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Morphological and Molecular Characterisation of Coccidia (*Eimeria* spp.) in Kiwi (*Apteryx* spp.)

A thesis presented in partial fulfilment

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Abstract

The coccidia of kiwi were first reported in the 1970s; however, in-depth, morphological descriptions of oocysts and endogenous stages were not described until 2013. The development and success of Operation Nest Egg (ONE) between 1994 to the present provided the ideal environment for the proliferation of coccidia, i.e., increased density of immunologically naïve hosts. These conditions led to an increase in morbidity and mortality of kiwi chicks due to high coccidial burdens. Current methods of detection and treatment rely heavily on husbandry practices and, potentially, over medication with coccidiocides. Treatment-resistant species of coccidia may exacerbate these problems causing increased cost of frequent hospitalisation and additional supportive care required by kiwi with high coccidial burdens. This thesis advances the ability to quantify oocyst loads in kiwi droppings by determining that a modified Mini-FLOTAC protocol is more accurate than the current method used in New Zealand diagnostic labs. This alternative protocol has the added benefit of not requiring large centrifuges, potentially enabling practitioners to determine coccidial burdens on site. The morphological descriptions of oocysts are continued in brown kiwi and established in Haast tokoeka. The four morphotypes previously described were represented in the brown kiwi dataset; however, *E. paraurii* was not described in Haast tokoeka. A new morphotype similar to *E. kiwii* was described in both brown kiwi and Haast tokoeka. This thesis provides the first genetic data at the mitochondrial cytochrome *c* oxidase I gene, which was initially amplified from the morphologically described species using Sanger sequencing. A larger dataset that included brown kiwi, Haast tokoeka, rowi, and a great spotted kiwi was targeted and sequenced using Illumina amplicon technology. This in-

depth analysis allowed for the detection of the full variation of sequences within the samples, informing the development of diagnostic testing, pathogenicity studies, and treatment efficacy monitoring in the future. Further, extensive optimisation of extraction protocols provides key guidelines for breaking open unsporulated oocyst walls to ensure all the oocysts in a sample are represented in the results.

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Publications

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Coker SM, Pomroy W, McInnes K, Vallee E, Howe L, Morgan KJ (2018) Coccidia in kiwi: microscopic methods of detection. Postgraduate Research Colloquium. Palmerston North, New Zealand.

Preface

My interest in parasites began as an undergraduate in Virginia, USA, with a misidentification of a blue bird, an experience that introduced me to the fascinating world of ornithology and, soon thereafter at the University of Georgia (Athens, GA, USA), the broader dimensions of wildlife disease. I am particularly interested in merging traditional parasitology with modern genetic technologies in order to gain further understanding of the changing dynamics between wildlife and their parasites in relation to human intervention. Accordingly, the intense management of kiwi and the potential impact of altered host-parasite interactions drew my interest in this project. Further, I firmly believe technology should complement rather than replace current systems on disease detection and management, which aligns perfectly with the overall goals of this project to continue both morphological and molecular characterisation of kiwi coccidia. My personal goal for this PhD was to develop a diagnostic tool for direct use in conservation management of kiwi. Unsurprisingly, several unexpected complications arose; however, I believe the results of this thesis bring kiwi conservation several steps closer to understanding the evolving dynamics of kiwi coccidia and how to overcome the issues currently faced by the Operation Nest Egg program.

Thesis structure and format

Kiwi are an iconic part of New Zealand culture; they are the national bird and are internationally renowned for their unique characteristics. Conservation of this taonga (treasure) has significantly slowed the decline of kiwi numbers. However, a large part of their management relies on human intervention. This intervention has led to threats from coccidia, density-dependent parasites that cause severe disease and mortality. Identification of these parasites is unusually difficult, in part due to the host diversity, management, and conservation status. Management of these parasites is potentially severely hindered without the ability to differentiate the many different species of coccidia that infect kiwi. Thus, the overarching aim of this thesis is to expand the characterisation of coccidia in kiwi with the goal of helping veterinarians and wildlife managers to make informed management decisions.

This thesis has four specific aims:

1. To improve microscopic detection and quantification of coccidia;
2. To broaden the morphological descriptions of coccidia species in kiwi, especially host species that are dependent on Operation Nest Egg;
3. To molecularly characterise coccidia species in kiwi; and
4. To develop a diagnostic tool to assist in making coccidia management decisions.

The first chapter of this thesis provides an introduction and literature review, with Chapter 7 providing a general discussion. Chapters 2-5 are experimental chapters intended for publication. Chapter 2 has been published in the journal *Parasitology Research*; Chapters 3 through 5 are being prepared for submission and have been written in the same journal format. Chapter 6 presents the systematic troubleshooting

and optimisation that was required to achieve sufficient genetic amplification; while this chapter is not intended for publication, it provides details that will inform future research in the field and is important to communicate.

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CHAPTER 1

Literature Review

Contribution of co-authors: All authors provided feedback on one or more drafts of this section.

1.1. Kiwi ecology and management

The interactions between hosts and their parasites comprise of complex signalling; immunological reactions and responses; and evolutionary dependency (Loye and Carroll 1995). While the effects of parasites can be deleterious to an individual host, under natural environmental pressures and within healthy ecosystems, parasites can increase the fitness and shift the evolutionary path of a host species or population (Betts et al. 2018; Christe et al. 2006; Papkou et al. 2016). With increased fragmentation and decreased genetic diversity of host populations, the deleterious aspects of parasite interactions become more common and a potential threat to the conservation of a host species (Loye and Carroll 1995). For instance, morbidity and mortality of kiwi (*Apteryx* spp.) that is associated with coccidiosis (disease caused by coccidia) is more often due to over exposure to coccidia caused by high density of immunologically compromised hosts, coinfection with another pathogen, poor environmental/nutritional conditions, or other stressors (e.g., translocation) that compromise the kiwi's immune response (Alnassan et al. 2014; Mengel et al. 2012; Sainsbury and Vaughan-Higgins 2012; Yabsley 2008). While these factors that contribute to increased incidence of coccidiosis can occur naturally (e.g., drought), they are more frequently associated with unpredictable anthropogenic interference (Christe et al. 2006). While kiwi/coccidia interactions have surely been impacted by loss of habitat and predation by introduced mammals, many of the issues that have led to this research began with increased management and conservation efforts (see Chapters 1.1.3.4 and 1.1.4 for more detail) (Christe et al. 2006; Craig et al. 2011; Miller and Pierce 1995; Robertson et al. 2011). In order to study the effects of these management practices, the parasites need to be thoroughly characterised and baseline knowledge of the diversity and parasite load expected in

healthy, functional ecosystems needs to be established for each kiwi species, population, or management group. This baseline is necessary to maintain the expected parasite biodiversity that led to the success of kiwi prior to human intervention and inform conservation decisions during translocations to and from offshore islands, wild populations, and captive populations. While it may seem preferred to treat or remove these parasites to prevent disease, it is important to remember that these parasites are important drivers of a balanced ecosystem and to instead strive for healthy host populations (captive and wild) that can continue to flourish under natural environmental pressures (Thomas et al. 2005; Windsor 1997). Additionally, arguments for the conservation of species for ethical and aesthetic reasons can equally be applied to the intrinsic value of parasite species (Gómez and Nichols 2013).

1.1.1. Kiwi taxonomy

Kiwi are endemic to New Zealand and share qualities with other ratites (e.g., emu) such as flightlessness, a flat sternum, small or unapparent wings, and powerful legs (Castro and Morris 2011; Roach 1952). Interestingly, based on mitochondrial data, kiwi are more closely related to extinct elephant birds (Aves: Aepyornithiformes), rather than the extinct moa (Aves: Dinornithiformes), a large ratite endemic to New Zealand (Figure 1.1.A.; Baker et al. 2014; Mitchell et al. 2014). Five species of kiwi are currently recognised; brown (*Apteryx mantelli*), rowi or Okarito (*Apteryx rowi*), tokoeka (*Apteryx australis*), great spotted (*Apteryx haastii*), and little spotted (*Apteryx owenii*) (Baker et al. 1995; Burbidge et al. 2003; del Hoyo et al. 2014). However, evidence suggests *A. mantelli* and *A. australis* contain populations with enough phylogenetic divergence that several subspecies classifications should be considered or, at least, managed separately

(Table 1.1.; Burbidge et al. 2003; Herbert and Daugherty 2002; Weir et al. 2016). Of these five species, brown kiwi, rowi and tokoeka genetically group together, while great and little spotted kiwi group together. Most recent estimates predict this initial split occurred around 3.85 (1.87 to 7.0, using 95% confidence intervals) million years ago (Ma) (Weir et al., 2016). At 1.56 (0.76 to 2.83) Ma, the tokoeka split from the group that would later split into rowi and brown kiwi at 1.12 (0.54 to 2.02) Ma (Weir et al., 2016). Finally, great and little spotted kiwi likely split 0.55 (0.27 to 0.99) Ma. All kiwi species are sexually dimorphic; females have larger bodies and longer bills, with which they probe for invertebrates and other food items (Castro and Morris 2011; Roach 1952). Kiwi use their strong legs and feet for protection, digging burrows for nesting, and roosting (Castro and Morris 2011; Roach 1952).

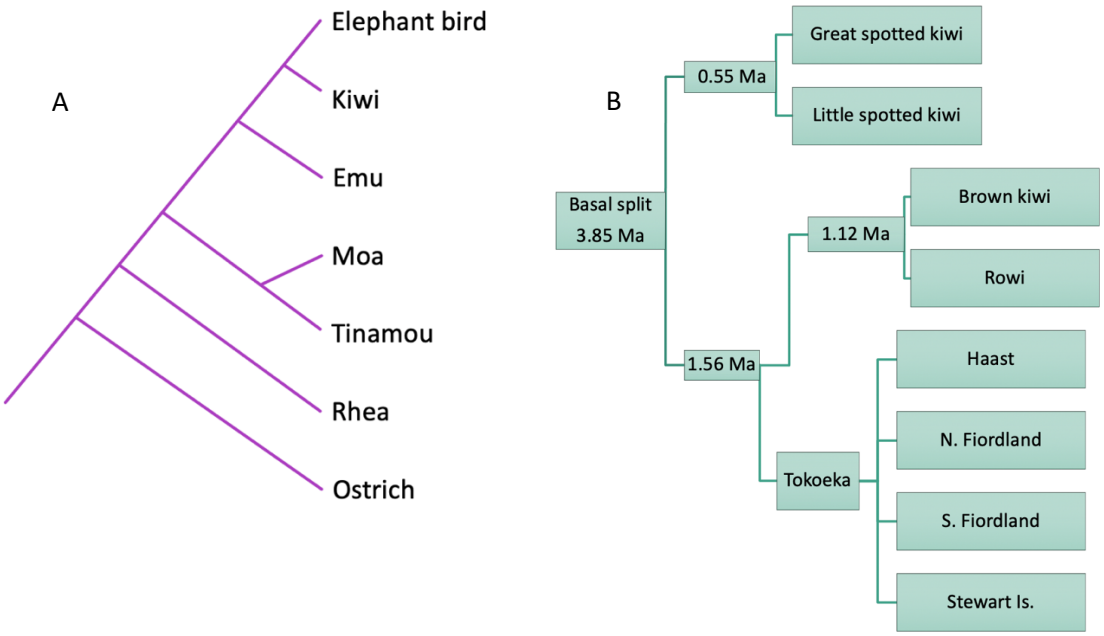


Figure 1.1. Kiwi phylogeny. A) Phylogenetic relationships of kiwi and other closely related species based on mitochondrial data (Baker et al. 2014; Mitchell et al. 2014). B) Phylogenetic relationships of currently recognised species and subspecies with estimates of when the splits occurred (Burbidge et al. 2003; Weir et al. 2016). Branch lengths are not to scale.

1.1.2. Kiwi breeding

Kiwi usually have two clutches from June to December with 1 to 2 eggs per clutch that they incubate for 75 to 91 days (Colbourne 2002; Heather and Robertson 2015). While there are exceptions, brown and little spotted kiwi males tend to incubate the eggs alone, whereas tokoeka, rowi, and great spotted kiwi share incubation with the female, other males, or close family members (Castro and Morris 2011; Colbourne 2002; McLennan 1988). Nesting in burrows helps control the temperature and humidity (Castro and Morris 2011; Colbourne 2002). Kiwi chicks are precocial, relying on residual

egg yolk for about a week, foraging around the nest for 10 to 60 days, and fledging when they reach 10 to 16% of adult body weight at around 70 days old (Heather and Robertson 2015; McLennan 1988; McLennan et al. 2004).

1.1.3. Kiwi conservation

1.1.3.1. Distribution

Brown kiwi are found in four main areas of the North Island with about 25,000 birds remaining (BirdLife International 2017; Innes et al. 2015). Rowi have populations in the Okarito Forest in the South Island and have also been introduced to Mana Island in the Cook Strait as well as Oruawairua and Motuara Islands in the Marlborough Sounds with a total population of 400 to 500 individuals (Heather and Robertson 2015; Innes et al. 2015). Tokoeka can be found on Stewart Island as well as Fiordland and the Haast region in the South Island with populations totalling about 11,000 (Heather and Robertson 2015). Great spotted kiwi have fragmented populations in the northern half of the South Island that total about 17,000 (Holzapfel et al. 2008). Little spotted kiwi are no longer present on the mainland but have been introduced over a wide range of offshore and mainland islands with Kapiti Island, Tiritiri Matangi Island, and Zealandia (Karori Sanctuary Trust, Wellington) being the most highly populated such that, as of 2013, there was a population of c.a. 1,700 (Heather and Robertson 2015). Table 1.1 details the current population distribution and conservation status.

Table 1.1 Population, distribution, and conservation status of all five species of kiwi.

Species	Sub-classification	Location[1,2,3,4,8]	Pop. Size (Year Estimated)	New Zealand Conservation Status[5]	IUCN Conservation Status[6]
Apteryx mantelli **(brown)**	North	Northland	24550 (2015)[7] 26550 (2015)[6]	At Risk: Declining	Vulnerable
	Coromandel	Coromandel Peninsula			
	East	East near Hawke's Bay and inland			
	West	Tongariro to the western coast			
Apteryx rowi **(rowi)**		Forest of Okarito on the South Island	400-500 (2013)[2,7]	Threatened: Nationally Vulnerable	Vulnerable
		Motuara and Oruawairua Islands in the Marlborough Sounds as well as Mana Island in the Cook Strait			
Apteryx australis **(tokoeka)**		Haast	900 (2013)[2]	Threatened: Nationally Critical	Vulnerable
		North Fiordland	9000 (2013)[2]	Threatened: Nationally Vulnerable	
	A. australis australis Shaw, 1813	Southern Fiordland		Threatened: Nationally Endangered	
	A. australis lawryi Rosthchild, 1893	Rakiura (Steward Island)	1200 (2013)[2]		
Apteryx haastii **(great spotted)**		Kahurangi National Park, the Paparoa Range, Arthur's Pass National Forest, and Nelson Lakes National Park	17000 (2008)[3]	Threatened: Nationally Vulnerable	Vulnerable
Apteryx owenii **(little spotted)**		Red Mercury Island	80 (2013)[2]	At Risk: Recovering	Near Threatened
		Hen Island	60 (2013)[2]		
		Long Island	50 (2013)[2]		
		Tiritiri Matangi Island	100 (2013)[2]		
		Zealandia (Wellington)	120 (2013)[2]		
		Chalky Island	40 (2013)[2]		
		Motuihe Island	40 (2013)[2]		
		Kapiti Island	1200 (2013)[2]		

[1]del Hoyo et al. (2014); [2] Heather and Robertson (2015); [3]Holzapfel et al. (2008); [4]Weir et al. (2016); [5]Robertson and Colbourne (2017); [6]BirdLife International (2017); [7] Innes et al. (2015); [8]BirdLife International (2020)

1.1.3.2. Threats

Population decline of kiwi has been linked to habitat loss and predation of adults and juveniles primarily by introduced predators, primarily stoats, but also dogs, cats, possums, weasels, and ferrets (Basse et al. 1999; McLennan et al. 2004; McLennan et al. 1996; Miller and Pierce 1995; Taborsky 1988). Due to their size, chicks remain extremely vulnerable to predation until they reach approximately 1 kg in weight (McLennan et al. 1996; Robertson et al. 2011). Without management to control predators, survival of chicks to this size is an estimated 10% (Robertson et al. 2011). Basse et al. (1999) calculated that stoat densities would need to be decreased to fewer than two per square kilometre for sufficient kiwi recruitment to sustain populations naturally.

1.1.3.3. *In situ* management

The threat introduced mammals pose to kiwi populations demonstrates a need for long term management or elimination of these pest species and is achieved through various combinations of ground based or aerial distributed toxins, trapping, or exclusion (e.g., predator proof fences or islands).

1.1.3.3.1. Toxins

Conservationists deliver toxins to kiwi reserves using a combination of several distribution methods and compounds that vary according to the terrain and resource availability. Robertson et al. (1999b) showed that 55 ha and 45 ha forests treated with ground-based cereal and jam bait stations laced with brodifacoum had significantly fewer ($p < 0.05$) kiwi chicks predated by mammals than 110 ha and 35 ha untreated

forests (28% and 56%, respectively). A combination of ground-based baiting using 1080 (sodium fluoroacetate) and brodifacoum laced cereal pellets for possums and rodents as well as trapping of cats and mustelids was used for predator control from 1996 to 2002 at the 445 ha Trounson Kauri Park in Northland (Gillies et al. 2003), which led to a mean yearly kiwi survival to 1 kg of 37.6%. Robertson et al. (2016) demonstrated that short, three cycle trapping targeted at rats and possums baited with 1080 prior to the kiwi breeding season increased survival of brown kiwi chicks to 6 months of age from 30% to 53% in the Riponui Scenic Reserve (175 ha) as well as from 45% to 62% in the Rarewarewa Scenic Reserve (76 ha). In the seven to eight years, respectively, prior to baiting with 1080, these reserves were heavily trapped to keep stoat populations down. Despite the rarely successful trapping of stoats, stoat-related kiwi deaths continued. Thus, the increase in chick survival was hypothesised to be due to secondary poisoning of untrappable stoats, which are selected for over long periods of trapping or when prey species are prevalent.

Aerial dispersal of toxic baits has been shown to be an effective and safe means of toxin distribution. For example, Empson and Miskelly (1999) detected an increase (though not significant) in kiwi populations after aerial dispersal of brodifacoum laced cereal pellets in 1997 on Kapiti Island (1965 ha) when compared with the previous three years of population estimates. Similarly, in a review of several Department of Conservation aerial dispersals of 1080 laced carrots and cereal pellets, Spurr and Powlesland (1997) did not report significant mortality in brown kiwi, little spotted kiwi, or great spotted kiwi (Pierce and Montgomery 1992). One death of a little spotted kiwi due to brodifacoum exposure from an aerial bait operation has been reported as a personal communication to Spurr

and Powlesland (1997), but no other distinct association has been established. The most comprehensive study to date by Robertson et al. (2019), demonstrated the population increases of brown kiwi after repeated 1080 aerial dispersal over a 20,000 ha area in the Tongariro Forest.

Controlling stoat and brushtail possum populations on the mainland using 1080 cereal pellets in ground based bait stations and brodifacoum cereal pellets dispersed by hand or aerially has shown to increase chick survival with no overall decline in kiwi health from exposure; however, accumulation of the brodifacoum toxin in the food chain over long periods of time has not been studied in depth and should remain a concern (Robertson et al. 1999a; Robertson and Colbourne 2001; Robertson et al. 1999b; Robertson et al. 1993; Robertson et al. 2016; Spurr and Powlesland 1997; Veltman and Westbrooke 2011).

1.1.3.3.2. Trapping

While potentially more time intensive, trapping has the benefit of being able to chiefly target predator species. Glen et al. (2012) demonstrated that continuous deployment and monitoring of 200 to 300 traps over a 6,000 ha peninsula sufficiently decreased the number of stoats (from a capture rate >0.325 in 2003 to <0.1 in 2011) to allow for an increase in the kiwi population (from 3.6 calls per hour in 2001 before trapping to 5.6 calls per hour in 2011). Similarly, Robertson and de Monchy (2012) demonstrated that large scale trapping (c.a. 12,000 to 18,500 ha) yielded population growths ranging from 0.6% and 2.9% on the South Island and 8.6% to 11.3% on the North Island; survival to

subadulthood (i.e., survival to 183 days) ranged from 15.1% to 32.2% on the South Island and 62.4% to 67.1% on the North Island. Tansell et al. (2016) compared trapped (1,400ha) and untrapped (1,600ha) areas and estimated the population growths of Fiordland tokoeka to be 1.21% and -1.64% respectively. Chick survival to subadulthood was markedly greater in the trapped area at 36.7% compared with 13.0% in the untrapped area.

1.1.3.3.3. Exclusion

A third method, exclusion, attempts to isolate kiwi from the predators by physical means such as isolation on an island or surrounded by a predator-proof fence. This method is the main source of protection currently used for *A. owenii*, which are confined to predator-free islands or fenced mainland sanctuaries (Heather and Robertson 2015). Unlike offshore islands, predator proofing on the mainland requires continuous, costly maintenance of fencing, which may have weak points that require intensive trapping to ensure a continually predator free environment (Keast et al. 2010). However, Clapperton and Day (2001) estimate that maintaining a predator free environment over a long period of time with an area >5,000 ha is more cost effective than baiting and trapping, especially when certain topography (e.g., peninsulas) can be utilised.

All of these *in situ* methods are useful tools in the management of kiwi; however, none are infallible and are often used in conjunction with *ex situ* management techniques.

1.1.3.4. *Ex situ* management

Operation Nest Egg (ONE) is a programme developed by the Department of Conservation in 1994 in response to the rapidly declining kiwi populations. Eggs or recently hatched chicks are taken from the wild into captivity. After hatching, chicks are raised individually in a hygienic brooder system on an artificial diet for approximately three weeks until they regain their hatch weight; then they are either kept in outdoor enclosures or sent to predator-free sites such as islands or fenced sanctuaries known as kiwi crèches. When these kiwi have reached a weight at which they can defend themselves from most predators (~1 kg), they are released back into the wild (Colbourne et al. 2005). Compared to pest trapping and baiting, the ONE programme produces the highest hatch success rate of up to 70% as well as chick survival to 183 days from 66.7 to 91.7% (McLennan et al. 2004; McLennan et al. 1996; Robertson et al. 2011; Robertson and de Monchy 2012). However, ONE is labour and resource intensive and currently only effective on a small scale of ~250 to 300 chicks per annum (K. McInnes pers. comm.). Regardless, ONE provides an effective method of establishing new populations, protecting the offspring of genetically important individual kiwi in the rowi and Haast tokoeka populations, and increasing population recovery rates in extant kiwi breeding populations. It remains a vital management tool while invasive mammals prey on kiwi chicks.

1.1.4. Disease

Captive rearing of kiwi chicks can lead to health issues. For example, while quicker hatch growth rates have been demonstrated in captive kiwi, accelerated weight gain so early in development can lead to bone deformities (Prier et al. 2013). Additionally, pathogens

such as *Mycobacterium* spp. (Davis et al. 1984), *Aspergillus fumigatus* (Glare et al. 2014), *Cryptococcus neoformans* (Hill et al. 1995), *Plasmodium* spp. (Alley et al. 2012; Banda et al. 2013), and avipoxvirus (Bean 2017) have been associated with morbidity and mortality in captive kiwi. This association between disease and captivity may be a product of increased monitoring, rather than a result of the captive environment. For example, with increased screening, *Plasmodium* spp. have been detected at a low prevalence in wild kiwi on Ponui Island (Howe et al. 2012) and Waimarino Forest (Sijbranda et al. 2016); neither documented morbidity nor mortality. Additionally, Ha et al. (2013) identified two subclades (B1 and A3) of avipoxvirus from two brown kiwi from Ponui Island. While increasing monitoring does increase the likelihood of detection of pathogens, there are extensive reports of coccidiosis in juvenile kiwi resulting in morbidity and mortality that are associated with ONE management practices. The outdoor enclosures are generally small (51 m^2 on average at The National Kiwi Hatchery), house two to three kiwi, and lead to the build-up of coccidia in the environment over time. While crèches encompass much larger areas (e.g., 16 ha at Cape Sanctuary, which holds 12 ONE kiwi juveniles at a time), practitioners frequently release kiwi in the same location every year and some provide supplemental feeding to ensure a softer release (K. McInnes, pers. comm.). Accordingly, young kiwi tend to stay in a small area near the release site, leading to a build-up of and exposure to high levels of environmental coccidial burdens. Exposure of juvenile kiwi to high levels of infectious coccidia oocysts, as is found in captivity and crèches; requires ongoing, intensive testing and treatment; has been shown to lead to severe morbidity and mortality; and will remain a significant continuing concern for kiwi conservation programmes (Alley et al.

2004; Boardman 1998; Boardman 1995; Morgan 2013; Robertson and Colbourne 2017; Thompson and Wright 1978).

1.2. Coccidia

Infection with coccidial parasites within captive facilities as well as in free ranging wild birds is common, rarely causing disease, i.e., coccidiosis (Morgan et al. 2014; Yabsley 2008). In the absence of clinical coccidiosis, infection with coccidia can compromise the immune response to other pathogens, increasing the likelihood of disease development (Alnassan et al. 2014; Chapman et al. 2002; Hegazy et al. 1999; Knight et al. 2018; Stockdale 1977). Accordingly, clearing even low- to mid-level infections would help ONE cope with other pathogens. The alternative is also true; given additional stress (e.g., translocation, lack of food), significant coccidiosis is more likely to develop (Gill and Paperna 2008; Knight et al. 2018; Sainsbury and Vaughan-Higgins 2012). For example, based on the analysis of 24 hazards including highly pathogenic avian influenza virus and *Aspergillus fumigatus* by Sainsbury and Vaughan-Higgins (2012), coccidia were found to represent the largest hazard during translocation of Eurasian Cranes (*Grus grus*). Translocation of kiwi regularly occurs; thus, infection with coccidia should be consistently considered and monitored.

1.2.1. Coccidia taxonomy

Coccidia in the genus *Eimeria* are Apicomplexan parasites in the family Eimeriidae and order Eucoccidiorida (Yabsley 2008). Eimeriid coccidia encompasses a wide variety of species and genera, the exact classifications of which have significantly fluctuated with

advancing technology (Tenter et al. 2002). For example, the classification established by Honigberg et al. (1964) was based on ultrastructure and occurred just as electron microscopy became established, but further changes occurred as technology developed, leading to further reclassification in the 1970s and regularly since (Ghimire 2010; Tenter et al. 2002). Rapid change is continuing based on the use of varying gene targets that affect the phylogenetics (Ghimire 2010; Tenter et al. 2002). These targets are continually examined and compared to determine which target or combination of targets leads to a phylogeny that makes biological sense and holds up with the continual addition of sequences from diverse locations and hosts.

The genus *Eimeria* typically forms oocysts containing four oocysts containing four sporocysts, each of which contains two sporozoites (conventionally referred to as 1:4:2 configuration. However, classifications based on morphology, which is frequently based on a single life stage, or those on single genes, such as the highly conserved nuclear 18S ribosomal DNA (rDNA) gene, sometimes do not provide sufficient differentiation (Ogedengbe et al. 2018; Tenter et al. 2002). Thus, while the four species of coccidia in *A. mantelli* (*Eimeria kiwii*, *E. apteryxii*, *E. mantellii*, and *E. paraurii*) that Morgan et al. (2017) morphologically described share many characteristics with other species of *Eimeria*, there may be precedence for a re-evaluation of phylogenetic classification based on mitochondrial DNA targets (Ogedengbe et al. 2018).

1.2.2. Life cycle of coccidia

Eimeria have direct life cycles with faecal-oral transmission, but their development a host is much more complex and varies among species. Yabsley (2008) and Blake and

Tomley (2014) provide summaries of *Eimeria* life cycles that can generally be applied across species. First after ingestion and mechanical disruption, infectious, sporulated oocysts release sporocysts. Enzymes in the digestive tract break open the sporocysts, releasing sporozoites, which invade host cells (e.g., intestinal epithelium) where they develop into trophozoites. Asexual replication (mergony, previously called schizogony) forms meronts, which undergo endogenous division to form merozoites. Depending on the species, two or more rounds of this asexual replication may occur. Sexual replication (gamogony) initiates after merozoites invade cells and develop into microgametocytes or macrogametocytes, which then develop into microgametes (male) and macrogametes (female), respectively. After this development, microgametes are released and fertilise macrogametes, forming a diploid zygote. After the zygote develops an outer wall and becomes an oocyst, the parasite is excreted with faecal material. Finally, sporogony (sporozoite formation) occurs under the right conditions, leading to the development of an infective, sporulated oocyst. Figure 1.2 visually summarises the *Eimeria* life cycle.

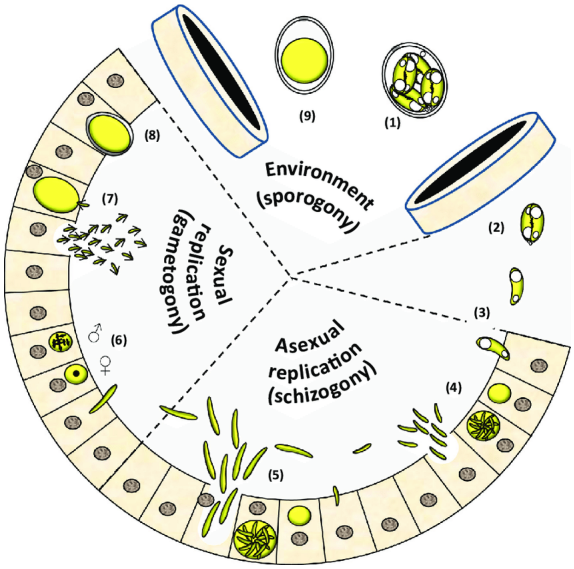


Figure 1.2. Generalised *Eimeria* life cycle. 1) sporulated oocyst; 2) sporocyst; 3) sporozoite; 4) trophozoite develops meront, and eventually release merozoites, which reinvade host cells; 5) second generation of merozoites develop and invade; 6) microgametocytes and macrogametocytes, which develop into microgametes and macrogametes, respectively; 7) fertilisation and development of zygotes; 8) zygote develops in an oocyst, forming a wall; 9) oocyst is shed. Interpreted with permission from Blake and Tomley (2014).

1.2.2.1 Host specificity

In general, *Eimeria* spp. maintain strong host specificity and are more likely to infect several closely related host species rather than switching hosts to other highly domesticated species (Bangoura and Daugschies 2018; Jirku et al. 2009; Megía-Palma et al. 2015). For example, *Eimeria bovis* does not infect sheep, nor do sheep coccidia infect cattle (Florin-Christensen and Schnittger 2018); however, *E. bovis* has been documented in wild species such as water buffalo (*Bubalus bubalis*), European bison (*Bison bonasus*), and American bison (*Bison bison*) (Dubey 2018; El-Alfy et al. 2019; Penzhorn et al. 1994; Pyziel and Demiaszkiewicz 2015). Similarly, the eight species of chicken *Eimeria* do not infect other avian species including farmed turkey (Florin-Christensen and Schnittger 2018; Kawahara et al. 2008; Vrba and Pakandl 2015). However, hooded cranes (*Grus monacha*), white-naped cranes (*Antigone vipio*), sandhill cranes (*Antigone canadensis*), and whooping cranes (*Grus americana*) have been shown using the internal transcribed spacer 2 (see Chapter 1.4.3.3) genetic data to be infected with *Eimeria gruis* and *E. reichenowi* (Honma et al. 2011a; Honma et al. 2011b; Novilla et al. 1981). Similarly, Vrba and Pakandl (2015) experimentally demonstrated the ability of three turkey (*Meleagris gallopavo*) coccidia to infect other hosts; *Eimeria dispersa* in northern bobwhite (*Colinus virginianus*); *Eimeria meleagridis* in grey partridge (*Perdix perdix*); and *Eimeria innocua* in both northern bobwhite and grey partridge were able to complete their life cycles.

Eimeria zygodontomyis, originally described in the hairy-tailed bolo mouse (*Necromys lasiurus*), was recently redescribed by de Santana Miglionico et al. (2020b) in a new,

closely related host, montane grass mouse (*Akodon montensis*). Similarly, *Eimeria vilasi* which was originally described in the eastern chipmunk (*Tamias striatus*), has now been described in a further two new hosts, round-tailed ground squirrel (*Xerospermophilus tereticaudus*) and Harris's antelope squirrel (*Ammospermophilus harrisii*) (Dorney 1962; Hnida 2019; Hnida and Flocken 2016), thereby increasing the number of species to host this parasite up to fourteen (Hill and Duszynski 1986; Ryan et al. 2001; Wilber et al. 1998). Mácová et al. (2018) provide molecular support for recent host-switching (as opposed to coevolution) of *Eimeria* between rodents, indicating the flexibility of some species of coccidia to expand to other closely related host-species. Similarly, studies on *Eimeria* of amphibians demonstrate similar host specificity, commonly infecting several host species within a genus (Hnida and Duszynski 1999; Jirku et al. 2009; Levine and Ivens 1988). Alternately, Hofmannová et al. (2016) demonstrated strong host specificity of *Eimeria* spp. that infect red squirrels (*Sciurus vulgaris*) native to Italy and introduced grey squirrels (*Scurius carolinensis*) and Pallas's squirrels (*Callosciurus erythraeus*). In this instance, all three squirrel species were hosts to a single morphotype; however, based on 18S rDNA, ITS-1 and ITS-2, as well as the mitochondrial cytochrome *c* oxidase I (COI) genes, this morphotype grouped strongly based on host species, leading the authors to predict these are morphologically cryptic species that are strongly host species specific.

The host-specificity of coccidia in kiwi has yet to be explored. Considering there are seven species of *Eimeria* that regularly affect domestic poultry, identifying further *Eimeria* spp. within all five species of kiwi in addition to the four described in brown kiwi

by Morgan et al. (2017) is highly likely (Florin-Christensen and Schnittger 2018; López-Osorio et al. 2020; Mehlhorn 2016). The five species of kiwi diverged from a common ancestor 3.8Ma (Weir et al. 2016); as kiwi diversified, it is only logical to assume their parasites likely experienced a range of evolutionary pressures as well. The immune responses to eimerian infection have been studied at length. Most importantly is the role of cell-mediated rather than humoral immune responses (Lillehoj 1987; Lillehoj 1991; Lillehoj 2007; Lillehoj and Lillehoj 2000; Rose 1987). While the latter does contribute at the time of infection, it has not been shown to strongly contribute to long-term immunity (Lillehoj 1987; Lillehoj 1991; Lillehoj 2007; Lillehoj and Lillehoj 2000). While a single, specific driver behind the most efficient cell-mediated responses has not been confirmed, genetic immune responses are key to developing an effective immune response and limiting the severity of coccidiosis (Lillehoj 1988; Yun et al. 2000). Accordingly, each species of kiwi likely responds differently to exposure to different parasite species with varying degrees of virulence, i.e., severity of disease. Further, immunity develops in response to each species a host is exposed to and is not reactive to any other coccidial species or, potentially, even strain (Barta et al. 1997; Lillehoj 2007; Yun et al. 2000). Thus, not only do different species of kiwi possess varying degrees of susceptibility to each species of *Eimeria*, but infection with a single species does not contribute to immunity to other species of *Eimeria*.

1.2.3. Treatment with toltrazuril

Effective treatment of coccidia in kiwi is a vital tool for ONE, especially when juvenile kiwi are transferred from the brooders to the outdoor enclosures, as well as at their release into either crèches or the wild. Department of Conservation (DOC) management

protocols recommend the use of toltrazuril at a dose rate of 20-25 mg/kg per os (PO) (Morgan 2008). This therapeutic treatment targets the coccidial apicoplast, a common organelle in apicomplexan parasites that provides essential metabolic functions (e.g., biosynthesis of fatty acids) and most likely originates from the endosymbiotic uptake of red algae, therefore containing a separate genome (Lim and McFadden 2010; Ralph et al. 2004; Wiesner et al. 2008). Accordingly, this plastid genome has been targeted for treatment with herbicides (e.g., aryloxyphenoxypropionate) and herbicide-derived compounds, such as toltrazuril (Hackstein et al. 1995; Wiesner et al. 2008; Zuther et al. 1999). Toltrazuril is a triazine compound that is a commonly used coccidiocide, which works to prevent or stop the development of apicomplexans by disrupting essential functions of the apicoplast as well as the mitochondrion (Darius et al. 2004b; Hackstein et al. 1995; Harder and Haberkorn 1989; Sykes and Papich 2014). In general, a single treatment has been shown to be effective in a range of host species, including birds. The development of intracellular stages of enteric coccidial infection tends to be inhibited severely by toltrazuril, thereby limiting damage to the host cells in the intestinal mucosa, renal tubules, and bile ducts (Alnassan et al. 2013; Bach et al. 2003; Darius et al. 2004b; Mehlhorn et al. 1984; Mitchell et al. 2005; Mitchell et al. 2003; Morgan et al. 2013; Mundt et al. 2005). While effective on many of these parasites, treatment with these coccidiocides may not be as effective against extraintestinal infections (Bangoura and Daugschies 2018; Haberkorn 1996; Taylor et al. 2019). Diclazuril, a related triazine compound has not been used to treat kiwi coccidia to the author's knowledge, although it is registered for use as an in-feed medication for chickens in New Zealand. Accordingly, toltrazuril together with its metabolite ponazuril (toltrazuril sulphone, which has never been approved for use in New Zealand), remains the focus of this literature review. In

New Zealand, as elsewhere, toltrazuril is commonly used to treat coccidiosis in domestic as well as commercial animals and has been a key component in coccidia management in captive kiwi for over 10 years (Morgan 2008).

1.2.3.1. Treatment regimes in mammals

In domestic mammals, treatment with toltrazuril prior (metaphylactic) and in response (therapeutic) to initial presentation of clinical signs of coccidiosis has been shown to be effective. A range of dose rates have been used in different hosts. A therapeutic dose of 20 mg/kg PO in naturally infected goat kids has been shown to significantly reduce nine species of caprine coccidia infection (Balicka-Ramisz 1999; Chartier et al. 1992; Iqbal et al. 2013). Additionally, treatment of kids and adult goats can help control levels of coccidial contamination in the environment and increase weight gain (Rehman et al. 2011). Similarly, metaphylactic treatment with toltrazuril at 20 mg/kg PO in lambs effectively decreased the shedding of *Eimeria ahsata*, *E. ovinoidalis*, as well as *E. crandallis* and led to an increase in live weight gain (Diaferia et al. 2013; Le Sueur et al. 2009; Mundt et al. 2009; Scala et al. 2014; Stafford et al. 1994). Toltrazuril at 20 mg/kg also effectively manages *Cystoisospora suis* (formerly *Isospora suis*) in piglets (Driesen et al. 1995; Joachim and Mundt 2011; Kreiner et al. 2011; Maes et al. 2007; Mundt et al. 2007; Rypula et al. 2012; Scala et al. 2009; Skampardonis et al. 2010; Streyl et al. 2015). Treatment of calves with toltrazuril at 15 mg/kg PO has been shown to be highly effective against *Eimeria zuernii*, *E. alabamensis*, and *E. bovis* (Daugschies and Najdrowski 2005; Epe et al. 2005; Jonsson et al. 2011; Mundt et al. 2005; Mundt et al. 2003). In mice, metaphylactic treatment (10 or 20 mg/kg PO) with ponazuril prevented clinical signs from infection with *Toxoplasma gondii* (Mitchell et al. 2004); two

metaphylactic doses of toltrazuril at 5 mg/kg PO one week apart is sufficient to treat experimental infections of *Eimeria falciformis* (Steinfelder et al. 2005). Furthermore, while not eliminatory, toltrazuril and ponazuril can reduce *Neospora caninum* loads in mice, lambs, and calves as well as lower abortion rates associated with neosporosis in cattle (Canatan et al. 2014; Cuteri et al. 2005; Darius et al. 2004a; Darius et al. 2004b; Gottstein et al. 2001; Gottstein et al. 2005; Kritzner et al. 2002; Qian et al. 2015; Strohbusch et al. 2009; Syed-Hussain et al. 2015). At 10 to 20 mg/kg PO, toltrazuril is an appropriate metaphylactic or therapeutic treatment for *Cystoisopora canis*, *C. burrowsi,* and *C. ohioensis* in dogs (Altreuther et al. 2011a; Altreuther et al. 2011b; Buehl et al. 2006; Daugschies et al. 2000). Similarly, Reinemeyer et al. (2007) found a therapeutic, 30mg/kg PO dose of ponazuril significantly decreased *C. canis* shedding in puppies; however, at this dose, Litster et al. (2014) did not find a significant decrease in *Cystoisopora* spp. infection rates in dogs and cats with therapeutic treatment of ponazuril. Finally, metaphylactic and therapeutic treatment in experimentally infected rabbits effectively treats *Eimeria stiedae*, *E. flavescens*, *E. intestinalis*, *E. magna*, and *E. perforans* (Cam et al. 2008; Peeters and Geeroms 1986). Toltrazuril and its metabolites are successfully and effectively used in mammal species.

1.2.3.2. Treatment regimes in birds

Toltrazuril has been shown to be equally effective in treating enteric coccidiosis in domestic avian species with a wide range of efficacy. Metaphylactic and therapeutic treatments administered for chicken (*Gallus gallus*) coccidia (*Eimeria tenella*, *E. brunetti*, *E. perfringens*, *E. maxima*) have been shown to be effective at 7 mg/kg PO, especially when treated at or before three days post infection (Alnassan et al. 2013; Claeskens et

al. 2007; Kandeel 2011; Laczay et al. 1995; Mathis et al. 2003; Mathis et al. 2004; Vertommen et al. 1990). In mule ducks (*Cairina moschata* × *Anas platyrhynchos*), Reynaud et al. (1999) demonstrated the efficacy of toltrazuril on *Eimeria mulardi* when administered two days post infection at 7 mg/kg PO; however, administration five days post infection was far less effective, indicating that toltrazuril may inhibit growth of early stages of development more effectively. In domestic pigeons (*Columba livia domestica*), metaphylactic treatment with a single dose at 20 mg/kg PO sufficiently suppressed oocyst shedding of *Eimeria labbeana* and *Eimeria columbarum* (Krautwald-Junghanns et al. 2009; Van Reeth and Vercruysse 1993; Vercruysse 1990). In Japanese quail (*Coturnix japonica*), treatment with toltrazuril at 25 mg/kg enables mucosal regeneration associated with *Eimeria tsunodai* and *Eimeria bateri* subclinical infection (Sokół et al. 2015); however, therapeutic treatment at this dosage does not necessarily eliminate quail coccidia and may have negative side effects, such as bile ductule epithelium cell necrosis (Gesek et al. 2015).

1.2.3.3. Kiwi management implications

DOC Operation Nest Egg best practice necessitates treatment with toltrazuril with 20 to 25 mg/kg PO as a means to decrease parasite loads in captive-managed kiwi chicks when infection loads of ≥ 2,000 unspeciated oocysts per gram are detected or at the presentation of clinical signs associated with faecal coccidia (Morgan 2008). With continual reinfection occurring, some birds receive almost weekly doses of toltrazuril during their 3-to-6-month juvenile growing period after they leave the brooder (Bassett 2012; Colbourne et al. 2005; K. McInnes pers. comm.; Morgan 2008). While resistance to toltrazuril has been documented in *C. suis* from Holland (Shrestha et al. 2017) and in

strains of *Eimeria acervulina* and *E. tenella* in chickens (Lan et al. 2017; Stephan et al. 1997; Vertommen et al. 1990), the evidence is limited compared to other anticoccidials used in chickens and pigs (Arabkhazaeli et al. 2013; Martin et al. 1997; Sykes and Papich 2014; Tan et al. 2017). However, the apparent low efficacy of current treatment protocols in young kiwis has been reported and remains a concern in at least some ONE rearing facilities (Taylor et al. 2019). The other consideration is that sufficient exposure to coccidia is required during this juvenile phase to sufficiently stimulate the development of cell-mediated immunity as low levels of coccidia commonly infect kiwi in the wild (Jakob-Hoff 2001; Jakob-Hoff et al. 1999; Yun et al. 2000). Thus, exposure to infective oocysts in kiwi chicks is vital to successful captive management. Reintroduction of coccidia-naïve kiwi to the wild may risk development of disease if exposed to significant environmental levels of coccidia at a time of high stress to individual birds. Some reports suggest that metaphylactic treatment with toltrazuril may inhibit the development of immunity, presumably due to effective suppression of the coccidia (Alnassan et al. 2013). For example, calves given metaphylactic treatment with toltrazuril were likely to be reinfected, which was suggested to be due to toltrazuril preventing calves from acquiring sufficient immunity to bovine coccidia (Alnassan et al. 2013; Philippe et al. 2014; Zechner et al. 2015). Nevertheless, several authors have suggested that although toltrazuril stops development of intracellular stages, hosts are still exposed to sufficient intracellular exposure (Greif 2000; Mundt et al. 2007; Peeters and Geeroms 1986; Steinfelder et al. 2005). Understanding the efficacy and potential implications of treatment protocols with toltrazuril can prevent the development of severe clinical disease in young kiwi as well as ensure the acquisition of sufficient immunity to kiwi coccidia. However, the efficacies of these treatment protocols vary

with species of coccidia, thus the *Eimeria* infecting kiwi need to be identified and characterised to allow for effective monitoring. In addition, the efficacy of toltrazuril against extraintestinal species of coccidia is yet to be reported.

1.3. Morphological characterisation of kiwi coccidia

At this time, identification of the coccidial parasites of kiwi relies on the characterisation of sporulated oocysts. Table 1.2 references, to the author's knowledge, all the documentation of novel descriptions of *Eimeria* species published in the last five years. These 43 papers contain descriptions of 62 novel species and all use morphological characterisation of the sporulated oocyst. Additionally, Thomas and Gardner (2015) and Jankovsky et al. (2017) could not provide descriptions of sporulated oocysts but provided all the morphological detail available and are also included. Only twelve papers (28%) provide molecular characterisation for 13 novel species (21%). This summary demonstrates the heavy reliance on morphological data in characterising coccidia. *Eimeria* are differentiated from other genera by four sporocysts that each contain two sporozoites (Yabsley 2008). Using this stage of development, four species of *Eimeria* have been described in kiwi (Morgan et al. 2017). *Eimeria paraurii* is ovoid (32.2 × 19.8 μm; 1.6 length to width, LW, ratio) with a rough, striated outer wall and a micropyle (a thin region on the oocyst wall; Mehlhorn 2016) visible microscopically (Figure 1.3A). *Eimeria apteryxii* is ovoid to pyriform (23.9 × 14.9 μm; 1.6 L/W ratio) with a smooth wall and visible micropyle (Figure 1.3B). *Eimeria mantelii* is ovoid and tapers to a narrow end (17.8×10.7 μm; 1.7 L/W ratio) with a smooth wall and no visible micropyle (Figure 1.3C).

Eimeria kiwii is spherical to subspherical (14.8 × 13.9 μm; 1.1 L/W ratio) with a striated wall and no visible micropyle (Figure 1.3D).

While oocyst length and width are extremely valuable, the length/width ratio provides a quantitative reference to the shape of the oocysts, allowing for additional statistical comparisons. Another means of comparison is the site of infection of endogenous stages in their hosts. For example, one of the main differentiating characteristics of *Eimeria dispersa* and *Eimeria innocua* in turkeys is the anatomical section of the intestines they invade (Vrba and Pakandl 2014). Based on endogenous stages alone, Morgan et al. (2012; 2013) was able to hypothesise the number of species of coccidia within the available samples. While this information is vital to understand the pathogenicity and virulence of a particular parasite in a particular host species, this data can only be collected post-mortem, thus, is limited to rare opportunistic observations. Another factor that could provide helpful information is the prepatent period, i.e., the time between inoculation and shedding of oocysts (Bangoura and Daugschies 2018). The prepatent periods of coccidia can vary tremendously (Table 1.3) but could provide keys to differentiating species of *Eimeria* in both crèche and captive settings. There is no data on the prepatent periods of coccidia in kiwi.

Table 1.2. Use of morphology and genetic data in novel descriptions of *Eimeria* species published from 2015 to 2020.

***Eimeria* n. sp.**	**Host**	**Location**	**Morph.**	**Gen.**	**Reference**
E. tsogoi	Midday gerbil, *Meriones meridianus*	Mongolia	+	-	Jensen et al. (2015)
E. pileata	Rufous-capped brushfinch, *Atlapetes pileatus*	Mexico	+	-	Soriano-Vargas et al. (2015)
***Eimeria* sp.**	Plains pocket mouse, *Perognathus flavescens*	USA	+	-	Thomas and Gardner (2015)*
E. lyoni	Mourning dove, *Zenaida macroura carolinensis*	USA	+	-	Yabsley et al. (2015)
E. collieie	southwestern snake-necked turtle, *Chelodina colliei*	Australia	+	+	Yang et al. (2015b)
E. donaldi ***E. nyumbu*** ***E. burchelli*** ***E. sokoine***	Blue wildebeest, *Connochaetes taurinus*	Tanzania	+	-	Debenham et al. (2016)
E. pavota ***E. egyptica***	Indian peafowl, *Pavo cristatus*	Egypt	+	-	El-Shahawy (2016)
E. barbarae	Barbary partridge, *Alectoris barbara*	Spain	+	-	Fernandez-Alvarez et al. (2016)
E. angustus	Southern brown bandicoot, *Isoodon obesulus*	Australia	+	+	Hillman et al. (2016)
E. schneideri	Common flat-tail gecko, *Uroplatus fimbriatus;* Satanic leaf-tailed gecko, Uroplatus phantasticus	Madagascar	+	-	McAllister et al. (2016)
E. tuttui	Barren-ground caribou, *Rangifer tarandus groenlandicus;* Caribou, *Rangifer tarandus tarandus*	Greenland	+	-	Skirnisson and Cuyler (2016)
E. burdi	Burrowing bettong, *Bettongia lesueur*	Australia	+	-	Slapeta et al. (2016)
E. antidorcasi ***E. versfeldi*** ***E. gasawayi***	Springbok, *Antidorcas marsupialis*	Namibia	+	-	Turner et al. (2016)
E. jiangi	Przewalski's gazelle, *Procapra przewalskii*	China	+	-	Wang et al. (2016)
E. labbeana-like	Domestic pigeon, *Columba livia domestica*	Australia	+	-	Yang et al. (2016a)
E. purpureicephali	Red-capped parrot, *Purpureicephalus spurius*	Australia	+	-	Yang et al. (2016d)
E. nhecolandensis ***E. jansenae*** ***E. fosteri***	Paraguayan punaré, *Thrichomys fosteri*	Brazil	+	-	Barreto et al. (2017)

E. corumbaensis	Broad-headed spiny rat, *Clyomys laticeps*	Brazil China	+ +	- +	Barreto et al. (2017) Cui et al. (2017)
E. kongi	Earopean rabbit, *Oryctolagus cuniculus*				
***Eimeria* sp.**	Great-horned owl, *Bubo virginianus*	USA	-	+	Jankovsky et al. (2017)†
E. iovai	De Vis's emo skink, *Emoia pallidiceps;* Littoral whiptail-skink, *Emoia atrocostata*	Papua New Guinea	+	-	McAllister et al. (2017a)
E. kirkpatricki	Littoral whiptail-skink, *Emoia atrocostata;* Copper-tailed skink, *Emoia cyanura;* dark-bellied copper-striped skink, *Emoia impar;* Loveridge's emo skink, *Emoia loveridgei;* De Vis's emo skink, *Emoia pallidiceps*	Papua New Guinea USA	+ +	- -	McAllister et al. (2017a) McAllister et al. (2017b)
E. stevejayuptoni ***E. emoia***	Shrub whiptail-skink, *Emoia longicauda*				
E. doddi	Ornate box turtle, *Terrapene ornata*				
E. tkachi	Southern short-tailed shrew, *Blarina carolinensis*	USA	+	-	McAllister and Seville (2017)
E. sassei	Eastern small-footed bat, *Myotis leibii*	USA	+	-	McAllister et al. (2017c)
E. taggarti	Yellow-footed antechinus**,** *Antechinus flavipes*	Australia	+	+	Amery-Gale et al. (2018)
E. akodonensis	Montane grass mouse, *Akodon montensis*	Brazil	+	-	de Santana Miglionico et al. (2018)
E. syrichta ***E. boholensis***	Philippine tarsier, *Tarsius syrichta*	Philippines	+	+	Hofmannová et al. (2018)
***Eimeria* sp.**	Takahē, *Porphyrio hochstetteri*	New Zealand	+	-	Jolly (2018)‡
E. raichoi	Rock ptarmigan, *Lagopus muta japonica*	Japan	+	+	Matsubayashi et al. (2018)
E. dunni	Southern black racer, *Coluber constrictor priapus*	USA	+	-	McAllister et al. (2018)
E. vison-like	American mink, *Neovison vison*	Denmark	+	+	Petersen et al. (2018)
E. psittacarae	White-eyed parakeet, *Psittacara leucophthalmus*	Brazil	+	-	Tucunduva et al. (2018)
E. pantholopensis ***E. wudaoliangensis*** ***E. hodgsonii*** ***E. schalleri*** ***E. sui***	Tibetan antelope, *Pantholops hodgsonii*	China	+	-	Cao et al. (2019)

E. ammospermophili	Harris's antelope squirrel, *Ammospermophilus harrisii*	USA	+	-	Hnida (2019)
E. hochatownensis	Barn swallow, *Hirundo rustica*	USA	+	-	McAllister and Hnida (2019)
E. lukfataensis	Eastern gray squirrel, *Sciurus carolinensis*	USA	+	-	McAllister et al. (2019)
E. aegoliusia	Northern saw-whet owl, *Aegolius acadicus*	Mexico	+	-	Medina et al. (2019)
E. woyliei	brush-tailed bettong, *Bettongia penicillata*	Australia	+	+	Northover et al. (2019)
E. riparii	Mouse-eared bat, *Myotis riparius* Handley	Brazil	+	-	de Santana Miglionico et al. (2020a)
E. montensis ***E. uricanensis*** ***E. parnasiensis***	Montane grass mouse, *Akodon montensis*	Brazil	+	-	de Santana Miglionico et al. (2020b)
E. melogale	Javan ferret-badger, *Melogale orientalis*	Czech Republic	+	+	Hofmannová et al. (2020)
E. machardyi	Eastern woodrat *Neotoma floridana*	USA	+	-	McAllister and Hnida (2020)
E. tamimi	Arabian Rock Hyrax, *Procavia capensis jayakari*	Saudi Arabia	+	+	Mohammed et al. (2020)
E. columbinae	Ruddy ground dove, *Columbina talpacoti*	Brazil	+	+	Ortúzar-Ferreira et al. (2020)

*This study does not identify this *Eimeria* as novel, as the oocysts did not sporulate completely, thereby limiting the information available to the authors. This study is included in this Table as an example of a scenario similar to kiwi coccidia.

†This study only had access to endogenous stages of this *Eimeria* and combined molecular characterisation with endogenous characteristics of documented coccidia from this host, leading them to conclude that this coccidia was likely to be a novel species but declined to definitively say without sporulated oocysts.

‡Not peer reviewed.

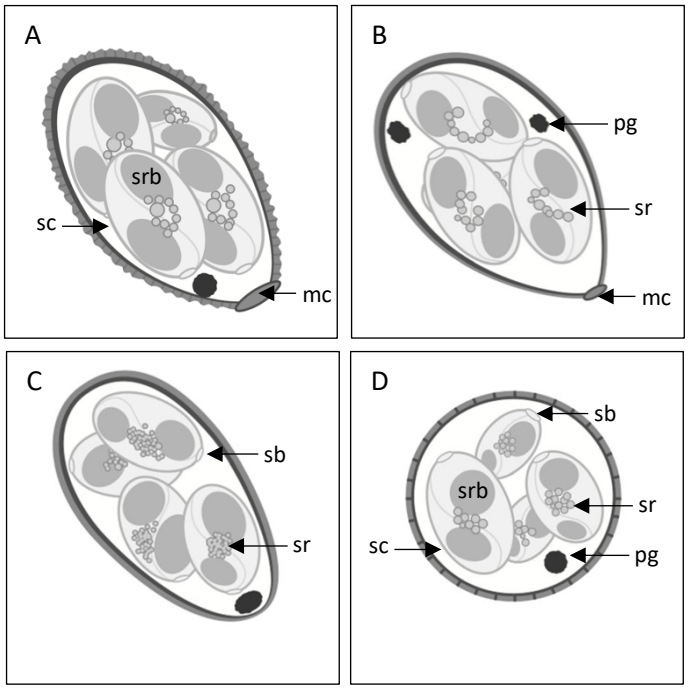


Figure 1.3. Line drawings of sporulated oocysts for A) *Eimeria praurii*; B) *Eimeria apteryxii*; C) *Eimeria mantelii*; D) *Eimeria kiwii*. Polar granules (pg), micropyles (mc), Steida bodies (sb), sporocysts (sc), sporocyst residuum (sr), and sporozoite refractile bodies (srb) are indicated. Interpreted with permission from Morgan *et al.* (2017).

Table 1.3. Prepatent period lengths and sporulation rates of common domestic and agricultural *Eimeria*. This table was interpreted from Bangoura and Daugschies (2018) and edited to focus on most relevant host and coccidial species.

Host	Species	Sporulation (h)	Prepatency (d)
Cattle	*E. bovis*	48-72	21-23
	E. zuernii	48-240	15-22
	E. alabamensis	96-192	6-11
Sheep	*E. ovinoidalis*	24-72	12-15
	E. ashata	48-72	18-21
Goat	*E. arloingi*	24-96	14-20
	E. christenseni	72-144	14-23
Hog	*E. neodebliecki*	13 days	10
	E. debliecki	6-9 days	7
Chicken	*E. necatrix*	18-24	5-7
	E. maxima	30-48	5-6
	E. acervulina	24	4
	E. tenella	18-48	6-7
	E. mitis	18-24	4-5

	E. praecox	48	4
	E. brunetti	24-48	5
Turkey	*E. adenoeides*	24	4.5-5.5
	E. gallopavonis	24	6-7
Rabbit	*E. media*	48	5-7
	E. intestinalis	72-96	9-10
Pigeon	*E. labbeana*	up to 3 days	6-8
Goose	*E. truncata*	24-120	5-14
	E. nocens	60-72	4-9
Duck	*E. mulardi*	24-72	5.5-7
Horse	*E. leuckarti*	15-41	31-34
Guinea Pig	*E. caviae*	2-11	7-11

1.3.1. Sporulation of *Eimeria*

Prior to sporulation, the excreted oocysts are not infective and contain a single sporont with few distinguishing characteristics visible under a standard microscope. The sporont (Figure 1.4), containing a nucleus and other vital organelles, undergoes sporogony (Bangoura and Daugschies 2018; Sibert and Speer 1980). During this process in *Eimeria*, four sporocysts develop, each containing two sporozoites (Figure 1.5). Each sporocyst contains a residuum that likely provides key nutrients for the sporozoites and is necessary to maintain viability over an extended period of time (Bangoura and Daugschies 2018; Seemann et al. 2012). Temperature can affect the rate or success of sporogony; in general oocysts have evolved to endure in their natural environments (Bertram et al. 2015; Landers 1953). Thus, storage and sporulation procedures should reflect the environmental conditions these species would typically encounter in order

to maintain viability. Table 1.3 summarises sporulation times of some common *Eimeria* species.

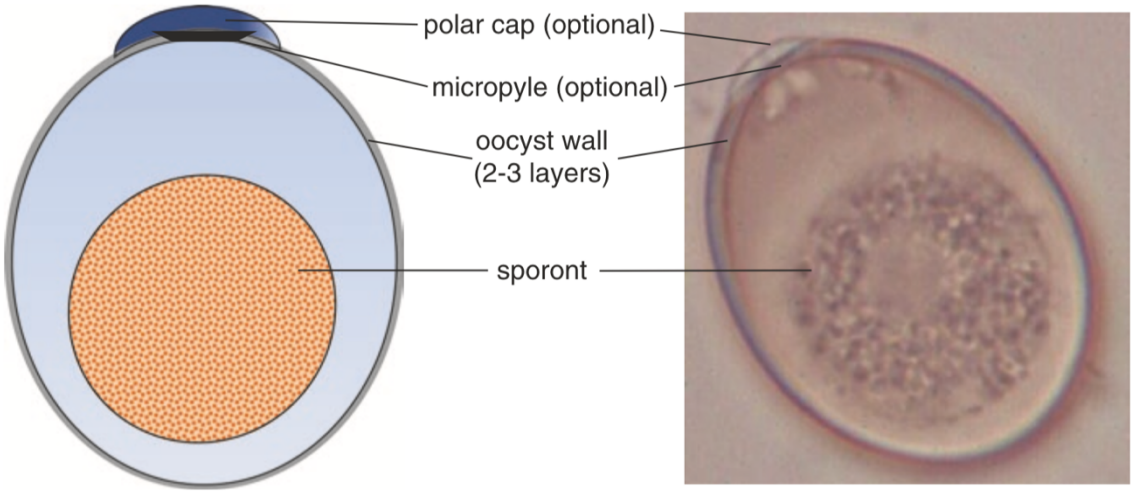


Figure 1.4. Example of unsporulated oocyst (*Eimeria brasiliensis* from cattle) containing a sporont and few other distinguishing features. Reproduced by permission of Springer Nature from Bangoura and Daugschies (2018).

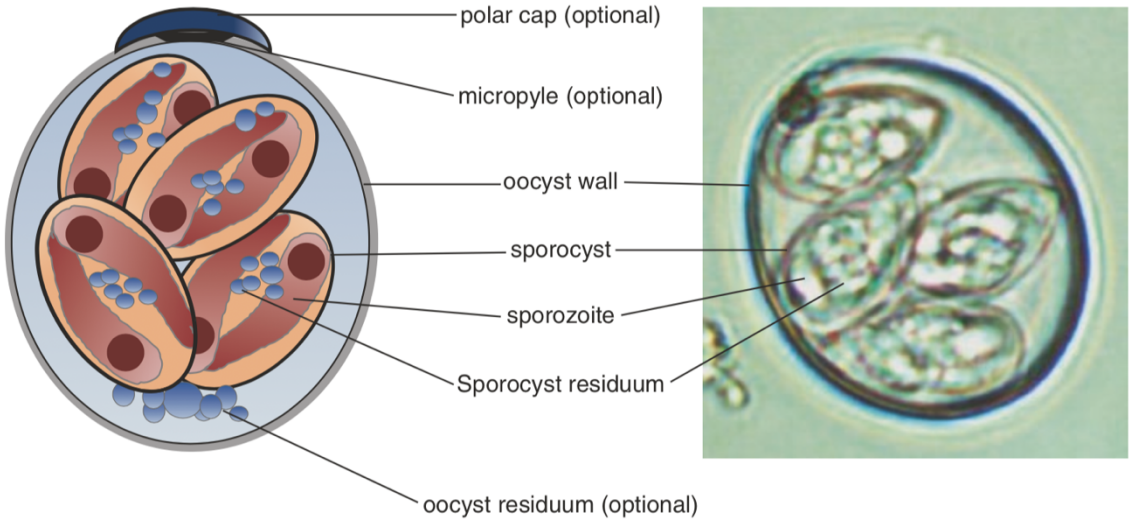


Figure 1.5. Distinguishing morphological features of a sporulated oocyst (*Eimeria maxima* from a chicken). Reproduced by permission of Springer Nature from Bangoura and Daugschies (2018).

1.3.2. Oocyst isolation

Isolation of oocysts from faecal material before or after sporulation is accomplished using a flotation solution combined with centrifugation and other apparatuses to separate the debris from the oocysts. The technique relies on the salt or sugar flotation solution having a specific gravity higher than that of the oocysts and other faecal material (Daugschies et al. 1999; Yabsley 2008). The minimum specific gravity (SG) necessary to float coccidial oocysts is 1.10 (Anderson and Rings 2008). A wide variety of different flotation media have been used. Sodium chloride (NaCl, 1.20 SG) is commonly used and is effective for helminth eggs and larvae as well as coccidial oocysts; however, the high osmotic pressure can lead to a distortion of oocysts (Bauer et al. 2010; Cantacessi et al. 2008; Cringoli et al. 2017; Cringoli et al. 2010; Dolnik et al. 2009; El-Sherry et al. 2014; Jeanes et al. 2013; Jonsson et al. 2011; Mundt et al. 2003; Norton and Chard 2009; Ogedengbe et al. 2013; Peek et al. 2017; Silva et al. 2013). Similarly, Sheather (1923) describes the development and use of a sucrose solution (1.2 to 1.25 SG) for isolation of coccidia in dogs, cats, pigs, an elephant, a squirrel, a pheasant, and a pigeon (11.5 to 50 μm in length). Fernandez-Alvarez et al. (2016) used this solution to detect *Eimeria* oocysts (16 to 23 μm) from a Barbary partridge (*Alectoris barbara*). Morgan et al. (2017) used Sheather's sugar to isolate oocysts from brown kiwi (9.4 to 44 μm in length). Zinc sulphate (1.18 to 1.2 SG) has been used for helminth eggs (Bauer et al. 2010; Lyons et al. 1972; Watson 1947). Further, zinc sulphate is the standard solution for diagnostic screening at the Massey University School of Veterinary Science Parasitology Lab (W. Pomroy, pers. comm.) as it does not exert extreme osmotic pressure on oocysts. Similarly, magnesium sulphate (1.28 SG) has been used to isolate nematode eggs (65 to 90 μm in length) in sheep, dogs, chickens as well as *Eimeria*

mulardi oocysts (19 to 23 μm in length) from hybrid ducks (Bauer et al. 2010; Chauve et al. 1994; Craig 2009; Cringoli et al. 2010; Mehlhorn 2016; Vadlejch et al. 2011; Zenner et al. 2002).

ONE facilities monitor levels of coccidial burdens in individual kiwi or groups of kiwi occupying a single pen by submitting fresh faecal samples to diagnostic laboratories for modified centrifugal faecal flotation (CFF) (Bassett 2012; Egwang and Slocombe 1982; Ministry of Agriculture 1986). Similar to CFF, the McMaster method, in which aliquots of faecal samples homogenised in flotation solution are transferred into a slide-like counting chamber, is used for detection of larvae and nematode strongylid eggs and coccidia in a variety of animals; coccidia in domestic and wild birds, pigs, dogs, rabbits, cattle and goats*;* and nematode eggs in horses, sheep and dogs (e.g., Alnassan et al. 2013; Altreuther et al. 2011a; Altreuther et al. 2011b; Bach et al. 2003; Balicka-Ramisz 1999; Bauer et al. 2010; Bortoluzzi et al. 2018; Bosco et al. 2018; Buehl et al. 2006; Cam et al. 2008; Chartier et al. 1992; de Souza Rodrigues et al. 2016; Egwang and Slocombe 1981; Epe et al. 2005; Fei et al. 2013; Fernandez-Alvarez et al. 2016; Gerhold et al. 2010; Gerhold et al. 2011; Silva et al. 2013; Vadlejch et al. 2011). However, studies have shown that a relatively new flotation/counting chamber (mini-FLOTAC; Cringoli et al. 2017; Cringoli et al. 2010) is more accurate and precise than the McMaster method for the detection of nematode eggs in horses and sheep nematode larvae (Bauer et al. 2010; Bosco et al. 2018; Noel et al. 2017; Ramos et al. 2018; Scare et al. 2017), as well as *Eimeria* spp. in poultry (Bortoluzzi et al. 2018) and goats (Silva et al. 2013).

1.3.3. Oocyst sporulation

Sporulation is the process by which *Eimeria* oocysts develop sporocysts then infective sporozoite stages necessary for morphological characterisation (see Chapter 1.3.1). The ideal conditions for sporulation vary from species to species, and if environmental factors (e.g., humidity, temperature, oxygen) are too harsh, sporulation can be slowed, but halting the development of all the oocysts in a sample is unlikely (Bangoura and Daugschies 2018; Chapman et al. 2013; Marquardt et al. 1960; Parker and Jones 1990; Venkateswara Rao et al. 2015; Yabsley 2008).

Following oocyst detection, faecal samples are usually mixed with 2% w/v potassium dichromate ($K_2Cr_2O_7$) or solutions containing antifungal and antibacterial agents at a ratio of at least one part faeces to five parts $K_2Cr_2O_7$ (Duszynski and Wilber 1997). Broadly speaking, an incubation at 20 to 23°C over a period of 1 to 3 days (see Table 1.3) leads to sporulation (Bangoura and Daugschies 2018; Duszynski and Wilber 1997; Williams et al. 2010).

1.3.4. Limitations of morphology

While sporulated oocysts provide key, identifiable characteristics and are easily isolated from the host, relying completely on morphology together with knowledge of the host species (see Chapter 1.2.2.1) can be misleading (Carreno and Barta 1999; Duszynski and Wilber 1997; Hillebrand et al. 2001; Hofmannová et al. 2016; Long and Joyner 1984). For example, Hofmannová et al. (2016) morphologically identified a morphotype of *Eimeria* as being the same in three species of squirrels (one native, two introduced) and was

shown to be three genetically distinct species of *Eimeria*. Further, key morphological characteristics can be affected by processing as well as storage conditions, which can contribute to overlooking genetically cryptic species (Al-Habsi et al. 2017; Duszynski and Wilber 1997; Ogedengbe et al. 2011a; Ogedengbe et al. 2018).

For brown kiwi, Morgan (2013) reported difficulties sporulating coccidia oocysts, which adds another limiting factor to identifying oocysts. When sporulation is unreliable, morphologically similar species may remain undifferentiated or species that sporulate more easily may be preferentially identified (Mehlhorn 2016). Such difficulties are not uncommon and molecular markers have the potential to help differentiate apicomplexan species and identify the presence of drug resistant strains (Joachim et al. 1996; Tan et al. 2017; Yabsley 2008). While *E. kiwii* and *E. paraurii* are very distinct and identifiable without sporulation, *E. mantellii* and *E. apteryxii* are close enough in size that differentiation could be hindered without further oocyst development. Additionally, other morphologically similar species that have yet to be identified may be missed without reliable sporulation techniques or methods of molecular identification.

To date, the morphological descriptions of coccidia in kiwi currently available are based on samples from six brown kiwi from only two locations. It would be expected that there could be more diversity of coccidia across New Zealand (Morgan 2013; Morgan et al. 2017). However, ONE operations have moved kiwi between and within the islands of New Zealand, as well as housed and reared kiwi of two or more taxa or species in the same facility (K. McInnes, pers. comm.). This overlap may have led to exposure to

parasites with which certain species are less immunologically adapted to cope (see Chapter 1.2.2.1; Lillehoj 1988; Yun et al. 2000). For example, brown kiwi kept in nocturnal houses on the South Island may have brought species of *Eimeria* that the Haast tokoeka have never been exposed to before human intervention, and therefore could theoretically cause more severe disease in tokoeka than in brown kiwi, if there is some innate immunity of the natural host of the parasite. Thus, in depth characterisation is needed to ensure identification of pathogenic species of *Eimeria* are monitored at an appropriate level for the host species.

1.4. Molecular characterisation

1.4.1. The genomes of *Eimeria*

1.4.1.1. Nuclear genome

The eimerian nuclear genome is comprised of fourteen chromosomes (Blake et al. 2020; del Cacho et al. 2005; Shirley 2000). Only nine genomes of *Eimeria* (*Eimeria tenella, E. maxima, E. necatrix, E. acervulina, E. brunetti, E. mitis, E. praecox, E. nieschulzi,* and *E. falciformis*) have been documented with varying degrees of depth (Blake et al. 2012; Blake et al. 2020; Heitlinger et al. 2014; Reid et al. 2014; Shirley 2000; Wiedmer et al. 2017). The few sites that have been routinely used for detection and characterisation of *Eimeria* spp. consist of small subunit (SSU) and large subunit (LSU) ribosomal RNA (rRNA) genes as well as two internal transcribed spacers (ITS) (Beck et al. 2009; Lavrinienko et al. 2020; Morrison et al. 2004). These rRNA (18S, 5.8S, 28S) and ITS (1 and 2) genes are sequentially located, in *E. tenella*, on chromosome 12 (Figure 1.6; Lavrinienko et al. 2020; Shirley 2000). The copy-number of this ribosomal DNA (rDNA) sequence likely

varies greatly within and between species (Kobayashi 2014; Lavrinienko et al. 2020; Prokopowich et al. 2003). For example, *Plasmodium falciparum* contains 5-8 copies of the 18S gene (Mercereau-Puijalon et al. 2002) and *Toxoplasma gondii* contains 110 copies per tachyzoite (Guay et al. 1993). Thus, while *Eimeria tenella* has been reported to have 140 copies of the 18S in each haploid genome (i.e., 1120 copies per sporulated oocyst; Lim et al. 2012; Yang et al. 2014c), other species of *Eimeria* likely vary in the number of copies of this gene complex. The repetitive nature of this gene makes it more easily detectable via polymerase chain reaction; however, this also affects the usefulness of this target for quantitative studies as the number of repeats can change over time within a single cell (Kobayashi 2014; Lavrinienko et al. 2020).

Figure 1.6. Schematic of nuclear ribosomal DNA in coccidia (Lavrinienko et al. 2020; Shirley 2000). The SSU rRNA gene complex (18S-ITS1-5.8S-ITS-2) can repeat many times. Lengths of genes are not perfectly to scale.

1.4.1.2. Mitochondrial genome

The mitochondrial genome of *Eimeria* is about 6,200 bp long with three protein-coding genes: cytochrome *b*; cytochrome *c* oxidase subunit I (COI); and cytochrome *c* oxidase subunit III (Blake et al. 2020; Hafeez et al. 2015b; Hikosaka et al. 2011). Fragments encoding the LSU and SSU rRNA are spread between the protein-coding regions. The number of these rRNA fragments ranges between *Eimeria* species. For example, 15 LSU and 11 SSU rDNA fragments have been reported from some *Eimeria* species of turkeys

(*Meleagris gallopavo*), whereas *Eimeria innocua* contains 19 LSU and 14 SSU rRNA fragments (Hafeez et al. 2015b; Ogedengbe et al. 2014). These are arranged in a linear, concatemeric formation, such that the layout described in Figure 1.7 repeats (Hafeez et al. 2015b; Hikosaka et al. 2011). Hikosaka et al. (2011) estimated that there are about 50 copies of the mitochondrial genome for each haploid nuclear genome in *Eimeria tenella*; however, Heitlinger et al. (2014) estimated about 180 copies per *Eimeria falciformis* cell.

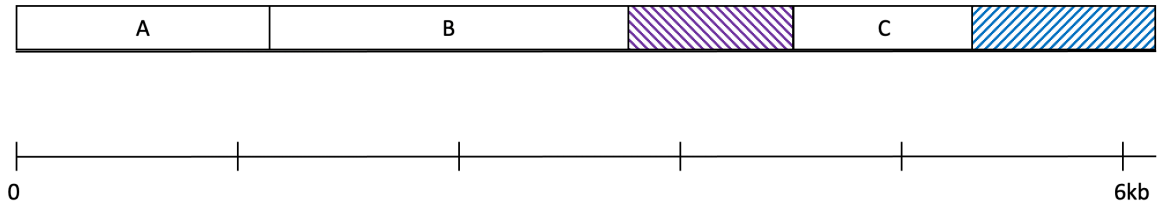


Figure 1.7. Map of the eimerian mitochondrial genome (Hafeez et al. 2015b). This genome contains three protein-coding regions: A) cytochrome *b*; B) cytochrome *c* oxidase subunit I; and C) cytochrome *c* oxidase subunit III. The purple and blue sections contain rRNA gene fragments, the number of which vary by species of *Eimeria*.

1.4.1.3. Apicoplast genome

The apicoplast, a non-photosynthetic chloroplast, i.e., a plastid, contains a circular genome about 35,000 base pairs long (Blake et al. 2020; Cai et al. 2003; Douglas 1999; Wiesner et al. 2008). This genome codes for tRNA, SSU rRNA fragments, LSU rRNA fragments, as well as several types of proteins (Arisue and Hashimoto 2015; Cai et al. 2003). *Eimeria falciformis* contains a predicted 18-19 copies per cell (Heitlinger et al. 2014). Few studies have used this genome for phylogenetic studies. Though this genome

remains a source of potential species-specific detection, nuclear and mitochondrial genome targets remain the most useful for phylogenetic comparisons (Blake et al. 2020; Jarquín-Díaz et al. 2019).

1.4.2. Genetic targets overview

Identifying genetic differences by sequencing of parasite DNA can be used to help differentiate between *Eimeria* species and also detect variations within species (i.e., different strains). This requires identifying and utilising appropriate molecular targets as well as level of analysis and is used in conjunction with morphological and epidemiological characteristics to provide accurate identification and classification of apicomplexans. For example, Carreno et al. (1999) molecularly demonstrated using the SSU rDNA that *Cryptosporidium*, an oocyst-forming apicomplexan parasite with morphological developmental characteristics similar to coccidia, is more closely related to gregarines in the subclass Gregarinasina that form neither cysts nor oocysts (Mehlhorn 2016). In contrast, however, more in-depth, larger-scale analysis of SSU rDNA in *Eimeria* demonstrated less variability than may be required to reliably inform phylogenetic comparisons of *Eimeria* and other closely related genera (Morrison et al. 2004). This review addresses relevant rDNA targets that have been frequently used in coccidial identification (either alone or in conjunction with other genes), thereby providing a larger dataset for comparison.

Several other genes have been shown to be useful in studying *Eimeria* as amplification-based detection and sequencing technologies have advanced (Beck et al. 2009; Jatau et

al. 2016). These genes include the internal transcriber spacer genes and the mitochondrial cytochrome *c* oxidase subunit 1. The use of standardised morphological descriptions in conjunction with informative molecular targets ensures the detection of genetically or morphologically cryptic species (Adl et al. 2007; Clark et al. 2016; Duszynski and Wilber 1997; Hofmannová et al. 2016; Jatau et al. 2016; Yabsley 2008; Yabsley and Gibbs 2006). Determining which target will be appropriate for identifying kiwi coccidia is key for differentiation and, therefore, management of these parasites. This challenge is approached by finding targets that have been useful for other, potentially closely related, species of *Eimeria*.

1.4.3. Conventional polymerase chain reaction

Mullis et al. (1986) provides a summary of conventional polymerase chain reactions (PCR). Briefly, PCR is a method of targeting regions of DNA from 100 to thousands of base pairs using primers, which are short (~20 bp) single strands of DNA that are specific to the target region. The target DNA in the sample without amplification is present in such small quantities that it is not able to be differentiated from non-target DNA. So, the target-specific primers are paired with an enzyme that extends DNA only from the ends of the primers. Through a series of temperature fluctuations (i.e., thermocycling), the enzymes replicate DNA only at the target sites until there is a large amount of the target. The amplified target is of a known length and is then detectable through gel electrophoresis and sequencing (see Chapter 1.5). The typical conventional PCR is illustrated in Figure 1.8. The double stranded DNA is denatured at 94-98°C, allowing for the primers to anneal to the target site at ~55 to 70°C. The temperature is then brought up to 68 to 72°C to allow the enzyme (usually *Taq* DNA polymerase) to extend the DNA

from the primer (extension). This cycle is then repeated up to 40 times, increasing the amount of target DNA exponentially each cycle. This method is frequently used for molecular characterisation of coccidia targeting the 18S, 28S, 5.8S, ITS-1, and/or ITS-2 regions of rDNA (Figure 1.5), which have been useful to explore the phylogenetic relationships among apicomplexans (Barta 1997; Tenter et al. 2002).

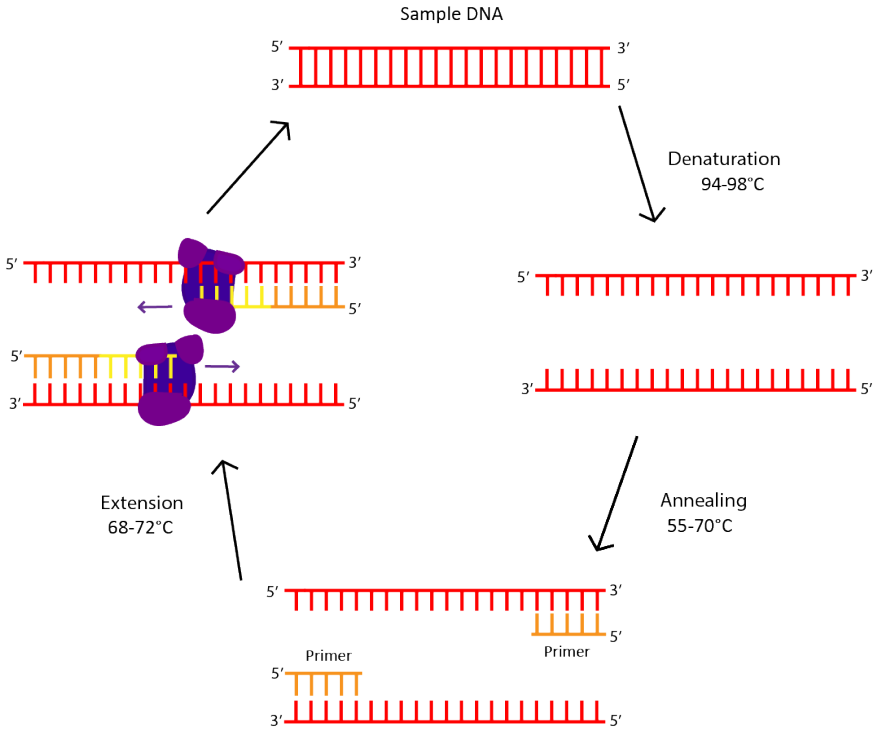


Figure 1.8. Summary of a generalised conventional polymerase chain reaction. Adapted from https://www.thermofisher.com/nz/en/home/life-science/cloning/cloning-learning-center/invitrogen-school-of-molecular-biology/pcr-education/pcr-reagents-enzymes/pcr-cycling-considerations.html

1.4.3.1. 18S rDNA

While the copy number may vary greatly between species of *Eimeria*, the 18S rDNA is a major source of phylogenetic comparisons among eukaryotes (Hall and Beiko 2018;

Kobayashi 2014). Barta et al. (1997) used the full 18S rDNA (~2,000 bp) to phylogenetically distinguish eight *Eimeria* spp. (*Eimeria acervulina*, *Eimeria brunetti*, *Eimeria maxima*, *Eimeria mitis*, *Eimeria praecox*, *Eimeria necatrix*, *Eimeria mivati,* and *Eimeria tenella*) of chickens. Power et al. (2009) developed 18S rDNA primers (1620 bp) for *Eimeria trichosuri* in marsupials. These primers have subsequently been used in nested and one-step protocols designed for *Eimeria* in macropods (Yang et al. 2012); *Eimeria haematoid* in a rainbow lorikeet (*Trichoglossus haematodus)* (Yang et al. 2015d); *Eimeria purpureicephali* in a red-capped parrot (*Purpureicephalus spurius*) (Yang et al. 2016d); *Choleoeimeria pogonae* in a western bearded dragon (*Pogona minor minor*) (Yang et al. 2016c); and *Isospora manorinae* in a yellow-throated miner (*Manorina flavigula wayensis*) (Yang et al. 2017). Schwarz et al. (2009) also developed a protocol to target the 18S rDNA gene (1790 bp) of coccidia in chickens, which Jeanes et al. (2013) subsequently applied to identify *Eimeria crecis* and *Eimeria nenei* in corncrakes (~1100 bp). Relman et al. (1996) developed a nested protocol to target *Cyclospora* spp. in humans. From this, Bertram et al. (2015) used the outer primer pair to amplify a 348 bp region of the 18S rDNA of *E. gruis* and *E. reichenowi* in whooping cranes. Likewise, Jenkins et al. (2006) developed a primer set that targeted 335 bp of the 18S rDNA region, which also amplified a 350 bp region in brown kiwi *Eimeria* spp. (Morgan 2013). More recently, Jankovsky et al. (2017) used a primer set designed to target a fragment of the 18S rDNA region (468 bp) in *Cyclospora* (Yamada et al. 2014) to molecularly characterise a novel *Eimeria* sp. in two great-horned owls (*Bubo virginianus*). However, despite this gene target proving useful in various studies, it has a limitation in that it is only able to identify coccidia to the genus level; therefore, the 18S rDNA gene is useful for broad classification, but likely would not differentiate between species of kiwi coccidia.

1.4.3.2. 28S rDNA

Similar to the 18S rDNA region, Mugridge et al. (1999) developed primers that amplified about 1500 bp of the 28S rDNA region of coccidia of the *Sarcocystis* genus, which have subsequently been applied to identify *Isospora* spp. (Schrenzel et al. 2005). More recently, these primers have been used as external primers for nested PCR protocols (yielding about 1400 bp) to target *Eimeria paludosa* in a dusky moorhen (*Gallinula tenebrosa*) (Yang et al. 2014a); *Isospora anthochaerae* in a red wattlebird (*Anthochaera carunculata*) (Yang et al. 2014b); *Eimeria labbeana-like* in a domestic pigeon (*Columba livia domestica*) (Yang et al. 2016a); and *Isospora serinuse* in a domestic canary (*Serinus canaria forma domestica*) (Yang et al. 2015a). Matsubara et al. (2017) analysed 18S (1,162 bp) and 28S (598 bp) rDNA of an *Isospora* sp. in a domestic pigeon. However, in these studies, these targets did not provide sufficient data to phylogenetically characterise these any further, indicating additional genes should be considered. In contrast, phylogenetic analysis of the 18S rDNA region (~1,700 bp) identified six groups within *Eimeria reichenowi* in red-crowned cranes (*Grus japonensis*), hooded cranes (*Grus monachal*), and white-naped cranes (*Grus vipio*) (Honma et al. 2011b). Combined with host specificity data, this analysis suggests that this coccidia, which had been classified using morphologic criteria, may be made up of several cryptic species (Honma et al. 2011b). The 28S rDNA region contributes valuable data for at least genus level and potentially species level differentiation when molecularly characterising coccidia and should be considered for genetic analysis.

1.4.3.3. Internal transcribed spacers 1 and 2

The high copy number of the 18S rDNA complex makes these spacers another candidate for easy detection of *Eimeria*. Furthermore, as these targets are flanked by the 18S and 28S rDNA regions and separated by the 5.8S (Figure 1.6) rDNA region, the ITS regions can be amplified with flanking genus-specific primers that can overlap the length of one or both genes. As introns they are able to be more diverse between species and more likely to be conserved within a species (Kumar et al. 2015). Consequently, analysis of the ITS-1 and ITS-2 regions, which evolve more quickly than 18S, 5.8S, and 28S rDNA, allows for phylogenetic comparisons within a species and have been used extensively for chicken coccidia (Barta et al. 1998; Kumar et al. 2015; Schnitzler et al. 1998; White et al. 1990). For example, when these regions were targeted using denaturing polyacrylamide gel electrophoresis and single-strand conformation polymorphism analysis, Woods et al. (2000a) established DNA profiles that differentiate *E. acervulina*, *E. brunetti*, *E. maxima*, *E. necatrix*, and *E. tenella*. Using these primers for PCR, the ITS-1 and ITS-2 regions have been used for identification of these five *Eimeria* spp. in chickens (Woods et al. 2000b) as well as *Eimeria crecis* and *Eimeria nenei* in corncrakes (Jeanes et al. 2013). When Morgan (2013) applied ITS-1 primers developed by Schnitzler et al. (1998) for poultry coccidia, amplicons ranged from 380 to 700 bp; however, only a 362 bp amplicon from brown kiwi (n = 3) could be sequenced. Similarly, Lew et al. (2003) found strains of *E. maxima* in chickens in Australia that were not amplified by chicken *Eimeria* specific primers (Barta et al. 1998; Schnitzler et al. 1998) and subsequently designed a nested PCR protocol targeting the coccidial ITS-1 (~400 to 600 bp) region to amplify Australian strains *Eimeria* spp. in chickens. This nested primer set successfully differentiated between the Australian-type and US-type strains of *E. maxima* and subsequently

detected eight species of *Eimeria* in chickens in northern India (Kumar et al. 2015; Kumar et al. 2014). Alternately, when Morgan (2013) applied the Lew et al. (2003) genus specific primer set to kiwi faecal samples containing mixed populations of coccidia, PCR product lengths ranged from 220 to 600 bp in positive samples (n = 17), none of which yielded clean sequences. When this primer set was used as inner primers in a nested protocol combined with outer primers designed by Honma et al. (2011a), 30 cloned amplicons sequenced successfully (127 to 205 bp); however, no single oocysts from kiwi droppings were successfully amplified with this nested protocol (Morgan 2013). Interestingly, Bertram et al. (2015) successfully amplified the ITS-1 and -2 regions (466 bp and 400 bp, respectively) in *E. gruis* and *E. reichenowi* from whooping cranes (*Grus americana*) using primers developed for chicken coccidia (Honma et al. 2011a; Schnitzler et al. 1998; Woods et al. 2000b). Unfortunately, these targets can yield such high sequence variation that relying on ITS-1 and ITS-2 sequence data alone may not provide useful phylogenetic comparisons among genetically diverse species (Lew et al. 2003; Motriuk-Smith et al. 2011).

Although the ITS-2 region can be highly variable, it still provides a well-studied target that is useful for comparisons when combined with other targets or methods. Using primers developed by Woods et al. (2000a) that target the ITS-2 region (~400 to 620 bp) of *Eimeria* spp., Gasser et al. (2005) applied capillary electrophoresis to the PCR products from laboratory strains of *E. tenella*, *E. praecox*, *E. necatrix*, *E. mitis*, *E. brunetti*, *E. maxima*, and *E. acervulina*. This technique demonstrated the variability of this target within a species and established DNA-profiles for these strains. Morris et al. (2007) used these profiles to study the epidemiology of *Eimeria* spp. in Australian broiler flocks.

Additionally, this protocol was modified into a nested protocol and applied to *Eimeria gruis* and *E. reichenowi* in hooded and white-naped cranes and was a useful tool for identifying differences in coccidian community composition between different host species. In this study, the generated profiles using short operational taxonomic units (OTUs; i.e., a genetic level of distinction used to compare and study genetically close individuals), demonstrated considerable variability in ITS-2 length (377 to 533 bp) even within a single oocyst (Honma et al. 2011a; Honma et al. 2011b). Similarly, Morgan (2013) applied this nested PCR protocol to kiwi *Eimeria* spp. and used standard gel electrophoresis and Sanger sequencing for analysis; sequences were highly variable (60 to 714 bp), and cloned products had a 31.4% (n = 137) sequencing success rate. Analysing species diversity in coccidia at the ITS regions may best be approached using small OTU profiling rather than data from a longer sequence. However, as Morgan (2013) found these locations to be highly variable as well as unreliable, other loci need to be considered.

1.4.3.4. Mitochondrial cytochrome c oxidase subunit 1 (COI)

The COI gene is about 1,500 bp long with, in the case of *Eimeria tenella*, about 50 repeats per haploid genome (i.e., 400 per oocyst; Hikosaka et al. 2011; Ogedengbe et al. 2013). This high copy number as well as sufficient interspecific and low intraspecific variability in this gene make it a useful target in DNA barcoding (Chapman et al. 2013; Hikosaka et al. 2011). In combination with other regions such as the 18S and 28S loci, the COI gene has been used to genetically compare a wide range of avian coccidia. El-Sherry et al. (2013) developed two primer sets for *Eimeria adenoeides* (803 bp product) and *Eimeria meleagrimitis* (513 bp) in turkeys that have been used to directly sequence single

oocysts (El-Sherry et al. 2014). El-Sherry et al. (2015) then redesigned primers that amplified about 500 bp in five species of turkey coccidia that also amplified chicken *Eimeria* spp. (Hafeez et al. 2015a). Similarly, Ogedengbe et al. (2011b) developed primers for partial (780 bp) amplification of the COI gene in chicken coccidia, which also amplified *Isospora manorinae* in a yellow-throated miner (Yang et al. 2016b). However, these primers did not work for an eimeriid parasite in a king's skink (*Egernia kingie*), thus Yang et al. (2013) developed new primers (465 bp), which, in addition to the Ogedengbe et al. (2011b) primer set, worked on *Isospora serinuse* in a domestic canary (Yang et al. 2015a); *Eimeria paludosa* (467 bp) in a dusky moorhen (Yang et al. 2014a); and *Eimeria haematoid* (496 bp) in a rainbow lorikeet (Yang et al. 2015d). By using the forward primer from Ogedengbe et al. (2011b) and developing a reverse primer, Illera et al. (2015) amplified a partial COI (648 bp) fragment from an *Isospora* sp. in spectacled warblers (*Sylvia conspicillata*). While the COI gene has provided an excellent target for phylogenetic analysis of diverse parasites, often some optimisation is required to determine appropriate primer sets. Dolnik et al. (2009) developed partial (250 bp) COI primers specifically for *E. tenella*, which was successfully applied to *Isospora anthochaerae* in a red wattlebird (Yang et al. 2014b) as well as *Isospora streperae* in a grey currawong (*Strepera versicolour plumbea*) (Yang et al. 2015c). Additionally, these primers have been used in combination with Ogedengbe et al. (2011b) and Yang et al. (2013) primers in nested protocols for *Eimeria labbeana-like* (723 bp) in a domestic pigeon (Yang et al. 2016a), *I. neochmiae* (725 bp) in a red-browed finch (*Neochmia temporalis*) (Yang et al. 2016e), and *Eimeria purpureicephali* (723 bp) in a red-capped parrot (Yang et al. 2016d). The diversity of parasites and hosts these primer sets have

been applied to suggests that this target may be excellent for characterisation of newly described coccidian species, especially when sequenced directly.

1.4.4. Real-Time PCR

These mitochondrial targets may provide kiwi researchers and practitioners useful epidemiological information regarding which species are present in both individual birds and populations. However, the wait associated with sending samples for Sanger sequencing after initial microscopic detection, extraction, amplification, and electrophoresis is likely to prevent meaningful management decisions being made in a timely manner. Real-time PCR (RT-PCR) may provide a faster solution for differentiating species of coccidia in kiwi. This method of PCR employs either probes or melting curves to identify multiple targets in a sample (Mussack et al. 2020). The probes and melting curves are detected as amplification occurs through the release of fluorescent molecules that are detected in increasing strengths as the target amplifies, allowing for relative quantification of target and, by extension, infection load (Higuchi et al. 1993; Landgraf et al. 1991; Mardis 2008). This method has proven useful in distinguishing and quantifying chicken *Eimeria* spp. in particular and should be considered for research and diagnostics of kiwi coccidia (Peek et al. 2017; Velkers et al. 2010; Vrba et al. 2010).

1.4.5. Loop-mediated isothermal amplification

Unfortunately, RT-PCR can be difficult to implement in a field setting. On the other hand, loop-mediated isothermal amplification (LAMP) assays do not require delicate equipment for amplification or detection. LAMP uses *bst* DNA polymerase, which decreases the cost of target

amplification by avoiding the need for thermocycling and increases specificity by targeting six distinct regions of a sequence, which can be more sensitive than conventional PCR (Barkway et al. 2011; Fallahi et al. 2015; Huang et al. 2016; Karanis et al. 2007; Nagamine et al. 2002; Notomi et al. 2015; Zhuo et al. 2015). The formation of magnesium pyrophosphate marks amplification, allowing for simpler interpretation of results in the form of turbidity and can be recorded in real-time for quantification (Mori et al. 2004; Mori et al. 2001). Figure 1.9 summarises the LAMP protocol. LAMP has been used to detect *Plasmodium* spp. (Aydin-Schmidt et al. 2017; Perera et al. 2017; Vallejo et al. 2015), *Toxoplasma gondii* (Abdoli et al. 2016; Fallahi et al. 2015; Trisciuoglio et al. 2015; Zhuo et al. 2015), *C. suis* (targeting the 18S rDNA gene) (Huang et al. 2016), and *Cryptosporidium* spp. (Karanis et al. 2007; Koloren et al. 2011; Mahmoudi et al. 2013). The simplicity of this method lends itself to large scale use in the poultry industry, allowing for on-site, rapid testing, and has been developed for seven species of chicken *Eimeria* (Barkway et al. 2011; Barkway et al. 2015). Barkway et al. (2011) initially targeted the ITS-1 region; however, due to a decrease in specificity they determined the use of a several small (10-20 bp) to create profiles for each species for quantitative PCR (Vrba et al. 2010) was more appropriate. If a simple, reliable DNA extraction from oocysts could be developed, LAMP could revolutionise how ONE facilities diagnose and, therefore, manage coccidiosis in kiwi, thus should be considered for future use.

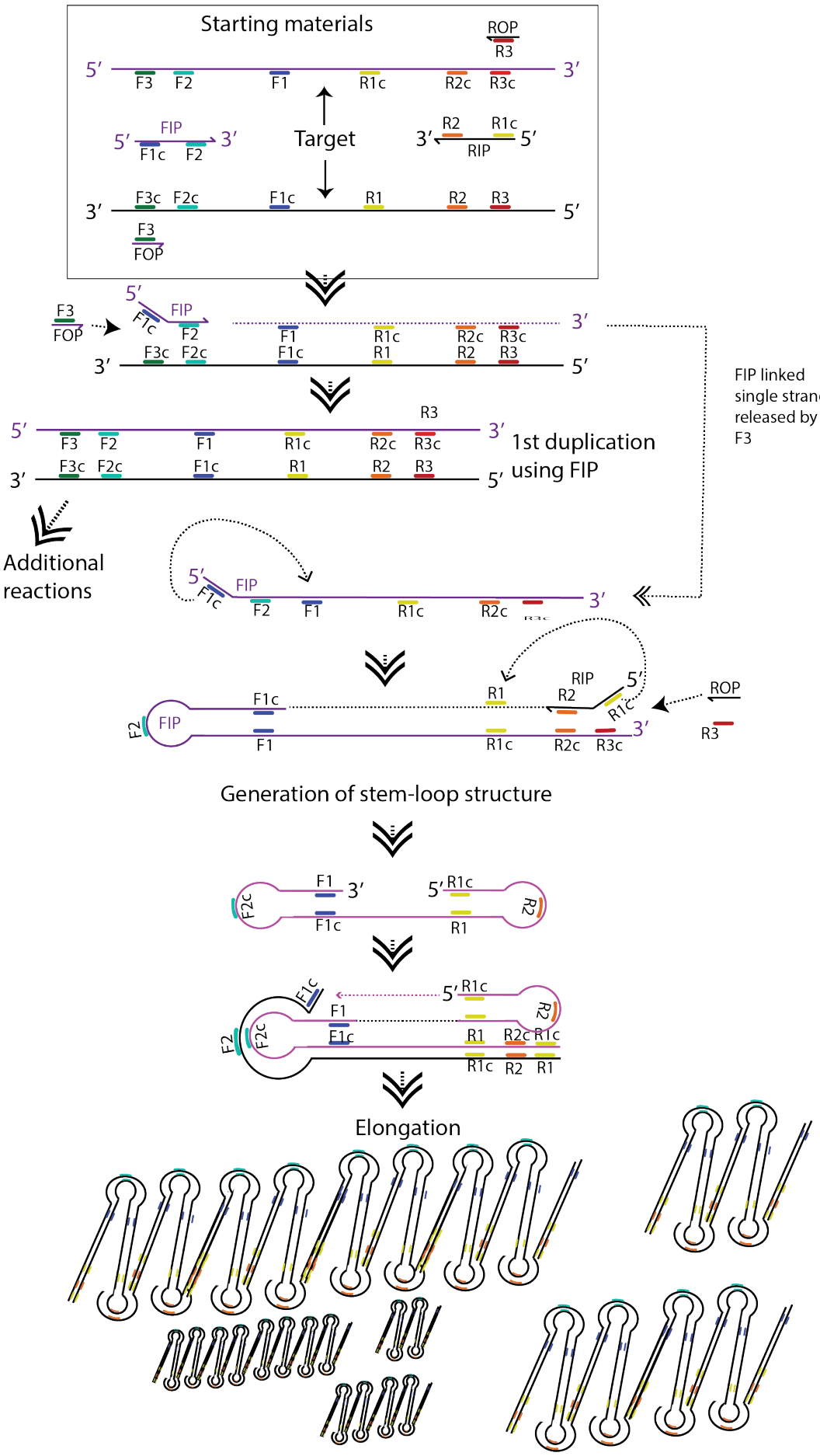

Starting materials
ROP
R3
5′
3′
F3
F2
F1
R1c
R2c
R3c
FIP
F1c
F2
Target
R2
R1c
RIP
F3c
F2c
R1
FOP
FIP linked single strand released by F3
1st duplication using FIP
Additional reactions
RIP
ROP
Generation of stem-loop structure
Elongation

Figure 1.9. Simplified illustration of how loop-mediated isothermal amplification works. Three sets of binding sites are used to amplify the target DNA. Two binding sites on the forward inner primer (FIP) and reverse inner primer (RIP) lay down on the starting single strands of complementary DNA and make an initial duplication. Then the F/R3 primers start another amplification outside the F/RIPs, releasing the original strand. This strand with the F/RIP primers loops onto itself, creating a stem-loop structure, which yields extensive elongation and duplication of the target DNA. Adapted from Notomi et al. (2015).

1.4.6. Whole genome sequencing

As an alternative to sequencing specific genes, whole genome sequencing (WGS) could provide insight into not only species differentiation, but also to identify targets that definitively distinguish one species of kiwi *Eimeria* from another. The whole genomes (51.8 Mb, *E. tenella*) of seven species of chicken *Eimeria* (Reid et al. 2014) have been analysed, as these parasites regularly impact the poultry industry worldwide. This process is costly yet has the potential to provide crucial information for the treatment and regulation of these is parasites. However, when the goal is identification and differentiation of closely related species, whole genome sequencing is not cost effective as very few regions would be useful.

Molecularly characterising coccidia ideally differentiates morphologically indistinct species quickly, at a large scale, and from samples of various sources and qualities. Thus, few have targeted avian coccidial species for whole genome sequencing, which requires high effort and time, single strain lineage, and provides vast amounts of conserved regions that are not useful for differentiation. Alternatively, sequencing the whole mitochondrial genome (~6.2 kb in *Eimeria* spp.) could identify gene targets that provide phylogenetically meaningful comparisons at a much lower cost (Chapman et al. 2013). Ogedengbe et al. (2013) developed primers for *E.*

mitis based on partial COI sequences and used a primer walking strategy, applying Sanger sequencing (see Chapter 1.5.1) directly to PCR products and clones. This primer walking method was also used by Ogedengbe et al. (2014) after developing two genera specific primer sets that amplified *E. dispersa*, *E. meleagrimitis*, *E. meleagridis*, *E. adenoeides*, and *E. gallopavonis* in turkeys. This protocol also has been also applied to *Eimeria innocua* from turkeys and an *Isospora* (Hafeez et al. 2015b; Ogedengbe et al. 2015). Yang et al. (2017) used this protocol for *I. serinuse* in a canary using cloning and compared it to using second generation sequencing (SGS, see Chapter 1.5.2) of the *I. manorinae* genome in a yellow-throated miner. Based on this comparison, SGS provided a stronger analysis of the variation within a population. Hinsu et al. (2018) detected two cryptic genotypes of chicken *Eimeria* utilising SGS, providing a valuable tool for screening and detection of coccidia in commercial and small-scale farms.

1.4.7. Application of molecular sequencing for coccidia of kiwi

While sequencing the entire mitochondrial genome from each species of kiwi coccidia would determine appropriate genes for use in experimental and routine/diagnostic applications, the associated cost would greatly hinder the number of reactions performed, thereby limiting the variation sampled. As very few species of *Eimeria* have been analysed this way, few phylogenetic comparisons could be made. Further, only one gene with the appropriate variation is needed to develop a diagnostic test, which is the overarching goal of this research. Thus, the COI gene, which has been shown to be useful in differentiating species of *Eimeria* from a wide range of hosts, combined with the 18S rDNA, which has a large dataset for comparison, has the greatest potential to provide the data required to assist in making informed management decisions. In order to determine the usefulness of the COI gene, several kinds of sequencing need to be

considered for analysis. Based on these COI sequencing results, both RT-PCR and LAMP have the potential to enable diagnostic identification of kiwi coccidia. The main factor that impacts which method is more appropriate is the number of highly conserved regions within the COI.

1.5. Sequencing and computational genomics

This section summarises the types of sequencing in current use and in developmental stages. Table 1.4 provides a cursory glance at the pros and cons of each generation of sequencing. Second and third generation sequencing both have a wide range of technologies under development. Second generation has advanced enough for the most commercially available technologies; Illumina amplicon sequencing, a kind of second (or next) generation sequencing, was the main focus of this literature review.

Table 1.4. Summary of the pros and cons of each generation of sequencing.

Feature	Sanger/First Generation	Next/Second Generation	Third Generation
Read length	800 bp	100-800 bp	Up to 900 kb
Accuracy	High	Low-high	Low
Speed	Slow	Fast	Fast
Cost (per sample)	High	Low	High
Computer resources	Low	High	High

(Ambardar et al. 2016; Arulandhu et al. 2018; El Bairi et al. 2020; Liu et al. 2012; Metzker 2010; Xie et al. 2020)

1.5.1. First generation/Sanger sequencing

The most commercially accessible sequencing (i.e., Sanger sequencing or first-generation sequencing; FGS) technology employs terminating inhibitors (ddNTP) that

stop amplification, leading to segments of different length in a given target (Metzker 2005; Quail et al. 2012; Sanger et al. 1977). The position of each dNTP in a target is determined using four reactions, each with a different fluorescently marked ddNTP; the products are then separated by length using gel/capillary electrophoresis and base-calling technologies (Dovichi and Zhang 2000; Metzker 2005). Systems have been developed to be fully automated with high resolution, allowing for accessible, high-throughput genotyping (Dovichi and Zhang 2000; Metzker 2005). However, only the most common sequence is represented, leading to a loss in detecting diversity (Altimari et al. 2013; Cereb et al. 2015; Magalhaes et al. 2015). Many applications, such as routine diagnostics, regularly use FGS. However, the cost effectiveness decreases as the number of samples increase and/or the variation within the target sequence increases.

1.5.2. Second generation sequencing

Second generation sequencing (SGS) is commonly referred to as Next Generation Sequencing (NGS), which has started to become a misnomer as sequencing technologies advance. Therefore, for the purpose of this literature review SGS will be used. SGS offers a more cost-effective option that allows for detection of underrepresented sequences, as all SGS involves clonal amplification in many individualised locations prior to sequencing (Altimari et al. 2013; Ambardar et al. 2016; van Dijk et al. 2014a). A wide variety of SGS platforms, i.e., instruments, are available that combine distinct sequencing and detection methods. Sequencing is accomplished either through hybridisation/ligation or synthesis (El Bairi et al. 2020). Briefly, hybridisation/ligation sequencing uses DNA ligase to attach probes containing short (1 to 2) base pair sequences at a primer site. The full target sequence is achieved by starting with primers

of different lengths and detection of the fluorescent label as it is cleaved from the probe (e.g., the SOLiD system; Liu et al. 2012). Similar to Sanger sequencing, the sequencing by synthesis methods use DNA polymerase rather than DNA ligase. During synthesis of the target, these methods detect light emission with the release of a pyrophosphate (i.e., pyrosequencing methodology; Fakruddin et al. 2012); changes in pH and the increase in voltage associated with the release of a hydrogen ion with the incorporation of a base (e.g., Ion Torrent platform; Eid et al. 2009); or fluorescently labelled, reversible terminators (e.g., Illumina platforms; Guo et al. 2008; Kawashima et al. 2002).

1.5.2.1 Sequencing by reversible termination

Illumina sequencing is the dominant supplier in commercially available SGS as it is the only system that uses paired-end sequencing with high-cost efficiency (Ambardar et al. 2016; El Bairi et al. 2020; Kawashima et al. 2002). Figure 1.10 summarises the Illumina workflow. Briefly, Illumina sequencing requires four steps: library preparation, cluster amplification, sequencing, and alignment/data analysis.

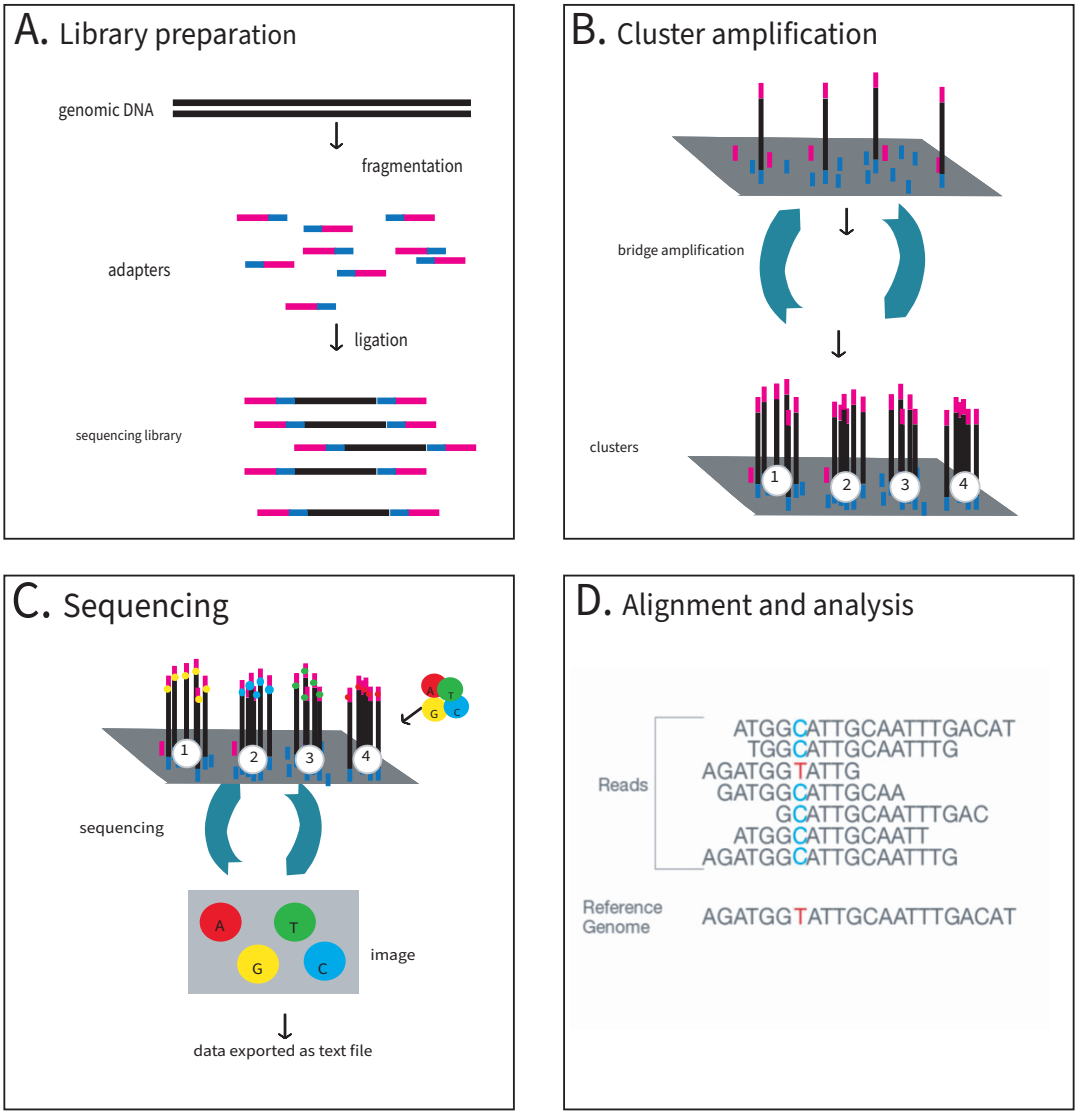


Figure 1.10. Summary of the Illumina workflow. All Illumina sequencing technology begins with A) Library Preparation, which varies based on the intended target; followed by B) Cluster Amplification, clonally amplifying individual sequences; then C) Sequencing using fluorescent, reversible terminators; and lastly the raw data is D) Aligned and Analysed (adapted from https://www.illumina.com/content/dam/illumina-marketing/documents/products/illumina_sequencing_introduction.pdf).

1.5.2.2. Amplicon library preparation

These steps of library preparation can vary significantly with the target sequence (e.g., *de novo* sequencing of a novel genome and amplicon sequencing of a known, 300 bp

target require different approaches). However, in general, library preparation incorporates oligonucleotides into the DNA that identify the source sample and allow for adhesion to the sequencing plate (Adessi et al. 2000; Filges et al. 2019). Figure 1.11 summarises the steps for Illumina library preparation.

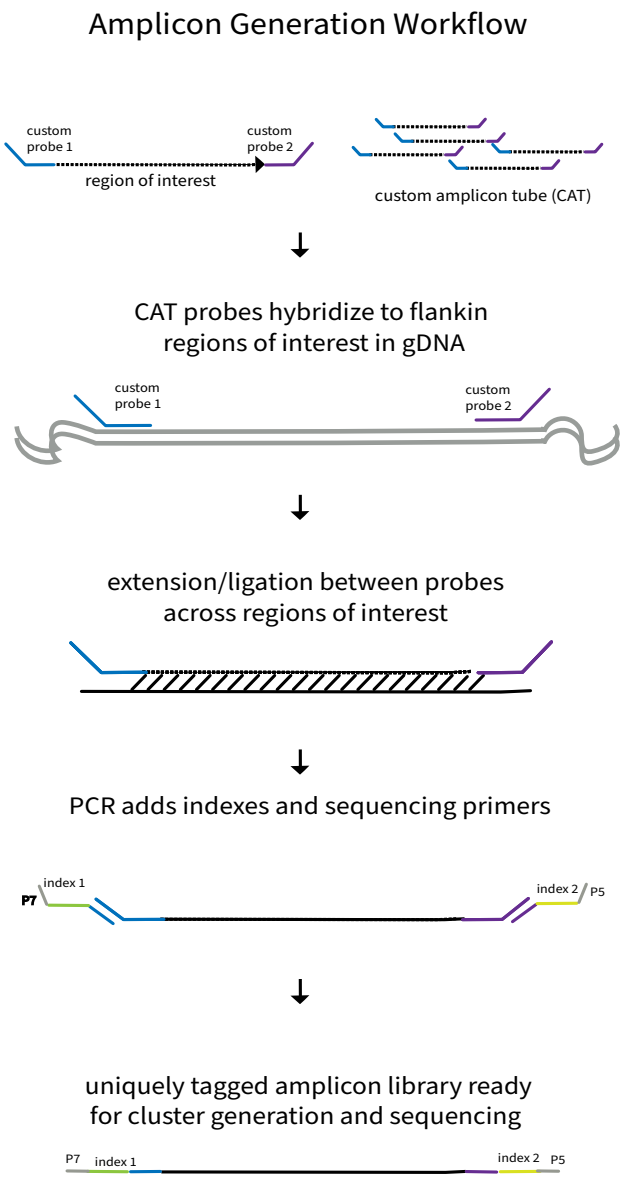


Figure 1.11. Summary of the library preparation for Illumina amplicon sequencing. (Adapted from https://www.illumina.com/content/dam/illumina-marketing/documents/products/illumina_sequencing_introduction.pdf)

1.5.2.3. Illumina amplicon sequencing

After the indexed samples are spread over and adhered to the plate, which is coated with oligonucleotides complimentary to the adapters, each sequence is clonally amplified via “bridge” amplification that forms clusters of identical sequences (Figure 1.10.B.; Bentley et al. 2008; Berglund et al. 2011; Filges et al. 2019). These clusters are then sequenced using fluorescently labelled bases; as each base is incorporated images are taken each cycle to determine the colour of fluorescence at that position, allowing for base calling at each cluster (Figure 1.10.C). Raw sequences can then be aligned and analysed.

1.5.2.4. Analysis of Illumina sequencing data

The journey from the raw sequence data generated by SGS sequencing to phylogenetic analysis requires the employment of an entire field of science (i.e., bioinformatics). While processing the single Sanger sequence detected in each sample by hand can be a long procedure depending on the sample size and target length, doing the same for Illumina, which can yield millions of reads, is impractical. Further, as the Illumina protocol is amplification based, analysis of the vast number of sequences it produces must account for errors associated with amplification (Ambardar et al. 2016; Arulandhu et al. 2018; Berglund et al. 2011). Unlike Sanger sequencing, which detects only the prominent sequence or sequences, the likelihood of detecting single base errors is guaranteed (Ambardar et al. 2016; Arulandhu et al. 2018; Berglund et al. 2011; Filges et al. 2019). Thus, many programmes (e.g., QIIME2; Bolyen et al. 2019) are continually

being developed to process, denoise, cluster unique sequences (i.e., operational taxonomic units, OTUs) into larger OTU groups that are more likely to represent the "true" sequence. Bioinformaticians continually improve upon methods of clustering to account for the errors that occur in amplification-based sequencing (Filges et al. 2019).

The clustering of samples presents a particularly interesting aspect of bioinformatics, as how the sequences are clustered can change the results drastically. The main goal of clustering is to determine which sequences are actually different from one another. Thus, the level or percentage of similarity determines the number of OTUs present in each sample. Determining the appropriate level of percent identity can drastically change between gene targets, taxonomic groups, and depth of sequencing. Accordingly, determining the appropriate level of clustering when describing a novel species of parasite that is a member of the largest genus in the family Eimeriidae can be challenging (Ogedengbe et al. 2011a). At the COI gene, the percent identity between morphologically similar species varies tremendously. For example, *E. dispersa* (HG793048.1) and *E. innocua* (HG793049.1), that infect turkeys are 97.7% similar at the 484 bp target amplified by the nested protocol cited in Chapter 1.4.1.4 (Vrba and Pakandl 2014; Yang et al. 2013; Yang et al. 2015a; Yang et al. 2014a; Yang et al. 2015d). Further, the bovine parasites, *E. bovis* (KT184372) and *E. zuernii* (HM771687.1), are 96.9% similar. Conversely, *E. gruis* (MF503489.1) and *E. reichenowi* (MF503493.1) are only 89.7% similar (Liang et al. 2018). Based on these values, we determined the best route is to conduct clustering at 90% to 99% at 1% intervals. Doing so allows for a comparison of groupings based on biological as well as statistical likelihood. As no coccidia from wild hosts in New Zealand have ever been analysed at the COI gene, our

analysis must rely on biological factors (such as morphology and geography) to assist in determining taxonomic units (Megía-Palma et al. 2015).

1.5.2.5. Computational genomics

The Quantitative Insights Into Microbial Ecology 2 (QIIME2) environment has been developed as a tool for making robust bioinformatic analysis accessible to biologists and other end-users (Bolyen et al. 2019). This tool is free to use with tutorials available for each step of the workflow and options that accommodate degrees of computational power. Additional plug-ins (i.e., tools) can be developed and contributed to the QIIME2 community, allowing for the accommodation of many fields of study to utilise this programme. The QIIME2 software and tools are installed and run in Anaconda on a virtual machine, thereby allowing anyone with a computer with sufficient storage space the ability to run the package (Hall and Beiko 2018). Furthermore, the data produced in the QIIME2 software is in part produced in a format that is able to be viewed online (http://view.qiime2.org) and is accompanied by all the information needed to repeat or cite the workflow used.

1.5.2.5.1. Illumina data analysis workflow

The QIIME2 ecosystem's most streamlined and accessible workflow analyses Illumina sequence data targeting the 16S rDNA gene (Hall and Beiko 2018). However, one of the key features of QIIME2 is that the data generated in each step is exported in a format that can be used in any other plugin or viewed online. For the purpose of this thesis, the workflow summarised here follows Illumina amplicon sequencing output of genes without an established reference database preloaded in QIIME2. The first step,

demultiplexing, separates the paired-end sequence (forward and reverse) reads from the Illumina output. These reads are then denoised, which involves identification and removal of errors from the analysis. The remaining sequences are compiled back into pair-end sequences. A set of reference sequences are introduced to QIIME2 and used to cluster and phylogenetically analyse the sample sequences.

1.5.2.5.2. Demultiplexing

Illumina sequencing results are usually returned to the user as paired-end sequence reads in FASTQ format. These sequences are still mixed together and unlinked to the metadata (multiplexed). The sequence data are imported with the metadata file into the QIIME2 environment, and the “q2-demux” plugin is used to demultiplex the paired-end sequence reads (Bokulich et al. 2018b; Bolyen et al. 2019). This takes all the raw sequences and identifies which sequences were from each sample.

1.5.2.5.3. Denoising

As for all sequencing, some level of error is expected. How these errors are accounted for have strong implications downstream (Fischer-Hwang et al. 2019). Many tools for a variety of programmes have been developed that are compatible with the QIIME2 environment. For example, the “DADA2” plugin was developed as an R package and has been a useful tool in QIIME2 workflows (Bokulich et al. 2018b; Bolyen et al. 2019; Callahan et al. 2016; Hall and Beiko 2018; Hernandez-Gomez et al. 2020). Here the “DADA2” plugin removes sequences that appear only once, and therefore, likely contain read errors; detects and removes chimeras (a mistake in amplicon sequencing in which

two single stranded templates anneal together, acting as primers and producing a novel sequence not seen in nature); and removes sequences with high levels of background signals (Callahan et al. 2016). Within this process, sequence lengths should be trimmed to avoid including sections of sequence more prone to errors: at a certain distance from the primer, the quality of sequence tends to decline (Caporaso et al. 2011; Hall and Beiko 2018). These denoised sequences are realigned into paired-end reads.

1.5.2.5.4. Reference database

In order to organise these sequences into meaningful groups, the reads need to be compared to reference sequences. When there is not an established database in the QIIME2 package (as is the case with the apicomplexan COI gene), a reference database needs to be created and integrated into the software. This integration employs machine learning in which the user provides QIIME2 a dataset comprised of, in this case, sequences from known phylogenetic origins (i.e. supervised learning) (Pedregosa et al. 2011).

Applicable reference sequences that cover the target amplicon are aligned, trimmed, and exported in the correct format for the library or plugin. One example of such a library is the Scikit-Learn library (Pedregosa et al. 2011), which offers several plugins that use machine learning for classifying unknown sequences. These plugins teach these machines particular parameters that provide taxonomic information that allows the machine to identify and classify novel taxonomic groups. The “q2-feature-classifier”

plugin includes several taxonomic classification tools, including a naive Bayes classifier that has been shown to be the most accurate to date (Bokulich et al. 2018b).

1.5.2.5.5. Clustering

At this point in the workflow, the sample sequences have been demultiplexed, denoised, errors removed, and organised into pair-end reads. These sequences are further narrowed into smaller groups (operational taxonomic units; OTUs) based on a specified sequence similarity using the "q2-vsearch" plugin (https://github.com/qiime2/q2-vsearch). The output is a dataset of OTUs using the "q2-feature-table" plugin, which creates a matrix of the abundance of each OTU present in each sample. This data matrix is in the BIOM format, which condenses datasets with large amounts of (especially negative) abundance data; can be converted into other formats; and is compatible with multiple platforms (McDonald et al. 2012). This format decreases the amount of data and, therefore, computing power required in downstream applications. Each individual paired-end read is now organised into OTUs. Further, a database containing reference sequences has been compiled and imported to the QIIME2 environment. In order to provide a taxonomic comparison, the OTUs need to be compared to the reference dataset. Briefly, the "cd-hit" package provides a fast solution that uses an algorithm that compares the similarity of sequences without the sequences needing to be aligned (Fu et al. 2012; Huang et al. 2010; Li and Godzik 2006; Li et al. 2001; Li et al. 2002). Instead, it organises the reference sequences into a table, starting with the longest. Each sample OTU is compared to the first reference sequence using a word counting algorithm, which identifies whether the OTU is similar enough to be aligned with the reference sequence

or should remain separate. The results of this clustering are then used to create taxonomic comparisons.

1.5.2.5.6. Viewing

The exportation and viewing of analytical outcomes are one of the main advantages of QIIME2. The two main types of files associated with QIIME2 data are artefacts (.qza) and visualisations (.qzv) files (Bolyen et al. 2019; Hall and Beiko 2018). The first contains all the information (i.e., data, metadata and provenance) about the analysis performed; the latter contains data for easily transportable visualisation methodology. Each of these files can be stored completely outside the QIIME2 environment and viewed from any computer with internet access on https://view.qiime2.org. For example, the sample OTUs can be viewed as a taxa summary bar plot using the "q2-taxa" plugin (https://github.com/qiime2/q2-taxa). After clustering using the cd-hit package, sample OTUs and reference sequences can be aligned in Geneious or other sequence data software for further downstream analysis.

1.5.3. Third generation sequencing

Third Generation Sequencing (TGS) focuses on increasing the read length and decreasing the sample preparation. Several approaches develop real time, single strand sequencing capabilities in order to avoid the amplification bias associated with FGS and SGS (e.g., Pacific Biosystems single molecule real time sequencing, Helicos single molecule sequencing, and Nanopore DNA sequencing; Ambardar et al. 2016; El Bairi et al. 2020;

Lu et al. 2016; van Dijk et al. 2014a; Xie et al. 2020). These systems employ sequencing by synthesis; require little to no indexing/library preparation; and have the potential to provide long read lengths (Ambardar et al. 2016; El Bairi et al. 2020). Other approaches (Complete Genomics Technology and GnuBIO) focus on increased coverage and simplified workflow (Ambardar et al. 2016; El Bairi et al. 2020). The main limitation to TGS at this time is that bases are misidentified at high rates (Ambardar et al. 2016).

1.6. Conclusions

Coccidia will be an ongoing consideration for ONE management of kiwi, especially during translocations from brooders to crèche or island to island. The only morphological and genetic descriptions from sporulated oocysts have been from brown kiwi, likely leaving significant amounts of variation undocumented. This variation could have vital implications for small, isolated populations or species of kiwi that may be exposed to novel species of *Eimeria*. Further, while enteric, renal, hepatic, pulmonary, and splenic coccidiosis has been described, little is known about which morphotype or genotype is linked to each form of disease. The ITS-1 and -2 regions of rDNA were too variable, whereas the 18S region was not variable enough to begin differentiating causative agents of each kind of coccidiosis. A different approach is needed, therefore, to expedite the process of connecting oocyst morphotypes to internal stages of development (i.e., types of coccidiosis).

This thesis, therefore, determined to expand morphological characterisations of coccidia in brown kiwi and Haast tokoeka to establish a stronger foundation for connecting external and internal stages of development. Additionally, a portion of the COI gene was chosen for in-depth SGS sequencing to not only gain an understanding of

how kiwi coccidia phylogenetically relate to other *Eimeria*, but also to identify conserved and variable regions within this gene. This robust analysis will hopefully provide the diagnostic capability to determine links between internal stages of coccidia, and therefore virulence, with morphotypes. In the long term, this link could inform management decisions based on morphology rather than abundance alone, thereby reducing the overuse of coccidiocides and increasing the quality of care.

CHAPTER 2

Comparing the Mini-FLOTAC and centrifugal faecal flotation for the detection of coccidia (*Eimeria* spp.) in kiwi (*Apteryx mantelli*)

Contribution of co-authors: Drs Pomroy and Morgan contributed to experimental design. Dr Emillie Vallee was instrumental in developing and interpreting the statistical analyses. Dr McInnes helped focus this research for practical use and was instrumental in obtaining research materials. Dr Howe guided the development of this research into a short communication intended for Parasitology Research. All authors provided feedback on one or more drafts of this manuscript. See Appendix G.

The following chapter has been published in *Parasitology Research* (2020) as a short communication. Detection of coccidia in a sample is the first step to identification and research. Thus, this research was undertaken to ensure the detection and quantification of coccidia used for this study was the most accurate possible. The Massey Parasitology Laboratory method (centrifugal faecal flotation, CFF) has been used for decades with little changes (pers. comm. W. Pomroy). Before beginning sensitive molecular characterisations, a comparison of the CFF to a new method commonly used for other coccidia in domestic hosts was necessary to ensure accurate comparisons between parasite load and species composition could be made.

Publication: Coker SM, Pomroy WE, Howe L, McInnes K, Vallee E, Morgan KJ (2020) Comparing the Mini-FLOTAC and centrifugal faecal flotation for the detection of coccidia (*Eimeria* spp.) in kiwi (*Apteryx mantelli*). Parasitology Research 119:4287–4290

2.1. Abstract

Coccidia (*Eimeria* spp.) in brown kiwi (*Apteryx mantelli*) cause significant morbidity and mortality in captive rearing facilities. Monitoring the abundance of this parasite in individual birds is crucial for successful management of kiwi. This research compares the abilities of centrifugal faecal flotations (CFF) and a modified Mini-FLOTAC protocol to detect oocysts. We hypothesised that the Mini-FLOTAC would detect higher oocyst counts. Kiwi dropping samples (n = 10) were homogenised in $MgSO_4$ (SG 1.28) and oocyst counts made with CFFs and Mini-FLOTAC counting chambers, with three replicates for each method. For CFF, 0.5g of droppings were examined using standard

methods. Mini-FLOTAC counts were made using a modified sample preparation compared with the manufacturer's protocol but still used a 1:20 dilution of droppings. Oocysts were quantified using light microscopy at 100-200× magnification. A linear mixed-effects model by REML showed that oocyst per gram estimates via the Mini-FLOTAC method were 3.4 times higher (95% CI 2.5-4.6, $p<0.01$) than the CFF results. This increased detection likely represents a more accurate estimation of parasite shedding and should be considered for use in research or applications requiring more accuracy, cost-effectiveness, or accessibility than the CFF provides.

2.2. Introduction

Brown kiwi (*Apteryx mantelli*), a ratite endemic to New Zealand, are listed as "Vulnerable" under the IUCN classification system with an estimated population of 26,550 as of 2015 (BirdLife International 2017). Predation of adults and juveniles by introduced mammals have played a major role in population decline; however, individuals greater than 800 grams body weight are far less likely to be preyed upon (Basse et al. 1999; McLennan et al. 2004; McLennan et al. 1996; Miller and Pierce 1995; Taborsky 1988). To help kiwi achieve this size, baiting, trapping and predator exclusion fencing are used to decrease the density of predators in kiwi management areas (Glen et al. 2012; Robertson et al. 1999a; Robertson et al. 1999b; Robertson et al. 2011). An additional crucial management resource is "Operation Nest Egg" (ONE), a programme that brings eggs and recently hatched wild chicks into captivity, where they are raised in a predator free environment until they reach at least 1kg before being released back to the wild (Colbourne et al. 2005). Compared to unmanaged kiwi, this program leads to a

significant increase from 52% to 62% hatching success and from 11% to 81% survival to 6 months of age (Colbourne et al. 2005). Unfortunately, due to a build-up of environmentally resilient oocysts (*Eimeria* spp.) in captive facilities, severe morbidity and mortality due to coccidiosis occurs in these captive juvenile kiwi (Morgan 2013; Thompson and Wright 1978; Williams 2001). Morgan et al. (2017) identified four novel species of *Eimeria* from six individual brown kiwi. *Eimeria* (Apicomplexa: Eimeriidae) are intracellular parasites with direct faecal-oral transmission and are commonly associated with captive avian hosts, likely due to a build-up of environmentally resilient oocysts (the infectious stage of coccidia) in captive facilities, which can lead to more severe disease than seen in wild hosts (Ruff and Wilkins 1987; Williams 2001; Yabsley 2008). ONE facilities monitor coccidial burdens by submitting kiwi droppings to diagnostic laboratories for centrifugal faecal flotation (CFF; Bassett 2012). Recent studies have shown that a new multivalent technique for qualitative and quantitative copromicroscopic diagnosis (Mini-FLOTAC; Cringoli et al. 2017; Cringoli et al. 2010) is more accurate and precise than the McMaster's method in which aliquots of faecal samples homogenised in flotation solution are transferred into a counting chamber for the detection of nematode eggs in horses and sheep (Bosco et al. 2018) as well as *Eimeria* spp. oocysts in poultry (Bortoluzzi et al. 2018). However, the main limitation of the Mini-FLOTAC method is the amount of time it takes to read (Bortoluzzi et al. 2018). Unfortunately, very few studies have made direct comparisons between the mini-FLOTAC and CFF for detection of Apicomplexa (Soares et al. 2020), with Hussein et al. (2017) supporting a small increase in detection of *Giardia* using a CFF technique.

Importantly, the Mini-FLOTAC technique, unlike CFF, does not require centrifuges that need technical support, maintenance, and storage space (Cringoli et al. 2017). Accordingly, this method has the potential of being utilised at remote field stations, allowing for the prompt detection of high coccidial burdens prior to translocation of kiwi. Thus, the purpose of this research is to determine if an abbreviated, modified version of the Mini-FLOTAC technique detects significantly more kiwi coccidia oocysts (11.6 to 44μm in length and 10.6 to 23μm in width) than the CFF (Morgan et al. 2017). This would determine the suitability of this technique for field stations or labs with limited technical facilities and personnel but still prefer to provide more accurate results.

2.3. Methods

2.3.1. Sample collection

From November 2017 to January 2018, ONE kiwi droppings were collected at The National Kiwi Hatchery (n = 7) in Rotorua, where these birds were being reared in small (26-75m^2) outside enclosures with 1-2 birds each. Five or more droppings from up to a 12-hour time period were pooled and forwarded to Massey University within 48 hours of being passed. Additional individual samples were collected from The Cape Sanctuary (n = 3) crèche in the Hawke's Bay, where these birds are free to roam within the 25,000 ha enclosure. Droppings were collected while the birds were in hand or fresh from the roost and forwarded to Massey University within a month of being passed. All samples were stored at room temperature.

2.3.2. Study design

Each sample was homogenised in the container in which it was sent by stirring with a single-use, wooden stirrer for at least 30 seconds. A 2g aliquot of this sample was mixed with 38 ml of magnesium sulphate solution ($MgSO_4$, SG 1.28; Appendix B.1) in a small bowl and passed through a sieve (aperture 0.5mm). This 1:20 dilution was then used to count oocysts using either a CFF (Appendix B.2) or with a Mini-FLOTAC chamber (Appendix B.3). While continuously stirring back and forwards, three 1 ml aliquots were taken to fill Mini-FLOTAC chambers, and three 10 ml aliquots were transferred to 15 ml test tubes for CFF. Replicates were dispersed alternately between each method.

2.3.3. Mini-FLOTAC

Mini-FLOTAC chambers were prepared with modifications to the manufacturer's instructions (Cringoli et al. 2017). Briefly, the 1 ml aliquot of the homogenised sample was introduced into the Mini-FLOTAC chambers while holding the chamber on an angle to avoid the formation of air bubbles. After sitting for ten minutes, the chambers were rotated 90°C to separate the upper and lower chambers. If the oocyst count was >5,000 OPG, every other grid of nonadjacent quarters was counted at 100-200 × magnification.

2.3.4. Centrifugal faecal flotation

The CFFs were modified from the protocol described by the Ministry of Agriculture (1986) at a 1:30 dilution and prepared by adding further $MgSO_4$ to the 15 ml test tubes until a convex meniscus was formed. A 22 mm × 22 mm glass cover slip was placed on top and each test tube was then centrifuged for five minutes at 314 × g. The cover slip

was removed vertically and placed on a slide. Oocysts were counted on one single strip, one field of view (FOV) wide, in the middle of the coverslip at 300 × magnification. At this magnification, the FOV was 0.7 mm in diameter.

2.3.5. Oocyst load calculations

If the total of one millilitre at a 1:20 dilution is viewed in a full chamber of the Mini-FLOTAC then the number of oocysts counted is multiplied by 20 to calculate the oocysts per gram (OPG). When a quarter of the chamber is viewed, this multiplication factor increased to 80. When calculating the OPG from the CFF, the number of oocysts that float to the top are considered the total number of oocysts within the sample. At 300× magnification, there are the equivalent of 31.4 FOV across a 22 mm wide coverslip; thus, the count in one strip is adjusted by the following calculation: $\left[\frac{no.oocysts \times 31.4\ FOV}{weight\ of\ sample}\right]$. When the oocyst load was >40,000 OPG only three FOV were counted and averaged to calculate the OPG using $\left[\frac{no.oocysts\ per\ FOV \times 1257\ FOV}{weight\ of\ sample}\right]$ as per standard Massey University Parasitology Lab protocol. Raw data is reported in Appendix B.4.

2.3.6. Statistical analyses and interpretation

A linear mixed-effects model fit by REML was performed using RStudio 1.0.153. The outcome was the log transformed oocyst count and the method was used as a fixed effect, so that the relationship between the log-transformed concentration measured by CFF and FLOTAC can be expressed as in equation [1]. A random effect for sample was used to account for multiple measurements on the same sample.

$$log_e(FLOTAC) = log_e(CFF) + \beta_{Method}$$ [1]

For comparison, the average log counts were then transformed to the original scale by exponentiation equation [1] as shown in equation [2].

$$FLOTAC = CFF \times e^{\beta_{Method}}$$ [2]

2.4. Results and Discussion

The Mini-FLOTAC almost always (26/30) detected more oocysts than the CFF (Figure 2.1). Table 2.1 reports the results of the mixed-effect linear model, which indicates that the log-transformed count increases by 1.18 with the Mini-FLOTAC. This coefficient was back transformed to the original scale using equation [2], which show that the Mini-FLOTAC detects on average 3.2 times more (95% CI 2.4-4.5, $p<0.01$) oocysts than the CFF. This increase in oocyst detection suggests that the Mini-FLOTAC more accurately quantifies oocyst loads in fresh kiwi samples; the more oocysts detected, the more likely the count is closer to the true OPG since there is no chance of a false positive. This improvement likely reflects a lower multiplication factor and the disruptive vertical motion used in the CFF reducing the number of transferred oocysts, but this may not entirely account for the difference (Bauer et al. 2010). The FLOTAC mode of isolation has been shown to be more accurate and precise than the alternative McMaster method (Bauer et al. 2010; Bortoluzzi et al. 2018; Bosco et al. 2018; Silva et al. 2013). Regardless, most (90%) of the variation in the oocyst counts occurred between samples, suggesting high repeatability (Table 2.1). However, caution is advised as the direct relationship between the Mini-FLOTAC and the CFF seems to weaken with extremely low and high counts (Figure 2.1).

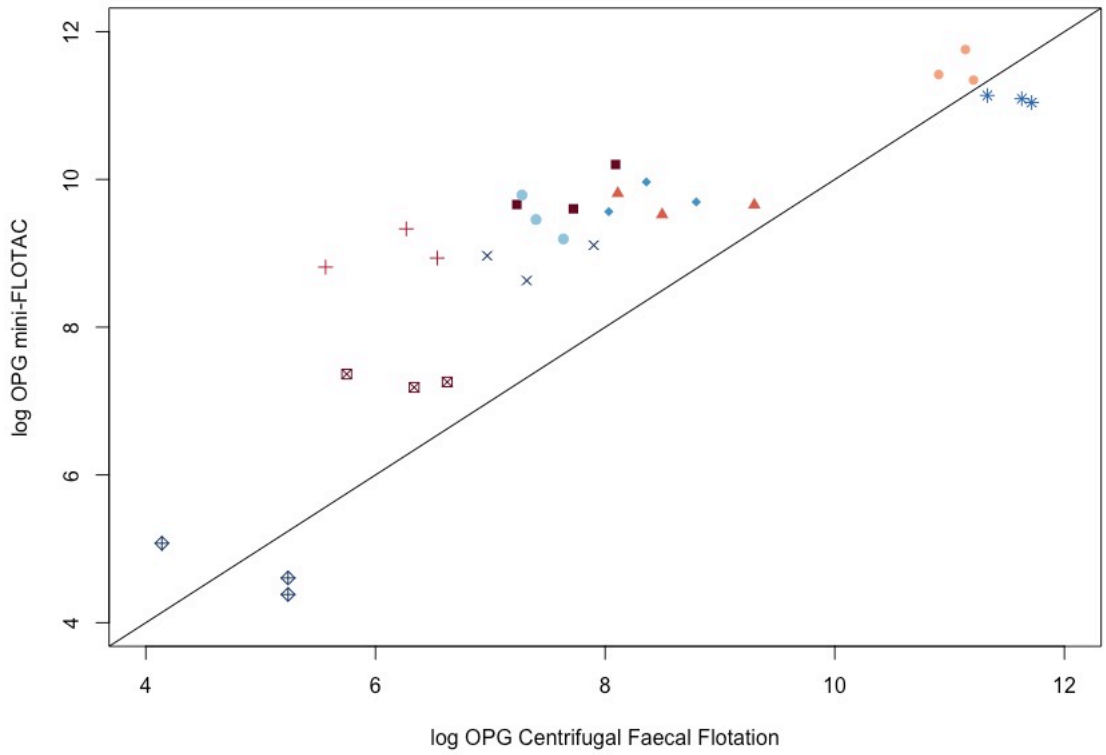


Figure 2.1. The difference in detection between centrifugal faecal flotation (CFF) and the Mini-FLOTAC. The black line indicates x = y. Each colour represents a sample (n = 10), with each Mini-FLOTAC replicate (n = 3) paired with CFF replicates (n = 3) in the order prepared.

Table 2.1. Linear mixed effects analysis results comparing the log_e transformed oocyst per gram counts made by the Mini-FLOTAC and centrifugal faecal flotation.

Fixed effects

	Beta	SE	p-value
Intercept	7.94	0.62	<0.01
Method:			
CFF	Ref.		
FLOTAC	1.18	0.16	<0.01

Random effects

Variance	Between sample	Within sample
	3.76	0.41

A limitation of the Mini-FLOTAC is the time required for accurate detection of oocysts when examining samples with high levels of debris (Bortoluzzi et al. 2018). When using CFF, samples can be diluted after transferring onto the slide to allow for more accurate reading as needed, whereas the Mini-FLOTAC cannot be further diluted after loading into the flotation chamber. Further, kiwi samples are commonly ≤0.5g, and all the faeces is used in one test; thus, only one Mini-FLOTAC assay can be performed, limiting further diagnostics.

The use of the Mini-FLOTAC is suggested for use in research that requires precise examination and characterisation of *Eimeria* spp. in kiwi (e.g., treatment efficacy). However, increased accuracy is not necessarily required for clinical decisions on treatment of coccidial infections in individual kiwi; the ability of CFF to detect large

burdens of coccidia may be sufficient for this purpose with the advantage of being a faster test.

Nevertheless, the Mini-FLOTAC apparatus provides a cost-effective and relatively easily performed test for onsite diagnostics that would eliminate shipping costs and delays. Modifications were made to the manufacturer's sample preparation protocol (Cringoli et al. 2017) in order to accommodate such a scenario. Magnesium sulphate solution ($MgSO_4$, SG 1.28) was chosen as it gave an appropriate balance between higher specific gravity and less osmotic pressure when used with the original FLOTAC apparatus (Bauer et al. 2010). Further, it is less viscous than a sugar solution (Bauer et al. 2010); requires ingredients, i.e. epsom salts, that are more easily obtained than zinc sulphate; and is more easily stored and transported without attracting pests, as may be the case with sugar. In addition, compensations for time, such as reading only one chamber rather than two (Bortoluzzi et al. 2018), is likely to occur in routine diagnostics, especially as testing capabilities shift to onsite, busy practitioners. A further modification used was a mesh with twice the aperture width recommended (Cringoli et al. 2017); however, this proved adequate for reducing debris in kiwi samples and did not limit the loading of the Mini-FLOTAC or reading the resulting preparation. In summary, this research demonstrates that even a procedure that varies from the validated protocol, the Mini-FLOTAC still provides a more accurate estimate of oocyst load.

2.5. Acknowledgements

We would like to thank the team at The National Kiwi Hatchery and The Cape Sanctuary for providing samples as well as Barbara Adlington and Anne Tunnicliffe in the Massey University parasitology laboratory for technical support.

CHAPTER 3

Morphological and molecular characterisation of coccidia in brown kiwi (*Apteryx mantelli*)

Contribution of co-authors: Dr McInnes was instrumental in communicating with practitioners and obtaining research materials. Dr Morgan assisted in the research design and lab protocols. Dr Pomroy contributed to developing the protocol for sporulation and morphological analysis. Dr Howe helped establish appropriate genetic targets. Further, Drs Howe and Biggs contributed to the genetic analysis and communication of the results. Emillie Vallee was instrumental in developing and interpreting the statistical analyses. All authors provided feedback on one or more drafts of this manuscript. See Appendix G.

The following chapter applies the Mini-FLOTAC in a research scenario and builds on the descriptions of coccidia in brown kiwi by Morgan et al. (2017). While it is intended for publication in Parasitology Research and has been formatted accordingly, additional details have been included for clarity for the purpose of this thesis.

3.1. Abstract

To prevent predation of brown kiwi (*Apteryx mantelli*) by introduced mammals, Operation Nest Egg (ONE) raises chicks and juveniles in outdoor, predator-proof enclosures/crèches until they are large enough to defend themselves from introduced predators. These facilities experience environmental accumulation of coccidial oocysts, which may lead to severe morbidity and mortality of these young kiwi. Four species of coccidia have been morphologically described from sporulated oocysts as well as additional opportunistic descriptions of endogenous stages. This research continues the morphological descriptions of these *Eimeria* spp. and provides the first genetic characterisation targeting the mitochondrial cytochrome *c* oxidase I (COI) gene. Pooled kiwi droppings were collected from ONE facilities and screened for coccidia using the Mini-FLOTAC apparatus with magnesium sulphate solution (SG 1.28). Positive samples were sporulated in 2% aqueous (w/v) potassium dichromate ($K_2Cr_2O_7$) at room temperature. Sporulated oocysts were cleaned and imaged under oil immersion; measurements were taken using ImageJ, v1.51s. DNA was extracted using the ZR Quick-DNA Fecal/Soil Microbe DNA Miniprep Kit, modified with additional steps to break open the oocysts. An approximately 350 bp region of the 18S rDNA and a 465 bp portion of the COI gene were targeted with conventional PCR and Sanger sequencing. Sequences

were trimmed, aligned to reference sequences, and used to build a neighbour-joining consensus tree. Seven samples from five locations were chosen for this study; 191 individual oocysts were measured and all samples contained coinfections. A novel morphotype (M5) is morphologically described. Similar to *Eimeria kiwi*, M5 is circular to elliptical (14.609 × 13.890 μm) and with a nonvisible micropyle and absent micropylar cap and oocyst residuum. The main distinction of M5 is the smooth wall. Whilst the molecular protocol targeting 18S rDNA was too nonspecific, analysis of the COI gene provided enough differentiation to confirm the genus and novelty of these coccidia. Five of the seven sequences contained ambiguous bases indicating mixed species infections. With the exception of one sample (OW01, which resulted in a sequence most similar to a mouse *Eimeria*), all kiwi coccidia sequences clustered into three clades separate from other *Eimeria*. Based on these findings, it was determined there are at least five morphotypes of *Eimeria* that infect brown kiwi and co-infections are common at the ONE facilities surveyed. The COI amplicon targeted for this study provided sufficient differentiation from other members of this genus. Sanger sequencing yielded ambiguous bases, indicating the need for more in-depth sequencing.

3.2. Introduction

Juvenile brown kiwi (*Apteryx mantelli*) in Operation Nest Egg (ONE) facilities suffer morbidity and mortality from coccidiosis caused by *Eimeria* spp., delaying this programme's ability to release kiwi into the wild (Morgan et al. 2012; Morgan et al. 2013). Morgan et al. (2017) presents descriptions of four species (*Eimeria mantellii, Eimeria kiwii, Eimeria paraurii, Eimeria apteryxii*) from sporulated oocysts shed by

captive brown kiwi (*Apteryx mantelli*, n = 6) from the ONE sites Pūkaha National Wildlife Centre (40°43'35.0"S, 175°38'23.6"E) and Maungatautari Ecological Island (38°01’33.13’’S, 175°33’57.94’’E). Other reports of coccidia in kiwi include four unsporulated morphotypes described by Thompson and Wright (1978); three morphologically distinct gametocytes found in the intestinal tract (Morgan et al. 2012); one gametocyte reported in the renal system; and meronts in multiple visceral organs (Morgan et al. 2013). These reports likely overlap, describing stages of the same morphotype; however, these connections require further verification.

Accurate detection of coccidia in individual birds provides vital information regarding treatment and control of disease within kiwi rearing facilities. More detailed identification of coccidia to a species level would enable key insights for management of disease within and between kiwi populations, including issues such as treatment efficacy; translocation of disease between populations; and determination of pathogenicity of individual parasite species (Morgan et al. 2013; Morgan et al. 2014; Taylor et al. 2019). When, for example, a brown kiwi tests positive for a particular species of *Eimeria* that is known to be highly pathogenic in brown kiwi, decisions around management can be made. These decisions may include a delay to a planned translocation of that individual as the stress involved with this may increase their chances of experiencing severe clinical disease post-release.

The majority (73%) of all species of coccidia described in the last five years have been classified only on morphological descriptions, with only 16% providing both exogenous

and endogenous descriptions (see Chapter 1.3, Table 1.2). This method of discrimination is limited by morphological similarities between oocysts of different *Eimeria* spp. and does not provide any information regarding endogenous development and subsequent pathology. In order to definitively connect clinical disease with sporulated morphotypes, experimental infection and pathogenicity trials have been undertaken in domestic host species, such as the Barbay partridge (*Alectoris barbara*) and chickens (*Gallus gallus domesticus*) (Fernandez-Alvarez et al. 2016; Jenkins et al. 2017). However, this approach is not possible in species such as the brown kiwi, which, from a conservation perspective, are considered to be nationally "At Risk: Declining" (Robertson et al. 2017). The use of molecular tools could be an effective approach to link morphological identification with key questions about the epidemiology, virulence, and pathogenicity of these species. A previous study by Morgan (2013), explored this approach and used Sanger sequencing to target the 18S rRNA, ITS-1, and ITS-2 genes. Whilst 18S sequences confirmed the genus, the ITS-1 and -2 genes were found to be extremely variable in length (ITS-1, 195-515 bp; ITS-2, 60-714 bp). While some variation in target sites is needed to differentiate species, discerning the points of variation that differentiate genetically distinct species of *Eimeria* can be exceedingly difficult especially if the target gene varies greatly within a single species of coccidia. Therefore, the ultimate aim of developing a diagnostic test using these targets to determine the presence or absence of one or more species in a single sample could be extremely complicated. Other gene targets may provide a more reliable means of differentiation and comparison to other species of *Eimeria*.

The following research presents additional morphological descriptions of oocysts shed by brown kiwi and documents a novel morphotype using the guidelines suggested by

Duszynski and Wilber (1997). Additionally, partial 18S rDNA and mitochondrial cytochrome *c* oxidase I (COI) genes are analysed with Sanger sequencing.

3.3. Methods

3.3.1. Sample collection

From 2017 to 2018, faecal samples from brown kiwi (*A. mantelli*) were collected from routine diagnostic and transportation health screenings. On arrival at Massey University, all dropping samples were assigned a code based on location as well as an identification number that reflects the individual kiwi host or group of hosts, if known. If unknown, a new identification number was assigned to that sample. Repeat sampling from the same host(s) were differentiated by the date of submission. All samples positive for coccidia were processed as described below. Samples were chosen for this study based on a combination of factors: location, the success of oocyst sporulation, oocyst load (oocysts per gram; OPG), amount of sample remaining, and diversity of coccidial morphotypes.

3.3.2. Oocyst detection

Samples were screened for coccidia using the Mini-FLOTAC technique, as described in Chapter 2 (Appendix B.3).

3.3.3. Sporulation and storage

The oocysts were sporulated as described by Duszynski and Wilber (1997). Briefly, small aliquots of droppings were mixed with 2% aqueous (w/v) potassium dichromate

($K_2Cr_2O_7$, Appendix C.1) at a minimum 1:5 ratio. After distributing these mixtures thinly over the bottom of Petri dishes (100 mm x 15 mm) to ensure oxygenation, the samples were incubated for 15 days at room temperature. Distilled water was added as needed to keep them moist, and they were regularly agitated for gas exchange during the incubation period. Sporulated oocysts were stored at room temperature in 25 ml, sealed flasks. See Appendix C.2 for more detail.

3.3.4. Sporulated oocyst isolation and measurement

One millilitre aliquots in 1.5 ml microcentrifuge tubes were spun at 18800 x g for 7 minutes to form a pellet. The supernatant was removed and replaced with 1 ml of dH_2O, vortexed, then centrifuged again. This rinsing was repeating until the supernatant was colourless (usually 4-6 times), then 500 μl magnesium sulphate solution ($MgSO_4$, SG 1.28) was added instead of dH_2O (Appendix C.3). The sample was then homogenised with a disposable pipette and left to rest for at least 1 minute. Drops of this mixture were suspended from the underside of a coverslip that was carefully placed on a cavity slide (Figure 3.1). The coverslip was temporarily adhered to the cavity ring using water (Appendix C.4).

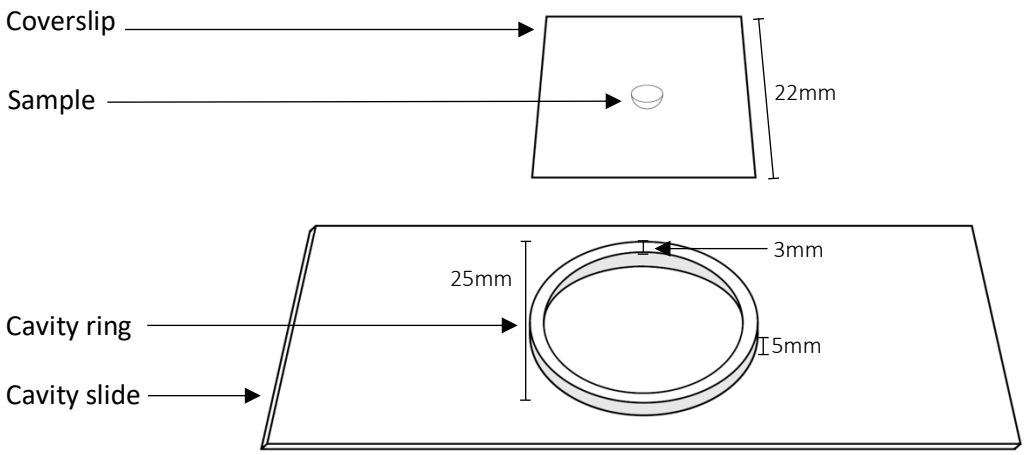


Figure 3.1. Diagram of oocyst isolation for imaging. The oocysts in the sample float in the $MgSO_4$ (SG 1.28), bringing them up to the coverslip and allowing for imagery under oil immersion (1,000x).

Using a light microscope (LEICA DM750) at 1,000× magnification with a LEICA ICC50W mounted camera (Leica Microsystems GmbH, Wetzlar, Germany) calibrated with a 1.0 mm stage micrometre (Olympus Optical Co. Ltd., Tokyo, Japan), pictures of individual oocysts were taken under oil immersion at multiple focal points (Appendix C.5). Morphological features were measured using ImageJ, v1.51s (Schneider et al. 2012) and described for up to 84 oocysts (Anonymous 1962; Duszynski and Wilber 1997; Appendix C.5). Sporocysts were only measured if the entire length was in focus. Type imaging was performed on an Olympus IX83 microscope outfitted with differential interference contrast (DIC) optics using a 100× (NA1.4) objective lens. All images were captured with a Retiga 6000 monochrome camera (QImaging) controlled by cellSens Dimension software (v1.18; Olympus) with a 2x2 bin resulting in apparent pixel sizes of 90.8 nm.

3.3.5. Molecular analysis

3.3.5.1. Controls

Immucox® Breeders and Layers (Pacificvet, Christchurch, New Zealand) live vaccine (sample IMMU) that contains *Eimeria acervulina, E. maxima, E. necatrix,* and *E. tenella* was used as a positive control for the PCR reactions. Sterile water controls were included as negative controls for the extraction process and PCR reactions. In addition, a dropping from a wild kereru (*Hemiphaga novaeseelandiae*) from Pūkaha National Wildlife Centre containing previously undescribed *Eimeria* spp. oocysts was included to test the specificity of the PCR reactions.

3.3.5.2. Extraction of DNA

Aliquots (0.15 g) of samples that were included in the morphological descriptions (n = 7) were incubated at -80°C for 12-24 hours; cycled three times between liquid nitrogen and 100°C for five minutes at each temperature; and incubated overnight with 40 μl Proteinase K (ThermoFisher Scientific, Waltham, MA, USA) at 56°C to break open the unsporulated oocyst walls (see Chapter 6). DNA was extracted using the ZR Quick-DNA Fecal/Soil Microbe DNA Miniprep Kit (Zymo Research, Orange County, CA, USA) according to the manufacturer's instructions (Appendix C.6).

3.3.5.3. 18S rDNA amplification

The 18S rRNA gene was amplified using a 25 μl reaction with 1 × PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, primers E18SF and E18SR each at 0.5 mM, 0.2 mg/mL BSA, 2 U

Platinum *Taq* DNA Polymerase (Invitrogen, ThermoFisher Scientific) with an initial denaturation at 95°C for 7 min; 40 cycles of 95°C for 20 sec, 60°C for 30 sec, and 72°C for 90 sec; and a final extension at 72°C for 10 min (Jenkins et al. 2006; Appendix C.7; Table 3.1).

3.3.5.4. COI DNA amplification

A nested PCR protocol targeting the mitochondrial cytochrome *c* oxidase I (COI) region was adapted from Yang et al. (2013) and (Ogedengbe et al. 2011b) (see Appendix C.8; Table 3.1). The primary amplification was a 50 μl reaction with 1 × PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, primers Cocci_COI_F and Cocci_COI_Rev each at 1.0 mM, 0.2 mg/mL BSA, and 2 U Platinum *Taq* DNA Polymerase (Invitrogen) with an initial denaturation at 96°C for 5 min; 40 cycles of 94°C for 20 sec, 59°C for 30 sec, and 72°C for 90 sec; and a final extension at 72°C for 10 min. The secondary protocol used a 50 μl reaction with 1 × PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 mM COIF2, 0.2 mM COIR2, 0.2 mg/mL BSA, and 2 U Platinum *Taq* DNA Polymerase (Invitrogen). The secondary conditions had an initial denaturation at 96°C for 5 min; 40 cycles of 94°C for 20 sec, 54°C for 30 sec, and 72°C for 90 sec; and a final extension at 72°C for 10 min (Yang et al. 2013).

Table 3.1. Primer sets used for amplification of partial COI genes from kiwi coccidia.

Gene Target		Primer Name	Primer Sequence	Amp. Size	Reference
18S		E18SF E18SR	5'- CGG TGA AAC TGC GAA TGG CTC A -3' 5'- GCC TTC CTT AGA TGT GGT AGC C -3'	~350	Jenkins et al. (2006)
COI	1'	Cocci_COI_For Cocci_COI_Rev	5'- GGT TCA GGT GTT GGT TGG AC -3' 5'- AAT CCA ATA ACC GCA CCA AG -3'	~780	Ogedengbe et al. (2011b)
	2'	COIF2 COIR2	5'- TAA GTA CAT CCC TAA TGT C -3' 5'- GTC ATC ATA TGR TGT GCC CA -3'	~465	Yang et al. (2013)

3.3.5.5. Sequencing

Amplicons were run on a 1.5% agarose gel containing Invitrogen UltraPure Agarose (ThermoFisher Scientific, Waltham, MA, USA) and visualised with RedSafe Nucleic Acid Staining Solution (iNtRON Biotechnology, Gyeonggi-do, South Korea). Samples with bands at the correct size (~350 bp for 18S; ~465 bp for COI) were purified using PureLink PCR Purification Kit (Invitrogen) and submitted to the Waikato DNA Sequencing Service (The University of Waikato, Hamilton, NZ) for initial Sanger sequencing. If one or both of the reads had an ABI % quality score below 50%, the samples were reamplified and sent to the Massey Genome Service (Massey University, Palmerston North, NZ) for resequencing (Appendix C.9).

3.3.5.6. COI phylogenetic analysis

The COI sequences obtained in this study and 61 reference sequences (including *Eimeria* spp., *Toxoplasma gondii*, *Neospora caninum*, *Isospora* sp., and *Hammondia* spp.) obtained from the GenBank database were aligned using Clustal W (Thompson et al.

1994) and trimmed to 497 bases (including spaces) using Geneious, v11.0.5 (Biomatters, Auckland, New Zealand). This alignment was used to build a neighbour-joining consensus tree in Geneious; the Jukes-Cantor distance model with 1,000 replicates was used to calculate branch length. *Toxoplasma gondii* served as an outgroup.

3.4. Results

3.4.1. Sample collection

Seven *A. mantelli* samples from five locations were utilised for this study. All seven samples were pooled droppings that were collected from outdoor enclosures using sanitary, single-use collection bottles (see Table 3.2). Pūkaha National Wildlife Centre, Mount Bruce, Wairarapa (40°43'35.0"S, 175°38'23.6"E; n = 2); The National Kiwi Hatchery Aotearoa, Rotorua (38°06'33.5"S, 176°13'14.3"E; n = 2); Nga Manu Nature Reserve, Waikanae (40°51’40”S, 175°03’40”E; n = 1); and Orana Wildlife Park, Christchurch (43°28’02”S, 172°27’40”E; n = 1) house ONE kiwi in enclosures containing one or two individuals at a time prior to release into a crèche. Warrenheip, Cambridge/Waikato (37°56'19.7"S 175°43'08.5"E; n = 1) is a 16-hectare crèche that can accept up to 12 juvenile kiwi at a time (K McInnes, pers. comm.). All samples were kept at room temperature and sent to Massey University, Palmerston North, New Zealand within one month of collection.

Table 3.2. Kiwi (*Apteryx mantelli*) dropping samples used for morphological and molecular characterisation of kiwi *Eimeria*.

Sample	Host Species	Status (IN/PO)	Island (N/S)	Region	Location	Year	Month	Age
MB06	Brown	IN	N	Manawatu	Pūkaha	2017	12	adult
MB16	Brown	IN	N	Manawatu	Pūkaha	2018	10	juv
NM01	Brown	IN	N	Manawatu	Nga Manu Nature Reserve	2018	12	ND
OW01	Brown	PO	S	Canterbury	Orana Wildlife Park	2017	8	adult
RSB06	Brown	IN	N	Bay of Plenty	NKHA	2018	4	ND
WB01*	Brown	IN	N	Bay of Plenty	NKHA	2018	2	juv
WP01	Brown	IN	N	Waikato	Warrenheip	2017	10	juv

*WB01 is a sample collected from a bird infected at The National Kiwi Hatchery Aotearoa that had been sent to Wildbase Hospital, Massey University, Palmerston North for treatment for high coccidial burdens. Pūkaha refers to Pūkaha National Wildlife Centre; NKHA refers to The National Kiwi Hatchery Aotearoa. "IN" refers to samples from individual kiwi dropping; "PO" refers to samples that were pooled from multiple droppings.

3.4.2. Morphology

Overall, 191 individual oocysts from pooled brown kiwi droppings (n = 7) were measured for this study (Table 3.3). All samples (7/7) contained coinfections, ranging from two to five morphotypes per sample, and all previously described *Eimeria* spp. were identified (Morgan et al., 2017). No single morphotype was associated with a particular sample. The only sample that contained five morphotypes (WP01), included a single oocyst resembling the previously characterised *E. paraurii* (Morgan et al. 2017).

Of the oocysts characterised for this study, 84 morphologically similar oocysts, named M5 for the purpose of this study, did not fit the descriptions of the species reported by Morgan et al. (2017). While similar to *E*. *kiwii*, this novel M5 morphotype has a smooth rather than striated wall. This qualitative distinction was reliably discernible under oil immersion and occasionally discernible at lower magnifications. In addition to this novel morphotype, the measurements of *E. mantellii* and *E. apteryxii* taken in the present study showed these morphotypes to be less distinguished by size than previously described (Figure 3.2; Table 3.4). The average dimensions of *E. mantellii* were reported to be 17.9 × 10.7 μm with a 1.7 length/width (L/W) ratio (Morgan et al. 2017), whereas the current research reports a mean of 18.8 × 13.9 μm and a L/W ratio of 1.4. While the sizes of these two reports are relatively similar, the L/W ratio provides a better metric for shape and indicates an overall difference. This inconsistency is illustrated in the increasing overlap in L/W ratio between *E. mantellii* and *E. apteryxii* (Figure 3.2c).

Table 3.3. The number of *Eimeria* oocysts measured from brown kiwi (*Apteryx mantelli*) collected from 2017-2018 in New Zealand.

***E*. species**	**MB06**	**MB16**	**NM01**	**OW01**	**RSB06**	**WB01**	**WP01**	**Total**
E. kiwii	1	-	2	2	16	3	10	34
E. mantellii	-	7	1	1	-	2	2	13
E. apteryxii	4	35	1	8	-	7	4	59
E. paraurii	-	-	-	-	-	-	1	1
M5*	1	5	-	4	51	1	22	84
Total	6	47	4	15	67	13	39	191

* M5 is a morphotype not consistent with previously reported morphotypes.

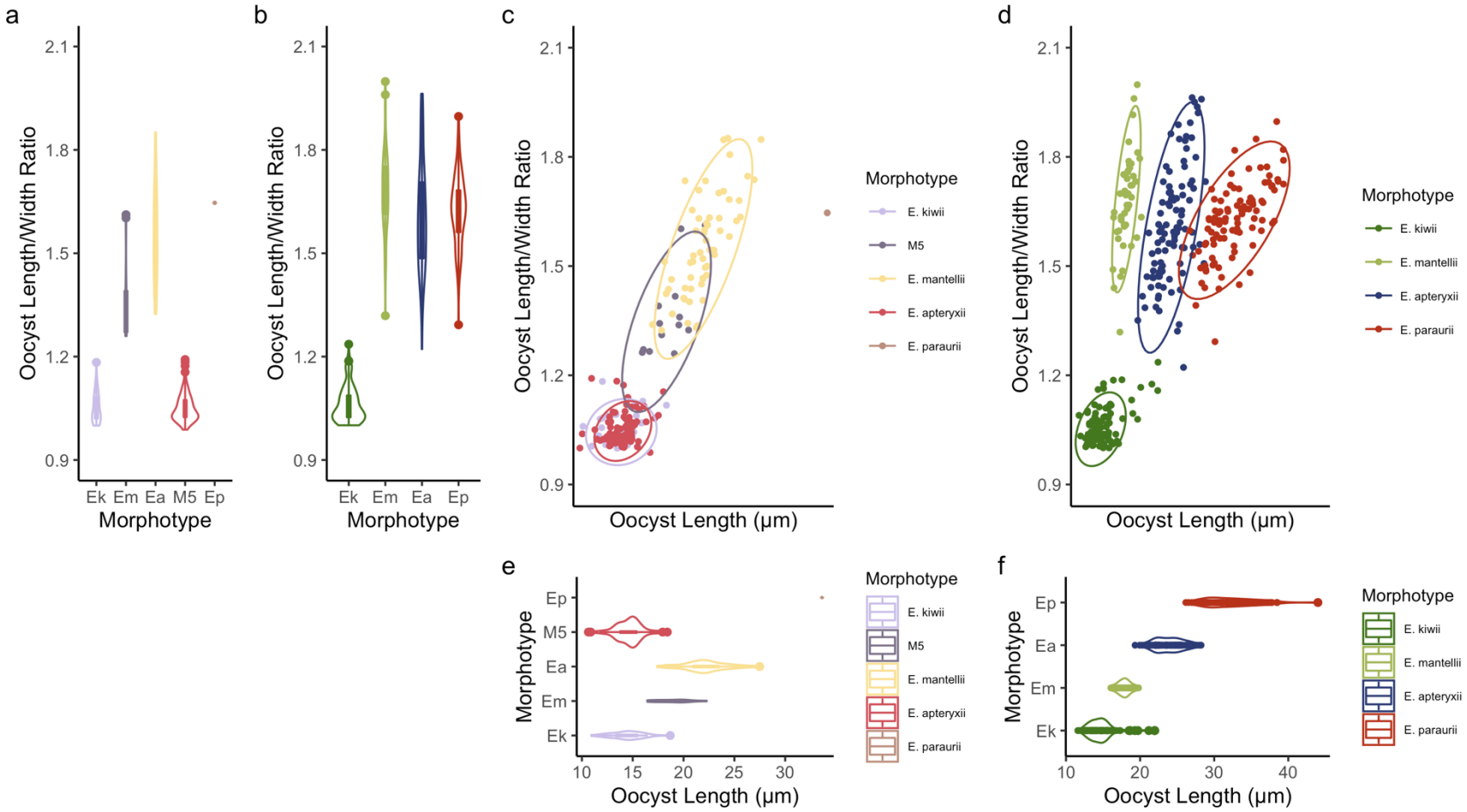

a
b
c
d
e
f
Oocyst Length/Width Ratio
Morphotype
Ek Em Ea M5 Ep
Oocyst Length (µm)
Morphotype
E. kiwii
M5
E. mantellii
E. apteryxii
E. paraurii

Figure 3.2. Six plots comparing coccidia from brown kiwi (Apteryx *mantelli*) this study and Morgan et al. (2017). Plots “a”, “c” and “e” are from this study whereas plots “b”, “d” and “f” are from Morgan et al. (2017). Plots “a” and “b” are violin (density) plots comparing the oocyst length/width ratios. Plots “c” and “d” are scatterplots sorted by oocyst length and length/width ratio with data. Plots “e” and “f” are violin (density) plots comparing the oocyst length of morphotypes. Morphotypes *E. kiwii* and M2 are distinguished by a striated and smooth wall, respectively. *E. mantellii* and *E. apteryxii* are distinguished by size and the absence and presence of a visible micropyle, respectively.

Table 3.4. Comparison of the morphometrics of kiwi *Eimeria* in the present study (A) and those reported by Morgan et al. (2017) (B). Values not reported in Morgan et al. (2017) were retrieved from Morgan (2013).

		Oo. Length x Width (avg) μm	**Oo. L/W (avg)**	**Sporocyst Length x Width (avg) μm**	**Sporocyst L/W (avg)**	**Wall Width (avg) μm**	**No. PG (avg)**	**PG Length x Wdith (avg) μm**	**Micropyle Width (avg) μm**
E. kiwii	A (n = 34)	10.62-18.67 x 12.70-16.72 (16.74 x 13.74)	1.00-1.18 (1.05)	6.74-12.03 x 4.13-6.42 (9.05 x 4.98)	1.36-2.33 (1.83)	0.55-0.93 (0.72)	1	1.11-2.81 x 0.90-2.37 (2.09 x 1.63)	Absent
	B (n = 100)	11.59-21.98 x 10.64-18.95 (14.78 x 13.91)	1.00-1.24 (1.06)	6.5–13.6 x 3.6–7.4 (9.37 x 4.91)	1.5-2.3 (1.9)	0.51-1.16 (0.79)	1-2	1.5-2.0 × 1.0-2.1 (2.1 × 1.6)	Absent
E. mantellii	A (n = 13)	16.44-22.28 x 12.50-15.66 (18.85 x 13.90)	1.26-1.61 (1.36)	7.79-12.46 x 4.74-6.69 (10.89 x 5.67)	1.44-2.21 (1.92)	0.53-0.88 (0.72)	1-5 (2.667)	0.64-2.42 x 0.56-2.18 (1.51 x 1.13)	Absent
	B (n = 50)	16.06-19.79 x 9.60-12.87 (17.85 x 10.67)	1.39-2.00 (1.68)	7.9–10.0 x 3.9–5.2 (9.15 x 4.51)	1.6-2.4 (2.0)	0.42-0.78 (0.62)	1-2	0.9-2.1 × 0.4-1.6 (1.5 × 1.1)	Absent
E. apteryxii	A (n = 59)	19.23-27.48 x 12.70-16.14 (22.23 x 14.38)	1.32-1.85 (1.56)	8.49-13.55 x 3.78-9.03 (11.35 x 5.85)	1.18-3.27 (1.96)	0.43- 0.92 (0.70)	1-7 (2.900)	0.64-3.20 x 0.50-2.44 (1.64 x 1.15)	1.11-3.20 (1.95)
	B (n = 100)	19.33-28.23 x 12.71-20.74 (23.90 x 14.93)	1.22-1.96 (1.61)	8.0–17.4 x 5.0–8.3 (11.71 x 6.03)	1.4-2.4 (1.9)	0.60-1.24 (0.82)	1-7	1.2-2.9 × 0.8-2.1 (2.2 × 1.3)	1.6-2.4 (2.0)
E. paraurii	A (n = 1)	33.63 x 20.45 (N/A)	1.65 (N/A)	17.83 x 8.00 (N/A)	2.23 (N/A)	1.14 (N/A)	1 (N/A)	3.16 x 2.80 (N/A)	3.14 (N/A)
	B (n = 100)	26.17-44.00 x 16.39-23.00 (32.23 x 19.76)	1.29-2.28 (1.63)	11.7-20.6 x 6.8–9.2 (16.2 x 7.9)	1.5-2.3 (2.0)	0.77-1.70 (1.23)	1-2	Not measured	Absent
M5	A (n = 84)	10.64-18.40 x 9.84-17.16 (14.61 x 13.89)	1.00-1.19 (1.05)	5.75-11.45 x 3.60-6.75 (9.43 x 5.08)	1.35-2.17 (1.86) n = 106	0.45-0.91 (0.67)	1	1.14 -3.26 x 0.85-2.37 (2.33 x 1.80)	Absent

3.4.2.1. Morphological descriptions

3.4.2.1.1. Morphotype 5 – (Figure 3.3)

Type host: Brown kiwi, *Apteryx mantelli* (Burbidge et al. 2003), Juvenile.

Type locality: Pūkaha National Wildlife Centre, Wairarapa, New Zealand (40°43'35.0"S, 175°38'23.6"E); Warrenheip, Waikato, New Zealand (37°56'19.7"S, 175°43'08.5"E); and The National Kiwi Hatchery Aotearoa, Rotorua, New Zealand (38°06'33.5"S, 176°13'14.3"E).

Type material: Sporulated oocysts are in the process of deposition in the Museum of New Zealand Te Papa Tongarewa (pending at time of submission). Prevalence: 86% (in 6 of 7 specimens).

Sporulation time: Exogenous. All oocysts were passed unsporulated and sporulated within 15 days at room temperature.

Site of infection: Unknown; retrieved from droppings.

Sporulated oocyst: Oocyst shape (n = 84) circular to elliptic: 10.638-18.397 × 9.839-17.159 μm (14.609 × 13.890 μm); length/width (L/W) ratio 1.000-1.191 (1.054). Smooth wall, 0.45-0.914 μm (0.666 μm). Micropyle, micropylar cap, and oocyst residuum absent. 1 polar granule present.

Sporocyst: Sporocysts (n = 106) 4, 5.751-11.454 × 3.596-6.746 μm (9.434× 5.082 μm); length/width (L/W) ratio 1.345-2.172 (1.862); Stieda body present; sporocyst residuum present, generally clumped.

Sporozoite: Two sporozoites, not measured; large sporozoite refractile body at base.

Taxonomic remarks: This is similar to *Eimeria kiwii* described by Morgan et al. (2017); however, this species is characterised by a smooth wall rather than a striated wall.

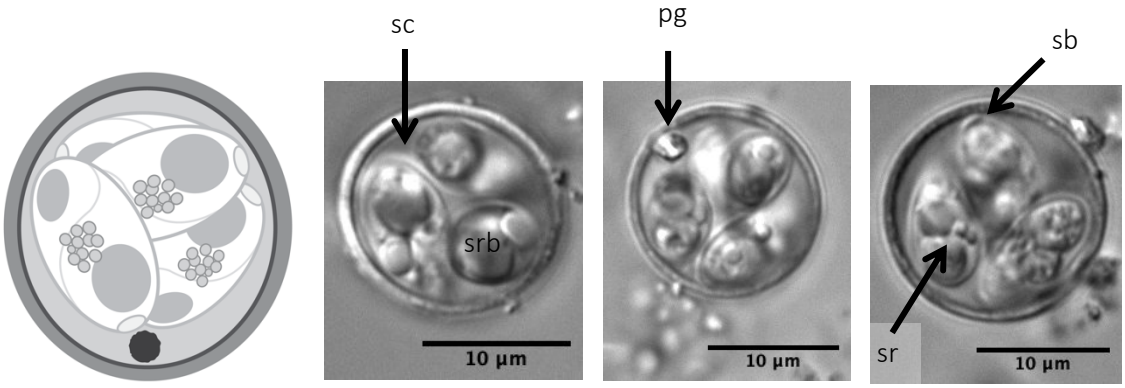


Figure 3.3. Line drawing and pictograph of the novel morphotype (M5, n = 84) reported in brown kiwi (*Apteryx mantelli*). Key: sc = sporocyst, srb = sporozoite refractile body, pg = polar granule, sr = sporocyst residuum, sb = Stieda body.

3.4.3. Molecular analysis

3.4.3.1. 18S rDNA

Due to low-quality amplicon sequences using the 18S rDNA primers, only MB16 returned a BLAST® search result relating to the genus *Eimeria* (96% homology *Eimeria bukidnonensis,* GenBank AB769601). Thus, no further analysis of the 18S sequences were pursued.

3.4.3.2 COI gene

The COI protocol provided sufficient amplification to yield high-quality chromatograms although overlapping peaks, indicative of co-infection with multiple *Eimeria* spp., were present in three of seven samples. Consensus sequences with the ambiguous bases included (Figure 3.4) confirmed that the sequences generated from the coccidia from all seven fell within the genus *Eimeria* (Table 3.5; Figure 3.5).

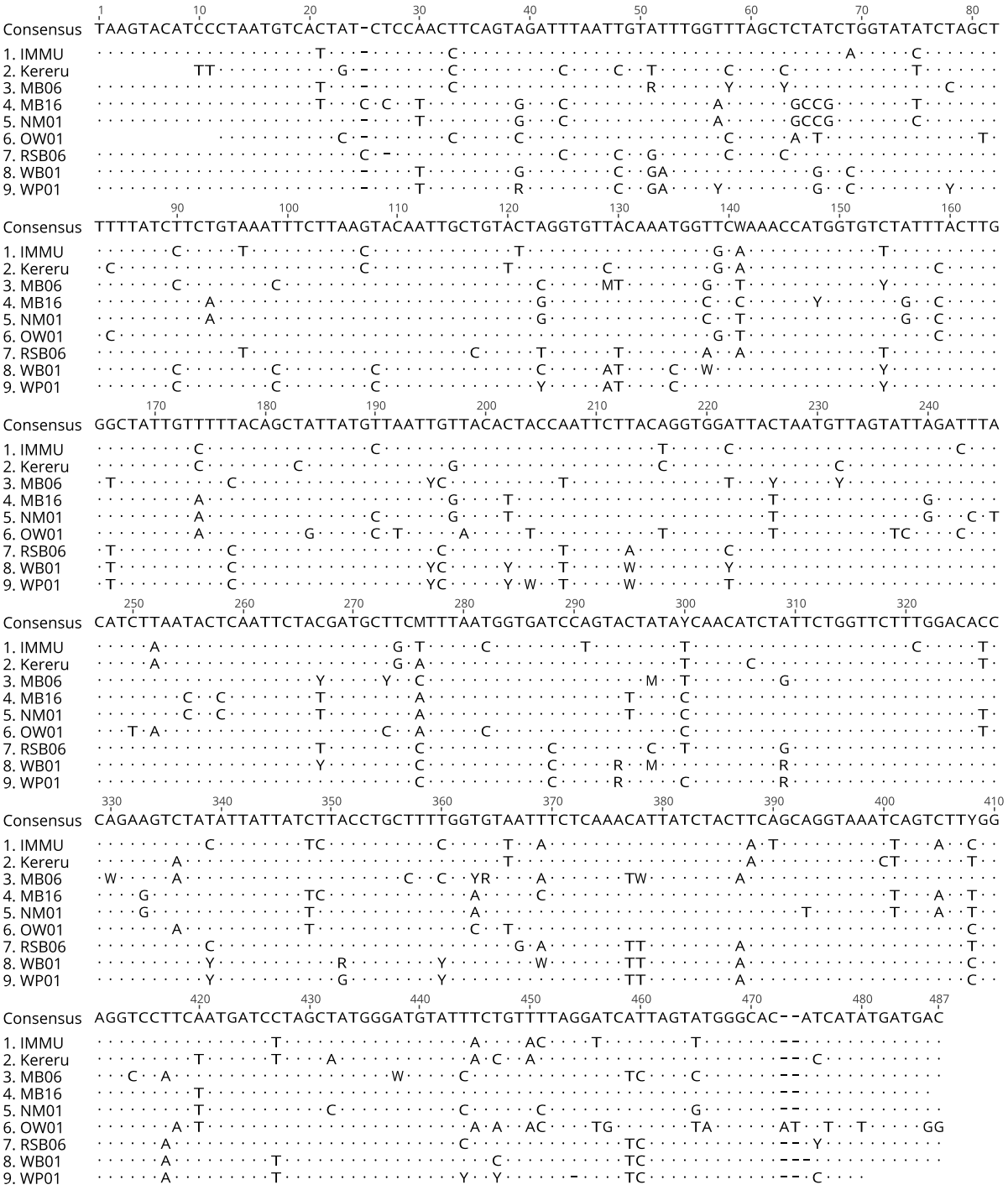


Figure 3.4. Eight Sanger sequencing COI results from juvenile brown kiwi (*Apteryx mantelli*) coccidia collected during 2017-2018 from captive-rearing facilities. IMMU = 'Immucox® Breeders and Layers' live vaccine that contains *Eimeria acervulina, E. maxima, E. necatrix* and *E. tenella* (Pacificvet, Christchurch, New Zealand) used as a reference sequence. Kereru = coccidia from a kereru from Pūkaha National Wildlife Centre. Dots indicate sequence homology, dashes indicate gaps.

However, the sequence obtained from sample OW01, which was collected from Orana Wildlife Park in Christchurch, was 99% similar to a mouse coccidia (*Eimeria ferrisi,* GenBank MH777579.1; Table 3.6) and grouped with *Eimeria falciformis* (GenBank HM771682.1), another species of mouse coccidia. Thus, with the exception of OW01, which was likely environmental contamination, the remaining six kiwi coccidia sequences fell into three main clusters, which have been grouped into clusters A-C (Figure 3.5). This clustering includes the ambiguous bases, ranging from 1 (0.2%) to 17 (3.5%), that occur in five of these sequences (Table 3.5).

Cluster A contained MB16 and NM01, which were 97% homologous (Table 3.6). The samples in this cluster originated from the Manawatu region. Cluster B contained WB01 and WP01, which shared 97% identity; both originated from the Waikato region. Cluster C, which contained MB06 and RSB06, was the most divergent; these sequences were no more than 93% homologous with each other or any other sample from this study.

With the exception of the OW01, the kiwi samples did not share strong homology with records documented in GenBank at this COI site (Table 3.6). The most homologous sequence was from MB16, which was 91% homologous with an *Eimeria mundayi* (GenBank MK202808.1) from a woylie (*Bettongia penicillata*) in Australia.

A BLAST search of the kereru sequence yielded a 93.23% homology with an *Eimeria* sp. from a western capercaillie (Tetrao urogallus; GenBank MG595963.1; Stenzel et al.

2019) and, in the phylogenetic tree (Figure 3.5), was most homologous (92.83%) to *E. bubonis*, though it did not group with any other *Eimeria*. The Immucox® grouped with *Eimeria acervulina* (Figure 3.5) with 99.79% homology with Australian isolates GenBank KX094946.1, KX094947.1 KX094948.1, HM771673.1, HQ702479.1, FJ236428.1, and EF158855.1 (Lin et al. 2011; Morgan and Godwin 2017; Ogedengbe et al. 2011a; Schwarz et al. 2009).

Table 3.5. Mitochondrial cytochrome *c* oxidase I consensus Sanger sequencing quality results and top BLAST results from brown kiwi (*Apteryx mantelli*) coccidia sampling from 2017-2018.

Sample	ABI % qual. scores (F, R)	Seq. length (bp)	Ambiguous Bases % (bp)	BLAST® result	% Identity
MB06	74.3, 31.6	487	3.5 (17)	*Eimeria* sp. MK315212.1	86
MB16	91.2, 57.7	484	0.2 (1)	*Eimeria mundayi* MK202808.1	91
NM01	89.9, 67.2	484	0	*Lankesterella* sp. KT369005.1	90
OW01	70.9	472	0	*Eimeria ferrisi* MH777579.1	99
RSB06	85.6, 83.4	484	0.2 (1)	*Eimeria* sp. MF496271.1	87
WB01	72.6, 77.6	483	3.3 (16)	*Isospora* sp. MK573842.1	85
WP01	56.3, 53.2	476	3.3 (16)	*Eimeria piriformes* JQ993698.1	85
Kereru	85.8, 87.8	445	0	*Eimeria* sp. MG595963.1	93
IMMU*	88.9, 48.9	445	0	*Eimeria acervulina* KX094948.1	100

* IMMU is the Immucox® Breeders and Layers live vaccine that contains *Eimeria acervulina, E. maxima, E. necatrix,* and *E. tenella.*

Table 3.6. Mitochondrial cytochrome *c* oxidase I consensus Sanger sequencing percent (%) identify results from brown kiwi (*Apteryx mantelli*) coccidia samples collected from 2017-2018.

	MB06	MB16	NM01	OW01	RSB06	WB01	WP01	IMMU	Kereru
MB06		87	86	84	93	93	92	87	86
MB16	87		97	86	87	87	86	87	87
NM01	86	97		87	86	86	86	87	87
OW01	84	86	87		83	83	83	88	88
RSB06	93	87	86	83		93	93	87	87
WB01	93	87	86	83	93		97	86	85
WP01	92	86	86	83	93	97		86	85
IMMU*	87	87	87	88	87	86	86		90
Kereru	86	87	87	88	87	85	85	90	

* IMMU is the Immucox® Breeders and Layers live vaccine that contains *Eimeria acervulina, E. maxima, E. necatrix,* and *E. tenella.*

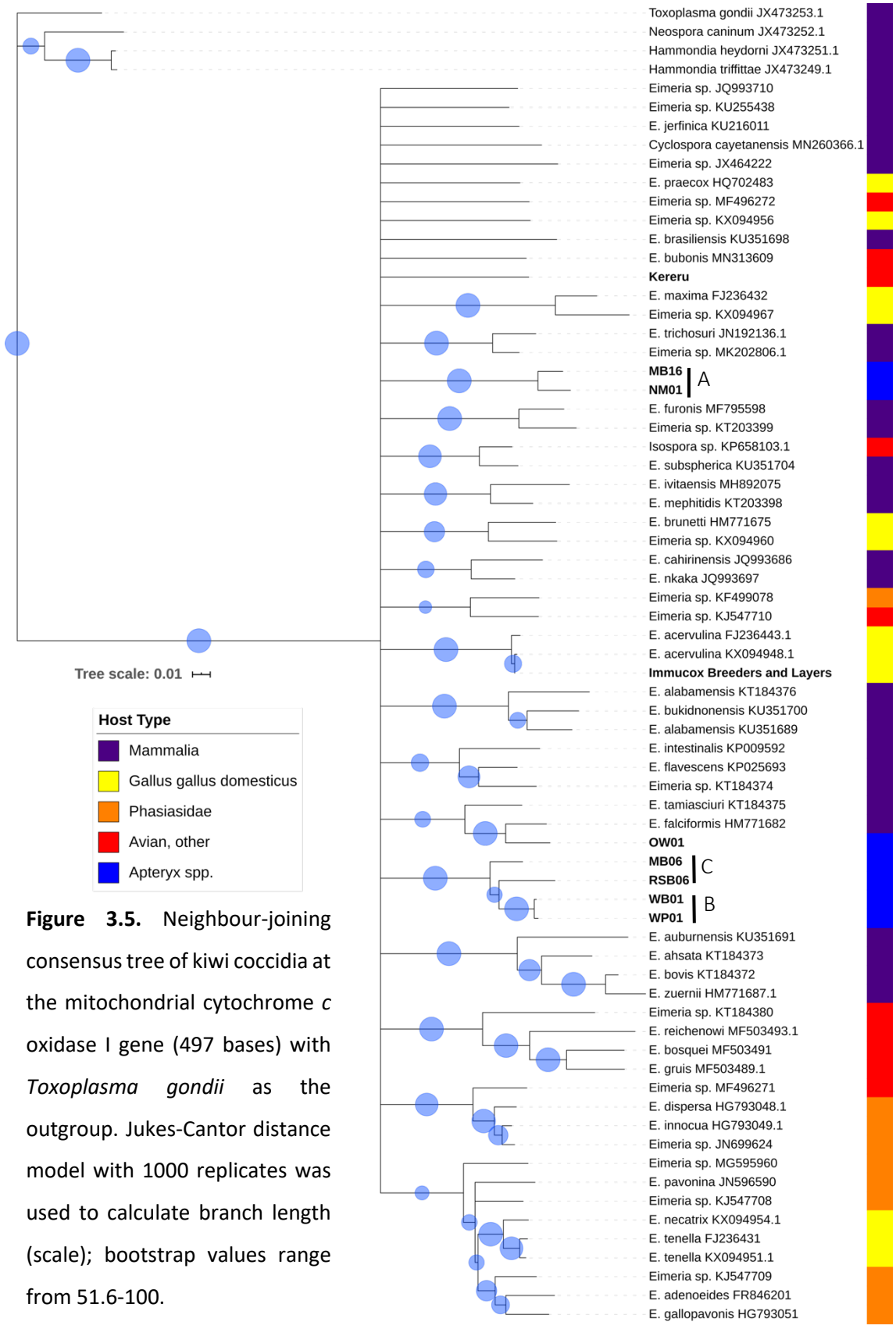


Figure 3.5. Neighbour-joining consensus tree of kiwi coccidia at the mitochondrial cytochrome *c* oxidase I gene (497 bases) with *Toxoplasma gondii* as the outgroup. Jukes-Cantor distance model with 1000 replicates was used to calculate branch length (scale); bootstrap values range from 51.6-100.

3.5. Discussion

3.5.1. Morphology

In this study, five *Eimeria* morphotypes were reported from the seven pooled faecal samples from brown kiwi with one novel morphotype (M5) described. Despite the size and shape variation of *E. mantellii* (Table 3.1) compared with previously described examples, it is likely these represent the same morphospecies. This study, as well as Morgan et al. (2017), used limited host sample sizes and geographic locations; thus, the addition of this data should be considered a continuation of the characterisations of these species as the differences may reflect natural variation. It should be noted that the change in size and shape of this morphotype blurs the lines between previously described *E. mantellii* and *E. apteryxii* (Figure 3.2). The main distinction between examples of these morphotypes from the present report relies heavily on the absence/presence, respectively, of a visible micropyle. The possibility remains that the present or previous study relies on technology (such as imaging software) that increases or decreases the likelihood of micropyles remaining unobserved. Differences in oocyst isolation for imaging may also have had an impact on characterisation. For example, the integrity of oocysts may be affected by the osmotic pressure of the flotation solution, an effect that increases the longer the oocysts are exposed (Ballweber et al. 2014; Zajac and Conboy 2012).

Similarly, the addition of the M5 morphotype in this report could reflect the difference in methods used by Morgan et al. (2017), rather than the absence of this morphotype

in the previous dataset. Nevertheless, the clear differences between the smooth and rough walls confirm the presence of at least five morphotypes in brown kiwi.

Interestingly, only one *E. paraurii* oocyst was identified in these samples, whereas it was identified in all (6/6) samples examined by Morgan et al. (2017). *E. mantellii* was present in 71% (5/7) of samples examined in this study and only 17% (1/6) previously. Alternatively, the prevalence of *E. apteryxii* and *E. kiwii* were similar (86% and 100%, respectively) between these reports (Morgan et al. 2017). This variation in prevalence indicates the species composition of coccidia infecting kiwi varies over time and location.

3.5.2. Molecular analysis

The amplification of the COI using these primers successfully amplified *Eimeria* from both the Immucox and the kereru. The Immucox contains *Eimeria acervulina, E. maxima, E. necatrix,* and *E. tenella* from chickens. Based on published mitochondrial genomes, these species are conserved and complement the COIR2 binding site. In the middle of the COIF2 binding site (more than 5 bp from either end), all these species contain one or two mismatches; it was unknown how flexible this primer would be under these conditions to accommodate certain mismatches. *E. maxima* and *E. tenella* contain two mismatches (G:A, G:T) from the primer site and were least likely to be amplified. *E. necatrix* contains a G:A mismatch; *E. acervulina* contains a G:T mismatch. A G to A bp mismatch has been shown to reduce the amplification by 100 fold, whereas a G to T

mismatch has been shown to have a much lower effect (Kwok et al. 1990). This likely explains the detection of only *E. acervulina* with these primers. The kereru, under brief microscopic observation, was observed to only contain a single morphotype of *Eimeria*; thus, this sample may contain a single species, leading to the detection of an unambiguous sequence.

The differentiation provided by the COI confirms the presence of novel species of *Eimeria* in brown kiwi. The sequences grouped in clusters A-C (Figure 3.5) potentially representing two or more of the morphospecies reported here. Unfortunately, Sanger sequencing tends to lead to the detection of only the most common sequence (Altimari et al. 2013; Cereb et al. 2015; Magalhaes et al. 2015). However, combining the morphology data (Table 3.2) with the percent identities of the most common sequence (Table 3.5) may enable predictions to be made between a morphotype and a particular sequence. For example, the samples in clade A, MB16 and NM01, were the most similar with a 97% identity. Sample MB16 contained predominately *E. apteryxii* (74%, 35/47 oocysts), whereas sample NM01 only contained 25% (1/4 oocysts) of *E. apteryxii.* Thus, the MB16 sequence may represent *E. apteryxii*, as it contained only a single ambiguous base. On the other hand, the NMO1 sequence had no ambiguous bases and contained three morphotypes. Sanger sequencing has been shown to preferentially yield the most dominant sequence, leading to missed variation (Altimari et al. 2013; Cereb et al. 2015; Magalhaes et al. 2015). This tendency is further illustrated by the OW01 sample, which yielded an unambiguous sequence from mouse coccidia even though microscopically, the sample contained four kiwi *Eimeria* morphotypes (Table 3.2). The oocyst length of

Eimeria ferrisi from mice ranges from 12-22 μm (17 μm) with a width ranging from 11-18μm (Ankrom et al. 1975). The length to width ratio of this species is 1.22 (1 to 1.6) and spherical to ovoid with a smooth wall. The sporocysts of *E. ferrisi* measure 8-11 μm (17 μm) in length and 5-7 μm (5.9 μm) in width. This description most resembled M5 oocysts, however, while the oocyst and sporocyst sizes were similar, the L/W ratio of the M5 morphotype ranged from 1.00-1.19 (1.05). The difference in oocyst L/W ratio as well as the description of M5 in other kiwi faecal samples, led the author to believe these oocysts were properly morphologically identified as M5. Interestingly, the COI sequence from this sample was clean with no overlapping peaks. This preferential sequencing of a non-target species of *Eimeria* demonstrates the importance of using several methods for the identification of oocysts in novel hosts.

Half (n = 3) of the samples successfully sequenced for the COI target contained many ambiguous regions, supporting the presence of co-infection. The similarity (88.9%) between the sequences enabled enough variation between the samples to provide meaningful comparisons between species of kiwi coccidia in brown kiwi, while remaining conserved enough for further diagnostic testing development and use in comparison to other *Eimeria*. The small sample size and the number of ambiguities in the COI sequencing results encourages further research into the genetic variation of these kiwi *Eimeria*. Increasing the sample size and range would encourage a greater representation of the diversity of coccidia infecting brown kiwi. A deeper sequencing analysis (e.g., Next Generation Sequencing) is encouraged to produce a truer

representation of the variation within and between samples, especially as producing a guaranteed, single-species infection in naturally infected kiwi is unlikely.

In the absence of experimentally infected kiwi, the best replacement would be single oocyst sequencing, which allows for definitive connections between morphotypes and particular sequences. However, extracting DNA from a single oocyst can be unreliable (Morgan 2013). Amplifying the DNA from a single oocyst has been approached several ways. The first relies on the dilution of a high concentration of oocysts to the equivalent dilution of a single oocyst (Molloy et al. 1998). Alternatively, the dilution of DNA (e.g., *Eimeria* in chickens; Vrba et al. 2010) to the concentration of a single haploid eimerian cell can also be used; however, this method may be more useful for testing the reliability of a particular amplification assay, rather than the reliable amplification of a single oocyst. Unfortunately, this dilution method is imprecise; with each dilution, the errors associated with manipulating small volumes increases, leading to progressively poorer estimates of true concentrations (Grgicak et al. 2010). Thus, physical visualisation and manipulation of individual oocysts provides a potential solution. The main tools that have allowed for the development of this method are micropipettes and micromanipulators. For example, Dolnik et al. (2009) used microscopic confirmation and micropipettes to isolate individual *Isospora* oocysts for amplification. This resulted in 72% (n = 39) successful, unambiguous sequences. Similarly, Sturbaum et al. (2001) described a method using a micropipette, micromanipulator, as well as microscopic confirmation to isolate *Cryptosporidium parvum*, which have oocysts up to 6 μm in diameter, with an 89% success rate. The lower success rate of detection and unknown

reliability of the selected primers to amplify *Eimeria* endemic to New Zealand led the authors to pursue the more reliable form of extraction (i.e., many oocysts at a time). This removed controllable factors of uncertainty. For example, even if a single oocyst is isolated from a sample, the possibility remains that liquid in the sample contained DNA from another, morphologically distinct oocyst. Thus, as an initial description, the authors believed it more important to establish the ability to detect these *Eimeria* and to provide baseline molecular references for future comparison.

3.5.4. Management implications

Future morphological research should focus on describing coccidia in other species of kiwi. For example, Haast tokoeka (*A. australis* "Haast") also rely heavily on ONE for continued population growth and are commonly raised in facilities that house brown kiwi on site. Determining whether these species of coccidia also infect other kiwi species would determine the extent to which these parasites need to be managed. The host specificity of kiwi coccidia is currently unknown.

Additionally, as rowi (*Apteryx rowi*) are most closely related to brown kiwi (Weir et al. 2016), *Eimeria* that can parasitise brown kiwi are most likely to be capable of reproducing in rowi. There are a number of examples where a species of *Eimeria* is capable of infecting closely related host species (e.g., *Eimeria gruis* and *Eimeria reichenowi* in hooded cranes, white-naped cranes, sandhill cranes, and whooping cranes; Honma et al. 2011a; Honma et al. 2011b; Novilla et al. 1981; see Chapter 1.2.2.1

for more detail). While the last known land bridge between the North and South Island occurred only 20,000 years ago, the glacial period drove rapid diversification of kiwi through geographic isolation and bottlenecking (Weir et al. 2016). During periods of geographic isolation and diversification, it would be expected for the coccidia of kiwi to also diversify. Whether or not some (or all) of these parasites differentiated enough to become specific to a particular species of kiwi has yet to be determined.

3.5.5. Conclusions

This study provides insight into the genetic variation of coccidia in kiwi and demonstrates the complexity of studying coccidia in kiwi. The samples included in this study are all from a small number of kiwi in captivity or crèches and, therefore, likely do not reflect the variation found throughout New Zealand. Identifying coccidia in wild populations of high conservation value would ensure novel parasites were not introduced into naïve populations through the release of captive-reared kiwi potentially carrying coccidia. Further, ONE facilities frequently house multiple species of kiwi, and knowledge of host-specificity will identify the risk of exposure of kiwi to novel, highly pathogenic species of *Eimeria* via cross contamination of enclosures. This introduction to naïve populations could not only lead to decreased reproductive success of the kiwi but could also lead to the introduction of an invasive parasite that could outcompete the endemic *Eimeria*. Therefore, characterisation of coccidia in other kiwi species should be a priority. Additionally, the development of rapid, non-invasive, highly specific tools to monitor treatment efficacy is vital to ensuring ONE uses resources wisely. Such a tool needs to account for as much of the variation in kiwi coccidia as possible to ensure rare,

but highly virulent species are not overlooked. This variation is likely to be missed unless more in-depth sequencing technologies (i.e., Second Generation Sequencing) are used.

CHAPTER 4

Morphological and molecular characterisation of coccidia in Haast tokoeka (*Apteryx australis* "Haast")

Contribution of co-authors: Dr McInnes was instrumental in communicating with practitioners and obtaining research materials. Dr Morgan assisted in the research design and lab protocols. Dr Pomroy contributed to developing the protocol for sporulation and morphological analysis. Dr Howe helped establish appropriate genetic targets. Further, Drs Howe and Biggs contributed to the genetic analysis and communication of the results. Emillie Vallee was instrumental in developing and interpreting the statistical analyses. All authors provided feedback on one or more drafts of this manuscript. See Appendix G.

The following chapter builds on the work in Chapter 3 and represents the first characterisation of coccidia in Haast tokoeka (*A. australis* "Haast"). As it is convention to identify coccidia separately for each host species, this chapter has duplication from Chapter 3 and has been similarly formatted (Parasitology Research journal) for consistency.

4.1. Abstract

Operation Nest Egg (ONE) raises young Haast tokoeka (*Apteryx australis* "Haast") in outdoor, predator-proof enclosures until large enough to defend themselves from introduced predators. Coccidial oocysts can build up in these enclosures, exposing immunologically naïve kiwi to high parasite loads. While five species of *Eimeria* have been morphologically identified in brown kiwi (*Apteryx mantelli*) from sporulated oocysts, and genetically, a ~465 bp portion of the mitochondrial cytochrome *c* oxidase I (COI) gene from six brown kiwi droppings have been successfully Sanger sequenced, only endogenous stages have been opportunistically described in Haast tokoeka. This research provides the first morphological and molecular characterisation of coccidia in Haast tokoeka. Samples from six Haast tokoeka from three locations were used for this study; conventional, nested PCR was used to amplify the ~465 bp portion of the COI gene. These amplicons were analysed using Sanger sequencing and compared to brown kiwi *Eimeria* (see Chapter 3).

Morphological descriptions of sporulated oocysts (N = 366) yielded four morphotypes. M1 (n = 102) was circular to elliptic with an average oocyst length and width of 14.73 × 13.37μm and a striated wall. This morphotype was very similar to *Eimeria kiwi* described from brown kiwi. M2 (n = 107) was circular to elliptic with an average oocyst length and width of 14.3 × 13.4μm and a smooth wall. The M2 morphotype was very similar to M5 described from brown kiwi. M3 (n = 50) was elliptic to ovate with an average oocyst length and width of 19.6 x 13.0μm and a smooth wall. This morphotype was most similar to *Eimeria mantellii* described from brown kiwi. M4 (n = 107) was elliptic to ovate with an average oocyst length and width of 21.7 × 13.1 μm and a smooth wall. The M4 morphotype was similar to *Eimeria apteryxii* described from brown kiwi. All samples contained multiple morphotypes. Molecularly, the Haast tokoeka *Eimeria* were 90% similar to each other as well as the brown kiwi. The kiwi *Eimeria* formed a clade separate from the reference sequences and further clustered into five main clusters, each of which included one or more samples from both from Haast tokoeka and brown kiwi.

4.2. Introduction

Brown kiwi (*Apteryx mantelli*) and Haast tokoeka (*Apteryx australis* "Haast"), one of the rarest kiwi species, rely heavily on Operation Nest Egg (ONE) to hatch and raise chicks in captive and crèche facilities. Brown kiwi and the three subspecies of tokoeka (i.e., *A. australis* 'Haast', *A. australis australis*, and *A. australis lawryi*) are morphologically similar, though brown kiwi and rowi (*Apteryx rowi*) are more closely genetically related (Weir et al., 2016). Based on the most recent estimates, tokoeka split 1.56 (0.76–2.83) million years ago (Ma) from the group that would later split into brown kiwi and rowi at

1.12 (0.54–2.02) Ma (Weir et al., 2016). The last land bridge between the North (with brown kiwi) and South Islands (with tokoeka and rowi) occurred ~20,000 years ago, with geographic separation of rowi and Haast tokoeka on the South Island driven by the mountainous landscape (Weir et al., 2016). While overlap of the parasites of Haast tokoeka, rowi, and brown kiwi may have occurred with conservation management practices, the host specificity of the coccidia of kiwi is unknown.

Coccidia (*Eimeria* species) can cause significant morbidity and mortality in crèche- and captive-reared kiwi (*Apteryx* species), making it more difficult to maintain chick health and achieve necessary weight gain (Morgan et al. 2012; Morgan et al. 2013). Morgan et al. (2017) provided the first morphological description of sporulated oocysts in kiwi and reported four species (*Eimeria mantellii*, *E. kiwii*, *E. paraurii*, *E. apteryxii*) from six captive brown kiwi (*Apteryx mantelli*) from two locations. Chapter 3 expands on the characterisation of coccidia in brown kiwi and suggests a fifth morphotype. While Morgan et al. (2013) documented coccidia gametocytes in the kidney of an individual Haast tokoeka, no reports have described sporulated oocysts from any of the three subspecies of tokoeka. Further, these coccidia have not been characterised using any molecular techniques.

Identifying species of coccidia in kiwi is a key step toward answering key management questions, including pathogenicity (Morgan et al. 2013; Morgan et al. 2014) and treatment efficacy (Taylor et al. 2019). This research contributes the first morphological and molecular characterisation of sporulated oocysts from tokoeka (*Apteryx australis*

"Haast") using guidelines suggested by Duszynski and Wilber (1997) as well as Sanger sequencing of the 18S rRNA gene and mitochondrial cytochrome *c* oxidase I gene targets.

4.3. Methods

4.3.1. Sample collection/sources

From 2017 to 2018, Haast tokoeka droppings were collected for routine faecal diagnostic and pre-translocation health screenings. On intake at Massey University, all samples were assigned the code "HA" and an identification number that reflects the individual kiwi host or group of hosts, if known. If unknown, a new identification number was assigned to that sample. Repeat sampling from the same host(s) were differentiated by the date of submission. All samples positive for coccidia were processed as described below. Samples were chosen for this study based on a combination of factors: location, the success of oocyst sporulation, oocyst load (oocysts per gram; OPG), amount of sample remaining after flotation, and diversity of coccidial morphotypes.

Based on these factors, six Haast tokoeka samples from three locations were utilised for this study (see Table 4.1). All six samples were individual droppings that were collected from outdoor enclosures/crèches or translocation boxes using sanitary, single-use collection bottles. The samples were collected into sterile collection tubes and stored at room temperature (RT) for up to one month before being shipped to Massey University, Palmerston North, New Zealand.

Table 4.1. List of sources of Haast tokoeka dropping samples from the South Island.

Sample	Region	Location	Year	Month	Age
HA01	Westland	Haast	2017	11	ND
HA04	Southland	Rona Island	2017	10	ND
HA14	West Coast	Haast	2018	2	chick
HA16	Southland	Rona Island	2018	10	juv
HA27	Southland	Rona Island	2018	10	chick
HA30	Westland	WCWC	2018	11	ND

Host species is Haast tokoeka (*Apteryx australis* "Haast"). "ND" indicates when the age of the kiwi at the time of collection was unknown or unprovided. A chick is a kiwi less than 2 months old, and a juvenile (juv) is from 2 months up to 18 months. Sample refers to the code assigned to dropping on arrival to Massey University, where "HA" refers to Haast tokoeka and the number refers to a particular individual kiwi. "WCWC" refers to the West Coast Wildlife Centre in Franz Josef.

4.3.2. Oocyst detection

Samples were screened for coccidia using the mini-FLOTAC technique, as described in Chapter 2 (Appendix B.3).

4.3.3. Sporulation and storage

Oocysts were sporulated and stored according to Chapter 3 (Duszynski and Wilber 1997). In brief, droppings were mixed with 2% aqueous (w/v) potassium dichromate ($K_2Cr_2O_7$, Appendix C.1) at a minimum 1:5 ratio and thinly spread over the bottom of Petri dishes (100 mm x 15 mm). After 15 days at room temperature, sporulated oocysts were transferred and stored at room temperature in 25 ml, sealed flasks.

4.3.4. Isolation

Oocysts were cleaned and isolated as described in Chapter 3. After the $K_2Cr_2O_7$ was removed, magnesium sulphate solution ($MgSO_4$, SG 1.28) was added to suspend the oocysts (Appendix C.3). Drops of this mixture were suspended from the underside of a coverslip that was carefully placed on a cavity slide (Appendix C.4).

4.3.4. Imaging and measuring

Imaging and measuring parameters were the same as described in Chapter 3 (Appendix C.5). Morphological features were measured and described for 50 to 107 oocysts for each described morphotype (Anonymous 1962; Duszynski and Wilber 1997).

4.3.5. Morphology clustering

Oocysts were removed from analysis when the feature used for clustering was not measurable due to poor positioning during imaging. First for initial direct comparison of size and shape, the scatter plots of the oocysts length (x-axis) and length/width ratio (y-axis) were made of the Haast tokoeka. Another scatter plot was then made with the addition of the brown kiwi coccidia described to date (Chapter 3; Morgan et al., 2013). To determine whether or not the four Haast tokoeka could be statistically differentiated, K means clustering was completed in RStudio version 1.0.153 (RStudio Team 2016). This clustering was performed using several combinations of characteristics (e.g., length/width sporocyst ratio and presence/absence of a micropyle); the strongest grouping is reported. Finally, violin plots of the oocyst and sporocyst length/width ratios

were created to compare Haast tokoeka coccidia morphotypes with the most synonymous brown kiwi coccidia morphotypes/species.

4.3.6. Phylogenetic analysis

The DNA was extracted as described in Chapter 3 (Appendix C.6). See Chapter 3 for amplification protocols. Sequencing was carried out in accordance with Chapter 3 (Appendix C.9). The same control measures were taken as described in Chapter 3. The eight COI sequences obtained in this study, the seven sequences from brown kiwi *Eimeria* obtained for Chapter 3, and 61 reference sequences (including *Eimeria* spp., *Toxoplasma gondii*, *Neospora caninum*, *Isospora* sp., and *Hammondia* spp.) obtained from the GenBank database were aligned using Clustal W (Thompson et al. 1994) and trimmed to 497 bases using Geneious v11.0.5 (Biomatters, Auckland, New Zealand). This alignment was used to build a neighbour-joining consensus tree in Geneious; the Jukes-Cantor distance model with 1000 replicates was used to calculate branch length. *Toxoplasma gondii* served as an outgroup.

4.4. Results

4.4.1. Morphology

Within 15 days in 2.5% $K_2Cr_2O_7$ at room temperature, 50-90% of oocysts had sporulated in all the samples. In total, 366 oocysts were measured with 47% from Rona Island, 31% from Haast, and 22% from the West Coast Wildlife Centre (WCWC) in Franz Josef (Table 4.2). Four distinct morphotypes were identified (M1, M2, M3, M4; Figure 4.2). When

samples were examined microscopically, all samples contained coinfections with all morphotypes present at each location.

Table 4.2. Summary of oocyst morphotypes (M1, M2, M3, M4) measured from each Haast tokoeka dropping sample and locations.

Sample	M1	M2	M3	M4	Location	Total
HA01	12	2	7	16	Haast	37
HA14	65	5	5	0	Haast	75
HA04	15	1	0	1	Rona Island	17
HA16	1	35	0	1	Rona Island	37
HA27	6	15	31	68	Rona Island	120
HA30	3	49	7	21	WCWC	80
Total	102	107	50	107		N = 366

Sample refers to the code assigned to dropping on arrival to Massey University, where "HA" refers to Haast tokoeka and the number refers to a particular individual kiwi. "WCWC" refers to the West Coast Wildlife Centre in Franz Josef.

4.4.1.1 Morphology clustering

The data from remaining 362 oocysts were examined to identify different morphotypes. Scatterplot using oocyst length and the oocyst length/width ratio as well as qualitative characteristics grouped the oocysts into four morphotypes (Figure 4.1A); similar groupings appear when plotted with the brown kiwi coccidia data presented in Chapter 3 (Figure 4.1B).

Further analysis was undertaken using K means clustering assuming three groups; Morphotypes 1 and 2 were assumed to cluster together since the main differentiating

feature is the presence/absence of striations in the wall. The resulting figure (4.2) showed that the length/width ratio was key to differentiating Morphotypes 1/2 from the other morphotypes. Morphotypes 3 and 4 clustered separately when the presence/absence of visible micropyle was used as the main source of differentiation between the two.

In order to further compare the differences between the Haast tokoeka and brown kiwi coccidia; Figure 4.3 provides side by side comparisons of each morphotype from the Haast tokoeka with the most synonymous brown kiwi coccidia morphotypes. Figure 4.3A compares the oocyst length/width ratios; Figure 4.3B compares the sporocyst length/width ratios.

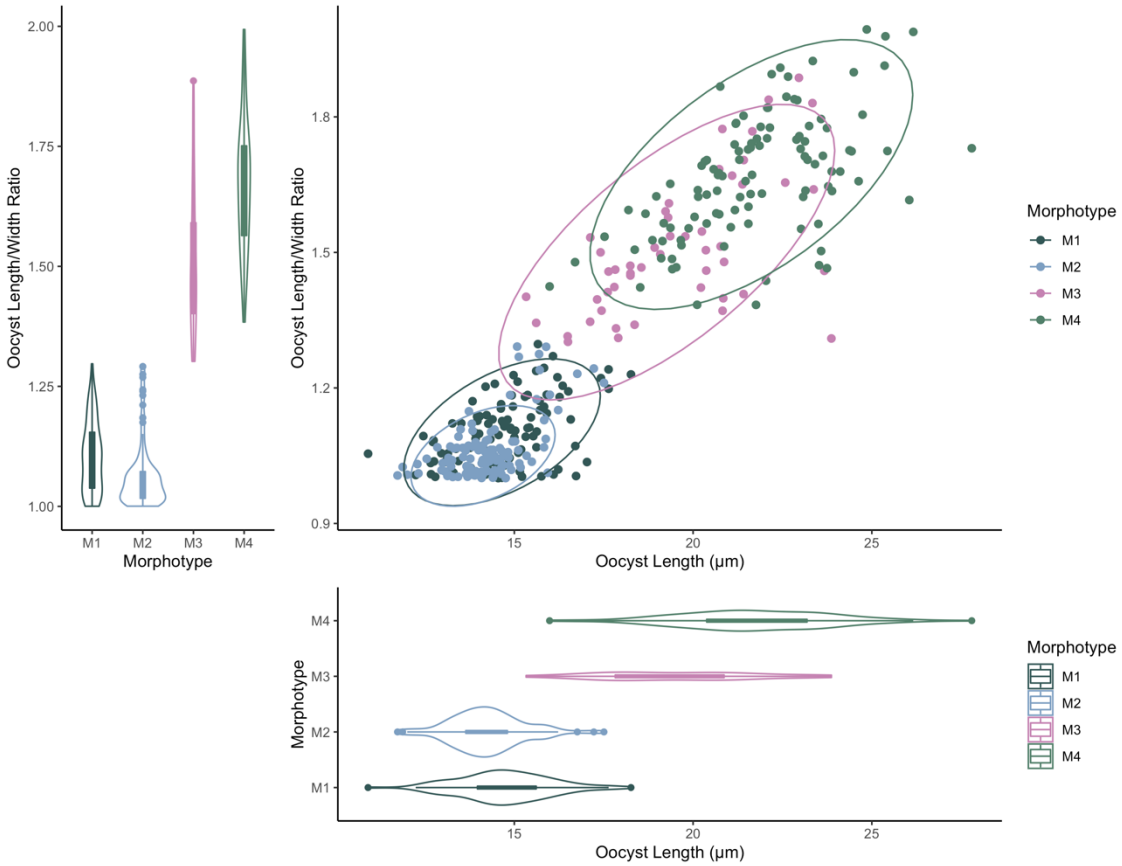


Figure 4.1.A. Three plots comparing coccidia from Haast tokoeka (*Apteryx australis* "Haast"). The four sporulated oocyst morphotypes (M1, n = 107; M2, n = 111; M3, n = 51; M4, n = 115) described from Haast tokoeka (*Apteryx australis* "Haast") droppings collected from the South Island of New Zealand. Plot A) Violin (density) plot comparing the oocyst length/width ratios. Plot B) Scatter plot sorted by oocyst length and length/width ratio with data ellipses. Plot C) Violin (density) plot comparing the oocyst length of morphotypes described from Haast tokoeka (*Apteryx australis* "Haast"). M1 and M2 are distinguished by a striated and smooth wall, respectively. M3 and M4 are distinguished by size and the absence and presence of a visible micropyle, respectively.

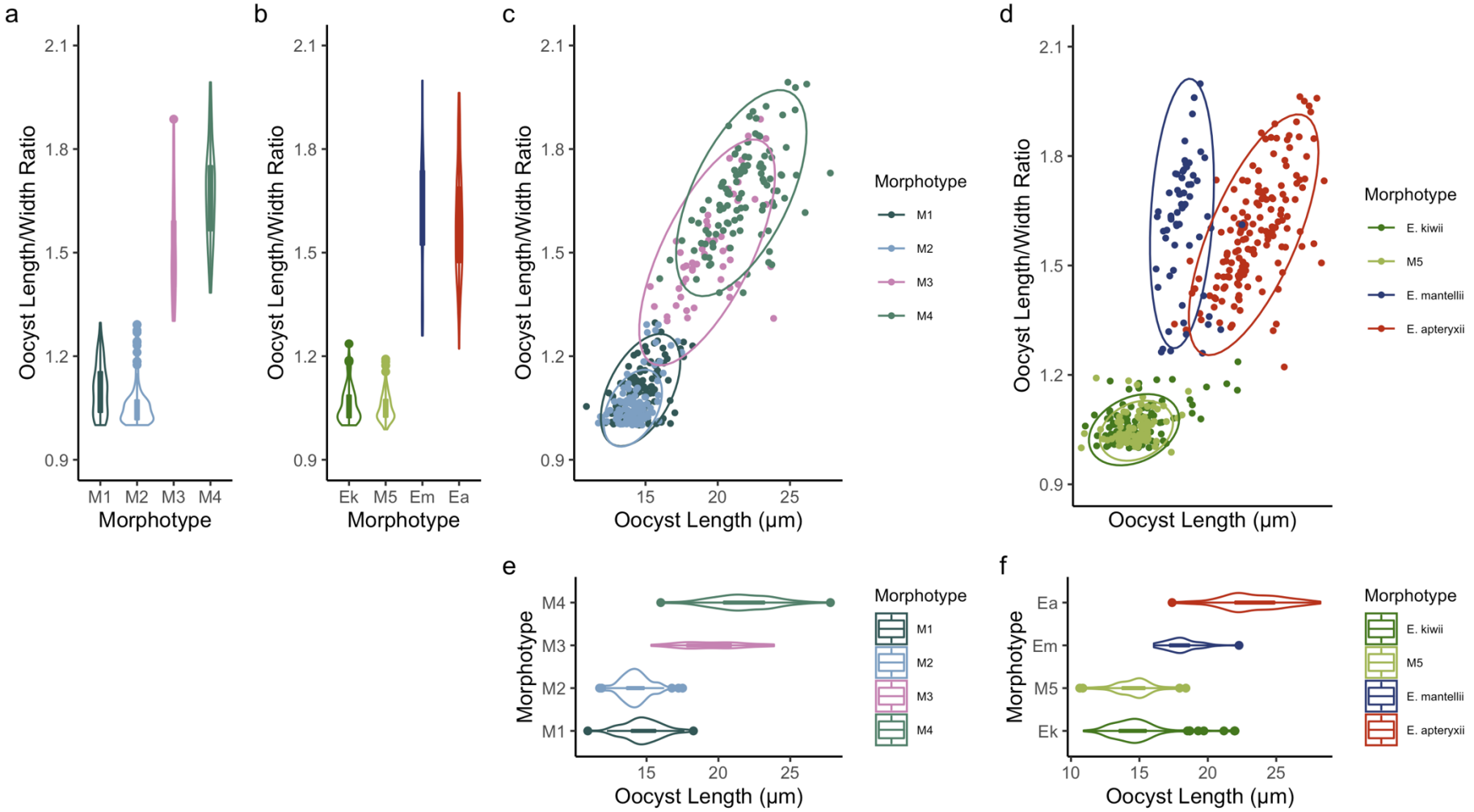
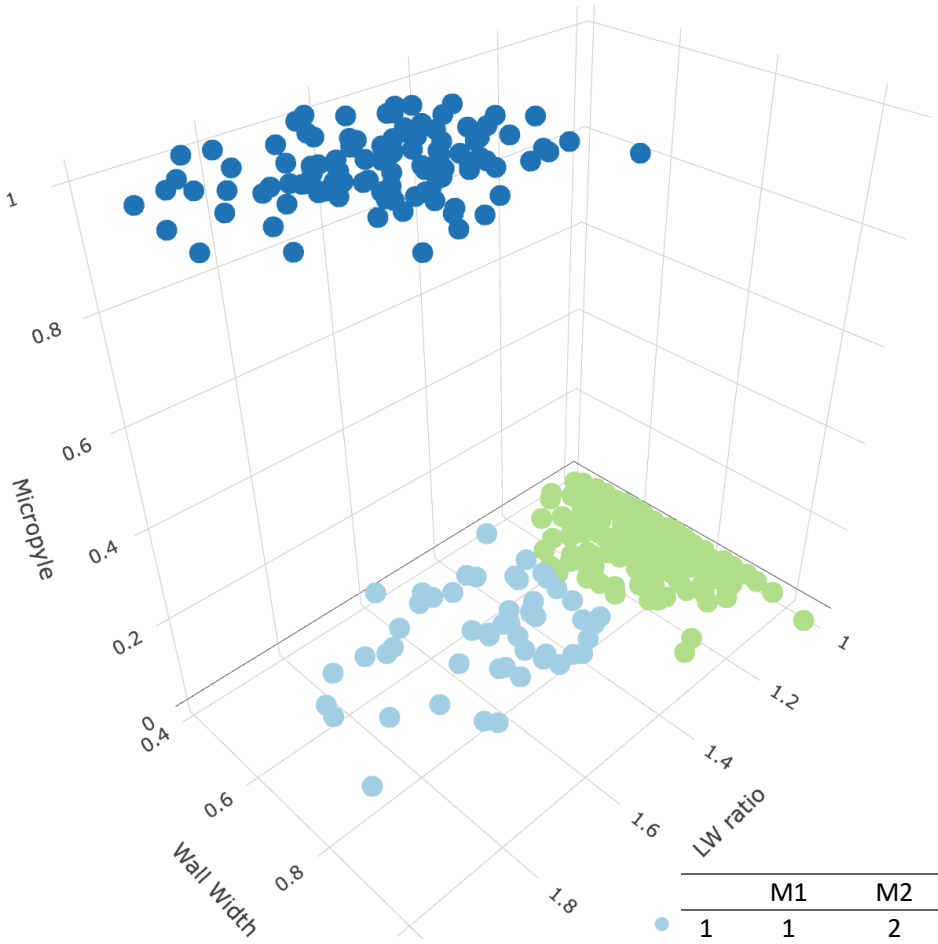

a
b
c
d
e
f
Oocyst Length/Width Ratio
Morphotype
Oocyst Length (µm)
M1
M2
M3
M4
Ek
M5
Em
Ea
E. kiwii
E. mantellii
E. apteryxii

Figure 4.1.B. Six plots comparing coccidia from two host species. The four sporulated oocyst morphotypes (M1, n = 107; M2, n = 111; M3, n = 51; M4, n = 115) described from Haast tokoeka (*Apteryx australis* "Haast") are compared with four synonymous morphotypes (*Eimeria kiwii,* n = 134; M5, n = 84; *Eimeria mantellii,* n = 63*; Eimeria apteryxii,* n = 159) previously described from brown kiwi (*Apteryx mantelli*). Plots "a", "c" and "e" are from tokoeka whereas plots "b", "d" and "f" are from brown kiwi. Plots "a" and "b" are violin (density) plots comparing the oocyst length/width ratios. Plots "c" and "d" are scatterplots sorted by oocyst length and length/width ratio with data. Plots "e" and "f" are violin (density) plots comparing the oocyst length of morphotypes. M1/*E. kiwii* and M2/M5 are distinguished by a striated and smooth wall, respectively. M3/*E. mantellii* and M4/*E. apteryxii* are distinguished by size and the absence and presence of a visible micropyle, respectively.



	M1	M2	M3	M4
1	1	2	49	0
2	0	0	0	106
3	99	105	0	0

Figure 4.2. K means clustering of kiwi (*Apteryx australis* Haast) coccidia, assuming 3 clusters. The presence/absence of a visible micropyle, oocyst wall width, and the oocyst length/width ratio were used to differentiate the morphotypes. Each colour represents a cluster (1-3).

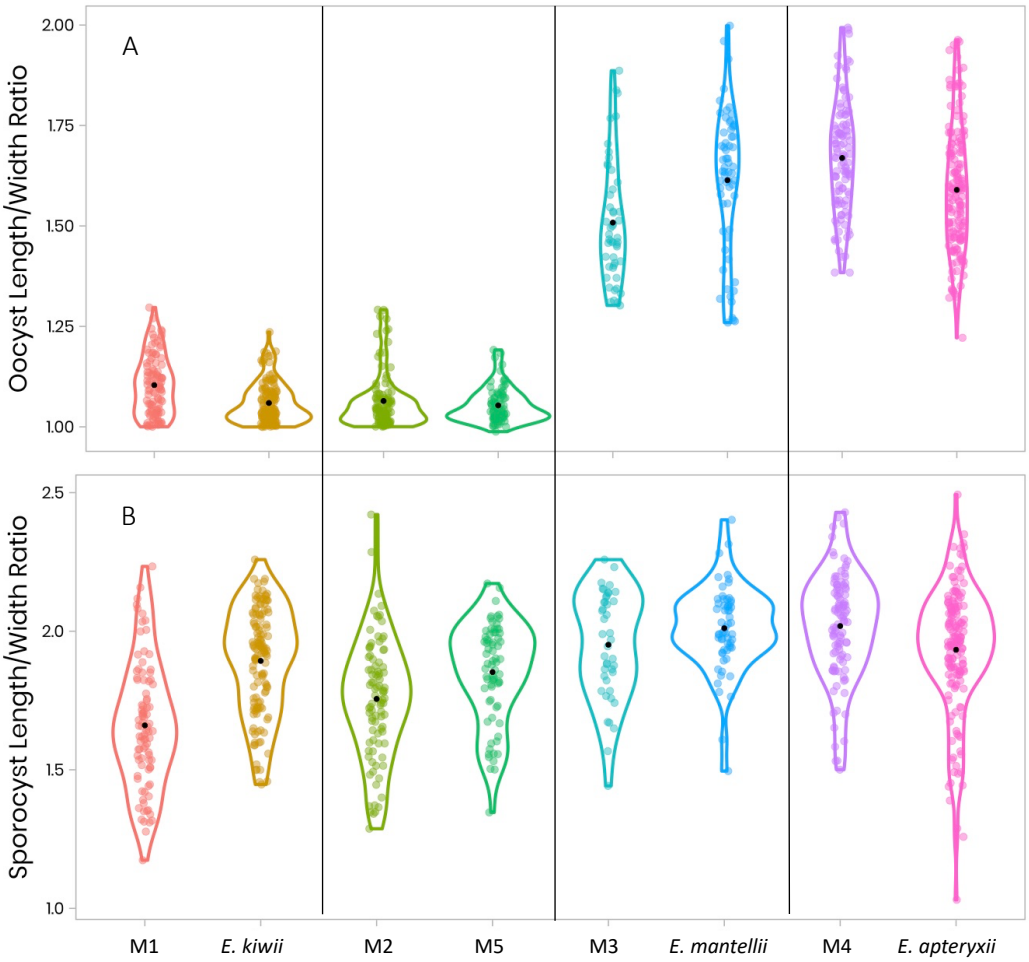


Figure 4.3. Violin plots comparing the (A) oocyst length/width ratios and (B) sporocyst length/width ratios. Morphotypes 1-4 (M1-4) were described from Haast tokoeka kiwi (*Apteryx australis* 'Haast') from the South Island of New Zealand. The remaining *Eimeria* spp. and Morphotype 5 (M5) were described from brown kiwi (*Apteryx mantelli*) from the North Island of New Zealand. These morphotypes are grouped into synonymous pairs.

4.4.1.2. Morphological descriptions

The follow descriptions provide the range of measurements of oocyst features with the average in parentheses.

4.4.1.2.1. Morphotype 1 (Figure 4.4)

Type host: Haast tokoeka, *Apteryx australis* "Haast" (Burbidge et al. 2003) Juvenile.

Type locality: Rona Island, Southland, New Zealand (-45.49371°S, 167.53986°E); Haast, Westland, New Zealand (-43.88007°S, 169.03302°E); and West Coast Wildlife Centre, Franz Joseph Glacier, Westland, New Zealand (-43.38869°S, 170.18243°E).

Type material: Sporulated oocysts are in the process of deposition in the Museum of New Zealand Te Papa Tongarewa (pending at time of submission). Prevalence: 100% (in 6 of 6 specimens).

Sporulation time: Exogenous. All oocysts were passed unsporulated and fully sporulated within 15 days at room temperature.

Site of infection: Unknown; retrieved from faeces.

Sporulated oocyst: Oocyst shape (n = 102) circular to elliptic: 10.91–18.263 × 10.347–16.643 μm (14.73 × 13.37μm), length/width (L/W) ratio 1.00–1.29 (1.1). Wall striated, 0.48–1.06 μm (0.71 μm). Micropyle, micropylar cap, and oocyst residuum absent. 1 polar granule present.

Sporocyst: Sporocysts (n = 144) 4, elliptical, 6.088–10.955 × 3.76–6.96 μm (8.26 × 5.00 μm); L/W ratio 1.17–2.24 (1.66); Stieda body present; sporocyst residuum present, generally clumped.

Sporozoite: Two sporozoites, not measured; large sporozoite refractile body at base.

Taxonomic remarks: This morphotype resembles *Eimeria kiwii* as described by Morgan et al. (2017) in brown kiwi (*A. mantelli*). See Chapter 3 for additional documentation.

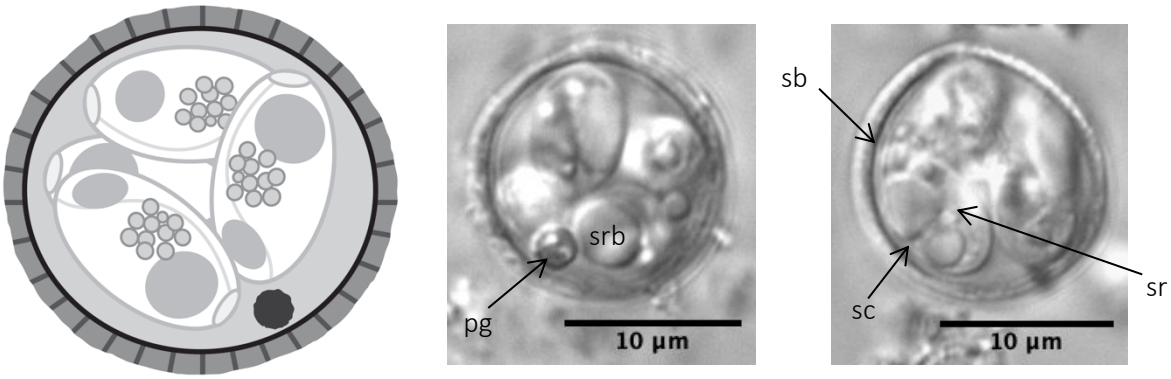


Figure 4.4. Line drawing and pictograph of "Morphotype 1" collected from Haast tokoeka (*Apteryx australis* 'Haast') droppings from the South Island of New Zealand. Key: sc = sporocyst, srb = sporozoite refractile body, pg = polar granule, sr = sporocyst residuum, sb = Stieda body.

4.4.1.2.2. Morphotype 2 (Figure 4.5)

Type host: Tokoeka, *Apteryx australis* "Haast" (Burbidge et al. 2003). Juvenile.

Type locality: Rona Island, Southland, New Zealand (-45.49371°S, 167.53986°E); Haast, Westland, New Zealand (-43.88007°S, 169.03302°E); and West Coast Wildlife Centre, Franz Joseph Glacier, Westland, New Zealand (-43.38869°S, 170.18243°E).

Type material: Sporulated oocysts are in the process of deposition in the Museum of New Zealand Te Papa Tongarewa (pending at time of submission). Prevalence: 100% (in 6 of 6 specimens).

Sporulation time: Exogenous. All oocysts were passed unsporulated and fully sporulated within 15 days at room temperature.

Site of infection: Unknown; retrieved from faeces.

Sporulated oocyst: Oocyst shape (n = 107) circular to elliptic: 11.7–17.5 × 11.6–15.7 μm (14.3 × 13.4 μm), length/width (L/W) ratio 1.0–1.3 (1.1). Wall smooth, 0.4–1.0 μm (0.7 μm). Micropyle absent, micropylar cap, and oocyst residuum absent. 1–2 polar granule(s) present.

Sporocyst: Sporocysts (n = 135) 4, elliptical, 6.3–12.5 × 3.9–6.2 μm (8.8 × 5.0 μm); L/W ratio 1.3–2.3 (1.76); Stieda body present; sporocyst residuum present, generally clumped.

Sporozoite: Two sporozoites, not measured; large sporozoite refractile body at base.

Taxonomic remarks: This morphotype resembles both *Eimeria kiwii* as described by Morgan et al. (2017) in brown kiwi (*A. mantelli*), and Morphotype 1 above (Figure 4.3); however, it differs in that it has a smooth, rather than striated wall. This morphotype resembles "Morphotype 5" as described in Chapter 3 of this thesis.

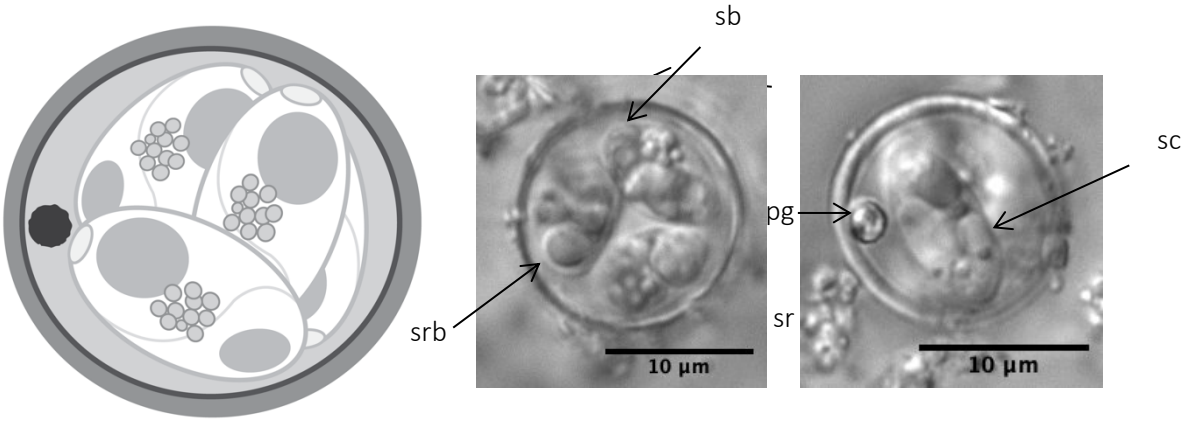


Figure 4.5. Line drawing and pictograph of "Morphotype 2" collected from Haast tokoeka (*Apteryx australis* 'Haast') droppings from the South Island of New Zealand. Key: sc = sporocyst, srb = sporozoite refractile body, pg = polar granule, sr = sporocyst residuum, sb = Stieda body.

4.4.1.2.3. Morphotype 3 (Figure 4.6)

Type host: Tokoeka, *Apteryx australis* "Haast" (Burbidge et al. 2003). Juvenile.

Type locality: Rona Island, Southland, New Zealand (-45.49371°S, 167.53986°E); Haast, Westland, New Zealand (-43.88007°S, 169.03302°E); and West Coast Wildlife Centre, Franz Joseph Glacier, Westland, New Zealand (-43.38869°S, 170.18243°E).

Type material: Sporulated oocysts are in the process of deposition in the Museum of New Zealand Te Papa Tongarewa (pending at time of submission). Prevalence: 67% (in 4 of 6 specimens).

Sporulation time: Exogenous. All oocysts were passed unsporulated and fully sporulated within 15 days at room temperature.

Site of infection: Unknown; retrieved from droppings.

Sporulated oocyst: Oocyst shape (n = 50) elliptic to ovate: 15.3–23.9 × 10.9–18.2 μm (19.6 × 13.0 μm), length/width (L/W) ratio 1.30–1.89 (1.51). Wall smooth, 0.41–0.82 μm (0.66 μm). Micropyle, micropylar cap, and oocyst residuum absent. 1–2 polar granule(s) present.

Sporocyst: Sporocysts (n = 82) 4, elliptical, 8.0–12.7 × 4.2–6.6 μm (10.4 × 5.3 μm); L/W ratio 1.4–2.4 (2.0); Stieda body present; sporocyst residuum present, generally clumped.

Sporozoite: Two sporozoites, not measured; large sporozoite refractile body at base.

Taxonomic remarks: This morphotype resembles *Eimeria mantellii* as described by Morgan et al. (2017) in brown kiwi (*A. mantelli*). See Chapter 3 for additional documentation.

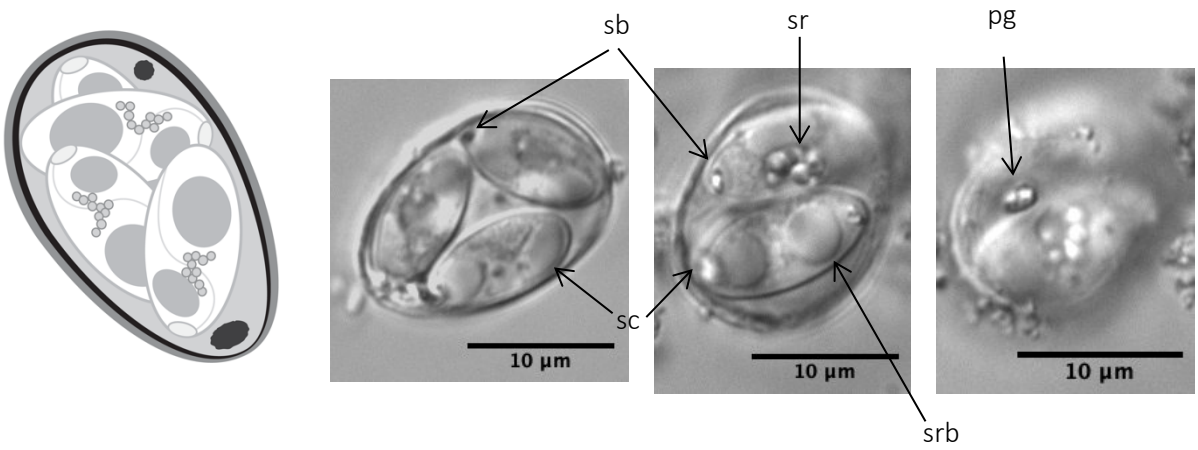


Figure 4.6. Line drawing and pictograph of "Morphotype 3" collected from Haast tokoeka (A*pteryx australis* 'Haast') droppings from the South Island of New Zealand. Key: sc = sporocyst, srb = sporozoite refractile body, pg = polar granule, sr = sporocyst residuum, sb = Stieda body.

4.4.1.2.4. Morphotype 4 (Figure 4.7)

Type host: Tokoeka, *Apteryx australis* "Haast" (Burbidge et al. 2003). Juvenile.

Type locality: Rona Island, Southland, New Zealand (-45.49371°S, 167.53986°E); Haast, Westland, New Zealand (-43.88007°S, 169.03302°E); and West Coast Wildlife Centre, Franz Joseph Glacier, Westland, New Zealand (-43.38869°S, 170.18243°E).

Type material: Sporulated oocysts are in the process of deposition in the Museum of New Zealand Te Papa Tongarewa (pending at time of submission). Prevalence: 83% (in 5 of 6 specimens).

Sporulation time: Exogenous. All oocysts were passed unsporulated and fully sporulated within 15 days at room temperature.

Site of infection: Unknown; retrieved from droppings.

Sporulated oocyst: Oocyst shape (n = 107) elliptic to ovate: 16.0–27.8 × 11.1–16.2 μm (21.7 × 13.1 μm), length/width (L/W) ratio 1.4–2.0 (1.7). Wall smooth, 0.4–0.9 μm (0.6 μm). Micropyle present, 2.5–5.6 μm (4.1 μm), micropylar cap and oocyst residuum absent. 1–2 polar granule(s) present.

Sporocyst: Sporocysts (n = 217) 4, elliptical, 8.5–13.1 × 4.3–7.3 μm (11.0 × 5.5 μm); L/W ratio 1.5–2.5 (2.0); Stieda body present; sporocyst residuum present, generally clumped.

Sporozoite: Two sporozoites, not measured; large sporozoite refractile body at base.

Taxonomic remarks: This morphotype resembles *Eimeria apteryxii* as described by Morgan et al. (2017) in brown kiwi (*A. mantelli*). See Chapter 3 for additional documentation.

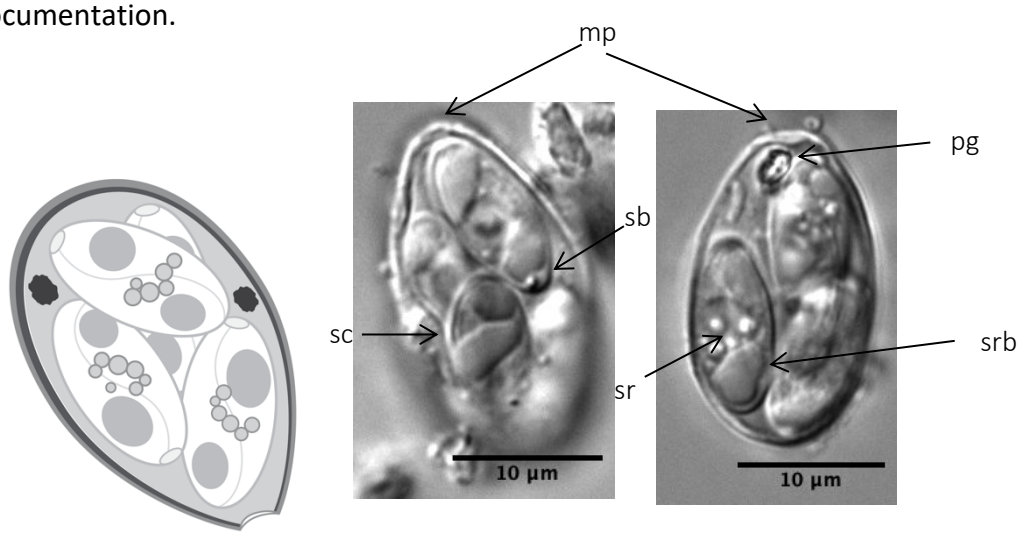


Figure 4.7. Line drawing and pictograph of "Morphotype 4" collected from Haast tokoeka (*Apteryx australis* 'Haast') droppings from the South Island of New Zealand. Characterised by a smooth wall (w), present micropyle, 1-2 polar granules, and a L/W ratio of 1.67. Key: sc = sporocyst, srb = sporozoite refractile body, pg = polar granule, sr = sporocyst residuum, sb = Stieda body.

4.4.2. Sequencing and phylogenetic analysis

4.4.2.1 18S rDNA

The 18S rDNA gene yielded highly unreliable sequencing results from the kiwi coccidia samples as well as the controls, despite repeated sequencing attempts. As the COI provides more variation for comparison and successfully identifies the novel kiwi coccidia as *Eimeria*, further troubleshooting for 18S analysis was not pursued.

4.4.2.2. COI gene

The COI Sanger sequencing yielded high quality results, which are summarised in Table 4.3. Four of the six samples were sent to the Massey Genome Service (Massey University, Palmerston North) for resequencing to increase the quality of the consensus sequence. Table 4.4 presents the best quality score recovered from each direction and includes the results from brown kiwi coccidia from Chapter 3 ("MB" refers to Pūkaha National Wildlife Centre; "NM" refers to Nga Manu Nature Reserve; "RSB" refers to The National Kiwi Hatchery Aotearoa; "WB" refers to a sample collected from a bird infected The National Kiwi Hatchery Aotearoa that had been sent to Wildbase Hospital, Massey University, Palmerston North for treatment for high coccidial burdens; "WP" refers to Warrenheip). HA14, HA27, and HA30 contained ambiguous bases, indicating mixed species infections. Consensus sequences were compared to the nucleotide NCBI database using the BLAST function; no result from this search came back with a sequence homology result greater than 91%. These Haast tokoeka coccidia shared a 90% identity among themselves as well as with the brown kiwi coccidia. In addition, the coccidia from both brown and Haast tokoeka kiwi formed a large clade separate from

the reference sequences (Figure 4.8). This clade has been broken up into five clusters (A-E). In Cluster A, HA01 and MB16 were 97% identical (Table 4.5) and the top BLAST result for both was *Eimeria mundayi* (GenBank MK202808.1) from a woylie (*Bettongia penicillata*) (Northover et al. 2019). NM01 was 97% homologous to MB16 and 95% homologous to HA01. The second cluster ("B") comprised only of HA27, which was grouped separately as it shared only a 91-92% identity with the samples in cluster A.

Cluster C contained HA30 and MB06, which were 96% identical with less than 90% identity with the sequences in the BLAST database. The three samples (HA04, HA14, and RSB06) in cluster D were 97-99% homologous; the top BLAST result for HA04 and RSB06 was an unpublished and unclassified species of *Eimeria* (GenBank MF496271.1). The second highest result for these was an unclassified *Isospora* sp. (GenBank MK573842.1), which was the top result for the last sample in the cluster, HA14. This *Isospora* sp. (GenBank MK573842.1) was also the top result for a brown kiwi sample, WB01, from cluster E. The other brown kiwi coccidia sample from cluster E, WP01, shared a 97% identity to WB01, and had a top BLAST result of *Eimeria piriformis* (GenBank JQ993698.1) from a European rabbit (*Oryctolagus cuniculus*). The Haast tokoeka sample from cluster E, HA16, was only 94% homologous to WP01 and WB01; the BLAST search yielded an 87% identity with and unspecified *Eimeria* sp. (GenBank MG595962.1) from a western capercaillie (*Tetrao urogallus*).

Table 4.3. Kiwi (*Apteryx australis* "Haast") coccidia consensus COI Sanger sequencing results from individual droppings collected from 2017-2018.

Sample	ABI % qual. scores (F, R)	Seq. length (bp)	Ambiguous Bases % (bp)	BLAST result	% Identity
HA01	35.7, 0	454	0	*Eimeria mundayi* MK202808.1	90.93
HA04	91, 72.1	484	0	*Eimeria* sp. MF496271.1	86.91
HA14	56.9, 37	484	3.7 (18)	*Isospora* sp. MK573842.1	85.19
HA16	83.1, 84.5	484	0	*Eimeria* sp. MG595962.1	86.55
HA27	48.4, 13.1	485	11.5 (56)	*Eimeria* sp. MG595961.1	82.75
HA30	91, 31.1	485	0.2 (1)	*Isospora coronoideae* MK867778.1	88.25
Kereru	85.8, 87.8	475	*0*	*Eimeria* sp. MG595963.1	93.41
IMMU	88.9, 84.3	484	*0*	*Eimeria acervulina* KX094948.1	100

"HA" refers to Haast tokoeka and the number refers to a particular individual kiwi. "IMMU" Immucox Breeders and Layers live vaccine that contains *Eimeria acervulina, E. maxima, E. necatrix,* and *E. tenella.* "Kereru" refers to coccidia isolated from a *Hemiphaga novaeseelandiae* dropping from Pūkaha National Wildlife Centre.

Table 4.4. Kiwi (*Apteryx* spp.) coccidia (*Eimeria* spp.) consensus COI Sanger sequencing percent identity comparison from individual droppings collected in 2017-2018 from various locations in New Zealand.

	HA01	HA04	HA14	HA16	HA27	HA30	MB06	MB16	NM01	RSB06	WB01	WP01
HA01		85	86	86	91	85	85	97	95	85	85	85
HA04	85		97	93	88	93	93	87	86	99	93	93
HA14	86	97		93	88	92	92	87	86	97	92	91
HA16	86	93	93		89	95	94	87	86	93	94	94
HA27	91	88	88	89		91	89	92	91	88	88	87
HA30	85	93	92	95	91		96	87	86	93	92	92
MB06	85	93	92	94	89	96		87	86	93	93	92
MB16	97	87	87	87	92	87	87		97	87	87	86
NM01	95	86	86	86	91	86	86	97		86	86	86
RSB06	85	99	97	93	88	93	93	87	86		93	93
WB01	85	93	92	94	88	92	93	87	86	93		97
WP01	85	93	91	94	87	92	92	86	86	93	97	

The two-letter code refers to the species or location of the host and the number refers to a particular individual kiwi. “HA” refers to Haast tokoeka (*Apteryx australis* ‘Haast’) collected from the South Island of New Zealand. All other samples are from droppings shed by brown kiwi (*Apteryx mantelli*) from the North Island of New Zealand. “MB” refers to Pūkaha National Wildlife Centre; “NM” refers to Nga Manu Nature Reserve; “RSB” refers to The National Kiwi Hatchery Aotearoa; “WB” refers to a sample collected from a bird infected The National Kiwi Hatchery Aotearoa that had been sent to Wildbase Hospital, Massey University, Palmerston North for treatment for high coccidial burdens; “WP” refers to Warrenheip.

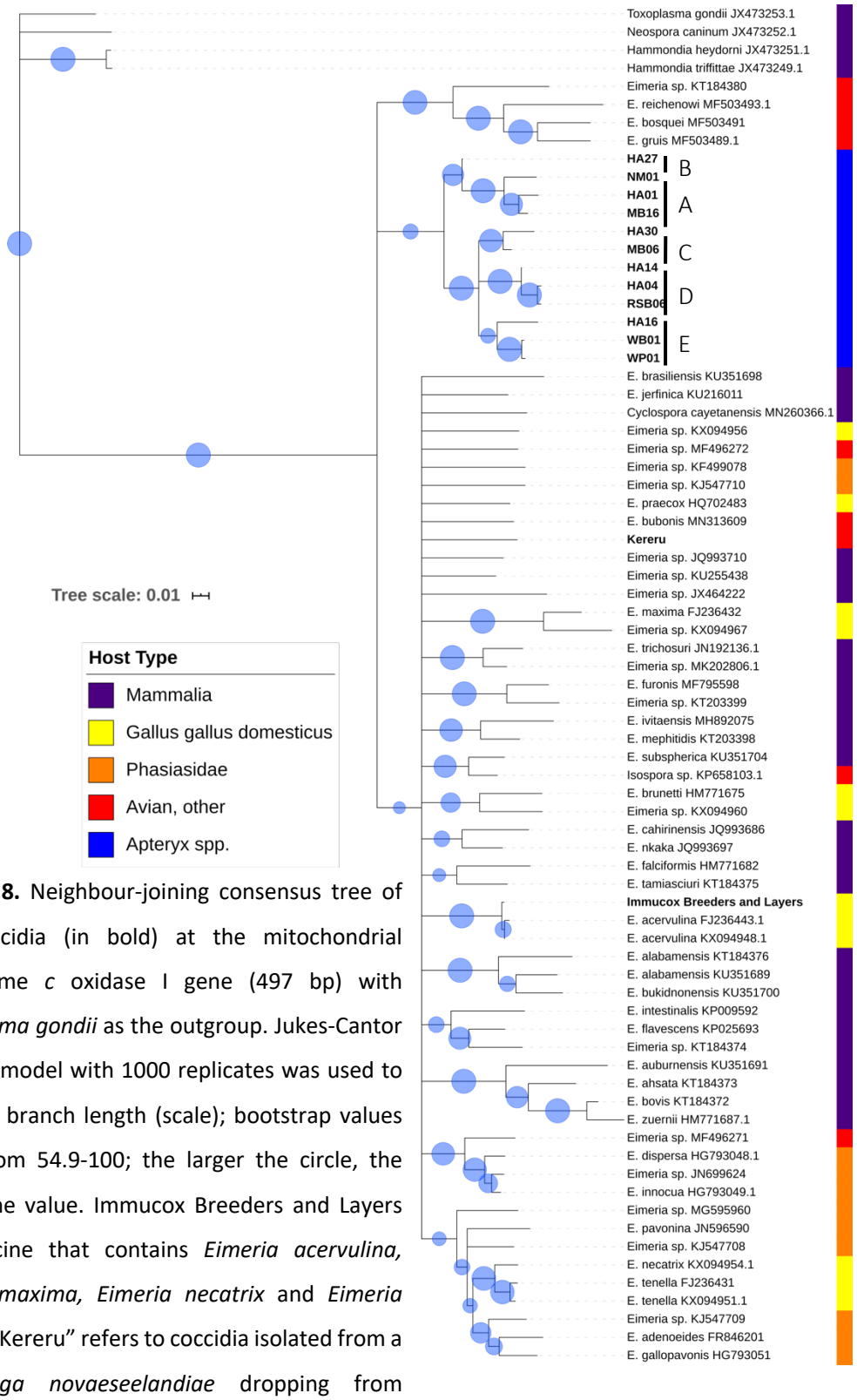


Figure 4.8. Neighbour-joining consensus tree of kiwi coccidia (in bold) at the mitochondrial cytochrome *c* oxidase I gene (497 bp) with *Toxoplasma gondii* as the outgroup. Jukes-Cantor distance model with 1000 replicates was used to calculate branch length (scale); bootstrap values range from 54.9-100; the larger the circle, the higher the value. Immucox Breeders and Layers live vaccine that contains *Eimeria acervulina, Eimeria maxima, Eimeria necatrix* and *Eimeria tenella.* "Kereru" refers to coccidia isolated from a *Hemiphaga novaeseelandiae* dropping from Pūkaha National Wildlife Centre.

4.5. Discussion

This is the first description of *Eimeria* spp. in Haast tokoeka (*A. Australia* "Haast"). Findings of this study suggest there are at least four distinct *Eimeria* morphotypes that infect Haast tokoeka (Figures 4.1A and 4.1B), although it is currently unknown if some or all of these are the same species as those previously described in brown kiwi (*A. mantelli*). The morphotypes described here greatly resemble previously described species from brown kiwi (Morgan et al. 2017, see Chapter 3). Morphotype 1 was most similar to *E. kiwii,* Morphotype 2 to Morphotype 5, Morphotype 3 to *E. mantellii*, and Morphotype 4 to *E. apteryxii*. Describing *Eimeria* from a novel host species is insufficient to declare the coccidia species novel (Duszynski and Wilber 1997); therefore, the authors consider these morphologically described species to be synonymous. These coccidia are from two species of kiwi that have been geographically separated for ~20,000 years, with speciation between the tokoeka and brown kiwi branches occurring 0.76–2.83 Ma (Weir et al. 2016). However, *Eimeria* sp. host switching has been documented between closely related rodent hosts and could have occurred prior to the final split of the main islands (Mácová et al. 2018). Further, while kiwi practitioners now take care to prevent overlap of kiwi species or the transfer of coccidia, prior to Tennyson et al. (2003) tokoeka and rowi were considered the same species of kiwi. Thus, ONE facilities have held both species simultaneously (K. McInnes, personal comm.), potentially leading to a transfer of coccidia between species since ONE began in 1994 (Colbourne et al. 2005). These hosts may be biologically similar enough to share eimeriid parasites, which are frequently able to infect closely related hosts (Duszynski and Wilber 1997). For example, *Eimeria gruis* and *Eimeria reichenowi* have been identified morphologically and molecularly in hooded cranes (*Grus monacha*), whooping cranes

(*Grus americana*), white-naped cranes (*Antigone vipio*), and sandhill cranes (*Antigone canadensis*) (Honma et al. 2011a; Honma et al. 2011b; Novilla et al. 1981). Similarly, based on morphological descriptions, *Eimeria vilasi* can infect fourteen species of mice (Dorney 1962; Hnida and Flocken 2016; Ryan et al. 2001; Wilber et al. 1998). On the other hand, a newly described morphotype of *Eimeria* in Italy was morphologically identical in red squirrels (*Sciurus vulgaris*), grey squirrels (*Sciurus carolinensis*), and Pallas's squirrels (*Callosciurus erythraeus*), but molecularly monoxenous and distinct (Hofmannová et al. 2016). This recent finding strongly suggests that molecular markers can make key distinctions and confirm documentations, thus encouraging the use of multiple tool approach to identify *Eimeria* spp.

Furthermore, the phylogenetic analysis (Figure 4.8) strongly suggests the coccidia of kiwi are genetically distinct from other species of *Eimeria* described to date. While the nucleotide BLAST results varied between *Eimeria* and *Isospora* spp., the percent identities reached no higher than 91%. This low homology to documented coccidia suggests these kiwi coccidia are likely endemic to New Zealand.

The morphological and phylogenetic similarities suggest one or more of the species of kiwi *Eimeria* might infect both brown kiwi and Haast tokoeka. Unfortunately, a definitive link between morphotypes and haplotypes cannot be made as all the Haast tokoeka and brown kiwi samples contained multiple morphotypes. For example, isolates MB06 (brown kiwi, refer to Chapter 3) and HA30 (Haast tokoeka) share 96% identity, which is likely due to co-infections within the samples. Of the six oocysts described from sample

MB06, four were *E. apteryxii*, one was *E. kiwii*, and one was an M5. Of the oocysts described in sample HA30 (Table 4.2), 4% (3/80) were M1, 61% (49/80) were M2, 9% (7/80) were M3, and 26% (21/80) were M4. Interestingly, MB06 contained 17 (3.5% of 485 bp) while the HA30 COI sequence contained only one ambiguous base. The variety represented in these samples makes it difficult to connect a single morphotype to the prominent HA30 sequence. Similarly, isolates RSB06 and HA04 share 99% identity, with 0.2% and 0% ambiguities, respectively. Morphologically, samples RSB06 from brown kiwi predominantly contained Morphotype 5 (synonymous to Morphotype 2 in Haast tokoeka; see Chapter 3), whereas the majority of oocysts described in the HA04 sample were Morphotype 1, which is most synonymous to *E. kiwii*. Therefore, while these two isolates had high sequence identity, it is still possible that these two morphotypes could still be distinct genetically, as both samples RSB06 and HA04 contained coinfections. Interestingly, isolate HA14 shares a 97% identity to isolates RSB06/HA04 with 3.7% ambiguous bases, and 87% of the oocysts described were Morphotype 1. This similarity could suggest this haplotype is more likely associated with Morphotype 1, syn. *E. kiwii*, which may have been preferentially amplified in the RSB06 sample. Without significantly increasing the sample size and/or conducting single oocysts sequencing, the most concrete conclusion that can be made about the coccidia in kiwi is that they are distinct from other *Eimeria* (Figure 4.8). Any connections that can be hypothesised from this study require further verification.

As observed with the molecular analysis of the coccidia from the brown kiwi (Chapter 3), half (3 of 6) of the sequencing results from the tokoeka samples contained

ambiguous bases. One of the tokoeka samples contained a single ambiguous base, indicating that there are only two dominate sequences this sample. The other two sequences range from 18 to 56 ambiguous bases. Each time there is an ambiguous base, the possible number of actual sequences increases; if each ambiguous base has only two options, the number of possible sequences is 2^n, where *n* is the number of ambiguous bases. Thus, a sample containing 16 ambiguous bases has a possible 262,144 combinations. The likelihood that there are that many "true" sequences in the sample is very low; however, determining which combinations of bases are actually present is vital to advancing the diagnostic testing abilities. On the other hand, all the samples that had no ambiguous bases contained multiple morphotypes. Given the variation seen in other closely related species of *Eimeria* at this target, two morphologically distinct species are expected to exhibit differences. For example, *E. dispersa* (HG793048.1) and *E. innocua* (HG793049.1) in turkeys are 97.7% similar at this target (Vrba and Pakandl 2014; Yang et al. 2013; Yang et al. 2015a; Yang et al. 2014a; Yang et al. 2015d); whereas *E. bovis* (KT184372) and *E. zuernii* (HM771687.1) are 96.9% similar. Accordingly, Sanger sequencing of single oocysts or Next Generation sequencing should be pursued.

4.5.1. Conclusions

While sequencing of single oocysts using methods similar to those described by Dolnik et al. (2009) or Sturbaum et al. (2001) could provide a clear connection between a morphotype and a sequence, a large number of samples would need to be analysed to begin to understand the genetic variation of these endemic *Eimeria*. Next Generation sequencing would be the most cost-effective step to gain a more complete picture of

the variation found in kiwi coccidia. Future research should endeavour to use more in-depth sequencing technologies in order to confirm the four species morphologically described here are indeed the same as those described in brown kiwi (Morgan et al. 2017, see Chapter 3). Further, this analysis could enable the development of a diagnostic test would detect less numerous and potentially more pathogenic species of coccidia.

4.6. Acknowledgments

The authors would like to acknowledge Dr Matthew Savoian at the Manawatu Microscopy & Imaging Centre for guidance and assistance capturing type images of oocysts. Further, the authors would like to thank the team at Blacksheepdesign, Palmerston North, for designing the oocyst line drawings.

CHAPTER 5

The use of Illumina amplicon sequencing to detect variation in the COI gene of *Eimeria* spp. from captive and wild kiwi (*Apteryx* spp.)

Contribution of co-authors: Dr McInnes was instrumental in obtaining research materials. Dr Howe provided assistance in protocol optimisation. Dr Biggs contributed extensive, robust bioinformatic processing of Illumina data and ensured that the downstream analyses were appropriate and that results were communicated accurately. All authors provided feedback on one or more drafts of this manuscript. See Appendix G.

The following chapter increases the sample size, host species variation, geographic variation and sequencing depth to analyse the COI gene of kiwi coccidia. While it is intended for publication in *Frontiers in Zoology*, additional details have been included for clarity for the purpose of this thesis.

5.1. Abstract

To prevent predation of kiwi (*Apteryx* spp.) by introduced mammals, Operation Nest Egg (ONE) raises chicks and juveniles in outdoor, predator-proof enclosures/crèches until they are large enough to defend themselves from introduced predators. These facilities experience environmental accumulation of coccidial oocysts, which may lead to severe morbidity and mortality of these young kiwi. Five species of coccidia from have been morphologically described from sporulated oocysts from Haast tokoeka (*Apteryx australis* 'Haast') and brown kiwi (*Apteryx mantelli*) as well as additional opportunistic descriptions of endogenous stages. This research provides the first in-depth genetic characterisation of the mitochondrial cytochrome *c* oxidase I (COI) gene. Pooled and individual droppings (n = 50) from four species of kiwi were collected from ONE facilities and wild locations and screened for coccidia using the Mini-FLOTAC apparatus with magnesium sulphate solution (SG 1.28). Positive samples were incubated at -80°C and DNA was extracted using the ZR Quick-DNA Fecal/Soil Microbe DNA Miniprep Kit, modified with additional steps to break open the oocysts. An approximately 484 bp portion of the COI gene was amplified with conventional PCR and sequenced using a 2 × 300 Illumina MiSeq run. The Illumina run yielded 13,102,879 reads; two samples were excluded due to low reads. Raw sequences were demultiplexed, denoised, and realigned in QIIME2. Clustering into OTUs was performed at 90-100% identity at 1% increments,

and the feature table was produced in the BIOM format. Apicomplexan reference taxa were clustered and merged with the sample data and using the cd-hit package. The results were visualised at http://view.qiime2.org and in alluvial plots; clustering at 95% homology led to the most sequences to be classifiable to the OTU (n = 49) level, thereby maintaining the highest level of variation. Phylogenetic comparisons of the 19 most abundant OTUs (which account for 99.9% of the sequences) were made using a Naive Bayes classifier with the reference sequences; OTUs were aligned and trees generated in Geneious using the Tamura-Nei Distance Model and the Neighbour-Joining Method. Kiwi *Eimeria* OTUs grouped into three main clades separate from the reference sequences, with three OTUs not grouping with any other OTU. All samples contained more than one OTU with no significant trends connecting particular OTUs to a metadata grouping (host species, location, season, etc.) of kiwi. Accordingly, no definite connections could be made between a particular morphotype and an OTU. However, the variation represented in these samples enables extensive opportunities for connections to be made in the future.

5.2. Introduction

Kiwi (*Apteryx* spp.) are small, nocturnal, burrow-nesting ratites that are endemic to New Zealand and of high conservation value (Germano et al. 2018; Heather and Robertson 2015). In response to high predation by introduced mammals, the Department of Conservation and Kiwis for Kiwi implemented Operation Nest Egg (ONE), which raises chicks in predator-free environments until they are large enough (at least 1 kg) to defend themselves (Colbourne et al. 2005; McLennan et al. 2004). While coccidiosis has been

documented in wild kiwi (Morgan et al. 2012; Morgan et al. 2013), severe clinical disease and mortality is commonly associated with ONE sites in which young, immunologically naïve birds are exposed to unnaturally high environmental loads of coccidia (Morgan 2013). Four species of *Eimeria* have been morphologically identified in brown kiwi (*Apteryx mantelli*, n = 6; syn. North Island brown kiwi) at two ONE locations, indicating a potentially high diversity of coccidia (Morgan 2017). Chapter 3 introduced the morphological characterisation of coccidia in brown kiwi, while chapter 4 provided the first morphological descriptions of coccidia from Haast tokoeka (*A. australis* "Haast"; syn. Southern brown kiwi). Morgan (2013) successfully sequenced the 18S rRNA of coccidial DNA from brown kiwi, confirming they belong in the genus *Eimeria*. However, attempts to differentiate further targeting the ITS-1 and ITS-2 genes was unreliable and highly variable.

In order to study these parasites in these threatened species, Chapters 3 and 4 of this thesis explored the use of a nested conventional PCR protocol paired with Sanger sequencing to target the mitochondrial cytochrome *c* oxidase I (COI) gene. This gene was chosen because it has been shown to provide enough variation to differentiate closely related species of coccidia while also containing conserved regions that could allow for the development of species-specific diagnostic tests (Chapman et al. 2013; Hikosaka et al. 2011). Such testing could identify species that cause severe disease in kiwi and link exogenous stages (oocysts) with endogenous stages (meront and gamonts), leading to information about virulence. However, relying on Sanger sequencing alone, which yields only the most common sequence, could easily lead to overlooked variation and misrepresentation of species composition (Altimari et al. 2013; Cereb et al. 2015;

Magalhaes et al. 2015). On the other hand, Illumina amplicon sequencing provides a much more in-depth sequencing that yields sequences that appear anywhere from once to tens of thousands of times (Ambardar et al. 2016; Arulandhu et al. 2018; Berglund et al. 2011; Filges et al. 2019). By sequencing the coccidia in droppings from several host species and locations, the genetic variation and distribution of coccidia in ONE and wild locations can begin to be associated with oocyst morphotypes, internal stages, severe cases of coccidiosis, and drug resistance.

Thus, the specific aim of this chapter is to increase the depth of sequencing at the COI gene using Illumina amplicon technology to provide a greater understanding of the distribution and variation of these parasites, thereby allowing for the development of more sensitive and specific testing protocols.

5.3. Methods

5.3.1. Faecal sample collection

From July 2017 to April 2019, fresh faecal samples (52) were collected in sterile tubes from birds in hand, roosts, or enclosures and sent at ambient temperature to Massey University at the earliest convenience. The Mini-FLOTAC (Cringoli et al. 2017) or standard centrifugal flotation procedures were used to visually identify samples positive for coccidia. Table 5.1 summarises the samples used in this study. The brown kiwi is the main focus of this research since the ONE network dedicated to the management of the four taxa of brown kiwi is extensive; however, three other kiwi species were also included. Haast tokoeka were included as this subspecies of tokoeka kiwi relies heavily on ONE, are commonly held or raised in facilities where brown kiwi are also present,

and are frequently moved throughout the South Island. Opportunistically collected rowi (*A. rowi*) and great spotted kiwi (*A. haastii*) samples were also included. Two extractions from the same MB15 sample were conducted and included separately to test the reliability of the extraction method. The Immucox® Breeders and Layers (Pacificvet, New Zealand) live vaccine served as a positive control that contains *Eimeria acervulina, E. maxima, E. necatrix,* and *E. tenella*; an *Eimeria* sample from a kereru (*Hemiphaga novaeseelandiae*) served as an outgroup. Additionally, Sanger sequencing (Chapters 3 and 4) of a subset of samples was used for comparison.

Table 5.1. Summary of samples and metadata used for Next Generation Sequencing of a 484 bp region of the mitochondrial cytochrome c oxidase subunit 1 (COI) gene.

ID	Host Species	IN/ PO	Island (N/S)	Region	Location	Year	Month	Age
BU01	Brown	IN	N	Northland	Auckland Zoo	2019	5	ND
CS01	Brown	IN	N	Hawke's Bay	Cape Sanctuary	2017	11	juv
CS17	Brown	IN	N	Hawke's Bay	Cape Sanctuary	2018	3	juv
CS30	Brown	IN	N	Hawke's Bay	Cape Sanctuary	2018	12	juv
OH02	Brown	IN	N	Northland	Motuarohia Island	2019	1	ND
OH03	Brown	IN	N	Northland	Motuarohia Island	2019	1	ND
RU01	Brown	IN	N	Northland	Moturua Island	2019	1	ND
RU04	Brown	IN	N	Northland	Moturua Island	2019	1	ND
MG01	Brown	IN	N	Waikato	Mt. Maunga-tautari	2018	11	ND
MG02	Brown	IN	N	Waikato	Mt. Maunga-tautari	2018	11	ND
MG03	Brown	IN	N	Waikato	Mt. Maunga-tautari	2018	11	ND
NM01	Brown	IN	N	Manawatu	Nga Manu Nature Reserve	2018	12	ND
RS01	Brown	PO	N	Bay of Plenty	NKHA	2017	11	chick
RS12	Brown	PO	N	Bay of Plenty	NKHA	2017	12	chick
RS13	Brown	PO	N	Bay of Plenty	NKHA	2018	1	chick

RS16	Brown	IN	N	Bay of Plenty	NKHA	2018	1	chick
RS18	Brown	IN	N	Bay of Plenty	NKHA	2018	4	ND
RSB05	Brown	IN	N	Bay of Plenty	NKHA	2018	4	ND
RSB06	Brown	IN	N	Bay of Plenty	NKHA	2018	4	ND
WB01	Brown	IN	N	Bay of Plenty	NKHA	2018	2	juv
OW01	Brown	PO	S	Canterbury	Orana Wildlife Park	2017	8	adult
WB05	Brown	IN	N	Waikato	Otorohanga Kiwi House	2018	3	juv
PN01	Brown	IN	N	Northland†	Ponui Island	2018	9	ND
MB06	Brown	IN	N	Manawatu	Pūkaha	2017	12	adult
MB12	Brown	IN	N	Manawatu	Pūkaha	2018	4	adult
MB14	Brown	IN	N	Manawatu	Pūkaha	2018	6	chick
MB15*	Brown	PO	N	Manawatu	Pūkaha	2018	10	juv
MB16	Brown	IN	N	Manawatu	Pūkaha	2018	10	juv
PC02	Brown	IN	N	Northland	Purerua	2019	5	ND
RA02	Brown	IN	N	Northland	Rakauman-gamanga	2019	1	ND
RA06	Brown	IN	N	Northland	Rakauman-gamanga	2019	1	ND
WI02	Brown	IN	N	Waikato	Wairakei Golf Sanctuary	2018	11	juv
WP09	Brown	IN	N	Waikato	Warrenheip	2019	8	juv
WP01	Brown	IN	N	Waikato	Warrenheip	2017	10	juv
WP08	Brown	IN	N	Waikato	Warrenheip	2018	2	ND
WS11	Brown	IN	N	Hawke's Bay	Westshore Wildlife Reserve	2018	12	ND
IMMU	Chicken	N/A	N/A	N/A	N/A	N/A	N/A	N/A
GSK01	GSK	IN	S	Tasman	Kahurangi National Park	2019	2	ND
MB10	Kereru	IN	N	Manawatu	Pūkaha	2018	4	ND
RW13	Rowi	IN	N	Wellington	Mana Island	2019	5	ND
RW16	Rowi	IN	S	Westland	Okarito	2019	3	ND
WK01	Rowi	IN	S	Canterbury	Willowbank Wildlife Reserve	2017	11	ND
HA08	Tokoeka	IN	S	West Coast	Franz Joseph	2018	1	juv
HA30	Tokoeka	IN	S	West Coast	Franz Joseph	2018	11	ND
HA14	Tokoeka	IN	S	West Coast	Haast	2018	2	chick
HA01	Tokoeka	IN	S	Westland	Haast	2017	11	ND
HA28	Tokoeka	IN	S	Otago	Orokonui Ecosanctuary	2018	10	juv
HA04	Tokoeka	IN	S	Southland	Rona Island	2017	10	ND
HA09	Tokoeka	IN	S	Southland	Rona Island	2018	9	juv
HA16	Tokoeka	IN	S	Southland	Rona Island	2018	10	juv

HA21	Tokoeka	IN	S	Southland	Rona Island	2018	10	juv
HA24	Tokoeka	IN	S	Southland	Rona Island	2018	9	ND
HA27	Tokoeka	IN	S	Southland	Rona Island	2018	10	chick

Host species refer to brown kiwi (*Apteryx mantelli,* Brown), great spotted kiwi (*Apteryx haastii*, GSK), Haast Tokoeka (*Apteryx australis* "Haast", Tokoeka), kereru (*Hemiphaga novaeseelandiae,* Kereru), and rowi (*Apteryx rowi*, Rowi). "IN/PO refers to whether the sample was from a single shed of faeces (IN) or if multiple sheds were pooled together (PO). "ND" indicates when the age of the kiwi at the time of collection was unknown or unprovided. *Sample MB15 was extracted twice in separate batches to examine the repeatability of the extraction and sequencing protocols. A chick is a kiwi less than 2 months old; a juvenile (juv) is from 2 months up to 18 months; and an adult is older that than 18 months. "Pūkaha" refers to Pūkaha National Wildlife Centre. "NKHA" refers to The National Kiwi Hatchery Aotearoa.

5.3.2. Extraction of DNA from oocysts

To break the environmentally resistant, unsporulated oocyst walls, aliquots (0.15 g) of these samples (n = 54) were incubated at -80°C for ≥12 hours; cycled three times between five minutes in liquid nitrogen and five minutes at 100°C; and then incubated overnight with 40 μl proteinase K (Promega) at 56°C. DNA was extracted using the ZR Quick-DNA Faecal/Soil Microbe DNA Miniprep Kit (Zymo Research, Orange, CA, USA) according to the manufacturer's instructions.

5.3.3. DNA amplification

A nested PCR protocol targeting the mitochondrial cytochrome *c* oxidase I (COI) region was used for amplification (Figure 5.1). The primary amplification was a 50 μl reaction with 1 × PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 1.0 mM of each primer (Table 5.2), 1 × BSA, 2 U Platinum *Taq* (Invitrogen) with an initial denaturation at 96°C for 5 min; 40 cycles of 94°C for 20 sec, 59°C for 30 sec, and 72°C for 90 sec; and a final extension at 72°C for 10 min (Ogedengbe et al. 2011b).

The secondary protocol, adapted from Yang et al. (2013), used a 50 μl reaction with 1 × PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 mM of each primer (Table 5.2), 1 × BSA, 2 U Platinum *Taq* (Invitrogen). The secondary conditions had an initial denaturation at 96°C for 5 min; 40 cycles of 94°C for 20 sec, 45°C for 30 sec, and 72°C for 90 sec; and a final extension at 72°C for 10 min.

Table 5.2. Primers used for nested amplification of a portion of the mitochondrial cytochrome *c* oxidase I (COI) region of *Eimeria* from kiwi (*Apteryx* spp.) prior to Illumina library preparation and amplicon sequencing. The Illumina adaptor portions of the secondary primers are underlined.

	Primer Name	Primer Sequence	Amp. Size (bp)	Reference
1'	Cocci_COI_For Cocci_COI_Rev	5'- GGT TCA GGT GTT GGT TGG AC -3' 5'- AAT CCA ATA ACC GCA CCA AG -3'	~780	Ogedengbe et al. (2011b)
2'	Illumina_COIF2 Illumina_COIR2	5'- <u>TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG</u> TAA GTA CAT CCC TAA TGT C -3' 5'- <u>GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA G</u>GT CAT CAT ATG RTG TGC CCA -3'	~465	Yang et al. (2013)

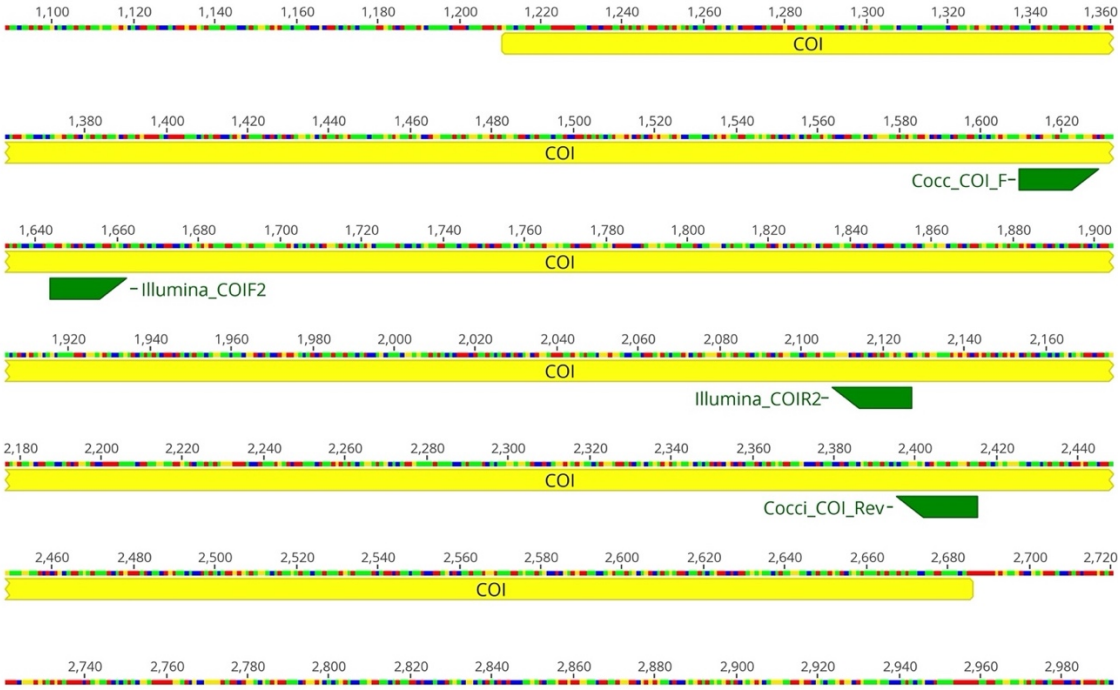


Figure 5.1. Excerpt of the COI gene (base pairs 1211-2686; yellow) of *Eimeria acervulina* from chickens as described by Lin et al. (2011). Primer binding regions (green) demonstrate the locations of the nested primers adapted from Ogedengbe et al. (2011b) and Yang et al. (2013). The outer primer pair, Cocc_COI_F/R, are both 20 bp long and target a 766 bp fragment (806 bp with the primers). The Illumina_COIF2/R2 (19 bp and 20 bp primer binding sites with an additional 33 bp and 34 bp adaptors, respectively) target a 445 bp fragment (484 bp with the primers) nested within the outer primer pair.

5.3.4. Controls

Immucox® Breeders and Layers (Pacificvet, Christchurch, New Zealand) live vaccine that contains *Eimeria acervulina, E. maxima, E. necatrix,* and *E. tenella* was used as a positive control for the PCR reactions (sample IMMU). Sterile water controls were included as negative controls for the extraction process and PCR reactions. In addition, a dropping from a wild kereru (*Hemiphaga novaeseelandiae*) from Pūkaha National Wildlife Centre, Mount Bruce, Wairarapa containing previously undescribed *Eimeria* spp. oocysts was included to test the specificity of the PCR reactions.

5.3.5. Sequencing

Amplicons from this nested protocol were run on a 1.5% (w/v) agarose gel; samples with bands at about 550 bp in length (450 bp amplicon, a 52 bp forward primer and 54 bp reverse primer including the Illumina adaptors). Amplicons were cleaned with the Invitrogen PureLink PCR Purification Kit (ThermoFisher Scientific, Waltham, MA, USA). The DNA concentration and quality were assessed using a Qubit Fluorometer (ThermoFisher Scientific, Waltham, MA, USA). Samples with $\geq$ 5 ng/μl of DNA (n = 38) were diluted with PCR grade water to 5 ng/μl; samples with $\leq$ 5 ng/μl of DNA (n =1 6) were prepared in duplicate to ensure the amount of DNA yielded enough sequences to be included in analysis. These amplified samples were submitted to the Massey Genome Service (Massey University, Palmerston North). Library preparation was carried out with the Illumina Two Step PCR Amplicon Approach using the Illumina Nextera XT™ DNA Primers for the second PCR step. Illumina sequencing was performed on the lllumina MiSeq™ V3 (2 $\times$ 300 base PE) with a 20% PhiX control library. Demultiplexed sequences were returned for analysis.

5.3.6. Analysis

5.3.6.1. QIIME2

Bioinformatic analyses were performed in the Quantitative Insights Into Microbial Ecology 2 (QIIME2) 2019.1 environment (Bolyen et al. 2019). First, the “q2demux” plugin was used for demultiplexing the paired-end sequence reads, taking all the raw sequences and identifying which sequences were from each sample (Bokulich et al. 2018b; Bolyen et al. 2019). Then the “DADA2” plugin was used to denoise the data set

(Bokulich et al. 2018b; Bolyen et al. 2019; Callahan et al. 2016; Hall and Beiko 2018; Hernandez-Gomez et al. 2020). This process includes trimming the ends of sequences to avoid areas furthest from the primers that are most likely to contain read errors (trunc_len_f:295 and trunc_len_r:280) (Caporaso et al. 2011; Hall and Beiko 2018); removing sequences with high levels of background noise that makes it difficult to determine the true sequence; and removing unique sequences that appear only once. Additionally, chimeras are detected and removed to avoid false positive novel sequences. Lastly, paired-end reads are realigned and compiled into a table using the BIOM format (McDonald et al. 2012).

5.3.6.2. Clustering

Clustering into OTUs was performed at 90-100% identity at 1% increments using the "q2-vsearch" plugin (https://github.com/qiime2/q2-vsearch). The feature table was produced with the "q2-feature-table" plugin in the BIOM format (McDonald et al. 2012). Apicomplexan reference sequences (n = 157, see Appendix E.2), which include *Eimeria* from a range of mammalian and avian species retrieved from GenBank, were clustered and merged with the sample data and using the cd-hit package (Fu et al. 2012; Huang et al. 2010; Li and Godzik 2006; Li et al. 2001; Li et al. 2002).

5.3.6.3. Visualisation and statistics

5.3.6.3.1. Alluvial plots

Alluvial plots to visualise the changes in individual sequence clustering were generated in R version 3.6.1 (R Core Team 2019).

5.3.6.3.2. Distribution

Phylogenetic and non-phylogenetic diversity indices were conducted in QIIME2 using the “q2-diversity” plugin (Bolyen et al. 2019; Faith et al. 1987; Halko et al. 2011; Katoh and Standley 2013; Kruskal and Wallis 1952; Legendre and Legendre 2012; Weiss et al. 2017). Alpha diversities (Pielou's evenness index and Faith’s phylogenetic diversities) were calculated using non-parametric, one-way ANOVAs (Katoh and Standley 2013; Kruskal and Wallis 1952; Lane 1991; McKinney 2010; Weiss et al. 2017). Beta diversity was analysed using the Jaccard distance index and the Bray-Curtis dissimilarity index (Callahan et al. 2016; Faith et al. 1987; Halko et al. 2011; Katoh and Standley 2013; Lane 1991; Legendre and Legendre 2012; McDonald et al. 2012; Price et al. 2010; Weiss et al. 2017). Principal Coordinate Analyses (PCA) were generated based on weighted and unweighted UniFrac indices as well as the Bray-Curtis dissimilarity index (Callahan et al. 2016; Chang et al. 2011; Chen et al. 2012; Faith et al. 1987; Halko et al. 2011; Lane 1991; Legendre and Legendre 2012; Lozupone and Knight 2005; Lozupone et al. 2007; McDonald et al. 2012; McDonald et al. 2018; Price et al. 2010; Vázquez-Baeza et al. 2017; Vázquez-Baeza et al. 2013; Vrba and Pakandl 2014; Weiss et al. 2017). Visualisations of the result were generated using the “EMPeror” plugin and viewed on http://view.qiime2.org (Vázquez-Baeza et al. 2017; Vázquez-Baeza et al. 2013). Significant/informative plots of analysis are shown in the results.

5.3.6.3.3. Abundance trends

The sample data was further classified into species and location metadata groupings to highlight abundance and distribution trends (Table 5.3). Within these groups, the most abundant OTU was highlighted to help identify trends.

5.3.6.3.4. Paired sample comparison

Samples MB15A and MB15B were collected from a single homogenised sample collected from Pūkaha National Wildlife Centre in 2018. These two aliquots were extracted, amplified, and sequenced separately. In order to compare the reproducibility of the workflow used for this study, the number of clustered OTUs, the diversity, and evenness were compared between these samples.

5.3.6.3.4.1. Pearson's Chi-Squared

Both true abundance and proportional abundance were analysed using Pearson's chi-squared in R version 3.6.1 (R Core Team 2019). Proportional abundance data was transformed by multiplying the proportion of each OTU by 1,000. A chi-squared analysis was performed to determine if there was a significant difference between the two.

5.3.6.3.4.2. Shannon's indices

Shannon's diversity and evenness were calculated by hand to determine which sample contained great diversity and evenness.

5.3.6.3.5. Phylogeny

The "classify-sklearn" plugin was taught with a Naive Bayes classifier using closely related *Eimeria* spp. with COI sequences in GenBank (Pedregosa et al. 2011). Taxonomic support for the samples was provided by the "q2-feature-classifier" plugin (Bokulich et

al. 2018a). Results at each percent identity were exported as taxa summary bar plots using the "q2-taxa" plugin and viewed on http://view.qiime2.org. The representative sequences from this clustering were aligned and trees were generated in Geneious v11.0.5 (Biomatters, Auckland, New Zealand) using the Tamura-Nei Distance Model and the Neighbour-Joining Method.

5.3.6.3.6. Comparison to Sanger sequencing

Sample RS01 was amplified using the Sanger sequencing protocol described in Chapter 3 (Appendix C.8) and submitted to the Waikato DNA Sequencing Service (The University of Waikato, Hamilton, NZ) for Sanger sequencing. This sequence was aligned with the two most abundant OTUs present in RS01 according to the data generated by the Illumina amplicon sequencing using ClustalW (Thompson et al. 1994) and trimmed to 488 bp (including spaces) using Geneious. An OTU not present in the RS01 sample according to Illumina sequencing was also included in the alignment.

5.4. Results

5.4.1. Illumina

The Illumina run yielded a total of 13,102,879 reads (Figure 5.2). Of the 54 samples (52 from individual kiwi, and 2 duplicates of MB15), only two samples (PN01 and WK01) were excluded from the analysis due to low reads; PN01 had been run with two reactions due to low DNA concentration, whereas WK01 had been normalised to 5 ng/μl. Denoising yielded 903 unique sequences (i.e., operational taxonomic units; OTUs). Each OTU was assigned a two to three letter code based on abundance. For

example, “s__aa” was the most abundant OTU prior to clustering. It is important to be aware that as the OTUs are clustered, the representative OTU of a given cluster can change, leading to OTU clusters with names that no longer represent the relative abundance.

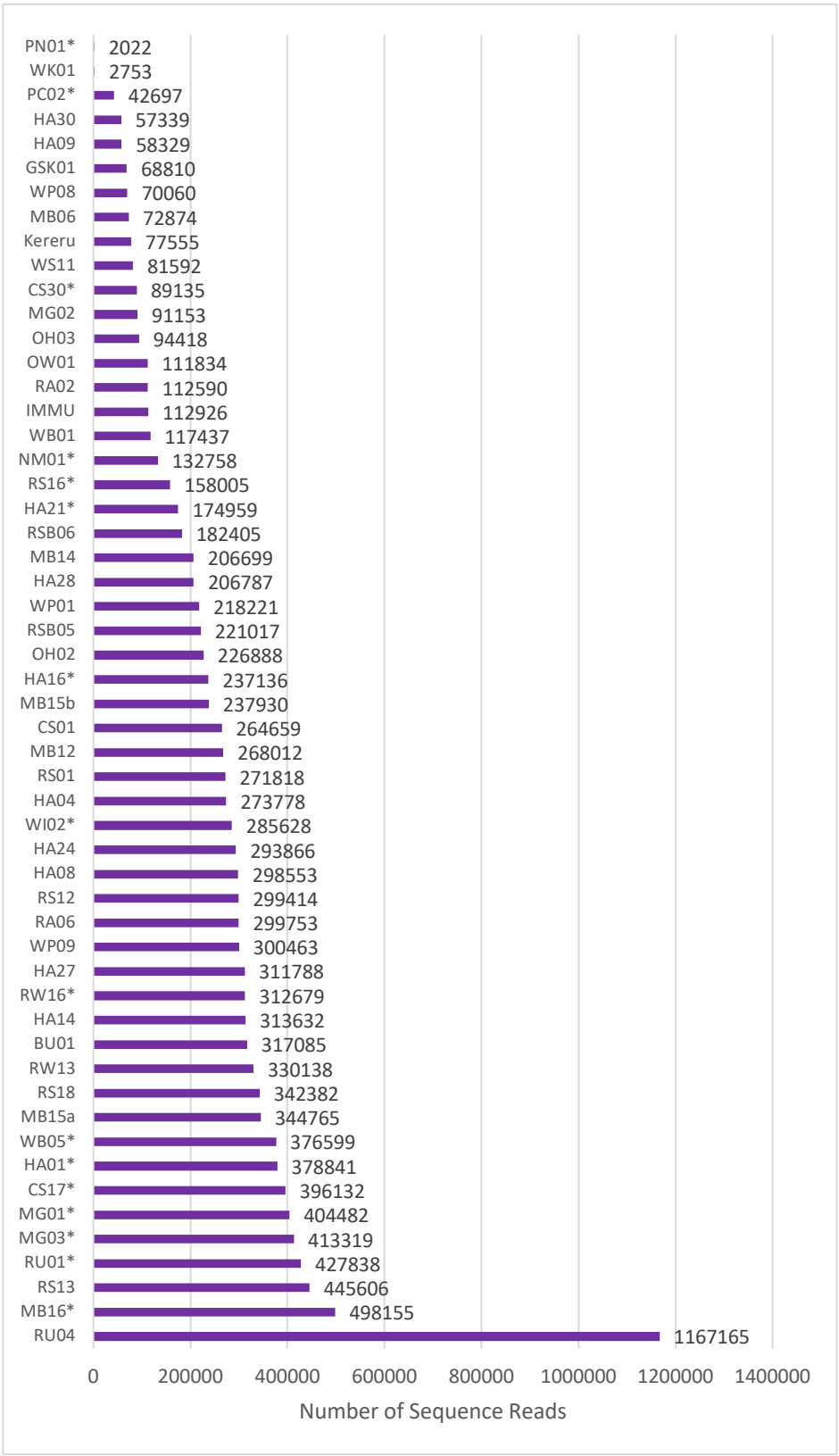


Figure 5.2. Illumina sequencing of kiwi (*Apteryx* sp.) coccidia COI gene (~484 bp) count results. Sample indicated with an asterisk (*) are the total of two sequencing reactions. MB15a and MB15b are two extractions from the same pooled sample.

5.4.2. Clustering

The number of clusters change drastically between 90 and 100% clustering in both samples and reference sequences (Figure 5.3). However, the main difference is the number of clusters present at 100% identity. At 100% identity, there were 903 OTUs present in the Illumina data, which drops below 200 OTUs at 99% clustering.

The taxonomic bar plots (Figure 5.4) demonstrate the change in the abundance of the "Genus" cluster at 90-98% identity in 1% increments. In this figure, only the clusters containing a number of OTUs greater than the "Genus" cluster are coloured. In most instances, the "Genus" cluster has a high relative abundance; at 95%, however, the clusters with a higher abundance than the "Genus" cluster accounts for almost all the sequences present in the entire run.

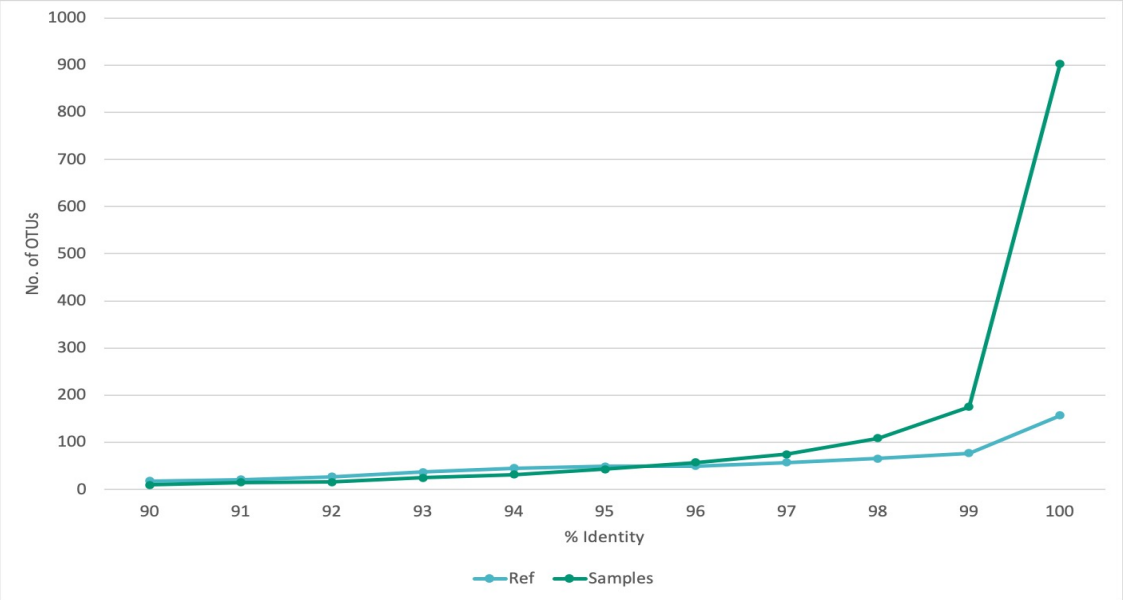


Figure 5.3. The number of operational taxonomic units (OTUs) from kiwi dropping samples compared to reference Apicomplexan sequences retrieved from GenBank based on clustering at 90-100% identity. Clustering was conducted on the reference sequences independently to form a reference database. Sample sequences were clustered under most abundant representative OTU. All clustering was performed in the QIIME2 environment.

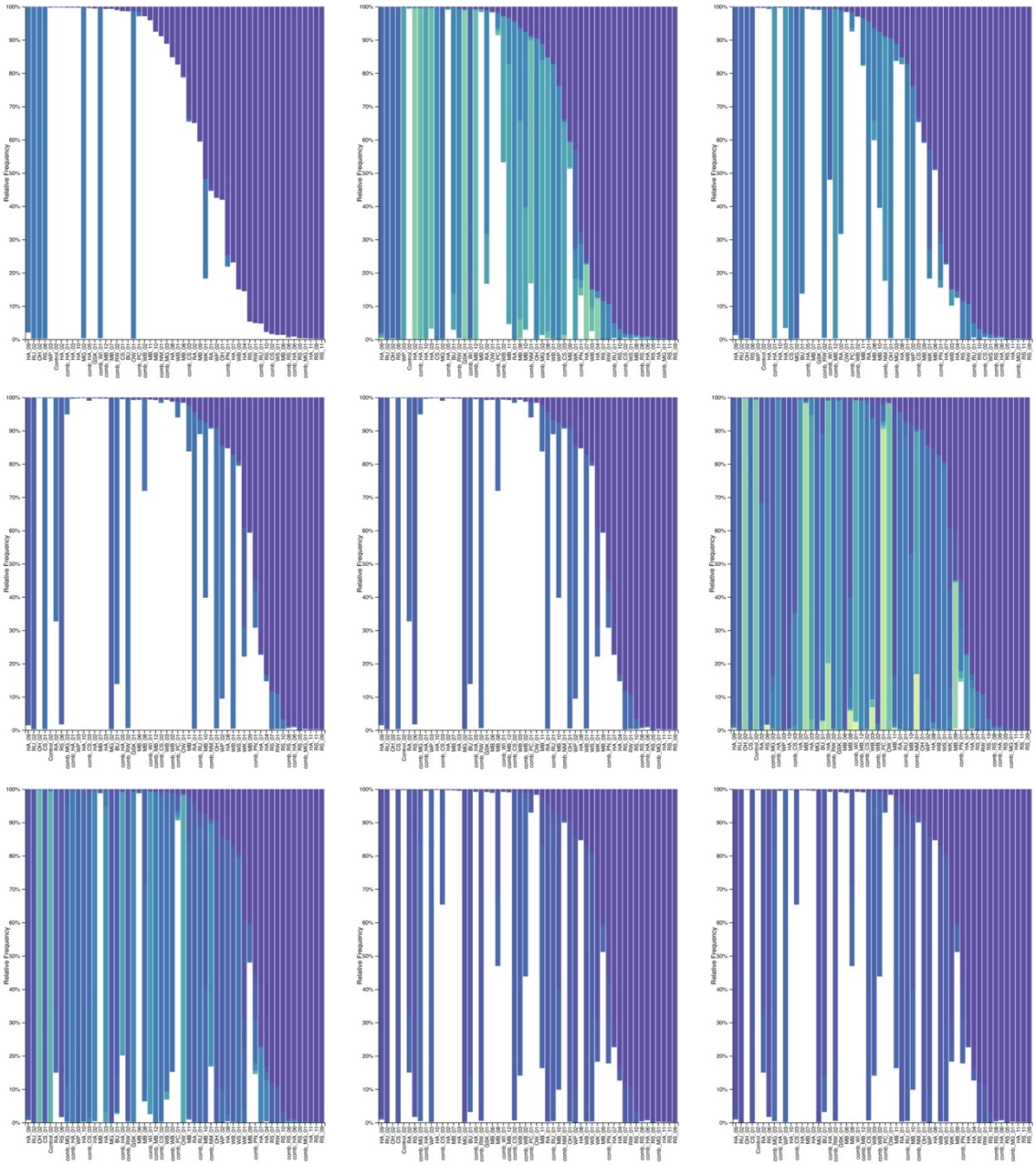


Figure 5.4. An illustration of how the representation of the most common OTUs change in each kiwi faecal sample (x-axis) based on clustering of increasing percent identity (90-98% identity from top left to bottom right). Each colour, ranging from the darkest purple to the lightest green, represents a different OTU; the darker the colour the more abundant (relative abundance, y-axis). White indicates the OTUs that could only be clustered to the level of *Eimeria* and those less abundant.

5.4.3. Visualisation and statistics

5.4.3.1. Alluvial plots

The first alluvial plot (Figure 5.5) includes sequences that were present in the samples more than three times clustered at 91-98% identity in 1% increments. The yellow demonstrates a cluster that remains relatively distinct, with few sequences moving in and out of this cluster between clustering percentages. Sequences in red, however, move from one cluster to another from 91-94%, until many are stabilised in the s__ad OTU that appears at 95% identity. This figure demonstrates how individual sequences move between groups as the clustering is performed at various percent identities. The second alluvial plot (Figure 5.6) highlights the sequences (in purple) grouped at 95% identity into the "Genus" cluster, illustrating where each individual sequence is grouped at other percent identities. This plot emphasises the low abundance in the "Genus" cluster at 95% identity compared to other percent identities (see Appendix E.3). The combination of bar and alluvial plots led to the selection of 95% identity as the most appropriate to represent the variation in kiwi *Eimeria* spp. at this COI target. At 95% identity there were 49 reference OTUs as well as 49 OTUs in the 54 samples submitted for Illumina amplicon sequencing.

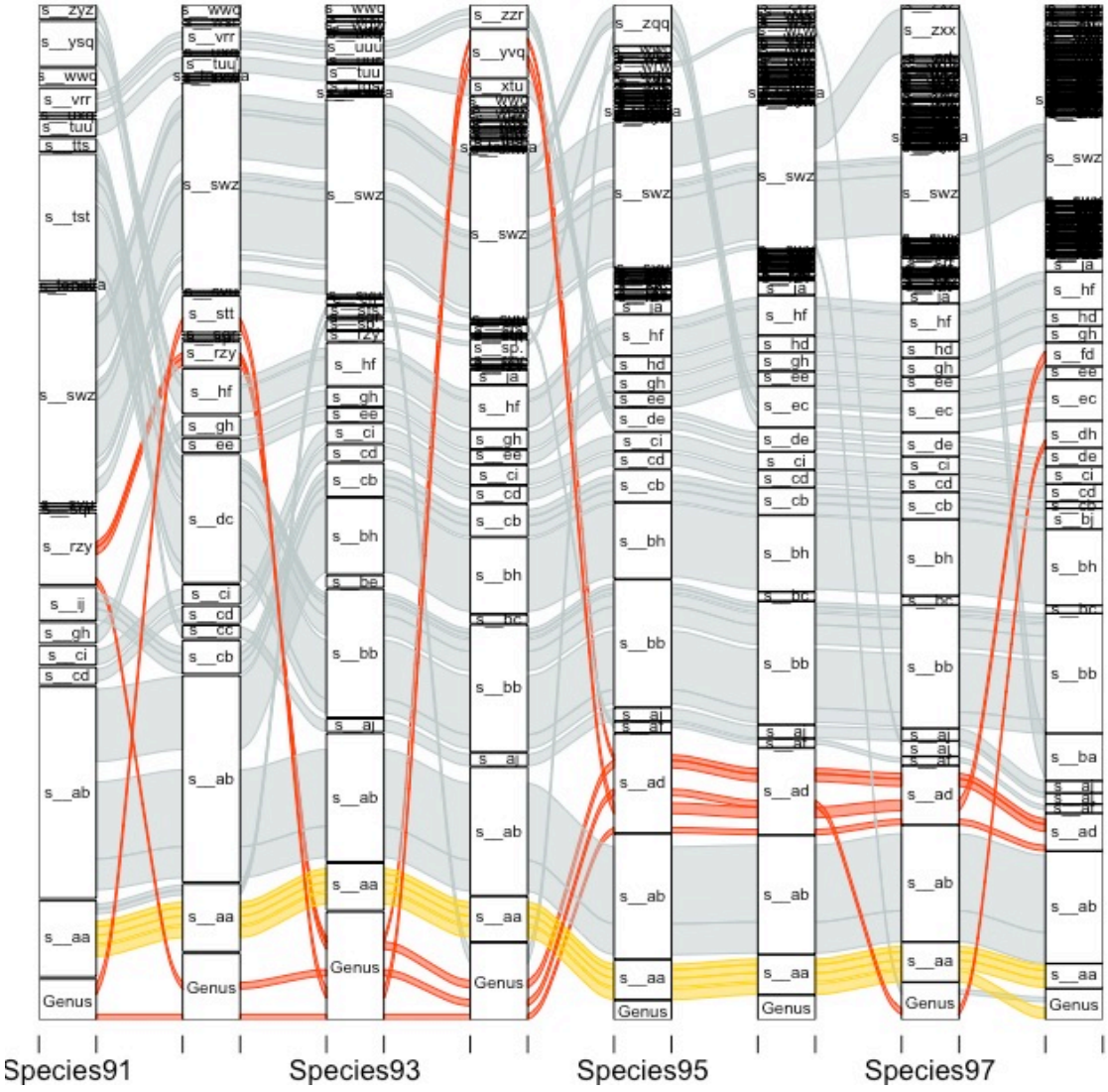


Figure 5.5. Alluvial plot illustrating the change in clustering from 91% to 98% identity in 1% intervals. Each column has boxes representing the relative abundance of each cluster. This simplified version of the clustering results highlights how unique sequences can be reorganised between clusters.

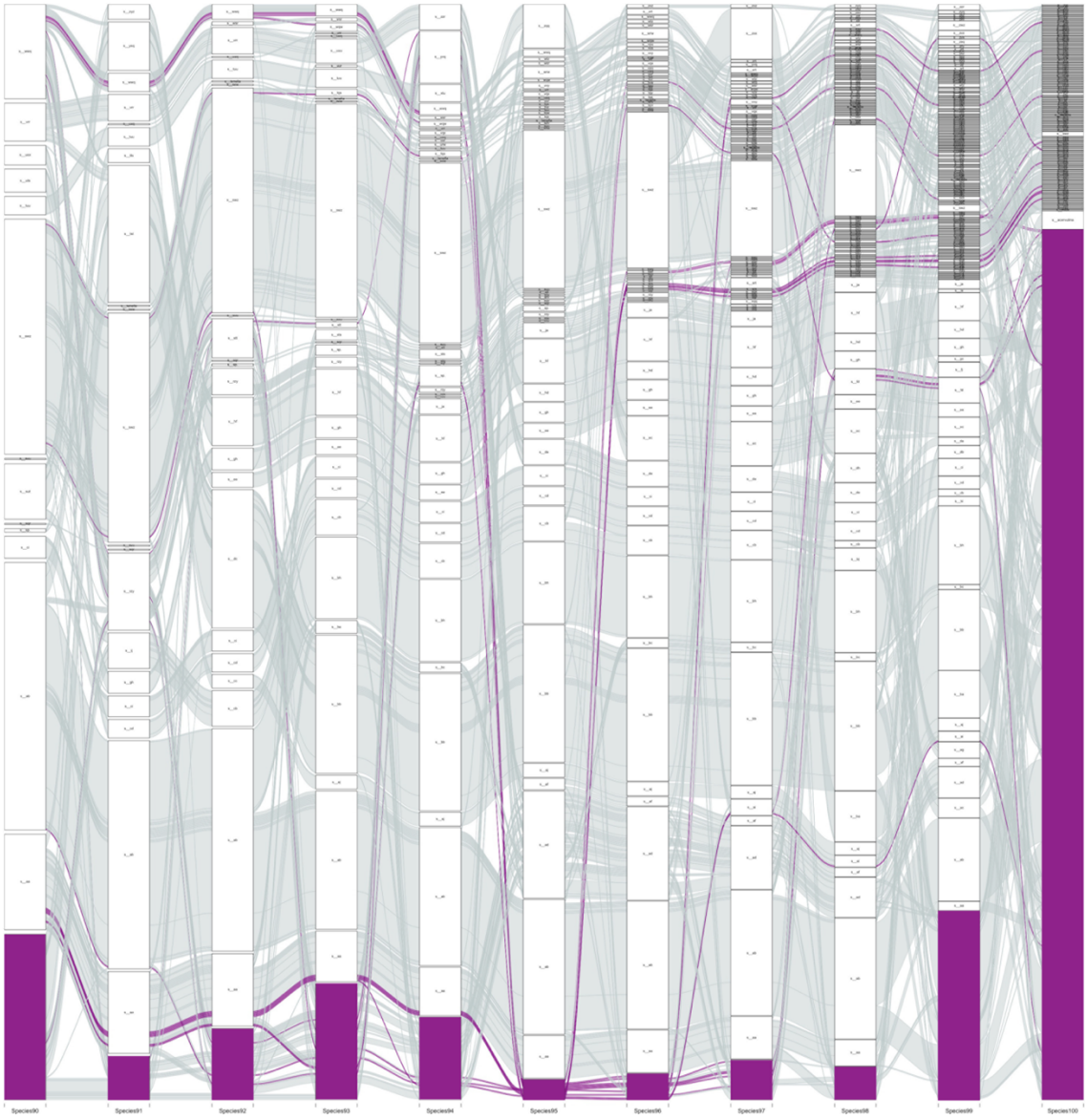


Figure 5.6. An alluvial plot illustrating the change in clustering from 90% to 100% identity in 1% intervals. Each column has boxes representing the relative abundance of each cluster. This plot demonstrates the complexity and fluidity of clustering and the impact choosing an inappropriate percent identity can have on the overall results. The purple cluster are sequences that could not be clustered beyond the level of *Eimeria* genus. The purple lines indicate the paths of species unspecified at 95% identity.

5.4.3.2. Distribution

When visualised as a heatmap (Figure 5.7), each host metadata group (Table 5.3) is dominated by one to two OTUs. The two control groups were dominated by a single OTU that was not proportionally abundant in any other group. The Br_OrWiPa_CAN_SI group was the only kiwi group that was dominated by an OTU that was not relatively abundant in any other group. All other groups contained two or more OTUs, with one obviously dominant OTU. With few exceptions, OTUs occur in multiple groups, with no obvious trend in host or location.

Table 5.3. Metadata group codes for visualisation of kiwi coccidia Illumina sequencing results.

Category	Abbreviation	Meaning
Host Species	Br	Brown kiwi (Apteryx mantelli)
	GSK	Great spotted kiwi
	Ro	Rowi
	To	Haast tokoeka
	Kereru	New Zealand pigeon
	Immucox	Immucox brooder vaccination for chicken Eimeria
Submitter	AuZoo	Auckland Zoo
	CaSa	Cape Sanctuary
	MoIs	Motuarohia Island
	MotIs	Moturua Island
	MtMa	Mt. Manganui
	NgMa	Nga Manu Nature Reserve
	OrWiPa	Orana Wildlife Park
	PuMtBr	Pūkaha Mt. Bruce
	Pure	Purerua
	Raka	Rakaumangamanga
	RaSp	The National Kiwi Hatchery
	Wai	Wairakei Golf Sanctuary
	Ware	Warrenheip
	West	Westshore Wildlife Reserve
	KaNaPa	Kahurangi National Park
	MaIs	Mana Island
	Okar	Okarito
	FrJoGl	Franz Josef Glacier
	Ha	Haast
	OrEc	Orokonui Ecosanctuary
	RoIs	Rona Island
	PacificVet	Immucox vaccine
Region	AKL	Auckland
	HWB	Hawke's Bay
	NTH	Northland
	WAI	Waikato
	MNW	Manawatu
	CAN	Canterbury
	BOP	Bay of Plenty
	TAS	Tasman
	WEL	Wellington
	WES	West Coast
	OTA	Otago
	STH	Southland
Island	NI	North Island
	SI	South Island

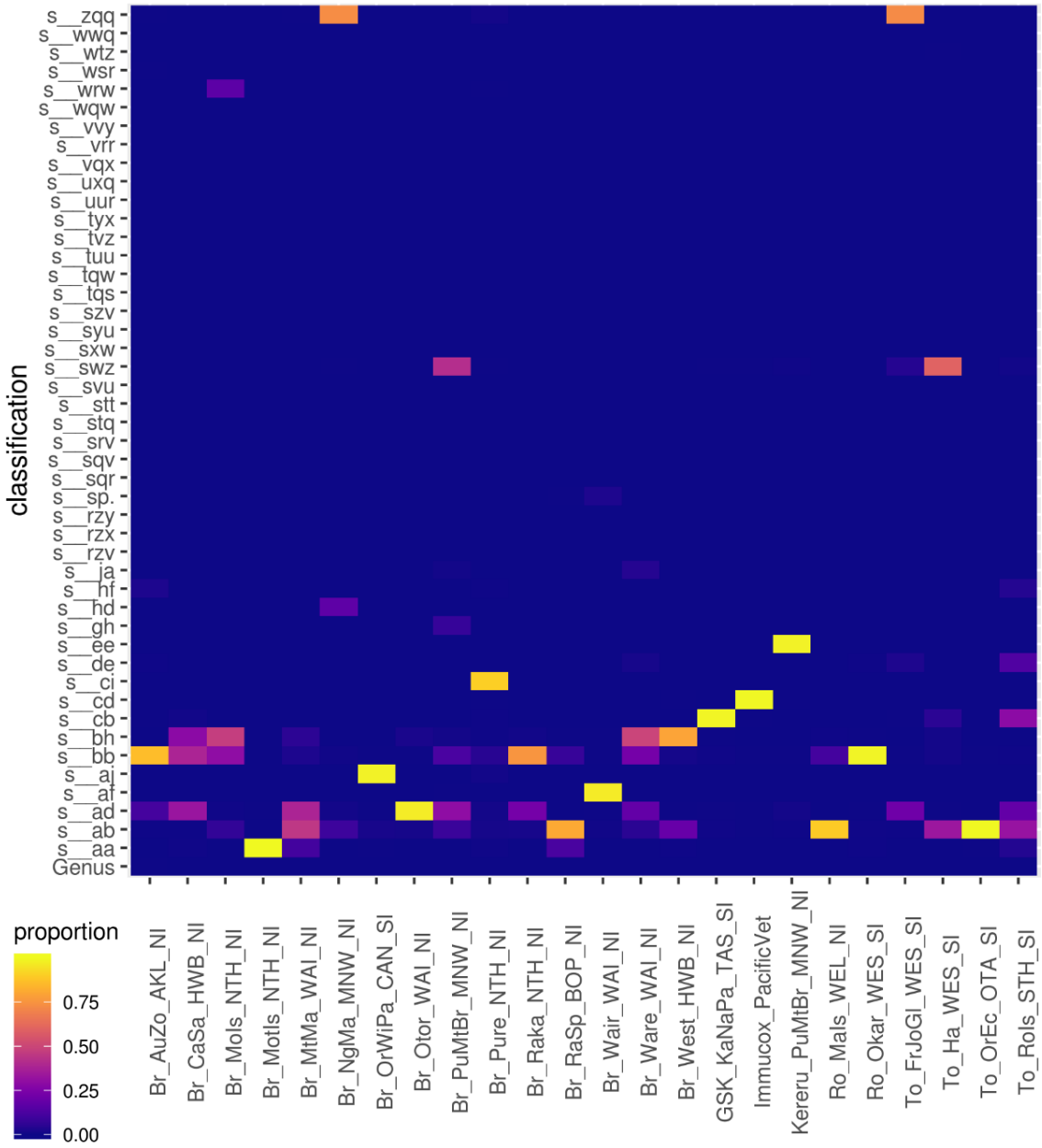


Figure 5.7. Heatmap illustrating the proportional abundance of sequences generated by Illumina amplicon sequencing and clustered at 95% identity. Samples are grouped into relevant metadata groups (x-axis) and named as a combination of species, submitter, region and island delineated by underscores. The codes are shown in Table 5.3. The y-axis are the OTUs after clustering and labelled with automatically generated codes.

5.4.3.2.1. Statistics

The most informative metadata groupings were host species and region. There were no significant differences in Faith's Phylogenetic Differences (not shown; $p>0.05$) nor the

evenness by host (Figure 5.8A; $p>0.05$); however, by region, the Manawatu had significantly ($p<0.05$) higher evenness than the Bay of Plenty, Northland, and Waikato (Figure 5.8B). The PCA based on the Jaccard similarity index yielded no component of interest (the most informative component contributed less than 6% explanation for variation). The three most informative axes in the PCA based on the Bray-Curtis diversity index (Figure 5.9) accounted for less than 30% of the observed variation. The three most informative axes in the PCA based on the unweighted UniFrac index accounted for about 44% of the observed variation (Figure 5.10).

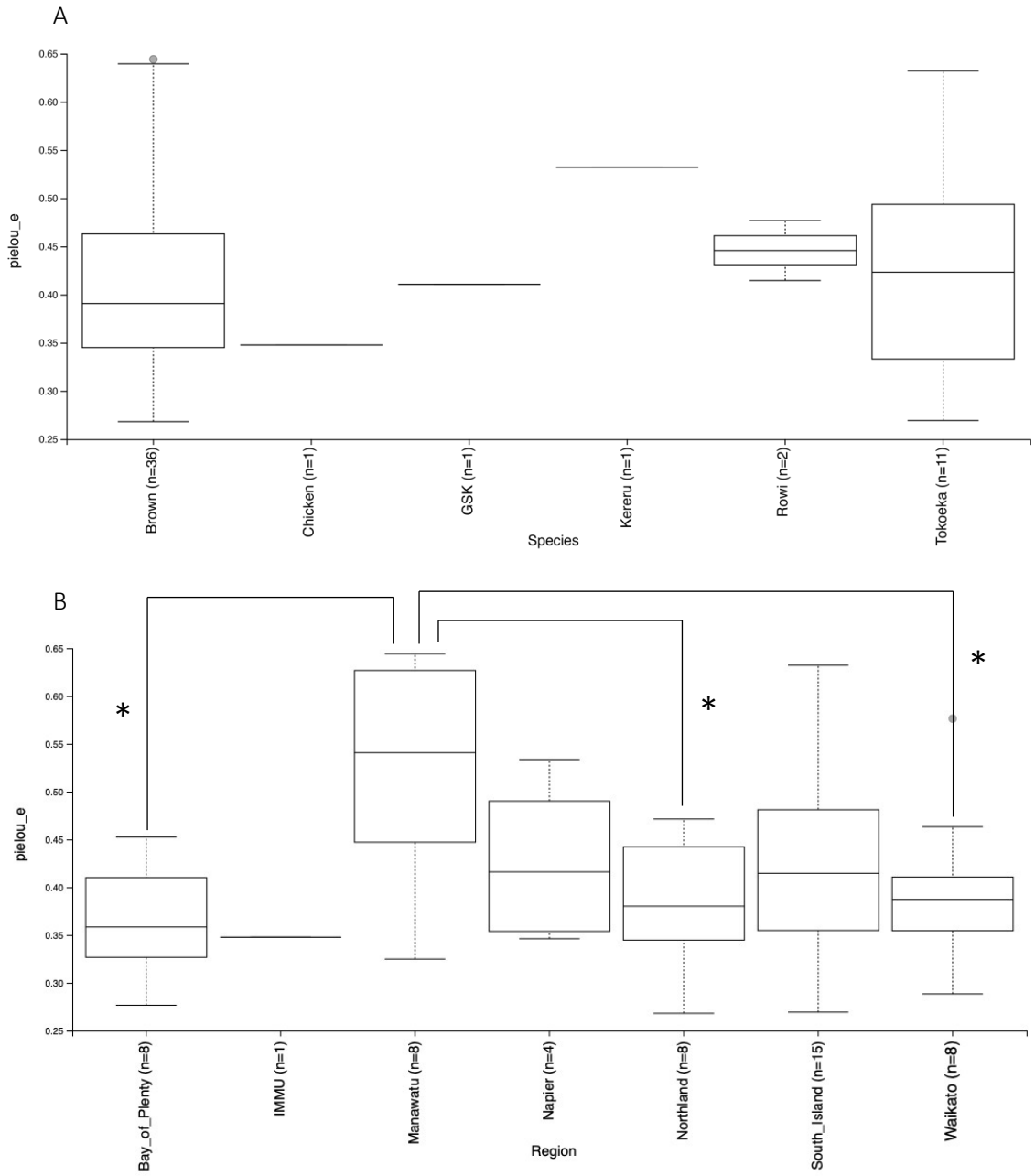


Figure 5.8. Boxplot comparing the Pielou's evenness index values (y-axis) of Illumina sequence data retrieved from eimerian oocysts in bird droppings. Plot (A) compares host species (x-axis). "Brown" refers to brown kiwi (*Apteryx mantelli*); "Chicken" refers to the Immucox Breeders and Layers live vaccine; "GSK" refers to great spotted kiwi (*Apteryx haastii*); "Kereru" refers to the New Zealand pigeon (*Hemiphaga novaeseelandiae*); "Rowi" refers to the rowi (*Apteryx rowi*); "Tokoeka" refers to Haast tokoeka (*Apteryx australis* 'Haast'). Plot (B) compares the region (x-axis) of sample collection. IMMU refers to the Immucox Breeders and Layers live vaccine. *Indicates significant differences ($p<0.05$) between two groups.

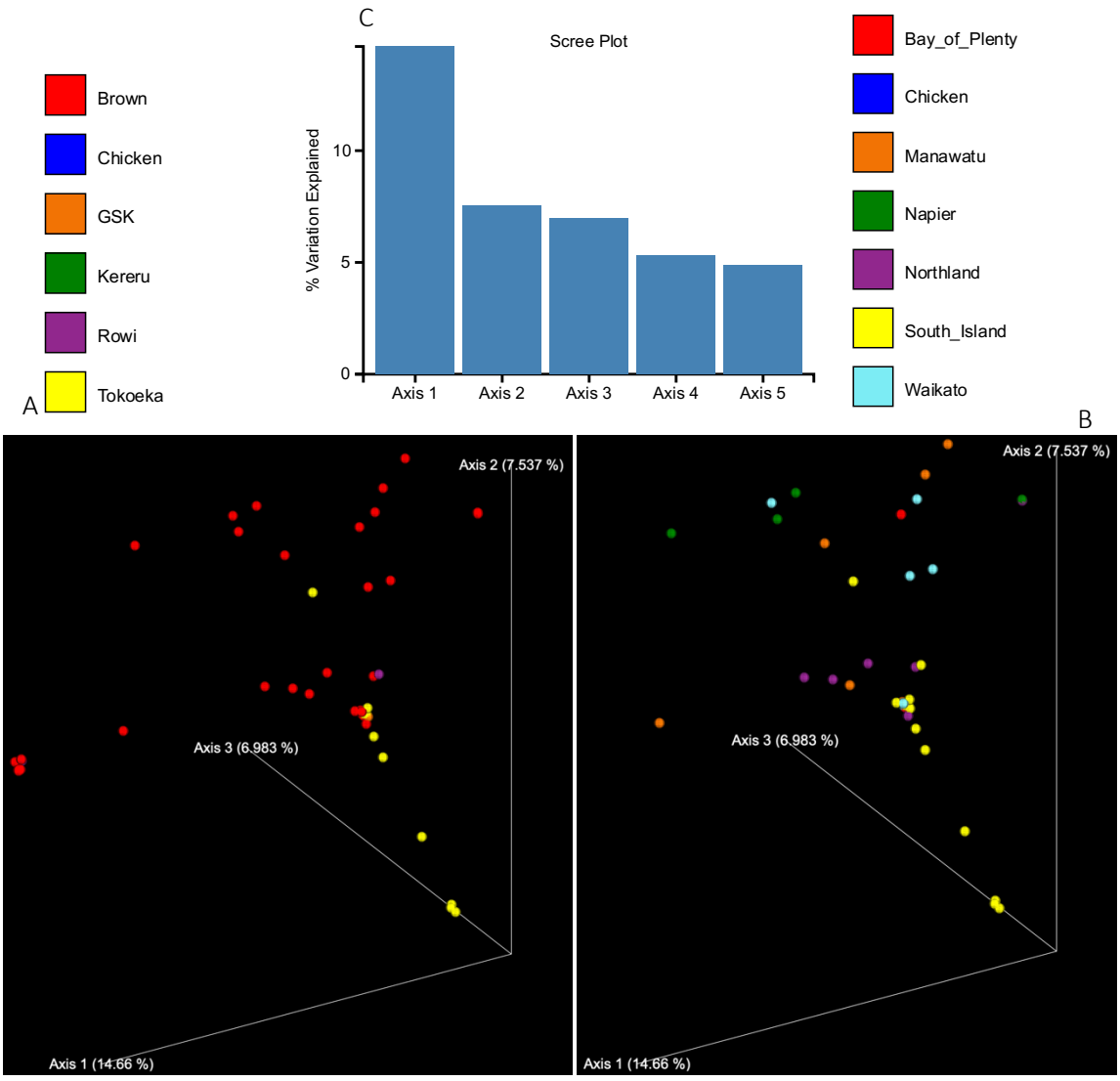


Figure 5.9. Principal coordinate analysis plots using the Bray-Curtis diversity index calculated from Illumina amplicon sequencing data. (A) compares host species. "Brown" refers to brown kiwi (*Apteryx mantelli*); "Chicken" refers to the Immucox Breeders and Layers live vaccine; "GSK" refers to great spotted kiwi (*Apteryx haastii*); Kereru refers to the New Zealand pigeon (*Hemiphaga novaeseelandiae); "*Rowi" refers to the rowi (*Apteryx rowi*); "Tokoeka" refers to Haast tokoeka (*Apteryx australis* 'Haast'). Plot (B) compares the regions of sample collection in New Zealand. Plot (C) shows the top axes that account for the % variation of the sequences.

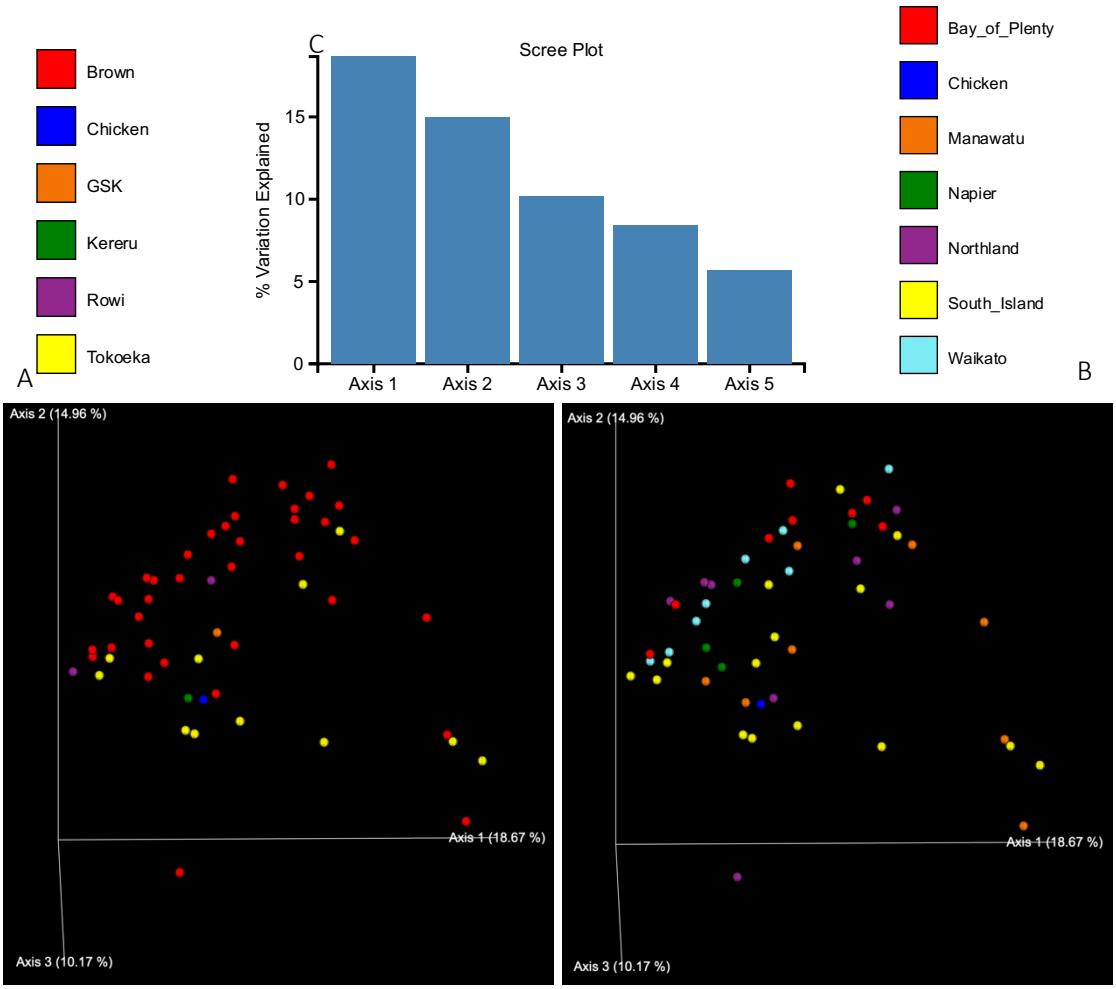


Figure 5.10. Principal coordinate analysis plots calculated using the unweighted UniFrac diversity index calculated from Illumina amplicon sequencing data. (A) compares host species. "Brown" refers to brown kiwi (*Apteryx mantelli*); "Chicken" refers to the Immucox Breeders and Layers live vaccine; "GSK" refers to great spotted kiwi (*Apteryx haastii*); Kereru refers to the New Zealand pigeon (*Hemiphaga novaeseelandiae); "*Rowi" refers to the rowi (*Apteryx rowi*); "Tokoeka" refers to Haast tokoeka (*Apteryx australis* 'Haast'). Plot (B) compares the regions of sample collection in New Zealand. Plot (C) shows the top axes that account for the % variation of the sequences.

5.4.3.3. Paired sample comparison

MB15A yielded 98,905 sequences and 8 OTUs. MB15B yielded 46,728 sequences and 11 OTUs. Figure 5.11 is an alignment of each unique sequence found in both MB15A and B; these are labelled as the OTU into which each sequence has been clustered at 95% identity.

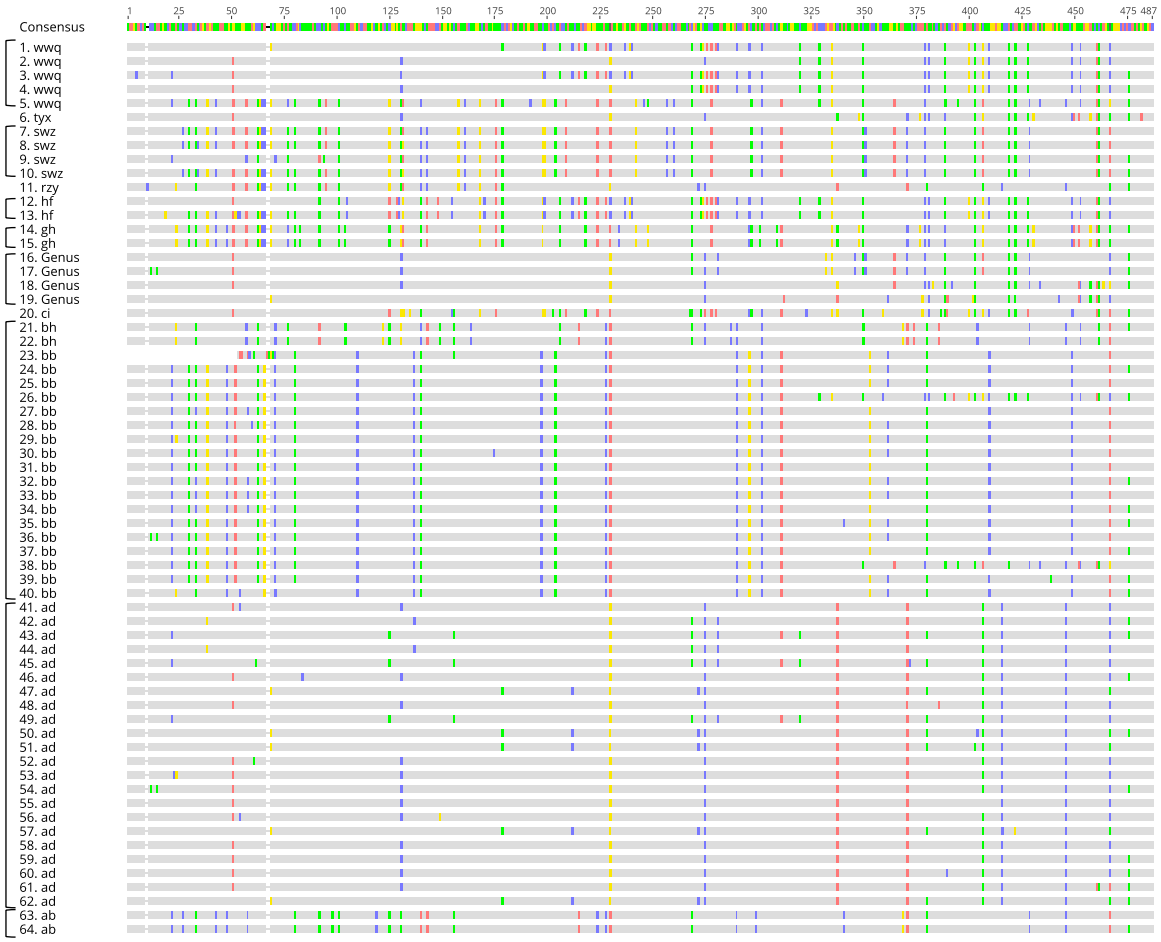


Figure 5.11. Consensus alignment unique sequences obtained from two paired samples, each aliquoted from a single brown kiwi dropping generated using ClustalW in Geneious, v11.0. Each sequence represents a unique sequence obtained from each sample. Sequence names were replaced with the two-letter code associated with the representative sample after clustering with the full dataset of other kiwi (*Apteryx* spp.) *Eimeria* at 95%. Gray areas indicate nucleotides that match the consensus sequence; coloured lines indicate nucleotides that do not match the consensus sequence. Brackets indicate sequences that have been grouped into the same cluster.

5.4.3.3.1. Pearson's Chi-Squared

Table 5.4A reports the proportional abundance of the sequences that were detected in the samples "MB15A" and "MB15B". Table 5.4B reports the proportional abundance multiplied by 1,000 and rounded to whole numbers; zeros were excluded from the analysis. This transformation was to enable the chi-squared analysis, which yielded: X-squared = 413.56 (df = 8, p-value < 0.01).

A chi-squared analysis was also performed on non-transformed abundance data using the three most abundant sequences in these MB15 aliquots (s__ab, s__ad, and s__bb). In MB15A, 98,905 sequences were obtained; 46,710 sequences were obtained from MB15B (Table 5.2). This analysis yielded: chi-squared = 24036 (df = 2, p-value < 0.01).

Table 5.4. Proportional abundance of sequences detected by Illumina amplicon sequencing from two aliquots from sample MB15). (A) Raw proportional data from aliquots MB15A and B. (B) Proportional data transformed to allow for chi-squared analysis.

A	MB15A	MB15B
Genus	0	3.85E-04
s__ab	0.07463728	0.02371169
s__ad	0.39459077	0.81415853
s__bb	0.52729387	0.13867488
s__bh	8.29E-04	0.01046482
s__ci	0	1.50E-04
s__gh	1.62E-04	0.00395908
s__hf	0	0.00126263
s__rzy	1.01E-05	0
s__swz	0.00224458	0.00383068
s__tyx	0	2.35E-04
s__wwq	2.33E-04	0.00237545
s__zqq	0	7.92E-04

B	MB15A	MB15B
Genus	0	0
s__ab	75	24
s__ad	395	814
s__bb	527	139
s__bh	1	10
s__ci	0	0
s__gh	0	4
s__hf	0	1
s__rzy	0	0
s__swz	2	4
s__tyx	0	0
s__wwq	0	2
s__zqq	0	1

5.4.3.3.2. Shannon's indices

Table 5.5 reports the diversity (H) and evenness (SEI) of MB15A and MB15B.

Table 5.5. Shannon's diversity (H) and evenness (SEI) indices between two Illumina amplicon sequencing reactions aliquoted from the same, homogenised sample prior to DNA extraction.

Index	**MB15A**	**MB15B**
H	0.92	0.66
SEI	0.44	0.26

5.4.3.3. Phylogeny

The reference sequences grouped well by host type and apicomplexan genus or species (Figure 5.12), with the exception of the *Isospora* sp. from a canary (GenBank KP658103.1) and *Eimeria subsherica* (GenBank KU315704), which grouped together. Two clades (A and B) have been labelled for comparison in Figure 5.9, which included the reference sequences as well as the OTUs from the kiwi droppings. With the exception of clades A and B, all the reference sequences grouped the same in both trees. The 19 most abundant OTUs, which accounts for 99.9% of the sequences, were used for phylogenetic analysis. With the addition of this Illumina sequencing data (Figure 5.13), clade A incorporated s__cd, which was most associated with the Immucox control, whereas clade B incorporated s__aj, the most abundant in sample OW01, which was been identified as *Eimeria ferrisi* (GenBank MH777579.1, 99.8% homology) from mice (Chapter 3). Figure 5.14 shows the consensus tree without the reference sequences; this tree is included to help highlight the relationships among the kiwi samples and compare the abundance data represented in the heatmaps.

Four OTUs did not group with other sequences. OTU s__aa, which was the third most abundant, s__wrw; s__af; and s__ee. OTU s__ee was mainly associated with the kereru, appearing in other groups only at very low levels of relative abundance.

Clade C, comprising of the OTUs s__gh, s__hf, and s__ci, was most closely related to clade D, comprising of s__ja, s__cb, s__swz (the sixth most abundant OTU), s__hd, and s__zqq. Clade E included s__zqq, s__ad (the second most abundant), s__bb (the fourth most abundant and the only OTU to be found in all samples), s__de, s__bh, and s__ab (the most abundant overall).

To further assist in analysis, the reference sequences were removed in Figure 5.10 to illustrate the distribution and variation found in each metadata host groups. OTUs s__ad, s__bb, and s__ad were present in all the groups. OTU s__bh was present in all groups except Br_OrWiPa_CAN_SI and the kereru sample.

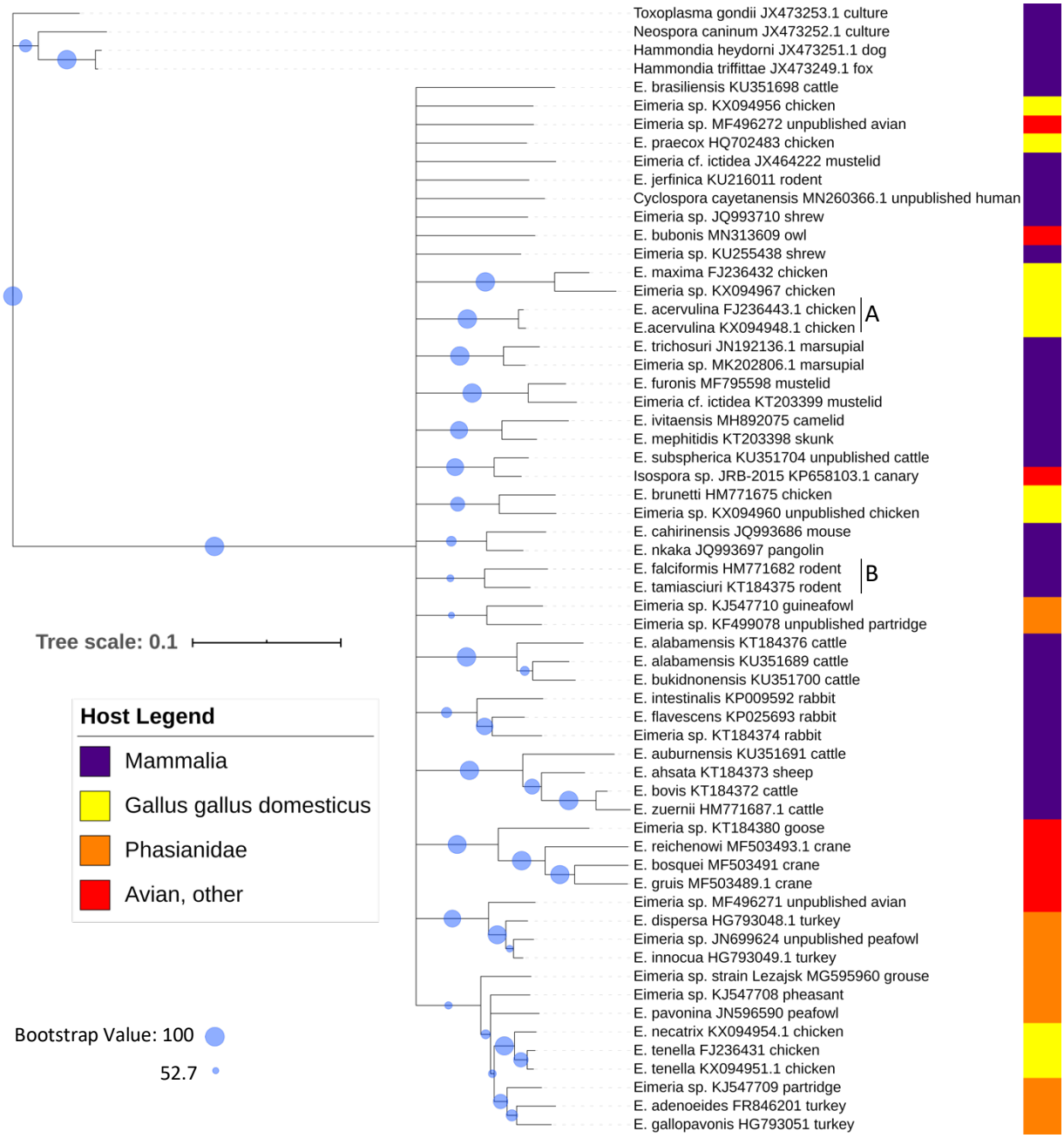


Figure 5.12. Reference sequence phylogeny at 95% identity built with the Tamura-Nei Distance Model and the Neighbour-Joining Method to determine how major groups are related to each other. The blue spheres represent bootstrap values which range from 52.7-100%. *Toxoplasma gondii* JX473253.1 was used as an outgroup. Purple = mammal; Yellow = chicken; Orange = Phasianidae; Red = other avian.

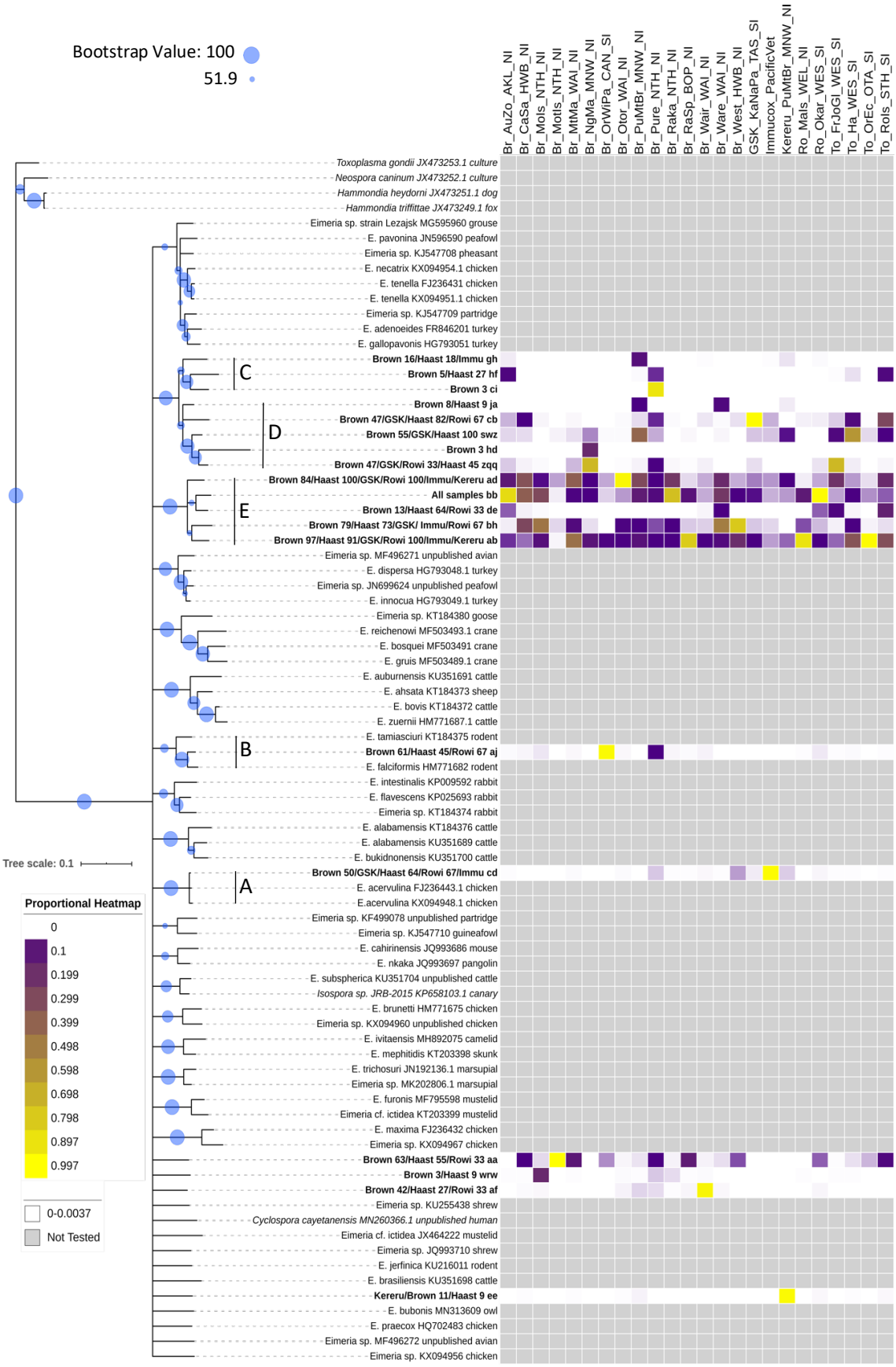

Bootstrap Value: 100
51.9
Br_AuZo_AKL_NI
Br_CaSa_HWB_NI
Br_Mols_NTH_NI
Br_Motls_NTH_NI
Br_MtMa_WAI_NI
Br_NgMa_MNW_NI
Br_OrWiPa_CAN_SI
Br_Otor_WAI_NI
Br_PuMtBr_MNW_NI
Br_Pure_NTH_NI
Br_Raka_NTH_NI
Br_RaSp_BOP_NI
Br_Wair_WAI_NI
Br_Ware_WAI_NI
Br_West_HWB_NI
GSK_KaNaPa_TAS_SI
Immucox_PacificVet
Kereru_PuMtBr_MNW_NI
Ro_Mals_WEL_NI
Ro_Okar_WES_SI
To_FrJoGl_WES_SI
To_Ha_WES_SI
To_OrEc_OTA_SI
To_Rols_STH_SI
Toxoplasma gondii JX473253.1 culture
Neospora caninum JX473252.1 culture
Hammondia heydorni JX473251.1 dog
Hammondia triffittae JX473249.1 fox
Eimeria sp. strain Lezajsk MG595960 grouse
E. pavonina JN596590 peafowl
Eimeria sp. KJ547708 pheasant
E. necatrix KX094954.1 chicken
E. tenella FJ236431 chicken
E. tenella KX094951.1 chicken
Eimeria sp. KJ547709 partridge
E. adenoeides FR846201 turkey
E. gallopavonis HG793051 turkey
Brown 16/Haast 18/Immu gh
Brown 5/Haast 27 hf
Brown 3 ci
Brown 8/Haast 9 ja
Brown 47/GSK/Haast 82/Rowi 67 cb
Brown 55/GSK/Haast 100 swz
Brown 3 hd
Brown 47/GSK/Rowi 33/Haast 45 zqq
Brown 84/Haast 100/GSK/Rowi 100/Immu/Kereru ad
All samples bb
Brown 13/Haast 64/Rowi 33 de
Brown 79/Haast 73/GSK/ Immu/Rowi 67 bh
Brown 97/Haast 91/GSK/Rowi 100/Immu/Kereru ab
Eimeria sp. MF496271 unpublished avian
E. dispersa HG793048.1 turkey
Eimeria sp. JN699624 unpublished peafowl
E. innocua HG793049.1 turkey
Eimeria sp. KT184380 goose
E. reichenowi MF503493.1 crane
E. bosquei MF503491 crane
E. gruis MF503489.1 crane
E. auburnensis KU351691 cattle
E. ahsata KT184373 sheep
E. bovis KT184372 cattle
E. zuernii HM771687.1 cattle
E. tamiasciuri KT184375 rodent
Brown 61/Haast 45/Rowi 67 aj
E. falciformis HM771682 rodent
E. intestinalis KP009592 rabbit
E. flavescens KP025693 rabbit
Eimeria sp. KT184374 rabbit
E. alabamensis KT184376 cattle
E. alabamensis KU351689 cattle
E. bukidnonensis KU351700 cattle
Brown 50/GSK/Haast 64/Rowi 67/Immu cd
E. acervulina FJ236443.1 chicken
E.acervulina KX094948.1 chicken
Eimeria sp. KF499078 unpublished partridge
Eimeria sp. KJ547710 guineafowl
E. cahirinensis JQ993686 mouse
E. nkaka JQ993697 pangolin
E. subspherica KU351704 unpublished cattle
Isospora sp. JRB-2015 KP658103.1 canary
E. brunetti HM771675 chicken
Eimeria sp. KX094960 unpublished chicken
E. ivitaensis MH892075 camelid
E. mephitidis KT203398 skunk
E. trichosuri JN192136.1 marsupial
Eimeria sp. MK202806.1 marsupial
E. furonis MF795598 mustelid
Eimeria cf. ictidea KT203399 mustelid
E. maxima FJ236432 chicken
Eimeria sp. KX094967 chicken
Brown 63/Haast 55/Rowi 33 aa
Brown 3/Haast 9 wrw
Brown 42/Haast 27/Rowi 33 af
Eimeria sp. KU255438 shrew
Cyclospora cayetanensis MN260366.1 unpublished human
Eimeria cf. ictidea JX464222 mustelid
Eimeria sp. JQ993710 shrew
E. jerfinica KU216011 rodent
E. brasiliensis KU351698 cattle
Kereru/Brown 11/Haast 9 ee
E. bubonis MN313609 owl
E. praecox HQ702483 chicken
Eimeria sp. MF496272 unpublished avian
Eimeria sp. KX094956 chicken
A
B
C
D
E
Tree scale: 0.1
Proportional Heatmap
0
0.1
0.199
0.299
0.399
0.498
0.598
0.698
0.798
0.897
0.997
0-0.0037
Not Tested

Figure 5.13. Consensus tree built with the Tamura-Nei Distance Model and the Neighbour-Joining Method using the 19 most abundant kiwi *Eimeria* OTUs generated by Illumina amplicon sequencing and clustered at 95% identity in QIIME2. Sequences from this study (in bold) are labelled with the host species in which it was found, followed by the proportion of samples from each species in which it was present, and concluded with a randomly assigned alphabetical code. The blue spheres represent bootstrap values which range from 51.9-100%. *Toxoplasma gondii* JX473253.1 was used as an outgroup. The heatmap reflects the proportions of each sequence found in each metadata group (x-axis; Table 5.3).

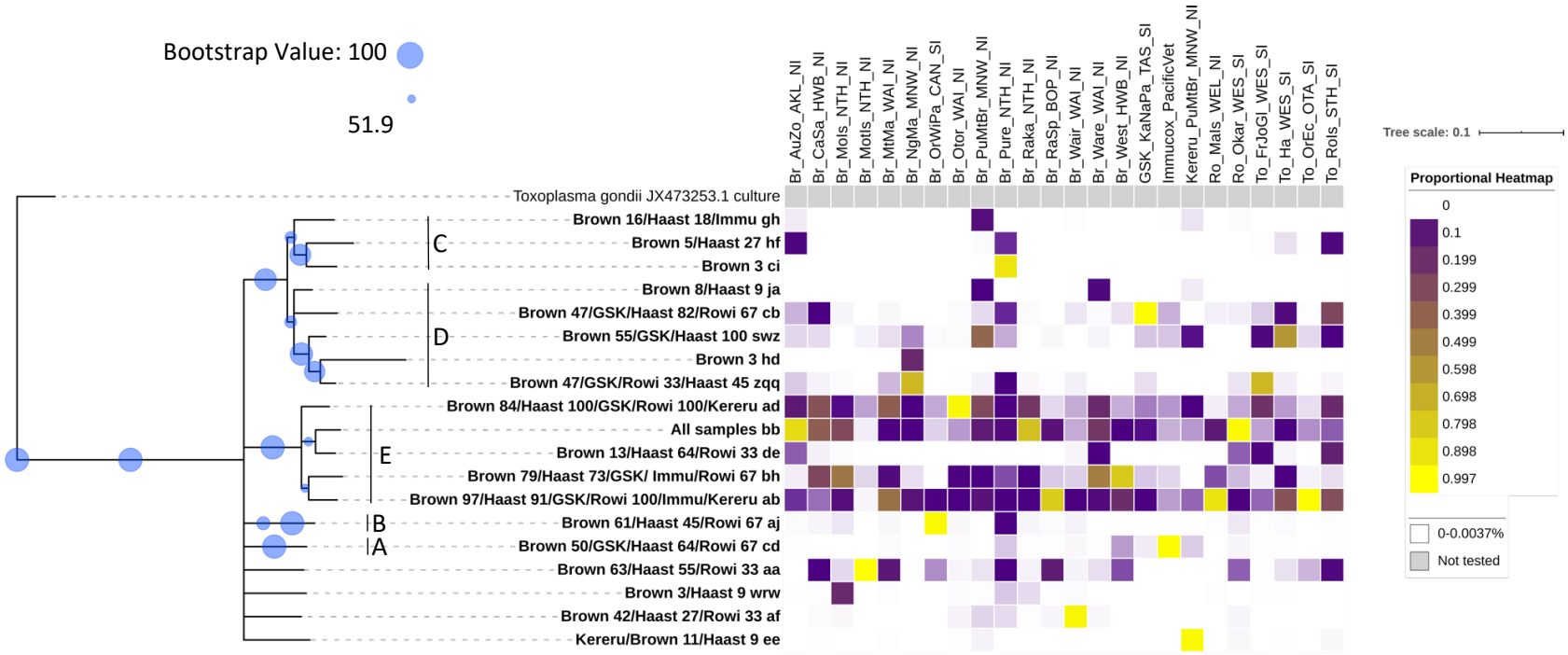


Figure 5.14. Consensus tree built with the Tamura-Nei Distance Model and the Neighbour-Joining Method using the 19 most abundant kiwi *Eimeria* sequences generated by Illumina amplicon sequencing and clustered at 95% identity in QIIME2. Sequences labelled with the host species in which it was found ("Brown" = brown kiwi, *Apteryx mantelli*; "Haast" = Haast tokoeka, *Apteryx australis* 'Haast'; "Rowi" = rowi, *Apteryx rowii*), followed by the proportion of samples from each species in which it was present, and concluded with a randomly assigned alphabetical code. The blue spheres represent bootstrap values which range from 51.9-100%; the larger the circle, the higher the bootstrap value. *Toxoplasma gondii* JX473253.1 was used as an outgroup. The heatmap reflects the proportions of each sequence found in each metadata group (x-axis; Table 5.3). Clade A is the sequence most similar to *Eimeria acervulina* isolated from the Immucox Breeders and Layers live vaccine. Clade B is most similar to *Eimeria ferrisi* from mice. Clades C, D, and, E are the largest groupings of *Eimeria* oocysts isolated from kiwi (*Apteryx* spp.) droppings collected from New Zealand.

5.4.3.4. Comparison to Sanger sequencing

Sanger sequencing of RS01 led to a sequence with no ambiguous bases assigned by Geneious. However, low signals that are present in the background on both directions were present, indicating the possibility of a coinfection. The OTU s__ab was 100% homologous to the dominant Sanger sequence; OTU s__bb was 92% homologous with s__ab and matched positions of background signals in RS01. OTU s__cd was 85% homologous to s__ab and not consistent with background signals. Figure 5.15 provides an excerpt from the alignment of these sequences.

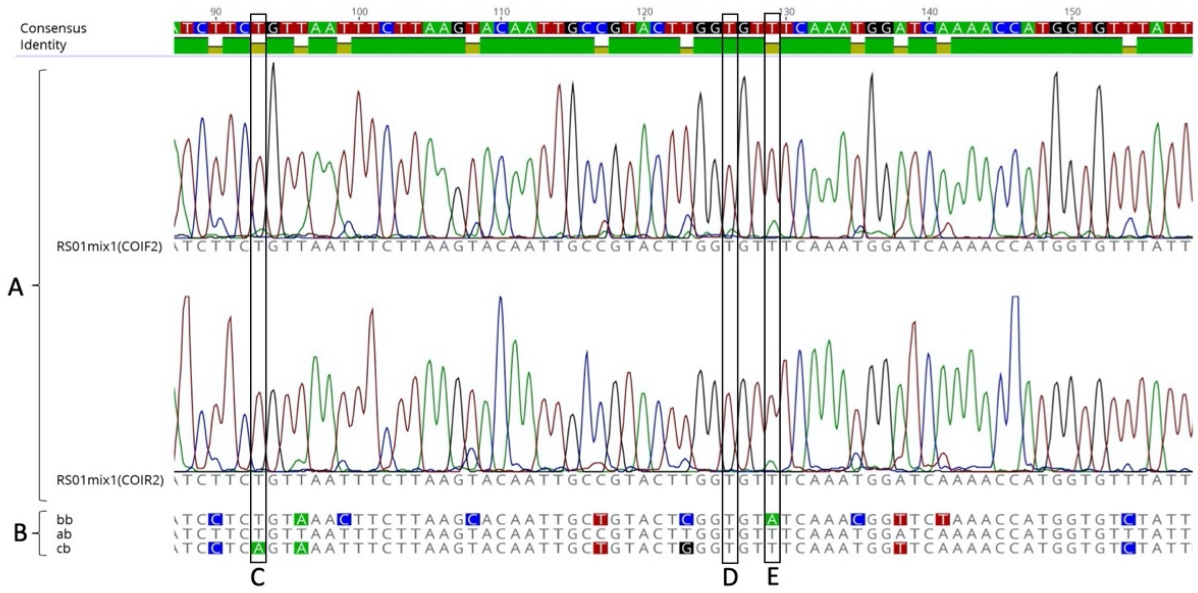


Figure 5.15. Chromatogram of next generation sequencing detecting variation in a kiwi (*Apteryx mantelli*) faecal sample that is cryptic in Sanger sequencing. A) Forward and reverse alignment from Sanger sequencing of the RS01mix1 sample. B) Sequences from Illumina amplicon sequencing. In the RS01 sample, "bb" was present at a low percentage, "ab" was present at a high percentage, and "cb" was not present. C) An example nucleotide position demonstrating that the "cb" sequence is not visible in the Sanger sequencing results. D) An example nucleotide position of how background noise can show up on a chromatogram, having the potential to confuse Sanger sequencing results. E) An ideal example of a nucleotide position showing how a sequence present at lower quantities can appear in Sanger sequencing results.

5.5. Discussion

Figure 5.15 illustrates the importance of utilising deeper sequencing techniques to detect variation in a population. In this case, the kiwi faecal sample RS01 contains two main sequences. The more abundant sequence was detected by Sanger sequencing as well as Illumina; however, the less abundant sequence was overpowered during Sanger sequencing. Frequently, these points of variation can be accounted for with ambiguous base calling; however, this can get complicated with highly variable sequences as well as the presence of three or more sequences, as is seen in several samples analysed in this study. This particular example helps validate the Illumina methodology as well as justify the use of more advanced sequencing technologies.

The Illumina sequencing data provided a dependable overview of the variation of this portion of the COI gene in the *Eimeria* spp. within this sample set. Clustering at 95% led to seven clades (OTUs that grouped together or OTUs that did not group with any other; Figures 5.13 and 5.14). Interestingly, one or more OTU from each clade were detected in more than one kiwi species, suggesting that the host specificity of these coccidia may be less specific than previously believed. This finding supports the morphotypes described from Haast tokoeka as being synonymous with morphotypes described from brown kiwi, and additional species (e.g., *Eimeria paraurii)* may be described from tokoeka in the future. Furthermore, there may be additional morphotypes yet to be described in brown kiwi, or one or more of the clades could represent cryptic species.

Without a doubt, the results of this sequencing yielded significant findings and sheds a light on the complexity of the host-parasite interactions occurring in kiwi. However, it

should be kept in mind that the sample set used was limited in geographic range and heavily biased towards brown kiwi as well as captive individuals. Furthermore, it should be kept in mind that Illumina amplicon sequencing still requires several steps of amplification, which inevitably leads to amplification bias (Ambardar et al. 2016; Vermeulen et al. 2016). Thus, the data provided by the analysis should be one of guidance for future study, rather than a definitive report of genetic variation or expected species distribution.

5.5.1. Abundance

Abundance of a particular species, morphotype, or genotype is frequently of high concern for diagnosticians and other practitioners; for example, a particularly abundant parasite in an individual host can point to increased virulence or drug resistance of that particular parasite. However, interpreting the data on abundance of different OTU's from Illumina amplicon sequencing requires careful consideration of a number of potential confounding factors and cross-reference to relevant information on the sample and host. Determining the significance of a particular OTU based on number of reads alone can be misleading as the total number of reads from each sample can vary drastically. For example, in this study, the Illumina sequencing resulted in 2,022 reads from PN01 and 1,157,165 reads from RU04 (Figure 5.2). This variation can be due to varying levels of targets, the condition of the sample, and the amount of inhibition during the initial PCR amplification steps. Accordingly, the relative/proportional abundance (as is used for phylogenetic and statistical analysis in this chapter) of an OTU in a particular sample or location can be more informative than overall abundance. For example, if you compare two individual kiwi and collect a dropping sample from each at

the same time on the same day from two different locations, it is very unlikely that both were exposed to identical levels and species composition of oocysts at exactly the same times. Therefore, the time at which the sample was collected will reflect not only species composition, but also the stage of infection.

Another issue of relying on abundance to inform significance lies in amplification bias. While Illumina sequencing technologies help ensure this bias is avoided during library preparation (van Dijk et al. 2014a; van Dijk et al. 2014b), the conventional PCR steps required for sample preparation can still lead to preferential amplification of specific sequences. This study utilised a nested PCR protocol, leading to two rounds of amplification in which a particular sequence may have been amplified more efficiently than others, thereby leading to an over representation of a particular sequence. Thus, while it may be tempting to assume that the most abundant sequence was caused by a higher parasite burden in a given host, this may not be the case. This was demonstrated by samples MB15A and MB15B, which were aliquots from the same sample and yielded significantly different results.

To confound this further, a higher parasite burden does not necessarily lead to increased virulence (i.e. severity of disease and impact on host fitness; Read 1994), particularly for wild kiwi. The greatest threat to wild kiwi populations is predation by introduced mammals. While predation of adults by dogs remains a source of significant loss, young, small kiwi are susceptible to stoats, cats, possums, weasels, and ferrets in addition to dogs (Basse et al. 1999; McLennan et al. 2004; McLennan et al. 1996; Miller and Pierce 1995; Taborsky 1988). The greatest defence a kiwi chick has against these predators is

to grow as quickly as possible. Thus, a coccidial genotype that slows the growth of a kiwi chick may be considered to be highly significant despite causing small direct impact on the host. While an impact on growth may be attributed to high infection loads, it is not necessarily linked to high virulence and causing morbidity and mortality. Previous pathological studies have indicated that at least some enteric species of coccidia in kiwi are highly fecund, as demonstrated by the presence of large macromeronts in the lamina propria (Morgan et al. 2012). Indeed, genotypes of particularly high abundance may have comparatively low virulence, which would allow the host to tolerate infection longer, leading to greater parasite success (Boots et al. 2009; Ebert 1994; Lively and Dybdahl 2000; Read 1994). It is therefore more likely for kiwi to be able to tolerate higher levels of infection of a less virulent, very common genotype of *Eimeria*. A high tolerance would further allow greater spread over a geographic area, increasing the likelihood of exposure of new hosts. As kiwi in the wild are at low densities and frequently maintain distinct territories (Potter 1990; Taborsky and Taborsky 1999), increased exposure to new hosts may be a defining adaptation of more successful genotypes. Unfortunately, it may be the case that in ONE facilities, where the density of kiwi is unnaturally high, the rate of exposure is also artificially high, leading to greater mortality rates due to coccidiosis.

It is important to remember, therefore, that genotypes that present with the highest infection loads may not be the only genotype of particular importance. Histological examination of historical samples from kiwi submitted for postmortem examination yielded three morphologically distinct gametocytes found in the intestinal tract of brown kiwi (Morgan et al. 2012) as well as signs of renal, splenic, pulmonary, and hepatic

coccidiosis in kiwi (Morgan et al. 2013). There are likely two or more genotypes associated with each morphotype described. The overarching goal of increasing the fitness of kiwi populations can be improved by determining the pathogenicity (i.e., the ability to cause disease) and virulence (i.e., the severity of disease) of coccidian genotypes (Read 1994). Knowing whether a particular genotype is able to cause one or more forms of coccidiosis could help practitioners determine when treatment is necessary. For example, if a particular genotype associated with severe enteric coccidiosis as well as potential for pulmonary, renal, or splenic disease, is detected, that kiwi may be prioritised for hospitalisation or supportive care over a kiwi that is infected with a genotype only associated with mild enteric coccidiosis. Thus, the sequences generated by the Illumina sequencing need to be analysed not only for abundance, but also for establishing relevant genotypes that can begin to be linked to various morphotypes.

5.5.2. Clustering

Because Illumina sequencing results in such large amounts of OTU data, clustering based on percentage similarity is an essential component of interpretation of the data. In this study it was used to examine the sequencing of *Eimeria* spp. from droppings from multiple host species and geographic ranges to assist in the understanding of the genetic variation and distribution of coccidia in ONE locations.

In other avian host species, this 484 bp COI target in *E. dispersa* (HG793048.1) and *E. innocua* (HG793049.1) that infect turkeys are 97.7% similar (Vrba and Pakandl 2014; Yang et al. 2013; Yang et al. 2015a; Yang et al. 2014a; Yang et al. 2015d), whereas *E.*

gruis (MF503489.1) and *E. reichenowi* (MF503493.1) in black-necked cranes (*Grus nigricollis*) are only 89.7% similar (Liang et al. 2018). A crucial distinction between turkeys and cranes is the level of management. Vrba and Pakandl (2014) studied turkey bred for the poultry industry, potentially leading to high selection for very few genotypes of *Eimeria*. On the other hand, Liang et al. (2018) studied wild cranes and collected samples from a nature reserve, where selection for more variation in the parasite population is more likely to occur. With several exceptions, the kiwi samples used in this study were collected from young kiwi in ONE facilities; however, it is important to note that these kiwi are, for the most part, bred in the wild. All the samples from Northland as well as RW13 and RW16 were collected from the wild, frequently from offshore islands. This is similar to tokoeka samples collected from Rona Island, except that that this is an ONE crèche, meaning all the birds on Rona Island were sourced from ONE facilities. Thus, while kiwi are not nearly as domesticated as turkeys, the kiwi samples used for this study are much more heavily managed than the black-necked cranes. It is perhaps expected, therefore, for an appropriate homology to lie somewhere between 90%, as these parasites are still under wild species selection pressures, and 98%, as some strong management pressures have been placed on certain parasite populations. Theoretically, when clustering is performed at greater homology, more variation should be represented. Clustering a 95% classifies the highest number of OTUs, thereby preserving the greatest variation (Figures 5.4-5.6); this preservation allows for greater resolution for future research into identifying biologically relevant genotypes.

5.5.3. Distribution

At 100% identity, 903 unique sequences were present in the samples (Figure 5.3). At 95% identity, there were 42 OTUs present in the samples. Unexpectedly, there was no obvious association between the location, host species, time of year, or age and the detection of any OTU (section 5.3.3.2.1). This result could be attributed to uneven and opportunistic sampling of locations, ages, and host species. A single genotype of coccidia infecting all species of kiwi was unexpected (Figure 5.14). While kiwi populations have fluctuated for millions of years between isolation and overlapping geographically, it is interesting that the coccidia have not evolved more host specificity over the 2.5 million years of separation between the two main islands (Weir et al. 2016). The continual fluctuations in geographic overlap caused by shifting glacial patterns may have selected for coccidia that can infect multiple species of kiwi without being so virulent as to cause acute coccidiosis. Alternatively, the most closely related kiwi species (brown kiwi, rowi, and tokoeka) may not have been diverged enough for their eimeriid genotypes to no longer be able to infect other host species. This hypothesis would explain the wide distribution of genotypes. There is some anecdotal evidence to suggest that Haast may be more susceptible to a particular genotype from rowi, suggesting that selection among rowi did not prevent infection in Haast tokoeka, but did increase the parasite virulence in this particular host species. In this instance, at an ONE facility that raised Haast tokoeka and rowi in adjacent pens, after periods of flooding there were increased incidents of severe coccidiosis but only in the Haast (pers. comm. Jacinda Amey). This facility has since been closed and Haast tokoeka and rowi are now raised in facilities less prone to flooding; however, the potential for overlapping of coccidia from the most common host species to a more susceptible host still remains and should be considered.

While no OTU was only found in one host species or location, there does usually appear to be only one to three relatively abundant (>0.1%) OTUs in a given group. While abundance of a particular OTU is not the only measure of importance, it does provide some guidance for which OTUs may be easiest to detect to begin assigning particular OTUs to a morphotype, host susceptibility, and virulence.

5.5.4. Phylogeny

In order to simplify the analysis of the phylogeny, only the OTUs that were more abundant than the "genus" group was included. This selection led to the inclusion of 19 out of 49 OTUs at 95% identity. These 19 OTUs included 91% (n = 903) of the unique sequences produced by Illumina sequencing. Of the six OTUs that did not group with other kiwi OTUs (Figure 5.9), one was from the Immucox vaccine (s__cd, clade A), one from a mouse (s__aj, clade B), and one from the kereru (s__ee). As can be easily seen in Figure 5.10, all three of these OTUs are also present in multiple other metagroups. It is likely that the presence of these OTUs in kiwi samples is due to contamination of the sample at the time of collection. Kereru and mice can freely travel in and out of kiwi enclosures and habitat throughout New Zealand. Meanwhile, it is not unimaginable that *Eimeria acervulina* oocysts or DNA could be present in the environment. It was not unexpected that only *E. acervulina* to be amplified from the five *Eimeria* spp. present in the Immucox vaccine as these primers target a portion of the COI gene that is severely inhibited by a G:A mismatch (Kwok et al. 1990).

Without these three control OTUs, there remains three individual OTUs and three main clades (C and D, as well as E; Figures 5.9 and 5.10) of kiwi coccidia. None of these OTUs

grouped with other *Eimeria*, confirming the novelty of these species. It is currently unknown whether these five clades correspond with the five previously described morphotypes (see Chapters 3 and 4; Morgan et al. 2017), especially as the two largest clades can be further broken down into smaller clades, as is demonstrated with clades C and D. Interestingly, clade E is more common across all metagroups and may be correlated with *E. kiwii* or Morphotypes 2/5, as these tend to be the very prevalent morphotypes (see Chapters 3 and 4; Morgan et al. 2017).

5.5.5. Statistics

The only statistically significant trend identified in this dataset is shown in Figure 5.8B, which demonstrates that the samples from Manawatu region contained a more evenly distributed species composition than the Bay of Plenty, Northland, and Waikato regions. This more even distribution means that the Manawatu was less dominated by a particular OTU than the other regions. It is difficult to say why the Manawatu region would be less dominated by a single OTU than the other regions. The Waikato region samples were collected from ONE facilities that crèche kiwi directly after release from the National Kiwi Hatchery Aotearoa (NKHA) in the Bay of Plenty. Thus, it is likely the difference between the Manawatu region and the Waikato region stems from the difference between the Manawatu and the Bay of Plenty region. The Bay of Plenty samples were exclusively collected from NKHA; the Manawatu samples were collected from Pūkaha National Wildlife Centre and Nga Manu Nature Reserve. The species composition is likely to contain higher levels of an OTU that is resistant to treatment; in the absence of resistance, therefore, the species composition would be expected to be more evenly distributed (Melville et al. 2016). It is possible, therefore, that the coccidia

at NKHA have developed some resistance to toltrazuril, whereas resistance may be lower in the Manawatu region. However, increased resistance would likely not explain the difference between the Manawatu and Northland samples. Of the nine Northland samples, only one was collected from an ONE site (Auckland Zoo). All other samples were collected from wild, and, therefore, untreated populations; five samples were collected from offshore islands. It is more likely that when kiwi were moved to these offshore islands, the kiwi *Eimeria* population experienced a significant bottleneck, leading to a low variation in the parasite populations and the observed decrease in genetic variation (Nadler 1995; Papkou et al. 2016).

The other most informative index was the unweighted UniFrac, which uses the phylogenetic distances of the sequences within the samples to calculate differences between the samples (Figure 5.10; Lozupone and Knight 2005). The main group that stood out from the others was the brown kiwi group (Figure 5.10A). Unfortunately, this trend is likely due to the over-representation of brown kiwi samples over all other host species. While the weighted UniFrac did yield a principal component that accounted for about 88% of variation, this was likely extremely skewed by sequence abundance, rather than diversity as no grouping led to a reasonable explanation.

5.5.6. Conclusions

This chapter presents the first in depth sequencing of *Eimeria* spp. from kiwi (*Apteryx* spp.). The use of Illumina amplicon sequencing of a portion of the mitochondrial COI gene has allowed for genetic comparison of these parasites infecting four species of kiwi located across the North and South Islands of New Zealand. The analysis of this data has

led to an impressively complex web of genetic variation and similarity within and between species and regions. The overarching trends were caused by the larger sample size of brown kiwi, which is a direct result of accessibility dependant on ONE and DOC practices as well as host population size. Using Illumina amplicon sequencing has provided a glimpse into the genetic variation of the *Eimeria* spp. that infect kiwi in New Zealand.

This deep sequencing will provide much needed information for the development of a rapid diagnostic test that will help practitioners make informed management decisions by ensuring such a test will not miss rare, but potentially highly pathogenic or virulent strains of coccidia. The gene target used in this study provides sufficient variation to differentiate species of kiwi *Eimeria* and could also be useful for other New Zealand avifauna. Additional testing of rowi, great spotted kiwi, and little spotted kiwi would be beneficial to apply this technique more widely across all kiwi species and locations to inform optimal parasite management in ONE operations. As conservation efforts continue to expand, the likelihood of overlapping these parasite/host species grows, potentially leading to exposure to species of coccidia that are potentially more pathogenic in certain host species than others.

Moreover, future research should also use this gene target and methodology on single oocysts as well as historical, formalin-fixed paraffin embedded samples from kiwi with clinical coccidiosis (Morgan et al. 2012; Morgan et al. 2013). The results of this analysis would help connect sequences to morphospecies and pathogenicities, respectively.

CHAPTER 6

Preliminary research into vital kiwi coccidia attributes and a real-time diagnostic assay proof of concept

Contribution of co-authors: Dr Howe guided the development and organisation of this chapter. All authors provided feedback on one or more drafts of this section.

6.1. Chapter overview

The specific aim of this chapter is to communicate the technical difficulties, protocol optimisations that arose during this project and preliminary diagnostic assay development data. Every year, Operation Nest Egg (ONE) uses vital resources addressing coccidiosis in kiwi; thus, including this information helps future researchers to understand the rationalisations for the methodologies selected and to make informed decisions for continuing the genetic and morphological analysis of kiwi coccidia. Overcoming these obstacles will assist in accomplishing the long-term goal of developing diagnostic tests to differentiate species of coccidia to address broad epidemiology and pathogenicity questions.

Accordingly, this chapter includes data accumulated over the entire course of this thesis and comprises four distinct parts. Part 1 provides preliminary data on the prepatent period of kiwi *Eimeria* spp. Part 2 provides information on the unpredictability of sporulating these coccidia and demonstrates the impact sporulation makes on successful DNA extraction from the oocysts. Part 3 provides detail on the optimisations and troubleshooting required for DNA extraction and amplification of oocyst DNA. Finally, Part 4 presents a proof of concept for the development of rapid, specific diagnostic testing using the NGS data generated for Chapter 5 of this thesis.

Part 1: Preliminary study to determine prepatent period

6.2. Introduction

The prepatent period of coccidia is the time between a host's exposure to sporulated oocysts and the time the first oocysts are excreted in the droppings (Bangoura and Daugschies 2018; Dorney 1962; Edgar 1955; Mesfin and Bellamy 1978). Documenting the prepatent periods of coccidia provides an additional means of biological differentiation (Allen and Fetterer 2002). Further, knowing the prepatent period can ensure screening for coccidial burdens is conducted at an appropriate time post-exposure (Bangoura and Daugschies 2018). If diagnostic screening is conducted at an inappropriate time, then the severity of infection may be misrepresented (Bangoura and Daugschies 2018). In addition, in the case of perceived persistent infection, knowledge of the prepatent period will assist in differentiation between reinfection and inefficacy of anti-coccidial medication. The length of prepatent periods is currently unknown in kiwi *Eimeria* spp. To date, the majority of research has focused on establishing morphological and genetic profiles, which is important as closely related species can vary in their prepatency and patency periods (Bangoura and Daugschies 2018).

In domestic hosts, the ability to characterise pathogenicity, virulence, treatment efficacy, as well as prepatent and patency periods of *Eimeria* spp. heavily relies on experimental trials (e.g., Ankrom et al. 1975; Buehl et al. 2006; Edgar 1955). These trials involve infecting a host with a known quantity of sporulated oocysts of an unknown level of pathogenicity and virulence. While kiwi eggs are regularly removed from the wild and raised in sanitary brooder rooms, the conservation status of this bird (especially certain

species such as the little spotted kiwi, *Apteryx owenii*) essentially outweighs the benefit of such an experiment. However, at the National Kiwi Hatchery Aotearoa (NKHR) in Rotorua (as well as other captive rearing facilities), chicks leaving the coccidia-free brooder room are transferred to outdoor enclosures, where naïve birds are exposed to potentially infective coccidia (Morgan 2013; Taylor et al. 2019). This exposure requires close monitoring, as disease can cause significant morbidity in some kiwi chicks due to high coccidial burdens (Morgan 2013). This pilot study was undertaken with the knowledge that exposure to eimerian oocysts for the first time is not guaranteed to occur at the same time for all kiwi at NKHA. The shock of transitioning from an indoor brooder to an outdoor enclosure may cause some chicks to start probe feeding later than others, or some may rely more on the supplemental food provided on a daily basis. Additionally, the number of viable, infective oocysts a chick is exposed to likely varies greatly depending on pen use and varying environmental conditions. Further, at the time of this study, NKHA collected and pooled samples shed overnight outside of the roost box. Because of the pronounced circadian rhythm of oocyst shed demonstrated in brown kiwi, this method of collection is likely to lead to samples in which the oocyst load is greatly diluted (Taylor et al. 2018). Accordingly, the intent of this research was to attempt to get initial data of the prepatent periods of the coccidia in kiwi with the future aim of providing an additional tool for coccidia collection and differentiation.

6.2.1. Brief methodology

Pooled samples were collected from NKHA enclosures (but outside the roost box) in sterile collection bottles during the 2017-2018 hatching season. The aim was to get a single sample from each enclosure at approximately days 7 (sample 1) and 21 (sample

2) post transfer from the brooders into the outdoor enclosures. It was understood that NKHA operates on a busy daily schedule and that samples would be collected as close to the target dates as possible. While release into the enclosures does not guarantee exposure to infective oocysts at the same time for all chicks, this is the best approximation available in the ONE network. Oocyst loads (oocysts per gram, OPG) were detected using the Mini-FLOTAC apparatus and protocol described in Chapter 2 (Appendix B.3).

6.2.2. Results

Twenty-nine samples were collected from 17 enclosures during the study period (Table 6.1). Samples were collected between days 6-9 (Sample 1, n = 15) and then again between days 19-29 (Sample 2, n = 14), although some repeat collections were missed. This collection resulted in days 6-9 and 19-29 being represented and some repeat measures missed completely. Of the 17 enclosures represented, 12 had repeated collections. Table 6.1 summarises the results; the second sample has been combined into a single column for simplicity as all that were tested were positive for coccidia. Of the 15 samples collected between days 6-9, three samples were positive for coccidia. Of the 14 samples collected between days 19-29, 100% of the samples were positive for coccidia with an overall average of 19,300 OPG.

Table 6.1. Counts of oocysts per grams detected in pooled brown kiwi (*Apteryx mantelli*) droppings opportunistically collected on known days post exposure at NKHA.

Enclosure	Day 6	Day 7	Day 8	Day 9	Days 20-29
A	-	0	-	-	19147
B	-	-	-	0	-
C	-	-	0	-	15840
D	-	0	-	-	101120
E	0	-	-	-	8534
F	-	-	0	-	11200
G	-	-	0	-	17253
H	-	0	-	-	7493
I	-	-	0	-	10320
J	0	-	-	-	-
K	-	-	-	560	-
L	-	-	-	0	5520
M	-	-	-	-	33920
N	220	-	-	-	6000
O	-	-	-	-	20480
P	-	40	-	-	12240
Q	-	0	-	-	980

A (-) indicates that a sample was not collected from that enclosure that day.

6.2.3. Discussion

This data presents the first approximation of the prepatent period of kiwi coccidia and provides baseline information to allow for more in-depth studies in future. The detection of oocysts as early as six days post movement to outdoor enclosures, when kiwi are likely to be first exposed to coccidial oocysts, indicates that future research into the prepatent period should focus on at least days 5 through 10, as the prepatent period is defined as the time to the first day oocyst shedding is observed (Bangoura and Daugschies 2018; Dorney 1962; Edgar 1955; Mesfin and Bellamy 1978). Several factors need to be accounted for in order to establish a true prepatency timeline. For example, each sample used in this study is a pooled sample of all the droppings from a single

enclosure shed over the entire night-time period. Pooling the sample can greatly dilute the detected OPG, as the number of oocysts shed over time varies drastically (Taylor et al. 2018). Additionally, while NKHA prefers to introduce two kiwi into a pen on the same day, this is not always the case. Moving kiwi from the brooder room to the outdoor enclosures is determined as the point when that kiwi regains hatch weight (the weight at the time of hatching). It is not always the case that two chicks destined for the same enclosure after transfer from the brooder room reach hatch weight on the same day, leading to the kiwi in each enclosure potentially being exposed to coccidia on different days.

Part 2: Sporulation of kiwi coccidial oocysts

6.3. Introduction

Unfortunately, unsporulated oocysts have few distinguishing features (see Figures 1.3 and 1.4) and thus, in the absence of molecular diagnostics, identification of coccidia relies heavily on characteristics of sporulated oocysts (Duszynski 2011). This characterisation process is further hampered by limitations on experimental design caused by the conservation status of kiwi. The ideal conditions for experimentally sporulating coccidial oocysts generally require retrieving fresh droppings within a few minutes after leaving the host and bringing the oocysts directly into a controlled environment (Norton and Chard 2009). This cannot be realistically achieved with kiwi, as experimentally infecting and housing kiwi for continuous sample collection may interfere with conservation outcomes and is unlikely to receive necessary permit and ethics approvals. Further, while characteristics of internal stages and prepatency can be

useful (e.g., differentiating *Eimeria innocua* and *Eimeria dispersa*; Vrba and Pakandl 2014), the most common source of morphospecies descriptions relies on oocysts that readily sporulate in 2% w/v potassium dichromate (Duszynski 2011; Yabsley 2008). This single factor has limited characterisation of coccidia in kiwi since the first documentation of unsporulated oocysts by Thompson and Wright (1978). Standard sporulation techniques and conditions tend to be less reliable for kiwi coccidia compared to other species of coccidia (Duszynski and Wilber 1997; Morgan 2013). Morgan et al. (2017) demonstrated the ability to sporulate these oocysts; however, the reported unpredictability may be due to less-than-ideal transportation or sporulation conditions (Duszynski and Wilber 1997; Morgan 2013; Thompson and Wright 1978). The species descriptions provided by Morgan et al. (2017) states that the sporulation time of the brown kiwi *Eimeria* species was approximately 10 days. However, knowing that the samples used for this thesis would vary in sampling and shipping conditions, the author decided additional data on the range of sporulation time under "normal" conditions warranted investigation. The main goal of this preliminary investigation was to determine if 10 days was sufficient for >90% of oocysts to sporulate, thus facilitating the methodology for identification and extraction experiments (Allen and Fetterer 2002; Hammond et al. 1973; Lee and Dorney 1971; Prowse 1991; Ryan 2002; Ryley and Robinson 1976)

6.3.1. Methods

Kiwi dropping samples (n = 7 separate samples) submitted from 22/8/17 to 31/8/17 to the Parasitology Lab at Massey University that tested positive for coccidia were set to sporulate ("Day 0") no more than five days after collection. The oocysts were sporulated

in 2% aqueous (w/v) potassium dichromate ($K_2Cr_2O_7$) at room temperature as described by Duszynski and Wilber (1997) and Morgan (2013), see Chapter 3 and Appendix C.2 for more detail. Each day for a total of 11 days, a drop of the sample was placed on a slide and examined for oocysts under 600X magnification. All observed oocysts were recorded as sporulated or unsporulated. Experimentally determining the sporulation rate of kiwi coccidia was not a primary goal of this thesis therefore, based on Morgan (2013), daily observations of samples for sporulation was limited to 11 days.

6.3.2. Results

In total, 695 oocysts were observed over 11 days of observation (Figure 6.1). On Day 11, about 80% (60/71) of the oocysts on that day were sporulated.

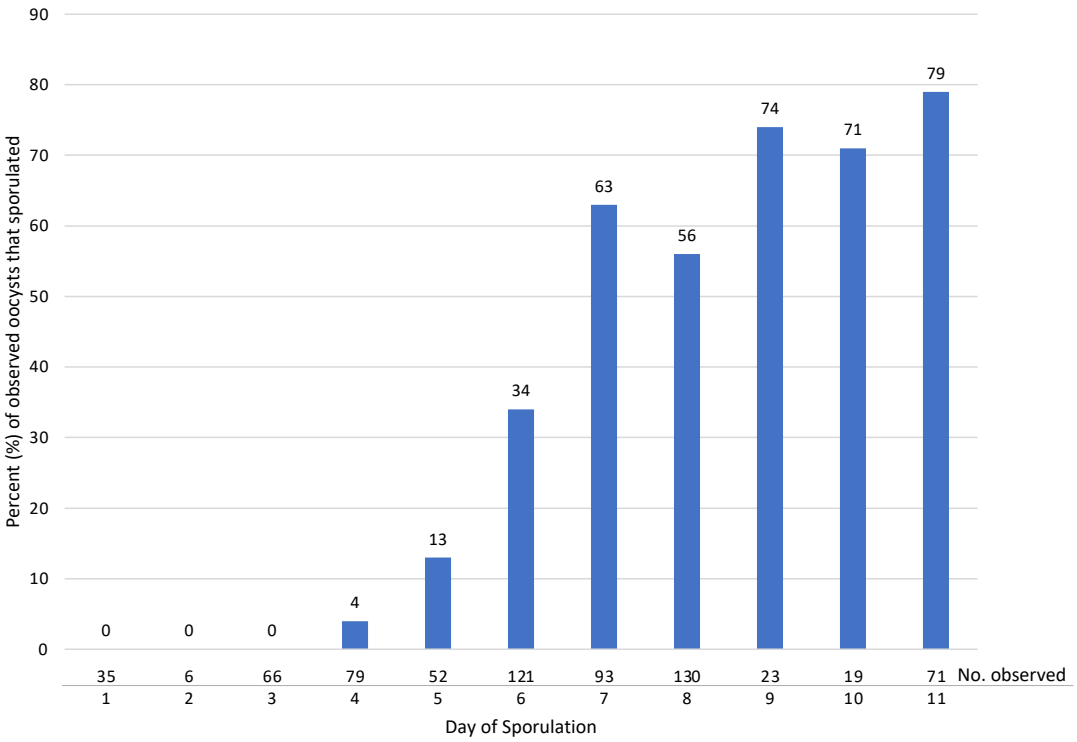


Figure 6.1. Sporulation success of kiwi (*Apteryx mantelli*) coccidia (*Eimeria* spp.) oocysts.

6.3.3. Discussion

The sporulation time for a given species of coccidia is frequently reported as a length of time (hours or days) or range of time (e.g., Al-Habsi et al. 2017; Waldenstedt et al. 2001; Yang et al. 2016a; Yang et al. 2016d). When the percent of oocysts that have sporulated is reported with the sporulation time, usually the time at which 90% of oocysts have successfully sporulated is reported (Allen and Fetterer 2002; Hammond et al. 1973; Lee and Dorney 1971; Prowse 1991; Ryan 2002; Ryley and Robinson 1976). With only about an 80% sporulation rate after 10 days, samples collected from kiwi should be allowed to sporulate longer than the ten days suggested by Morgan (2013).

Experimentally determining the best conditions for sporulation would help ensure all morphospecies were properly identified and characterised. Kiwi *Eimeria* spp. sporulation conditions may require specific levels of humidity, light, temperature, salinity, or other chemical cues. For example, similar problems with reliable sporulation of penguin (*Pygoscelis* spp.) coccidia have also been reported (Golemansky 2011). Interestingly, penguin (*Spheniscus demersus*) coccidia may sporulate more reliably in saltwater than potassium dichromate (M J Yabsley, pers. comm.). Without further optimisation, the potential to preferentially sporulate some species of *Eimeria* in kiwi will persist. As kiwi are nocturnal and live in dark, damp, temperate forests, these *Eimeria* spp. may prefer higher humidity and lower temperatures and light than other coccidia (Castro and Morris 2011; Heather and Robertson 2015).

On the other hand, from a diagnostic point of view, sporulation of coccidia to morphologically identify them is time intensive and not feasible for ONE movements of kiwi (10-15 days; see Chapters 3-4, Duszynski and Wilber 1997; Morgan 2013). The conditions of transportation of kiwi faecal samples varies based on the location, time of year, and submitter; potentially leading to conditions that kiwi *Eimeria* oocysts cannot withstand. These conditions would be difficult to alter for routine diagnostic testing; thus, accommodations need to be made in other ways. While morphological identification relies heavily on sporulation, identification using genetic markers does not. Despite sporulated oocysts containing more copies of DNA (Lim et al. 2012; Yang et al. 2014c), genetic differentiation is a much more reliable method than microscopic identification. However, the main barrier to PCR-based methods is the tough oocyst wall that thins during sporulation to allow for the release of sporocysts after ingestion by a host. Thus, this research focused instead on troubleshooting extracting DNA from unsporulated oocysts and amplifying markers for reliable differentiation.

Part 3. Molecular detection troubleshooting

6.4. Introduction

The first molecular characterisation of coccidia in kiwi was conducted by Morgan et al. (2013), used the 18S rRNA gene to confirm the genus of kiwi coccidia to be *Eimeria* and explored the use of the ITS-1 and -2 regions for speciation. DNA was obtained using sporulated oocysts and extracted using a micropestle for mechanical disruption followed by a standard column extraction protocol.

For this thesis, conventional PCR optimisation was undertaken prior to widespread sample collection after the initial planning stages of this project began in September 2017. As the overall goal of continuing genetic characterisation was to build on the genetic work by Morgan et al. (2013), this research began by recreating the DNA extraction, and 18S rDNA and ITS-2 PCR protocols.

6.2.1 Methods and Results for the 18S and ITS-2 regions

A brown kiwi (MB51) faecal sample from Pūkaha National Wildlife Centre that was previously processed and sporulated by Kerri Morgan was used for these initial comparisons. Figure 6.2 illustrates the variable results targeting the ITS-2 using the primers designed by Woods et al. (2000b) and contamination associated with reusable micropestles to break open oocysts prior to extraction. After replacing the extraction water, a second DNA extraction and ITS-2 PCR was completed, resulting in a very weak positive in the sample (Figure 6.3). Further, while the extraction negative control was successfully negative, contamination was observed in the PCR water control. The frustration at these results combined with the knowledge of Dr Morgan's similar issues (Morgan 2013) and the theory that other gene targets could prove more useful, led the project to move forward to the 18S rDNA protocol (Jenkins et al. 2006) and testing of the Zymo Research (ZR) Quick DNA Fecal/Soil miniprep kit in October 2017. Figure 6.4 illustrates the 18S rDNA amplification results (primers *Eimeria* 18SF/R, Appendix C.7) of a pooled brown kiwi faecal sample (MB01) extraction using a different extraction protocol and a positive and extraction negative control using the Morgan et al. (2013) PCR protocol. These results supported the contamination involved the use of the micropestle to extract DNA from kiwi coccidia. However, as 18S rRNA genes are present

in all eukaryotic cells, contamination may have also been attributed an increased likelihood of detecting environmental sources of 18S rDNA (Hadziavdic et al. 2014; Hall and Beiko 2018). Therefore, priority shifted to SGS targeting the COI gene, which is more specific to *Eimeria* and, therefore, less likely to be detected from the laboratory environment as well as provide more useful phylogenetic comparisons. Accordingly, time and resources were then focused on sample processing and development of the COI protocols.

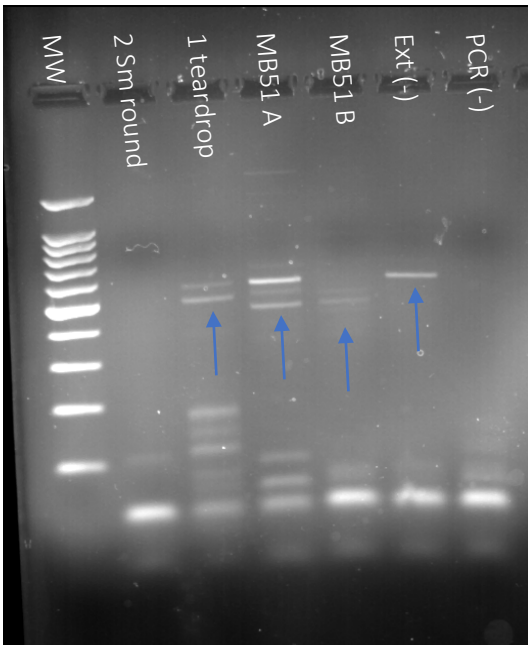


Figure 6.2. Gel electrophoresis results targeting the ITS-2 region of kiwi coccidia from brown kiwi (*A. mantelli*). The "MW" is the molecular weight marker: a 100 bp ladder. The first two samples are isolated oocysts that could not be reliably differentiated at the magnification used. All samples are from an environmental sample collected at Pūkaha National Wildlife Centre in 2012. The presence of bands (e.g., arrows) at or above ~400 bp indicates amplification.

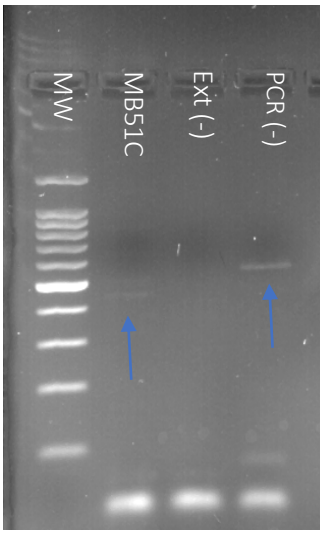


Figure 6.3. Gel electrophoresis results targeting the ITS-2 region of kiwi coccidia from brown kiwi (*A. mantelli*). The "MW" is the molecular weight marker: a 100 bp ladder. The sample, "MB51C", is a mixed species environmental sample collected at Pūkaha National Wildlife Centre in 2012. The presence of a band(s) (e.g., arrows) indicates amplification.

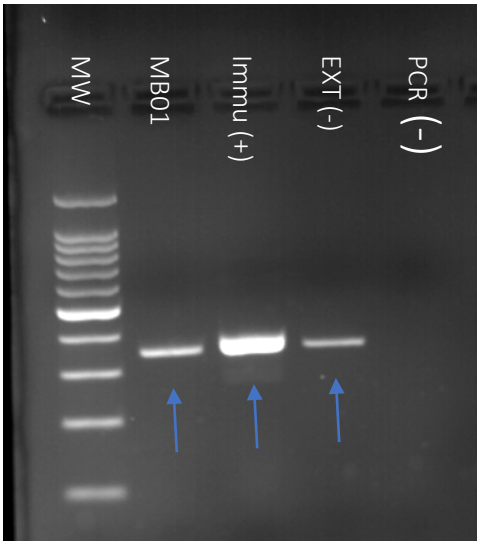


Figure 6.4. Gel electrophoresis results targeting the 18S rDNA region of kiwi coccidia from brown kiwi (*A. mantelli*). The "MW" is the molecular weight marker: a 100 bp ladder. The sample, MB01 is a pooled, cleaned, sporulated environmental faecal sample from Pūkaha National Wildlife Centre. The presence of a band(s) (e.g., arrows) indicates amplification.

6.4.2. Methods and Results for Mitochondrial COI gene

Samples used for this troubleshooting were extracted using the published, standard ZR Quick DNA Fecal/Soil miniprep kit protocol (Appendix C.6).

Initial DNA extraction from about 3,000 oocysts with the published ZR protocol (Appendix C.6) of a mixed species from Pūkaha National Wildlife Centre (MB) and the Immucox® Breeders and Layers (Pacificvet, Christchurch, New Zealand) yielded 18.4 ng/μl and 1.05 ng/μl, respectively. The COIF2/R2 PCR protocol detailed in Chapters 3 and 4 for COI amplification (Appendix C.8; Ogedengbe et al. 2011a; Yang et al. 2013) successfully amplified the vaccine, but not the kiwi sample (Table 6.2). In order to rule out if there was something wrong with that sample (for example, too much inhibition from too much non-target DNA), 10 single species or mixed species dropping samples (Pūkaha National Wildlife Centre: n = 3 single oocyst; n = 2 mixed species; The National Kiwi Hatchery: n = 1 mixed species; Cape Sanctuary: n = 3 single oocysts; Immucox vaccine: n = 1 mixed species) were DNA extracted using the published ZR fecal protocol.

The COIF2/R2 protocol amplified only the “RS01mix1” sample (Figure 6.5), but not the positive control, “Immu (+)”, which was the same sample used in the first COI test or any other kiwi coccidia sample, leading to the hypothesis that this COI protocol using the COPIF2/R2 is too unpredictable/ insensitive for kiwi coccidia.

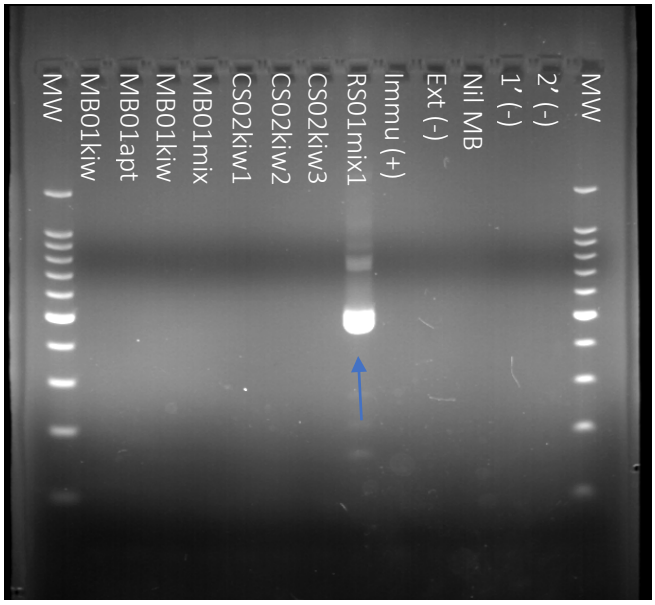


Figure 6.5. COI results of the second test run using extractions from single and mixed kiwi coccidia. The presence of a band (arrows) indicates amplification.

In response, the alternative protocol targeting a longer (~720 bp) COI target using the outer primers Cocci_COI_For/ COX tenella R2 and inner primers COIF2/COX tenella R2 was tested (Appendix F.1; Dolnik et al. 2009; Ogedengbe et al. 2011b; Yang et al. 2013). Similar issues of insensitivity/inconsistency persisted with extensive troubleshooting and optimisation required. The goal of pursuing NGS required the shorter COI target to be the focus, as 400-500 bp is the current limit of the commercially available, cost-effective, Illumina amplicon technology. Table 6.2 summarises the results of each step of the troubleshooting and optimisation process from July 2017 to November 2018 with notes to indicate the reasoning for the next steps and/or issues to be addressed. These steps include the process to adapt the short COI amplicon to the Illumina library preparation process (see Chapter 1.5.2.2). The table ends when the main focus of the

optimisation shifted more towards the extraction process than the PCR optimisation, though these optimisations were strongly intertwined. The samples that had been successfully amplified using the Illumina_COIF2/R2 were used for a test NGS run that moved the project towards a completion.

Table 6.2. Optimisation and troubleshooting of PCR protocols targeting the mitochondrial COI gene of kiwi coccidia. These processes reflect the overarching goals of this project and illustrate the long process required to adapt a conventional amplification and next generation sequencing to novel apicomplexans in hosts of high conservation value. DNA was extracted using the ZR Quick DNA Fecal/Soil miniprep published protocol unless otherwise noted. The 445 bp and 725 bp COI targets followed the protocols described by Yang et al. (2013) and Yang et al. (2016a), respectively (Appendices C.8 and F.1). COI targets that are approximately 485 bp long reflects the addition of the Illumina adaptors. Where a nested protocol is used, modifications to the primary PCR are noted with (1') and modification to the secondary/nested PCR are noted with (2').

Date	Target bp	Note	ext (-)	1' PCR (-)	2' PCR (-)	Immu (+)	MB01	MB01oo1	MB01oo2	MB01oo3	MB01oo4	CS02oo1	CS02oo2	CS02oo3	RS01mix1	CS14 (9oo)	CS14 (18oo)	CS14 (45oo)	CS14 (90oo)	CS14 (450oo)	CS14 (900oo)	CS14 (3600)	CS14 (3600) 2.5ul	MB51-2012C
7/12/17	445		-	-	-	++	-																	
15/12/17	445		-	-	-	-	-	-	-	-	-	-	-	-	++									
19/12/17	725	25 ul rxn	-	-	-	++	-	-	-	-	-	-	-	-	++									
23/2/18	725	50 ul rxn	-	-	-	++		-																
23/2/18	725	1ul, 2.5ul, and 5 ul	-	-	-	++		-							++									
10/4/18	800 1'	Dilution series	-	-		+									++	-	-	-	-	-	-	++		
11/4/18	725	Does adding 2' help? - no	-	-	-	++									++	-	-	-	-	-	-			
12/4/18	725	Does adding 1' DNA help?	-	-	-										+	-	-	-	-	-	-			
13/4/18	725	Varied amount of 1' used	-	-	-	++									++							+	++	
17/4/18	445		-	-	-										++							++		
16/5/18	445	Freeze/boil x1	-	-	-	++										-	-	-	++	++				
1/7/18	445	Freeze/boil x3	-	-	-	++										-	-	-	-	-				
4/7/18	485	Illumina	-	-	-	++										-		-	-	-				
1/8/18	485	Lower primer concentration	-	-	-	++	-								++									-
5/8/18	485	Lower primer concentration	-	-	-	++													+	+				
16/8/2018	445	Lower primer concentration	-	-	-	++										-	-	-	-	-				

6.4.3. Optimization of DNA extraction protocols

Each sample used in the PCR troubleshooting described thus far involved samples with known quantities of oocysts, i.e., all negative amplification results were false negatives. Thus, as the PCR optimisation and troubleshooting continually yielded unpredictable results, the focus shifted to include steps to improve the extraction protocol.

Extraction of DNA relies on the lysis of the cells containing the target DNA. In the case of coccidia samples retrieved from droppings, the target DNA is encapsulated in a robust wall that is intended to withstand harsh environmental conditions (Bangoura and Daugschies 2018; Seemann et al. 2012; Stenzel et al. 2019). This wall is unlike the membranes of other cell types (e.g., intracellular coccidial stages, blood, muscle, brain) that require minor mechanical disruption (e.g., cutting into small pieces and/or rapid mixing) combined with incubation in a mixture of lysis buffer and proteases (Hall and Beiko 2018; Polley et al. 2010). The development that occurs during sporulation, weakens the walls, allowing for disruption in a bird host's crop or gizzard (Blake and Tomley 2014). Coccidial DNA extraction method optimisations usually address sporulated, rather than unsporulated oocysts. For example, Tang et al. (2018) demonstrated the use of a sodium hypochlorite incubation to break the sporulated oocyst walls of *Eimeria tenella* and *Eimeria intestinalis*. Extracting DNA from unsporulated oocysts tends to be a lengthy ordeal, with low evidence of success. For example, Stenzel et al. (2019) attempted to extract DNA from capercaillie (*Tetrao urogallus*) and black grouse (*Tetrao tetrix*) dropping samples (n = 49) that were microscopically positive for *Eimeria*, but only 8 samples tested positive using PCR. Similar issues have been reported in other studies of coccidia from undomesticated

hosts (Honma et al. 2011a). Stenzel et al. (2019) attributed this poor amplification rate to possible difficultly in DNA extraction, with most samples remaining unsporulated, despite following standard sporulation guidelines. They used an extraction protocol with additional steps prior to extraction with a standard DNeasy mini kit (Qiagen, Germany). First the oocysts were rinsed in 0.9% sodium chloride and then transferred to lysis buffer. Steel beads were then added to this mixture, which was then homogenised in a mechanical tissue disrupter. After incubation on ice, this disruption was performed again prior to extraction with the Qiagen kit.

6.4.3.2. Extraction methods/results

6.4.3.2.1. Extraction kit

The ZR Quick DNA Fecal/Soil miniprep (Zymo Research Corporation, Irvine, CA) used for this project uses mechanical disruption rather than the more commonly used chemical disruption in standard DNA extraction. The mechanical disruption employs silicon beads in single use tubes that are rapidly shaken in a bead beater/tissue lysis chamber where the beads break open cell walls, allowing for the lysis buffer to further lyse the cells in later steps. This kit was selected for this project because it is validated for *Cryptococcus neoformans*, a yeast with extremely thick capsule-like wall that is very difficult to lyse (Bolano et al. 2001) and has been shown to be the best bacterial manual DNA extraction option compared to other faecal DNA extraction kits (Claassen et al. 2013). This kit has also been shown to be effective on *Cryptosporidium parvum*, an apicomplexan closely related to *Eimeria* and other coccidia. Unfortunately, this species of *Cryptosporidium* leave hosts as sporulated oocysts, whereas *Eimeria* oocysts sporulate after excretion

(Daugschies and Najdrowski 2005; Lendner and Daugschies 2014; Mehlhorn 2016; Walker et al. 2013; Yabsley 2008). As optimisation of extraction methods using this kit was from sporulated eimeriid oocysts (e.g., Tang et al. 2018), further validation was required to confirm whether mechanical disruption alone is sufficient to break open unsporulated oocysts.

6.4.3.2.2. Additions to the ZR protocol

As illustrated in Table 6.2, adjusting the PCR protocol and conditions brought few improvements in amplification success. The first main addition to the ZR published protocol was the addition of 3 cycles of freeze in liquid nitrogen (LIN) and then transfer to 100°C for 5min each prior to mechanical disruption. Next, incubation at 56°C with proteinase K overnight after the freeze/boil and prior to mechanical disruption was added. After this, however, a trend emerged that led to the hypothesis that perhaps the main difference between success and failure is the presence of enough sporulated oocysts for detection. Thus, in Table 6.2, samples shift to compare sporulated and unsporulated samples (see the "*"). While amplification success increased, results were still unpredictable, especially with the inclusion of the Illumina adaptor.

6.4.3.2.3. Bovine serum albumin

As of the last PCR included in Table 6.3, a series of troubleshooting experiments focusing on the PCR was conducted due to an inability to determine the cause of certain samples amplifying regularly and others not under the same conditions. The main samples that highlighted this issue were MB15 and MB16. These samples were collected on the same

day from adjacent enclosures at Pūkaha National Wildlife Centre and each contained over 2 million oocysts per gram. Interestingly, however, MB15 amplified using the primers with Illumina adaptors whereas MB16 did not. A series of optimisations included varying the $MgCl_2$ concentrations, DNA concentrations, and annealing temperatures in many combinations. In the end, lowering the $MgCl_2$, decreasing the annealing temperature, and, crucially, adding 10% bovine serum albumin (BSA) led to successful amplification of both MB15 and MB16. BSA has been shown to greatly decrease the effects of inhibitors in PCR (Kreader 1996), indicated that some of the dropping samples may contain more inhibitors than others. Thus, BSA was incorporated into PCR protocol for the COI primers with Illumina adaptors.

6.4.3.2.4 Final optimisation and sample selection for Illumina sequencing

After incorporating BSA and optimising the PCR amplification protocol, it was hypothesised that DNA from sporulated oocysts was far easier to extract than from unsporulated oocysts in fresh faecal samples. However, unsporulated oocysts from samples that had been aliquoted and set aside for long-term storage in the -80°C freezer were also more easily amplified. Therefore, samples that were stored in the -80°C freezer with higher oocyst counts were prioritised. Unfortunately, the majority of samples had extremely low OPG values and were unsuitable for extraction without extensive cleaning, concentration, and additional screening.

Table 6.3. DNA extraction optimisation from *Apteryx* spp. *Eimeria.* All extractions here (*) and later include 3 cycles of 5 min in liquid nitrogen to 100°C, then incubation at 56°C with proteinase k overnight, followed by 3 cycles of mechanical disruption at top speed for 3 minutes.

Date	Target bp	Note	ext (-)	1' PCR (-)	2' PCR (-)	Immu (+)	MB01	MB15	CS17	WB02	RS10	RS15	WS02	WS04	CS14	RS18 spor	RS18 un	MB1 spor	MB15 un	MB16 spor	MB16 un	WP01 spor	WP01 un	RSB06 un	HA01	HA14	HA16	HA27	RSB06	MB12	WB09	HA24
13/11/18	445	Freeze/boil (FB)	-	-	-	+ +	-	-	-	-	-	-	-	-																		
13/11/18	485	FB	-	-	-	+ +	-	-	-	-	-	-	-	-																		
15/11/18	485	FB, Increase DNA	-	-	-	+ +	-	-	-	-	-	-	-	-																		
15/11/18	485	FB, lower primer conc	-	-	-	+ +	-	-	-	-	-	-	-	-																		
15/11/18	485	FB, lower primer conc, increase DNA	-	-	-	+ +	-	-	-	-	-	-	-	-																		
13/12/18	445	FB, proteinase K, disruption	-	-	-	+ +							-		-																	
13/12/18	445	FB, disruption, Proteinase K,	-	-	-	+ +							-		-																	
***18/1/19**	445	Sporulated versus unsporulated	-	-	-	+ +										+ +	+	+ +	-	+	-												
21/1/19	485	For NGS	-	-	-	+ +										+ +	-	+ +		-													
1/2/19	445	Spor/unspor	-	-	-	+ +														+		+	-	+ +								
4/2/19	485	For NGS	-	-	-	+ +														-		-		-								
BSA/Annealing temp/primer concentration optimisation for primers with Illumina adaptors																																
15/3/19	485	Check using BSA, etc	-	-	-	+ +							-							+		+										
9/4/19	445/ 485	-80°C	-	-	-	+ +			+ +																+	+ +	+	+ +	+ +	+ +	+	+ +

6.4.4. Discussion

While the summation of all these steps sufficiently broke open the oocyst walls, there was evidence that a -80°C incubation step prior to mechanical disruption could be effective and remains to be further explored and refined. Such a single incubation step prior to extraction would allow for more timely diagnostics and needs to be tested in combination with the other individual treatments. The success of DNA extraction yielding amplifiable DNA was demonstrated by the success of the PCR rather than just quantification of DNA. The ZR Quick DNA Fecal/Soil miniprep kit was chosen for its validation on *Cryptococcus neoformans* and efficacy on *Cryptosporidium parvum*; however, other tests should be considered for use. Automated/magnetic bead extraction techniques in combination with one or more steps discussed here could remove inhibitors that prevents detection of coccidial DNA (Claassen et al. 2013). The use of a sodium hypochlorite incubation, which has been shown to be useful for sporulated *Eimeria* oocysts from chickens, may be a reliable replacement for incubation at -80°C (Tang et al. 2018). This chemical disruption method requires less expensive equipment than necessary for the maintenance of a -80°C freezer.

6.5. PCR protocol development

6.5.1. Introduction

The long-term goal of this thesis is to provide kiwi practitioners with tools that help them make sound management decisions. This chapter communicates the first attempts to develop such a tool on a real-time PCR platform using the NGS results (Chapter 5). Real-time polymerase chain reaction (see Chapter 1.4.2.) has the potential as a rapid

diagnostic test that could differentiate between species of kiwi coccidia. The purpose of this section is to provide a proof of concept for using NGS generated sequences to design primers, troubleshoot a real-time PCR protocol, and produce reliable results. Achieving sufficient amplification and differentiation of specific OTUs demonstrates the ability to use the results generated by Illumina sequencing to answer questions about coccidia in kiwi beyond presence/absence.

6.5.2. Methods and Results

6.5.2.1. Sample selection

Five kiwi coccidia DNA extractions were chosen from the samples used for Illumina amplicon sequencing (Chapter 5). Four samples containing mostly a single sequence and one sample that contained three of the four were selected (Table 6.4). See Chapter 5, Table 5.1 for more in-depth information about the metadata associated with each sample.

Table 6.4. Most abundant OTU detected in each sample used to design primers and troubleshoot a real-time PCR protocol.

Sample Id	s__bb	s__ab	s__ad	s__aa
CS01	x			
HA09				x
MB06			x	
RSB06		x		
MB15B		x	x	x

6.6.2.2. Primer design

Sequences s__bb, s__ab, s__ad, and s__aa were aligned in Geneious v11.0.5 (Biomatters, Auckland, New Zealand) and two conserved areas were chosen based on GC content, length of conserved region, and length of target fragment. One reverse primer, CokerR3, was chosen to be paired with COIF2, and one forward primer, Coker F2, for pairing with COIR2 (Table 6.5; Yang et al. 2013). Locations of the primers in the 484 bp COI fragment is illustrated in Figure 6.6.

Table 6.5. Primers selected for qPCR diagnostic testing based on 97% identity of a 484 bp region of the mitochondrial COI gene.

Primer Name	F/R	Source	Sequence	Target (bp)
COIF2	F	Yang et al. (2013)	5' TAAGTACATCCCTAATGTC 3'	127
CokerR3	R	Chapter 5	5' ACACCDAGWACRGCAATTGT 3'	
CokerF2	F	Chapter 5	5' AYGATGCYTCYTTTAATGGTGA 3'	220
COIR2	R	Yang et al. (2013)	5' GTCATCATATGRTGTGCCCA 3'	

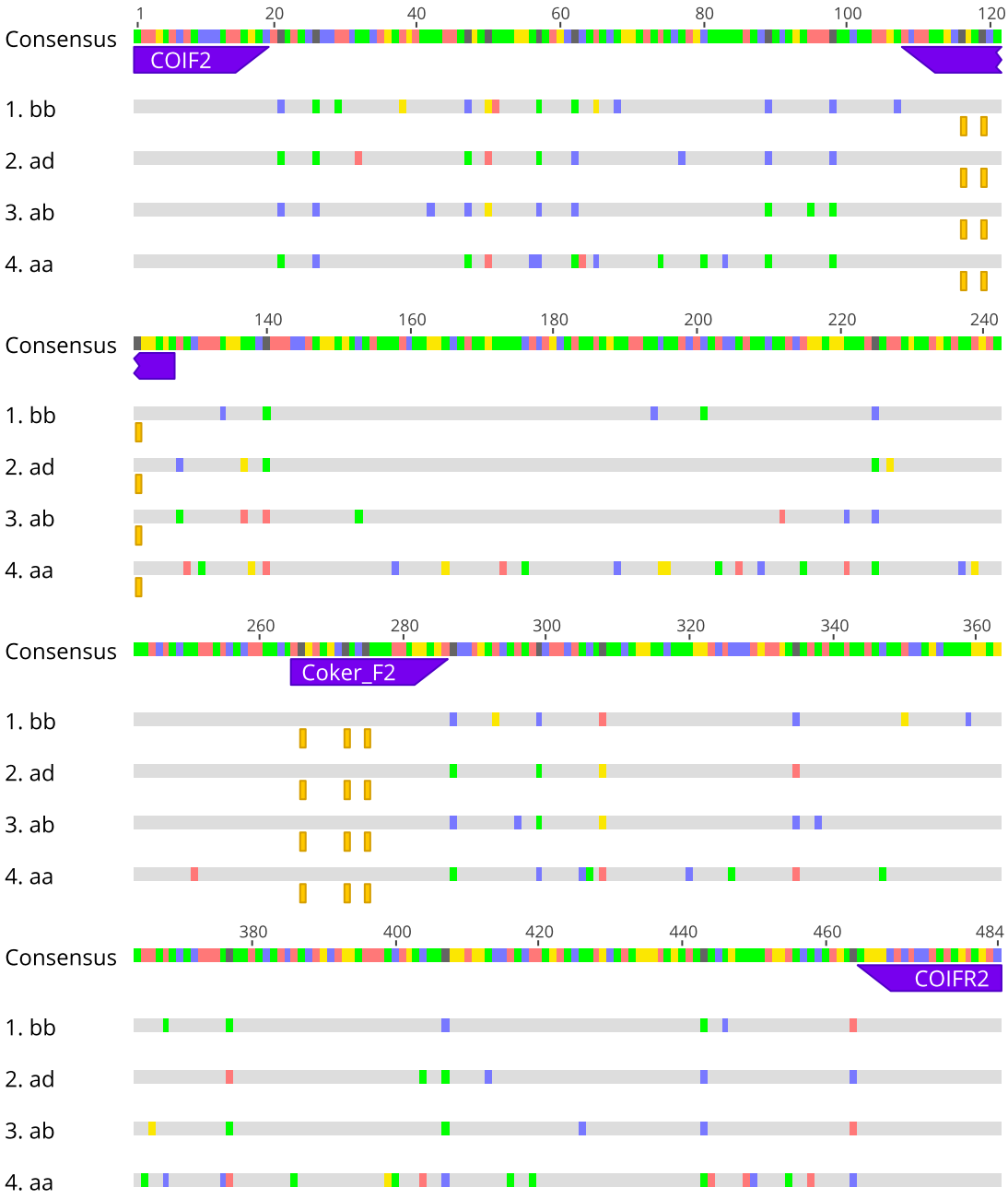


Figure 6.6. Consensus alignment of the four most abundant OTUs clustered at 95% generated by Illumina amplicon sequencing targeting the mitochondrial COI gene of *Eimeria* spp. in kiwi (*Apteryx* spp.). Grey areas indicate nucleotides that match the consensus sequence. Coloured areas indicated nucleotide that do not match the consensus sequence. Purple annotations indicate the locations of two primer pairs selected for developing real-time PCR protocols. The yellow bars under the sequences within the range of the primer bind sites are locations of ambiguous bases.

6.6.2.3. Conventional PCR and Sanger sequencing

Prior to troubleshooting on RT-PCR, a conventional PCR using these primer sets was performed to determine if one primer set would be easier to optimise. The protocol was adapted from Yang et al. (2013) and used a 50 μl reaction with 1 $\times$ PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 μM of each primer (Table 6.5), 2 U Platinum *Taq* (Invitrogen, ThermoFisher Scientific, Waltham, MA, USA). The PCR conditions had an initial denaturation at 94°C for 2 min; 35 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec; and a final extension at 72°C for 10 min.

6.6.2.3.1. Controls

Immucox® Breeders and Layers (Pacificvet, Christchurch, New Zealand) live vaccine (sample IMMU) that contains *Eimeria acervulina, E.maxima, E. necatrix,* and *E. tenella* was used as a non-target DNA control for the PCR reactions. Sterile water controls were included as negative controls for the extraction process and PCR reactions.

6.6.2.3.2. Conventional PCR results

Amplicons were run on a 1.5% agarose gel (Figure 6.7) containing Invitrogen UltraPure Agarose (ThermoFisher Scientific, Waltham, MA, USA) and visualised with RedSafe Nucleic Acid Staining Solution (iNtRON Biotechnology, Gyeonggi-do, South Korea).

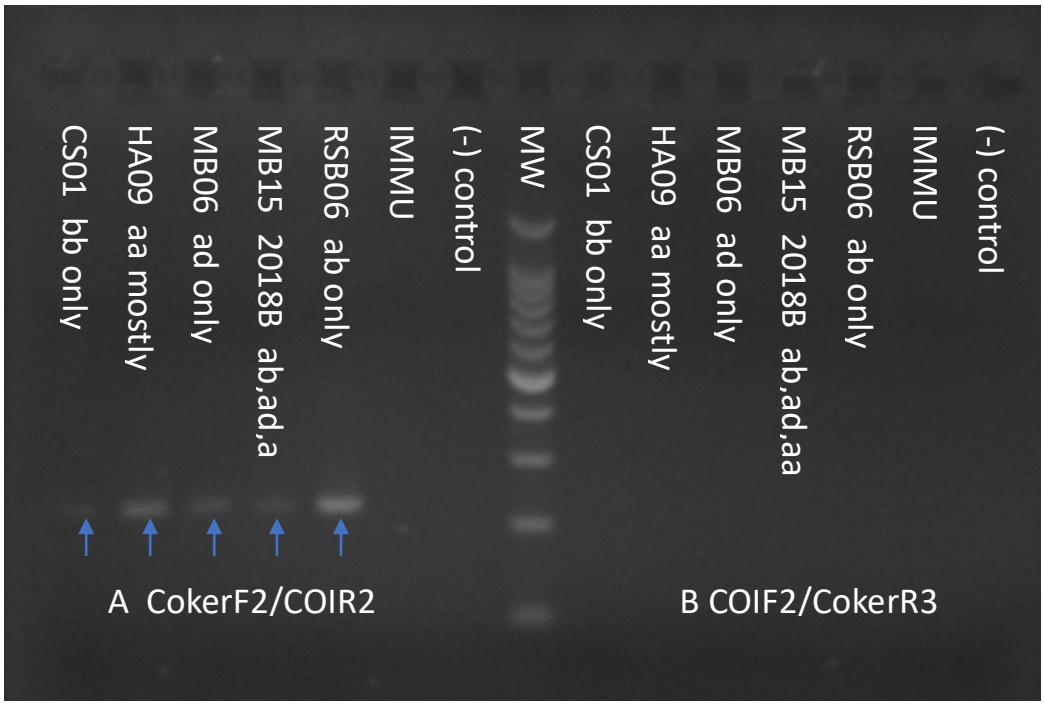


Figure 6.7. Gel electrophoresis image of the amplification results of two primers sets targeting the COI gene of kiwi (*Apteryx* spp.) *Eimeria* spp. These primers were based on Illumina amplicon results from a large region. The presence of a band (arrows) indicates amplification.

6.6.2.3.3. Sanger sequencing

As only the CokerF2/COIR2 primer combination successfully amplified the kiwi coccidia samples and did not appear to amplify the non-target Immucox live vaccine DNA, the amplicons were purified using the PureLink PCR Purification Kit (Invitrogen, ThermoFisher Scientific, Waltham, MA, USA) and submitted to the Massey Genome Service (Massey University, Palmerston North, NZ) for Sanger sequencing (Appendix C.9).

Figure 6.8 illustrates the results of the Sanger sequencing. The COIR2 matched all the sequences; the newly designed forward primer matched all but one nucleotide in MB06 in the middle of the primer. Each kiwi sample is ordered and bracketed with the main

OTU it should contain. CS01 and s__bb shared 100% homology; HA09 and s__aa shared 87% homology; MB06 and s__ad shared 86% homology; and RSB06 and s__ab shared 93% homology. Mb15B shared 92%, 86%, 95%, and 94% with s__bb, s__aa, s__ad, and s__ab, respectively.

Figure 6.8. A consensus alignment of *Eimeria* spp. sequences obtained from kiwi droppings. The consensus was generated using ClustalW in Geneious, v11.0. Sequences 3, 5, 7, 9, and 11 were sequenced used Sanger sequencing. Sequences 4, 6, 8, and 10 are the top four most abundant OTUs generated by Illumina amplicon sequencing. Each bracket indicates which OTU comprised the majority of that kiwi dropping sample in the NGS sequencing. MB15 mainly comprised of s__aa, s__ab, and s__ad. Dots indicate nucleotides that match the consensus sequence. Coloured areas indicated nucleotide that do not match the consensus sequence, and ones in grey indicate ambiguous base calls. Purple annotations indicate the locations of two primer pairs selected for developing real-time PCR protocols.

6.6.2.4. Real-Time PCR

Three main options exist for diagnostic test development. First, conventional PCR, while generally easily accessible to most labs, would require either differentiation based on amplicon size or through sequencing. Considering the size (484 bp) of the COI amplicon analysed in this thesis and the number of OTUs detected (Chapter 5), it would be difficult to reliably identify and differentiate OTUs, especially in the presence of mixed species infections. Alternatively, sending the amplicons for sequencing would slow the process considerably. The next option, loop-mediated isothermal amplification (LAMP) assays, provide a very cost-effective option that does not require a molecular lab with a thermocycler. However, this method requires three sets of primers, with each set requiring troubleshooting and optimising. Thus, while this method has immense potential in future applications, it is not ideal for a basic proof of concept.

Real-time PCR (RT-PCR) uses a single primer set of either additional, sequence specific, fluorescent primers (i.e., probe) or non-sequence specific, fluorescent dyes (e.g., SYBR Green) that bind to double stranded DNA. The use of probes for quantitative RT-PCR (qPCR) would allow for the detection of oocyst load as well as enabling the differentiation of up to four OTUs in a single reaction. Unfortunately, probes are very expensive and add an additional level of optimisation (Gašparič et al. 2010; Papin et al. 2004). Intercalating DNA fluorescent dyes bind to DNA without being sequence specific; as the amount of DNA increases, the stronger the fluorescent signal (Giglio et al. 2003; Ririe et al. 1997; Wittwer et al. 1997). This non-specificity can be overcome to a certain extent by detecting the melting temperature (T_m) of the amplified targets with a high-resolution melt approach (Giglio et al. 2003; Ririe et al. 1997; Wittwer et al. 1997). The

T_m changes with the GC content of each sequence often providing enough temperature differences in the target sequences that the OTUs could be differentiated based on the melting temperatures. Thus, at this time, RT-PCR using an intercalating dye was chosen as the ideal option to provide a proof of concept for the development of a rapid diagnostic test.

6.6.2.4.1. SYBR Green

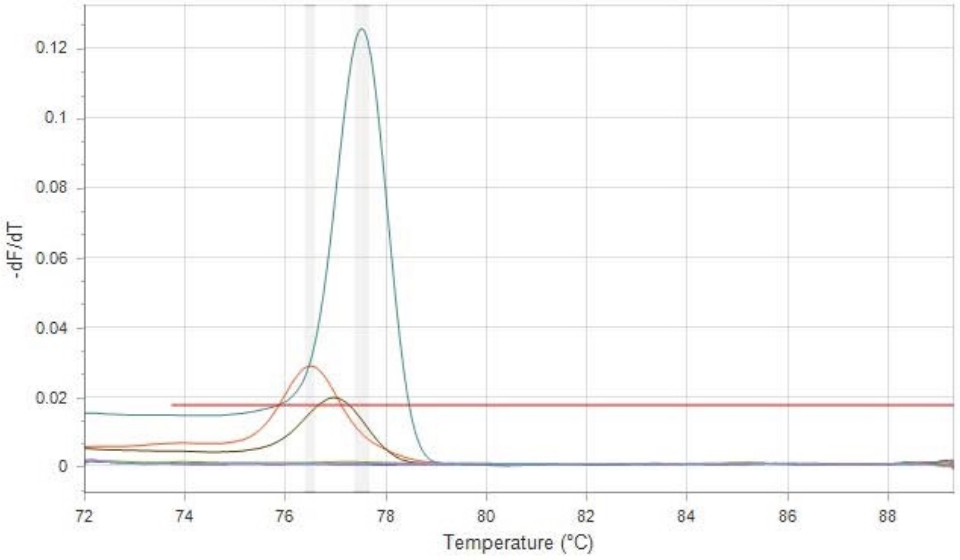
The most readily available and versatile dye, SYBR Green, is double-stranded (ds) DNA specific and cost effective (Gašparič et al. 2010; Papin et al. 2004; Ririe et al. 1997; Wittwer et al. 1997). For this proof of concept, the SYBR Green Universal Master Mix (Applied Biosystems, Foster City, CA) was used with the protocol described in Table 6.6 with melting steps from 70.5°C to 90°C at 0.1°C/sec. Figure 6.9 illustrates the melting temperature output. All but one sample (RSB06, blue) had very low levels of amplification; however, there was potential for differentiation. Optimisation began with lowering the level of DNA in the reactions, followed by a series of annealing temperature and primer concentration adjustments. While each step of optimisation taken yielded consistent or better results, Figure 6.10 illustrates that even as amplification increased, the ability to definitively differentiate the four sequences was unlikely.

Table 6.6. RT-PCR diagnostic testing protocol and conditions to target the mitochondrial COI gene of *Eimeria* using newly designed primers. These primers were based on Illumina amplicon data and using SYBR Green fluorescent dye with a high-resolution melt.

Protocol		
Reactions	Final Conc.	Volume per reaction (μL)
2X qPCR SYBR mix	1X	10
Forward (10 μM stock)	250 nM	0.5
Reverse (10 μM stock)	250 nM	0.5
PCR grade water	-	7
Sample	-	2
RT-PCR conditions		
1 cycle:	Temp	Time
Initial denaturation	95°C	5 min
40 cycles:		
Denaturation	95°C	15 sec
Annealing	55°C	30 sec
Extension and Tm read	72°C	30 sec

Target	coker_coccidia_test 1 → New Target
Invert Data	No
Threshold	0.018 starting at 73.73°C

Genotype	Melt Range (°C)
Genotype mb06	76.48 ±0.10
Genotype rsb06	77.52 ±0.15

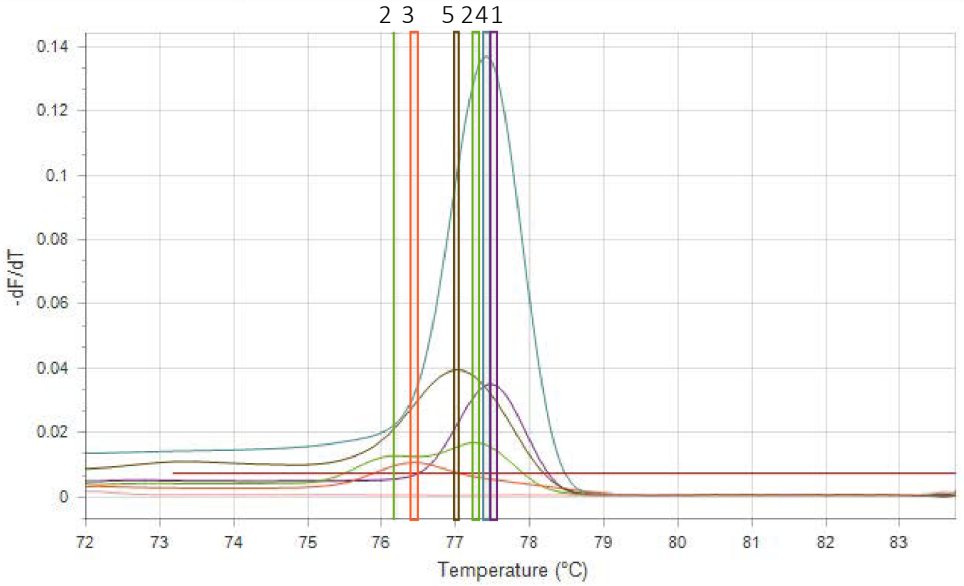


Well	Colour	Sample Name	Tm (°C)	Genotype
1		CS01		
2		HA09		
3		MB06	76.50	Genotype mb06
4		RSB06	77.51	Genotype rsb06
5		MB15 2018B	76.96	
6		IMMU		
7		neg		

Figure 6.9. Melting curve report from RT-PCR targeting *Eimeria* from kiwi (*Apteryx* spp.) using SYBR Green as the fluorescent dye prior to optimisation.

Target	coker_coccidia_test 1 → New Target
Invert Data	No
Threshold	0.007 starting at 73.18 °C

Genotype	Melt Range (°C)
RSB06	77.41 ± 0.04
CS01	77.50 ± 0.04
MB15	77.03 ± 0.04
HA09	77.27 ± 0.04 , 76.18 ± 0.04
MB06	76.43 ± 0.04



Well	Colour	Sample Name	Tm (°C)	Genotype
1		CS01	77.47	CS01
2		HA09	77.23	
3		MB06	76.44	MB06
4		RSB06	77.41	RSB06
5		MB15	73.37 , 77.03	MB15
6		neg		

Figure 6.10. Melting curve report from RT-PCR targeting *Eimeria* from kiwi (*Apteryx* spp.) using SYBR Green as the fluorescent dye after optimising.

6.6.2.4.2. SYTO9

Though still widely used, SYBR Green dyes have been shown to preferentially bind to certain GC rich products, can potentially inhibit PCR, and substantially affect the T_m of amplicons (Giglio et al. 2003; Gudnason et al. 2007; Monis et al. 2005). While SYTO9 is generally used as a gel electrophoresis dye, its effectiveness in RT-PCR, especially

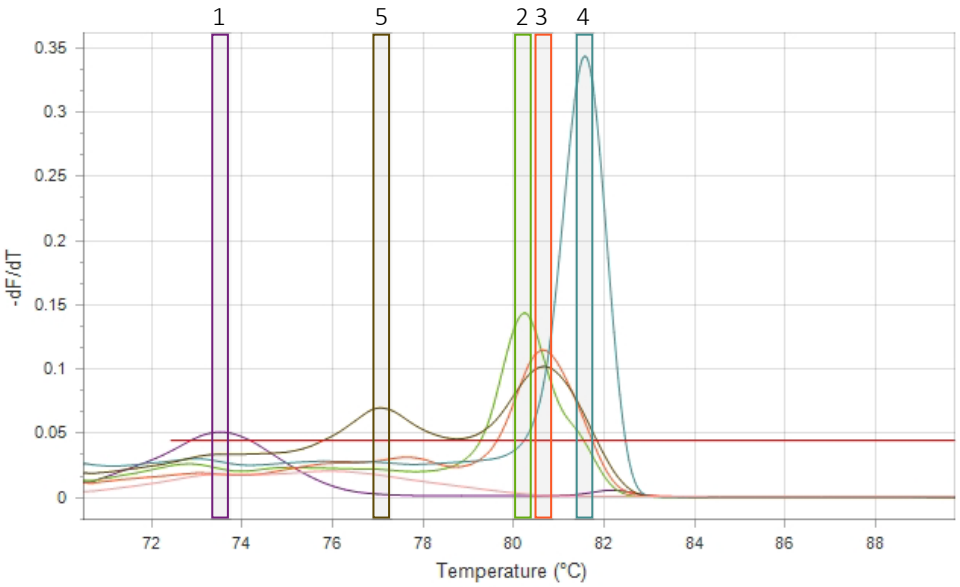
compared to SYBR Green, has been demonstrated for multiplexing and quantitative, diagnostic assays (Gašparič et al. 2010; Gudnason et al. 2007; Jansson et al. 2017; Li et al. 2020; Monis et al. 2005; Oscorbin et al. 2016). While the reason for the increased efficiency is not certain, it is likely due to the chemical structure of SYTO dyes and how they interact with the DNA (Eischeid 2011; Haugland et al. 1995; Monis et al. 2005). As these dyes are not primarily marketed for RT-PCR assays, optimised master mix kits are not commercially available. This study used the SYTO 9 Green Fluorescent Nucleic Acid Stain (Invitrogen, ThermoFisher Scientific, Waltham, MA, USA) with the protocol and conditions reported in Table 6.7 with melts from 70.5°C to 90°C at 0.1°C/sec. This protocol resulted in much more efficient amplification and five melting curves that are differentiable from each other (Figure 6.11). CS01, HA09, MB06, and RSB06 each primarily contained a single OTU (s__bb, s__aa, s__ad, and s__ab, respectively) based on the NGS data reported in Chapter 5. MB15 contained primarily an almost equivalent amount of s__aa, s__ad, and s__ab OTUs and had one peak that aligned with MB06/s__ad and one peak that did not match other samples.

Table 6.7 RT-PCR diagnostic testing protocol and conditions to target the mitochondrial COI gene of *Eimeria* using newly designed primers. These primers were based on Illumina amplicon data and using SYTO9 fluorescent dye with a high-resolution melt.

Protocol		
Reactions	Final Conc.	Volume per reaction (μL)
10XPCR buffer	1X	2
$MgCl_2$ (50 mM)	1.5 mM	0.6
dNTPs (10 mM)	0.4 mM	0.8
Forward (10 μM stock)	250 nM	0.8
Reverse (10 μM stock)	250 nM	0.8
Syto9	0.15 mM	0.6
PCR grade water	-	12.68
Taq	0.6 U	0.12
Sample	-	1.6
RT-PCR Conditions		
1 cycle:	Temp	Time
Initial denaturation	95°C	5 min
40 cycles:		
Denaturation	95°C	15 sec
Annealing	55°C	30 sec
Extension and Tm read	72°C	30 sec

Target	coker_coccidia_test 1 New Target
Invert Data	No
Threshold	0.045 starting at 72.43°C

Genotype	Melt Range (°C)
aa	80.22 ± 0.22
bb	73.53 ± 0.22
ad	80.66 ± 0.22
ab	81.56 ± 0.22
MB15	77.09 ± 0.22



Well	Colour	Sample Name	Tm (°C)	Genotype
1		CS01 1:10	73.52	bb
2		HA09 1:20	80.24	aa
3		MB06 1:2	80.67	ad
4		RSB06	81.57	ab
5		MB15	77.08, 80.68	ad, MB15
6		neg		

Figure 6.11. Melting curve report from RT-PCR targeting *Eimeria* from kiwi (*Apteryx* spp.) using SYTO9 as the fluorescent dye.

6.6.2. Discussion

The use of the NGS sequencing data to develop PCR primers, sequence the amplicons, and differentiate between OTUs based on melting curve provides a solid proof of concept for more in-depth analyses. For example, the test demonstrated here could be used on single oocysts, historical histological samples, and fresh tissue samples to

establish the pathogenicity and virulence of these genotypes. Additionally, sequences can begin to be connected with morphotype, allowing for the management decisions to be made based on virulence, rather than infection load alone. Figure 6.12 illustrates a generalisation of how real-time diagnostic protocols could be incorporated into a kiwi sampling protocol. The figure indicates parts of the protocol that require additional optimisation. First, after the collection of samples, storage (1) and postage (2) times vary from population or facility, depending on how the samples are used and the remoteness of the population. Unfortunately, little may be able to be done about this, except establishing the use of RT-PCR in more laboratories around New Zealand. While additional care may be taken in some circumstances to ensure the samples remain moist as close to ambient temperature as possible, this may be difficult to reliably achieve. Additionally, the ideal conditions of sporulation (3) need to be determined as well as whether or not the -80°C incubation (4) alone is enough for breaking open the oocyst walls or if the freeze/boil and incubation with proteinase k can be circumvented. The RT-PCR protocols (5) need further development, as the proof of concept presented here only addresses the four most abundant OTUs.

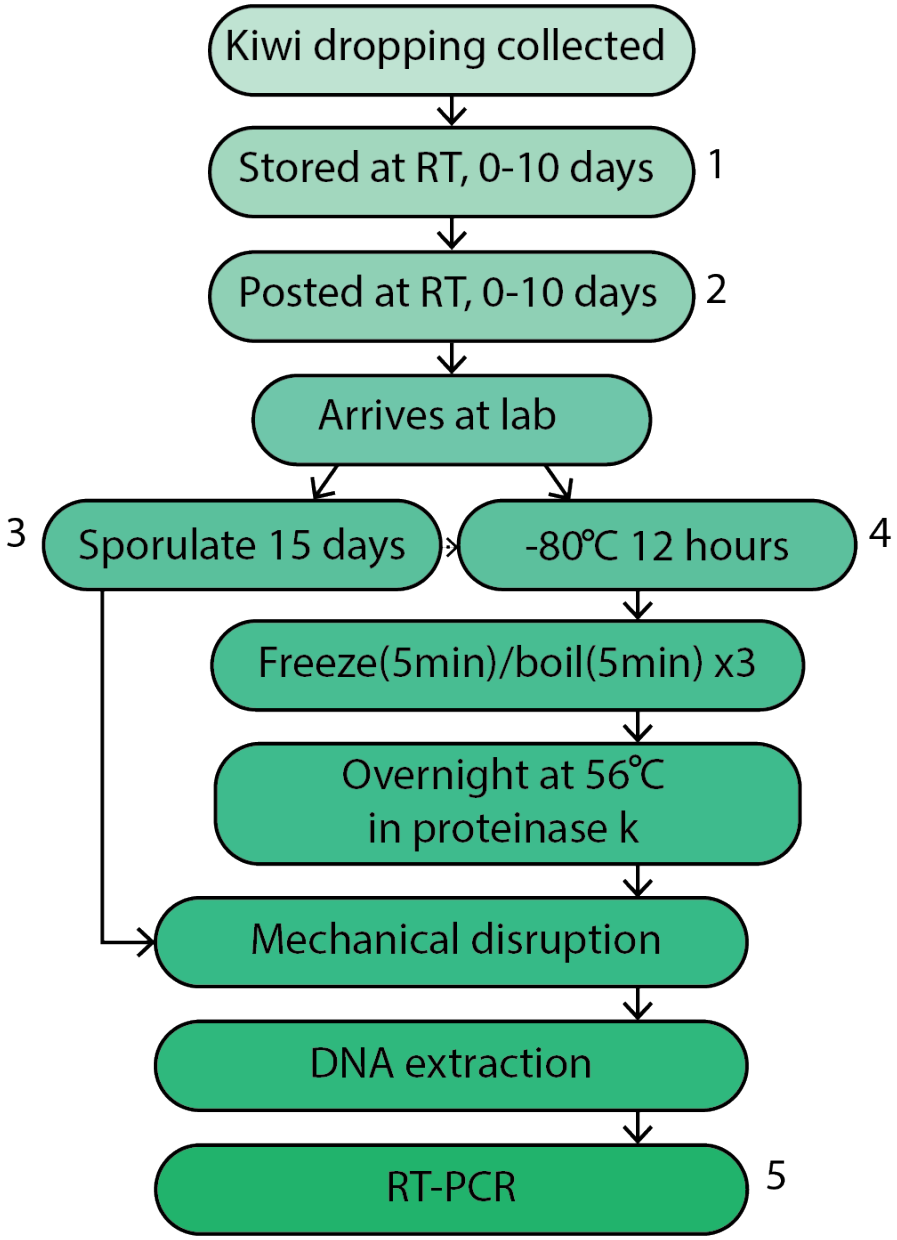


Figure 6.12. A flowchart illustrating how RT-PCR diagnostics and research could be integrated into a kiwi sampling protocol. The incubation of sporulated oocysts is suggested for consistency but may not be required. Numbers indicate areas that require improvements. 1) Storing of sample prior to postage should be kept to minimum, especially with the intent of sporulation. 2) Postage times vary tremendously, especially from remote areas. 3) The ideal conditions of sporulation need to be determined. 4) whether or not the -80°C incubation is enough for breaking open the oocyst walls. 5) RT-PCR protocols need further development.

CHAPTER 7

General Discussion

Contribution of co-authors: All authors provided feedback on one or more drafts of this section.

7.1. Introduction

Conservation of kiwi in New Zealand has been a focus for several decades due to declining numbers of all species. It has required swift actions to prevent extinction and preserve as much diversity as possible. Predation of kiwi chicks by introduced predators, in particular, has been the main concern. Accordingly, Operation Nest Egg (ONE) initiatives and facilities have increasingly been a significant tool used to supplement and establish populations (Bassett 2012; Colbourne et al. 2005; Innes et al. 2015). However, this results in young kiwi being housed at a higher density than is expected in the wild until they reach a suitable body weight to allow release back into the wild (K. McInnes, pers. comm.; Morgan et al. 2012; Morgan et al. 2013). As for farmed domestic livestock, a concentration of young animals in one system can result in health issues (Bangoura and Daugschies 2018; Chapman et al. 2013; Daugschies and Najdrowski 2005; Yabsley 2008). A health issue that has emerged for kiwi in ONE facilities is coccidiosis (Alley et al. 2004; Boardman 1998; Boardman 1995; Morgan 2013; Robertson and Colbourne 2017). As coccidia naturally infect wild kiwi (Thompson and Wright 1978), researching and managing coccidiosis in kiwi was not identified as a high priority by ONE; however, more recently, ONE management has focussed on developing effective coccidia management protocols as a priority, as this parasite has since been shown to hamper ONE progress (Germano et al. 2018). To date, descriptions of several life stages of brown kiwi coccidia have provided essential groundwork (Morgan 2013; Morgan et al. 2012; Morgan et al. 2013; Morgan et al. 2014; Morgan et al. 2017). In this series of publications, histological descriptions established the likelihood that several coccidia species infect kiwi with varying pathogenicity (Morgan et al. 2012; Morgan et al. 2013). At that time, morphological species descriptions determined the presence of four

species of *Eimeria* in six brown kiwi, hinting at the high diversity of parasites likely to infect kiwi (Morgan et al. 2017). Further, Morgan (2013) determined the ITS-1 and -2 to be too highly variable in these species to make useful phylogenetic comparisons which indicated that other targets needed to be developed as molecular tools for use in further studies and to be used as diagnostic tools.

As indicated in Chapter 1, there were four specific aims for this thesis. The first was to improve microscopic detection and quantification of coccidia. This aim was addressed in Chapter 2, which determined the improved accuracy of the Mini-FLOTAC apparatus for detecting and quantifying the coccidia in kiwi. The second aim was to broaden the morphological descriptions of coccidia in kiwi, especially for species dependent on ONE. Accordingly, Chapter 3 extended morphological descriptions of coccidia in brown kiwi to new areas and formally described a novel morphotype. Further, Chapter 4 presented the first in-depth morphological descriptions of coccidia in Haast tokoeka. The third aim was to develop molecular tools to assist with characterising coccidia species in kiwi. To begin addressing this aim, Chapters 3 and 4 provided the first genetic data from the mitochondrial cytochrome *c* oxidase I (COI) gene, which allowed for phylogenetic comparisons with other species of *Eimeria* in other hosts. To improve the depth of analysis, Chapter 5 used a modern, robust sequencing technology (Illumina amplicon sequencing) to illustrate the variation present at the COI site within samples from brown kiwi, Haast tokoeka, rowi, and one sample from a great spotted kiwi. The last aim of this thesis was to develop a diagnostic tool to assist in making management decisions. Correspondingly, Chapter 6 presents a proof of concept in which the results from the Illumina sequencing were used to develop a real-time PCR melting curve analysis that

detects and differentiates between the four most abundant genetic types of *Eimeria* detected in Chapter 5.

To frame further discussion a decision-tree flow chart for managing coccidiosis in kiwi is shown in Figure 7.1. This flow chart includes three main scenarios (Figure 7.1 A, B, C) in which kiwi practitioners seek information on the infection status of coccidia in a particular kiwi; each scenario seeks different information for management decisions (Morgan 2008; Robertson and Colbourne 2017).

The first scenario (Figure 7.1 A) occurs when a wild (or captive) kiwi is found dead or dies soon after discovery (e.g., during hospitalisation), and a necropsy is performed to determine the cause of death. Thus far, the protocol in such situations has been to record the cause of death and any other incidental findings, and then store the samples and information for future use. To further our understanding of coccidia, if the bird is infected with coccidia it is important to document what organs are affected by the parasites and the extent of damage. Recording these details provides crucial information for determining whether a particular species of coccidia is especially virulent and could potentially poses a higher risk to wild or managed kiwi populations. These preserved samples and histological slides are submitted to the DOC Wildlife Pathology Database ("HUIA") database which is available for further study. This was the source for the description of coccidia lesions by Morgan et al. (2012; 2013). The analysis of these samples thus far, by necessity has been descriptive involving morphology and pathology. However, opportunistic observations of endogenous stages of coccidia, while valuable,

do not allow a direct linkage with the morphologically described external stages. Application of a suitable molecular tool should begin to link these stages.

In the second of these scenarios (Figure 7.1 B), a kiwi in captivity or in an ONE facility begins to lose weight and/or presents with other clinical signs (e.g., lethargy or decreased appetite; Yabsley 2008). In such scenarios, droppings are collected from that individual and sent to a parasitology lab for faecal flotation and parasitological analysis (Morgan 2008; Robertson and Colbourne 2017). The current recommendation by the DOC is to treat any bird that has an oocyst load greater than or equal to 2,000 oocysts per gram with toltrazuril (Morgan 2008). However, this cut-off point is known to be arbitrarily based with little scientific basis. ONE facilities that knowingly have high environmental burdens in their enclosures prophylactically treat their kiwi with toltrazuril (pers. comm, K. McInnes). Unfortunately, several problems exist with these protocols. First, Taylor et al. (2019) provided evidence for toltrazuril resistance in kiwi coccidia in an ONE facility; therefore, treatment may not only be ineffective, but also exacerbate the problem in the long term if used incorrectly. Second, Morgan et al. (2013) documented extra-intestinal coccidia life stages in kiwi, and toltrazuril is at least less effective, if not completely ineffective, at treating extra-intestinal stages (Bangoura and Daugschies 2018; Haberkorn 1996; Taylor et al. 2019). Further, Taylor et al. (2018) documents a circadian rhythm in oocyst shedding, which peaks during the early hours of the morning; thus, when practitioners pool several droppings into a single tube to send for testing, a true representation of the kiwi's infection status could be severely diluted and, therefore, understated. In addition, faecal oocyst counts over a 24-hour period demonstrate such variability from the complete absence of coccidial oocysts to

figures in the tens of thousands, that analysis of a single sample is problematic if collected at the wrong time of the day. Lastly, oocysts within a sample are generally not counted separately based on morphology; even if/when they are for research purposes, for example, the virulence of each morphotype is undocumented. Consequently, the cut-off point is independent of the *Eimeria* species involved which may have differing pathogenicity. Again, suitable molecular tools combined with improved faecal diagnostics and timing of collecting samples will improve the accuracy of this approach.

In the third scenario (Figure 7.1 C), kiwi within an ONE chick enclosure receive a general health screening or an individual ONE adult kiwi is being moved from one facility/island to another and receives a general health screening. This scenario proceeds similarly to the second scenario in that droppings are collected for faecal flotation; however, in the case of translocation, additional swabs may also be taken. Generally speaking, screening of the population prior to translocation would occur, with the individual birds that are being transported tested after translocation (Robertson and Colbourne 2017). Regardless, at present commercial laboratories only report the quantity of oocysts per gram of faeces, and nothing about oocyst morphology. Depending on the remoteness of the location of collection, it may take days to over a week before a parasitology lab receives the sample, leading to the birds that could have high loads of parasites released without knowledge of the infection status. The development of improved faecal diagnostics and on-site molecular diagnostic procedures would greatly assist in these circumstances. In the case of a scheduled routine health screening of a chick at an ONE facility, pooled samples from the enclosures are sent to the closest parasitology lab for screening.

As indicated, the research presented in this thesis seeks to improve the efficiency and quality of coccidia testing in all of these scenarios by 1) demonstrating the improved accuracy of the Mini-FLOTAC apparatus over standard centrifugal faecal flotation; 2) providing morphological descriptions of coccidia in brown kiwi and, for the first time, Haast tokoeka; and 3) analysing a phylogenetically informative gene in-depth. This thesis also includes key data for designing future research into kiwi coccidia; including an example of a genetic-based, rapid diagnostic test based on a small fraction of the data generated by Illumina Sequencing. This chapter will discuss how each section of this thesis contributes to the overarching goal of informing management decisions for ONE and wild kiwi.

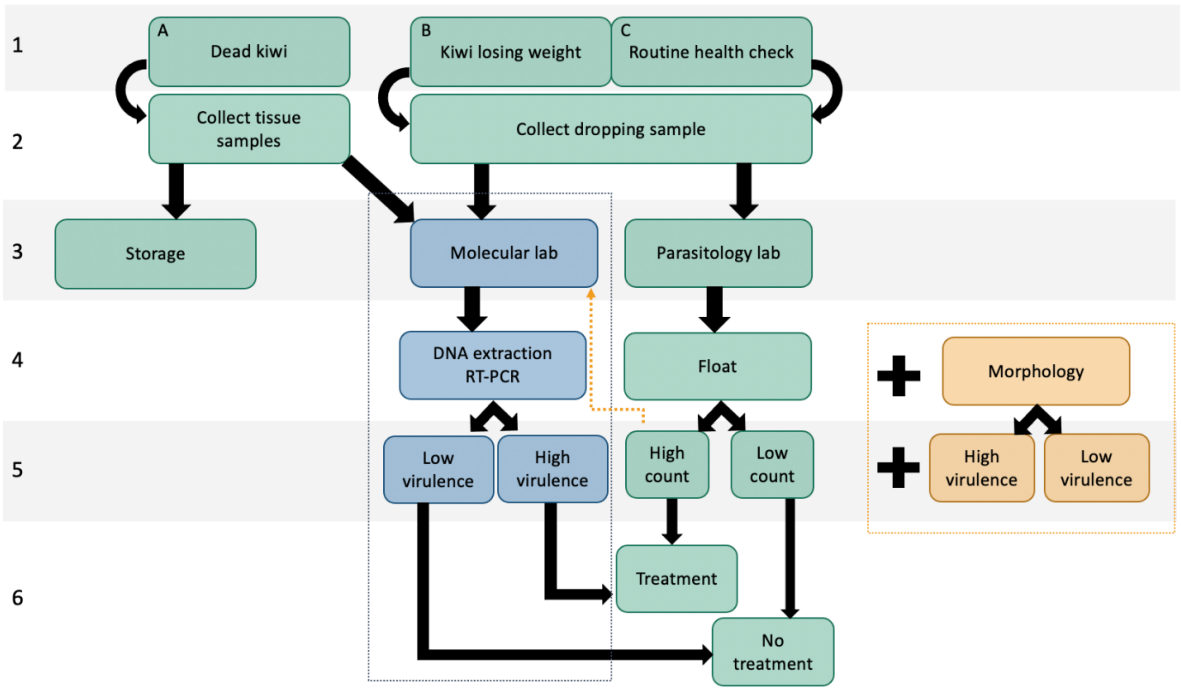


Figure 7.1. This flowchart illustrates current workflow options for kiwi practitioners in monitoring and treating coccidia in kiwi (green). Blue and orange options for each step present possible alternative routes to be used instead of or in parallel with the current protocol. 1) The presentation of a dead kiwi, routine health checks, and unexpected weight loss are the main reasons a practitioner would collect samples from kiwi. 2) Tissue samples are collected and preserved form dead kiwi after a post-mortem. Droppings are collected for health checks and weight loss. 3) Samples from dead kiwi are stored in the HUIA wildlife database for future research. Dropping samples from kiwi in captivity or during translocations are currently sent to parasitology labs for (4) faecal flotation. Then the practitioner decides, based on the recommendations from the Department of Conservation, whether the count is high enough (5) to warrant treatment (6). The blue is a potential workflow given the development of various RT-PCR tests that differentiate genotypes of low and high virulence. This genetic information could be used to inform the yellow workflow, allowing for a broader range of diagnostic reporting in parasitology labs as well. Another option (yellow arrow) is when a sample yields a high count, an aliquot could be sent for molecular analysis.

7.2. Improving the accuracy of traditional screening

New Zealand diagnostic laboratories regularly use centrifugal faecal flotation (CFF) to determine the parasite load (oocysts per gram, OPG) in kiwi. The results of the CFF currently determines the treatment and management routes for kiwi in captivity and prior to translocation (WE Pomroy, pers. comm.). However, this protocol has been demonstrated to be less accurate and precise than the Mini-FLOTAC (Cringoli et al. 2017; Cringoli et al. 2010) in the detection of nematode eggs in horses and sheep (Bauer et al. 2010; Bosco et al. 2018; Noel et al. 2017; Ramos et al. 2018; Scare et al. 2017), nematode larvae (Ramos et al. 2018), as well as *Eimeria* spp. in poultry (Bortoluzzi et al. 2018) and goats (Silva et al. 2013). This thesis compared CFF with the Mini-FLOTAC in faecal kiwi coccidia oocyst detection and established the use of the Mini-FLOTAC apparatus as a more accurate estimation of OPG in kiwi droppings. The data presented ensure that this method is a viable option under the specific conditions used in New Zealand diagnostic labs and encourages its widespread adoption. This combined with more appropriate timing of collection of faecal samples should improve this diagnostic approach.

7.2.1. Management application – Scenario C – Routine Health Checks

The improvements the Mini-FLOTAC bring to kiwi coccidia management most greatly impacts scenario C (Figure 7.1 C) by providing a more accurate method for estimating OPG, which is key to determining infection status and treatment efficacy. Hence, the Mini-FLOTAC protocol can be used to further improve the workflow of this scenario linking infection load and clinical signs to help inform practitioners when treatment with toltrazuril is sufficient or if additional intervention should be sought. The current

recommendation from DOC is that a “high count” that warrants treatment is greater than or equal to 2,000 OPG using a standard faecal floatation, which is equivalent (based on Chapter 2) to 8,000 OPG using the Mini-FLOTAC. For reference, the highest count in this study reached over 65,000 OPG and the highest count the author has ever encountered was over 3 million OPG. Both of these counts were performed for routine health checks; neither bird showed clinical signs. This illustrates the limitations of this simplistic approach to just base management decisions on oocyst count alone. Nevertheless, the Mini-FLOTAC protocol would be a perfect tool for systematically comparing the effect of collection technique, location, and timing on the detected infection load and linking these to the level at which clinical signs may be expected and the efficacy of treatment with toltrazuril. This comparison could prevent dosing too frequently, and, therefore, the unnecessary development of toltrazuril resistance while still enabling treatment before clinical signs become severe.

7.3. Expanding morphological characterisation of sporulated oocysts

For the purpose of this discussion, the newly described morphotypes (M1-5) from Chapters 3 and 4 will be referred to HT (i.e., Haast tokoeka), M1-4 (e.g., M1 = HTM1), and BK (brown kiwi) M5.

Morphological studies of coccidia provide key information for identification and classification of these parasites and remains a primary tool in species descriptions, despite the increasing use and development of genetic tools (Duszynski 2015; El-Alfy et al. 2019; Matsubara et al. 2017; McAllister and Duszynski 2019; Miles et al. 2019;

Morgan et al. 2017; Northover et al. 2019; Williams et al. 2010; Woodyard et al. 2019). Screening methods rely heavily on microscopy, which frequently provides the only morphological data on coccidia species infecting wildlife (Bandoni and Duszynski 1988; Duszynski 2015; Morgan et al. 2017; Taylor et al. 2019; Williams et al. 2010).

Chapter 3 demonstrates the ability of at least five species of coccidia to infect brown kiwi. The newly described morphotype, BKM5, mainly differs from *E. kiwii* previously described in that it possesses a smooth wall rather than a rough/striated wall. This small, yet significant distinction may not have been detected by Morgan et al. (2017) due to differences in methodology, as photographing this morphological feature can be difficult. Specifically, the ability to distinguish between the differences in wall types can vary as the plane of focus is moved in and out. Thus, definitively characterising a rough versus a smooth wall can require several intentionally captured photographs. Further, the strength or quality of the striation can vary between individual oocysts, creating a gradient between smooth and striated. For this thesis, obviously striated oocysts were labelled as *E. kiwii* and less pronounced or ambiguous examples were labelled BKM5. This qualitative range of characterisation indicates that morphology alone may not be enough for unambiguous characterisation.

Additionally, the descriptions of oocysts provided in Chapter 4 show that there are at least four species of *Eimeria* that infect Haast tokoeka. Morphologically, these are very similar to *Eimeria* documented in brown kiwi. Based on the results from Chapter 5, the author believes that HTM1 is synonymous with *Eimeria kiwii*; HTM2 is synonymous with

BKM5; HTM3 is synonymous with *Eimeria mantellii*; and that HTM4 is synonymous with *Eimeria apteryxii*. However, the author also recognises the limited number of droppings examined and, therefore, encourages continued work to describe sporulated oocysts from Haast tokoeka. The samples used for morphological descriptions from both Haast tokoeka and brown kiwi thus far have been collected from a very small subset of ONE facilities. While obtaining samples from wild populations will be more difficult, doing so could demonstrate whether morphotypes have crossed between these two kiwi species. In evolutionary terms these birds have been geographically separated for ~20,000 years (Weir et al. 2016). It seems likely that these may have originally been the same but may have separated when the two kiwi species evolved separately or may have cross-infected after this time and subsequently developed as separate species. Whether these types in these two kiwi species are truly distinct remains to be confirmed.

7.3.1. Management application

By expanding the dataset of brown kiwi *Eimeria* and establishing baseline records on Haast tokoeka coccidia, this thesis sought to encourage the continued use of morphology as an informative, accurate tool for facilitating effective management. Specifically, the ability to screen for changes in morphological species composition with basic laboratory equipment such as the Mini-FLOTAC apparatus and a microscope will likely remain a vital tool for kiwi practitioners. In particular, a key with more thorough morphological descriptions is a first step towards connecting morphotypes with differences in virulence. While sporulating oocysts in a laboratory setting does require more time and safety equipment, screening for unsporulated oocysts may be enough for certain management decisions. For example, if a correlation can be made between

morphological shape (e.g., oval or round and the level of virulence), then morphological notations on the shape (round or oval) of unsporulated oocysts would increase the opportunity for practitioners to apply correct measurements. For instance, a sample that results in a midrange parasite load of a more virulent oocyst type may lead practitioners to treat the kiwi, while midrange parasite load of the less virulent types may not result in treatment. This additional information may seem minor, but the occurrence of toltrazuril-resistant *Eimeria* in recent years suggests that avoiding over medication will prevent exacerbating the problem (Taylor et al. 2019). For instance, when a kiwi begins to show clinical signs such as losing weight, a high or even low-moderate oocyst count may warrant treatment if the morphotype present is known to be of high virulence (Scenario B). Coccidia are known to be prevalent within established ONE facilities and infection with coccidia can compromise the immune response to other pathogens, hence, even if the clinical signs are mainly associated with another pathogen, treating for the coccidia helps the kiwi fight other infections (Alnassan et al. 2014; Chapman et al. 2002; Hegazy et al. 1999; Stockdale 1977). Knowing whether or not the kiwi is infected with a highly virulent morphotype would enable veterinarians to prioritise treatment for coccidiosis. On the other hand, if the kiwi is infected with a morphotype known to have a low virulence in that host species and the infection load is moderate, the veterinarians can decide not to initiate treatment, but focus the treatment on other agents causing disease, and indicate when husbandry issues need to be addressed. In-depth understanding of the morphological species of coccidia associated with levels of virulence in a given host species would significantly improve the long-term management of coccidia in ONE facilities such as routine health checks (Scenario C). Figure 7.1 illustrates in green the current morphological workflow, that is

limited exclusively by intensity of infection. In yellow is a complementary workflow that uses both infection load and species composition. Establishing a morphological profile, even at the most basic oocyst size and shape level would help improve the information provided to practitioners.

This discussion highlights the need to more clearly describe the differences in virulence between *Eimeria* species in different kiwi hosts. Further examination of dead kiwi (Scenario A) would be key to determining the virulence of each morphotype. During a post-mortem of every kiwi, samples from each tissue are collected and preserved for long-term storage. Coccidia oocysts prior to excretion can often be described histologically from thin sections of these preserved tissues although these are often distorted due to fixation (Morgan et al. 2012; Morgan et al. 2013). Having a strong understanding of the morphological species that infect each species of kiwi can help draw connections to descriptions of unsporulated oocysts in post-mortem cases.

7.4. Expanding molecular characterisation of kiwi *Eimeria*

In kiwi, morphological identification is currently limited to descriptions of extracellular stages of development. While morphology provides vital information on coccidia species descriptions, molecular markers continue to influence taxonomic standings of coccidia species (Ghimire 2010; Tenter et al. 2002) and can illuminate the presence of cryptic species (Bandoni and Duszynski 1988; Clark et al. 2016; Duszynski 2015). Thus, while morphological descriptions of endogenous stages of development have been provided by Morgan et al. (2012, 2013) and in this thesis, making a connection between

sporulated and unsporulated oocysts is not definitive without conducting experimental trials, which is impractical in threatened species such as kiwi. Consequently, the ability to routinely detect genetic variation would enable more accurate monitoring and management by differentiating *Eimeria* species at all life stages of the parasite. Thus, developing genetic-based tests allows a more in-depth analysis into the life cycles and epidemiological characteristics of kiwi *Eimeria* spp. This thesis focused on establishing the foundation for the development of such a test by targeting a portion of the COI gene using the Illumina MiSeq platform. Illumina amplicon technology was used as it provides the most cost effective, accurate, and robust analysis that is commercially available and ensures that a molecular test based on this amplicon is sensitive enough to differentiate each species of coccidia present in the samples (Ambardar et al. 2016; Kawashima et al. 2002). In the present studies, this analysis led to 903 operational taxonomic units (OTUs) that clustered into 49 OTUs at 95% homology.

However, as the samples were collected opportunistically, the dataset used for this thesis was heavily skewed towards brown kiwi in the Waikato and Hawke's Bay regions, as well as Haast tokoeka on Rona Island. While this bias was largely unavoidable considering the accessibility of samples, the geographic spread of samples was not representative of kiwi distribution, and this should be addressed in future research. Increasing the geographic and species representation could provide for a more accurate representation of the distribution of coccidia species. This is needed to ensure that a future test can pick up all genotypes present across all populations of kiwi. One option for a larger dataset is to pool faecal samples from each location (or metadata group) into a single extraction, potentially allowing better representation of the true variation.

In addition, applying SGS to single oocysts would be a promising way to confirm links between genotype, morphotype, and virulence. This would be key for furthering the application of molecular assays of samples in improving the decision making for when and how to treat coccidia.

7.4.1. Proof of concept

In order to develop an amplification test that accurately detects and characterises different coccidia OTUs, Illumina data at 95% identity was used to determine a range of possible primer sites. The real-time PCR (RT-PCR) SYTO9 proof of concept protocol presented in Chapter 6 can determine a kiwi's infection status of four OTUs. This RT-PCR protocol most reliably differentiates these OTUs when one is dominant. This protocol demonstrates the reliability of using the data generated by Illumina sequencing and, thus, prompts the continued optimisation of this protocol as well as the development of additional diagnostic assays using real-time PCR technologies.

Additionally, other technologies, such as loop-mediated isothermal amplification (LAMP), should be considered for future work. LAMP removes the requirement for access to thermocyclers and -20°C freezers; thus, a successful LAMP assay would avoid the lengthy process involved in achieving a diagnostic result. However, the need for three primer sets can be an inhibiting factor for development of a successful LAMP protocol (Notomi et al. 2015; Notomi et al. 2000). Perhaps the best scenario is using real-time PCR technologies to first identify the more pathogenic genotypes of coccidia.

Then those specific genotypes could be targeted for LAMP assay development and potentially brought to more remote field settings.

7.4.2. Current and future management implications – scenarios A-C

With the data provided by this thesis, several important steps have been taken to get closer to understanding the genetic, morphologic and epidemiologic variation observed in kiwi coccidia. The newly developed RT-PCR protocol could be used to (1) test single oocysts to begin establishing which morphotypes are associated with four of the most abundant OTUs; (2) initiate the development of quantitative protocols as well as additional OTU-specific protocols; and (3) start linking historical and current clinical cases to OTUs, potentially identifying the OTUs that are highly virulent or cause a particular kind of coccidiosis. Based on the latter, the author suggests that samples submitted for the first scenario (Figure 7.1 A) could be sent to the molecular lab for testing and/or Sanger sequencing, rather than storage (which is the current default). Given the development of additional tests, the life cycles of kiwi *Eimeria* spp. could then be described in detail, documenting which tissue each species is able to invade.

In terms of the second and third scenarios described above (Figure 7.1 B and C), the developed test could be used to determine the presence/absence of these specific OTUs. Similarly, samples in the current workflow (Figure 7.1 B and C in green) where the oocysts are either ambiguous morphologically and/or are on the edge between a high and low infection load could be sent through a molecular workflow (Figure 7.1 in blue) to help make an informed decision. Ideally, these results could be combined with the

coccidia infection load and the level of clinical signs shown to approach determining the level of virulence for these OTUs.

7.5. Future research

7.5.1. Sporulation of kiwi *Eimeria* oocysts

Difficulty in sporulating kiwi oocysts has been a problem since their first description (Thompson and Wright 1978). It remains an important limiting factor in kiwi coccidia research since this impairs morphological characterisation as well as DNA extraction for molecular analyses. Accordingly, establishing a reliable sporulation protocol remains an important focus for future studies. Why it is so difficult to induce the various species of kiwi *Eimeria* to sporulate remains a mystery. The first documentation of coccidia in kiwi included measurements for only unsporulated oocysts (Thompson and Wright 1978). Morgan et al. (2012, 2013) initially described endogenous stages of coccidia and followed with descriptions of sporulated oocysts in Morgan et al. (2017). The protocols used by Morgan (2013) as well as for the research presented herein follow the method described by Duszynski and Wilber (1997), which generally considered standardised and efficient for sporulation (e.g., Carrera-Játiva et al. 2018; Miles et al. 2019; Northover et al. 2019; Pyziel and Demiaszkiewicz 2015; Waldenstedt et al. 2001; Yang et al. 2016a), though some exceptions have been noted in penguins (*Eudyptula minor*) and puffins (*Fratercula arctica*) (Golemansky 2011; Obendorf and McColl 1980). As observed by Morgan (2013), it was extremely difficult to predict whether or not the oocysts in a kiwi sample would sporulate, an attribute unusual for coccidia in general. In nature, most *Eimeria* oocysts sporulate spontaneously given appropriate temperatures for the

relevant species and there are many reports of samples sporulating more or less efficiently under particular laboratory conditions (e.g., Pyziel and Demiaszkiewicz 2015; Waldenstedt et al. 2001). However, for whatever reason all kiwi *Eimeria* oocysts are reluctant to sporulate spontaneously or under "ideal" laboratory conditions. An experimental trial that compares humidity, temperature, light exposure as well as sample freshness and transportation technique could provide consistent, reliable advice on oocyst preparation and preservation. There clearly will be conditions that these oocysts prefer, and the conditions of these trials should reflect the biology of the parasites in their natural environment. For example, unlike most birds, kiwi nest and frequently sleep in burrows that potentially maintain microclimates different than above ground (Birchard et al. 1984). Thus, temperature, humidity, and oxygen concentrations fluctuations during storage, transport, and incubation may affect the development of these species.

7.5.2. DNA extraction

Closely linked to the issue of sporulation is the issue of DNA extraction from, especially, unsporulated oocysts. As described in Chapter 6.3, the addition of a 24-hour incubation at -80°C led to successful, reliable extraction of DNA from sporulated and unsporulated oocysts. Unfortunately, the time and equipment required for the 24 hour -80°C incubation and overnight chemical disruption in addition to the Zymo Research Quick DNA Fecal/Soil miniprep (ZR Quick) severely restricts its use to well-equipped laboratories and the time required to achieve a result. That is, the time and effort required to extract the DNA may completely defeat the purpose of a quick molecular diagnostic tool, and thus may restrict its use to research. Hence, additional research to

determine a simpler, more rapid protocol is required rather than the 24 hour, -80°C incubation prior to using the ZR Quick protocol.

7.5.3. Toltrazuril resistance

Even though large-scale pest control has been identified as the most efficient method for increasing kiwi population success (Germano et al. 2018; Innes et al. 2015; Robertson and de Monchy 2012), ONE will likely remain a key tool for kiwi management over the years to come. Thus, arguably the main focus on kiwi coccidia research is whether or not the current treatment and husbandry protocols are effectively managing coccidia in kiwi. ONE sites rely heavily on prophylactic treatment with toltrazuril to keep infection levels low while allowing for exposure and consequent immunological development in juveniles. While resistance to toltrazuril has been documented in one facility already (Taylor et al. 2019), additional research is needed to investigate toltrazuril resistance to understand the impact current management protocols are having on the coccidia in other management facilities. Other coccidiocides or coccidiostats (e.g., diclazuril) should also be considered for use in rotation with toltrazuril to decrease preferential selection for resistance. In addition, toltrazuril is theoretically only effective enterically (Bangoura and Daugschies 2018; Haberkorn 1996; Taylor et al. 2019), and Morgan et al. (2013) suggests there to be at least two species of kiwi coccidia that reproduce extra-intestinally. In order to test the efficacy of toltrazuril on extra-intestinal parasites in kiwi, however, the morphotypes or OTUs that infect extra-intestinally need to be identified.

7.5.4. The next step in coccidia genetics and genomics

Second and, especially, third generation sequencing technology is evolving rapidly. While developing diagnostic assays remain the goal for coccidia research, second generation, and especially Illumina sequencing on the MiSeq platform may remain the technology of greatest use. The main limitations of this second generation sequencing technology are the short read lengths and amplification bias; however, with a $\leq$1% error rate, it is unlikely that this platform will phase out any time soon (Arulandhu et al. 2018; El Bairi et al. 2020). Alternatively, the long read lengths of third generation sequencing technology will also be a major advantage, especially as error rates are decreased (El Bairi et al. 2020; Lu et al. 2016). Combining these technologies to use the advantages of both technologies have been demonstrated to be a possible next step in genomic research (e.g., Chen et al. 2020; Nygaard et al. 2020). Assembling a full mitochondrial genome from kiwi coccidia is well within the length limitations of third generation sequencing; combining these technologies could provide an even more economical solution than assembly with Illumina alone. While these technologies are not widely available commercially, several platforms are quickly setting a higher standard in sequencing capabilities (El Bairi et al. 2020).

7.5.5. One Health considerations

The current focus of kiwi conservation in regard to coccidia is treatment and husbandry. However, it needs to be acknowledged that coccidia have evolved with kiwi and are participants in this endemic ecosystem and disrupting this ecosystem with cross infections between host species and elimination of parasites from a host population can

have long-lasting negative impacts (Christe et al. 2006; Loye and Carroll 1995; Papkou et al. 2016; Sasal et al. 2000; Thomas et al. 2005). It is possible that cross infection of coccidia between kiwi species has already occurred; however, this requires further investigation to prevent future cross infections. On the other hand, but equally important, is whether or not newly established populations of kiwi, especially on offshore islands, have retained the species richness of their parasite communities (Christe et al. 2006; Papkou et al. 2016; Thomas et al. 2005; Windsor 1997). As noted in Chapter 5, one of the only significant differences between groups was between the Manawatu samples, which was not dominated by a particular OTU, and the Northland groups, which was dominated by a few OTUs. This difference may be due to a bottleneck of parasite species that occurred when birds were introduced to the islands sampled (Papkou et al. 2016). This suggestion is made with the caveat that the data collection for this chapter was primarily opportunistic, with the goal of an initial assessment of the variation found across New Zealand, and especially the North Island. Accordingly, it is extremely difficult to draw robust comparisons between the species richness of especially offshore islands (as many have been isolated longer than onshore islands) and free roaming populations. Regardless, preserving parasite species richness is key to conservation, as it is unclear what crucial roles coccidia have played and will continue to play in the evolution of kiwi (Papkou et al. 2016; Thomas et al. 2005; Windsor 1997).

7.6. Concluding remark

This thesis has provided fundamental groundwork towards effectively identifying and, therefore, managing coccidia in kiwi. Use of traditional morphological descriptions

together with modern DNA amplification and sequencing technology provides significant contributions to the scientific literature. The data and analysis presented here facilitates more in-depth, specific studies of this parasite and the disease it causes in New Zealand's national birds.

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APPENDICES

Appendix A

Index

Term	Abbreviation	Definition	Citation
Loop-Mediated Isothermal Amplification	LAMP	A method of gene amplification that uses Bst DNA polymerase, which is stable at room temperature, and up to 6 primers.	Notomi et al. (2015); Notomi et al. (2000)
Operation Nest Egg	ONE	A program that removes wild kiwi chicks and raises them in a predator free environment until they are large enough to defend themselves.	Colbourne et al. (2005); Germano et al. (2018)
Eimeria	N/A	Genus of coccidial parasites known to infect kiwi.	Morgan (2013); Morgan et al. (2017)
Polymerase Chain Reaction	PCR	Method of DNA amplification that utilises *Taq* DNA polymerase for synthesis of genes.	
Liquid Nitrogen	LIN, N_2	The element, Nitrogen, cooled to its liquid state at -160°C.	
Quantitative Polymerase Chain Reaction	qPCR	Method of rapid nucleotide amplification using fluorescence dyes.	
Specific Gravity	SG	Density of a liquid compared to water.	Stauffer et al. (2008)
Ribosomal DNA	rDNA	DNA that codes ribosomal RNA.	
Internal Transcribed Spacer 1	ITS-1	DNA situated between the 18S rDNA and 5.8S rDNA	
Apicomplexan	N/A	Organisms or features of the phylum Apicomplexa	Mehlhorn (2016)

Internal Transcribed Spacer 2	ITS-2	DNA situated between the 5.8S rDNA and 28S rDNA	
Distilled Water	dH2O	Vaporised water that is recondensed into a separate container to remove impurities.	

Appendix B

Appendix B.1

Standard Operating Procedure

Preparation of Magnesium sulphate ($MgSO_4$, SG 1.28)

Purpose:

To prepare MgSO4 (SG 1.28) for oocyst flotation.

Materials:

- Epsom Salt ($MgSO_4$)
- 1 L bottle
- Warm Water
- Scale
- Metal spatula
- Stir bar
- Stirrer

Safety:

$MgSO_4$ is toxic if inhaled or ingested. Be sure to use gloves or wash hands thoroughly after contact.

Procedure:

1. On the bench, add 500 g Epsom salt ($MgSO_4$) to the bottle.
2. Add 500 mL warm water.
3. Stir at maximum speed until completely mixed.
4. Store on the benchtop at room temperature with the lid securely fastened.

Appendix B.2

Standard Operating Procedure

Centrifugal Faecal Flotation

Purpose:

To conduct oocyst counts on fresh faecal samples from kiwi.

Materials:

- 15 ml test tubes
- Test tube rack
- Steel bowls
- Sieve (0.5 mm)
- Spoon
- 33% Zinc sulphate
- Centrifuge
- Glass slide
- 18 mm^2 glass cover slip
- Microscope
- 3 ml transfer pipettes

Safety:

Zinc sulphate is harmful if ingested and can cause skin and eye irritation.

Procedure:

1. Homogenise the fresh faecal sample with the stirrer.
2. Add $\leq$0.5 g fresh faecal sample to sieve placed inside metal bowl.
3. Add 10-12 ml of zinc sulphate and homogenise with the spoon.
4. Transfer mixture to a test tube.
5. Use a transfer pipette to add enough zinc sulphate to create a convex meniscus.
6. Place cover slip on top.
7. Centrifuge for 5 min at 1200 rpm.
8. Transfer coverslip to slide for examination under microscope.
9. Count oocysts in one strip at 300 $\times$, multiply by 25 and divide by the weight to calculate OPG.
10. Save excess sample for sporulation/molecular diagnostics.

Appendix B.3

Standard Operating Procedure

Mini-FLOTAC

Purpose:

To conduct oocyst counts on fresh faecal samples from kiwi.

Materials:

- Steel bowls
- Sieve (0.5 mm)
- Spoon
- Wooden stirrer
- Graduated cylinder
- Scale
- Microscope
- 3 ml transfer pipettes
- miniFLOTAC
- miniFLOTAC stage adapter
- $MgSO_4$ (1.28 SG)

Safety:

$MgSO_4$ is harmful if ingested and can cause skin and eye irritation. Be sure to use standard PPE at all times.

Procedure:

1. Homogenise the fresh faecal sample with the stirrer.
2. Add 0.5 g fresh faecal sample to sieve placed inside metal bowl.
3. Add 9.5 ml $MgSO_4$ (1:20 dilution).
4. Homogenise with the spoon.
5. While stirring continuously, retrieve 1 ml with a pipette.
6. Fill one flotation chamber at a 45° angle to avoid air bubbles.
7. Rest flat for at least 10 minutes.
8. Transfer the sample by turning the reading disc 90° clockwise using the key.
9. Remove key and secure on the microscope stage using the adapter.
10. Count oocysts in counting chamber and multiply by 20 to calculate the OPG.
11. Save excess sample for sporulation/molecular diagnostics.

Appendix B.4

Raw Data

Comparing the Mini-FLOTAC with Centrifugal Faecal Flotation

ID	Date tested	Date collected	FLOTAC 1 OPG	FLOTAC 2 OPG	FLOTAC 3 OPG	FLOTAC Average OPG	CFF 1 OPG	CFF 2 OPG	CFF 3 OPG	CFF AVG OPG	Sender
RS01	7.11.17	2.11.17	26960	14800	15680	19146.6667	3265.6	2260.8	1381.6	2302.66667	NKHA
RS04	28.11.17	22.11.17	127760	84480	91120	101120	53901.3228	57838.76	42726.6614	51488.9147	NKHA
RS03	27.11.17	22.11.17	18240	13680	15600	15840	3328.4	4898.4	10927.2	6384.66667	NKHA
RS05	4.12.17	30.11.17	11280	7600	6720	8533.33333	527.52	690.8	261.248	493.189333	NKHA
RS09	21.12.17	14.12.17	9040	7840	5600	7493.33333	2700.4	1067.6	1507.2	1758.4	NKHA
RS08	22.12.17	19.22.17	21280	16240	14240	17253.3333	4270.4	6594	3077.2	4647.2	NKHA
RS14	2.1.17	28.12.17	17840	12800	9840	13493.3333	1444.4	1632.8	2072.4	1716.53333	NKHA
CS02	5.1.18	29.12.17	1580	1420	1320	1440	314	753.6	565.2	544.266667	Cape Sanctuary
CS01	6.1.18	28.12.17	62320	65680	68640	65546.6667	95970.1408	88082.1408	65233.76	83095.3472	Cape Sanctuary
CS13	6.1.18	29.12.17	160	80	100	113.333333	62.8	188.4	188.4	146.533333	Cape Sanctuary

Appendix C.1

Standard Operating Procedure

Potassium dichromate preparation (2%)

Purpose:

To prepare 2% Potassium dichromate ($K_2Cr_2O_7$, CAS Number-7778-50-9, Fisher Chemical, ThermoFisher Scientific, Waltham, MA, USA, MW = 294.185 g/mol) solution for use in oocyst sporulation.

Materials:

- Potassium dichromate ($K_2Cr_2O_7$)
- 250 ml bottle
- Water
- Scales
- Metal spatula

Safety:

$K_2Cr_2O_7$ is a corrosive oxidiser; contact via skin or inhalation is acutely dangerous. Ensure to work in fume hood at all times and use gloves in addition to standard PPE.

Procedure:

1. In the fume hood, add 4 g $K_2Cr_2O_7$ to the bottle.
2. Add 200 mL water.
3. Mix and store with the lid on in the fume hood.

Appendix C.2

Standard Operating Procedure

Oocyst Sporulation and storage

Purpose:

To sporulate oocysts for morphology and long-term storage

Materials:

- Petri dishes
- Wooden spatula
- 3 ml transfer pipettes
- 2% Potassium dichromate ($K_2Cr_2O_7$) solution ($K_2Cr_2O_7$, CAS Number-7778-50-9, Fisher Chemical, ThermoFisher Scientific, Waltham, MA, USA, MW = 294.185 g/mol)
- 25 ml storage flasks
- Room temperature (RT) large, water-proof storage container (e.g., Sistema)
- 1.5 mL microcentrifuge tubes
- -80°C storage container

Safety:

$K_2Cr_2O_7$ is a corrosive oxidiser; contact via skin or inhalation is acutely dangerous. Ensure

to work in fume hood at all times and use gloves in addition to standard PPE.

Procedure:

1. Using a wooden spatula, aliquot some of the homogenised faecal sample to a labelled microcentrifuge tube. Store at -80°C.
2. Then aliquot small amounts of the sample (~0.1 g) to labelled petri dishes.
3. In the fume hood, add enough $K_2Cr_2O_7$ to cover the bottom of the petri dish and at least a 1:5 ratio (faeces:$K_2Cr_2O_7$).
4. Homogenise faeces in the $K_2Cr_2O_7$ solution and spread over the bottom of the petri dish to ensure oxygenation.
4. Keep moist at RT for 15-20 days.
5. Transfer sample to 25 ml storage flasks and store in a water-proof storage container at RT.

Appendix C.3

Standard Operating Procedure

Oocyst cleaning

Purpose:

To clean $K_2Cr_2O_7$ solution off oocysts for analysis.

Materials:

- Sporulated or unsporulated oocysts in $K_2Cr_2O_7$ solution
- 1.5 ml microcentrifuge tubes, standard
- Single-use, disposable pipettes
- Microcentrifuge
- Nitrile gloves
- Sterile water
- 100-1000 μl micropipette tips
- 100-1000 μl micropipette
- Chemical waste disposal
- Fume hood
- Permanent marker
- Vortex

Safety:

$K_2Cr_2O_7$ is a corrosive oxidiser; contact via skin or inhalation is acutely dangerous. Ensure

to work in fume hood at all times and use gloves in addition to standard PPE.

Procedure:

1. In the fume hood, aliquot 1-1.5 ml of sporulated or unsporulated oocysts in $K_2Cr_2O_7$ solution into labelled microcentrifuge tubes using the single-use pipets.
2. Spin the samples at 18800 x g for 7 minutes.
3. In the fume hood, dispose of the supernatant in the chemical waste container.
4. Add 1 ml water and vortex thoroughly.
5. Repeat steps 2-4 at least two more times or until clear.
6. When clear, do not add water unless required.

Appendix C.4

Standard Operating Procedure

Oocyst Imaging

Purpose:

To take images at high enough magnification and quality to measure and categorise the physical features for morphotyping *Eimeria* oocysts.

Materials:

- Sporulated oocyst samples clean, undiluted of $K_2Cr_2O_7$ (See Appendix C.3)
- Single-use, disposable pipettes
- 1.5 ml microcentrifuge tubes, standard
- Sterile water
- $MgSO_4$ (1.28 SG)
- 1.0 mm stage micrometre
- LEICA DM750 microscope
- Computer with the Leica LAS EZ imaging software
- Coverslips (22 mm^2)
- Cavity slide
- Biohazard waste disposal
- Paper towels
- Lens wipes
- Immersion oil

Safety:

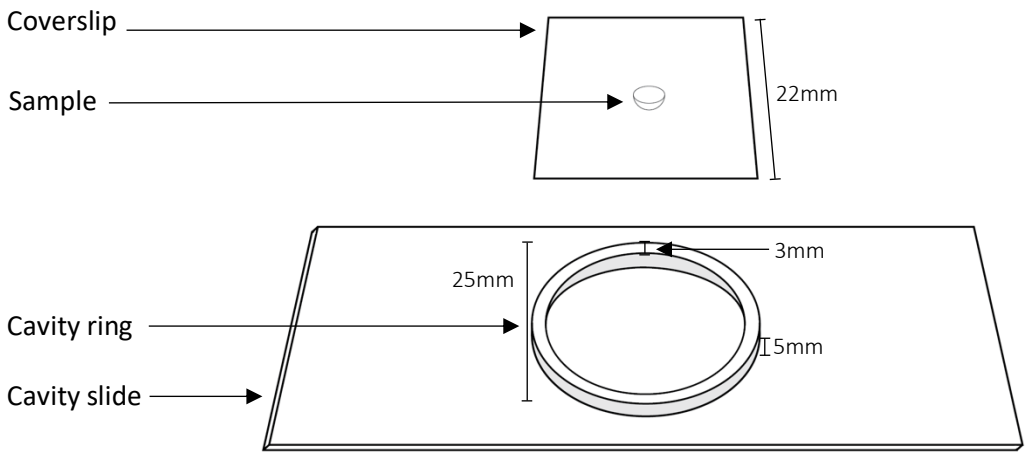
$MgSO_4$ is harmful if ingested and can cause skin and eye irritation. Be sure to use standard PPE at all times.

Procedure:

- Calibrate the LEICA DM750 microscope to the imaging software with the 1.0 mm stage micrometre.
- Add 500 μl magnesium sulphate solution to the sample using a single-use, disposable pipette.
- Mix gently using the pipette and allow to rest for 1 minute to allow for the oocysts to float to the top and other debris to sink.
- While waiting, add four, evenly distributed, droplets of water around the rim of the cavity ring.

- Targeting the top of the sample, remove a small aliquot of the sample and suspend a drop on the bottom of a coverslip (see diagram).
- Place the coverslip, sample side down onto the coverslip and remove excess water with a paper towel so that the coverslip adheres to the ring rather than floats atop.
- Place the slide onto the microscope and let it rest for a few seconds.
- Site the microscope into 100 × magnification.
- Take multiple pictures of each oocysts of important features (listed below) at multiple depths.
    - Wall
    - Micropyle (if present)
    - Sporocysts (if entire length is in focus)
    - Polar granules
- Save at least one image with a scale from each set of images.
- Save all images with an individual oocyst ID.

Diagram of oocyst isolation for imaging.



Appendix C.5

Standard Operating Procedure

Oocyst Measuring

Purpose:

To measure oocyst features from images of sporulated oocysts.

Materials:

- ImageJ and compatible operating system
- Excel
- Images of oocysts

Safety:

There are no safety concerns.

Procedure:

1. Import all the images from one oocyst at a time.
2. Set the ImageJ universal scale using the image with the set scale.
3. Measure each feature using the image with the sharpest, highest quality.
    a. Suggested: fill the line to track which features you have measured.
4. Record measurements in excel.

Appendix C.6

Standard Operating Procedure

Modified Zymo Research Quick-DNA Fecal/Soil Microbe DNA extraction

Purpose:

To extract DNA from sporulated and unsporulated oocysts for PCR.

Materials:

- Cleaned sporulated oocysts (see cleaning oocyst cleaning SOP, Appendix C.3) or unsporulated oocysts that have been stored at -80°C for ≥12 hours.
- Zymo Research Quick-DNA Fecal/Soil Microbe DNA Miniprep Kit (Zymo Research, Orange County, CA, USA)
- Liquid nitrogen (LIN)
- Thermos
- Heat block
- 1.5 ml microcentrifuge tubes, locking lids
- 1.5 ml microcentrifuge tubes, standard
- Forceps
- Proteinase K
- 56°C water bath
- Bead beater
- 100-1000 μl micropipette tips
- 100-1000 μl micropipette

Safety:

LIN is extremely dangerous and should be handled with care. Wear thermal gloves and protective face gear when aliquoting and use only in well ventilated areas. Avoid direct contact with skin and inhalation of fumes.

Buffers in the Zymo extraction kit are flammable and/or corrosive and can be harmful if swallowed, inhaled or splashed in the eyes. Keep away from flames and avoid inhaling the fumes. Wear proper PPE at all times.

Procedure:

1. Prepare sample in a 1.5 ml microcentrifuge tubes with locking lids
    a. Thaw and aliquot $\leq$0.15 g faecal sample from -80°C freezer
    OR
    b. Aliquot $\leq$200 μl cleaned, sporulated oocysts.
2. Freeze/boil
    a. Submerge the samples in LIN for five minutes.
    b. Use forceps to transfer samples to the heat block at 100°C for five minutes.
    c. Repeat three times.
3. Spin down the samples.
4. Overnight incubation
    a. Add 200 μl Bead Beating Buffer from the Zymo extraction kit.
    b. Add 40 μl proteinase k.
    c. Vortex vigorously.
    d. Spin down very briefly.
    e. Mix gently.
    f. Incubate overnight in the 56°C water bath.
5. Zymo extraction
    a. Vortex the sample and spin down briefly.
    b. Using a 1000 μl tip that has been trimmed (if necessary), transfer the sample to the bead tube.
    c. Continue as per the Zymo kit instructions.

Appendix C.7

Standard Operation Procedure

Ribosomal DNA 18S gene amplification

Target Sequence: Ribosomal DNA 18S gene

Primer Pair: Eimeria 18S-F/R

Reference: Jenkins et al. (2006), Morgan (2013)

Eimeria 18S-F Sequence (5'-3'): CGGTGAAACTGCGAATGGCTCA

Eimeria 18S-R Sequence (5'-3'): GCCTTCCTTAGATGTGGTAGCC

PCR Mixture (25μl):

*10X Buffer	2.5 μl
*MgCl2 (50 mM)	1.5 μl
*dNTPs (10 mM)	1.0 μl
E18SF (10 μM)	0.5 μl
E18SR (10 μM)	0.5 μl
*Platinum Taq	0.2 μl
Sterile Water	16.8 μl
1' product	2 μl

Program:

1 cycle	Initial Denaturation	95°C	7 min
40 cycles	Denaturation	95°C	20 sec
	Annealing	50°C	30 sec
	Extension	72°C	60 sec
1 cycle	Final Extension	72°C	5 min

* ThermoFisher Scientific, Waltham, MA, USA

Appendix C.8

Standard Operating Procedure

Nested Mitochondrial cytochrome *c* oxidase subunit I gene amplification

Target Sequence: Mitochondrial cytochrome *c* oxidase subunit 1 (COI) gene

Primary Primer Pair: Cocci_COI_For/Cocci_COI_Rev

Primary Product Length: ~780 bp

Reference: Ogedengbe et al. (2011b)

Cocci_COI_For: 5'- GGT TCA GGT GTT GGT TGG AC -3'

Cocci_COI_Rev: 5'- AAT CCA ATA ACC GCA CCA AG -3'

PCR Mixture (50 μl):

*10X Buffer	5.0 μl
*$MgCl_2$ (50 mM)	2.5 μl
*dNTPs (10 mM)	1.0 μl
COIF2 (10 μM)	2.5 μl
COIR2 (10 μM)	2.5 μl
*1X BSA	5.0 μl
*Platinum Taq	0.2 μl
Sterile Water	30.3 μl
1' product	1 μl

Program:

1 cycle	Initial Denaturation	96C	5 min
40 cycles	Denaturation	94C	20 sec
	Annealing	59C	30 sec
	Extension	72C	90 sec
1 cycle	Final Extension	72C	10 min

* ThermoFisher Scientific, Waltham, MA, USA

Secondary Primer Pair: COIF2/R2

Product Length: ~460 bp

Reference: Yang et al. (2013)

COIF2 Sequence (5'-3'): TAA GTA CAT CCC TAA TGT C

COIR2 Sequence (5'-3'): GTC ATC ATA TGR TGT GCC CA

PCR Mixture (50 μl):

*10X Buffer	5.0 μl
*$MgCl_2$ (50 mM)	2.5 μl
*dNTPs (10 mM)	1.0 μl
COIF2 (10 μM)	5.0 μl
COIR2 (10 μM)	5.0 μl
*1X BSA	5.0 μl
*Platinum Taq	0.2 μl
Sterile Water	25.3 μl
1' product	1 μl

Program:

1 cycle	Initial Denaturation	96°C	5 min
40 cycles	Denaturation	94°C	20 sec
	Annealing	54°C	30 sec
	Extension	72°C	90 sec
1 cycle	Final Extension	72°C	10 min

*ThermoFisher Scientific, Waltham, MA, USA

Appendix C.9

Brown Kiwi Morphology Measurements

Image/Oocyst ID	Oocyst length (µm)	Oocyst width (µm)	Oocyst L/W	Sporocyst Avg length (µm)	Sporocyst Avg width (µm)	Sporocyst L/W	Wall width (µm)	Micropyle width (µm)	Polar Granule length (µm)	Polar Granule width (µm)	No. of Polar Granules	Wall Description	Morphotype
MB06_29_12_18_1	20.94	14.26	1.47	9.75	5.98	1.63	0.69	1.61	1.47	1.25	1	smooth	*E. apteryxii*
MB06_29_12_18_2	22.49	14.99	1.50	10.92	8.47	1.29	0.95	2.33	0.91	0.83	5	smooth	*E. apteryxii*
MB06_29_12_18_4	23.98	14.06	1.71	11.85	6.99	1.69	0.82	2.01	2.02	1.61	2	smooth	*E. apteryxii*
MB06_29_12_18_6	19.23	14.05	1.37	10.01	6.39	1.57	0.71	2.22	2.32	2.03	1	smooth	*E. apteryxii*
MB16_23_10_18_10	21.19	13.86	1.53	11.83	5.88	2.01	0.68	1.61	1.82	1.19	3	smooth	*E. apteryxii*
MB16_23_10_18_12	21.70	12.70	1.71	9.82	5.27	1.86	0.65	1.29	2.76	1.47	1	smooth	*E. apteryxii*
MB16_23_10_18_13	27.48	14.87	1.85	12.32	6.27	1.97	0.63		1.86	1.65	3	smooth	*E. apteryxii*
MB16_23_10_18_14	20.96	14.58	1.44	10.40	5.80	1.79	0.83	2.31	2.39	1.18	3	smooth	*E. apteryxii*
MB16_23_10_18_15	24.57	13.60	1.81	10.61	5.40	1.96	0.83	2.05	1.45	1.05	4	smooth	*E. apteryxii*
MB16_23_10_18_16	22.15	13.93	1.59	11.54	6.01	1.92	0.78	1.11	2.17	1.23	2	smooth	*E. apteryxii*
MB16_23_10_18_17	20.29	14.37	1.41	11.48	5.37	2.14	0.79	1.18	0.99	0.83	6	smooth	*E. apteryxii*

MB16_23_10_18_18	21.57	16.14	1.34	10.38	6.08	1.71	0.61	1.92	2.24	1.73	1	smooth	*E. apteryxii*
MB16_23_10_18_19	23.56	13.85	1.70	12.52	5.90	2.12	0.75	1.97	1.23	0.81	3	smooth	*E. apteryxii*
MB16_23_10_18_2	21.08	12.75	1.65	12.14	6.03	2.01	0.67	1.45	1.85	1.77	2	smooth	*E. apteryxii*
MB16_23_10_18_20	23.99	12.99	1.85	11.53	5.57	2.07	0.55		0.98	0.72	4	smooth	*E. apteryxii*
MB16_23_10_18_200	23.68	14.83	1.60				0.62	2.79	1.23	0.82	5	smooth	*E. apteryxii*
MB16_23_10_18_201	22.08	15.07	1.47	11.22	5.86	1.92	0.71	2.35	1.45	1.14	2	smooth	*E. apteryxii*
MB16_23_10_18_202	23.10	14.96	1.54	12.57	5.96	2.11	0.62	2.25	1.50	1.01	2	smooth	*E. apteryxii*
MB16_23_10_18_203	18.66	13.10	1.42	10.28	4.82	2.13	0.61	2.56	1.90	1.68	1	smooth	*E. apteryxii*
MB16_23_10_18_207	18.87	13.14	1.44	10.36	5.03	2.06	0.62	1.83	1.25	1.07	3	smooth	*E. apteryxii*
MB16_23_10_18_208	26.83	15.45	1.74	12.73	6.07	2.10	0.73	2.62	1.51	1.06	4	smooth	*E. apteryxii*
MB16_23_10_18_209	22.23	13.90	1.60	12.11	6.03	2.01	0.77	2.27	1.54	1.18	4	smooth	*E. apteryxii*
MB16_23_10_18_25	25.44	15.14	1.68	11.91	6.57	1.81	0.77	1.54	1.13	0.65	4	smooth	*E. apteryxii*
MB16_23_10_18_26	23.59	14.46	1.63	11.57	6.01	1.92	0.88	1.52	1.90	0.85	7	smooth	*E. apteryxii*

MB16_23_10_18_27	21.14	13.98	1.51	11.94	4.79	2.49	0.67	1.26	1.28	0.95	2	smooth	*E. apteryxii*
MB16_23_10_18_28	21.44	13.43	1.60	11.54	5.57	2.07	0.64	1.28	1.44	0.90	2	smooth	*E. apteryxii*
MB16_23_10_18_29	20.37	14.46	1.41	10.78	5.77	1.87	0.66	1.71	1.29	1.25	4	smooth	*E. apteryxii*
MB16_23_10_18_3	22.51	13.96	1.61	11.98	5.27	2.27	0.68	1.63	1.73	1.15	4	smooth	*E. apteryxii*
MB16_23_10_18_30	22.08	15.70	1.41	12.09	6.03	2.01	0.63	1.30	1.66	1.11	4	smooth	*E. apteryxii*
MB16_23_10_18_31	24.39	13.17	1.85	11.80	5.28	2.24	0.75	2.01	1.09	0.60	9	smooth	*E. apteryxii*
MB16_23_10_18_33	24.51	14.57	1.68	13.55	5.95	2.28	0.61	1.35	1.38	0.91	3	smooth	*E. apteryxii*
MB16_23_10_18_34	26.22	15.01	1.75	12.29	6.26	1.96	0.84	2.17	1.72	0.86	2	smooth	*E. apteryxii*
MB16_23_10_18_35	18.23	13.77	1.32	11.48	5.66	2.03	0.84	1.96	1.99	1.00	3	smooth	*E. apteryxii*
MB16_23_10_18_4	17.39	12.98	1.34	5.25	5.09	1.03	0.52	1.67	1.72	1.20	1	smooth	*E. apteryxii*
MB16_23_10_18_5	20.83	14.86	1.40	11.89	6.00	1.98	0.68	1.97	1.42	1.08	3	smooth	*E. apteryxii*
MB16_23_10_18_6	19.16	13.09	1.46	10.12	5.18	1.95	0.69	2.13	1.37	0.96	3	smooth	*E. apteryxii*
MB16_23_10_18_7	22.71	14.70	1.54	12.08	5.50	2.20	0.55	1.49	1.30	0.79	4	smooth	*E. apteryxii*
MB16_23_10_18_8	21.95	15.13	1.45	11.48	6.41	1.79	0.65	2.54	1.49	1.05	3	smooth	*E. apteryxii*
MB16_23_10_18_9	22.36	14.24	1.57	12.63	5.86	2.16	0.60	2.79	2.18	1.58	3	smooth	*E. apteryxii*
NM01_22_18_17_1	20.42	14.19	1.44	11.04	5.93	1.86	0.92	1.41	1.87	1.46	1	smooth	*E. apteryxii*
OW01_27_8_17_1	24.53	15.51	1.58	12.12	6.73	1.80	0.69	3.08	2.05	1.20	2	smooth	*E. apteryxii*

OW01_27_8_17_2				11.41	5.33	2.14	0.82	2.44	1.53	1.10		smooth	*E. apteryxii*
OW01_27_8_17_20	20.66	15.39	1.34	10.97	5.63	1.95	0.64	1.79	1.17	1.14	1	smooth	*E. apteryxii*
OW01_27_8_17_22	18.51	12.24	1.51	10.45	5.12	2.04	0.60	2.21	1.42	1.02	5	smooth	*E. apteryxii*
OW01_27_8_17_23	25.20	15.52	1.62	10.73	6.69	1.60	0.69	2.37	1.49	1.12	5	smooth	*E. apteryxii*
OW01_27_8_17_24	20.91	13.85	1.51	10.58	5.84	1.81	0.71	1.96	1.61	1.07	6	smooth	*E. apteryxii*
OW01_27_8_17_5	21.70	13.30	1.63	10.57	5.39	1.96	0.66	2.61	1.21	1.11	7	smooth	*E. apteryxii*
OW01_27_8_17_7	20.41	11.77	1.73	10.63	5.32	2.00	0.75	2.28	1.10	0.91		smooth	*E. apteryxii*
WB01_22_3_18_1	23.15	15.12	1.53				0.78	1.90	2.20	1.63	1	smooth	*E. apteryxii*
WB01_22_3_18_10	22.07	14.52	1.52	9.38	7.46	1.26	0.50	1.73	2.28	1.68	1	smooth	*E. apteryxii*
WB01_22_3_18_11	22.42	15.20	1.48	11.19	5.75	1.94	0.69	1.75	1.84	1.41	2	smooth	*E. apteryxii*
WB01_22_3_18_13	21.46	16.13	1.33	9.78	6.46	1.51	0.62	3.20	3.20	1.40	1	smooth	*E. apteryxii*
WB01_22_3_18_6	25.56	15.64	1.63	11.03	5.94	1.86	0.80	2.06	2.32	1.23	1	smooth	*E. apteryxii*
WB01_22_3_18_7	21.82	14.19	1.54	9.09	6.29	1.44	0.43	1.99	1.64	1.12	4	smooth	*E. apteryxii*
WB01_22_3_18_9	22.68	13.89	1.63	11.06	6.32	1.75	0.50	1.99	2.23	1.96	1	smooth	*E. apteryxii*
WP01_30_10_17_18	22.41	13.85	1.62	12.56	5.42	2.32	0.84	1.86	2.19	1.21	2	smooth	*E. apteryxii*
WP01_30_10_17_19	22.31	12.87	1.73	11.59	5.51	2.10	0.58	1.73	1.87	2.44	1	smooth	*E. apteryxii*
WP01_30_10_17_21	22.29	14.90	1.50	12.02	5.61	2.14	0.68	2.46	1.83	1.16	4	smooth	*E. apteryxii*

WP01_30_10_17_31	22.07	15.05	1.47	11.10	6.28	1.77	0.89	2.02	1.40	0.98	6	smooth	*E. apteryxii*
MB06_29_12_18_5	18.69	16.72	1.12	11.93	6.33	1.89	0.78		1.81	1.30	1	striated	*E. kiwii*
NM01_22_18_17_2	16.47	15.39	1.07	11.03	5.73	1.93	0.79				1	striated	*E. kiwii*
NM01_22_18_17_3	15.06	14.94	1.01	8.11	4.65	1.74	0.68		1.30	1.04	1	striated	*E. kiwii*
OW01_27_8_17_4	16.00	15.94	1.00				0.76		2.53	2.01	1	striated	*E. kiwii*
OW01_27_8_17_6	12.53	11.60	1.08	7.58	4.63	1.64	0.50		1.68	1.46	1	striated	*E. kiwii*
RSB06_30_4_18_10	15.66	15.00	1.04				0.86		2.96	1.93	1	striated	*E. kiwii*
RSB06_30_4_18_11	14.01	12.82	1.09	9.18	4.90	1.87	0.81		2.61	2.13	1	striated	*E. kiwii*
RSB06_30_4_18_12	15.49	14.59	1.06	9.27	4.76	1.95	0.86					striated	*E. kiwii*
RSB06_30_4_18_13	14.69	14.32	1.03	9.62	4.59	2.10	0.84		2.19	1.64	1	striated	*E. kiwii*
RSB06_30_4_18_14	14.46	14.00	1.03	9.26	4.82	1.92	0.73		2.81	2.16	1	striated	*E. kiwii*
RSB06_30_4_18_15	15.18	14.82	1.02	7.76	4.98	1.56	0.64					striated	*E. kiwii*
RSB06_30_4_18_16	14.19	13.80	1.03	9.85	5.06	1.95	0.73					striated	*E. kiwii*
RSB06_30_4_18_17	15.89	14.59	1.09	10.84	5.26	2.06	0.74		2.61	2.32	1	striated	*E. kiwii*
RSB06_30_4_18_202	14.11	14.11	1.00				0.69		2.17	1.71	1	striated	*E. kiwii*
RSB06_30_4_18_203	14.41	14.35	1.00	8.73	5.07	1.72	0.71		2.58	1.78	1	striated	*E. kiwii*
RSB06_30_4_18_212	16.33	15.28	1.07	9.98	5.71	1.75	0.93		2.70	2.37	1	striated	*E. kiwii*

RSB06_30_4_18_239	15.34	15.04	1.02	10.36	5.61	1.85	0.73		2.59	1.82	1	striated	*E. kiwii*
RSB06_30_4_18_34	13.83	12.65	1.09	9.21	5.37	1.72	0.67		1.98	1.12	1	striated	*E. kiwii*
RSB06_30_4_18_35	15.83	14.11	1.12	10.76	5.26	2.04	0.72		1.96	1.23	1	striated	*E. kiwii*
RSB06_30_4_18_6	13.03	12.91	1.01				0.62		1.77	1.69	1	striated	*E. kiwii*
RSB06_30_4_18_9	14.31	14.01	1.02	9.79	4.66	2.10	0.85					striated	*E. kiwii*
WB01_22_3_18_4	16.49	14.61	1.13	8.55	5.91	1.45	0.59		1.25	1.00	1	striated	*E. kiwii*
WB01_22_3_18_5	14.74	14.42	1.02	8.22	5.64	1.46	0.64		1.44	1.08	1	striated	*E. kiwii*
WB01_22_3_18_8	14.55	14.32	1.02	10.08	4.64	2.17	0.77				1	striated	*E. kiwii*
WP01_30_10_17_12	11.75	11.69	1.01	7.17	4.25	1.69	0.59		1.68	1.37	1	striated	*E. kiwii*
WP01_30_10_17_13	12.39	11.99	1.03	7.29	5.00	1.46	0.69		2.26	1.88	1	striated	*E. kiwii*
WP01_30_10_17_14	12.73	11.77	1.08	7.84	4.90	1.60	0.64		1.94	1.25	1	striated	*E. kiwii*
WP01_30_10_17_32	12.73	10.76	1.18	7.89	4.35	1.82	0.73		1.11	0.90	1	striated	*E. kiwii*
WP01_30_10_17_33	12.67	11.99	1.06	8.84	4.39	2.02	0.66		2.16	1.08	1	striated	*E. kiwii*
WP01_30_10_17_34	14.16	13.96	1.01	8.30	4.30	1.93	0.81		2.37	2.14	1	striated	*E. kiwii*
WP01_30_10_17_36	13.22	12.14	1.09	6.78	4.52	1.50	0.74		2.14	1.84	1	striated	*E. kiwii*
WP01_30_10_17_4	14.56	13.97	1.04	6.91	4.21	1.64	0.64		2.51	2.29	1	striated	*E. kiwii*
WP01_30_10_17_6	14.91	14.43	1.03	9.42	4.41	2.14	0.73		2.46	2.00	1	striated	*E. kiwii*

WP01_30_10_17_7	10.93	10.31	1.06				0.55		1.94	1.55	1	striated	*E. kiwii*
MB16_23_10_18_1	19.84	14.82	1.34	11.32	5.73	1.97	0.88		1.82	1.16	3	smooth	*E. mantellii*
MB16_23_10_18_204	16.54	13.02	1.27	9.68	5.26	1.84	0.72		1.54	0.88	1	smooth	*E. mantellii*
MB16_23_10_18_205	19.50	13.76	1.42				0.76		1.80	1.55	1	smooth	*E. mantellii*
MB16_23_10_18_21	22.28	13.82	1.61	11.95	5.62	2.13	0.61		1.28	0.67	2	smooth	*E. mantellii*
MB16_23_10_18_22	19.42	15.42	1.26	12.36	6.20	1.99	0.73		1.91	1.41	2	smooth	*E. mantellii*
MB16_23_10_18_23	18.24	13.91	1.31	10.47	5.65	1.85	0.73		1.43	1.19	5	smooth	*E. mantellii*
MB16_23_10_18_32	20.04	12.50	1.60	11.53	5.51	2.09	0.73		2.16	1.56	1	smooth	*E. mantellii*
NM01_22_18_17_4	17.95	12.91	1.39	9.32	4.92	1.89	0.69		0.97	0.71	5	smooth	*E. mantellii*
OW01_27_8_17_19	19.91	14.64	1.36	10.88	5.66	1.92	0.80		1.74	1.26		smooth	*E. mantellii*
WB01_22_3_18_2	18.06	13.45	1.34	8.12	5.43	1.50	0.79		2.04	1.58	1	smooth	*E. mantellii*
WB01_22_3_18_3	20.74	15.66	1.32	11.78	6.20	1.90	0.80		1.22	1.11	1	smooth	*E. mantellii*
WP01_30_10_17_20	16.44	13.02	1.26	9.96	5.15	1.93	0.53		2.42	2.18	1	smooth	*E. mantellii*
WP01_30_10_17_45	17.11	13.52	1.27	10.30	5.33	1.93	0.73		1.25	0.90	4	smooth	*E. mantellii*
WP01_30_10_17_1	33.63	20.43	1.65	17.83	8.00	2.23	1.14	3.14	3.16	2.80	1	striated	*E. paraurii*
MB06_29_12_18_3	17.93	16.45	1.09	11.29	6.75	1.67	0.73		2.02	1.89	1	smooth	M5

MB16_23_10_18_11	11.72	9.84	1.19	7.58	3.80	1.99	0.65		2.22	1.30	1	smooth	M5
MB16_23_10_18_206	13.36	13.10	1.02				0.63		1.14	1.03	1	smooth	M5
MB16_23_10_18_210	10.64	10.64	1.00	7.06	4.08	1.73	0.45		1.61	1.21	1	smooth	M5
MB16_23_10_18_211	16.81	15.68	1.07				0.65		2.66	1.47	1	smooth	M5
MB16_23_10_18_24	10.87	10.46	1.04	5.75	4.27	1.35	0.53		1.41	1.36	1	smooth	M5
OW01_27_8_17_18	18.40	15.93	1.16	10.42	5.76	1.81	0.70		1.48	1.56	1	smooth	M5
OW01_27_8_17_21	13.15	12.75	1.03	8.05	4.97	1.62	0.66		1.43	1.10	1	smooth	M5
OW01_27_8_17_25	17.16	17.37	0.99	9.29	6.19	1.50	0.74		1.69	1.23	2	smooth	M5
OW01_27_8_17_3	13.77	13.28	1.04	8.62	4.92	1.75	0.71		2.28	1.87	1	smooth	M5
RSB06_30_4_18_1	15.93	14.85	1.07	11.45	5.60	2.05	0.79		2.54	1.38	1	smooth	M5
RSB06_30_4_18_2	14.84	13.48	1.10	10.57	5.26	2.01	0.69		2.49	1.73	1	smooth	M5
RSB06_30_4_18_200	13.48	12.76	1.06	9.01	4.97	1.81	0.67		1.27	1.13	1	smooth	M5
RSB06_30_4_18_201	13.46	13.37	1.01	8.55	4.64	1.84	0.71		2.28	1.82	1	smooth	M5
RSB06_30_4_18_204	14.70	14.26	1.03	9.26	4.87	1.90	0.80		2.31	2.20	1	smooth	M5
RSB06_30_4_18_205	15.10	13.25	1.14	9.28	4.80	1.94	0.59		2.70	1.87	1	smooth	M5

RSB06_30_4_18_206	14.16	13.45	1.05				0.57				1	smooth	M5
RSB06_30_4_18_207	13.64	13.31	1.02	8.89	4.61	1.93	0.73		2.53	2.28	1	smooth	M5
RSB06_30_4_18_208	14.99	13.90	1.08				0.57		2.47	1.98	1	smooth	M5
RSB06_30_4_18_209	15.41	14.81	1.04	10.53	5.37	1.96	0.68		2.45	1.81	1	smooth	M5
RSB06_30_4_18_210	15.04	14.30	1.05	10.35	5.15	2.01	0.56		2.55	1.86	1	smooth	M5
RSB06_30_4_18_211	14.70	14.40	1.02	10.81	5.38	2.01	0.66		2.39	1.64	1	smooth	M5
RSB06_30_4_18_213	15.99	15.93	1.00				0.68		2.36	1.71	1	smooth	M5
RSB06_30_4_18_214	15.73	14.78	1.06				0.76		2.67	2.01	1	smooth	M5
RSB06_30_4_18_215	14.56	14.51	1.00	10.40	5.06	2.05	0.65		2.57	2.26	1	smooth	M5
RSB06_30_4_18_216	15.21	14.85	1.02	9.78	5.34	1.83	0.87		2.58	2.29	1	smooth	M5
RSB06_30_4_18_217	14.74	13.57	1.09	10.35	5.27	1.96	0.59		3.20	2.23	1	smooth	M5
RSB06_30_4_18_218	14.00	13.61	1.03	9.39	4.90	1.92	0.76		2.45	1.68	1	smooth	M5
RSB06_30_4_18_219	14.12	13.37	1.06	9.96	5.30	1.88	0.68		1.41	1.27	1	smooth	M5
RSB06_30_4_18_220	14.86	13.74	1.08	9.70	4.93	1.97	0.53				1	smooth	M5
RSB06_30_4_18_221	13.65	13.39	1.02				0.56		2.87	2.06	1	smooth	M5
RSB06_30_4_18_222	14.41	13.69	1.05	9.98	5.11	1.95	0.62		2.13	1.81	1	smooth	M5
RSB06_30_4_18_223	15.50	14.65	1.06	10.02	5.57	1.80	0.73		3.26	1.40	1	smooth	M5

RSB06_30_4_18_224	15.48	15.12	1.02	10.86	5.34	2.03	0.67		2.58	2.32	1	smooth	M5
RSB06_30_4_18_225	14.89	14.52	1.03	10.33	5.16	2.00	0.73		2.37	1.81	1	smooth	M5
RSB06_30_4_18_226	14.43	13.97	1.03				0.81				1	smooth	M5
RSB06_30_4_18_227	16.16	14.60	1.11	10.71	5.86	1.83	0.61		2.10	1.83	1	smooth	M5
RSB06_30_4_18_228	15.22	14.03	1.08	8.95	5.74	1.56	0.70		2.46	2.32	1	smooth	M5
RSB06_30_4_18_229	14.76	14.41	1.02				0.83		2.81	2.13	1	smooth	M5
RSB06_30_4_18_230	15.43	13.86	1.11	10.14	4.93	2.06	0.68		2.84	2.05	1	smooth	M5
RSB06_30_4_18_231	14.79	14.67	1.01	9.44	4.77	1.98	0.67		2.61	2.23	1	smooth	M5
RSB06_30_4_18_232	15.06	14.13	1.07	10.66	5.21	2.05	0.64		2.55	2.15	1	smooth	M5
RSB06_30_4_18_233	15.43	14.78	1.04	10.01	5.58	1.79	0.71		1.69	1.38	1	smooth	M5
RSB06_30_4_18_234	15.37	15.00	1.02	10.41	5.22	2.00	0.72		1.99	1.26	1	smooth	M5
RSB06_30_4_18_235	14.31	14.11	1.01				0.71		2.30	1.85	1	striated	M5
RSB06_30_4_18_236	15.05	14.73	1.02				0.64		2.70	2.28	1	smooth	M5
RSB06_30_4_18_238	15.21	14.74	1.03	10.92	5.86	1.86	0.67		2.56	1.86	1	smooth	M5
RSB06_30_4_18_240	15.24	14.40	1.06	10.44	5.36	1.95	0.59		2.31	1.99	1	smooth	M5
RSB06_30_4_18_241	13.93	13.75	1.01	10.01	5.07	1.98	0.65		2.42	1.59	1	smooth	M5
RSB06_30_4_18_242	15.44	14.41	1.07	10.49	4.83	2.17	0.63		2.48	2.04	1	smooth	M5

RSB06_30_4_18_243	14.74	14.71	1.00	9.86	5.40	1.83	0.63		2.80	2.12	1	smooth	M5
RSB06_30_4_18_244	13.70	13.37	1.02	9.24	4.63	2.00	0.64		2.96	1.72	1	smooth	M5
RSB06_30_4_18_245	15.57	14.66	1.06	10.11	5.32	1.90	0.65		2.21	2.04	1	smooth	M5
RSB06_30_4_18_246	13.42	12.91	1.04	8.71	4.63	1.88	0.61				1	smooth	M5
RSB06_30_4_18_247	14.99	13.88	1.08	9.66	4.76	2.03	0.56		2.96	1.96	1	smooth	M5
RSB06_30_4_18_248	15.42	14.81	1.04	9.68	5.21	1.86	0.64		2.78	2.19	1	smooth	M5
RSB06_30_4_18_3	16.08	15.80	1.02	10.31	5.02	2.05	0.91		2.14	1.73	1	smooth	M5
RSB06_30_4_18_4	14.91	14.07	1.06	9.18	5.03	1.82	0.79		2.19	1.55	1	smooth	M5
RSB06_30_4_18_5	14.61	14.30	1.02				0.69				1	smooth	M5
RSB06_30_4_18_7	14.73	14.55	1.01	10.70	5.46	1.96	0.58		2.68	1.74	1	smooth	M5
RSB06_30_4_18_8	13.29	12.41	1.07	8.67	4.78	1.81	0.76		2.07	1.47	1	smooth	M5
WB01_22_3_18_12	17.61	16.28	1.08	11.45	6.64	1.73	0.74		2.24	1.59	1	smooth	M5
WP01_30_10_17_10	15.46	14.97	1.03	9.05	4.93	1.84	0.64		2.61	2.16	1	smooth	M5
WP01_30_10_17_11	12.85	12.54	1.02	7.93	4.70	1.69	0.61		2.36	2.13	1	smooth	M5
WP01_30_10_17_15	13.51	12.60	1.07	8.19	4.91	1.67	0.73		1.66	1.62	1	smooth	M5
WP01_30_10_17_16	13.40	12.91	1.04	9.15	4.60	1.99	0.69		2.12	1.99	1	smooth	M5
WP01_30_10_17_17	14.52	14.33	1.01	9.14	4.84	1.89	0.61		2.63	2.28	1	smooth	M5

WP01_30_10_17_2	13.82	13.46	1.03	8.65	4.95	1.75	0.69		2.50	1.73	1	smooth	M5
WP01_30_10_17_3	13.40	12.34	1.09	8.13	5.31	1.53	0.49		1.83	1.80	1	smooth	M5
WP01_30_10_17_37	14.07	11.88	1.18	8.63	4.56	1.89	0.67		2.57	1.61	1	smooth	M5
WP01_30_10_17_40	12.53	12.27	1.02	7.79	4.24	1.84	0.52		2.22	1.43	1	smooth	M5
WP01_30_10_17_41	15.87	14.26	1.11	11.36	5.27	2.16	0.83		2.74	2.20	1	smooth	M5
WP01_30_10_17_42	12.24	11.94	1.02	8.10	3.84	2.11	0.49		2.26	1.37	1	smooth	M5
WP01_30_10_17_43	14.69	12.52	1.17	9.64	5.30	1.82	0.47		2.07	1.89	1	smooth	M5
WP01_30_10_17_46	13.72	12.48	1.10	7.61	4.83	1.57	0.65		2.48	2.00	1	smooth	M5
WP01_30_10_17_47	16.60	14.88	1.12	8.49	5.65	1.50	0.71		2.46	1.70	1	smooth	M5
WP01_30_10_17_49	13.90	12.67	1.10	8.12	5.22	1.56	0.59		2.21	1.80	1	smooth	M5
WP01_30_10_17_5	14.85	14.33	1.04	9.51	4.86	1.96	0.70		2.22	1.58	1	smooth	M5
WP01_30_10_17_56	14.07	13.48	1.04	9.49	6.14	1.55	0.73		2.61	2.11	1	smooth	M5
WP01_30_10_17_58	13.38	13.30	1.01	8.13	5.23	1.56	0.59		2.21	1.97	1	smooth	M5
WP01_30_10_17_59	14.98	13.38	1.12	8.75	5.46	1.60	0.69		2.57	2.37	1	smooth	M5
WP01_30_10_17_60	15.12	14.60	1.04	8.34	5.23	1.59	0.72		2.76	1.86	1	smooth	M5
WP01_30_10_17_8	12.03	11.46	1.05	7.79	3.88	2.01	0.56		1.93	1.14	1	smooth	M5
WP01_30_10_17_9	15.37	15.09	1.02	9.98	5.08	1.96	0.77		2.58	1.63	1	smooth	M5

Appendix D.1.

Haast Tokoeka Morphology Measurements

Image/Oocyst ID	Oocyst length (µm)	Oocyst width (µm)	Oocyst L/W	Sporocyst Avg Length (µm)	Sporocyst Avg Width (µm)	Sporocyst L/W	Wall Width (µm)	Micropyle Width (µm)	Polar Granule Length (µm)	Polar Granule Length (µm)	No. Polar Granule	Wall Description	Morphotype
HA01_29_11_17_11	14.73	13.11	1.12	8.38	4.52	1.86	0.75		2.39	1.89	1.00	striated	M1
HA01_29_11_17_12	14.72	13.21	1.11	8.29	5.12	1.62	0.77		2.11	1.88	1.00	striated	M1
HA01_29_11_17_15	15.83	13.67	1.16	7.22	5.65	1.28	0.78		2.00	1.40	1.00	striated	M1
HA01_29_11_17_16	15.19	14.28	1.06	8.75	5.64	1.55	0.68		1.95	1.55	1.00	striated	M1
HA01_29_11_17_2	14.39	12.73	1.13	8.07	5.03	1.60	0.68		2.01	1.50	1.00	striated	M1
HA01_29_11_17_20	14.33	13.19	1.09	8.39	5.32	1.58	0.69		1.79	1.65	1.00	striated	M1
HA01_29_11_17_3	15.11	14.30	1.06	7.30	5.56	1.31	0.82		1.88	1.41	1.00	striated	M1
HA01_29_11_17_30	16.27	13.79	1.18	7.70	5.70	1.35	0.77		2.06	1.74	1.00	striated	M1
HA01_29_11_17_33	14.69	13.09	1.12	8.57	5.23	1.64	0.68		2.09	1.98	1.00	striated	M1
HA01_29_11_17_37	16.35	13.57	1.20	8.80	4.20	2.10	0.81		2.18	2.16	1.00	striated	M1
HA01_29_11_17_7	16.51	13.85	1.19	7.91	5.47	1.45	0.82		2.28	1.72	1.00	striated	M1

HA01_29_11_17_8	14.66	13.01	1.13	9.30	5.37	1.73	0.68		2.01	1.97	1.00	striated	M1
HA04_11_10_17_1	12.75	12.29	1.04	6.67	5.07	1.32	0.58		1.63	1.04	1.00	striated	M1
HA04_11_10_17_11	12.46	11.39	1.09	8.02	4.77	1.68	0.73		1.50	1.12	1.00	striated	M1
HA04_11_10_17_12	12.67	12.56	1.01	8.05	4.99	1.61	0.60		1.57	1.51	1.00	striated	M1
HA04_11_10_17_13	13.52	12.05	1.12	8.20	4.50	1.82	0.82		1.89	1.66	1.00	striated	M1
HA04_11_10_17_14	10.91	10.35	1.05	6.87	3.76	1.82	0.53		1.32	1.11	1.00	striated	M1
HA04_11_10_17_16	13.30	13.14	1.01	8.42	4.81	1.75	0.70		2.32	1.81	1.00	striated	M1
HA04_11_10_17_17	14.46	11.96	1.21	7.79	5.17	1.51	0.67		2.09	1.79	1.00	striated	M1
HA04_11_10_17_2	13.13	12.57	1.04	7.46	4.64	1.61	0.73		1.43	1.12	1.00	striated	M1
HA04_11_10_17_3	14.87	14.23	1.05	9.95	4.70	2.12	0.80		1.70	1.41	1.00	striated	M1
HA04_11_10_17_4	13.83	11.82	1.17	9.58	5.78	1.66	0.56		1.28	0.73	1.00	striated	M1
HA04_11_10_17_5	13.05	12.60	1.04	7.39	4.42	1.67			1.78	1.76	1.00	striated	M1
HA04_11_10_17_6	12.72	11.78	1.08	8.16	4.79	1.70	0.79		1.61	1.43	1.00	striated	M1
HA04_11_10_17_7	13.37	12.55	1.07	6.55	4.75	1.38	0.76		1.87	1.52	1.00	striated	M1
HA04_11_10_17_8	14.27	12.74	1.12	8.18	4.72	1.73	0.70		1.42	1.03	1.00	striated	M1
HA04_11_10_17_9	14.02	12.76	1.10	8.43	5.09	1.66	0.75		1.77	1.52	1.00	striated	M1
HA14_21_2_18_1	14.56	13.29	1.10	7.05	5.26	1.34	0.74		1.77	1.42	1.00	striated	M1

HA14_21_2_18_10	14.90	13.40	1.11				0.87		2.00	1.82	1.00	striated	M1
HA14_21_2_18_11	12.74	11.76	1.08	6.83	4.45	1.54	0.66		1.76	1.58	1.00	striated	M1
HA14_21_2_18_12	12.96	12.28	1.06	6.42	4.28	1.50	0.63		1.87	1.63	1.00	striated	M1
HA14_21_2_18_13	14.62	12.67	1.15	7.39	5.00	1.48	0.66		1.82	1.76	1.00	striated	M1
HA14_21_2_18_14	15.37	12.98	1.18	6.97	5.01	1.39	0.62		2.16	1.69	1.00	striated	M1
HA14_21_2_18_15	15.72	13.66	1.15	6.85	5.03	1.36	0.73		2.64	1.80	1.00	striated	M1
HA14_21_2_18_17	14.65	14.64	1.00	8.86	4.62	1.92	0.71		2.39	1.39	1.00	striated	M1
HA14_21_2_18_18	15.61	12.61	1.24	8.96	4.93	1.82	0.71		1.77	1.59	1.00	striated	M1
HA14_21_2_18_19	14.03	12.34	1.14	8.33	4.83	1.73	0.76		1.94	1.46	1.00	striated	M1
HA14_21_2_18_2	15.83	12.72	1.24				0.68				1.00	striated	M1
HA14_21_2_18_20	14.93	12.61	1.18	7.38	5.46	1.35	0.62		2.02	1.58	1.00	striated	M1
HA14_21_2_18_21	14.85	14.24	1.04	9.33	5.01	1.86	0.76		2.43	1.40	1.00	striated	M1
HA14_21_2_18_22	14.63	12.57	1.16				0.79		1.88	1.63	1.00	striated	M1
HA14_21_2_18_23	16.58	14.67	1.13				0.74		2.19	1.88	1.00	striated	M1
HA14_21_2_18_24	17.03	16.44	1.04	7.59	5.75	1.32	1.06		1.91	1.86	1.00	striated	M1
HA14_21_2_18_28	15.32	14.03	1.09				0.86		1.70	1.70	1.00	striated	M1
HA14_21_2_18_29				8.29	5.06	1.64	0.71		2.02	1.80	1.00	striated	M1

HA14_21_2_18_3	13.97	12.39	1.13	8.95	4.83	1.85	0.90		2.08	1.68	1.00	striated	M1
HA14_21_2_18_30	14.20	14.08	1.01				0.93				1.00	striated	M1
HA14_21_2_18_31	16.71	15.59	1.07	9.97	5.35	1.86	0.90				1.00	striated	M1
HA14_21_2_18_32	14.96	12.91	1.16	8.65	5.27	1.64	0.75		1.95	1.86	1.00	striated	M1
HA14_21_2_18_34	12.24	12.16	1.01	6.57	4.33	1.52	0.52		1.52	1.78	1.00	striated	M1
HA14_21_2_18_35	12.79	12.75	1.00	7.59	5.40	1.40	0.57		1.76	1.71	1.00	striated	M1
HA14_21_2_18_36	16.73	16.64	1.01	9.63	5.64	1.71	0.87		2.36	2.09	1.00	striated	M1
HA14_21_2_18_37	16.23	15.92	1.02	9.14	5.15	1.78	0.87		2.50	2.19	1.00	striated	M1
HA14_21_2_18_38	15.42	13.86	1.11	8.94	4.47	2.00	0.78		2.26	1.57	1.00	striated	M1
HA14_21_2_18_39	14.51	14.09	1.03	8.87	5.32	1.67	0.70		2.42	1.83	1.00	striated	M1
HA14_21_2_18_4	13.76	12.89	1.07	7.59	4.50	1.69	0.76		2.06	1.57	1.00	striated	M1
HA14_21_2_18_40	15.03	13.59	1.11	8.41	4.87	1.72	0.71		1.70	1.55	1.00	striated	M1
HA14_21_2_18_41	15.76	15.69	1.00	8.81	5.84	1.51	0.90				1.00	striated	M1
HA14_21_2_18_42	15.37	13.83	1.11	8.51	4.85	1.76	0.79		1.70	1.55	1.00	striated	M1
HA14_21_2_18_43	14.27	14.00	1.02	8.09	4.78	1.69	0.81		2.18	2.07	1.00	striated	M1
HA14_21_2_18_44	14.90	13.45	1.11	9.85	5.21	1.89	0.58		2.09	2.96	1.00	striated	M1
HA14_21_2_18_45	15.66	12.07	1.30	8.82	4.82	1.83	0.60		1.97	1.85	1.00	striated	M1

HA14_21_2_18_47	15.91	13.42	1.18	8.23	4.84	1.70	0.64		1.64	0.73	1.00	striated	M1
HA14_21_2_18_48	14.20	11.80	1.20	7.74	4.92	1.57	0.54		1.97	1.79	1.00	striated	M1
HA14_21_2_18_49	15.62	14.61	1.07	10.03	4.92	2.04	0.81		2.62	1.71	1.00	striated	M1
HA14_21_2_18_5	12.66	12.25	1.03	7.28	4.47	1.63	0.69				1.00	striated	M1
HA14_21_2_18_50	16.08	12.66	1.27	9.47	5.56	1.70	0.60		1.88	1.58	1.00	striated	M1
HA14_21_2_18_51	15.91	13.91	1.14	10.96	6.96	1.57	0.58		2.09	1.98	1.00	striated	M1
HA14_21_2_18_53	15.47	13.63	1.13	8.58	5.17	1.66	0.51		1.84	1.49	1.00	striated	M1
HA14_21_2_18_54	14.34	13.85	1.04	7.86	5.10	1.54	0.67		1.49	1.46	1.00	striated	M1
HA14_21_2_18_55	15.71	13.41	1.17				0.48		2.10	1.98	1.00	striated	M1
HA14_21_2_18_56	15.16	14.94	1.01	8.08	5.10	1.58	0.60		2.44	2.05	1.00	striated	M1
HA14_21_2_18_57	17.42	14.26	1.22	8.48	5.52	1.54	0.62		1.95	1.76	1.00	striated	M1
HA14_21_2_18_59	13.90	12.62	1.10				0.54		2.13	1.30	1.00	striated	M1
HA14_21_2_18_6	13.14	12.46	1.05	8.87	4.26	2.08	0.73		2.26	1.49	1.00	striated	M1
HA14_21_2_18_61	17.64	14.21	1.24	7.96	5.42	1.47	0.68		1.81	1.42	1.00	striated	M1
HA14_21_2_18_62	13.96	12.28	1.14	6.92	5.11	1.35	0.53		1.96	1.87	1.00	striated	M1
HA14_21_2_18_63	17.64	14.72	1.20	8.43	5.49	1.54	0.64		1.82	1.78	1.00	striated	M1
HA14_21_2_18_64	13.27	11.83	1.12	6.96	4.45	1.56	0.62		1.55	1.30	1.00	striated	M1

HA14_21_2_18_65	15.22	15.16	1.00	9.92	4.44	2.23	0.84		2.43	1.94	1.00	striated	M1
HA14_21_2_18_66	16.21	13.24	1.22	8.34	5.37	1.55	0.73		1.91	1.54	1.00	striated	M1
HA14_21_2_18_67	14.17	14.04	1.01				0.69		2.14	1.88	1.00	striated	M1
HA14_21_2_18_68	18.26	14.85	1.23	7.75	5.46	1.42	0.94		2.36	1.86	1.00	striated	M1
HA14_21_2_18_69	14.81	13.11	1.13	7.55	5.31	1.42	0.85		1.94	1.49	1.00	striated	M1
HA14_21_2_18_7	15.00	13.76	1.09				0.91		2.69	1.77	1.00	striated	M1
HA14_21_2_18_70	14.23	12.48	1.14	8.52	5.28	1.61	0.68					striated	M1
HA14_21_2_18_71	14.23	13.82	1.03	8.89	4.37	2.03	0.68		2.59	1.69	1.00	striated	M1
HA14_21_2_18_73	15.30	12.96	1.18	6.09	5.19	1.17	0.77		1.80	1.78	1.00	striated	M1
HA14_21_2_18_74	13.44	12.94	1.04				0.67		1.64	1.59	1.00	striated	M1
HA14_21_2_18_77	15.53	14.21	1.09	8.63	5.33	1.62	0.79		1.60	1.19	1.00	striated	M1
HA14_21_2_18_8	14.98	12.19	1.23	7.18	5.48	1.31	0.67		1.65	1.36	1.00	striated	M1
HA14_21_2_18_9	15.09	12.43	1.21	7.52	4.71	1.59	0.61		1.94	1.52	1.00	striated	M1
HA16_17_10_18_13	14.23	13.46	1.06	7.31	5.07	1.44	0.50		2.36	2.80	1.00	striated	M1
HA27_17_10_18_27	14.17	13.33	1.06	9.14	4.24	2.16	0.86		2.28	1.67	1.00	striated	M1
HA27_17_10_18_33	14.12	13.58	1.04	7.65	4.46	1.72	0.57		1.96	1.83	1.00	striated	M1
HA27_17_10_18_37	13.78	13.46	1.02	8.94	4.64	1.93	0.71		2.12	1.88	1.00	striated	M1

HA27_17_10_18_41	13.28	13.09	1.01	9.00	4.94	1.82	0.67		1.97	1.55	1.00	striated	M1
HA27_17_10_18_43	14.55	14.55	1.00	10.02	5.41	1.85	0.71		2.03	1.71	1.00	striated	M1
HA27_17_10_18_51	13.92	12.80	1.09	9.34	5.16	1.81	0.58		2.09	1.82	1.00	striated	M1
HA30_27_11_18_54	15.63	14.16	1.10	9.60	4.79	2.00	0.71		2.47	2.46	1.00	striated	M1
HA30_27_11_18_63	16.10	15.96	1.01	8.83	4.61	1.92	0.70		2.63	2.36	1.00	striated	M1
HA30_27_11_18_7	14.71	14.31	1.03	10.16	4.93	2.06	0.61		2.20	1.76	1.00	striated	M1
HA01_29_11_17_32	17.51	14.46	1.21	11.05	5.98	1.85	0.64		2.40	1.80	1.00	smooth	M2
HA01_29_11_17_5	16.23	14.10	1.15	10.73	5.21	2.06	0.78		2.39	1.88	1.00	smooth	M2
HA04_11_10_17_10	15.08	11.68	1.29	9.04	4.40	2.05	0.55		2.02	1.29	1.00	smooth	M2
HA14_21_2_18_16	13.81	12.33	1.12	7.35	4.69	1.57	0.60		2.08	1.52	1.00	smooth	M2
HA14_21_2_18_25	15.13	11.92	1.27	7.64	5.04	1.51	0.95		1.83	1.68	1.00	smooth	M2
HA14_21_2_18_33	14.45	13.77	1.05	7.31	4.83	1.51	0.50		2.44	1.87	1.00	smooth	M2
HA14_21_2_18_72	14.76	14.75	1.00	9.02	4.64	1.94	0.71		2.21	1.71	1.00	smooth	M2
HA14_21_2_18_76	15.71	12.67	1.24	8.83	5.41	1.63	0.75		2.16	1.74	1.00	smooth	M2
HA16_17_10_18_1	13.06	12.86	1.02				0.46		1.94	1.70	1.00	smooth	M2
HA16_17_10_18_10	14.23	13.50	1.05	9.25	4.90	1.89	0.61		2.11	2.09	1.00	smooth	M2
HA16_17_10_18_11	14.31	13.79	1.04	7.84	5.25	1.49	0.74		3.03	2.55	1.00	smooth	M2

HA16_17_10_18_12	13.39	12.55	1.07	7.30	4.93	1.48	0.75		1.87	1.75	1.00	smooth	M2
HA16_17_10_18_14	11.88	11.59	1.03	7.74	4.54	1.71	0.44		1.67	1.63	1.00	smooth	M2
HA16_17_10_18_15	15.02	13.89	1.08	7.25	5.40	1.34	0.70		2.39	1.79	1.00	smooth	M2
HA16_17_10_18_17	13.56	12.70	1.07	9.21	5.10	1.81	0.55		1.80	1.44	1.00	smooth	M2
HA16_17_10_18_18	12.01	11.91	1.01	8.26	4.64	1.78	0.49		1.78	1.72	1.00	smooth	M2
HA16_17_10_18_19	12.55	12.03	1.04	7.86	4.56	1.72	0.54		2.03	1.57	1.00	smooth	M2
HA16_17_10_18_2	15.69	12.31	1.27	8.38	4.95	1.69	0.63		1.86	1.66	1.00	smooth	M2
HA16_17_10_18_20	13.94	13.83	1.01	8.97	5.42	1.66	0.62				1.00	smooth	M2
HA16_17_10_18_22	14.53	14.34	1.01	9.38	5.17	1.82	0.56		1.93	1.85	1.00	smooth	M2
HA16_17_10_18_24	13.92	13.71	1.02	9.11	4.60	1.98	0.64		2.43	2.12	1.00	smooth	M2
HA16_17_10_18_27	13.50	12.18	1.11	8.45	5.01	1.69	0.44		2.59	1.79	1.00	smooth	M2
HA16_17_10_18_28	13.92	12.89	1.08	8.77	5.50	1.60	0.69		2.11	1.82	1.00	smooth	M2
HA16_17_10_18_29	13.72	12.80	1.07	8.58	5.06	1.69	0.71		2.19	2.07	1.00	smooth	M2
HA16_17_10_18_3	12.97	12.74	1.02	8.26	4.72	1.75	0.76		1.71	1.63	1.00	smooth	M2
HA16_17_10_18_32	13.21	12.34	1.07				0.76		2.18	1.89	1.00	smooth	M2
HA16_17_10_18_33	13.57	12.96	1.05	7.92	4.83	1.64	0.55		2.34	1.70	1.00	smooth	M2
HA16_17_10_18_34	13.77	13.20	1.04	8.75	4.87	1.80	0.53		2.20	1.83	1.00	smooth	M2

HA16_17_10_18_35	13.17	13.07	1.01	8.27	4.94	1.67	0.72		1.99	1.66	1.00	smooth	M2
HA16_17_10_18_36	14.27	13.15	1.09	7.97	5.43	1.47	0.72		2.41	1.53	1.00	smooth	M2
HA16_17_10_18_37	13.44	12.30	1.09	8.41	4.56	1.84	0.43		1.81	1.41	1.00	smooth	M2
HA16_17_10_18_38	15.88	14.61	1.09	10.38	5.37	1.93	0.78		2.46	2.24	1.00	smooth	M2
HA16_17_10_18_39	13.16	12.59	1.05				0.64		2.34	1.61	1.00	smooth	M2
HA16_17_10_18_4	14.35	13.02	1.10	8.61	5.23	1.65	0.53		1.88	1.88	1.00	smooth	M2
HA16_17_10_18_40	14.59	14.39	1.01	9.37	5.59	1.68	0.53		2.29	1.83	1.00	smooth	M2
HA16_17_10_18_41	14.62	14.01	1.04				0.89		2.43	1.78	1.00	smooth	M2
HA16_17_10_18_42	14.34	13.49	1.06	7.00	5.20	1.35	0.76		2.13	1.93	1.00	smooth	M2
HA16_17_10_18_43	15.18	14.82	1.02	9.44	5.52	1.71	0.80		2.57	1.70	1.00	smooth	M2
HA16_17_10_18_5	11.73	11.66	1.01	7.10	2.93	2.42	0.66		1.90	1.56	1.00	smooth	M2
HA16_17_10_18_6	13.76	13.45	1.02	9.03	4.91	1.84	0.58		2.02	1.76	1.00	smooth	M2
HA16_17_10_18_7	13.84	12.93	1.07	8.49	4.88	1.74	0.64		2.22	1.79	1.00	smooth	M2
HA16_17_10_18_8	14.09	13.64	1.03	8.97	4.82	1.86	0.65		2.32	2.07	1.00	smooth	M2
HA16_17_10_18_9	13.00	12.58	1.03	6.57	4.70	1.40	0.74		1.79	1.47	1.00	smooth	M2
HA27_17_10_18_11	16.76	13.61	1.23	8.99	5.03	1.79	0.62		2.28	1.94	1.00	smooth	M2
HA27_17_10_18_12	15.89	12.31	1.29	9.68	5.58	1.73	0.43		1.40	1.16	1.00	smooth	M2

HA27_17_10_18_15	14.19	13.40	1.06	7.34	4.47	1.64	0.76		2.14	1.49	1.00	smooth	M2
HA27_17_10_18_21	12.29	11.91	1.03	7.51	3.93	1.91	0.61		1.91	1.77	1.00	smooth	M2
HA27_17_10_18_28	14.28	13.65	1.05	6.96	5.16	1.35	0.68		2.14	1.64	1.00	smooth	M2
HA27_17_10_18_30	13.28	13.24	1.00	8.39	4.82	1.74	0.71		2.32	2.10	1.00	smooth	M2
HA27_17_10_18_31	14.64	14.50	1.01				0.61		1.89	1.36	1.00	smooth	M2
HA27_17_10_18_34	14.13	13.17	1.07	8.43	5.24	1.61	0.56		2.14	1.50	1.00	smooth	M2
HA27_17_10_18_64	13.73	11.96	1.15				0.68		2.73	1.91	1.00	smooth	M2
HA27_17_10_18_67	15.62	13.30	1.17				0.83		2.30	1.21	1.00	smooth	M2
HA27_17_10_18_77	17.22	13.86	1.24	12.37	6.17	2.01	0.56		1.18	0.96	1.00	smooth	M2
HA27_17_10_18_8	13.23	12.89	1.03	8.39	4.77	1.76	0.75		2.03	1.87	1.00	smooth	M2
HA27_17_10_18_80	13.26	12.75	1.04	7.59	4.91	1.54	0.50		2.21	1.27	1.00	smooth	M2
HA27_17_10_18_90	13.65	13.52	1.01				0.55		2.42	1.35	1.00	smooth	M2
HA27_17_10_18_91	13.31	12.48	1.07	8.76	5.11	1.72	0.47		2.19	1.79	1.00	smooth	M2
HA30_27_11_18_1	14.21	13.12	1.08	8.34	4.67	1.78	0.86		2.13	1.85	1.00	smooth	M2
HA30_27_11_18_11	14.59	13.68	1.07				0.67		2.47	1.82	1.00	smooth	M2
HA30_27_11_18_12	14.19	13.77	1.03				0.72		2.18	2.05	1.00	smooth	M2
HA30_27_11_18_13	13.43	13.38	1.00				0.62		2.14	1.96	1.00	smooth	M2

HA30_27_11_18_16	13.70	13.45	1.02				0.76		1.36	1.14	1.00	smooth	M2
HA30_27_11_18_17	14.91	14.64	1.02	9.96	5.15	1.93	0.82		2.27	1.98	1.00	smooth	M2
HA30_27_11_18_18	14.64	13.80	1.06	7.07	5.16	1.37	0.71		2.03	1.65	1.00	smooth	M2
HA30_27_11_18_2	13.89	13.81	1.01	8.85	4.55	1.95	0.79		2.21	1.66	1.00	smooth	M2
HA30_27_11_18_20	14.87	14.46	1.03	8.94	5.86	1.53	0.68		2.61	1.48	1.00	smooth	M2
HA30_27_11_18_21	13.86	13.71	1.01	9.77	5.28	1.85	0.71		2.74	1.44	1.00	smooth	M2
HA30_27_11_18_22	13.66	13.31	1.03	10.23	5.46	1.87	0.67		2.91	1.93	1.00	smooth	M2
HA30_27_11_18_24	15.34	14.61	1.05	9.87	5.67	1.74	0.90		2.34	2.12	1.00	smooth	M2
HA30_27_11_18_25	15.94	15.74	1.01	9.47	4.99	1.90	0.97		2.76	1.93	1.00	smooth	M2
HA30_27_11_18_26	13.18	12.74	1.03				0.69		2.56	1.94	1.00	smooth	M2
HA30_27_11_18_27	14.42	14.34	1.01	8.46	5.47	1.55	0.74		2.43	1.85	1.00	smooth	M2
HA30_27_11_18_28	14.46	14.21	1.02	10.07	5.14	1.96	0.68		2.46	1.93	1.00	smooth	M2
HA30_27_11_18_3	12.85	12.02	1.07	8.80	4.21	2.09	0.50		2.19	2.00	1.00	smooth	M2
HA30_27_11_18_30	13.85	13.74	1.01	7.51	5.49	1.37	0.76		1.93	1.02	1.00	smooth	M2
HA30_27_11_18_31	13.13	13.06	1.01	9.12	4.27	2.14	0.58		1.85	0.58	1.00	smooth	M2
HA30_27_11_18_32	14.12	13.99	1.01	10.49	5.32	1.97	0.80		2.06	1.92	1.00	smooth	M2
HA30_27_11_18_33	14.68	14.27	1.03	9.89	5.45	1.81	0.60		2.32	2.11	1.00	smooth	M2

HA30_27_11_18_34	14.79	14.11	1.05	8.88	4.48	1.98	0.77		2.26	1.85	1.00	smooth	M2
HA30_27_11_18_35	14.50	13.95	1.04	9.83	5.07	1.94	0.60		2.12	1.90	1.00	smooth	M2
HA30_27_11_18_36	14.12	13.57	1.04	9.44	5.56	1.70	0.74		1.25	0.92	1.00	smooth	M2
HA30_27_11_18_38	14.09	14.08	1.00	9.55	5.31	1.80	0.60		2.46	2.22	1.00	smooth	M2
HA30_27_11_18_39	15.14	14.66	1.03	8.74	5.48	1.60	0.80		2.14	2.02	1.00	smooth	M2
HA30_27_11_18_4	13.64	12.85	1.06	9.47	4.96	1.91	0.61		2.23	1.20	1.00	smooth	M2
HA30_27_11_18_40	15.11	14.23	1.06	7.40	5.42	1.36	0.72		2.28	1.91	1.00	smooth	M2
HA30_27_11_18_41	14.84	14.18	1.05	10.17	5.47	1.86	0.79		2.40	2.06	1.00	smooth	M2
HA30_27_11_18_42	14.40	14.38	1.00				0.69		2.21	1.78	1.00	smooth	M2
HA30_27_11_18_44	14.88	12.57	1.18	6.33	4.92	1.29	0.64		1.97	1.87	1.00	smooth	M2
HA30_27_11_18_45	14.24	14.01	1.02	9.38	5.34	1.76	0.60		2.74	1.84	1.00	smooth	M2
HA30_27_11_18_46	14.41	13.55	1.06	9.10	5.15	1.77	0.52		1.95	1.78	1.00	smooth	M2
HA30_27_11_18_47	14.11	13.17	1.07	7.21	4.99	1.45	0.64		2.17	2.04	1.00	smooth	M2
HA30_27_11_18_48	14.64	14.30	1.02	10.39	5.06	2.06	0.58		2.14	2.05	1.00	smooth	M2
HA30_27_11_18_49	15.84	14.03	1.13	10.02	4.92	2.04	0.66		2.21	2.06	1.00	smooth	M2
HA30_27_11_18_5	12.32	12.19	1.01	9.10	4.93	1.84	0.71		1.70	1.82	1.00	smooth	M2
HA30_27_11_18_51	14.45	13.04	1.11	9.53	5.32	1.79	0.63		2.24	1.86	1.00	smooth	M2

HA30_27_11_18_52	14.08	13.88	1.01	7.87	4.92	1.60	0.49		2.44	1.78	1.00	smooth	M2
HA30_27_11_18_53	14.00	13.18	1.06	8.60	4.64	1.85	0.55		2.10	1.94	1.00	smooth	M2
HA30_27_11_18_55	15.49	14.94	1.04				0.67		2.73	2.07	1.00	smooth	M2
HA30_27_11_18_56	14.42	14.24	1.01	9.88	5.41	1.83	0.64		2.05	1.79	1.00	smooth	M2
HA30_27_11_18_59	13.83	12.52	1.11	8.78	5.09	1.73	0.58		2.12	1.88	1.00	smooth	M2
HA30_27_11_18_60	16.00	13.50	1.18	10.88	5.24	2.07	0.77		2.19	2.12	1.00	smooth	M2
HA30_27_11_18_62	14.83	14.01	1.06	8.08	5.15	1.57	0.65		2.79	2.30	1.00	smooth	M2
HA30_27_11_18_64	13.59	12.74	1.07	8.65	4.62	1.87	0.67		2.14	1.71	1.00	smooth	M2
HA30_27_11_18_65	15.83	14.81	1.07	9.91	4.34	2.29	0.65		2.05	2.02	1.00	smooth	M2
HA30_27_11_18_66	13.69	13.64	1.00	9.75	4.97	1.96	0.74		2.10	1.55	1.00	smooth	M2
HA30_27_11_18_67	14.95	13.83	1.08	9.68	5.37	1.80	0.52		2.81	1.78	1.00	smooth	M2
HA01_29_11_17_14	20.22	14.22	1.42	10.58	5.76	1.84	0.80		2.63	2.24	1.00	smooth	M3
HA01_29_11_17_21	17.80	12.50	1.42	8.29	5.03	1.65	0.63		1.82	1.20	1.00	smooth	M3
HA01_29_11_17_31	18.24	12.41	1.47	8.42	5.84	1.44	0.77		1.30	0.81	2.00	smooth	M3
HA01_29_11_17_35	19.78	12.88	1.54				0.49		1.55	1.20	1.00	smooth	M3
HA01_29_11_17_38				11.42	5.31	2.15	0.75		2.39	1.79	1.00	smooth	M3
HA01_29_11_17_4	23.37	14.26	1.64	9.19	4.96	1.85	0.82		1.42	1.23	4.00	smooth	M3

HA01_29_11_17_6	16.49	12.55	1.31	9.80	5.04	1.95	0.70				1.00	smooth	M3
HA14_21_2_18_26	15.61	11.62	1.34	8.78	4.92	1.78	0.57		1.71	1.66	1.00	smooth	M3
HA14_21_2_18_27	17.32	12.41	1.40	8.57	5.13	1.67	0.67		1.59	1.13	2.00	smooth	M3
HA14_21_2_18_75	15.33	10.94	1.40	8.53	4.54	1.88	0.52		1.64	1.58	1.00	smooth	M3
HA14_21_2_18_85	17.64	12.10	1.46	9.23	5.52	1.67	0.77		2.64	0.99	2.00	smooth	M3
HA14_21_2_18_86	17.85	13.40	1.33				0.78		2.17	1.89	1.00	smooth	M3
HA27_17_10_18_101	21.66	12.25	1.77				0.68		1.61	1.33	2.00	smooth	M3
HA27_17_10_18_107	20.73	12.31	1.68				0.72					smooth	M3
HA27_17_10_18_108	22.58	13.65	1.65	11.20	5.32	2.11	0.80		1.28	0.64	2.00	smooth	M3
HA27_17_10_18_112	19.33	12.01	1.61	11.12	5.22	2.13	0.52		1.21	1.05	1.00	smooth	M3
HA27_17_10_18_113	21.42	12.57	1.70	10.57	5.10	2.07	0.53		1.84	1.57	1.00	smooth	M3
HA27_17_10_18_118	19.08	12.76	1.50	10.38	5.22	1.99	0.65		1.04	0.46	1.00	smooth	M3
HA27_17_10_18_119	17.91	13.66	1.31	9.87	5.42	1.82	0.66		1.77	1.29	2.00	smooth	M3
HA27_17_10_18_122	17.82	12.19	1.46	11.40	5.05	2.26	0.70		1.63	0.96	3.00	smooth	M3
HA27_17_10_18_14	20.36	13.53	1.50	10.96	5.12	2.14	0.50		1.63	0.90	1.00	smooth	M3
HA27_17_10_18_16	20.25	13.09	1.55	11.43	5.12	2.23	0.73		1.55	0.97	3.00	smooth	M3
HA27_17_10_18_17	18.25	12.55	1.45	9.43	5.34	1.77	0.65		2.38	1.37	1.00	smooth	M3

HA27_17_10_18_19	20.77	13.73	1.51				0.62		2.24	1.21	1.00	smooth	M3
HA27_17_10_18_22	17.61	12.47	1.41	11.06	5.32	2.08	0.50		1.51	1.31	2.00	smooth	M3
HA27_17_10_18_26	20.82	11.74	1.77	10.62	5.04	2.11	0.52				1.00	smooth	M3
HA27_17_10_18_3	22.11	12.03	1.84	10.19	4.70	2.17	0.61		1.73	1.09	1.00	smooth	M3
HA27_17_10_18_32	18.25	12.59	1.45	10.43	5.49	1.90	0.66		2.39	1.70	1.00	smooth	M3
HA27_17_10_18_47	18.93	12.53	1.51	10.61	5.00	2.12	0.47		2.36	1.52	1.00	smooth	M3
HA27_17_10_18_49	21.38	12.95	1.65	11.54	5.39	2.14	0.55		1.76	1.51	2.00	smooth	M3
HA27_17_10_18_5	17.44	12.72	1.37	10.38	4.86	2.14	0.65		2.10	1.77	1.00	smooth	M3
HA27_17_10_18_50	17.12	12.72	1.35	10.05	5.26	1.91	0.58		1.29	1.18	1.00	smooth	M3
HA27_17_10_18_52	16.50	12.67	1.30	9.46	5.43	1.74	0.62				1.00	smooth	M3
HA27_17_10_18_53	17.41	11.61	1.50	9.68	5.20	1.86	0.66		1.41	0.91	2.00	smooth	M3
HA27_17_10_18_6	18.55	12.65	1.47	9.73	4.63	2.10	0.52		1.33	0.81	2.00	smooth	M3
HA27_17_10_18_7	19.36	12.61	1.54	10.95	5.04	2.17	0.74		2.52	2.15	1.00	smooth	M3
HA27_17_10_18_70	19.23	12.09	1.59				0.67		2.08	1.47	2.00	smooth	M3
HA27_17_10_18_73	17.13	11.17	1.53	9.16	5.21	1.76	0.77				1.00	smooth	M3
HA27_17_10_18_74	21.09	12.63	1.67	11.33	5.26	2.15	0.55		2.77	1.52	1.00	smooth	M3
HA27_17_10_18_83	19.30	12.24	1.58	11.42	5.45	2.10	0.41		1.49	1.15	3.00	smooth	M3

HA27_17_10_18_86	23.34	12.75	1.83				0.58		2.63	1.46	1.00	smooth	M3
HA27_17_10_18_97	22.96	12.17	1.89	11.30	5.53	2.04	0.77		1.40	1.51	1.00	smooth	M3
HA27_17_10_18_98	20.37	13.95	1.46	10.93	5.55	1.97	0.65		1.67	1.20	2.00	smooth	M3
HA30_27_11_18_107	18.36	13.71	1.34	12.31	6.56	1.88	0.75		2.40	1.79	2.00	smooth	M3
HA30_27_11_18_109	23.67	16.22	1.46	11.67	6.26	1.86	0.80		3.41	1.61	1.00	smooth	M3
HA30_27_11_18_71	20.83	15.19	1.37	9.79	6.25	1.57	0.80		2.47	2.27	3.00	smooth	M3
HA30_27_11_18_72	23.87	18.23	1.31	11.52	6.46	1.78	0.77		2.65	1.91	2.00	smooth	M3
HA30_27_11_18_74	21.41	15.21	1.41	11.26	5.98	1.88	0.82		1.49	1.11	2.00	smooth	M3
HA30_27_11_18_78	20.84	14.91	1.40	11.49	5.58	2.06	0.68		1.56	1.20	1.00	smooth	M3
HA30_27_11_18_8	20.87	14.11	1.48	11.19	5.61	1.99	0.73		0.89	0.60	7.00	smooth	M3
HA01_29_11_17_1	26.16	13.16	1.99	11.39	5.46	2.09	0.61	1.98	2.25	1.70	1.00	smooth	M4
HA01_29_11_17_10	24.43	14.17	1.72	11.39	5.96	1.91	0.67	2.12	2.04	1.32	1.00	smooth	M4
HA01_29_11_17_13	21.30	12.49	1.71	11.13	5.30	2.10	0.70	1.75	2.05	1.40	2.00	smooth	M4
HA01_29_11_17_17	21.06	13.53	1.56	11.05	5.85	1.89	0.73	1.58	1.17	0.87	2.00	smooth	M4
HA01_29_11_17_18	23.13	13.25	1.75	11.82	5.59	2.11	0.77	1.73	1.74	1.00	1.00	smooth	M4
HA01_29_11_17_19	21.65	12.95	1.67	10.69	5.46	1.96	0.61	1.37	2.44	1.35	1.00	smooth	M4
HA01_29_11_17_22	21.55	13.78	1.56	8.50	5.55	1.53	0.68	1.92	1.11	0.92	1.00	smooth	M4

HA01_29_11_17_23	23.41	13.81	1.70	11.14	6.07	1.83	0.78	2.14	1.79	1.63	1.00	smooth	M4
HA01_29_11_17_24	20.58	12.22	1.68	9.69	5.11	1.90	0.75	2.09	1.36	1.03	3.00	smooth	M4
HA01_29_11_17_25	24.12	14.36	1.68	11.62	5.77	2.01	0.86	2.00	2.49	1.45	1.00	smooth	M4
HA01_29_11_17_26	23.14	14.14	1.64	11.86	5.77	2.06	0.73	1.74	1.61	1.47	2.00	smooth	M4
HA01_29_11_17_27	25.36	13.25	1.91	12.11	5.41	2.24	0.77	2.26	2.56	1.78	1.00	smooth	M4
HA01_29_11_17_28	21.86	12.59	1.74	11.54	5.49	2.10	0.73	1.68	1.39	1.31	2.00	smooth	M4
HA01_29_11_17_29	23.58	13.13	1.80	11.93	5.25	2.27	0.60	1.71	1.98	1.18	2.00	smooth	M4
HA01_29_11_17_34	23.25	13.06	1.78	11.52	5.34	2.16	0.77	1.19	2.60	1.67	1.00	smooth	M4
HA01_29_11_17_9	21.17	13.28	1.59	10.42	5.86	1.78	0.74	1.25	2.94	1.56	1.00	smooth	M4
HA04_11_10_17_15	23.08	14.22	1.62	12.91	5.52	2.34	0.69	1.77	0.94	0.75	2.00	smooth	M4
HA16_17_10_18_16	15.99	11.22	1.42	8.83	5.19	1.70	0.61	2.53	1.63	1.51	1.00	smooth	M4
HA27_17_10_18_1	21.16	12.17	1.74	9.45	5.10	1.85	0.57	1.24	2.50	2.15	1.00	smooth	M4
HA27_17_10_18_10	20.33	11.95	1.70	10.79	5.33	2.03	0.58	1.90	1.78	1.30	1.00	smooth	M4
HA27_17_10_18_100	18.97	11.68	1.62	10.87	4.55	2.39	0.83	1.52	2.31	2.31	1.00	smooth	M4
HA27_17_10_18_102	20.04	12.70	1.58	12.04	5.27	2.28	0.65	1.75	2.19	1.21	2.00	smooth	M4
HA27_17_10_18_103	22.44	11.76	1.91	10.58	4.88	2.17	0.62	1.76	1.44	1.06	1.00	smooth	M4
HA27_17_10_18_104	20.82	12.47	1.67	10.38	5.20	2.00	0.47	1.81	2.29	1.46	1.00	smooth	M4

HA27_17_10_18_105	22.89	13.08	1.75	11.53	5.27	2.19	0.64	1.86	1.35	0.82	3.00	smooth	M4
HA27_17_10_18_106	20.73	13.09	1.58	11.18	5.37	2.08	0.73	2.30	2.81	2.21	1.00	smooth	M4
HA27_17_10_18_109	20.65	13.02	1.59	10.19	4.69	2.17	0.67	2.01	1.25	0.78	2.00	smooth	M4
HA27_17_10_18_110	18.19	11.42	1.59	9.53	4.46	2.14	0.57	1.99	1.19	0.92	2.00	smooth	M4
HA27_17_10_18_111	19.41	13.07	1.49	11.06	5.19	2.13	0.49	2.23	1.66	1.26	2.00	smooth	M4
HA27_17_10_18_114	21.28	12.34	1.72	11.28	5.13	2.20	0.61	1.87	2.25	1.74	1.00	smooth	M4
HA27_17_10_18_115	20.76	11.12	1.87				0.56	1.96	1.93	1.17	2.00	smooth	M4
HA27_17_10_18_116	21.89	12.31	1.78	10.60	4.87	2.18	0.65	1.91	2.11	1.78	1.00	smooth	M4
HA27_17_10_18_117	23.50	15.04	1.56	11.62	5.55	2.09	0.76		1.83	0.94	2.00	smooth	M4
HA27_17_10_18_120	21.19	11.87	1.78	9.73	4.90	1.98	0.61	2.25	1.81	1.58	2.00	smooth	M4
HA27_17_10_18_121	19.68	12.98	1.52	11.11	5.21	2.13	0.66	2.27	2.08	1.97	1.00	smooth	M4
HA27_17_10_18_123	19.88	12.80	1.55	11.32	5.15	2.20	0.71	2.59	1.85	1.50	2.00	smooth	M4
HA27_17_10_18_124	18.67	11.77	1.59	10.37	4.99	2.08	0.45	1.65	1.95	1.06	2.00	smooth	M4
HA27_17_10_18_13	21.61	12.48	1.73	9.14	5.02	1.82	0.74	1.80	2.20	1.52	1.00	smooth	M4
HA27_17_10_18_18	23.35	12.14	1.92	9.96	5.35	1.86	0.50	2.12	2.41	1.13	1.00	smooth	M4
HA27_17_10_18_2	21.93	13.45	1.63	11.62	4.89	2.38	0.68	1.73	1.49	0.91	3.00	smooth	M4
HA27_17_10_18_20	21.47	12.95	1.66	10.86	5.01	2.17	0.56	2.28	2.08	1.94	2.00	smooth	M4

HA27_17_10_18_23	21.54	12.47	1.73	10.67	5.18	2.06	0.47	1.79	2.58	1.82	1.00	smooth	M4
HA27_17_10_18_24	21.20	11.87	1.79	10.64	4.91	2.17	0.65	1.70	1.63	1.12	3.00	smooth	M4
HA27_17_10_18_25	20.73	12.37	1.68	10.05	5.05	1.99	0.67	2.44	1.66	1.23	3.00	smooth	M4
HA27_17_10_18_35	24.86	12.47	1.99	11.14	5.76	1.93	0.69	1.54			1.00	smooth	M4
HA27_17_10_18_36	20.38	12.52	1.63	11.44	5.16	2.22	0.41	1.59	2.42	1.40	1.00	smooth	M4
HA27_17_10_18_38	19.37	12.46	1.55	11.09	4.92	2.25	0.55	1.40	1.73	1.20	2.00	smooth	M4
HA27_17_10_18_39	22.66	11.99	1.89	10.05	5.16	1.95	0.69	1.17	2.77	1.47	1.00	smooth	M4
HA27_17_10_18_4	21.63	12.42	1.74	9.00	5.05	1.78	0.65	1.90	2.38	2.03	1.00	smooth	M4
HA27_17_10_18_40	20.13	12.29	1.64	11.06	4.59	2.41	0.53	2.22	1.18	0.80	3.00	smooth	M4
HA27_17_10_18_42	17.52	11.42	1.53	10.32	4.78	2.16	0.49	1.33	2.08	1.49	1.00	smooth	M4
HA27_17_10_18_44				10.03	4.79	2.09	0.64	1.43				smooth	M4
HA27_17_10_18_45	22.07	12.13	1.82	11.21	5.40	2.07	0.50	1.62	2.09	1.68	2.00	smooth	M4
HA27_17_10_18_46	21.41	11.88	1.80	11.11	5.71	1.94	0.43	1.30	2.10	1.39	2.00	smooth	M4
HA27_17_10_18_48	20.14	12.41	1.62	11.36	5.72	1.99	0.58	1.32	2.46	2.10	1.00	smooth	M4
HA27_17_10_18_54	22.61	12.26	1.84	10.80	5.23	2.07	0.41	1.49	1.72	1.32	2.00	smooth	M4
HA27_17_10_18_56	22.10	12.14	1.82	11.74	4.83	2.43	0.59	1.83	1.79	1.55	1.00	smooth	M4
HA27_17_10_18_57	22.20	11.72	1.89	10.77	5.19	2.08	0.48	1.34	1.57	1.28	1.00	smooth	M4

HA27_17_10_18_58	21.55	13.46	1.60	10.99	5.14	2.14	0.63	2.01	2.42	2.20	1.00	smooth	M4
HA27_17_10_18_59	21.79	12.44	1.75	10.97	5.45	2.01	0.64	1.81			1.00	smooth	M4
HA27_17_10_18_60	19.36	11.72	1.65	9.64	5.06	1.91	0.42	2.07	2.48	1.29	1.00	smooth	M4
HA27_17_10_18_62	20.24	11.96	1.69	11.08	5.28	2.10	0.81	2.65	2.14	1.46	1.00	smooth	M4
HA27_17_10_18_63	23.78	14.45	1.65	11.67	5.50	2.12	0.73	1.64	1.99	1.69	1.00	smooth	M4
HA27_17_10_18_65	22.97	13.07	1.76	10.89	5.27	2.07	0.54	2.20	1.67	0.94	1.00	smooth	M4
HA27_17_10_18_66	19.11	12.85	1.49				0.62	1.91	2.65	1.85	1.00	smooth	M4
HA27_17_10_18_68	18.37	12.20	1.51	9.43	4.82	1.96	0.47	2.22	1.87	1.10	2.00	smooth	M4
HA27_17_10_18_69	22.07	12.59	1.75	11.46	5.06	2.26	0.59	2.54	2.64	1.64	1.00	smooth	M4
HA27_17_10_18_71	24.49	12.90	1.90	11.26	5.38	2.09	0.53	1.51	2.00	1.90	1.00	smooth	M4
HA27_17_10_18_72	20.38	13.03	1.56	11.36	5.28	2.15	0.59	2.09	1.67	1.61	1.00	smooth	M4
HA27_17_10_18_75	21.61	13.26	1.63	11.23	5.48	2.05	0.62				1.00	smooth	M4
HA27_17_10_18_76	22.91	12.47	1.84	9.77	4.45	2.20	0.65	1.56	1.10	0.61	4.00	smooth	M4
HA27_17_10_18_78	22.83	12.42	1.84	11.42	5.61	2.03	0.60	1.93	2.51	2.30	1.00	smooth	M4
HA27_17_10_18_79	22.15	12.47	1.78	10.97	5.59	1.96	0.62	1.63	1.40	0.95	4.00	smooth	M4
HA27_17_10_18_81	18.52	13.02	1.42	11.00	5.12	2.15	0.76	1.77	1.43	1.04	4.00	smooth	M4
HA27_17_10_18_82	20.70	12.38	1.67	10.35	5.30	1.95	0.49	2.42	1.80	1.25	3.00	smooth	M4

HA27_17_10_18_84	21.32	13.14	1.62				0.61	1.86	2.05	0.96	1.00	smooth	M4
HA27_17_10_18_85	19.14	12.55	1.53	10.76	4.95	2.17	0.56	2.11	2.62	1.39	2.00	smooth	M4
HA27_17_10_18_87	19.42	13.27	1.46	10.91	5.35	2.04	0.63	2.22	2.45	1.46	1.00	smooth	M4
HA27_17_10_18_88	19.66	12.87	1.53	10.70	5.34	2.00	0.50	2.22	1.62	1.35	1.00	smooth	M4
HA27_17_10_18_89	20.67	12.63	1.64	10.92	5.52	1.98	0.69	2.10	1.41	1.27	1.00	smooth	M4
HA27_17_10_18_9	16.70	11.30	1.48	9.38	4.36	2.15	0.55	1.62	1.95	1.46	2.00	smooth	M4
HA27_17_10_18_92	19.53	13.31	1.47	11.65	5.88	1.98	0.75	1.54	1.93	0.97	1.00	smooth	M4
HA27_17_10_18_93	20.38	11.96	1.70	10.67	5.92	1.80	0.75		1.69	1.00	3.00	smooth	M4
HA27_17_10_18_94	19.18	12.26	1.56	10.19	4.62	2.21	0.57	2.13	1.76	1.35	2.00	smooth	M4
HA27_17_10_18_95	23.75	13.38	1.78	11.73	5.63	2.08	0.68	1.74	1.62	0.97	3.00	smooth	M4
HA27_17_10_18_96	18.86	12.35	1.53	10.88	4.88	2.23	0.59	1.77	2.25	1.40	1.00	smooth	M4
HA30_27_11_18_10	23.58	15.69	1.50	11.85	6.39	1.85	0.66	2.10	2.48	1.23	2.00	smooth	M4
HA30_27_11_18_14	23.13	13.50	1.71	10.27	6.49	1.58	0.59	1.98	1.52	0.96	4.00	smooth	M4
HA30_27_11_18_15	21.76	15.72	1.38	11.18	5.97	1.87	0.86	2.29	2.06	1.15	3.00	smooth	M4
HA30_27_11_18_19	20.87	13.79	1.51	11.39	6.12	1.86	0.77	1.44	1.87	1.17	2.00	smooth	M4
HA30_27_11_18_23	22.04	15.33	1.44	11.76	6.33	1.86	0.53	2.20	2.77	2.28	1.00	smooth	M4
HA30_27_11_18_29	23.01	13.31	1.73	10.55	6.19	1.70	0.58	1.45	1.99	1.44	1.00	smooth	M4

HA30_27_11_18_37	25.38	12.83	1.98	12.07	6.50	1.86	0.52	1.78				smooth	M4
HA30_27_11_18_43	23.89	14.60	1.64	12.08	6.47	1.87	0.54	2.29	1.74	1.42	3.00	smooth	M4
HA30_27_11_18_50	24.38	14.12	1.73	11.38	6.64	1.71	0.71	2.36	2.25	2.08	1.00	smooth	M4
HA30_27_11_18_57	23.63	13.79	1.71	11.90	6.07	1.96	0.67	2.27	2.66	2.41	1.00	smooth	M4
HA30_27_11_18_58	23.02	14.83	1.55	11.78	6.45	1.82	0.63	1.49	2.42	1.88	1.00	smooth	M4
HA30_27_11_18_6	24.63	14.86	1.66	11.63	5.82	2.00	0.63	2.04	1.90	1.45	2.00	smooth	M4
HA30_27_11_18_61	20.11	14.53	1.38	9.03	6.02	1.50	0.66	1.44	2.14	0.79	1.00	smooth	M4
HA30_27_11_18_68	23.20	13.61	1.71	11.35	5.91	1.92	0.82	2.10	2.14	1.06	2.00	smooth	M4
HA30_27_11_18_69	23.88	14.22	1.68	11.93	6.23	1.92	0.75	1.77	2.37	1.69	1.00	smooth	M4
HA30_27_11_18_73	26.05	16.12	1.62				0.71	2.46	2.05	1.11	3.00	smooth	M4
HA30_27_11_18_75	24.74	13.71	1.80	12.02	7.28	1.65	0.88	1.85	2.19	1.10	1.00	smooth	M4
HA30_27_11_18_76	23.52	15.99	1.47	12.56	6.18	2.03	0.77	2.50	2.00	1.38	2.00	smooth	M4
HA30_27_11_18_77	25.44	14.75	1.72	12.81	6.65	1.93	0.73	1.54	1.99	1.18	2.00	smooth	M4
HA30_27_11_18_79	27.79	16.06	1.73	11.07	6.91	1.60	0.86	2.51	2.26	1.55	3.00	smooth	M4
HA30_27_11_18_9	23.75	16.21	1.47	10.75	7.02	1.53	0.68	1.82	2.22	1.08	2.00	smooth	M4

Appendix E.1

Illumina Amplification SOP

Primary Primer Pair: Cocci_COI_For/Cocci_COI_Rev

Primary Product Length: ~780 bp

Reference: Ogedengbe et al. (2011b)

Cocci_COI_For: 5'- GGT TCA GGT GTT GGT TGG AC -3'

Cocci_COI_Rev: 5'- AAT CCA ATA ACC GCA CCA AG -3'

PCR Mixture (50μl):

10X Buffer	5.0 μl
MgCl2 (50 mM)	2.5 μl
dNTPs (10 mM)	1.0 μl
COIF2 (10 μM)	2.5 μl
COIR2 (10 μM)	2.5 μl
1X BSA	5.0 μl
Platinum Taq	0.2 μl
Sterile Water	30.3 μl
1' product	1 μl

Program:

1 cycle	Initial Denaturation	96°C	5 min
40 cycles	Denaturation	94°C	20 sec
	Annealing	59°C	30 sec
	Extension	72°C	90 sec
1 cycle	Final Extension	72°C	10 min

Secondary Primer Pair: Illumina_COIF2/R2

Product Length: ~460 bp

Reference: Yang et al. (2013)

COIF2 Sequence (5'-3'): TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG TAA GTA CAT CCC TAA TGT C

COIR2 Sequence (5'-3'): GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGT CAT CAT ATG RTG TGC CCA

PCR Mixture (50 μl):

*10X Buffer	5.0 μl
*$MgCl_2$ (50 mM)	1.5 μl
*dNTPs (10 mM)	1.0 μl
Illumina_COIF2 (10 μM)	1.0 μl
Illumina_COIR2 (10 μM)	1.0 μl
*1X BSA	5.0 μl
*Platinum Taq	0.2 μl
Sterile Water	34.3 μl
1' product	1 μl

Program:

1 cycle	Initial Denaturation	96C	5 min
40 cycles	Denaturation	94C	20 sec
	Annealing	45C	30 sec
	Extension	72C	90 sec
1 cycle	Final Extension	72C	10 min

*ThermoFisher Scientific, Waltham, MA, USA

Appendix E.2

Illumina Analysis Reference Sequences

Species of *Eimeria*	GenBank Accession Number	Species of *Eimeria*	GenBank Accession Number	Species of *Eimeria*	GenBank Accession Number
E. reichenowi	MF503493	***E. maxima***	FJ236456	***E. tenella***	FJ236426
E. gruis	MF503489	***E. maxima***	FJ236418	***E. tenella***	FJ236455
E. gruis	MF503490	***E. maxima***	FJ236412	***E. tenella***	FJ236398
E. bosquei	MF503491	***E. maxima***	FJ236454	***E. tenella***	FJ236403
E. bosquei	MF503492	***E. maxima***	FJ236459	***E. tenella***	FJ236405
Eimeria sp.	KT184380	***E. maxima***	FJ236380	***E. tenella***	FJ236384
Eimeria sp.	KT184379	***E. maxima***	FJ236439	***E. tenella***	FJ236400
E. cylindrica	KU351702	***E. maxima***	EU025107	***E. tenella***	FJ236444
E. cylindrica	KU351687	***E. maxima***	FJ236438	***E. tenella***	FJ236447
E. canadensis	KU351701	***E. maxima***	FJ236415	***E. tenella***	FJ236453
E. auburnensis	KU351691	***E. maxima***	FJ236410	***E. tenella***	FJ236421
E. auburnensis	KU351692	***E. maxima***	HQ702481	***E. tenella***	FJ236430
E. auburnensis	KU351693	***E. maxima***	FJ236391	***E. tenella***	FJ236383
E. ahsata	KT184373	***E. maxima***	FJ236389	***E. gallopavonis***	HG793051
E. illinoisensis	KU351703	***E. maxima***	FJ236407	***E. adenoeides***	FR846201
E. bovis	KT184372	***E. maxima***	FJ236401	***E. adenoeides***	KC346357
E. bovis	KU351694	***E. cf. mivati***	FJ236441	***Eimeria sp.***	HM117017
E. bovis	KU351696	***E. mitis***	JN864949	***Eimeria sp.***	HM117019
E. bovis	KU351695	***E. mitis***	KC409030	***Eimeria sp.***	KJ547709
E. alabamensis	KT184376	***E. mitis***	KC409029	***E. intestinalis***	KP009592
E. bukidnonensis	KU351700	***E. cf. mivati***	FJ236433	***Eimeria sp.***	KT184374
E. alabamensis	KU351689	***E. cf. mivati***	FJ236434	***E. irresidua***	KP025690
E. alabamensis	KU351688	***E. mitis***	HM771681	***E. flavescens***	KP025693
E. alabamensis	KU351690	***E. ivitaensis***	MH892075	***E. piriformis***	JQ993698
E. cf. tenggilingi	JX464222	***E. mephitidis***	KT203398	***E. subspherica***	KU351704
E. brasiliensis	KU351698	***E. trichosuri***	JN192136	***Eimeria sp.***	KU255438
E. brasiliensis	KU351699	***E. tamiasciuri***	KT184375	***E. bubonis***	MN313609
E. furonis	MF795598	***E. burdai***	JQ993709	***E. anseris***	MH758793
E. cf. ictidea	KT203399	***Eimeria sp.***	JQ993700	***E. acervulina***	FJ236420
Eimeria sp.	KX094967	***Eimeria sp.***	JQ993703	***E. acervulina***	FJ236419
E. maxima	FJ236432	***Eimeria sp.***	MH698544	***E. acervulina***	FJ236443
E. maxima	FJ236449	***Eimeria sp.***	MH698546	***E. acervulina***	HQ702479
E. maxima	FJ236442	***E. alorani***	JQ993701	***E. acervulina***	FJ236427
E. maxima	FJ236393	***E. vermiformis***	HM771683	***E. nkaka***	JQ993697
E. maxima	FJ236387	***Eimeria sp.***	MH698541	***E. cahirinensis***	JQ993686
E. maxima	FJ236435	***Eimeria sp.***	JQ993707	***E. cahirinensis***	JQ993687
E. maxima	FJ236448	***Eimeria sp.***	MH698543	***Eimeria sp.***	MF496271
E. maxima	FJ236437	***Eimeria sp.***	MH698540	***E. dispersa***	HG793048
E. maxima	FJ236451	***E. pavonina***	JN596590	***Eimeria sp.***	MH777502
E. maxima	FJ236402	***Eimeria sp.***	MG595960	***Eimeria sp.***	JN699624
E. maxima	FJ236417	***Eimeria sp.***	MG595957	***E. innocua***	HG793049
E. maxima	FJ236413	***Eimeria sp.***	MG595958	***Eimeria sp.***	MK315212
E. maxima	FJ236386	***Eimeria sp.***	KJ547708	***Eimeria sp.***	MG595961
E. maxima	FJ236416	***E. tenella***	FJ236431	***Eimeria sp.***	MG595963
E. maxima	KX094964	***E. tenella***	FJ236445	***Eimeria sp.***	JQ993710
E. maxima	FJ236406	***E. tenella***	FJ236397	***E. boholensis***	MH350860
E. maxima	FJ236388	***E. tenella***	FJ236399	***E. syrichta***	MH350859
E. maxima	FJ236436	***E. tenella***	FJ236425	***E. jerfinica***	KU216011
E. maxima	FJ236409	***E. tenella***	FJ236385	***Eimeria sp.***	KU255440
Eimeria sp.	KF499078	***E. praecox***	HQ702483	***E. jerfinica***	KU215493
Eimeria sp.	KJ547710	***E. brunetti***	HM771675	***E. jerfinica***	KU216032
Eimeria sp.	KX094956	***Eimeria sp.***	KX094960	***E. jerfinica***	KU215480
Eimeria sp.	MF496272				

Appendix E.3

Frequency of sequences grouped into each cluster at 90-100% identity.

Cluster	90	91	92	93	94	95	96	97	98
Genus	144	38	62	101	72	18	23	35	29
s__aa	83	71	63	44	42	37	37	37	23
s__ab	232	198	193	120	120	117	111	109	105
s__ad	0	0	0	0	0	93	81	55	35
s__af	0	0	0	0	0	10	8	8	8
s__ai	0	0	0	0	0	0	0	14	10
s__aj	0	0	0	12	12	12	12	11	11
s__ba	0	0	0	0	0	0	0	0	44
s__bb	0	0	0	120	119	119	115	115	112
s__bc	0	0	0	0	8	0	8	8	7
s__be	0	0	0	11	0	0	0	0	0
s__bh	0	0	0	71	71	71	71	71	71
s__bj	0	0	0	0	0	0	0	0	19
s__cb	0	0	31	31	30	30	25	25	6
s__cc	0	0	12	0	0	0	0	0	0
s__cd	0	16	16	16	16	16	16	16	16
s__ci	19	18	18	18	18	17	16	16	16
s__dc	0	0	120	0	0	0	0	0	0
s__de	0	0	0	0	0	22	22	22	17
s__dh	0	0	0	0	0	0	0	0	25
s__ec	0	0	0	0	0		38	38	38
s__ee	0	0	13	13	13	13	13	13	12
s__fd	0	0	0	0	0	0	0	0	22

s__gh	0	19	19	18	18	17	17	17	15
s__hd	0	0	0	0	0	15	15	15	15
s__hf	0	0	42	40	40	38	37	35	35
s__ij	0	31	0	0	0	0	0	0	0
s__ja	0	0	0	0	13	13	13	13	13
s__rzu	0	0	0	0	0	0	0	0	1
s__rzv	0	0	0	0	1	1	1	1	1
s__rzx	0	0	0	0	1	1	1	1	1
s__rzy	0	67	23	9	4	4	4	2	1
s__sp.	3	0	2	9	18	5	0	0	0
s__sqq	0	0	0	0	0	0	0	4	2
s__sqr	1	1	1	1	1	1	1	1	1
s__sqs	0	0	0	0	0	0	1	1	1
s__sqv	0	0	0	0	0	2	0	1	1
s__sqx	0	0	0	0	0	0	2	2	2
s__srr	0	0	0	0	0	0	0	0	1
s__srs	0	0	0	0	0	0	1	1	1
s__srt	0	0	0	0	0	0	0	9	0
s__srv	0	0	0	0	0	2	2	0	1
s__ssu	0	0	0	0	0	0	0	2	2
s__ssv	0	0	0	0	0	0	0	0	1
s__ssz	0	0	0	0	0	0	0	0	2
s__stq	0	0	0	0	1	1	1	1	1
s__sts	0	0	0	9	8	0	0	0	0
s__stt	0	0	34	5	1	1	1	1	1
s__stv	0	0	0	0	0	0	0	0	1

s__stw	0	0	0	0	0	0	0	0	2
s__stx	0	0	0	0	0	0	0	0	1
s__stz	0	0	0	0	0	0	0	1	1
s__sur	0	0	0	0	0	0	0	0	1
s__sus	0	0	0	0	0	0	0	0	1
s__sut	48	0	0	0	0	0	0	1	1
s__suu	0	0	0	0	0	0	0	0	1
s__suy	0	0	0	0	0	0	0	0	2
s__svu	1	1	1	1	1	1	1	1	1
s__svw	0	0	0	0	0	0	0	0	1
s__svz	0	0	0	0	0	0	0	1	1
s__swr	0	0	0	0	0	0	2	2	2
s__sws	0	0	0	0	0	0	0	1	1
s__swy	0	0	0	0	0	0	1	1	1
s__swz	204	199	195	185	156	137	135	83	79
s__sxs	0	0	0	0	0	0	0	0	1
s__sxt	0	0	0	0	0	0	0	0	1
s__sxw	0	1	1	1	1	1	1	1	1
s__sxx	0	0	0	0	0	0	0	0	3
s__syr	0	0	0	0	0	0	0	1	1
s__syu	0	0	0	0	0	1	1	1	1
s__syv	0	0	0	0	0	0	3	2	1
s__syw	0	0	0	0	0	0	0	1	1
s__szv	0	0	0	0	0	2	1	1	1
s__tenella	0	1	1	1	1	1	1	1	1
s__tqs	0	0	0	6	5	3	3	1	1

s__tqw	0	0	0	0	0	2	0	2	1
s__tqy	0	0	0	0	0	0	3	1	1
s__tqz	0	0	0	0	0	0	1	1	1
s__tst	0	119	0	0	0	0	0	2	2
s__tsy	0	0	0	0	0	0	1	1	1
s__tts	0	12	0	0	0	0	0	1	1
s__ttt	0	0	0	0	0	0	0	0	2
s__tuu	16	16	16	16	1	1	1	1	1
s__tvw	0	0	0	0	0	0	2	1	1
s__tvz	0	0	0	0	0	2	2	2	2
s__tyx	0	0	0	0	0	0	1	1	1
s__tzx	0	0	0	0	0	1	0	0	0
s__tzz	0	0	0	0	0	0	0	0	2
s__uqq	0	0	0	0	0	0	0	0	1
s__urq	0	0	0	0	0	0	0	0	1
s__urs	0	0	0	0	0	0	0	0	1
s__urw	0	0	0	0	3	0	0	1	1
s__uts	20	0	0	0	0	0	0	0	1
s__uur	0	0	0	2	2	2	2	2	1
s__uuu	0	0	0	21	0	0	0	0	0
s__uxq	0	1	1	1	1	1	1	1	1
s__uxx	17	0	0	0	0	0	0	0	0
s__uyq	0	0	0	0	0	0	0	0	1
s__uyu	0	0	0	0	0	0	3	1	1
s__vqx	0	0	0	0	4	4	4	4	2
s__vrr	33	23	23	1	1	1	1	1	1

s__vuw	0	0	0	0	0	0	1	1	1
s__vvu	0	0	0	0	0	0	0	5	0
s__vvy	0	0	0	0	0	5	5	0	2
s__vwr	0	0	0	0	0	0	0	1	1
s__vys	0	0	0	0	0	0	2	2	1
s__vyu	0	0	0	0	0	0	3	0	0
s__vzy	0	0	0	0	0	0	0	2	2
s__wqw	0	0	0	7	5	2	2	2	1
s__wrw	0	0	0	0	0	10	9	0	1
s__wsr	0	0	3	3	3	3	3	3	3
s__wtq	0	0	0	0	0	0	0	2	2
s__wtz	0	0	0	0	0	3	3	3	3
s__wuq	0	0	0	0	0	0	0	1	0
s__wwq	82	16	13	10	10	6	3	1	1
s__wzr	0	0	0	0	0	0	0	0	2
s__xqt	0	0	0	0	0	0	0	0	1
s__xqu	0	0	0	0	0	0	0	0	1
s__xrt	0	0	0	0	0	0	5	5	5
s__xtu	0	0	0	0	15	0	0	0	0
s__xxv	0	0	0	0	0	0	0	0	1
s__ysq	0	42	0	0	0	0	0	0	0
s__yvq	0	0	0	0	45	0	0	3	0
s__zqq	0	0	0	0	0	38	0	0	0
s__zrr	0	0	0	0	22	0	0	0	0
s__zrt	0	0	0	0	0	0	0	2	2
s__zxv	0	0	0	0	0	0	0	0	3

s__zxx	0	0	0	0	0	0	0	44	0
s__zxz	0	0	0	0	0	0	3	3	2
s__zyq	0	0	0	0	0	0	0	0	3
s__zyz	0	13	0	0	0	0	0	0	0
s__xtr	0	0	0	0	0	0	0	0	2

Appendix F.1

Standard Operating Procedure

Nested Mitochondrial cytochrome *c* oxidase subunit I gene amplification Option 2

Target Sequence: Mitochondrial cytochrome *c* oxidase subunit 1 (COI) gene

Primary Primer Pair: Cocci_COI_For/Cocci_COI_Rev

Primary Product Length: ~790 bp

Reference: Ogedengbe et al. (2011b)

Cocci_COI_For: 5'- GGT TCA GGT GTT GGT TGG AC -3'

COX tenella R: 5'- CCAAGAGATAATACRAARTGGAA -3'

PCR Mixture (50μl):

*10X Buffer	5.0 μl
*$MgCl_2$ (50 mM)	1.5 μl
*dNTPs (10 mM)	2.0 μl
COIF2 (10 μM)	2 μl
COIR2 (10 μM)	2 μl
*Platinum Taq	0.2 μl
Sterile Water	31.3 μl
Sample	5 μl

Program:

1 cycle	Initial Denaturation	96°C	5 min
40 cycles	Denaturation	94°C	20 sec
	Annealing	55°C	30 sec
	Extension	72°C	90 sec
1 cycle	Final Extension	72°C	10 min

* ThermoFisher Scientific, Waltham, MA, USA

Secondary Primer Pair: COIF2/R2

Product Length: ~460 bp

Reference: Yang et al. (2013) and Dolnik et al. (2009)

COIF2 Sequence (5'-3'): TAA GTA CAT CCC TAA TGT C

COX tenella R2 (5'-3'): ATAGTATGTATCATGTARWGCAA

PCR Mixture (50μl):

*10X Buffer	5.0 μl
*$MgCl_2$ (50 mM)	1.5 μl
*dNTPs (10 mM)	2.0 μl
COIF2 (10 μM)	2.0 μl
COIR2 (10 μM)	2.0 μl
*Platinum Taq	0.2 μl
Sterile Water	36.3 μl
1' product	1 μl

Program:

1 cycle	Initial Denaturation	96°C	5 min
40 cycles	Denaturation	94°C	20 sec
	Annealing	50°C	30 sec
	Extension	72°C	90 sec
1 cycle	Final Extension	72°C	10 min

* ThermoFisher Scientific, Waltham, MA, USA

Appendix G

DRC-16

The following documents are the DRC-16 forms for Chapters 2-5.

STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANU

ate and the candidate's Primary Supervisor, certify eir work being included in the thesis and they have ndicated below in the *Statement of Originality*.

e:	Sarah Coker
ıary Supervisor:	Assoc. Prof. Laryssa Howe

Output and full reference:

s, K., Vallee, E., & Morgan, K. J. (2020). Comparing the Mini-FLOTAC and centrifugal faecal flotation for the detection of coccidia (Eimeria spp.) in kiwi (A

s the Manuscript /Published work:	Chapt
ntage of the manuscript/Published Work that was d by the candidate:	80

he contribution that the candidate has made to the Manuscri

collected all the data, drafted the paper for submissi ack, and collaborated with co-authors.

intended for publication please indicate target journal:

ure:	Digitally s Date: 202
	24 November 2020
r's Signature:	Laryssa Howe Digitally signed DN: cn=Laryssa ou=School of Ve email=L.Howe@ Date: 2020.12.0
	9/12/20

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We, the candidate and the candidate's Primary Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of candidate:	Sarah Coker
Name/title of Primary Supervisor:	Assoc. Prof. Laryssa Howe
Name of Research Output and full reference:	
Morphological and Molecular Characterisation of Coccidia in Brown Kiwi (Apteryx mantelli)	
In which Chapter is the Manuscript /Published work:	Chapter 3
Please indicate:	
• The percentage of the manuscript/Published Work that was contributed by the candidate:	80
and	
• Describe the contribution that the candidate has made to the Manuscript/Published Work:	
The candidate collected all the data, collaborated/communicated with graphic design artists and statisticians, conducted statistical analyses, and drafted/revised the contents.	
For manuscripts intended for publication please indicate target journal:	
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Candidate's Signature:	Digitally signed by Sarah Coker Date: 2020.11.24 10:23:21 +13'00'
Date:	24 November 2020
Primary Supervisor's Signature:	Laryssa Howe Digitally signed by Laryssa Howe DN: cn=Laryssa Howe, c=NZ, o=Massey University, ou=School of Veterinary Science, email=L.Howe@massey.ac.nz Date: 2020.12.09 13:56:29 +13'00'
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Name/title of Primary Supervisor:	Assoc. Prof. Laryssa Howe
Name of Research Output and full reference:	
Morphological and Molecular Characterisation of Coccidia in Haast Tokoeka (Apteryx australis "Haast").	
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Date:	24 November 2020
Primary Supervisor's Signature:	Laryssa Howe Digitally signed by Laryssa Howe DN: cn=Laryssa Howe, c=NZ, o=Massey University, ou=School of Veterinary Science, email=L.Howe@massey.ac.nz Date: 2020.12.09 13:59:05 +13'00'
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Name/title of Primary Supervisor:	Assoc. Prof. Laryssa Howe
Name of Research Output and full reference:	
The Use of Illumina Amplicon Sequencing to Detect Variation in the COI gene of Eimeria spp. from captive and wild kiwi (Apteryx spp.).	
In which Chapter is the Manuscript /Published work:	Chapter 5
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The candidate collected all the data, conducted optimisation and troubleshooting of protocol, conducted statistical analyses, and drafted/revised the contents.	
For manuscripts intended for publication please indicate target journal:	
Frontiers in Zoology	
Candidate's Signature:	Digitally signed by Sarah Coker Date: 2020.12.08 11:26:42 +13'00'
Date:	8 December 2020
Primary Supervisor's Signature:	Laryssa Howe Digitally signed by Laryssa Howe DN: cn=Laryssa Howe, c=NZ, o=Massey University, ou=School of Veterinary Science, email=L.Howe@massey.ac.nz Date: 2020.12.09 13:57:00 +13'00'
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