The application of molecular diagnostic techniques based on DNA amplification is revolutionizing the study of wildlife disease ecology (Leroy et al. 2005, Li et al. 2005). Where previously we have often lacked the necessary tools to understand disease dynamics in natural host communities, or have been limited to costly and time-consuming approaches such as serology, Polymerase Chain Reaction (PCR) techniques (complemented by the sequencing of amplified products or the detection of molecular markers) now provide a relatively cheap and powerful tool for documenting and quantifying parasite transmission through often complex host/vector associations. The utility of such knowledge for formulating disease management strategies, both to control current outbreaks and pre-empt future ones, is clearly demonstrated by recent work on West Nile Virus in the United States (Kilpatrick et al. 2006), where molecular diagnostics proved invaluable in their role in parasite detection and identification.

Although such techniques are now commonly employed in the detection of parasites in vectors (e.g., Schall and Smith 2006), the additional identification of host blood meals (e.g., Ansell et al. 2000, Prior and Torr 2002, Molaei et al. 2006) from the same vector individuals has, to the best of our knowledge, only been employed in laboratory experimental studies (e.g., Fabian et al. 2004). However, the benefits of applying this approach to field surveys are clear, with the provision of direct evidence for likely transmission pathways, as opposed to inference from multiple data sources. Here we report such an application. Our focus was on the Culex mosquito vectors of Plasmodium avian malarial parasites in New Zealand. These protozoan parasites are believed to be introduced to New Zealand (Bennett et al. 1993), emerging at higher prevalence in recent decades (Tompkins and Gleeson 2006), and potentially impacting on the native bird community in a similar fashion to that observed in Hawaii (van Riper et al. 1986). Hence, in also being the first application of PCR for amplification of avian malaria parasites from a mosquito vector, the work reported here is an essential tool development for the understanding and management of avian malarial disease dynamics and the impact on New Zealand’s highly endangered endemic avifauna (Sekercioglu et al. 2004).

Mosquitoes were collected from eight pipe-traps (Nelson 1980) and ten black-painted resting boxes (Pletsch 1970) placed out for five nights in early December 2005, at a farm 10 km from Gisborne, New Zealand (E2938126, N6272445). Previous surveys indicated a high prevalence (4%) of Plasmodium infection in birds in this locality (Tompkins and Gleeson 2006). Only one blood-engorged female mosquito was collected during this period from a resting box and identified as the native Culex pervigilans using the taxonomic keys of Snell (2005). Collections, using E67 Model PR101 combination light and CO$_2$ traps (http://www.entosupplies.com.au), of an average of 146 non-engorged mosquitoes per trap per night (98% of which were identified as Culex sp.), clearly indicated the low success rate of pipe-traps and resting boxes for collecting blood-engorged females in our study.

As previous studies have shown the head and thorax to possess inhibitory properties for PCR (Arez et al. 2000), only the abdomen of the single blood-engorged mosquito was used for DNA extraction, performed using a modified phenol–chloroform method (Kikuchi and Fukatsu 2003). The abdomen was ground by plastic pestle in a micro-centrifuge tube containing 120 µl of warm (65°C) extraction buffer (0.5% SDS, 0.2 M NaCl, 25 mM EDTA, and 10 mM Tris pH 8.0). Proteinase K (0.02 mg/µl) was added to the sample, which was then incubated for 1 h at 50°C. Following incubation, 120 µl phenol:chloroform:iso-amyl alcohol (24:25:1) was added, and the sample centrifuged for 10 min at 14,000 rpm. The sample was then precipitated in 95% ethanol overnight in a −20°C freezer, centrifuged for another 10 min at 14,000 rpm, and washed in 70% ethanol. All the ethanol was carefully removed, and the air-dried pellet was re-suspended in 100 µl of hydration buffer (Aqua Pure genomic DNA extraction kit, BIORAD).

Parasite detection within the mosquito blood meal was carried out using PCR primers L15368 (5′AAA AAT ACC CT'T TCA TCC AAA TCT 3') and H15730 (5′CAT CCA ATC CAT AAT AAA GCA T 3'), designed to amplify an approximately 355-base-pair fragment of the mitochondrial cytochrome b gene of Plasmodium and Haemoproteus parasites as described in Ricklefs et al. (2004). The PCR reaction was carried out in a 25-µl volume containing 10× PCR buffer and 20 mM MgCl$_2$, 2 mM each of dNTPs, 0.2 mM each of primer L15368 and H15730 and 1.5 U/reaction
of Faststart Taq (Roche), and 5 µl of extracted DNA. The PCR was performed using the GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, U.S.A.). Amplification was performed using a program of initial denaturation of 4 min at 95° C, followed by 35 cycles of 30 s at 94° C for denaturation, 60 s at 48° C, extension for 1 min 30 s at 72° C, followed by a final extension for 3 min at 72° C.

PCR was used to amplify host DNA from the mosquito blood meal using primers AvctbF (5’GACTGTGACAAATCCGNTCCA 3’) and AvctbR (5’GGTCTTCTACCTHYGGYTTACAAGAC 3’), designed as avian-specific primers to amplify a 508-bp fragment of the mitochondrial cytochrome b gene (Molaei et al. 2006). The PCR reaction was carried out in a 25-µl volume containing 10× PCR buffer and 20 mM MgCl₂, 2 mM each of dNTPs, 0.2 mM each of primer AvctbF and AvctbR and 1.5 U reaction of Faststart Taq (Roche), and 1 µl of extracted DNA. Amplification consisted of initial denaturation for 4 min at 95° C, followed by 36 cycles of 30 s at 94° C for denaturation, 50 s at 60° C, extension for 1 min at 72° C, followed by a final extension for 3 min at 72° C.

Following both PCRs, 10 µl of resulting products were analyzed using 2% agarose gel in Tris acetate EDTA and visualized under UV light following ethidium bromide staining. Products were purified using the High Pure PCR Product Purification Kit (Roche Diagnostics), following the procedure outlined by the manufacturer, and direct sequencing of purified products was achieved using BigDye® Terminator Ready Reaction Cycle sequencing Kit Version 3.1 (Applied Biosystems). Primers used in the initial PCR reactions were also used for sequencing. Sequences were analyzed on a 3100 – Avant genetic Analyzer (Applied Biosystems, Hitachi), edited on SEQUENCER™ Version 4.5 (Gene Codes Corporation), and identified using the BLAST search algorithm on the NCBI GenBank nucleotide database.

Parasite and blood-meal mtDNA were both successfully amplified from the one blood-engorged female Culex pervigilans. The sample was positive for the presence of the parasite cytochrome b fragment, with a closest (99%) match to Plasmodium relictum. In addition, a c. 508-base-pair amplicon fragment was produced from the sample using the avian-specific primers, with a closest (99%) match to the introduced blackbird Turdus merula. All results were replicated using a duplicate round of PCR and sequencing. Hence, we demonstrate that this complete set of information can be obtained from the same vector individuals sampled from the field, and that this approach can be usefully employed in field surveys designed to identify potential parasite transmission pathways. This information, from just a single blood-engorged vector, has increased our understanding of avian malarial disease dynamics in New Zealand. Whereas previously the role of the native C. pervigilans as a vector for malarial parasites was merely hypothesized (Holder et al. 1999), we now have direct evidence for such a role being likely. In addition, with the inference that this native mosquito has picked up malarial parasites from an introduced bird (no other bird species DNA matches were found in the PCR product amplified), a potential transmission pathway for malarial parasites from introduced to native birds has been identified.

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REFERENCES CITED


