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ORIGINAL PAPER

Development of a rapid HRM qPCR for the diagnosis of the four most prevalent *Plasmodium* lineages in New Zealand

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Abstract Although wildlife rehabilitation and translocations are important tools in wildlife conservation in New Zealand, disease screening of birds has not been standardized. Additionally, the results of the screening programmes are often difficult to interpret due to missing disease data in resident or translocating avian populations. Molecular methods have become the most widespread method for diagnosing avian malaria (*Plasmodium* spp.) infections. However, these methods can be time-consuming, expensive and are less specific in diagnosing mixed infections. Thus, this study developed a new real-time PCR (qPCR) method that was able to detect and specifically identify infections of the three most common lineages of avian malaria in New Zealand (*Plasmodium* (Novyella) sp. SYAT05, *Plasmodium elongatum* GRW6 and *Plasmodium* spp. LINN1) as well as a less common, pathogenic *Plasmodium relictum* GRW4 lineage. The assay was also able to discern combinations of these parasites in the same sample and had a detection limit of five parasites per microlitre. Due to concerns relating to the presence of the potentially highly pathogenic *P. relictum* GRW4 lineage in avian populations, an additional confirmatory high resolution (HRM) qPCR was developed to distinguish between commonly identified *P. elongatum* GRW6 from *P. relictum* GRW4. The new qPCR assays were tested using tissue samples containing *Plasmodium* schizonts from three naturally infected dead birds resulting in the identified infection of *P. elongatum* GRW6. Thus, these rapid qPCR assays have shown to be cost-effective and rapid screening tools for the detection of *Plasmodium* infection in New Zealand native birds.

Keywords Avian malaria · Disease screening · High resolution melt (HRM) · New Zealand · *Plasmodium* · Real-time PCR

Introduction

Conservation translocations to preserve endangered species are becoming more significant worldwide as more restoration areas become available and captive breeding programmes for wildlife reintroductions become more successful. In New Zealand, reintroductions and translocations have become the preferred methods in species recovery programmes (Cunningham et al. 1996; Mathews et al. 2006) and advances in pest control have enabled the creation of large mainland islands that are either predator-free or intensively controlled (Craig et al. 2000). In addition, wildlife rehabilitation centres treat injured and sick endangered native birds for re-release back to the wild.

Disease screening for both wildlife translocations and rehabilitation in birds has not been standardized in New Zealand, and often there are no protocols in place on how to deal with infections if they are found. For example, birds infected with ubiquitous avian malaria parasites (*Plasmodium* spp.) have previously disrupted translocations of saddleback (*Philesturnus carunculatus rufusater*) (Thorne 2007) and hihi (*Nothiomystis cincta*) (Ewen et al. 2012) because there were no protocols to manage or

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assess the impact of such a finding. Currently, the consensus in managing translocated birds with *Plasmodium* infections in New Zealand is to translocate birds with low parasitaemia (chronically infected) or negative in PCR test ([1]pers. comm. K. McInnes, Department of Conservations 2015), since *Plasmodium* lineages reported in New Zealand are currently either thought to be native or are cosmopolitan and ubiquitous around the country (Schoener et al. 2014). However, each translocation needs to be viewed independently because the risks of disease transmission depend on different factors each time.

To date, avian malaria parasites belonging to 17 lineages have been found in 37 different bird species in New Zealand (Baron et al. 2014; Castro 2011; Howe et al. 2012; Schoener et al. 2014; Sturrock and Tompkins 2008; Tompkins and Glesson 2006). The most common lineages infecting endemic/native New Zealand bird species, and also introduced passerines, are *Plasmodium* (Huffia) *elongatum* lineages GRW06 and the closely related *Plasmodium* spp. LINN1 as well as *Plasmodium* (Novyella) sp. lineage SYAT05; with *P. elongatum* lineage GRW6 having the widest host range. Other lineages of *Plasmodium* detected in endemic species are *Plasmodium* (Haemamoeba) *relictum* lineages GRW4 and SGS1 (Castro et al. 2011; Ewen et al. 2012; Howe et al. 2012). Although all of these common *Plasmodium* lineages found in New Zealand appear to be low-level chronic infections, they can be pathogenic and are able to cause mortalities, particularly when birds are in an environment which increases stress levels, such as captive management or translocation events (Alley et al. 2008; Alley et al. 2010; Banda et al. 2013; Dinhopl et al. 2015). In past studies on experimentally infected birds, *P. elongatum* (Palinauskas et al. 2016) and *P. relictum* lineage GRW4 (Atkinson et al. 2000) have been found to be highly pathogenic parasites.

Currently, health screening of birds for *Plasmodium* spp. infection relies on either the examination of blood smears or a standard nested PCR protocol (Hellgren et al. 2004). While the examination of blood smears can be very accurate and can identify parasite species and even mixed infections, it requires an experienced operator and is time-expensive. It should also be noted that the identification of parasite morphology is often difficult as *Plasmodium* spp. are known for their cryptic diversity (Palinauskas et al. 2015). Sensitive conventional PCR assays with post-amplification sequencing can resolve these problems, but current PCR assays can underestimate mixed infections, which are common in the wild (Valkiūnas et al. 2006). Both molecular methods and microscopy can potentially miss low-level chronic infections (Fallon et al. 2003; Jarvi et al. 2002) because of varying assay sensitivity and examiner experience. Thus, the use of a combination method of both microscopy and PCR is recommended (Valkiūnas et al. 2006).

Recent advances in PCR technology and availability of real-time PCR (qPCR) and HRM (high resolution melt) machines have now provided a potential third option for the rapid diagnosis of *Plasmodium* spp. infections. These assays are in general faster, cheaper and more sensitive than conventional PCR, because they require less material (smaller reaction volumes) and forgo a sequencing step (Madigan et al. 2015). In addition, qPCR assays can be developed to detect multiple parasite species in one sample, preventing the need for post-amplification sequencing and potential amplification bias.

To date, two genes have been examined for creating a qPCR assay for the detection of avian *Plasmodium* spp. infections. Previously published protocols by Friedl and Groscurth (2012) using the plastid-like large subunit ribosomal-RNA (LSU-rRNA) gene and Bell et al. (2015) using a conserved region of the mitochondrial ribosomal DNA, have only been able to diagnose the presence or absence of *Plasmodium* spp. in the blood and tissue of New Zealand birds. However, none of these methods were able to specifically identify different lineages or mixed infections. For over 10 years, the cytochrome b gene has been the reference gene used for avian malaria research with a wealth of information and sequencing data of a continuously growing number of new lineages and species available online. However, in published qPCR protocols using the cytochrome b gene (Bentz et al. 2006; Njabo et al. 2011), the emphasis has been on diagnosing infection without differentiating between different parasite species. Still, using a different protocol, Knowles et al. (2011) were able to discern between two different species, *P. relictum* (lineages pSGS1 and pGRW11) and *Plasmodium circumflexum* (lineages pTURDUS1 and pBT7).

Thus, the aim of this study was to improve avian malaria screening by developing a real-time PCR (qPCR) technique that is able to detect and specifically identify infections and co-infections of the three most common lineages of avian malaria in New Zealand (*P.* (Novyella) sp. SYAT05, *P. elongatum* GRW6 and *P.* sp. LINN1) as well as the *P. relictum* GRW4 lineage.

Methods

Establishing positive controls

To develop positive controls for the three most common avian *Plasmodium* lineages in New Zealand, DNA extracted from blood samples from birds which had previously tested positive during diagnostic examination of a blood smear and PCR were used. These birds were one blackbird (*Turdus merula*), positive for *P.* spp. LINN1 (homology 100%, GenBank

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DQ847270), and two North Island saddleback (*Philesturnus rufusater*), one positive for *P. elongatum* GRW6 (homology 100%, GenBank DQ368381) and one positive for *P.* (Novyella) sp. SYAT05 (homology 100%, GenBank DQ847271). All birds were caught at Bushy Park/Wanganui in February 2013 during a study examining avian malaria under the Department of Conservation permit TW-32756-FAU and the approval by the Animal Ethics Committee at Massey University, MUAEC Protocol 11/59. Birds were banded under institutional banding permit No. 2012/009. In addition, one sample of blood from a bird experimentally infected with *P. relictum* GRW4 (GenBank AF254975) was provided by Carter Atkinson (U.S. Geological Survey, HI, USA). DNA was extracted from all blood samples using the DNeasy Blood and Tissue kit (Qiagen, Düsseldorf, Germany) according to the manufacturer's instructions for nucleated blood. DNA was stored at −20 °C until needed for molecular analysis.

Amplification and cloning of cytochrome *b* gene

Due to the availability of sequence data, the cytochrome *b* gene was determined to be the most suitable target gene for the development of the qPCR assay. The whole cytochrome *b* gene (1101 bp) was amplified by PCR from the positive control samples of *P.* sp. LINN1, *P. relictum* GRW4 and *P.* (Noyvella) sp. SYAT05 using the primers CytbFnew and CytbR (Table 1). The PCR reaction mix of 50-μl volume contained ~50 ng template DNA, 10X PCR Buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 μM of each primer and 1 unit of Platinum® Taq polymerase (Invitrogen, CA, USA). The cycling parameters were an initial denaturing at 94 °C for 4 min followed by 35 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min and a final extension step at 72 °C for 10 min. However, due to poor Cytb primer binding, the control for *P. elongatum* GRW6 was amplified with the first step of the nested PCR developed by (Hellgren et al. 2004) using the primers HaemF and HaemR2 (Table 1) to amplify a 480-bp sequence of the cytochrome b gene. PCR amplicons were run on a 1% (*w*/*v*) ultra-pure agarose gel (Invitrogen) containing ethidium bromide and visualized under UV light on a transilluminator. All positive PCR amplicons were purified (PureLink PCR purification kit, Invitrogen) and subjected to sequencing as described previously to confirm genomic sequence. Sequences were examined with Geneious™ (Biomatters, Auckland, New Zealand) and compared in a BLAST search to confirm correct target amplification.

Cloning of the PCR amplicons was performed using the pGEM T-easy vector kit (Promega, Madison, USA) according to the manufacturer's instructions. The PureLink® Quick Plasmid Miniprep Kit (Invitrogen) was used for DNA extraction of bacterial clones, eluted in 75 μl and stored at −20 °C until needed. Sequencing was again performed to confirm that the correct insert had been cloned as described previously. These clones were used as positive controls subsequently for qPCR.

Assessment of primers for qPCR

Various primer pairs within the cytochrome *b* gene, either previously published or generated using Geneious™ (Biomatters) for this study, were tested for their suitability to discriminate between the four target *Plasmodium* lineages. The two most promising primer pairs were HRMF/HaemR2 and Plas56F/PlasrevRT (Table 1). The primer pairs were chosen for testing if their expected amplicon products had between 50 and 250 base pairs and they showed a discriminatory DNA melting profile as assessed with the Oligocalc (http://www.basic.northwestern.edu/biotools/ oligocalc.html) initially based on cytochrome *b* gene sequence data of the four *Plasmodium* species available in GenBank (*P. relictum* GW4 AY733090, *P. elongatum* GRW6 DQ368381, *P.* spp. LINN1 DQ847270, *P.* (Novyella) sp. SYAT05 DQ847271) and subsequent sequencing confirmation of sequence homology of the four control isolates described earlier (Fig. 1).

Table 1 Cytochrome *b* gene primers used in this study

Primer name	Sequence	Position	Reference
CytbFnew (forward)	5′-GCACARTTAATTCATTATCC-3′	47–67	Designed in this study
CytbR (reverse)	5′-TTATATGTTTGCTTGGGAGCT-3′	1127–1148[a]	Musset et al. 2006
HaemF (forward)	5′-ATGGTGCTTTCGATATATGCATG-3′	257–280	Hellgren et al. 2004
HRMF (forward)	5′-CAGCTYTAAAAATACCCTTYTATCCA-3′	627–653	Njabo et al., 2011
HaemR2 (reverse)	5′-GCATTATCTGGATGTGATAATGGT-3′	730–754	Hellgren et al. 2004
Plas56F (forward)	5′-GTCAAATGAGTTTCTGGGGTGC-3′	407–429	Designed in this study
PlasrevRT (reverse)	5′-CCTAAAGGATTWGTGCTACC-3′	599–619	Designed in this study

[a] Predicted *Plasmodium* mitochondrion cytochrome *b* gene is 1130 bp

Source: GenBank AB250690, EF079654, LT594520

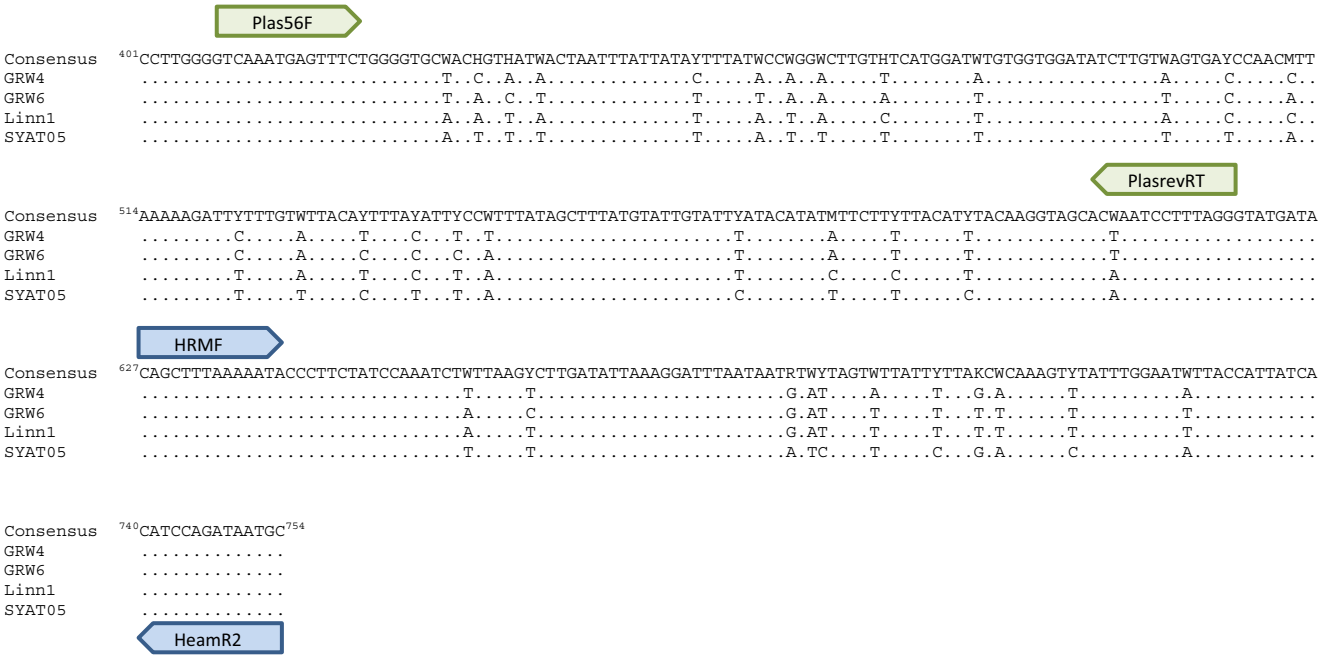


Fig. 1 The four *Plasmodium* lineages examined in alignment showing the differences in the DNA sequences in a 250-bp part of the cytochrome b gene flanked by the primer pairs HRMF/HaemR2 and Plas56F/PlasrevRT. GenBank reference numbers for sequences used: *P. elongatum* GRW6 DQ368381, *P. relictum* GRW4 AY733090, *P.* sp. LINN1 DQ847270, *P.* (Novyella) sp. SYAT05 DQ847271

All qPCR and HRM assays were performed on the Rotor-Gene 6000 platform (Corbett Life Science, Sydney, Australia). For the reactions, the MeltDoctor™ HRM Master Mix (Applied Biosystems, Foster City, USA) was used according to the manufacturer's instructions with primer concentrations of 0.3 μM. In order to assess the discriminatory ability of each primer pair combination, 1 ng DNA from each of the cloned controls was added to the reactions. The thermocycling parameters for the primer pair HRMF/HaemR2 were 10 min at 95 °C, then 35 cycles of 15 s at 95 °C and 10s at 55 °C followed by melt analysis of the 127-bp fragment. The PCR conditions for the primer pair Plas56F/PlasrevRT were as above but run for 45 cycles followed by a high resolution melt step of 65–75 °C with 0.1 °C increments pausing for 2 s per step. To test the detection of mixed infections, 1:1 mixes of the different positive controls (1 ng/μl) were prepared and tested with the primer pairs HRMF/HaemR2 and Plas56F/PlasrevRT.

Determination of qPCR detection limit

The detection limit of the qPCR with promising primer pair combinations was determined using seven duplicate 10-fold dilutions of the cloned controls as described previously. All seven dilutions had a Cq value (quantification cycle value) within the 35 (45 for Plas56F/PlasrevRT) thermo-cycles of qPCR and the standard curves were reproducible and had R^2 values of >95%. The concentration of *Plasmodium* DNA in each assay control sample was calculated from the standard curves. It was assumed that each *Plasmodium* parasite contained an average 10 copies of the mitochondrial cytochrome *b* gene. However, it should be noted that the copy number has been estimated between 3 and 20 per parasite, with sexual stages having up to three times higher copy numbers than those of asexual stages (Farrugia et al. 2011, Learngaramkul et al. 1999). Therefore, for the clones of *P.* spp. LINN1, GRW4 and SYAT05, 1 ng of control DNA is approximately 2.26×10^8 copies of vector plus insert and representative of 4.52×10^6 parasites. For clone GRW6, due to the shorter cloning fragment used, 1 ng of DNA is equivalent to 2.66×10^8 copies of vector plus insert and representative to 5.32×10^6 parasites.

Application to clinical samples

In order to assess the discriminatory ability of the qPCR-HRM for detecting *Plasmodium* spp. in clinical samples, tissues were collected from three avian malaria-infected birds that died in April 2015 at Wildbase Hospital at Massey University, Palmerston North. One was a North Island brown kiwi (*Apteryx mantelli*) and the other two were yellow-eyed penguins (YEP, *Megadyptes antipodes*) which were submitted for post-mortem to Wildbase Pathology, where tissue samples were collected shortly after death. Multiple tissues, including the lung, liver, spleen, heart, skeletal muscle, kidney, gastrointestinal tract and gonad, were fixed in 10% buffered formalin

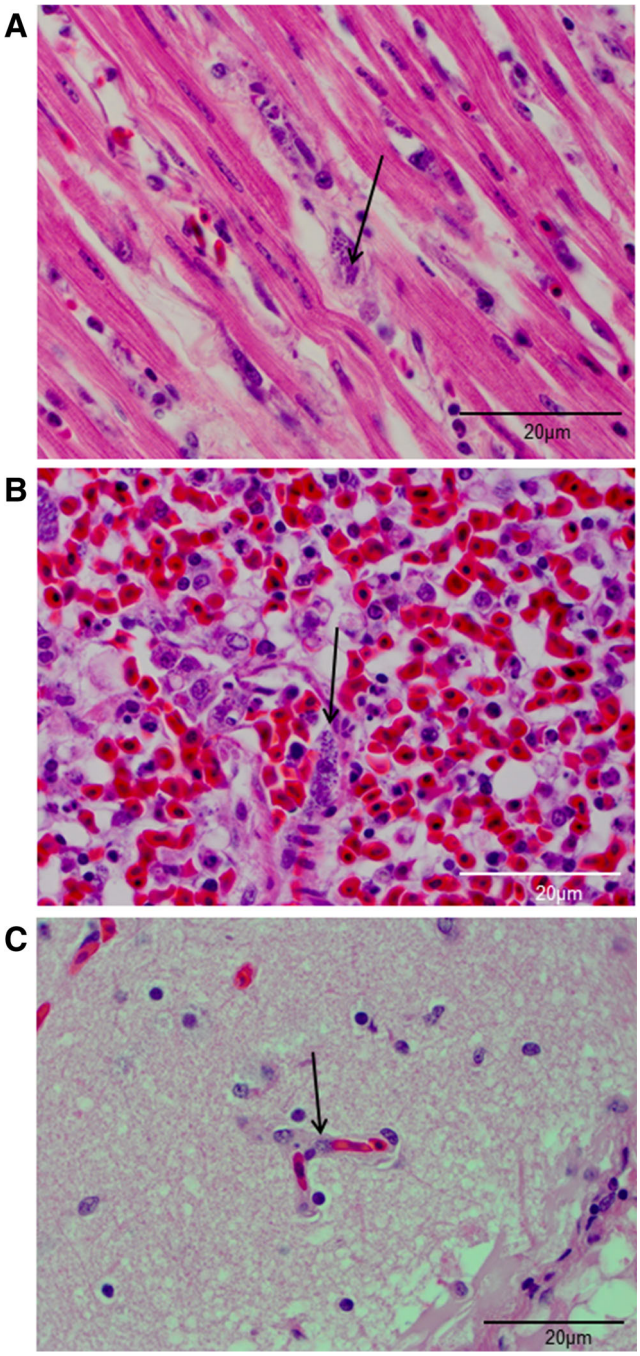


Fig. 2 *Plasmodium* spp. tissue schizogony (*arrow*) in the heart muscle of a kiwi (**a**); lung (**b**) and brain (**c**) of a yellow-eyed penguin

for histopathology or frozen for molecular analysis. All fixed tissues were routinely processed for histology by embedding in paraffin, cutting at 3 mm and staining with haematoxylin and eosin before light microscopic examination. Histopathology on all three birds showed large numbers of schizonts within the endothelial cells of the myocardium (kiwi, Fig. 2a) as well as the lung, liver and brain (YEP, Fig. 2b, c) which are indicative of *Plasmodium* spp. infection. DNA was extracted from these tissues and analysed with the qPCR and HRM protocols using the first primer pair HRMF/HaemR2 followed by Plas56F/PlasrevRT as described earlier using 20–50 ng of genomic DNA per 20 μl reaction volume.

Results

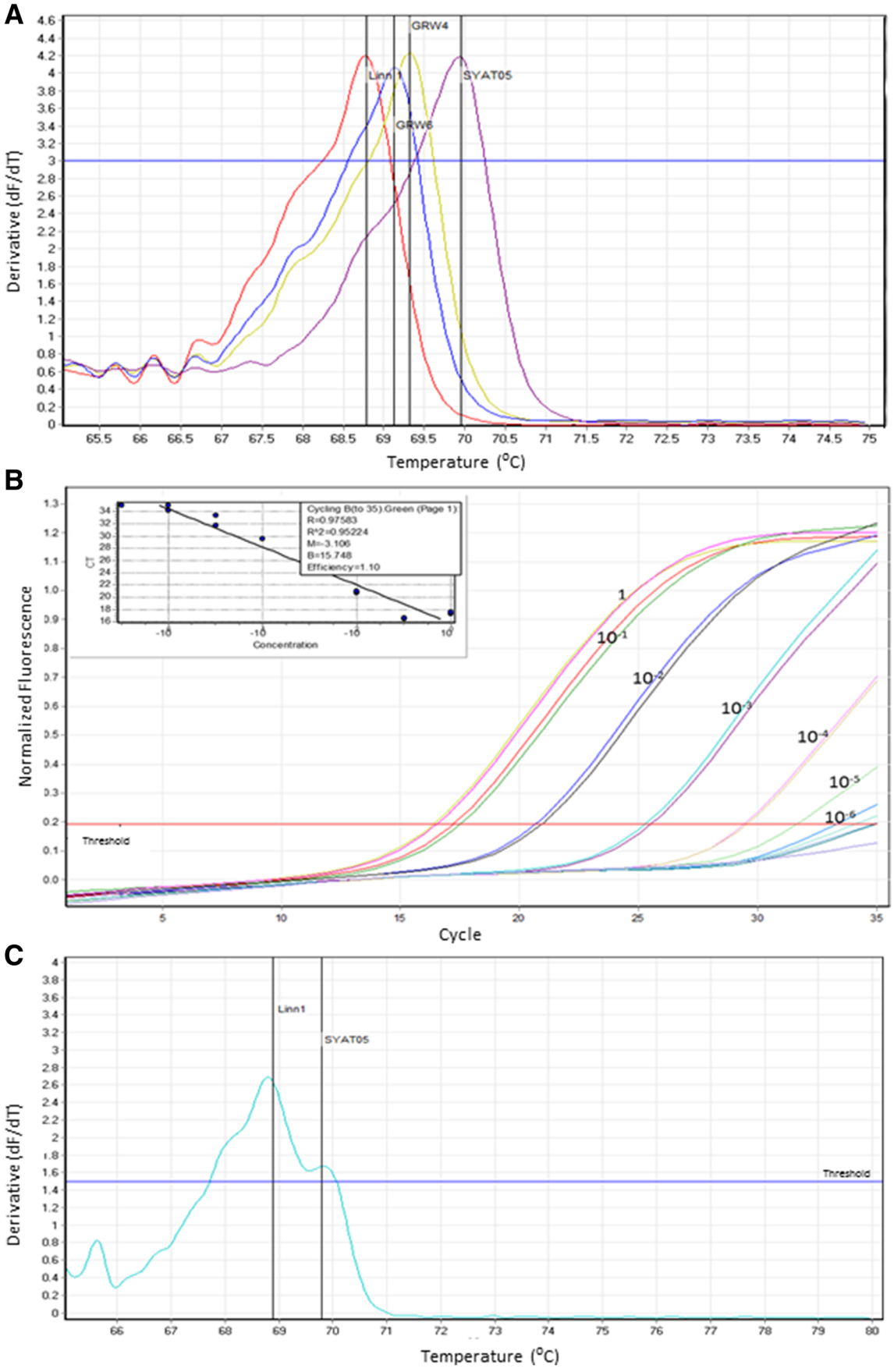
Assessment of primers

The most promising primer pairs (HRMF/HaemR2 and Plas56F/PlasrevRT) showed the best separation between the different melt peaks of the four examined *Plasmodium* lineages. Using the primers HRMF/HaemR2, the melt peak for the *Plasmodium* sp. lineage LINN1 was at 68.8 °C, for GRW6 at 69.1 °C, *P. relictum* GRW4 at 69.3 °C and *P.* (Novyella) sp. SYAT05 at 69.9 °C (Fig. 3a). However, since the commonly detected *P. elongatum* GRW6 and rarely detected *P. relictum* GRW4 only displayed a 0.2 °C separation in the melt and the potential devastating effects of an incursion of *P. relictum* GRW4, an additional confirmatory HRM-qPCR was developed (Fig. 4). Thus, using the Plas56F/PlasrevRT primer pair (211-bp fragment), the qPCR-HRM assay was able to amplify and definitively discriminate *P. elongatum* GRW6 from *P. relictum* GRW4 under high resolution melt (Fig. 4a).

Due to the possibility of various parasite life stages within avian blood and the assumption of an average cytochrome b copy number of 10 per parasite, the detection limit for the qPCR using the HRMF/HaemR2 primer pair was approximately 0.01 pg/μl, equal to about 24 parasites per microlitre DNA from all four cloned control lineages (Fig. 3b). The detection limit for the Plas56F/PlasrevRT primer pair was less sensitive at approximately 0.1 pg/μl, equivalent to approximately 250 parasites per microlitre DNA (Fig. 4b). In addition, successful differentiation using mixed combinations of controls, in equal concentrations, with both the primer pairs HRMF/HaemR2 (Fig. 3c) and Plas56F/PlasrevRT supported the possible use of the assays for detecting co-infecting *Plasmodium* spp. within a clinical sample.

Application to clinical samples

In the three clinical samples of deceased birds, it was possible to determine the lineage that had infected these birds using the qPCR assay. In DNA extracted from tissues, a qPCR melt peak indicating *P. elongatum* GRW6 was seen in all three cases using the primer pair HRMF/HaemR2 (Fig. 5a), with a confidence of 96.34 to 98.04%. This result was confirmed by running all three samples on the HRM programme using the primers Plas56F and PlasrevRT (Fig. 5b).


A
GRW4
Linn1
SYAT05
GRW6
Derivative (dF/dT)
Temperature (°C)
B
Cycling B(to 35) Green (Page 1)
R=0.97583
R^2=0.95224
M=-3.106
B=15.748
Efficiency=1.10
CT
Concentration
Normalized Fluorescence
1
10-1
10-2
10-3
10-4
10-5
10-6
Threshold
Cycle
C
Linn1
SYAT05
Threshold
Derivative (dF/dT)
Temperature (°C)

◀ **Fig. 3** QPCR melt protocol using primers HRMF and HaemR2. **a** Melt of all four tested *Plasmodium* lineages (*P. elongatum* GRW4, *P. relictum* GRW6, *P.* sp. Linn1 and *P.* (Novyella) sp. SYAT05). **b** Seven 10-fold serial dilutions of a 1 ng/μl standard starting at 0.1 ng/μl and standard curve (*inset*) for *P. relictum*. **c** Assessing a "mixed" infection of 1:1 (1 ng/μl) of *P.* sp. LINN1 and *P.* (Novyella) sp. SYAT05, showing a double peak at the melt temperatures of both lineages. Derivative (dF/dT) is defined as the negative derivative of fluorescence (dF) with respect to temperature (dT)

Discussion

The qPCR melt protocol using the primer pair HRMF and HaemR2 was able to discriminate between three of the main lineages present in New Zealand. An additional qPCR-HRM (primers Plas56F/PlasrevRT) was successfully used as a confirmation assay between *Plasmodium* lineages GRW4 and GRW6. Due to the selection of the two genetically variable cytochrome *b* regions, it is expected that the combination of the two assays will be able to identify additional isolates and between closely related *Plasmodium* lineages, such as the New Zealand native *Plasmodium* spp. PADOM02/HIHI01 (MalAvi database) and introduced *P.* sp. GRW4, once appropriate clone controls and melt profiles have been established. The differentiation of these lineages is of importance for New Zealand conservation, because although avian malaria parasites almost exclusively cause subclinical infections in areas with endemic infection, disease and mortality remain a possibility (Bennett et al. 1993; Valkiūnas, 2005). For example, in

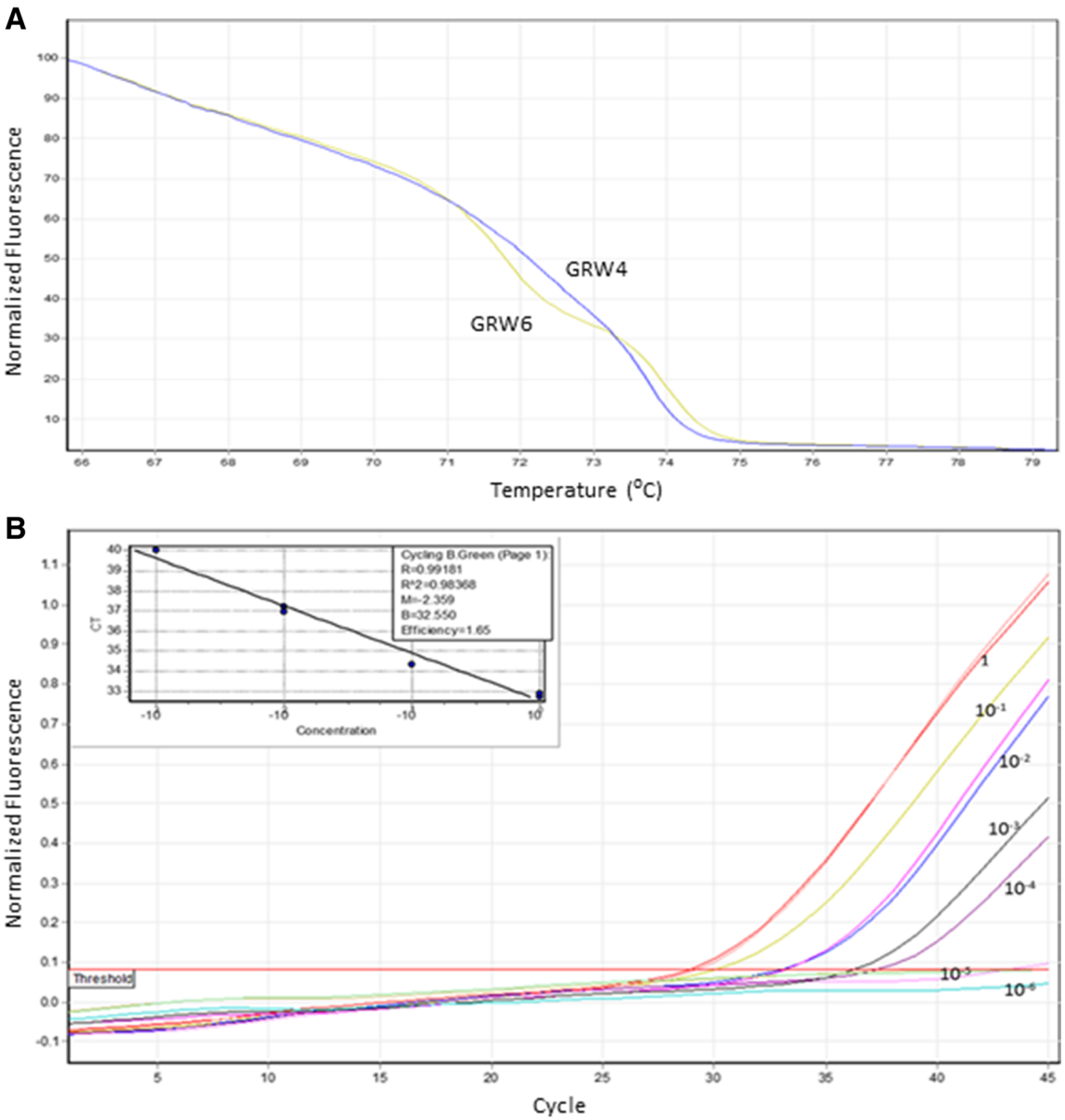


Fig. 4 QPCR HRM protocol using primer pair Plas56F and PlasrevRT. **a** High resolution melt discrimination between *P. elongatum* GRW6 and *P. relictum* GRW4; **b** Seven 10-fold serial dilutions of a 1 ng/μl standard starting at 0.1 ng/μl and standard curve (*inset*) for *P. relictum* GRW4

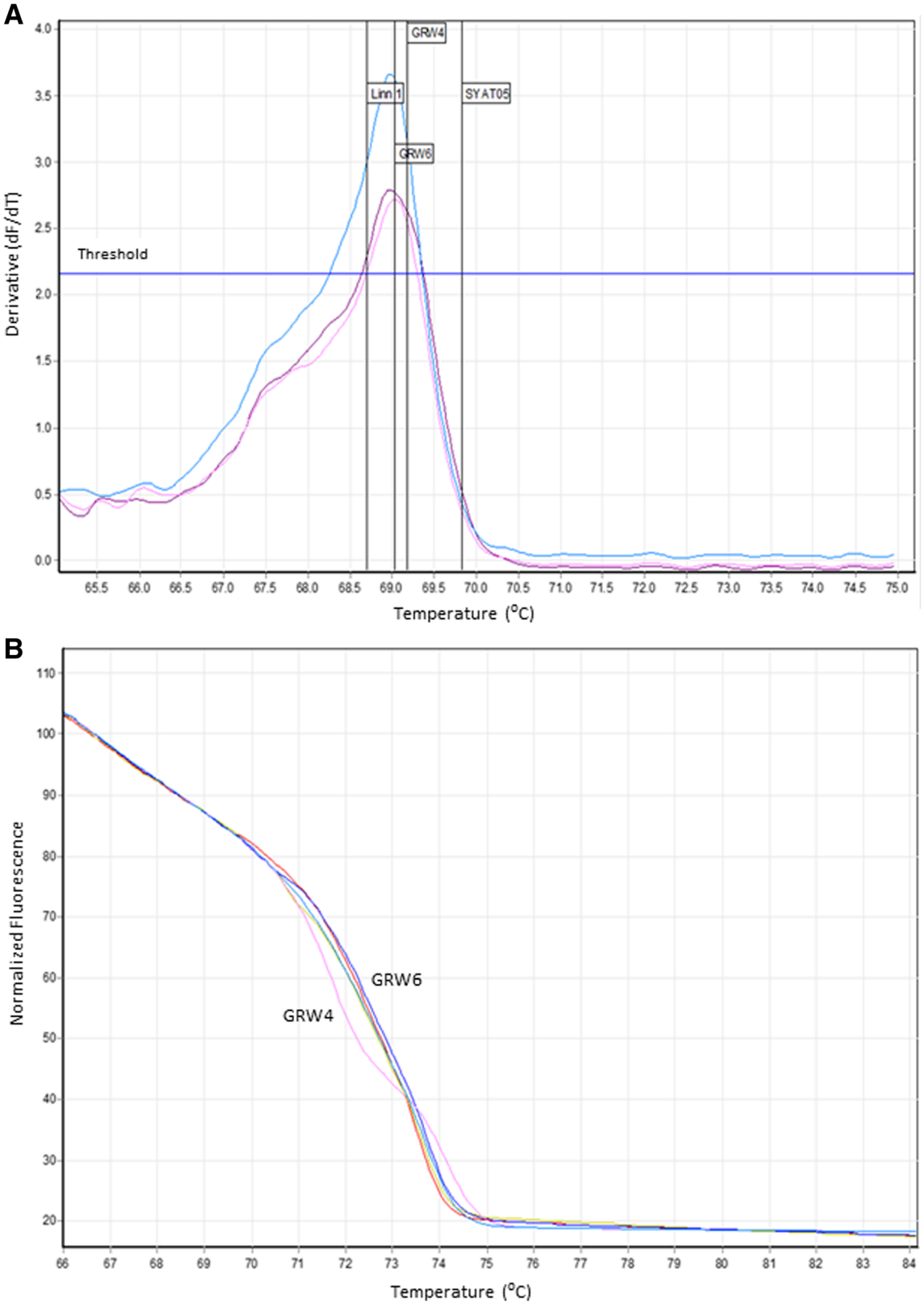


Fig. 5 Clinical application of qPCR assays. **a** Detecting the infecting lineage in three deceased birds, North Island brown kiwi (*purple*) and two yellow-eyed penguins (*light blue* and *pink*) using primers HRMF and HaemR2. **b** HRM using primer pair Plas56F and PlasrevRT of clinical samples Kiwi 28, YEP 35 and YEP 36; the samples are equivalent to the GRW6 baseline (*green*) compared to *P. relictum* GRW4 control (*pink*). Derivative (dF/dT) is defined as the negative derivative of fluorescence (dF) with respect to temperature (dT)

captive situations and wildlife rehabilitation, mortalities have been observed in endangered New Zealand birds, like the NI brown kiwi (Banda et al. 2013), mohua (Alley et al. 2008) and great spotted kiwi (Howe et al. 2012). Indeed, avian malaria has been widely recognized worldwide as one of the most significant infectious diseases in captive penguins and penguins in rehabilitation, with *P. relictum* and *P. elongatum* as the most important disease agents (Vanstreels et al. 2015; Parsons and Underhill 2005; Jones and Shellam 1999; Clarke and Kerry 1993). Therefore, the identification of *P. elongatum* GRW6 during this study in a deceased brown kiwi, as well as two yellow-eyed penguins is consistent with previous observations and it underlines the pathogenic nature of this parasite lineage to captive kept endangered birds in New Zealand.

The HRMF/HeamR2 assay showed good sensitivity detecting approximately 24 parasites per microliter of extracted genomic DNA. However, a higher level of sensitivity may be desirable for detecting the very common low chronic infections with *Plasmodium* spp. in native birds. The assay presented here appeared less sensitive when compared to other published molecular techniques, although direct comparison is difficult. There is currently only one report by Friedl and Gruscurth (2012) stating a detection limit of 0.1 blood parasites per 100 blood cells with their LSU-rRNA gene qPCR assay. Although, there have been several comparisons with the widely used nested PCR assays. For example, a qPCR assay targeting the cytochrome *b* gene developed by Njabo et al. (2011) was able to detect 17% more positives than the cytochrome *b*-nested PCR protocol described by Hellgren et al. (2004). Similarly, Knowles et al. (2011) noted a significantly higher sensitivity in their cytochrome *b* qPCR assay compared to a similar nested protocol (Waldenström et al. 2004). However, Bell et al. (2015) found no difference in sensitivity between their protocol and the established cytochrome *b*-nested PCR assay (Hellgren et al. 2004). Of note, only one of these previously published assays was able to reliably discern between different *Plasmodium* species (*P. relictum* and *P. circumflexum*) (Knowles et al. 2011).

One important advantage of the assay presented in this study is the potential to detect co-infections with two or more different *Plasmodium* species. Co-infections are common in wild birds and can be present in up to 80% of all infected birds in Europe (Jarvi et al. 2002; Valkiūnas et al. 2006). However, there is some discrepancy in the literature about the effect of co-infections with different lineages of *Plasmodium*, although co-infections are generally considered more virulent than infections with just a single lineage possibly due to sharing of limited physiological resources (Arriero and Moller. 2008; Atkinson and van Riper 1991; Marzal et al. 2008; Palinauskas et al. 2011; Vanstreels et al. 2015). For example, Palinauskas et al. (2011) found heavier parasitaemia (over 35 and up to 90% during peaks of blood cells infected) in three species of passerines experimentally co-infected with *P. relictum* SGS1 and *Plasmodium ashfordi* GRW2. Additionally, Dimitrov et al. (2015) reported that the success of experimental co-infections and subsequent parasitaemia depended on the avian species and *Plasmodium* lineages used. However, also reported a markedly higher parasitaemia in red crossbills (*Loxia curvirostra*) when co-infected with both *P. relictum* SGS1 and *P. ashfordi* GRW02 than with *P. relictum* SGS1 infection alone (Dimitrov et al. 2015).

Admittedly, the two assays presented in this study are only the beginning of our ability to detect and reliably discriminate between *Plasmodium* species and as new species or lineages are identified, new primer or primer-probe sites along the cytochrome *b* gene will be identified. The advantage of using the cytochome *b* gene for primer design is that in recent years, it has been used as a reference gene for avian haemosporidian parasites and therefore has the most extensive sequence data from different haemosporidian species and lineages available (Bensch et al. 2009). However, the cytochrome *b* gene in malarial parasites has a high AT content (approximately 73%), which causes difficulties in designing effective primers (Fallon et al. 2003). Therefore, as genetic data on other parasite genes becomes available, a multi-gene approach may need to be employed. For example, the *18S* gene is commonly used in human malaria diagnostics, but rarely used for the detection and speciating avian *Plasmodium* spp. (Richard et al. 2002). The *18S* gene also has a lower copy number than cytochrome *b* gene, ranging on average in *Plasmodium falciparum* between five (Tran et al. 2014) and six copies (Wampfler et al. 2013) per parasite, whereas mitochondrial cytochrome *b* gene has a range of three in asexual stages to as many as 20 copies depending on the life cycle stage (Farrugia et al. 2011; Learngaramkul et al. 1999). Recently, Hellgren et al. (2015) used the highly variable nuclear merozoite surface protein 1 (*MSP1*) gene, a gene linked to the invasion biology of the parasite, to discern different alleles in several distinctive cytochrome *b* haplotypes of *P. relictum*. This made it possible to record the genetic variation occurring in different *P. relictum* populations and how they are geographically distributed. Using the *MSP1* gene in a New Zealand setting would enable studies on the different populations of *Plasmodium* species and lineages in their geographical origins as well as finding local patterns of virulence and host resistance.

One problem encountered with using these genes is the lack of available avian parasite sequences on Genbank and therefore the ability to design primers for New Zealand lineages to reliably amplify these genes. For future studies, full genome sequencing of the different *Plasmodium* lineages found in New Zealand has to be performed to be able to design primers for a new assay. Next-generation sequencing techniques have already been used to document previously undetected levels of variation in a single *Plasmodium* lineage,

suggesting the origins and evolution of *P. relictum* GRW4 in Hawaii might be more complicated than previously recognized (Jarvi et al. 2013). A similar approach in New Zealand would provide invaluable information about host parasite interactions and evolution and might also help answering the question if the introduction of new *Plasmodium* lineages together with European birds in the late 1800s played a role in the disappearance of New Zealand's endemic species. However, it should be noted that the high diversity of avian haemosporidian parasites will make this task difficult and will need to be tailored to specific regions and/or avian species of interest or concern (Valkiūnas et al. 2014).

Despite these problems, the new assays described here produced fast and accurate data that can be used in rapid disease screening for *Plasmodium* parasites in native New Zealand birds suffering from acute infection with clinical symptoms as well as birds that have been found dead. These assays will be especially useful in a rehabilitation setting where the health of the birds is monitored on a regular basis enabling early detection of clinical symptoms and where treatment is available. The advantage of this method compared to standard PCR and microscopy is the speed, the lower cost, and the ability to detect mixed infections. Although these assays do not appear to be sensitive enough to diagnose birds for translocation with low parasitaemia, they will enable conservation managers to rapidly assess which *Plasmodium* species are present in outbreak events within monitored captive bird populations and thus assist with the development and implementation of control measures where possible.

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