAGTCAAGCTAACTAGAACGCT GTCAGTAGTGTGCCTCCGGGCGCACCTGA CGTTAGGAGTGGCGGGTGTCCTCACGGG TGCCCGTCACTTAGTTTGCCCTGCTTAGC GGGACAACTTGTGTTTAGCAAGATGAGAT TGAGCGATAACAGGTCCGTGATGCCCTTA GATGTTCTAGGCTGCACGCGTGCTAAATT	296	231	*Anopheles gambiae*

For sample PF_1/10_S2, the sequence is significantly shorter than expected and the quality is poor (indicated by a chromatogram with poorly defined peaks and signal to noise ratios of <15)(*PCR Troubleshooting Guide - UK*).
The sequence generated from sample PF_1/100_S2, which was closer to the expected length, revealed 96-99% sequence identity to *Anopheles gambiae*. This reflects primer binding vector rather than parasite DNA. When *Anopheles spp.* were excluded, no significant similarities were identified. As a result of these findings, it was concluded that the primers were not capable of binding *Plasmodium spp.* DNA with sufficient sensitivity and specificity and therefore an alternative primer target site was considered.

3.2.3 Design of novel primers targeting Plasmodium *Cytochrome B gene*

3.2.3.1 Alignment of Cytochrome B gene sequences

The alignment of cyt B generated using AliView (Larsson, 2014) is demonstrated in Figure 8, this alignment identified both conserved and variable regions (Figure 9). Primers were again designed to target conserved regions and to flank more variable regions, to facilitate subsequent development of species-specific probes.

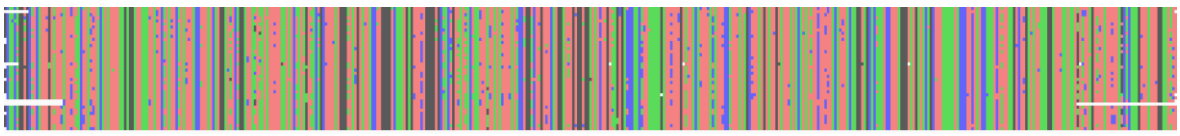

Figure 8 Alignment of *Plasmodium spp.* Cytochrome B gene sequences derived from the non-redundant NCBI GenBank database. Base pairs are identified by colour, adenine is green, thymine is red, guanine is grey, and cytosine is blue.

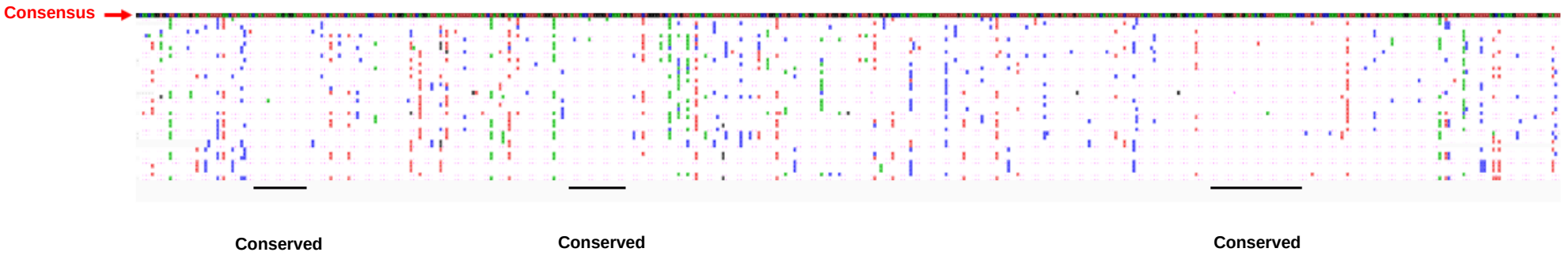

Figure 9 Alignment *Plasmodium spp*. Cytochrome B gene sequences from the non-redundant NCBI GenBank database, demonstrating variance from the consensus sequence (red arrow) and conserved regions (black lines) using AliView (Larsson, 2014).

3.2.3.2 Development of candidate primers targeting Cyt B

Twenty potential primer sites with no off-target binding were identified (Table 17). Three potential primer binding sites were identified as most suitable, based on their position within the sequence and the criteria defined in Section 2.4.3.1.

Table 17 Primers targeting Cytochrome B gene generated using Geneious Prime, including primer name, direction, sequence, length, the percentage of the sequence that comprises guanine or cytosine (%GC), the Hairpin Melting Temperature (HTm), Self Dimer Melting Temperature (SDTm) and Melting Temperature (Tm). Temperatures are given in º C.

Name	Direction	Sequence	Length	%GC	HTm	SDTm	Tm
370R	R	ACCCTAAAGGATTTGTGCTACC	22	45.5	39.6	None	58.0
369R	R	CCCTAAAGGATTTGTGCTACCTTG	24	45.8	41.1	None	59.6
258R	R	TGGGTCACTTACAAGATATCCACC	24	45.8	None	None	59.8
240R	R	TCCACCACAAATCCATGAAACAAG	24	41.7	None	None	60.0
239R	R	CCACCACAAATCCATGAAACAAG	23	43.5	None	None	58.7
237R	R	ACCACAAATCCATGAAACAAGTCC	24	41.7	None	None	60.0
236R	R	CCACAAATCCATGAAACAAGTCC	23	43.5	None	None	58.4
234R	R	ACAAATCCATGAAACAAGTCCAGG	24	41.7	None	None	59.7
186R	R	AACAGTAGCACCCCAGAAACTC	22	50.0	None	None	60.2
185R	R	ACAGTAGCACCCCAGAAACTC	21	52.4	None	None	59.7
165F	F	GAGTTTCTGGGGTGCTACTG	20	55.0	None	None	57.9
164F	F	TGAGTTTCTGGGGTGCTACTG	21	52.4	None	None	59.7
163F	F	ATGAGTTTCTGGGGTGCTACTG	22	50.0	None	None	60.0
159F	F	TCAAATGAGTTTCTGGGGTGC	21	47.6	None	None	58.8
158F	F	GTCAAATGAGTTTCTGGGGTGC	22	50.0	None	None	60.0
156F	F	GGGTCAAATGAGTTTCTGGGG	21	52.4	None	None	58.8
155F	F	GGGGTCAAATGAGTTTCTGGG	21	52.4	None	None	58.8
154F	F	TGGGGTCAAATGAGTTTCTGGG	22	50.0	None	None	60.5
137F	F	TGGGTTATGTATTACCTTGGGGTC	24	45.8	46.0	0.7	59.8
136F	F	ATGGGTTATGTATTACCTTGGGGTC	25	44.0	46.0	0.7	60.2

These were further optimised to best satisfy the criteria. The resultant putative primer sequences are demonstrated in Figure 10 and Table 18.

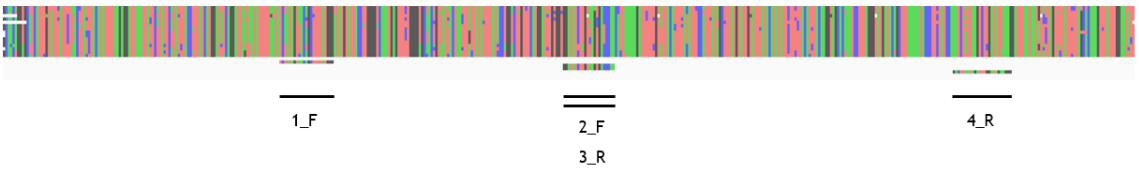

Figure 10 Alignment of *Plasmodium spp.* Cytochrome B gene sequences derived from the non-redundant NCBI GenBank database showing the binding locations of novel primers. Base pairs are identified by colour, adenine is green, thymine is red, guanine is grey, and cytosine is blue.

Table 18 Overview of novel primers targeting *Plasmodium spp.* Cytochrome B gene, outlining the sequence of each primer.

Primer Name	Sequence
>P_1_F	TCTATAGTAACAGCTTTTATGGG
>P_2_F	GGATATCTTGTAAGTGACCCAA
>P_3_R	TTGGGTCACTTACAAGATATCC
>P_4_R	CCCTTCTATCCAAATCTATTAAGTC

3.2.3.3 Trial of novel primers targeting the Cytochrome B gene

Trial of the inner round of the newly devised nested PCR on *Plasmodium relictum* and *Plasmodium falciparum* positive control material (FBPR and FBPF), demonstrated that amplicons generated using Set A (P_1_F as forward primer and >P_3_R as reverse primer), and the protocol outlined in section 2.4.3.3, demonstrated the most consistent, clear bands following gel electrophoresis. Similarly, samples incubated at 55 ^{0}C demonstrated the most consistent, clear bands following gel electrophoresis. Therefore, Set A primers and an annealing temperature of 55 ^{0}C were selected. None of the three primer pairs generated amplicons from *P. falciparum* positive control material.

3.2.3.4 Novel nested PCR trialled on extracted DNA

The optimised novel nested PCR targeting the cyt B gene incorporating published outer primers HaemNFI and HeamR2L for the initial reaction, followed by inner reaction using P_1_F and P_3_R was applied to DNA extracted from 10 frozen and 16 FFPE tissue sets from 25 individual birds. The final PCR product was subject to gel electrophoresis which demonstrated PCR product of expected length in all cases (Table 19). Sanger sequencing and BLAST analysis of product sequences identified *P. relictum* in 25/25 penguins and *P. matutinum* in 1/25. These results may indicate successful identification of *Plasmodium spp.* infection using the novel PCR in all penguin material examined, including identification of different *Plasmodium* species from both frozen and FFPE tissues in one individual (P10).

Table 19 BLAST identity of PCR products produced from DNA extracted from penguin tissues, using a novel PCR protocol incorporating the primers P_1_F and >P_3_R followed by Sanger sequencing. The tissue type (FFPE or frozen) is described, alongside the case identifier, penguin species (where known) and BLAST identity. Samples from birds suspected not to be infected with *Plasmodium* (P17 and P19) are highlighted in blue. NA indicates the tissue type was not available for this case.

Penguin Species		**Sample ID**	BLAST identity	
			FFPE	Frozen tissue
Macaroni	*Eudyptes chrysolophus*	P1	*P. relictum*	*NA*
Penguin	Unknown species	P2	*P. relictum*	*NA*
Penguin	Unknown species	P3	*P. relictum*	*NA*
Macaroni	*Eudyptes chrysolophus*	P4	*P. relictum*	*NA*
Macaroni	*Eudyptes chrysolophus*	P5	*P. relictum*	*NA*
Macaroni	*Eudyptes chrysolophus*	P6	*P. relictum*	*NA*
Humboldt's	*Spheniscus humboldti*	P7	*P. relictum*	*NA*
Macaroni	*Eudyptes chrysolophus*	P8	*P. relictum*	*NA*
Macaroni	*Eudyptes chrysolophus*	P9	*P. relictum*	*NA*

African	*Spheniscus demersus*	P10	*P. relictum*	*P. matutinum*
Macaroni	*Eudyptes chrysolophus*	P12	*P. relictum*	*NA*
Macaroni	*Eudyptes chrysolophus*	P13	*P. relictum*	*NA*
Humboldt's	*Spheniscus humboldti*	P14	*P. relictum*	*NA*
Macaroni	*Eudyptes chrysolophus*	P15	*NA*	*P. relictum*
Humboldt's	*Spheniscus humboldti*	P17	*NA*	*P. relictum*
Humboldt's	*Spheniscus humboldti*	P18	*NA*	*P. relictum*
Humboldt's	*Spheniscus humboldti*	P19	*NA*	*P. relictum*
Magellanic	*Spheniscus magellanicus*	P20	NA	*P. relictum*
Magellanic	*Spheniscus magellanicus*	P21	NA	*P. relictum*
Humboldt	*Spheniscus humboldti*	P22	NA	*P. relictum*
Humboldt	*Spheniscus humboldti*	P23	NA	*P. relictum*
Magellanic	*Spheniscus magellanicus*	P24	NA	*P. relictum*
Humboldt's	*Spheniscus humboldti*	H5	*P. relictum*	*NA*

Humboldt's	*Spheniscus humboldti*	H6	*P. relictum*	*NA*
Humboldt's	*Spheniscus humboldti*	H7	*P. relictum*	*NA*

3.3 Design of a novel *Plasmodium relictum* specific probe

3.3.1 Putative novel probes

The most variable section of consensus sequences in the region amplified by probes >P_1_F and >P_3_R was identified, the resultant candidate probe was designed to target the *P. relictum* gene in this region (Figure 11), and named 'P_Prob' (Table 20).

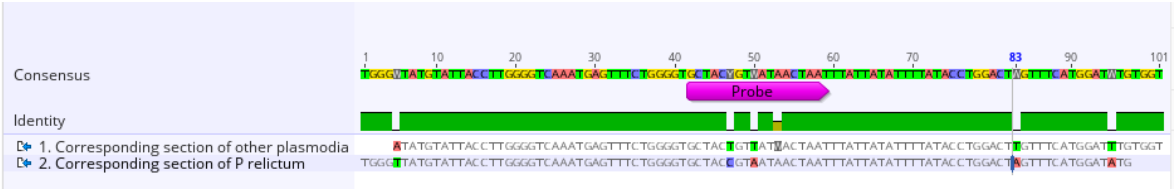

Figure 11 Location of the novel probe 'P-Prob' on the Cytochrome B gene consensus sequences for *P. relictum* and non-*relictum* Plasmodium species, indicating that the target binding site spans the region of most variation between the two sequences.

Table 20 Features of novel probe 'P-Prob' designed to target *P. relictum*, indicating the sequence, position on the target DNA, probe length, melting temperature (Tm), Hairpin melting temperature (HTm), Self Dimer melting temperature (SDTm) and the guanine and cytosine content of the probe sequence (%GC). All temperatures are given in ºC.

Name	Sequence	Position	Length	Tm	HTm	SDTm	%GC
P_Prob	GGGTGCTACCG TAATAACTAAT	38-59	22 bp	55.4	39.8	None	40.9

3.3.2 Testing the novel P. relictum *specific probe in a nested PCR*

The probe was initially synthesised as a primer ('P_Prob') and used in a nested PCR with either >P_3_R or >P_4_R. Detectable product was produced by both reactions (Table 21), however, the band quality on gel electrophoresis was better when the probe was paired with >P_3_R. In contrast, the sequence quality was significantly better using >P_4_R. BLAST analysis of the product generated using the probe paired with >P_4_R revealed 92% identity with *P.*

relictum, whilst the product generated using >P_3_R was of poor quality and did not generate significant results using BLAST.

Table 21 Outline of the products derived from PCR targeting the Cytochrome B gene using the novel probe sequence as a forward primer and >P3_R or >P_4_R as a reverse primer, applied to *Plasmodium relictum* positive control material (FBPR). The protocol, product sequence, length and BLAST identity are detailed. The expected amplicon lengths are 129 and 38 base pairs respectively.

Protocol	Sequence	Sequence length	BLAST result
PCR incorporating >P_4_R	ATATGGACTTTCTACATTTACAAGGTATCACAAATCCTTTAGGGTATGATACAGCTTTAAAAATACTACTAATACATTATTAAATCCTTTAATATCAAGACTTAATAGATTTGGATAGAAGGGAC	125 bp	*P. relictum*
PCR incorporating >P_3_R	GATCCTTTAAGGAATGATA	19 bp	None

3.3.3 Testing the novel P. relictum *specific probe in a heminested PCR*

3.3.3.1 Heminested PCR on positive control material

Since the resultant DNA fragment was short (approximately 125 base pairs) and therefore challenging to identify on gel electrophoresis, a heminested PCR was performed with HEAMNF as the forward primer in the initial round and P_Prob was used as a forward primer in the second round, with HEAMNR2 as the reverse primer in both rounds of the reaction. Positive control *P. relictum* (FBPR) and *P. falciparum* (FBPF) samples were tested. This generated significant product for FBPR 1/10 dilution, Sanger sequencing and subsequent BLAST analysis of the amplicon sequence revealed 99% query cover to *Plasmodium relictum*, indicating successful targeting of *P. relictum*. However, no amplicon was generated with the FBPF (*P. falciparum* control) samples.

3.3.3.2 Heminested PCR on DNA extracted from frozen tissue

Application of the novel Plasmodium-specific heminested PCR on DNA extracted from 9 frozen tissue samples followed by Sanger sequencing, revealed successful amplification of DNA in 8/9 cases (Table 22).

Table 22 Sanger sequencing results and BLAST identity of PCR products obtained from DNA extracted from frozen tissue samples using a novel heminested PCR including P_Prob targeting the Cytochrome B gene. HEAMNF was the forward primer in the initial round and P_Prob was used as a forward primer in the second round, HEAMNR2 was the reverse primer in both rounds of the reaction. Again, birds shaded in blue (P17 and P19) were not expected to be infected with *Plasmodium.* The expected amplicon size is approximately 300 base pairs.

Sample ID	Sequence	BLAST identity
P10	ACATATCTTGTAAGTGACCCAACCTTAAAAAGATTTTTTGTATTACATTTTACATTTCCATTTATAGCTTTATGTATTGTATTTATACATATCTTCTTCTTACATTTACAAGGTAGCACAAATCCTTTAGGGTATGATACAGCTTTAAAAATACCCTTCTATCCAAATCTATTAAGTCTTGATATTAAAGGATTTAATAATGTATTAGTTTTATTTTTATCTCAAAGTTTATTTGGAATTTTACCATTATCACATCCAGATAATGCAATCACAGTAGATAGATATGCTACACCTCTA	*P. matutinum*
P17	ATACAATAAAAAGATTCTTTGTACTACATTTTACATTTCCTTTTATAGCTTTATGTATTGTATTTATACATATTCTTTCTACATTTACAAGGTAGCACAAATCCTTTAGGGTATGATACAGCTTTAAAAATACCCTTCTATCCAAATCTTTTAAGTCTTGATATTAAAGGATTTAATAATGTATTAGTACTATTTTTAGCACAAAGTTTATTTGGAATACTACCATTATCACATCCAGATAATGCTATTACAGTAGATAGATATGCTACACCTCTAAG	*P. relictum*
P18	CAAAGCTTGTAAGTGACACCAACCTTAAAAAGATTCTTTGTACTACATTTTACATTTCCTTTTATAGCTTTATGTATTGTATTTATACATATATTCTTTCTACATTTACAAGGTAGCACAAATCCTTTAGGGTATGATACAGCTTTAAAAATACCCTTCTATCCAAATCTTTTAAGTCTTGATATTAAAGGATTTAATAATGTATTAGTACTATTTTTAGCACAAAGTTTATTTGGAATACTACCATTATCACATCCAGATAATGCTATTACAGTAGATAGATATCTTAC	*P. relictum*
P19	No meaningful sequence generated	
P20	GAAAAATCTTGTAAGTGACCCAACCTTAAAAAGATTCTTTGTACTACATTTTACATTTCCTTTTATAGCTTTATGTATTGTATTTATACATATATTCTTTCTACATTTACAAGGTAGCACAAATCCTTTAGGGTATGATACAGCTTTAAAAATACCCTTCTATCCAAATCTTTTAAGTCTTGATATTAAAGGATTTAATAATGTATTAGTACTATTTTTAGCACAAAGTTTATTTGGAATACTACCATTATCACATCCAGATAATGCTATTACAGTAGATAGATATGTTA	*P. relictum*
P21	GCTTGTAAGTGAACCAACCTTAAAAAGATTCTTTGTACTACATTTTACATTTCCTTTTATAGCTTTATGTATTGTATTTATACATATATTCTTTTTACATTTACAAGGTAGCACAAATCCTTTAGGGTATGATACAGCTTTAAAAATACCCTTCTATCCAAATCTTTTAAGTCTTGATATTAAAGGATTTAATAATGTATTAGTACTATTTTTAGCACAAAGTTTATTTGGAATACTACCATTATCACATCTAGATAATGCTATTACAGTAGATAGATATCTCA	*P. relictum*

P22	ACTTATAAAGATGCTTTGTACTACATTTTACATTTCCTTTTATAGCTTTATGTATTGTATTTAT ACATATATTCTTTCTACATTTACAAGGTAGCACAAATCCTTTAGGGTATGATACAGCTTTAAAA ATACCCTTCTATCCAAATCTTTTAAGTCTTGATATTAAAGGATTTAATAATGTATTAGTACTAT TTTTAGCACAAAGTTTATTTGGAATACTACCATTATCACATCCAGATAATGCTATTACAGTAGA TAGATATGCTACACCTCTA	*P. relictum*
P23	CGGCAGCACCTCTCTATAATTATAGAGATGCTTTGTACCTATTTTACATTTCCTTTTATAGCTT TATGTATTGTATTTATACATATATTCTTTCTACATTTACAAGGTAGCACAAATCCTTTAGGGTA TGATACAGCTTTAAAAATACCCTTCTATCCAAATCTTTTAAGTCTTGATATTAAAGGATTTAAT AATGTATTAGTACTATTTTTAGCACAAAGTTTATTTGGAATACTACCATTATCACATCCAGATA ATGCTATTACAGTAGATAGATATGCTACACCTCTAA	*P. relictum*
P24	TTGACAGCACCTCTCTATACTTAAAAAGATTCTTTGTACTACATTTTACATTTCCTTTTATAGC TTTATGTATTGTATTTATACATATATTCTTTCTACATTTACAAGGTAGCACAAATCCTTTAGGG TATGATACAGCTTTAAAAATACCCTTCTATCCAAATCTTTTAAGTCTTGATATTAAAGGATTTA ATAATGTATTAGTACTATTTTTAGCACAAAGTTTATTTGGAATACTACCATTATCACATCCAGA TAATGCTATTACAGTAGATAGATATGCTACACCTCTAAA	*P. relictum*

The results of the heminested PCR suggest that P_Prob was successful in targeting *Plasmodium spp*. however, the successful amplification of *Plasmodium matutinum* in sample P10 suggest that the probe may not be sufficiently specific to *Plasmodium relictum*. It also amplified *P. relictum* from P17, which was a bird not expected to be infected with *Plasmodium*.

The application of P_Prob on positive control material from *P. matutinum* (C7WBL), *P. vaughani* (C11-33) and *P. relictum* (MUNCH) revealed the following results (Table 23).

Table 23 Sanger sequencing results and BLAST identity of products of a novel heminested PCR including P_Prob targeting the cyt B gene applied to positive control material from *P. matutinum* (C7WBL), *P. vaughani* (C11-33) and *P. relictum* (MUNCH). In this nested PCR protocol, HEAMNF was the forward primer in the initial round and P_Prob was used as a forward primer in the second round, HEAMNR2 was the reverse primer in both rounds of the reaction. The expected amplicon size is approximately 300 base pairs.

Sample Name	Sequence	BLAST identity
C7WBL	ATAATCAAACCTGGGAACTAAGTTTCATGGGATATGTGGGGTGGGATATCTTGTAAGTGACCCAA CCTTAAAAAGATTCTTTGTACTACATTTTACATTCTCCTTTTATAGCTTTATGTATTGTATTTATAC ATATATTCTTTCTACATTTACAAGGTAGCACAAATCCTTTAGGGTATGATACAGCTTTAAAAATACC CTTCTATCCAAATCTTTTAAGTCTTGATATTAAAGGATTTAATAATGTATTAGTACTATTTTTAGCA CAAAGTTTATTTGGAATACTACCATTATCACATCCAGATAATGCTATTACAGTAGATAGATATGTTA CAACCCTCTACTAAAA	*P. relictum*

C11-33	AATTAAACTGGGACTTGATATCATGGTGATTTGTCGGTCGAATATCTTGTTAGTGATCCAACATTA AAAAGATTTTTTGTTTTACACTTTATATTTCCATTTATAGCTTTATGTATTGTATTCATACATATATT CTTTCTACATCTACAAGGTAGCACAAATCCTTTAGGGTATGATACAGCTTTAAAAATACCCTTCTAT CCAAATCTTTTAAGTCTTGATATTAAAGGATTTAATAATATTCTAGTTTTATTCTTAGCACAAAGTC TATTTGGAATATTACCATTATCACATCCAGATAATGCAATCACAGTAGATAGATATGCTACACCTCT AC	*P. vaughani*
MUNCH	CCTTCAAATATGGGAGTAGATAGATGATGCTACAGCACGACATATCTTGTAAGTGAACCAACCTTA AAAAGATTCTTTGTACTACATTTTACATTTCCTTTTATAGCTTTATGTATTGTATTTATACATATATT CTTTCTACATTTACAAGGTAGCACAAATCCTTTAGGGTATGATACAGCTTTAAAAATACCCTTCTAT CCAAATCTTTTAAGTCTTGATATTAAAGGATTTAATAATGTATTAGTACTATTTTTAGCACAAAGTT TATTTGGAATACTACCATTATCACATCCAGATAATGCTATTACAGTAGATAGATATGCTACACCTCT AA	*P. relictum*

Sample C7WBL was retested using a previously published PCR protocol targeting the 18S SSU rRNA gene (Feldman, Freed and Cann, 1995a), generating the following product;

CCGAATAACGAACGAGACTCTGGCATGCTAACTAGTTACGCGACCCCCGAGCGGTCGGCGTCCAACTTCTT AGAGGGACAAGTGGCGTTCAGCCACCCGAGATTGAGCAATAACAGGTCTGTGATGCCCTTAGATGTCCGGG GCCGCACGCGCGCTACACTGACTGGCTCAGCTTGTGCCTACCCTCCGCCGGCAGGCGCGGGTAACCCGTTG AACCCCATTCGTGATGGGGATCGGGGATTGCAATTCTTCCCCGTGAACGAGGAATTCCCAGTAAGCGCGGG TCATAAGCTCGCGTTGATTAAGTCCCTGCCCTTTGTACACACCGCCCGTCGCTCCTACCGATTGAAAGATA.

This sequence demonstrated 99.14% homology to *Hirundo spp.*, an avian genus comprising swallows and martins. This is considered the likely source of the control material. This again demonstrates a lack of specificity of this protocol.

3.4 Application of novel PCR in recent UK outbreak of avian malaria

3.4.1 Results of novel PCR protocol

The novel PCR protocol (which incorporates >P_1_F and >P_3_R) amplified DNA from all three recent outbreak cases, and all three amplicons were consistent with *P. relictum* following BLAST analysis (Table 24).

Table 24 Sanger Sequencing results and BLAST identity of PCR products derived using novel nested PCR protocol targeting the Cytochrome B gene and incorporating primers >P_1_F and >P_3_R, applied to samples obtained from penguins during a recent UK outbreak of avian malaria. HaemNFI and HeamR2L were used as outer primers, and >P_1_F

and >P_3_R as inner primers. An amplicon length of approximately 97 base pairs is expected.

Sample ID	Sequence	Blast identity
H5	GGGGTCAATGAGTTTCTGGGGTGCTACCGTAATAACTAATTTATTATATTTTATAC CTGGACTAGTTTCATGGATATGTGGTGGATATCTTGTAAGTGACCCAAAAGCGC	*P. relictum*
H6	GGGTCAATGAGTTTCTGGGGTGCTACCGTAATAACTAATTTATTATATTTTATACC TGGACTAGTTTCATGGATATGTGGTGGATATCTTGTAAGTGACCCAATGG	*P. relictum*
H7	GCGGTCAATGAGTTTCTGGGGTGCTACCGTAATAACTAATTTATTATATTTTATAC CTGGACTAGTTTCATGGATATGTGGTGGATATCTTGTAAGTGACCCAAAATTCTT	*P. relictum*

4. Discussion

4.1 Assessing success of DNA extraction

Established (and previously published) avian 'barcoding' primers (BirdF1 and COIbirdR2) used to test DNA extracted from chicken (*Gallus gallus)* liver and spleen yielded amplicons of expected length. However, testing of DNA obtained from lamb liver also generated amplicons of anticipated length. Repeating the PCR on DNA derived from equine blood did not yield detectable product. Amplification of DNA from the lamb sample is anticipated to be the result of contamination of the lamb sample with avian DNA as the PCR tests for each were performed in tandem. This hypothesis is supported by the absence of product from horse DNA. If required, DNA extraction from lamb liver could be repeated and this PCR test repeated in order to further investigate this theory.

The same PCR protocol did not yield detectable product from DNA extracted from the penguin samples used in this study. Bioinformatic assessment of primer sequences BirdR2 and COIBirdR2 indicates that they do not map to Cytochrome C Oxidase 1 (COI) sequences from penguin species examined in this study. Furthermore, neither BirdF1 and COIbirdR2 primer sequences exhibited significant similarity to *Sphenicidae spp.* on BLAST analysis. Therefore, the most likely explanation for the failure of the PCR to identify avian DNA in these samples this is that these primers do not bind the COI sequences of penguin species *Spheniscus demersus, Spheniscus humboldti* or *Eudyptes chrysolophus* (Figure 12). The COI gene is known to demonstrate significant diversity, indeed, this is a feature which makes it useful for barcoding (although this utility is not unquestioned) (Baker, Tavares and Elbourne, 2009), however this genetic variation in the target sequence may make these primers unreliable for use in penguins. Alternative barcoding primers have been successfully utilised in penguins, for example by Tavares and Baker (Tavares and Baker, 2008). The PCR protocol outline therein may be useful in assessing DNA extraction from penguin tissues in future, although confirmatory preliminary trials would be required first (Hebert et al., 2004; Tavares and Baker, 2008; Baker, Tavares and Elbourne, 2009; Lijtmaer et al., 2012).

Alternative possible explanations for the failure of the established avian primers to identify penguin DNA include factors relating to the template DNA, such as excessive degradation, poor purity, or excessively high concentration (the latter as excessive concentration of DNA can lead to failure of the PCR due to false priming and/or inhibition of diffusion of TAQ polymerase (Lorenz, 2012; *PCR Troubleshooting Guide - UK*)). To examine this possibility, we quantified DNA using fluoroscopy, which indicates concentration of DNA in the extracted sample (ng/ul), however, the number of DNA molecules containing the target sequence, is a more meaningful metric and was not directly assessed for these samples (Lorenz, 2012; *PCR Troubleshooting Guide - UK*). We also quantified DNA using spectrophotometry for comparison, however this is considered a less accurate method and therefore the results of fluoroscopy were considered more reliable (Paul et al., 2021).

Significant variation in DNA quantity was observed between samples. There are a number of possible explanations for this, including differences in DNA quantity and quality between samples, as well as variation in ease of extraction (in the authors' experience connective tissue is less readily digested during extraction, which means that DNA is typically less readily extracted from tissues with higher concentrations of connective tissue). In addition, DNA extracted from FFPE tissues is typically of lower molecular weight due to fragmentation, and may perform less reliably in molecular tests than equivalent frozen tissues (*QIAamp DNA FFPE Tissue Kit*). In this study, the concentration of DNA extracted from frozen tissues was consistently higher than that from FFPE tissues but given that the initial sample quantities were not equivalent, it is not possible to assess whether this finding reflects quality or quantity of DNA in the initial samples.

Finally, the GC% content of extracted DNA may alter the optimal PCR conditions, specifically, very high GC% content samples may require longer denaturation in order to separate DNA strands (*PCR Troubleshooting Guide - UK*). The GC% content of extracted DNA was not evaluated in this study, and therefore the impact of this remains equivocal.

In conclusion, several factors may explain the absence of product when screening extracted penguin DNA with established avian primers, however, given

successful quantification of DNA using other methods and subsequent successful PCR screening of this extracted material for parasite DNA using alternative primer sets, this finding is considered of minimal significance to the remainder of the results. Quantification indicated successful extraction from frozen and FFPE tissues in all cases, variation in concentration of extracted DNA was expected, and a number of possible explanations for this have been identified.

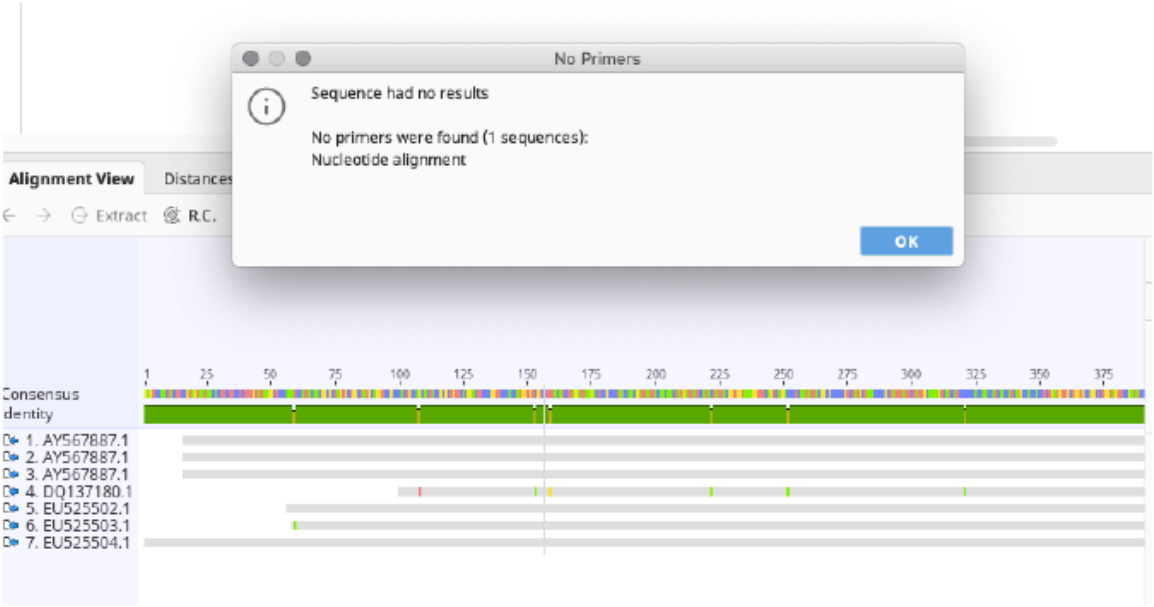

Figure 12 Bird_F1 primer fails to map to the consensus sequence of the Cytochrome C Oxidase 1 gene of *Spheniscus demersus*, Sp*heniscus humboldti* or *Eudyptes chrysolophus*.

4.2 Development of a novel PCR for *Plasmodium spp.*

4.2.1 Trials of previously published primers

The positive control material was trialled with two PCR protocols targeting the 18S rRNA gene (Feldman, Freed and Cann, 1995b; Fallon et al., 2003), and one targeting the cyt B gene (Snounou and Singh, 2002). The primers published by Fallon et al (Fallon et al., 2003) reliably identified *P. relictum* and *P. falciparum* DNA at all tested concentrations, proving that the positive control material was of sufficient quality for PCR analysis. Those published by Feldman (Feldman, Freed and Cann, 1995a) reliably identified *P. relictum* DNA at higher conventions, but amplified sequences from *A. gambiae* from *P. falciparum* positive control material, suggesting incomplete primer specificity and amplification of host DNA. Finally, the PCR protocol published by Snounou and Singh (Snounou and Singh, 2002) only generated perceptible product from the

most concentrated *P. falciparum* sample, which when sequenced revealed 100% identity with *P. ovale* (Acession No. MW426207.1), and 99.5% identity to *P. falciparum*. Possible reasons for this include mischaracterisation of the control tissue, mischaracterisation of the *P. ovale* sequence on the database, or sequence homology between this isolate of *P. falciparum* and the sample of *P. ovale* on the database (Bensch, Hellgren and Pérez-Tris, 2009). Given that the Feldman and Snounou and Singh published protocols successfully amplified expected target DNA, and the that the protocol published by Fallon et al reliably identified all of the positive control samples, the samples were deemed sufficient for use as positive controls.

Despite the promising findings reported in some of the published protocols, caution is warranted when extrapolating previously published findings, owing in part to genetic variation within *Plasmodium spp.* from different geographic regions. Correspondingly, Richard et al reported that the protocol published by Feldman et al failed to identify *Plasmodium spp.* infection in African birds, and speculated that this was due to failure of published primers to bind the African parasitic genome due to genetic variation (Richard et al., 2002). Therefore, any negative PCR findings generated from primers designed using birds from different geographic regions to the test subject(s) ought to be interpreted with care.

4.2.2 Design of novel primers

4.2.2.1 Nested PCR

Nested PCR protocols offer increased sensitivity and specificity compared with traditional PCR testing. This is particularly desirable in the context of avian malaria owing to the relatively low abundance of parasite DNA compared to host DNA and the (expected) variable quality of template DNA (as a result of freezing/formalin fixing and paraffin embedding which can damage DNA) (Snounou and Singh, 2002; Freed, 2006; Wanger et al., 2017; Shen, 2019). Therefore, despite the increased labour, time and reagent demands of nested PCRs, this approach was felt to be most appropriate for this study.

4.2.2.2 Design of novel primers targeting the 18S small sub-unit ribosomal RNA

The 18S small sub-unit ribosomal RNA (18S SSU rRNA) gene was historically a commonly used target for *Plasmodium spp*. PCR, and reports of 'genus-conserved' sequences which flank more variable regions suggest that this region may also be a useful target for a PCR with the ultimate aim of speciation (Field et al., 1988; Li et al., 1995). The 18S SSU rRNA gene (or 16s SSU rRNA in the case of prokaryotes) has traditionally been a commonly utilised target for speciation of a wide range of organisms, owing to its high copy number, ubiquity, and mutational rate which is low enough to remain conserved within species, but in many cases sufficient to allow distinction between species (Fox et al., 1977; Woese and Fox, 1977; Field et al., 1988). In the case of *Plasmodium spp*. initial alignment of 18S genome sequences indicated conserved and variable regions as reported, the two novel sets of nested primers were designed to target conserved regions and flank variable regions.

The first set of primers, 'Set 1', failed to yield detectable product when trialled on positive control *Plasmodium relictum* and *P. falciparum* DNA. Possible reasons for this include variation of template DNA from the consensus sequence (and therefore also the designed primer sequence), marked variation between expected and in vitro melting and binding characteristics, or excessively stringent PCR cycling conditions (Lorenz, 2012; *PCR Troubleshooting Guide - UK*). Degradation of DNA is also a possible contributory factor, however subsequent successful PCR testing of the samples suggests that poor DNA quality alone is not a sufficient explanation for the failure of this PCR.

The second set of primers 'Set 2' generated an amplicon similar to the expected length on gel electrophoresis. However, sequencing revealed that the product was shorter than anticipated (50 and 231 bp respectively, compared with the anticipated length of 296 bp). BLAST analysis of the longer product indicated homology to *Anopheles gambiae*, a known host of *P. falciparum,* and the presumptive source of control material in this case. The most likely explanation for these findings is that the newly designed PCR is not specific for *Plasmodium spp*. DNA and instead amplified sections of 18S SSU rRNA gene of the mosquito host. Mapping the novel primers to *Anopheles gambiae* whole genome revealed

2,202 possible binding sites for >1457_Inner_F (allowing for one mismatch in the binding region), but none for >1930_Inner_R under the same conditions, whilst 18 possible binding sites were identified in the latter when allowing for up to three mismatches. This finding does not exclude the possibility that Primer Set 2 could bind *P. falciparum,* as it my bind with lower avidity to the parasite than the host, therefore the host DNA would be preferentially amplified. Regardless, the primers did not demonstrate sufficient avidity to *P. falciparum* DNA.

Furthermore, this PCR did not amplify any product from the *P. relictum* sample. The most likely explanation is that the primers are not sufficiently sensitive to *P. relictum.* The absence of host DNA (in contrast to the findings from the *P. falciparum* sample*)* may suggest that this positive control material was not derived from a mosquito, or that genomic variation between *A. gambiae* and avian malaria host mosquitos is sufficient to prevent binding to the latter. Finally, as discussed for Set 1, DNA degradation is also a possible contributary factor (Lorenz, 2012; *PCR Troubleshooting Guide - UK*).

In conclusion, the novel primers designed to target the 18S SSU rRNA gene do not reliably amplify target DNA and are therefore unsuitable for diagnosis of avian malaria infection. Poor sensitivity of PCR protocols targeting the 18S SSU rRNA gene of *Plasmodium spp.* is a reported concern, and rather than redesign primers targeting the same region, it was decided, in line with the majority of recently published literature, to develop primers to target the cyt B gene (Ricklefs, Fallon and Bermingham, 2004a; Freed, 2006; Clark, Clegg and Lima, 2014; Dhatterwal, Mehrotra and Mehrotra, 2017; Pacheco et al., 2018a; Videvall, 2019).

4.2.2.3 Design of novel primers targeting Cytochrome B gene

The Cytochrome B gene (cyt B) is currently a widely used target in *Plasmodium spp.* diagnosis (Ricklefs, Fallon and Bermingham, 2004b; Freed, 2006; Bensch, Hellgren and Pérez-Tris, 2009; Pacheco et al., 2018b). The reasons for this are its ubiquity, high copy number, and maternal inheritance; the latter has been reported to increase its propensity to become homologous is reproductively isolated communities and at least partiality explains its utility in speciation studies (Ricklefs, Fallon and Bermingham, 2004b; Freed, 2006; Bensch, Hellgren

and Pérez-Tris, 2009; Pacheco et al., 2018b). The majority of more recently published PCR studies on the avian haemosporidia target cyt B, and a comparative analysis of PCRs which target the 18S SSU rRNA gene and cyt B indicated that that the latter are more reliable (Richard et al., 2002). Cytochrome B was therefore selected as a suitable candidate gene for PCR development in this study.

Alignment of *Plasmodium spp.* Cyt B sequences indicated that again suitable conserved and variable regions for primer targeting were present. Most published sequences were produced using the PCR protocol outlined by Bensch et al (or variants thereof) (Bensch, Hellgren and Pérez-Tris, 2009). Given the ubiquity of these primers in current speciation of avian-infective *Plasmodium spp.,* they were selected as outer primers for development of a novel nested PCR, whilst the inner primers were designed to flank variable regions of the consensus sequence (with the intention to target these with species-specific molecular methods subsequently). Assessment of three primer pairs generated from four novel primers (as putative inner primers for a nested PCR) revealed 'Pair A' (P_1_F and P_3_R) to be the most sensitive, however, none of the three primer pairs generated amplicons from *P. falciparum* positive control material. This may be because the primers were designed based on consensus sequences of avian infective malaria species and given that human infective species (such as *P. falciparum*) are reproductively isolated from avian infective species, sufficient genetic drift may have occurred such that the primers are unable to bind human-infective *Plasmodium spp.* genomes. Performing this PCR on a variety of human infective malaria samples would be required to further test this hypothesis.

4.2.2.4 Results of novel PCR applied to material extracted from FFPE and frozen tissues

Novel primers P_1_F and P_3_R were incorporated into a nested PCR and used to screen DNA extracted from penguins P10, P15 and P17-24. Sanger sequencing and BLAST analysis of the amplicon generated from frozen tissues P15 and P17-P24 were consistent with *P. relictum* on BLAST analysis, whilst P10 was consistent with *P. relictum* from FFPE and *P. matutinum* from frozen tissue. All

FFPE samples (P1-10, P12-14 and H5-7) yielded amplicons consistent with *P. relictum* infection when subject to BLAST analysis. Therefore, 25/25 penguins examined were positive for *P. relictum*, and 1/25 was positive for both *P. relictum* and *P. matutinum*. This is consistent with evidence which suggests *P. relictum* is the most common cause of avian malaria in Europe, likely reflecting that this particular *Plasmodium* species is most prevalent in native host birds, rather than a unique susceptibly of European penguins to *P. relictum* (Vanstreels et al., 2015). However, the novel protocol returned positive results from 2 control cases which had been diagnosed with other causes of death and were not anticipated to be infected with *Plasmodium*. The possible explanations for this are either that the cases were co-infected with *Plasmodium,* which, as discussed in the introduction, may be more common in birds with co-morbidities. However, particularly in the case of P19 which was a chick, the possibility of false positive results cannot be excluded. These may arise because of insufficient specificity of the PCR, or because of contamination of a negative sample with *Plasmodium* DNA during testing. Trialling the PCR on known uninfected (e.g. In ovo), penguin material would be essential to further assess the utility of this PCR.

Cases H5-7 (FFPE tissues obtained from a recent UK outbreak) had been speculatively diagnosed as avian malaria, but no definitive histological or cytological diagnosis had been made. All three cases produced sequences consentient with *P. relictum,* this may therefore be implicated as the cause of the outbreak. Again, this is plausible given reports of the ubiquity and virulence of *P. relictum* infections in Europe (Stidworthy and Denk, 2018; Hernandez-Colina et al., 2021). However, the absence of negative results from any of the tissues tested means that the specificity of the novel PCR test is in question, and therefore these findings must be interpreted with caution.

Formalin fixed paraffin embedded tissues yielded amplicons consistent with *P. relictum*. Again, the absence of a negative control means that these findings must be interpreted with care, however this may suggest suitability of the novel PCR protocol for use in FFPE tissues. FFPE tissues can be more difficult to assess than fresh or frozen material, owing to greater DNA fragmentation/damage. FFPE tissue is commonly stored by histopathology labs, is stable, and is relatively

easily shipped therefore utility of the test on FFPE material is a desirable trait for many diagnostic PCRs. Utility on FFPE tissues has been achieved for avian malaria tests in the past (Richard et al., 2002; Hellgren, Waldenström and Bensch, 2004; Alley et al., 2008; Dinhopl et al., 2011), and may be possible with this novel PCR. Again, repetition of the PCR using known negative control penguin tissue would be essential to further investigate this possibility.

4.3 Design of a novel *Plasmodium relictum* specific probe

There is currently no molecular method to identify the species of *Plasmodium* involved in avian malaria without sequencing of the PCR product. Given that *P. relictum* is the most common cause of avian malaria infection in the UK, and that it was also by far the most ubiquitous species in the samples analysed in this study (considering the caveats mentioned in the previous section), *P. relictum* was chosen as a suitable target for probe design. Comparison between the consensus genetic sequence of the expected *P. relictum* amplicon generated by the novel PCR with a consensus sequence of non-*relictum Plasmodium spp.* amplicon revealed minimal genetic variation (six base pairs), of three variant bases were included in the region targeted by the probe. Given that the probe sequence was untested, and that synthesis of a probe is significantly more costly than that of a primer, the probe was first assessed for suitability by synthesising the putative probe sequence as a primer. This primer when used in a nested and heminested PCR generated amplicons from *P. relictum* control material, and not from *P. falciparum* control material. This finding may be early indication of specificity of the probe within the genus *Plasmodium*.

The PCR protocol incorporating the novel *P. relictum*-specific probe sequence generated detectable product when used to screen DNA extracted from frozen tissues from birds known or highly suspected to be infected with *Plasmodium* (P10, P18, P20-24) and two birds not expected to be infected (P17 and P19). BLAST analysis of sequenced product demonstrated *Plasmodium relictum* in seven of the nine cases (consistent with findings of the novel PCR previously described), one sample (P19) did not generate an amplicon of sufficient quality to sequence, and one (P10) was consistent with *P. matutinum* (as previously discussed). The absence of product from case P19 may reflect an absence of

Plasmodium in this sample, as it is one of two bird not suspected to be infected, however this is in contrast to the findings described from the novel PCR previously, therefore repeat testing, and testing on known negative control material would be required to further investigate this theory.

Amplification of *P. matutinum* from case P10 is in line with the findings using the novel nested PCR discussed previously, however, this suggests that the probe is not specific to *P. relictum,* and is capable of binding other avian infective *Plasmodium spp.*. To further investigate this hypothesis, positive control material from *P. matutinum* and *P. vaughani* were screened using a nested PCR protocol incorporating the putative probe. In both cases this yielded detectable product, and when the amplicon from the *P. vaughani* sample was sequenced, it returned a result consistent with *P. vaughani*. This further indicates that the probe is not sufficiently specific to reliably identify *P. relictum*.

The product of the *P. matutinum* positive control material returned a sequence constituent with *P. relictum* on BLAST analysis. The most likely explanations for this are that either the positive control material or PCR reaction were contaminated with *P. relictum* DNA (either due to mixed infection in the avian host, or during handing and processing), or that the positive control sample had been misidentified. This hypothesis was further investigated using a published PCR (Feldman, Freed and Cann, 1995a) targeting the 18S SSU rRNA gene, the result of which was amplification of a sequence which was identified on BLAST analysis as having 99.14% identity to the 18S SSU rRNA gene of *Hirundo smithii*, the presumptive avian host from which the sample was collected. This finding provides further evidence of a lack of specificity of the primers published by Feldman et al, but it does not provide definitive evidence of the presence or the species of *Plasmodium* in positive control material C7WBL. Testing the sample with alternative primers may help to whether *Plasmodium spp.* are present in this sample, and which species may be involved, but is not likely to alter the conclusions of this study.

The probe did not generate significant product when used to screen *P. falciparum* positive control material, but did generate product from a number of avian-infective *Plasmodium spp.*. This likely reflects genetic variation between

P. falciparum and *Plasmodium spp.* which infect birds. As human-infective malaria parasites are generally highly host specific (not thought to infect birds), and avian infections are not considered zoonotic, the two groups of malaria are likely to have been reproductively isolated from each other for a considerable period. It is therefore logical to assume that they may have diverged genetically. Avian infective species on the other hand, are variably, and often poorly, specific to one (or a small group) of avian species, and therefore are less likely to have experience reproductive isolation from each other, and therefore less likely to have diverged. This is further complicated by the fact that *Plasmodium* species which infect birds are also much less well characterised than their human counterparts.

In conclusion, the novel probe is insufficiently specific for *P. relictum,* and is therefore unsuitable for speciation of *Plasmodium* infection in birds. The most likely reason for the failure of the probe to specifically identify *P. relictum* is the minimal genetic variation (6 bp) between the non-relictum *Plasmodium spp.* amplicon consensus and the *P. relictum* amplicon consensus. A low number of divergent base pairs between *Plasmodium spp.* means non-specific binding would only have to occur at a very low rate to yield false positive results from non-relictum *Plasmodium spp*.

It is also important to consider that when designing the novel probe, that the non-*relictum* sequence represents a consensus of 35 different sequences. Individual contributing species (such as *P. matutinum* in this case) may demonstrate greater homology to the *P. relictum* sequence than the consensus, again increasing the risk of non-specific binding. Furthermore, the current list of avian-infective *Plasmodium spp.* is considered incomplete, which means that the non-*relictum* consensus sequence does not represent an exhaustive summary of infective species, making definitive identification of specific probes difficult. Until there is a more definitive list of avian-infective *Plasmodium spp.* development of species-specific diagnostics is likely to remain an ongoing challenge.

4.4 Study limitations and further work

4.4.1 Absence of negative control material and risk of contamination

The major limitation encountered in this study was the absence of known negative control material. Samples from two birds (P17 and P19) which were suspected to have died of other causes and therefore unlikely to be infected with *Plasmodium* were examined, however in both cases the novel PCR protocol amplified a product consistent with *P. relictum,* whilst the probe-based PCR also amplified *P. relictum* from P17 but not P19. As discussed previously, this may be the result of contamination, however neither sample is known to be uninfected. Assessment of the suitability of the PCR and probe would be greatly enhanced if they were trialled on known negative penguin tissue; one possible option for this would be in ovo material.

The nature of PCR testing requires assiduous effort to prevent cross-contamination between samples. Whilst several steps were taken to avoid contamination, including using a separate freezer for samples and reagents, making the master mix first, then adding to each well prior to handling template material, use of separate pipette tips with filters between samples, regular cleaning of the bench and laboratory equipment, frequent changing of gloves, minimisation or aerosol by careful pipetting, handling of samples and use of centrifuge. Despite these efforts, a number of improvements could be made, for example the inclusion of three replicates for each sample, the use of non-avian negative control material throughout (from initial production of the FFPE scrolls to completion), and incorporation of negative control material (discussed previously). Finally, it would be preferable if different areas of the laboratory could be used for initial pre-PCR handling and post PCR handling, to reduce the risk of contamination of the pre-PCR samples with highly concentrated amplified product.

4.4.2 Incomplete characterisation of and genetic diversity within the avian haemosporidia

There is considerable genetic similarity between the members of the haemosporidia, and this lack of genetic diversity makes distinguishing *Plasmodium spp.* infection at a species, or indeed genus level, difficult (Hellgren, Waldenström and Bensch, 2004; Dinhopl et al., 2011; Clark, Clegg and Lima, 2014; Pacheco et al., 2018b; Ellis et al., 2020; Harl et al., 2020). This is considered the most substantial factor in the lack of specificity of the *P. relictum* probe in this study.

In addition, and as previously discussed, avian infective *Plasmodium spp.* have not been exhaustively described, and identification of previously undescribed extant species is considered likely (Clark, Clegg and Lima, 2014; Valkiūnas and Iezhova, 2018; Ellis et al., 2020; Harl et al., 2020). Furthermore, as described by Richard et al, *Plasmodium* of the same species can demonstrate geographic genetic variation, which means that primers developed using *Plasmodium* genomes from one geographical region may not be applicable elsewhere (Richard et al., 2002). Both the incomplete list of avian infective *Plasmodium spp.* and geographic genetic variance make development of a species-specific probe very challenging. Further work to assess novel primers or probes would ideally be utelise material from a variety of geographic regions, or be optimised for a single, defined region. The problem of characterisation of *Plasmodium* species is intrinsic to the nature and epidemiology of the parasite, and whilst work is ongoing to further our understanding, this is likely to remain a challenge for development of diagnostic PCRs for avian malaria.

4.4.3 Mixed infections

The novel PCR described in this study will not detect mixed infections. Instead, in the case of mixed infections, one of the infective *Plasmodium spp.* will be bound with greater avidity by the primers, and will therefore be amplified at a greater rate, thus resulting in a single species being identified. This is a well-recognised problem and possible solutions include combining PCR testing with microscopic methods (although this is hampered by the constrains of microscopy

as discussed previously) (Snounou et al., 1993; Ciloglu et al., 2018; Pacheco et al., 2018a). Future work could aim to develop probes specific to the 8 *Plasmodium spp*. currently known to infect penguins which could ultimately be combined to a developed multiplex PCR/probe analysis.

4.4.4 Number and origin of samples

The limited number of samples utilised in this study were all derived from penguins resident in the UK. As mentioned previously, this is a strength when developing a UK based PCR test. However, geographic variation in parasite genetics is expected and therefore testing primers (and probes) on these samples alone is not sufficient to demonstrate suitability for a wider geographical application. Future work to validate the novel PCR for wider use would need to be conducted on a larger international population of samples.

In addition, aspects of the signalment such as sex, age and/or species was not known for several cases. This limitation is inherent when using archival tissue from a diagnostic lab, as the information available for each case is limited to that provided on the initial submission form. Further work on this diagnostic PCR would ideally be based on tissue for which the host species is known, in order to assess viability of the test across host species.

Development of a penguin specific PCR, for example targeting the COI gene, would be valuable in verifying the origin of tissues, testing for PCR inhibitory substances and assessing the quality of extracted DNA, and would therefore be a highly useful undertaking.

4.4.5 Alternative target loci for primer and probe development

The two molecular target genes used in this study, 18S SSU rRNA gene and cyt B, did not prove viable for speciation of *Plasmodium spp*. infection. It is possible that alternative target sites within these genes may prove more fruitful, however, alterative target genes could also be considered in further work.

Cytochrome c oxidase subunit 1 (COX1) and Cytochrome c oxidase subunit 3 (COX3) have been proposed as possible alternative target sites for diagnostic haemosporidian PCR testing, indeed Pacheco et al reported selective amplification of *Plasmodium spp.* using COX1 targeting primers 'AE971/973' and 'AE965/966' (Pacheco et al., 2018b). Additionally, a number of heat shock proteins (HSP) have been used in the speciation of protozoa including *Leishmania* (HSP70) (Garcia et al., 2004). Furthermore, a number of heat shock proteins have also been studied in human malaria, although to the author's knowledge none have been used as diagnostic PCR targets (Su and Wellems, 1994; Das et al., 1997). GADPH genes have been used for the barcoding of trypanosomes, however these genes are relatively conserved and most valuable when used in combination with other barcoding genes (Hutchinson and Stevens, 2018). Finally, microsatellites have been used to characterise *P. falciparum,* where they demonstrated species specificity, and to assess intraspecific genetic diversity in human-infective malaria (Anderson et al., 1999; Greenhouse et al., 2006; Russell, Suwanarusk and Lek-Uthai, 2006). These microsatellite characteristics may be a useful area of research in avian malaria, although again the abundance of species to consider may make this approach more challenging than in human disease.

The possibility of using differential molecular weight of amplicons by exploiting insertions, deletions or restriction sites for species identification was also considered. However, suitable sites were not identified in the sequences targeted in this study. Nevertheless, such approaches may prove valuable in conjunction with alternative primer target sites.

4.4.5 Assessing other haemosporidia

Primers published by Bensch et al have been widely used to identify avian haemosporidia, and the results are the basis for a comprehensive database thereof (Bensch, Hellgren and Pérez-Tris, 2009). However, these primers have also been criticised for failure to identify haemosporidia to genus level (Pacheco et al., 2018b). Although the primers outlined in this study have been designed to specifically identify *Plasmodium spp.* they have not been trialled using positive control material from other members of the family, most pressingly

Leukocytozoon spp. or *Haemoproteus spp.* This would be a highly valuable undertaking, as if they are able to do so they may be candidate primers for genus-specific identification. Unfortunately, suitable positive control material was not available.

4.5 Conclusion

Avian malaria is a scourge on captive penguins in the UK, and rapid diagnosis of would enable preventative and supportive treatment to be delivered in a timely manner. Our current understating of *Plasmodium spp.* involved in causing disease in penguins is not exhaustive, and species-specific identification of causative *Plasmodium spp.* would be beneficial in understanding the epidemiology of infection and may have prognostic implications. This study successfully identified novel primers which may be used in a nested PCR to identify *Plasmodium spp.* infection. However, the unavailability of known negative control material makes assessment of the specificity of this protocol highly challenging. In addition, the amplicon generated by this novel PCR from *P. relictum* DNA was not sufficiently diverse to allow development of a species-specific probe, and therefore species-specific *P. relictum* diagnosis is not possible using this method. A range of possible targets for future development of species-specific diagnostics have been identified. Given the anticipated increased exposure of captive and wild penguins to avian malaria vector species in the context of global warming, advances in diagnostic speed and specificity are highly desirable.

Bibliography

Alley, M. et al. (2008) 'An outbreak of avian malaria in captive yellowheads/mohua (*Mohoua ochrocephala*)', *New Zealand Veterinary Journal*, 56(5), pp. 247–251. Available at: https://doi.org/10.1080/00480169.2008.36842.

Anderson, T.J.C. et al. (1999) 'Twelve microsatellite markers for characterization of *Plasmodium falciparum* from finger-prick blood samples', *Parasitology*, 119(2), pp. 113–125. Available at: https://doi.org/10.1017/S0031182099004552.

Asghar, M. et al. (2015) 'Chronic infection. Hidden costs of infection: chronic malaria accelerates telomere degradation and senescence in wild birds', *Science (New York, N.Y.)*, 347(6220), pp. 436–438. Available at: https://doi.org/10.1126/science.1261121.

Atkinson, C.T. (2008) 'Avian Malaria', in C.T. Atkinson, N.J. Thomas, and B. Hunter (eds) *Parasitic Diseases of Wild Birds*. First. Ames, Iowa: Wiley Blackwell, pp. 35–53.

Atkinson, C.T. et al. (2013) 'Experimental evidence for evolved tolerance to avian malaria in a wild population of low elevation Hawai'i 'Amakihi (*Hemignathus virens*)', *EcoHealth*, 10(4), pp. 366–375. Available at: https://doi.org/10.1007/s10393-013-0899-2.

Baker, A.J., Tavares, E.S. and Elbourne, R.F. (2009) 'Countering criticisms of single mitochondrial DNA gene barcoding in birds', *Molecular Ecology Resources*, 9(s1), pp. 257–268. Available at: https://doi.org/10.1111/j.1755-0998.2009.02650.x.

BBC News (2022) 'Dudley Zoo loses majority of penguin population to malaria', 7 January. Available at: https://www.bbc.com/news/uk-england-birmingham-59909321 (Accessed: 19 April 2022).

Bensch, S., Hellgren, O. and Pérez-Tris, J. (2009) 'MalAvi: a public database of malaria parasites and related haemosporidians in avian hosts based on mitochondrial cytochrome b lineages', *Molecular Ecology Resources*, 9(5), pp. 1353–1358. Available at: https://doi.org/10.1111/j.1755-0998.2009.02692.x.

'BirdLife International. 2020. *Eudyptes chrysocome*. The IUCN Red List of Threatened Species' (2020). International Union for Conservation of Nature and Natural Resources. Available at: https://dx.doi.org/10.2305/IUCN.UK.2020-3.RLTS.T22735250A182762377.en (Accessed: 8 June 2021).

'BirdLife International. *Aptenodytes patagonicus*. The IUCN Red List of Threatened Species' (2020). International Union for Conservation of Nature and Natural Resources. Available at: https://dx.doi.org/10.2305/IUCN.UK.2020-3.RLTS.T22697748A184637776.en (Accessed: 9 June 2021).

'BirdLife International. *Eudyptes chrysolophus*. The IUCN Red List of Threatened Species' (2020). International Union for Conservation of Nature and Natural Resources.

‘BirdLife International. *Eudyptes moseleyi*. The IUCN Red List of Threatened Species.’ (2020). International Union for Conservation of Nature and Natural Resources. Available at: https://dx.doi.org/10.2305/IUCN.UK.2020-3.RLTS.T22734408A184698049.en (Accessed: 8 June 2021).

‘BirdLife International. *Eudyptula minor*. The IUCN Red List of Threatened Species’ (2020). International Union for Conservation of Nature and Natural Resources. Available at: https://dx.doi.org/10.2305/IUCN.UK.2020-3.RLTS.T22697805A184753545.en (Accessed: 9 June 2021).

‘BirdLife International. *Megadyptes antipodes*. The IUCN Red List of Threatened Species’ (2020). International Union for Conservation of Nature and Natural Resources. Available at: . https://dx.doi.org/10.2305/IUCN.UK.2020-3.RLTS.T22697800A182703046.en (Accessed: 8 June 2021).

‘BirdLife International. *Pygoscelis adeliae*. The IUCN Red List of Threatened Species’ (2020). International Union for Conservation of Nature and Natural Resources. Available at: https://dx.doi.org/10.2305/IUCN.UK.2020-3.RLTS.T22697758A157660553.en.

‘BirdLife International. *Pygoscelis papua*. The IUCN Red List of Threatened Species’ (2020). International Union for Conservation of Nature and Natural Resources. Available at: https://dx.doi.org/10.2305/IUCN.UK.2020-3.RLTS.T22697755A157664581.en (Accessed: 9 June 2021).

‘BirdLife International. *Spheniscus demersus*. The IUCN Red List of Threatened Species.’ (2020). International Union for Conservation of Nature and Natural Resources. Available at: https://dx.doi.org/10.2305/IUCN.UK.2020-3.RLTS.T22697810A157423361.en (Accessed: 8 June 2021).

‘BirdLife International. *Spheniscus magellanicus*. The IUCN Red List of Threatened Species’ (2020). 2020 International Union for Conservation of Nature and Natural Resources. Available at: https://dx.doi.org/10.2305/IUCN.UK.2020-3.RLTS.T22697822A157428850.en.

‘BirdLife International. *Spheniscus mendiculus*. The IUCN Red List of Threatened Species.’ (2020). International Union for Conservation of Nature and Natural Resources. Available at: https://dx.doi.org/10.2305/IUCN.UK.2020-3.RLTS.T22697825A182729677.en.

Brossy, J.J. (1992) ‘Malaria in Wild and Captive Jackass Penguins Spheniscus Demersus Along the Southern African Coast’, *Ostrich*, 63(1), pp. 10–12. Available at: https://doi.org/10.1080/00306525.1992.9634174.

Ciloglu, A. et al. (2018) ‘A new one-step multiplex PCR assay for simultaneous detection and identification of avian haemosporidian parasites’, *Parasitology Research*, 118(1), pp. 191–201. Available at: https://doi.org/10.1007/s00436-018-6153-7.

Clark, N.J., Clegg, S.M. and Lima, M.R. (2014) ‘A review of global diversity in avian haemosporidians (*Plasmodium* and *Haemoproteus*: Haemosporida): new insights from molecular data’, *International Journal for Parasitology*, 44(5), pp. 329–338. Available at: https://doi.org/10.1016/j.ijpara.2014.01.004.

Cosgrove, C.L., Day, K.P. and Sheldon, B.C. (2006) 'Coamplification of *Leucocytozoon* by PCR diagnostic tests for avian malaria: a cautionary note', *The Journal of Parasitology*, 92(6), pp. 1362–1365. Available at: https://doi.org/10.1645/GE-879R.1.

Das, A. et al. (1997) 'Molecular characterization and ultrastructural localization of Plasmodium falciparum Hsp 601Note: Nucleotide sequence data reported in this paper is available in the GenBank™ database under accession No. U94594.1', *Molecular and Biochemical Parasitology*, 88(1), pp. 95–104. Available at: https://doi.org/10.1016/S0166-6851(97)00081-9.

Delhaye, J. et al. (2018) 'Avian malaria and bird humoral immune response', *Malaria Journal*, 17(1), p. 77. Available at: https://doi.org/10.1186/s12936-018-2219-3.

Dhatterwal, P., Mehrotra, S. and Mehrotra, R. (2017) 'Optimization of PCR conditions for amplifying an AT-rich amino acid transporter promoter sequence with high number of tandem repeats from *Arabidopsis thaliana*', *BMC Research Notes*, 10, p. 638. Available at: https://doi.org/10.1186/s13104-017-2982-1.

Dinhopl, N. et al. (2011) 'Application of in-situ hybridization for the detection and identification of avian malaria parasites in paraffin wax-embedded tissues from captive penguins', *Avian Pathology: Journal of the W.V.P.A*, 40(3), pp. 315–320. Available at: https://doi.org/10.1080/03079457.2011.569533.

Duffy, P.E. and Patrick Gorres, J. (2020) 'Malaria vaccines since 2000: progress, priorities, products', *npj Vaccines*, 5(1), pp. 1–9. Available at: https://doi.org/10.1038/s41541-020-0196-3.

Ellis, V. et al. (2020) 'Explaining prevalence, diversity, and host specificity in a community of avian haemosporidian parasites', *Oikos*, 129. Available at: https://doi.org/10.1111/oik.07280.

Fallon, S.M. et al. (2003) 'Detecting Avain Malaria: an Improved Polymerase Chain Reaction Diagnostic', *Journal of Parasitology*, 89(5), pp. 1044–1047. Available at: https://doi.org/10.1645/GE-3157.

Fantham, H.B. and Porter, A. (1944) 'On a Plasmodium (P*lasmodium relictum var. spheniscidæ, n. var.*), observed in four species of Penguins', *Proceedings of the Zoological Society of London*, 114(3), pp. 279–292. Available at: https://doi.org/10.1111/j.1096-3642.1944.tb00222.x.

Fecchio, A. et al. (2020) 'Evolutionary ecology, taxonomy, and systematics of avian malaria and related parasites', *Acta Tropica*, 204, p. 105364. Available at: https://doi.org/10.1016/j.actatropica.2020.105364.

Feldman, R.A., Freed, L.A. and Cann, R.L. (1995) 'A PCR test for avian malaria in Hawaiian birds', *Molecular Ecology*, 4(6), pp. 663–673. Available at: https://doi.org/10.1111/j.1365-294x.1995.tb00267.x.

Field, K.G. et al. (1988) 'Molecular Phylogeny of the Animal Kingdom', *Science*, 239(4841), pp. 748–753.

Fox, G.E. et al. (1977) 'Classification of methanogenic bacteria by 16S ribosomal RNA characterization', *Proceedings of the National Academy of Sciences of the United States of America*, 74(10), pp. 4537–4541.

Freed, L. (2006) 'DNA quality and accuracy of avian malaria PCR diagnostics: A review', *The Condor*, 108, pp. 459–473. Available at: https://doi.org/10.1650/0010-5422(2006)108[459:DQAAOA]2.0.CO;2.

Garamszegi, L.Z. (2011) 'Climate change increases the risk of malaria in birds', *Global Change Biology*, 17(5), pp. 1751–1759. Available at: https://doi.org/10.1111/j.1365-2486.2010.02346.x.

Garcia, L. et al. (2004) 'Culture-Independent Species Typing of Neotropical Leishmania for Clinical Validation of a PCR-Based Assay Targeting Heat Shock Protein 70 Genes', *Journal of Clinical Microbiology*, 42(5), pp. 2294–2297. Available at: https://doi.org/10.1128/JCM.42.5.2294-2297.2004.

Graczyk, T.K. et al. (1994) 'Characteristics of naturally acquired avian malaria infections in naive juvenile African black-footed penguins (*Spheniscus demersus*)', *Parasitology Research*, 80(8), pp. 634–637. Available at: https://doi.org/10.1007/BF00932944.

Graczyk, T.K., Brossy, J.J., et al. (1995) 'Avian Malaria Seroprevalence in Jackass Penguins (*Spheniscus demersus*) in South Africa', *The Journal of Parasitology*, 81(5), pp. 703–707. Available at: https://doi.org/10.2307/3283958.

Graczyk, T.K., Cranfield, M.R., et al. (1995) 'Detection of Avian Malaria Infections in Wild and Captive Penguins', *Journal of the Helminthological Society of Washington*, pp. 135–141.

Greenhouse, B. et al. (2006) 'Validation Of Microsattelite Markers For Use In Genotyping Polyclonal *Plasmodium falciparum* Infections', *The American journal of tropical medicine and hygiene*, 75(5), pp. 836–842.

Grim, K.C. et al. (2004) 'Preliminary results of an anticircumsporozoite DNA vaccine trial for protection against avian malaria in captive African black-footed penguins (*Spheniscus demersus*)', *Journal of Zoo and Wildlife Medicine: Official Publication of the American Association of Zoo Veterinarians*, 35(2), pp. 154–161. Available at: https://doi.org/10.1638/03-060.

Harl, J. et al. (2020) 'Geographic and host distribution of haemosporidian parasite lineages from birds of the family *Turdidae'*, *Malaria Journal*, 19(1), p. 335. Available at: https://doi.org/10.1186/s12936-020-03408-0.

Hebert, P.D.N. et al. (2004) 'Identification of Birds through DNA Barcodes', *PLOS Biology*, 2(10), p. e312. Available at: https://doi.org/10.1371/journal.pbio.0020312.

Hellgren, O. et al. (2015) 'Global phylogeography of the avian malaria pathogen *Plasmodium relictum* based on MSP1 allelic diversity', *Ecography*, 38(8), pp. 842–850. Available at: https://doi.org/10.1111/ecog.01158.

Hellgren, O., Waldenström, J. and Bensch, S. (2004) 'A New PCR Assay for Simultaneous Studies of *Leucocytozoon*, *Plasmodium*, and *Haemoproteus* from Avian Blood', *The Journal of Parasitology*, 90(4), pp. 797–802.

Hernandez-Colina, A. et al. (2021) 'Avian malaria affecting penguins in zoological gardens, aquariums and wildlife parks in the UK', *Veterinary Record*, n/a(n/a), p. e511. Available at: https://doi.org/10.1002/vetr.511.

Hutchinson, R. and Stevens, J.R. (2018) 'Barcoding in trypanosomes', *Parasitology*, 145(5), pp. 563–573. Available at: https://doi.org/10.1017/S0031182017002049.

Ishtiaq, F. (2021) 'Ecology and Evolution of Avian Malaria: Implications of Land Use Changes and Climate Change on Disease Dynamics', *Journal of the Indian Institute of Science*, 101(2), pp. 213–225. Available at: https://doi.org/10.1007/s41745-021-00235-3.

Iurescia, M. et al. (2021) '*Plasmodium matutinum* Transmitted by *Culex pipiens* as a Cause of Avian Malaria in Captive African Penguins (*Spheniscus demersus*) in Italy', *Frontiers in Veterinary Science*, 8, p. 621974. Available at: https://doi.org/10.3389/fvets.2021.621974.

Jansen van Rensburg, M. (2010) Parasitism, disease and breeding ecology of little blue penguins (*Eudyptula minor*) on Tiritiri Matangi Island, New Zealand : a thesis submitted in partial fulfilment of the requirements for the degree of Master of Science in Conservation Biology, Massey University, Auckland. Thesis. Massey University. Available at: https://mro.massey.ac.nz/handle/10179/1634 (Accessed: 29 November 2021).

Jarvi, S.I., Schultz, J.J. and Atkinson, C.T. (2002) 'PCR Diagnostics Underestimate the Prevalence of Avian Malaria (*Plasmodium relictum*) in Experimentally-Infected Passerines', *The Journal of Parasitology*, 88(1), pp. 153–158. Available at: https://doi.org/10.2307/3285407.

Knowles, S.C.L., Palinauskas, V. and Sheldon, B.C. (2010) 'Chronic malaria infections increase family inequalities and reduce parental fitness: experimental evidence from a wild bird population', *Journal of Evolutionary Biology*, 23(3), pp. 557–569. Available at: https://doi.org/10.1111/j.1420-9101.2009.01920.x.

Lapointe, D.A., Atkinson, C.T. and Samuel, M.D. (2012) 'Ecology and conservation biology of avian malaria', *Annals of the New York Academy of Sciences*, 1249, pp. 211–226. Available at: https://doi.org/10.1111/j.1749-6632.2011.06431.x.

Larsson, A. (2014) 'AliView: a fast and lightweight alignment viewer and editor for large datasets', *Bioinformatics*, 30(22), pp. 3276–3278. Available at: https://doi.org/10.1093/bioinformatics/btu531.

Levin, I. et al. (2009) '*Plasmodium* blood parasite found in endangered Galapagos penguins (*Spheniscus mendiculus*)', *Biological Conservation*, 142, pp. 3191–3195. Available at: https://doi.org/10.1016/j.biocon.2009.06.017.

Li, J. et al. (1995) '*Plasmodium*: genus-conserved primers for species identification and quantitation', *Experimental Parasitology*, 81(2), pp. 182–190. Available at: https://doi.org/10.1006/expr.1995.1107.

Liao, W. et al. (2017) 'Mitigating Future Avian Malaria Threats to Hawaiian Forest Birds from Climate Change', *PLOS ONE*, 12(1), p. e0168880. Available at: https://doi.org/10.1371/journal.pone.0168880.

Lijtmaer, D.A. et al. (2012) 'DNA Barcoding Birds: From Field Collection to Data Analysis', in W.J. Kress and D.L. Erickson (eds) *DNA Barcodes*. Totowa, NJ: Humana Press (Methods in Molecular Biology™), pp. 127–152. Available at: https://doi.org/10.1007/978-1-61779-591-6_7.

Loiseau, C. et al. (2013) 'Predictions of avian *Plasmodium* expansion under climate change', *Scientific Reports*, 3(1), p. 1126. Available at: https://doi.org/10.1038/srep01126.

Lorenz, T.C. (2012) 'Polymerase Chain Reaction: Basic Protocol Plus Troubleshooting and Optimization Strategies', *Journal of Visualized Experiments : JoVE*, (63), p. 3998. Available at: https://doi.org/10.3791/3998.

Maddocks, S. and Jenkins, R. (2017) 'Chapter 2 - Designing and Ordering Your Polymerase Chain Reaction Primers', in S. Maddocks and R. Jenkins (eds) *Understanding PCR*. Boston: Academic Press, pp. 11–30. Available at: https://doi.org/10.1016/B978-0-12-802683-0.00002-2.

McNamara, D.T. et al. (2004) 'Development of a Multiplex PCR-Ligase Detection Reaction Assay for Diagnosis of Infection by the Four Parasite Species Causing Malaria in Humans', *Journal of Clinical Microbiology*, 42(6), pp. 2403–2410. Available at: https://doi.org/10.1128/JCM.42.6.2403-2410.2004.

de Muro, M.A. (2005) 'Probe Design, Production, and Applications', in J.M. Walker and R. Rapley (eds) *Medical Biomethods Handbook*. Totowa, NJ: Humana Press (Springer Protocols Handbooks), pp. 13–23. Available at: https://doi.org/10.1385/1-59259-870-6:013.

Murray, C.K. et al. (2008) 'Update on Rapid Diagnostic Testing for Malaria', *Clinical Microbiology Reviews*, 21(1), pp. 97–110. Available at: https://doi.org/10.1128/CMR.00035-07.

Pacheco, M.A. et al. (2018a) 'Primers targeting mitochondrial genes of avian haemosporidians: PCR detection and differential DNA amplification of parasites belonging to different genera', *International journal for parasitology*, 48(8), pp. 657–670. Available at: https://doi.org/10.1016/j.ijpara.2018.02.003.

Pacheco, M.A. et al. (2018b) 'Primers targeting mitochondrial genes of avian haemosporidians: PCR detection and differential DNA amplification of parasites belonging to different genera', *International journal for parasitology*, 48(8), pp. 657–670. Available at: https://doi.org/10.1016/j.ijpara.2018.02.003.

Paul, P. et al. (2021) 'Comparison of Fluorometric and UV Spectrophotometric Findings for DNA Isolated From Formalin-Fixed Paraffin-Embedded Blocks, Fine Needle Aspiration Cytology Smears, and Blood', *Cureus*, 13(11), p. e19583. Available at: https://doi.org/10.7759/cureus.19583.

PCR Troubleshooting Guide - UK. Available at: //www.thermofisher.com/uk/en/home/life-science/cloning/cloning-learning-center/invitrogen-school-of-molecular-biology/pcr-education/pcr-reagents-enzymes/pcr-troubleshooting.html (Accessed: 25 April 2022).

Pfaller, M.A. (1991) 'Diagnostic applications of DNA probes', *Infection Control and Hospital Epidemiology*, 12(2), pp. 103–110. Available at: https://doi.org/10.1086/646296.

QIAamp DNA FFPE Tissue Kit. Available at: https://www.qiagen.com/us/products/discovery-and-translational-research/dna-rna-purification/dna-purification/genomic-dna/qiaamp-dna-ffpe-tissue-kit/ (Accessed: 25 April 2022).

'QIAamp DNA Mini Blood Mini Handbook - EN' (2016). QIAgen. Available at: file:///C:/Users/xxxxxxxx/Downloads/HB-0329-004-1102728-HB-QIAamp-DNA-Mini-Blood-Mini-0516-WW%20(6).pdf (Accessed: 20 July 2022).

'QIAquick® Spin Handbook' (2020). Qiagen.

Quillfeldt, P. et al. (2011) 'Prevalence of blood parasites in seabirds - a review', *Frontiers in Zoology*, 8(1), p. 26. Available at: https://doi.org/10.1186/1742-9994-8-26.

Richard, F.A. et al. (2002) 'A Comparative Analysis of PCR-Based Detection Methods for Avian Malaria', *Journal of Parasitology*, 88(4), pp. 819–822. Available at: https://doi.org/10.1645/0022-3395(2002)088[0819:ACAOPB]2.0.CO;2.

Richards, J.C. (1991) 'Gene probes', *Current Opinion in Biotechnology*, 2(1), pp. 76–85. Available at: https://doi.org/10.1016/0958-1669(91)90064-C.

Ricklefs, R.E., Fallon, S.M. and Bermingham, E. (2004) 'Evolutionary relationships, cospeciation, and host switching in avian malaria parasites', *Systematic Biology*, 53(1), pp. 111–119. Available at: https://doi.org/10.1080/10635150490264987.

Russell, B., Suwanarusk, R. and Lek-Uthai, U. (2006) '*Plasmodium vivax* genetic diversity: microsatellite length matters', *Trends in Parasitology*, 22(9), pp. 399–401. Available at: https://doi.org/10.1016/j.pt.2006.06.013.

Sallaberry-Pincheira, N. et al. (2015) 'Molecular Epidemiology of Avian Malaria in Wild Breeding Colonies of Humboldt and Magellanic Penguins in South America', *EcoHealth*, 12(2), pp. 267–277. Available at: https://doi.org/10.1007/s10393-014-0995-y.

Sheldon, B.C. and Verhulst, S. (1996) 'Ecological immunology: costly parasite defences and trade-offs in evolutionary ecology', *Trends in Ecology & Evolution*, 11(8), pp. 317–321. Available at: https://doi.org/10.1016/0169-5347(96)10039-2.

Shen, C.H. (2019) 'Chapter 9 - Amplification of Nucleic Acids', in C.-H. Shen (ed.) *Diagnostic Molecular Biology*. Academic Press, pp. 215–247. Available at: https://doi.org/10.1016/B978-0-12-802823-0.00009-2.

Sijbranda, D. et al. (2017) ‘Cases of mortality in little penguins (*Eudyptula minor*) in New Zealand associated with avian malaria’, *New Zealand Veterinary Journal*, 65(6), pp. 332–337. Available at: https://doi.org/10.1080/00480169.2017.1359124.

Snounou, G. et al. (1993) ‘High sensitivity of detection of human malaria parasites by the use of nested polymerase chain reaction’, *Molecular and Biochemical Parasitology*, 61(2), pp. 315–320. Available at: https://doi.org/10.1016/0166-6851(93)90077-B.

Snounou, G. and Singh, B. (2002) ‘Nested PCR Analysis of *Plasmodium* Parasites’, in Doolan, D. L., *Malaria Methods and Protocols*. New Jersey: Humana Press, pp. 189–204. Available at: https://doi.org/10.1385/1-59259-271-6:189.

Stidworthy, M. and Denk, D. (2018) ‘Sphenisciformes, Gaviiformes, Podicipediformes, Procellariformes, and Pelecaniformes’, in K.A. Terio, D. McAloose, and J. St. Leger (eds) *Pathology of Wildlife and Zoo Animals*. First. Oxford, United Kingdom: Elsevier, pp. 649–682.

Su, X. and Wellems, T.E. (1994) ‘Sequence, transcript characterization and polymorphisms of a *Plasmodium falciparum* gene belonging to the heat-shock protein (HSP) 90 family’, *Gene*, 151(1), pp. 225–230. Available at: https://doi.org/10.1016/0378-1119(94)90661-0.

Tavares, E.S. and Baker, A.J. (2008) ‘Single mitochondrial gene barcodes reliably identify sister-species in diverse clades of birds’, *BMC Evolutionary Biology*, 8(1), p. 81. Available at: https://doi.org/10.1186/1471-2148-8-81.

Valkiūnas, G. et al. (2005) ‘What distinguishes malaria parasites from other pigmented haemosporidians?’, *Trends in Parasitology*, 21(8), pp. 357–358. Available at: https://doi.org/10.1016/j.pt.2005.06.005.

Valkiūnas, G. and Iezhova, T.A. (2018) ‘Keys to the avian malaria parasites’, *Malaria Journal*, 17(1), p. 212. Available at: https://doi.org/10.1186/s12936-018-2359-5.

Vanstreels, R.E.T. et al. (2015) ‘Epidemiology and pathology of avian malaria in penguins undergoing rehabilitation in Brazil’, *Veterinary Research*, 46, p. 30. Available at: https://doi.org/10.1186/s13567-015-0160-9.

Vanstreels, R.E.T., Braga, É.M. and Catão-Dias, J.L. (2016) ‘Blood parasites of penguins: a critical review’, *Parasitology*, 143(8), pp. 931–956. Available at: https://doi.org/10.1017/S0031182016000251.

Videvall, E. (2018) ‘*Plasmodium* parasites of birds have the most AT-rich genes of eukaryotes’, *Microbial genomics*, 4(2). Available at: https://doi.org/10.1099/mgen.0.000150.

Videvall, E. (2019) ‘Genomic Advances in Avian Malaria Research’, *Trends in Parasitology*, 35(3), pp. 254–266. Available at: https://doi.org/10.1016/j.pt.2018.12.005.

Wanger, A. et al. (2017) ‘Chapter 12 - Overview of Molecular Diagnostics Principles’, in A. Wanger et al. (eds) *Microbiology and Molecular Diagnosis in*

Pathology. Elsevier, pp. 233–257. Available at: https://doi.org/10.1016/B978-0-12-805351-5.00012-0.

Weissenböck. H. (University of Veterinary Medicine, Vienna) (2022) ‘Personal Communication’.

Woese, C.R. and Fox, G.E. (1977) ‘Phylogenetic structure of the prokaryotic domain: The primary kingdoms’, *Proceedings of the National Academy of Sciences*, 74(11), pp. 5088–5090. Available at: https://doi.org/10.1073/pnas.74.11.5088.