POULTRY CRC LTD

FINAL REPORT

Sub-Project No: Morgan 1.2.3

PROJECT LEADER: Jess A T Morgan

Characterizing population structure and diversity of Australian *Eimeria*

DATE OF COMPLETION: 31/10/2013

ISBN 1 921010 21 5

Characterizing population structure and diversity of Australian *Eimeria*

Sub-Project No. Morgan 1.2.3

Researcher Contact Details

Dr Jess A T Morgan
Queensland Alliance for Agriculture and Food Innovation (QAAFI)
Queensland Biosciences Precinct
The University of Queensland
St Lucia Qld 4072

Phone: 07 3255 4527
Fax: 07 3346 2167
Mob: 0419 676 977
Email: jessica.morgan@uq.edu.au

Dr Rosamond Godwin
Department of Agriculture Fisheries and Forestry
Queensland Biosciences Precinct
The University of Queensland
St Lucia Qld 4072

Phone: 07 3255 4530
Fax: 07 3346 2167
Mob: 0421 546 118
Email: Rosamond.Godwin@daff.qld.gov.au

In submitting this report, the researcher has agreed to the Poultry CRC publishing this material in its edited form.

Poultry CRC Ltd Contact Details

PO Box U242
University of New England
ARMIDALE NSW 2351

Phone: 02 6773 3767
Fax: 02 6773 3050
Email: admin@poultrycrc.com.au
Website: http://www.poultrycrc.com.au

Published in 2013

Executive Summary

Coccidiosis is a disease of chickens caused by infection with parasites of the genus *Eimeria*. Poultry industries worldwide lose billions of dollars annually to the disease. Ten species of *Eimeria* have been identified from Australia. Control of the disease is by chemical coccidiostats and vaccines. Live *Eimeria* vaccines use low virulence strains to boost bird immunity to the disease. Although species and strains vary in their biology and pathogenicity, they are difficult to tell apart and mixed-species infections frequently occur. Genetic markers are routinely used to diagnose species. There are no genetic markers to differentiate among strains of *Eimeria*. Recent outbreaks on vaccinated farms have highlighted concerns that the vaccine strains may not be protecting birds sufficiently. Without a means of differentiating vaccine from wild strains it not possible to discern if the problem is at the farm level or if there is an issue with the vaccine. Accurate disease diagnosis is important to the poultry industry where coccidiosis is a costly and virtually ubiquitous problem. The aim of this project was to identify new genetic markers to differentiate among Australian strains of *Eimeria*. With these markers we were interested in assessing the national status of coccidiosis.

This project sampled chicken faeces, collected from every Australian state and territory, and screened them for *Eimeria*. Five nationwide chicken faecal sampling surveys were conducted over three years. In total, 260 samples were screened visually and genetically for infective *Eimeria* oocysts. The high prevalence of uncharacterised *Eimeria* species (operational taxonomic units – OTUs) in the samples led to the development of a new species-diagnostic genetic assay targeting the mitochondrial genome. Coccidiosis was more prevalent in commercial broiler flocks, 98%, than backyard flocks, 81%. Different species dominated in backyard (*E. mitis*, OTU-Y then *E. acervulina*) compared to commercial (*E. acervulina*, *E. maxima* then *E. mitis*) flocks. This shift in dominant species likely reflected bird age and the use of in-feed chemical coccidiostat shuttle programs used in commercial flocks. Backyard flocks may be acting as important reservoirs for *Eimeria* species. All species were widespread and collection location did not affect which *Eimeria* species were present. Screening caecal samples late in the study highlighted that this study has underestimated the national prevalence of *E. tenella*. A subset of strains was passaged through laboratory birds to obtain sufficient numbers of oocysts to cryopreserve for live storage in the DAFF-QAAFI *Eimeria* collection. Forty two new stabilates, containing 123 *Eimeria* infections, were added to the collection.

DNA regions of the mitochondrial and apicoplast genomes were sequenced to identify single nucleotide polymorphisms (SNPs) to differentiate species and strains of Australian *Eimeria*.

Genetic assays were developed using pure strains of the species first. *Eimeria* apicoplast DNA proved difficult to amplify and was too conserved for strain diagnostic assays. Complete mitochondrial genomes of 25 *Eimeria* isolates representing the 7 described species plus the three operational taxonomic species (OTU) were sequenced. Species within the genus diverge by 2 to 11.5% while maximum strain diversity within a species was 0.6%. Species-specific assays targeting the variable SNPs were then developed so that DNA extracted from mixed species infections could be screened. Overall mitochondrial diversity was lower than expected for all *Eimeria* species, however, this genetic marker proved useful for identifying the high occurrence of mixed strain infections of *E. acervulina*, *E. mitis*, *E. praecox* and OUT-Z in flocks. Within Australian populations the greatest mitochondrial diversity was detected among *E. mitis* strains and *E. maxima* was also quite diverse. In contrast, little genetic diversity was found among strains of *E. acervulina* and *E. tenella* despite screening a similar number of flocks.

Publicly available nuclear genome sequences for *E. tenella* and *E. maxima* were mined for highly repetitive DNA sequences, called microsatellites. Reduced representation, 454-next generation sequence libraries were sourced for *E. necatrix* and *E. acervulina* microsatellites. Slippage of the repeat elements during DNA replication produces differences in repeat copy number which can be used to characterise strains. One-hundred–and-one genetic assays targeting microsatellite loci were developed for *E. maxima*, *E. acervulina*, *E. tenella* and *E. necatrix*. Thirty-eight loci proved variable among Australian strains. A striking finding of this study was that despite being diploid organisms, all of the species, across all of the loci, were predominantly homozygous. The best explanation for this observation is that sexual reproduction of the parasite is predominantly through selfing. Inbreeding has caused a deficit of heterozygotes across all loci. For diagnostic purposes, excess homozygocity was an advantage. The majority of strains had unique genotypes. Every *E. maxima* strain was unique, and only one duplicate strain of *E. acervulina* and *E. necatrix* was found. This result was an extremely promising outcome for vaccine strain diagnostics. Strains of *E. maxima*, *E. acervulina* and *E. necatrix*, for both the Australian vaccine (Eimeriavax) and UK vaccine (Paracox), could be differentiated from all wild strains using, at most, two microsatellite loci. The Paracox Houghton strain of *E. tenella* was also unique but the Eimeriavax Redten strain of *E. tenella* could only be distinguished from two thirds of wild isolates.

Coccidiosis in Australian flocks does not correlate with a panmictic sweep of a single genetic variant of each species. A high level of genetic diversity was observed among strains with very few duplicate genotypes found. Although extensive genetic diversity exists, multivariate analyses of strain diversity within *E. acervulina E. necatrix*, *E. maxima* and *E. tenella*

displayed little, if any geographic grouping of strains. Temporal sampling of flocks provides strong evidence that *Eimeria* species are dynamic. Within as little as four months changes in species and strains were apparent. The lack of geographic structure in the genetic signal suggests that the overall spread of coccidiosis has been extensive despite on-farm biosecurity measures to contain the disease.

Future research priorities identified in this project include 1. characterizing the impact on industry of the three poorly understood operational taxonomic units, 2. measuring the true prevalence of *E. tenella* in commercial flocks by screening caecal samples and 3. further testing is needed to optimise the strain diagnostic assays for improved diagnosis of outbreaks in vaccinated flocks.

Contents

List of tables

List of figures

Introduction

Coccidiosis is a worldwide enteric disease of chickens caused by parasites of the genus *Eimeria*. Estimated yearly worldwide losses resulting from coccidiosis to the poultry industry are estimated to be over US$2.4 billion per annum (Shirley *et al.*, 2005). Seven species of *Eimeria* have been identified from Australia based on varying biology and pathogenicity (Callow, 1984). Different strains within a species can also vary greatly in their pathogenicity. Vaccines consisting of live attenuated *Eimeria* species (EimeriaVax 4M containing four species and Paracox-8 containing seven species with two strains of *E. maxima*) have been developed as a means of countering the increasing emergence of drug resistant strains. Attenuation of *Eimeria* involves the selection for individual oocysts that are shed first in the faeces resulting from a shorter prepatent period, a reduction in the number of asexual stages in the lifecycle, and reduced schizont and meront size all lower pathogenicity while maintaining immunogenicity (Jeffers, 1975). Molecular markers are now commonly used to identify species but, despite the urgent need to understand the pathogenic differences among strains, no genetic markers have been developed that can distinguish among strains of *Eimeria*. Recent outbreaks on vaccinated farms have highlighted concerns that the vaccine strains may not be protecting birds sufficiently (Industry *pers. comm.*). For most *Eimeria* species, it is not currently possible to distinguish vaccine strains from wild infections. Accurate disease diagnosis is important to the poultry industry where coccidiosis is a costly and virtually ubiquitous problem.

Mixed *Eimeria* species infections are common but overlapping morphological characters make species identification difficult (Figure 1). Molecular identification of species is more reliable but assays based on nuclear ribosomal RNA markers have been confounded by within strain variation. Non-nuclear genetic markers offer independent genomes that do not undergo recombination. As a result genes can acquire mutations more rapidly than nuclear DNA making them suitable for species and population genetic studies. There has been limited research to distinguish strains of *Eimeria.* A recent study by Schwarz *et al*. (2009a) of the mitochondrial cytochrome oxidase 1 (CO1) gene found that this marker separated American strains of *E. maxima*, *E. tenella* and *E. acervulina* by species. They also reported some intra-specific diversity within each of the species. Preliminary screening of non-nuclear genetic markers (mitochondrial DNA and apicoplast DNA) in Australian strains of *Eimeria* (Poultry CRC project 09-27) found that these genomes would be suitable for developing diagnostic assays for the seven species of *Eimeria* and that they may also contain enough mutations to distinguish among strains within a species.

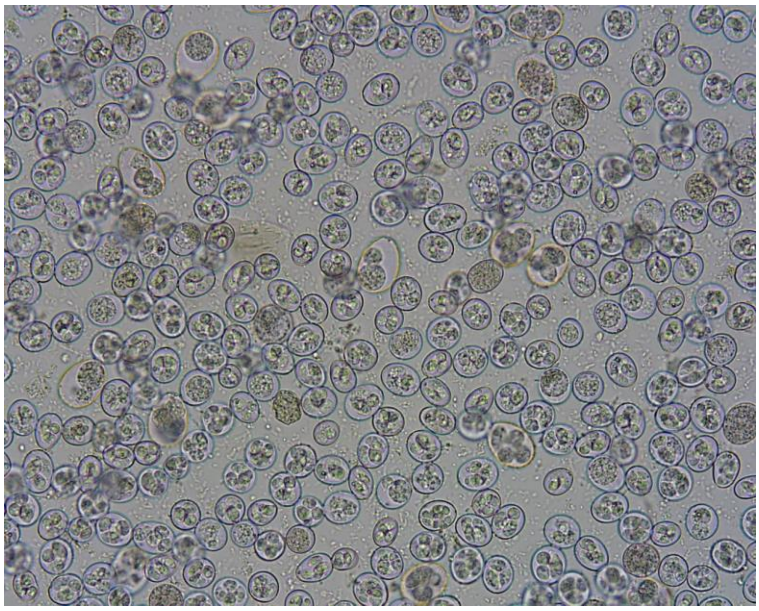


Figure 1 Microscopic image of oocysts purified from the faeces of a chicken with a mixed species infection of *Eimeria*. Photograph courtesy of Wayne Jorgensen.

Nuclear microsatellite markers are variable, generally non-coding, highly repetitive, short strings (2-6 base pairs) of DNA. They are often used as molecular markers in population genetics studies. Microsatellite markers were developed for *E. tenella* and *E. necatrix* in RIRDC project PRJ-002473. Twelve microsatellite markers were developed to distinguish among strains of *E. tenella* and six microsatellite markers were developed to distinguish among strains of *E. necatrix*. Of the nine strains of *E. tenella* screened, five unique haplotypes were identified. The Paracox vaccine Houghton strain of *E. tenella* had a unique haplotype but the 4M vaccine strain (Redten) grouped with two other strains from south east Queensland. Five unique *E. necatrix* haplotypes were identified and both vaccine strains, Mednec (4M) and Houghton strain of *E. necatrix* (Paracox) were distinct. Although sample numbers were low, strain relatedness mapped reasonably well to geographic origin. A limiting factor to both this study and the non-nuclear genome study, detailed in the paragraph above, was the paucity of strains. Sampling was limited to Queensland, Victoria and NSW with strains of some species only available from a single state. More widespread sampling of *Eimeria* strains was desperately needed to investigate Australia-wide strain differentiation.

Better diagnostic markers would allow improved coccidiosis management, resulting in more efficient chicken meat and egg production, improved animal welfare through control of the disease and greater consumer confidence from improved product image. The aim of this project was to characterise Australian strains of the seven species of *Eimeria* that infect chickens using genetic markers. To capture the genetic diversity of wild strains around the country, broad-scale sampling and screening of faeces from unvaccinated flocks was conducted bi-annually over 3 years from each Australian state and territory. For each strain

recovered a genetic profile was created based on mutations identified across the organism's three genomes (Figure 2). Regions of the mitochondrial and apicoplast genomes were sequenced for strains belonging to all species and microsatellite markers in the nuclear genome were screened for the four species of greatest economic importance (*E. tenella, E. necatrix*, *E. acervulina* and *E. maxima*). The microsatellite loci already characterised for *E. tenella* and *E. necatrix* were tested against *E. acervulina* and *E. maxima* and more loci were developed for all four species to provide greater resolving power.

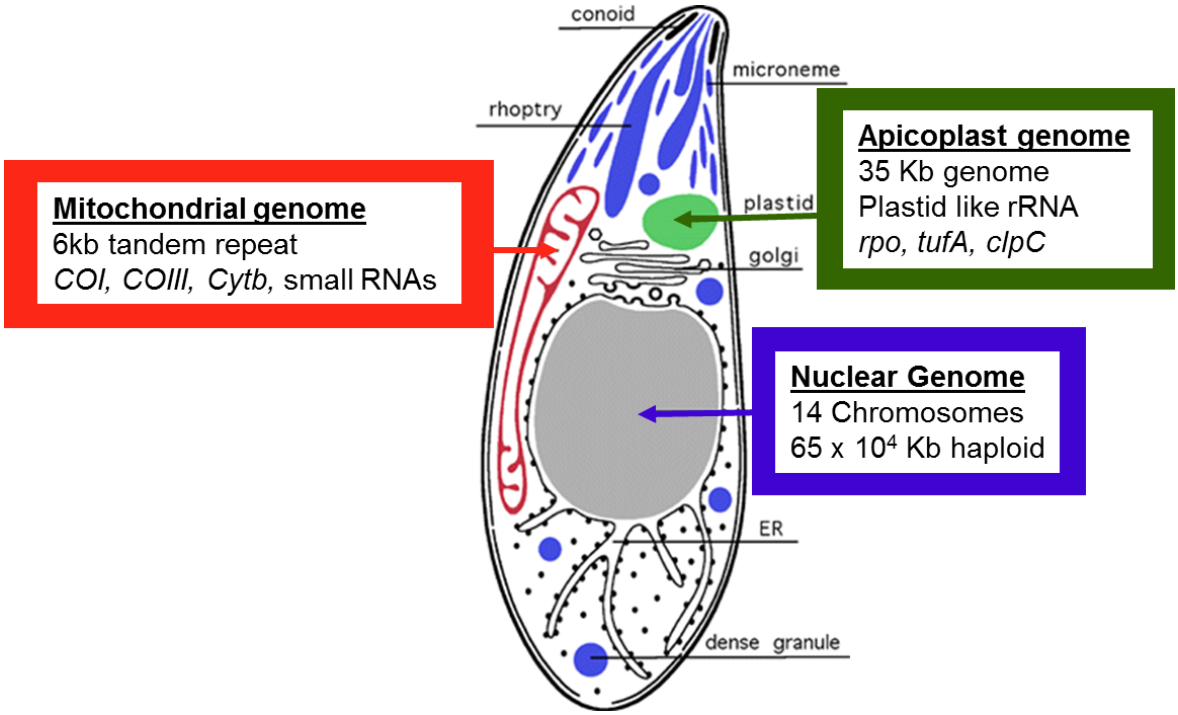


Figure 2 Schematic describing the cellular location of the three genomes of *Eimeria* (and other apicomplexans). Figure modified from http://webs.cb.uga.edu/~striepen/.

Investigating strain diversity using neutral genetic markers (DNA markers not under selection pressure) will provide information about how strains relate to one another. Understanding strain relatedness will give a large scale indication of how Australian strains of *Eimeria* have spread. Within Australia each species may be represented by one large panmictic population of *Eimeria* where outbreak strains sweep the country each year like a flu epidemic. Alternatively strains may show spatial diversity with geographic barriers maintaining unique assemblages. The temporal stability of strains is unknown; farms could have a high turnover of different strains or else stable populations might be maintained by continual cycling at background levels. Strain differences could influence how well a flock will respond to treatment. Distinguishing vaccine from wild strains of *Eimeria* would give commercial live vaccine companies an additional form of quality control for on farm monitoring.

Objectives

The project had the following specific objectives:

1. **Obtain strains of each of the seven species of *Eimeria* from every Australian state and territory**

   Over 3 years (5 sample time points) we hoped to find 5 strains per species, per state or territory. This objective may have been too optimistic for some of the rarer species e.g. *E. praecox* and *E. brunetti*. Faecal samples found positive for *Eimeria* from locations under-represented in the live *Eimeria* collection, were cycled through disease free birds to amplify the number of oocysts. A subsample of oocysts were cryopreserved to maintain the strain.

2. **Sequence 500 base pairs of mitochondrial DNA for strains of each of the seven species of chicken *Eimeria* from every Australian state and territory**

   The mitochondrial DNA sequences will be aligned and compared to identify species and strain specific mutations. A phylogenetic analysis will be conducted to investigate strain relatedness. This study will be the first to characterise the large scale genetic differences that exist among Australian strains of *Eimeria*.

3. **Sequence 500 base pairs of the apicoplast genome for strains of each of the seven species of chicken *Eimeria* from every Australian state and territory**

   As with the mitochondrial DNA, the apicoplast DNA sequences will be aligned and compared to identify species and strain specific mutations. A phylogenetic analysis will be conducted alone and on a concatenated data set to investigate strain relatedness. This study will be the first to characterise the large scale genetic differences that exist among Australian strains of *Eimeria*.

4. **Develop microsatellite markers to distinguish among strains of *E. acervulina* and *E. maxima***

   Identify and characterise at least five variable non-coding, highly repetitive, short strings of DNA called microsatellites in *E. acervulina* and *E. maxima*. Genotype the microsatellite loci for strains of *E. tenella* and *E. necatrix* (using existing loci) and *E. acervulina* and *E. maxima* from every Australian state and territory. Strains will be characterised by a combined genetic profile of DNA sequence based mutations (Objective 2) and locus specific microsatellite alleles. The ability to distinguish among strains and species of *Eimeria* will assist the poultry industry to track the occurrence and spread of coccidiosis and better monitor control measures.

5. **Temporal sampling from known locations where historical strains were isolated**
   Sample and real-time PCR screen faeces from flocks where historical *Eimeria* strains were collected. If samples are positive for the same species, cycle oocysts through disease free birds to amplify the oocysts and cryopreserve a subsample. Screen the strains from the two time points following objectives 2 - 4 above to determine if they are the same. These comparisons will assess how stable *Eimeria* strains are through time.

Methodology

Sampling

The Department of Agriculture Fisheries and Forestry (DAFF) (formerly DEEDI, formerly DPI&F), until 2011 based at the Animal Research Institute (ARI), but since 2011 located at The University of Queensland (UQ) in Brisbane, houses one of Australia's best collections of poultry *Eimeria* genomic DNA isolates (Table 1). Pure strains of each species were used for preliminary genetic marker testing and development.

Over the duration of the project Australia wide sampling of faeces from unvaccinated poultry farms and flocks was conducted twice per year for 3 years. Over each sampling period fresh faeces (pooled sample of roughly 50 grams) were collected from 5 unvaccinated flocks (mix of commercial broiler and back-yard) from every Australian state and territory. CRC collaborators were consulted, via email and at meetings, prior to collection to determine the best time of year for sampling (early and late summer). Samples were collected fresh for live transport to the laboratory for screening. Industry was also invited to submit faecal samples from farm outbreaks throughout the term of the project for screening.

Temporal sampling of historical isolates (where the origin of the sample was known and birds were still present) was also conducted. Mixed-species vaccine samples (Eimeriavax and Paracox) were kindly provided by industry.

Table 1 Species and strains of *Eimeria* in the DAFF genomic DNA collection.

Species	Strain	Purity	Geographical origin	Year collected
E. acervulina	Newace$^{\Psi}$	pure	Queensland, Aus	1993
	Medace	pure	Victoria, Aus	<1998*
	Ponace$^{\Psi}$	pure	Queensland, Aus	1994
	Olyace II	pure	Queensland, Aus	<1994*
	Royace$^{\Psi}$	pure	Queensland, Aus	1995
E. brunetti	Bowbru$^{\Psi}$	pure	New South Wales, Aus	<2001*
	Monbru$^{\Psi}$	pure	South Australia, Aus	1999
	Roybru$^{\Psi}$	pure	Queensland, Aus	1995
	Bonbru	pure	New South Wales, Aus	2000
	Badbru	pure	New South Wales, Aus	<2009*
	Ingbru	mixed	Victoria, Aus	1998
	Andbru	mixed	Queensland, Aus	<1995*
E. maxima	Medmax$^{\Psi}$	pure	Victoria, Aus	<2003*
	Ingmax	pure	Victoria, Aus	2002
	Logmax	pure	Queensland, Aus	1997
	ARI-M3-max$^{\Psi}$	mixed	Victoria, Aus	2002
	ARI-M12$^{\Psi}$	pure	New South Wales, Aus	2002
E. mitis	Jormit$^{\Psi}$	pure	Queensland, Aus	1995
	Kelmit$^{\Psi}$	pure	Queensland, Aus	1995
	Redmit$^{\Psi}$	pure	Queensland, Aus	1995
	Beemit	mixed	Queensland, Aus	1998
E. necatrix	Gatnec$^{\Psi}$	pure	Queensland, Aus	1996
	Mednec$^{\Psi}$	pure	Victoria, Aus	1996
	Gronec$^{\Psi}$	pure	Queensland, Aus	1996
	Kewnec	mixed	Western Australia, Aus	2009
	Ingnec	mixed	New South Wales, Aus	2009
	Macnec	mixed	Queensland, Aus	1995
E. praecox	Jorpra$^{\Psi}$	pure	Queensland, Aus	1995
	Ingpra$^{\Psi}$	mixed	New South Wales, Aus	1998
	Andpra$^{\Psi}$	mixed	Queensland, Aus	<1997*
	Medpra	mixed	Victoria, Aus	?
	Beapra	mixed	Queensland, Aus	<1996*
	ARI-M3-pra	mixed	Victoria, Aus	2002
E. tenella	Redten$^{\Psi}$	pure	Queensland, Aus	1995
	Darten$^{\Psi}$	pure	Queensland, Aus	1995
	Ingten$^{\Psi}$	mixed	New South Wales, Aus	1997
	Macten	mixed	Queensland, Aus	1995
	Medten	pure	Victoria, Aus	<1996*
	Narten	mixed	New South Wales, Aus	2009
	Orgten	mixed	Queensland, Aus	2009
OTU-X	X1$^{\Psi}$	pure	Victoria, Aus	<2006*
	X2	pure	?	<2007*
OTU-Y	Y1$^{\Psi}$	pure	Victoria, Aus	<2006*
OTU-Z	Z1$^{\Psi}$	pure	Victoria, Aus	<2006*
	Z2$^{\Psi}$	pure	New South Wales, Aus	<2006*

$^{\Psi}$ Strains used for mitochondrial genome sequencing and assay development

* First record of sample in database, collection date earlier

Faecal sample processing

Upon arrival at the laboratory the faeces were thoroughly mixed with a wooden applicator stick then 2 vials of 200 mg each were sub-sampled into 2 mL plastic screw cap tubes and stored at 4°C for subsequent DNA screening. The remaining faeces were transferred to 1 L plastic bottles containing 200 ml of 2% potassium dichromate and then placed on rollers for 3 days to sporulate any potential oocysts present. After three days of rolling, 5 mL of the slurry was sub-sampled and screened visually for oocysts using a modified Sheather's (Sheather, 1923) sugar flotation solution (Anderson, 1981). Faecal samples that were visually positive for *Eimeria* were filtered and oocysts were cleaned and purified using salt flotation following Jorgensen *et al.* (1997). Purified oocysts were resuspended in 5 mL 2% potassium dichromate and stored at 12°C. Oocysts were counted microscopically using a McMaster chamber (Hodgson, 1970).

Genomic DNA extraction

DNA was extracted from up to 1,000,000 purified oocysts following Morgan *et al*. (2009a). Briefly oocyst walls were cracked prior to lysis using a bead-beater and 1 mm glass beads then the lysate was extracted using a DNeasy Blood and Tissue kit (Qiagen, Valencia, CA, USA).

DNA was also extracted directly from 200 mg samples of faecal material using a QIAamp DNA Stool Mini Kit (Qiagen, Valencia, CA, USA) to remove PCR inhibitors (Morgan *et al.*, 2009a). A negative control was included in every extraction run.

Species diagnostics

Real-time PCR assay

Species diagnostic real-time PCR (rt-PCR) assays (Morgan *et al.*, 2009b) using species-specific TaqMan® MGB probes targeting the second internal transcribed spacer of nuclear ribosomal DNA (ITS2) were used to detect and quantify the seven described *Eimeria* species (Table 2). Screening was conducted using a Rotor-Gene 3000 (Corbett Research, Mortlake, NSW, Australia) and at the completion of the run the dynamic tube was turned on, the data was slope-corrected and the threshold line was set at 0.01. The point at which the curve crossed the threshold line, the cycle threshold (Ct) score, was recorded for each sample. A negative extraction, negative PCR control, and a positive sample control were run with each screening. Samples with Ct scores ≤35 were recorded as *Eimeria* positive infections.

Table 2 Primer and probe sequences for real-time PCR assays for the seven species of poultry *Eimeria*.

Species target	Primers	Sequence 5' to 3'
E. tenella & *E. necatrix*	Etene-ITS2F	TATGSTCCTTTCATTCBGAAAGAGA
E. maxima & *E. acervulina*	Emaac-ITS2F	CCTTTCGTYCAYGRAAGAGAT
E. brunetti & *E. acervulina*	Ebrac-ITS2F	CCTTTCGTCCAYGAAAGAGATA
E. praecox & *E. mitis*	Eprmi-ITS2F	CCYTTCGTTHAYGRAAGAGAT
All species reverse primer	E-28SR	CTCGMCTGATTTCAGGTCTA
Species target	TaqMan®Probe	Sequence 5' to 3'
E. tenella	tenFAM	AATGTTTTGAGCAGGGCTA
E. brunetti	bruFAM	AGTGCTACTGGTGGATAT
E. praecox	praFAM	AATGAACGATTCTAGCATGCA
E. maxima	maxFAM	TGTACTACTGAATTGTATCTCGGA
E. necatrix	necVIC	AATACGCACAGCACATGT
E. acervulina	aceVIC	CACTGGTGTATATCTCGAAAT
E. mitis	mitVIC	TTTTCCTGTTGTGAGTTGTGTGT

Capillary-electrophoresis assay (CE-assay)

Due to inconsistencies between visual oocyst screening and real-time PCR results a new diagnostic assay was developed for rapid screening of *Eimeria* DNA. The mitochondrial DNA PCR fragment length diagnostic assay uses PCR-coupled capillary electrophoresis. Generic primers targeting a non-coding region, just downstream of the 3' end of cytochrome oxidase III (*Cox*III), amplified length diagnostic fragments (174-197 bp depending on the species) (Table 3 and Table 4). The forward primer was given an M13 extension so that an additional 6-FAM-labeled M13 forward primer could be included in the reaction for product detection.

Table 3 Generic primers for mtDNA based PCR fragment length diagnostic CE-assay for *Eimeria* species of chickens.

Primer name	Direction	Sequence 5'-3'
M13EmtF	Forward	GAGCGGATAACAATTTCACACAGGCGTAAACATGCGAACTCACTTG
M13FAM	Forward	6FAM-GAGCGGATAACAATTTCACACAGG
EmtR	Reverse	GGATAYDTTGCATTATCCTATGC

Table 4 PCR fragment length differences (including primers) for mitochondrial DNA generic species detection CE-assay.

Species	Product size with M13 tag (bp)	Bin position in Genemapper (bp)
E. brunetti	198	198
OTU-Y	203	203
OTU-Z	205	205
E. acervulina	208	208
E. mitis	211	210
E. praecox type 1	214	213
E. praecox type 2	215	214
OTU-X	217	217
E. maxima	218	218
E. tenella	220	219
E. necatrix	221	220

A Multiplex PCR kit (Qiagen, Valencia, CA, USA) was used to perform PCRs. Undiluted genomic DNA was added if extracted directly from stool samples or diluted 1 in 10 or 1 in 100 if DNA was extracted from purified oocysts. PCRs were conducted in 6 µL volumes using 0.2 pmol M13EmtF, 2 pmol M13FAM, 2 pmol EmtR , 1 µL DNA, 0.6 µL 5x Q solution and 3 µL Qiagen Master Mix 2.5x. The following thermo-cycling conditions were used: 95ºC for 15 min (initial denaturation); 94ºC for 30 s (denaturation); 50ºC for 45 s (annealing); 72ºC for 90 s (extension) for 35 cycles followed by 72ºC for 45 min (to ensure complete extension) in a thermal cycler (Biorad DNA Engine Peltier).

Amplicons were then diluted 1 in 40 with water and 2 µL of each dilution were mixed with 10 µL formamide plus LIZ 500 size standard (1: 0.005 ratio) (Applied Biosystems, USA). Samples were denatured at 95ºC for 3 min 30s and then chilled on ice before being electrokinetically injected into a POP-7 polymer matrix using a 50cm, 16 capillary, 3130XL DNA Genetic Analyser (Applied Biosystems, USA). Electrophorectic profiles for individual samples were captured and .fsa files were analysed using Genemapper (v3.7, Applied Biosystems USA). Allele calling was based on samples achieving signal threshold of at least 100 relative fluorescence units (rfu). For the 3130XL Genetic Analyzer, saturation occurs at 8000 rfu and therefore any peaks of this size detected were off scale and required further dilution.

A mixed template positive control, containing all seven species (including two variants of *E. praecox*) and the three OTUs, was also included with each PCR. The positive control was prepared by individually extracting DNA from 10^5 oocysts of each species. The extracted DNA was then quantified using a Nanodrop 1000 spectrophotometer (Thermo Scientific,

USA). DNA concentration, rather than oocyst count, was used to quantify sample concentrations. This was due to inconsistent levels of oocyst sporulation among samples. An aliquot of each extraction was diluted to 1 ng μL^{-1} and 1 ng of each species was mixed together to give an 11 sample mix. One microlitre of this 11 sample mix was then used as the PCR positive control template which was included with every screen.

CE-assay specificity and sensitivity

The ability of the assay to PCR-amplify target fragments was first tested on pure DNA from two strains of each species of *Eimeria* and each of the OTUs (Table 1). The assay was then tested on a DNA mixture containing 0.1 ng of DNA from all seven *Eimeria* species, including two genetic length variants of *E. praecox*, and three OTUs making an 11 sample, 10 species mix.

The sensitivity of the assay was assessed in two ways, first to give an indication of the detection limit for DNA copy number and second, to estimate the assay's ability to detect DNA from decreasing numbers of oocysts in a sample such as those from field samples. In the first instance, DNA was extracted from 10^5 or 10^6 oocysts of each individual *Eimeria* species except OTU-Y for which there were insufficient oocysts. Both variants of *E. praecox* were also tested. This DNA was then diluted using a 10 fold serial dilution and oocyst equivalents ranging from 1000 down to 0.0001 were used as template for PCR. The resulting amplicons were then assessed with the genetic analyzer as described above.

Second a 10-fold serial dilution (10^5- 10^0) was made of pure *E. maxima* oocysts. DNA was then extracted from each of these oocyst dilutions and the resulting DNA was used for PCR. Other individual *Eimeria* species were not tested in this way owing to the limited availability of fresh pure oocysts.

CE-assay reproducibility

Reproducibility was tested by conducting the CE assay on the 11 sample mixed-template positive control on at least 20 different occasions. Additionally the assay was tested in triplicate on three replicate DNA extractions of the commercial vaccine Eimeriavax 4m (Bioproperties, Victoria, Australia) and two replicate DNA extractions of Paracox 8 (MSD Animal Health, UK). Both Eimeriavax 4m and Paracox 8 are live mixed-species vaccines containing precocious attenuated strains. Eimeriavax 4m contains *E. acervulina*, *E. maxima*, *E. tenella* and *E. necatrix* oocysts in a 1:2:3:2 ratio, while Paracox 8 contains *E. brunetti*, *E. acervulina*, *E. mitis*, *E. maxima*, *E. tenella*, *E. necatrix*, and *E. praecox* in a ratio of 1:5:10:3:5:5:1. It was anticipated that testing the reproducibility of the CE assay on

multispecies vaccine strains would give a good indication of the assay's performance for detecting mixed species infections in field samples.

Laboratory propagation of oocysts

Samples visually positive with moderate to high infection levels, and collected from locations poorly represented in the live oocysts collections, were selected for passaging through chickens to amplify the available number of oocysts. Animal ethics permission was obtained to infect up to sixty chickens per sampling period (Animal Ethics Approval SA 2011-02-345).

Animal housing

Birds were a cross of Rhode Island Red and Rhode Island White Bond's, obtained at one day old from a minimal disease flock. While research was conducted at the ARI facility the day old chicks were immediately placed into positive pressure isolators, with HEPA filtered air supply, in a designated clean chicken room (Figure 3a). In later trials based at CAAS Gatton, and then Pinjarra Hills, clean chickens were raised in large, disposable cardboard boxes (lawn-mower cartons) in a designated, heated (30-35°C) clean chicken room (Figure 3b). Strict biosecurity and hygiene measures were applied to avoid any contamination of the birds with *Eimeria* parasites. During rearing birds were given an in-feed coccidiostat [Cycostat 66 (robenidine hydrochloride 66g/kg)]. Faeces of the birds were screened weekly using oocyst sugar flotation (Anderson, 1981) to ensure they were *Eimeria* free prior to use in trials. Birds were reared for a minimum of three weeks then transferred to cages in isolated rooms prior to inoculation with field samples. All facilities and housing were approved by the Department of Primary Industries Animal Ethics Committee (ARI Animal Ethics # SA 2011-02-345).

Figure 3 Rearing of *Eimeria*-free chickens in a. positive pressure isolators at ARI or b. newspaper lined, disposable cardboard boxes with mesh covers at Gatton and Pinjarra Hills.

After each collection period 30 chickens were infected with 15 wild *Eimeria* samples. For each field sample two birds (three-week-old disease free chickens) were inoculated. It was not possible to house cages in 15 independent rooms, however, 8 independent rooms were available so samples were separated by state of origin (i.e. a room for NSW samples, another for WA samples etc) thus reducing the potential of cross-contamination to within state. Strict biosecurity and hygiene measures were put into place during propagation (gloves changed between cages; no unnecessary handling of birds; gloves, boots and lab coats changed between rooms, where possible different people dedicated to rooms). Propagation of the 15 samples was split into two rounds for reasons of practicality (roller space, processing time and additional biosecurity).

Birds were placed in cages (2 birds per cage) with wire mesh floors over trays to allow for easier access to faecal samples (Figure 4a). After a settling period of 3 days birds were inoculated with up to 1 million sporulated oocysts in 1mL PBS (phosphate buffered saline) with a disposable pipette (Figure 4b). Doses over 10,000 oocysts correspond to samples that had been stored for 10 months in a 12°C incubator prior to inoculation and were presumed to have suffered a significant decline in oocysts survival (Jeston *et al*., 2002). Trays were scraped on day 4 post inoculation, and faeces were collected until day 10 post inoculation. On day 10 birds were euthanized, faeces were collected in 1L plastic bottles and 300 mL of

2% potassium dichromate was added. The bottles were placed on rollers and rotated at 2 rpm for 72 hours to sporulate the oocysts. The slurry was then blended and sieved (1 mm mesh) to remove larger debris. Oocysts were then cleaned and purified using salt flotation (specific gravity = 1.2) as described in Jorgensen *et al.* (1997). Oocysts were counted using a McMaster chamber (Hodgson, 1970). Purified oocysts were stored in 2% potassium dichromate in a 12°C incubator prior to cryopreservation.

a b

Figure 4 Example of a. wire cages with faecal collection trays used for housing *Eimeria* infected chickens and b. inoculating birds with a disposable plastic pipette.

Waste removal and decontamination

Following completion of the experimental work, birds were euthanized via cervical dislocation (Animal Ethics Approval SA 2011-02-345). All birds and contaminated laboratory materials were secured in biosecurity bags and removed for incineration by a biohazard waste disposal contractor. All experimental facilities were decontaminated using Divosan Q-cide™ and washed with water (hot where available), cages, trays, and scrapers were additionally heat-treated in ovens at 80°C for a minimum of two hours.

Oocyst cryopreservation

For samples where more than 1 million oocysts were recovered following propagation, sporulation and separation, oocysts were cryopreserved in liquid nitrogen at 0.25–1 million oocysts per vial following Shirley (1995). Briefly oocysts were washed in Eagles minimum essential medium (MEM) to remove the potassium dichromate. Oocysts were then cracked using 2 mm glass beads using short 1 minute bursts of vortexing followed by microscopic examination. Once at least 90% of the cell walls were disrupted they were suspended in cryopreservative mixture containing 20% foetal calf serum (FCS) and 15% dimethyl sulphoxide (DMSO) and 65% of minimum essential medium (MEM) and 2% Antibiotic/

antimycotic (A/b). Tubes were frozen by placing on a polystyrene float above liquid nitrogen prior to storage in a cryotank at -180°C.

Population genetic marker screening

Mitochondrial DNA sequencing

Complete mitochondrial DNA genomes were sequenced for 3 strains of each of the seven species of *Eimeria* and the three OTU (species and strains see Table 1, primers see Table 5). The genomes were aligned to each other and to publically available sequences for other strains using ClustalX v1.8 (Thompson *et al.*, 1997). Within and between species diversity was determined using PAUP* (Swofford, 2002). Species-specific PCR primers (2 assays per species, Table 6) were designed by eye to span the most informative single nucleotide polymorphisms (SNP's) for characterising Australian strains.

Amplification reactions were carried out in 10 µl volumes containing 0.5 µM of each primer pair, combined with 10-100 ng of extracted DNA, 10x HotMaster Taq buffer (Eppendorf, Australia, containing 25 mM magnesium), 0.8 mM dNTP, and 0.05 units/µl of HotMaster Taq DNA polymerase (Eppendorf, Australia). Thermal cycling conditions consisted of an initial denaturation (95°C for 4 minutes) followed by 30 cycles of 95°C for 30 seconds, 47-57°C (for specific temperatures refer to Ta column in primer tables 5 and 6) for 30 seconds and 72°C for 1 minute 30 seconds, with a final extension step of 72°C for 7 minutes. Cycling was performed in a Biorad thermal cycler (DNA Engine Peltier). PCR products were viewed on 1.5% agarose TBE gels stained with GelRed (Biotium, USA). PCR products were concentrated and desalted prior to sequencing using Exosap-it® (USB Corporation distributed by GE Healthcare Bio-Sciences, Rydalmere NSW, Australia). Approximately 20 ng of DNA was used in standard ABI Dye Terminator sequencing reactions using Big Dye Vers 3.1 technology (Applied Biosystems, California, USA) and were run on an Applied Biosystems 3130XL Genetic Analyser (Griffith University DNA Sequencing Facility, Queensland, Australia). Sequence data was edited and aligned with Sequencher (Vers 4.8 Gene Codes Corporation, Ann Arbor, MI, USA).

A phylogenetic tree were constructed using maximum likelihood analysis in PAUP*, Vers 4.0b10 (Swofford, 2002). Before generating the tree a series of likelihood ratio tests were completed using Modeltest (Vers 3.04; Posada and Crandall, 1998) to determine the best nucleotide substitution model to use for the likelihood analysis. A general time reversible model (GTR) with estimates of invariant sites (I) and among site heterogeneity (G) was selected (summarized as GTR+I+G). The command line for PAUP analysis is below.

Lset Base = (0.3044 0.1686 0.1684) Nst=6 Rmat=(1.6668 2.6296 1.7918 0.3275 5.7404) Rates=gamma Shape=0.7714 Pinvar=0.4942;

Unweighted trees were found using heuristic searches with random sequence addition and tree-bisection-reconnection (TBR) branch swapping. Other settings used were Mulpars in effect, Maxtrees set to 200 and heuristic search repetitions were set to 1.

Table 5 Details of the generic primers designed to span the complete mitochondrial genome for the amplification of all *Eimeria* species of chicken.

Primer	Sequence 5' to 3'	Position	Ta*
E-mt-F1.1	TTAACACCTCCATGTCGGCTC	478	53
E-mt-R1	CTTTCCGGTTGTTTCCATCTC	1620	53
E-mt-F2	TGGGGATCCAATCCAGTGC	1516	53
E-mt-R2.1	CADATAGCTTCYACRAAATGCCA	2574	53
E-mt-F2.5	CTWTGGATTACAGGWYTACACTT	2428	53
E-mt-R2.5	TCGGGTAAATTCCGTCCTGC	3404	53
E-mt-F3	AGGGAAGTAAAGGTGCTCAG	3285	53
E-mt-R3	CCCCAGAAACTCATTTGACC	4358	53
E-mt-F4.1	GTTTATTATGTCTCAAGTGAGATC	3997	53
E-mt-R4.1	ATACCTAATTCYTTATGGTTTGC	5119	53
E-mt-F4.5	CAAGAAATTGYGCAACATCTTGG	4924	53
E-mt-R4.5	ACDGKCATCATATGRTGTGCC	5962	53
E-mt-F5	TGGTGATCCAGTATTATATCAAC	5795	53
E-mt-R5	GATAGGGAACAAACTGCCTCA	560	53

Ta= optimal annealing temperature for primers

Table 6 Species-specific primers for mtDNA strain differentiation within eight species of *Eimeria.* Primers flank single nucleotide polymorphisms (SNPs) in the mitochondrial genome. For some primers the penultimate 3' nucleotide was modified (italicized and marked in bold) to improve specificity.

Species Primer name	Sequence 5' to 3'	Position[Ψ]	Ta*	Target SNP	Product size bp
E. acervulina					
ace197F	TTTAAAAAATTAATTGGTTGTAT	75	47	1	439
ace197R	TCAGGGGTGTATGTAATG	474			
ace3430F2	AAATGAGGCTTGATGGTTA***A***G	3266	50	2	657
ace3430R2	AGAATCTTTTTAATGTAGGAC***C***G	3882			
E. maxima					
max3422F	GCAGTAGCGGTAATACTATA	3305	47	3	579
max3422R	AAACCTCCTAATAACCATGAA	3844			
max3560F3	ACTGGGGCGCTACTGTAA***C***C	4078	55	7	1048
max3560R3	ATCGGTACTAATAACAGTGATATG	5104			
E. necatrix					
nec946F2	AAGAAATTTTGGTTTCCCT***C***C	260	53	5	1261
nec946R2	CAGCTTCTCTGAATGTGA***T***A	1485			
nec5364F2	GATGCCGCTTTTAATGGTG***C***C	5247	57	2	668
nec5364R2	ACATTAAATCCTAGTAAGTGCA***C***A	5870			
E. tenella					
ten1210F	AAAAATTTTAGACTCTTTCTAA	1150	47	6	527
ten1210R	CCTTCAGTAGGACTGAAC	1638			
ten3468F	CGCTCTACCAATATTCGTTAT	3038	50	1	958
ten3468R	GAGCTACAAATGGAAGTACG	3956			
ten4274F	CTTTGTATTACATTTCGTACTT	3962	47	9	769
ten4274R	CTAATGCAACAACACGTAAC	4690			
E. brunetti					
bru621F	CCATATTTATACTAGAACGGTA	619	50	9	939
bru621R	AGTACTAATAACACCTAAACAG	1515			
bru5751F	CAATTATTGGTTTAATATGTGGC	5683	50	4	708
bru5751R	TTCAGGAGCAAACCGTTGAT	212			
E. mitis					
mit1623F	CAGGTCCGGTCCGATTG	1312	53	6	1028
mit1623R	ACAGTTATTTTTTAAATGACCGA	2340			
mit2816F	TGTCAAGTTCCTTTAATGTAGTTC	2467	57	11	1218
mit2816R	GGTGTACTTTTGTTTTAAATTTATACA	3685			
E. praecox					
Pra507F	TAAAGCACGAAATATCATGTGT	79	53	3	892
Pra507R	GCTTCCATTAATAAGAAAGTAT	971			
Pra4645F	GCAACATCTTGGAGTATTGC	4337	53	4	1223
Pra4645R2	GATAAAACTTAGAGCATACC***G***T	5560			
OTU-Z					
Z1660F	ATATAACTGATACAACTCTAATA	1602	53	1	421
Z1660R	ATGATTCTAATTGAGGTAATACTA	2023			
Z2496F	GTAGTTATCTCACAGCTTAG	2398	53	2	723
Z2496R	GTATAACCATAGGTTATTGTC	3121			

[Ψ] position: arbitrary start position in the middle of the Large Subunit RNA fragment G (the *Eimeria* mitochondrial genome was unannotated when sequencing commenced).
*Ta= optimal annealing temperature for primers

Apicoplast DNA sequencing

Primers spanning 2761bp of the apicoplast rpoC2 gene and 630 bp of the rpoB to ABC transporter genes were designed (Table 7) from the *E. tenella* apicoplast genome sequence (Cai *et al.*, 2003). PCR reaction volumes were as for the mtDNA assays above but due to the AT rich nature of the apicoplast the PCR thermocycling conditions were modified as follows. Tubes were denatured at 95°C for 4 minutes followed by 35 cycles of 95°C for 30 seconds, 50°C for 30 seconds and 60°C for 3 minutes, with a final extension step of 60°C for 7 minutes. PCR products were viewed on a 1.5% agarose TBE gel then cleaned and direct sequenced as described above for the mitochondrial DNA products.

Table 7 Primers for apicoplast gene amplification of *Eimeria* species. Species-specific primers flank single nucleotide polymorphisms (SNP) in the apicoplast genome. For some primers the penultimate 3' nucleotide was modified (italisized and marked in bold) to improve specificity.

Gene target Primer name	Sequence 5' to 3'	Position[Ψ]	Ta*	Target # SNP	Product size bp
RpoC2 gene generic primers					
RPOC2F1	TATATTTTAATAAAATGTCTATGCCA	19854	50		
RPOC2F2	AATTATTATTTATAAATTGTCTGTGT	20847	50		
RPOC2F3	CTTTTTATTTTATGGTATTTAACAC	21891	50		
RPOC2F4	AATTTCTATAAGAATTAATCCTG	20668	50		
RPOC2F5	CAGGAATAATAATATTATTAGGT	20431	50		
RPOC2F6	TATATTTTTATATAAAAATTCTCCAG	21677	50		
RPOC2F7	TAATATTCCTTTTCTTCCACCG	22537	50		
RPOC2R1	TAATTCCTAAAAATATAGTATCGC	20901	50		
RPOC2R2	AAGAACTTTTCATACAGGTGGAAC	22028	50		
RPOC2R3	TTACTTTTTTTTCTACAGGACCT	22995	50		
RPOC2R4	CTATATTATTTAATAAATATTTTCCG	21568	50		
RPOC2R5	GAGGTTATTTAGCAAATGCTACA	22615	50		
RpoB to ABC transporter gene generic primers					
api27700F	TTTTTCAATAGTATATTTTGGAATTTC	27700	50	10	630
api28330R	TACTTATAATTATACTGAATGTAATTC	28330	50		
RpoC2 gene *E. tenella*-specific primers					
ten1714F	TAATTTATTAATATATTTTCTATATG***T***G	21469	50	6	638
ten1714R	ATGTAGGTATTTTATCTGGGC***T***G	22093	50		
RpoC2 gene *E. necatrix*-specific primers		**Pair with**			
nec173R	TTTATATGCAAGATCAAGC***A***G	RpoC2F1	50	2	380
nec1057R	AATTATAATTTTAAATATATTTTAA***T***G	RpoC2F2	50	2	480

[Ψ] Position in Cai (2003).
*Ta= optimal annealing temperature for primers

Nuclear DNA microsatellites

Repetitive elements with sufficient flanking sequence to design primers were mined from the publically available *E. tenella* genome project (Houghton strain Wellcome Trust Sanger Institute UK: http://www.sanger.ac.uk/resources/downloads/protozoa/eimeria-tenella.html) and *E. maxima* genome project (Houghton strain Malaysia Genome Institute *Emax*DB: http://www.genomemalaysia.gov.my/emaxdb/). Attempts to amplify these loci in other

species were largely unsuccessful. Fortunately the authors sourced some additional funding (Morgan UQ start-up grant) to create and sequence a reduced representation, 454-next generation sequencing library for *E. necatrix* and *E. acervulina* (Authors, unpublished data). These libraries were mined for *E. necatrix* and *E. acervulina* microsatellite loci. Primers targeting 42 loci for *E. tenella* (Table 8), 60 loci for *E. maxima* (Table 9), 23 loci *for E. necatrix* (Table 10) and 22 loci for *E. acervulina* (Table 11) were designed using Primer 3 software (Rozen and Skaletsky, 2000).

Simple tri-nucleotide repeats were preferentially selected for ease of scoring following electrophoresis separation. Loci containing high numbers of repeat elements were also targeted because longer microsatellites are more likely to be mistranslated in DNA replication giving rise to more alleles. A small number of di-, and tetra- nucleotide repeats were included, and a few imperfect microsatellites (disrupted by base substitutions) were chosen when options were limited.

Rather than fluorescent labelling the forward primer at every locus the PCR reaction contains two forward primers. An M13 extension is added to the 5' end of loci specific forward primer. Including a fluorescent labelled M13 forward primer (FAM-GAG CGG ATA ACA ATT TCA CAC AG) in the PCR reaction enabled target DNA to be amplified and labelled for less cost (Schuelke, 2000). A Qiagen Multiplex PCR Kit (Qiagen, Valencia, CA, USA) was used to amplify the DNA in a final volume of 6 μl. PCR reactions contained 3 μl of 2 x Master Mix, 0.6 μl of 5 x Q solution, 20 nM forward primer with M13 extension, 100 nM reverse primer, 100nM FAM fluorescent dye labelled M13 primer (M13FAM) and approximately 20 ng of genomic DNA template. Microsatellite PCR amplifications were performed in a Biorad thermal cycler (DNA Engine Peltier). The DNA template and enzyme were denatured at 95^{o}C for 15 min, followed by 37 cycles consisting of 94^{o}C for 30 sec, 50-55^{o}C (for specific temperatures refer to Ta column in primer Tables 8-11) for 45 sec and 72^{o}C for 90 sec. To ensure consistent allele calling during genotyping, a final extension at 72^{o}C for 45 min was used to ensure complete addition of adenine to the PCR product. Products were separated via capillary on an ABI3130xl sequencer (Applied Biosystems, Foster City, CA, USA). Genotypes were scored and binned using ABI Genemapper 3.7 software (Applied Biosystems, Foster City, CA, USA).

Table 8 Primers and specifications for *E. tenella* microsatellite loci tested

Locus name Repeat$_{\#\ copies}$	Primer direction	Primer sequence 5' to 3' (M13=GAGCGGATAACAATTTCACACAGG)	Ta*	Product size bp
Etm 09t	F	M13-AATGGACCCACCATTGTGAT	55	396
TGC_{17}	R	CCGAAGTGCAGCAAAGCGAAAAACCCC		
Etm 13t	F	M13-CTGCGCGGCTTGTCGCA	55	280
TGC_{12}	R	ACAGCCCTCAGGAGCGCACA		
Etm 14t	F	M13-ATGCACATCGACACAACTCC	55	441
GCA_{12}	R	GGTTTACTTCCGCATCTTGC		
Etm 18	F	M13-CGAAGGAGACATTAGAGCCG	55	196
$WSB_{21}{}^{\Psi}$	R	TCCTTATAGTGAGTCAGGAAGCC		
Etm 19	F	M13-GCTCATCTTGCCTCCAACAC	55	159
GCT_{12}	R	AATTGCTCCTCAAGAGACACC		
ten00040	F	M13-TCTCCTTATAGGCGGAGCTG	50	177
GCT_{72}	R	CCTGACTCGCCTCTCAACTT		
ten01680	F	M13-CAAGAAGTTGGAAGTGGATCTG	50	235
GCA_{54}	R	GCACAGAAGTCAGCATCAGC		
ten02303	F	M13-TACGCACACGTCAGTGAAGG	50	181
GCT_{54}	R	GTGAGTGTTGTTCCTCTGCC		
ten02393	F	M13-AAGTCATCGGCAACAACCTG	50	183
GCA_{72}	R	GTGCTGTGTCTTCGTTGTGG		
ten02664	F	M13-CTGCGCTCTTCCACATCAAC	50	243
TGC_{45}	R	CAGCAGGATTAGAGTGGCACT		
ten02737	F	M13-CAAGTACCACACCTACACCACG	50	289
GCA_{81}	R	GGTAAGTGACTTCGTTGCAGC		
ten02895	F	M13-TGCTCTAGAGAGAACAGCAGC	50	234
AGC_{72}	R	CAATTCGTTGGACTCTGCTG		
ten03153	F	M13-ACAAGTCTTGGCGGTCTTG	50	274
TGC_{63}	R	CTGCTCAATGGACTTGGCA		
ten03434	F	M13-ACCAAGTTGCACATGCTCAG	50	200
GCA_{81}	R	GGCGCAAGCATTATTACCAT		
ten03438	F	M13-TGCTGCCAATTAATGTCTCC	50	259
CTG_{72}	R	GGAAGCCAACAGAGGAGCTA		
ten03997	F	M13-ACAGCAGCACAGCAGAGAAC	55	214
TGC_{54}	R	CAAGAAGTGGTCGAAGCCGT		
ten04556	F	M13-AACAAGGTAGCAGCGACACTC	50	202
GCT_{63}	R	CAGAAGCAGCAAGAGCTGCT		
ten05039	F	M13-TCACTTGCTGCTGTCGTCTG	55	228
GCT_{63}	R	GGACATGCTGGTGAAGACCT		
ten05645	F	M13-CTGAGCGAAGGAGACAACAG	50	218
CAG_{72}	R	CAAGACAAGTGAGGCAGCAA		
ten05838	F	M13-ATCGACGGAGCTTACAATCG	55	218
TGC_{54}	R	GAAGAGGAGGAGCAGCACAA		
ten06683	F	M13-TTCATCGACAAGCTCAGCAG	50	238
GCA_{54}	R	CTGCTGCCTCTTCATTGAGTT		
ten08884	F	M13-ATTAGCGCAGCAGCTACAGG	55	230

Locus name Repeat$_{\#\ copies}$	Primer direction	Primer sequence 5' to 3' (M13=GAGCGGATAACAATTTCACACAGG)	Ta*	Product size bp
AGC_{54}	R	GGCATTCTGAGGTGTACGTAC		
ten09245	F	M13-ACTCAAGTGCAGTGAGGCAG	55	219
GCA_{54}	R	CCTAATAGCTGCGGACACAA		
ten09728	F	M13-CTACTTCATTGGCTGCTGCG	50	241
CAG_{63}	R	GAGGTCAAGGCAGTCACCAT		
ten11885	F	M13-GGAATGTCGTAGTGCCTTGC	50	193
GCT_{54}	R	GTCGAACTCGCTGTCCATTA		
ten12461	F	M13-CATCTTCTAGCGGCAAGGTC	50	201
CTG_{54}	R	GGAGCCAATCAGTCCATCTG		

$^{\Psi}$ Microsatellite Etm18 is complex, WSB_{21} corresponds to $(TGC)_8ACT(TGC)_3ACT(TGC)_3TCG(TGC)_4$

*Ta= optimal annealing temperature for primers

Table 9 Primers and specifications for *E. maxima* microsatellite loci tested.

Locus name Repeat$_{\#\ copies}$	Primer direction	Primer sequence 5' to 3' (M13=GAGCGGATAACAATTTCACACAGG)	Ta*	Product size bp
max01638	F	M13-CGGCGATCTTGCCGTACACTGC	55	279
ACT_{64}	R	TCGCTGTTCAAATCCCCTGCTC		
max02036	F	M13-TTAAAGCCGCAGAGCTGCTAC	55	284
AAC_{28}	R	GGGAGTGTTTGCACCTGTTAG		
max02095	F	M13-ACTCTCCCTAACCAATTAGCCAG	55	172
CAG_{23}	R	TCTCCTTTGCATGCAGGCTGTG		
max02223	F	M13-AATTTCGCTACCTTTGGACCGT	55	304
AC_{50}	R	CACGATCAAAGATTCATGGGAG		
max02230	F	M13-CGAATATGTGCAGACACCAGG	55	265
AGC_{19}	R	CGTTTGGCTTTGGCTGAGGC		
max02306	F	M13-GCAATTGGCAGCTCCGTAGG	55	337
AC_{61}	R	GAATCCAACGTATGTCTTCGTG		
max02384	F	M13-CAGCGCACTTCCTTACATCAC	55	290
AC_{43}	R	GCGTTGTTACGTCTATGCATAC		
max02425	F	M13-TTACTTCGTATACACCTCGTTGT	55	255
AGC_{19}	R	TAGAGGGTAAGGCATTAAGACG		
max02511	F	M13-GAGTCCTATCTGTGGTCTATGAG	55	191
GYT_{26}	R	GGATAAAAGGACAAGGTATTAATCC		
max02830	F	M13-AGGTGACGACAGTTTTACATTTGG	55	160
AGC_{21}	R	CAAATTAATTGTCTATACACCCCAC		
max03351	F	M13-GCACCAGAACCTAGATAAACAC	55	395
$AATG_{53}$	R	TCGCCGGAATTGACACATAGC		
max03437	F	M13-ACAGCGATCAACCTCCTTCTCG	55	195
WGC_{22}	R	CTGCTGCTGTCATCATCTAATCC		
max03565A	F	M13-TGCTGACTTTGTCTATGGATACAG	55	199
AAC_{20}	R	TGCTGCAGTTCTTGCTGCGGC		
max03565B	F	M13-AGCTGCTGCTGCTCCCTCAG	55	171
AAC_{20}	R	TGCGGCTCCTGCTGCAGTAC		
max04369	F	M13-CTTAGATTTCCTTCTGCAACATC	55	277
AGC_{21}	R	GTTGCTGCTCCTGCATCTGC		
max04435	F	M13-AGTTAGTGAGGAAGCAGAAGAGC	55	213
GCT_{21}	R	CAACGGTTGCTGCTACCTAATAG		
max05657	F	M13-AAAGAATTGGAGAGCAGCAAGTTC	55	193
AGC_{26}	R	TATAGGGTATCTTAACGTGGATCC		
max07989	F	M13-AGGCCCACAAGCAGAGGCCG	55	203

Locus name Repeat$_{\#\ copies}$	Primer direction	Primer sequence 5' to 3' (M13=GAGCGGATAACAATTTCACACAGG)	Ta*	Product size bp
GGA_{23}	R	TGTAAGTTCATTAGGCAGAACACG		
max08489	F	M13-TTCTATTACGCAGACACCTGAGC	55	230
GCT_{21}	R	TTTGCAGATGCGCCACCAACAG		
max09657	F	M13-GCAGGAGCAGCAAGAGTATGAG	55	176
CTT_{20}	R	TTGCTGCTGCACCTGCTGCAG		
max09866	F	M13-GTTTAGGGTCTGCATTCCTTGAC	55	200
GCT_{34}	R	CTCTAAGCACTCCTTCCTGTGC		
max10581	F	M13-AACTGCTGCTCCTGTAACTCTA	55	233
AGC_{19}	R	GCAGATAACTCACAGAGACGC		
max11590	F	M13-GCTGCAGGAAGCAACATATAAC	55	188
AAC_{46}	R	CCTGCTGCTGTTGCTGAAACTG		
max13231	F	M13-CAGAAGGAGCAGCAGACAATG	55	278
AGC_{19}	R	CCAATCAGACAGTACTGTCCC		
max14162	F	M13-TCTACACACAATCATAACTCCTC	55	272
AGC_{23}	R	GCATTGATGCCGCCTTGTGC		
max15327	F	M13-CTTGTTGGTCATGTTGCTATTAAG	55	160
AGC_{21}	R	TCTTCTCTTCCTGTGTATTATTCC		
max17013	F	M13-TTTGTGTATGACATTAGAGGGAC	55	238
AAT_{19}	R	CATGGCCAGGTATCATTTCTTG		
max18007	F	M13-TCTTGCTGCTGCTGATGATTGTG	55	171
GCA_{21}	R	AATGTAGCTAATTGTCATCTAGGTC		
max19109	F	M13-GTCCACGCCAATTGCGCAAC	55	313
ACT_{31}	R	CAGCCTCTGGTTGTGGCGC		
max25273	F	M13-CCCTAAACCCTAATTAGCACCTC	55	194
$ATKT_{23}$	R	CGATTGCTTCGTACAACAATTGCC		

*Ta= optimal annealing temperature for primers

Table 10 Primers and specifications for *E. acervulina* microsatellite loci tested.

Locus name Repeat$_{\#\ copies}$	Primer direction	Primer sequence 5' to 3' (M13=GAGCGGATAACAATTTCACACAGG)	Ta*	Product size bp
Medace_0681	F	M13-ACGCCTCCTTTGTCTCTTCC	50	148
AGC_9	R	AGTGCAGCCGGAGAAGAC		
Medace_0994	F	M13-TATTCATCAGCAAGACGGCC	50	126
AGC_{12}	R	TTTGTGGTGTCTGCTGGAG		
Medace_1406	F	M13-AAAGTCGGCTCCTCTCGTG	50	136
AGC_9	R	GCAGCAAATCGAGCACAACC		
Medace_2532	F	M13-TGCACAGCTTCACCCAAATG	55	379
AT_{12}	R	AGTTGTATGCGTCGAAACCC		
Medace_2566	F	M13-CCTCTTCGCCTCCTTCTTTG	55	248
AGC_{12}	R	TGTGTATCGGCTTGTGAACG		
Medace_2826	F	M13-TCTCCTCATACTGGCAGTGC	55	191
AGC_9	R	GCACATTGTCTTGCTGCTCC		
Medace_2854	F	M13-CTAAACCCTCGCTGCCTTTG	50	252
AGC_9	R	AGCGACCAATTAAACTGCCG		
Medace_3286	F	M13-TGTCTGAGTCTCCATAGGGC	50	217
AGC_{10}	R	GCTACACCTCACCACAAGTC		
Medace_3375	F	M13-CTGTTAGCTTGCTTCACCGG	55	128
AGC_{11}	R	GCCTAAGAACTGCCGCATG		
Medace_3808	F	M13-CTCCAAACTCAGCAGACACC	55	219
AGC_{13}	R	CTTTGTTGTTTCGCCGGTTG		
Medace_3820	F	M13-TCTCTGCATGCCTCTTCCTC	55	225
AGC_{12}	R	GTCGACCACACTACTCTGGC		
Medace_3825	F	M13-AATGATTGGCTGCGCTTCTG	50	213
AGC_{13}	R	TCACGAGCACCTACAATCCC		
Medace_3837	F	M13-TGCTATTTGTGCTGTCTCCC	55	143
AGC_{14}	R	GTGCTTCTCCGTAACAGCATC		
Medace_4514	F	M13-ACTTGCACGAGTTGGGTTAAG	50	196
AT_{15}	R	AGATGTGGGAGGCGTTGAG		
Medace_4572	F	M13-ACTGAACGGACTCTGCTTCG	55	158
AGC_{10}	R	AATACTGGCTGCAATCCGTC		
Medace_4611	F	M13-CTTGCCTTTCTTGGTGGCTC	50	165
AGC_9	R	AGTGGCTGCATCGGATCC		
Medace_5458	F	M13-GGGCTAGCATAGGGTGAGAC	50	248
AT_{11}	R	ATCGCATTGTTGACTCAGCC		
Medace_5509	F	M13-AGCCTCATCGAATTGCTTGC	50	147
AGC_9	R	GTTTCTGTCCCTGCGAGTAG		
Medace_6013	F	M13-GTGCTGCACTTCTTAGGAGTG	50	145
AGC_{15}	R	CGAGTCGAGTCCTAGCTGC		
Royace_0884	F	M13-CGAAAGGAGACATAGCTGCC	55	142
AT_{11}	R	GTGCAGTTATCCATTTGTGCG		
Royace_1399	F	M13-CTTTCCGGTGCGTTCGTATC	55	259
AT_{13}	R	CATACCTAGCGGCACCAATG		
Royace_1567	F	M13-TGATACTCCTGCTGCTTCCC	50	152
AGC_{12}	R	CCATCCCACTAAGAGAGCGG		

*Ta= optimal annealing temperature for primers

Table 11 Primers and specifications for *E. necatrix* microsatellite loci tested.

Locus name Repeat$_{\#\ copies}$	Primer direction	Primer sequence 5' to 3' (M13=GAGCGGATAACAATTTCACACAGG)	Ta*	Product size bp
Etm13n	F	M13-GCGCGGCTCGTCGCG	50	287
TGC_{12}	R	CAGCYCTCAGGAGCGCATGT		
Etm24n	F	M13-CTCCGAAGCAAACGGGCCG	55	168
GCY_{25}	R	GCAGTGGCTGAAGCTCGCC		
Etm27n	F	M13-CAGGGCTTCCCTCCGAACATG	55	185
$AAACCCT_{11}$	R	ACACTTCTGACGCGCAGATGCTG		
Gronec_0358	F	M13-CACGTCGATGCCAACTTCTC	55	124
AGC_{14}	R	GCTGCTCATTCATACCACGC		
Gronec_0378	F	M13-GCTTGAATGGCTGCTGTTG	50	144
AGC_{10}	R	GAAGCACCAGATCAGCAACG		
Gronec_0479	F	M13-TTCCCGTGGCCTTCTATGAG	55	334
AGC_{11}	R	GGAGGTGGCTGGGTAAATTTC		
Gronec_0931	F	M13-GATGTGTCTGTTGCAGCGAC	50	162
AGC_{11}	R	CTCCAGCACCCACTCTCG		
Gronec_0937	F	M13-GGTAAATCCCGCGGCTGTAG	50	176
AGC_{11}	R	CCGCTTTGATTTGATTGCAGG		
Gronec_0997	F	M13-CAACTGTTGCTGGTGTTCCC	55	171
AGC_{9}	R	GCTGCTGCCTTTCTGACAG		
Gronec_1497	F	M13-CCAGGCAGAGAGGTTAGTCC	50	154
AGC_{20}	R	CTATGCGCCTTGGCCCTAG		
Gronec_1507	F	M13-TCCCTGTATGTGGCTTTCCC	55	208
AGC_{10}	R	ACTGCCACCAATACAGACCC		
Gronec_1591	F	M13-GGTAGAGAGCATGGGTAGGC	50	304
AC_{8}	R	CTCTTCTACAATACCTCGCTCC		
Gronec_1710	F	M13-CCGCTTTCTTCTGCTGCTC	50	159
AGC_{7}	R	GAAGAAGTTGGGAAGTGTTTGG		
Gronec_1824	F	M13-CTTGTTGGTCCCAGAACTGC	50	149
AGC_{13}	R	ACCTGGATGTCGATCTGCTG		
Gronec_1880	F	M13-AGAGGCAGTGCTTGAGTCTG	55	127
AGC_{9}	R	GAAGCTGTCCGTCTCAATCG		
Gronec_1909	F	M13-ACTTGCGACGGATCAAAGAAC	50	144
AGC_{6}	R	AAGCCACGCCATAGACCTTC		
Gronec_2028	F	M13-ACCTGAGACGCTACCTATGC	55	129
AGC_{13}	R	CTTAGCAGCACACTCGGG		
Mednec_0647	F	M13-CTCACAGCACAACACGAAATG	55	138
AGC_{12}	R	GGGAACAAAGAGACGATGGC		
Mednec_1399	F	M13-AGCTTAGTCTTTGTTGGTGCTG	50	129
AGC_{6}	R	AATCCTCACTGATCTCGGCG		
Mednec_1532	F	M13-GCGCACTCAATGTAATGCAC	50	131
AGC_{8}	R	TGCTACTGTGTCTTTGTGGC		
Mednec_2338	F	M13-GGCTTTCACTGTCCACCAAC	55	144
AC_{12}	R	CAGCTGCCACACACTGTTTG		
Mednec_2437	F	M13-CGGAGTGCACGGAAATCC	55	162
AGC_{12}	R	TTTAGGGTTTGGGCGTTTCG		
Mednec_2703	F	M13-CCAGCTTCAACGCAGAGATG	50	151
AGC_{8}	R	AGTGCAGAGCTAGACTTGGC		

*Ta= optimal annealing temperature for primers

Preliminary microsatellite loci screening

Microsatellite loci were tested and optimised on three to four pure isolates of each species. The initial denaturation temperature for amplification (PCR) was set at 55°C. Loci that were invariant across the four isolates were excluded from further screening. Primers that either failed, or performed badly, at this temperature were re-tested at annealing temperature 50°C. Loci that failed to amplify again, those that showed poor amplification success, those that amplified but produced multiple, unscorable peaks, and invariant loci were discarded from further screening. Primers that successfully amplified variable loci were then tested against a pure isolate of their sister-species (*E. maxima* with OTU-X and *E. necatrix* with *E. tenella*), and a mixed DNA template containing the remaining 8 non-target species, to assess primer specificity. Primers that amplified target species-specific products were then tested on field isolates containing the species of interest. Due to varying infection levels some PCR troubleshooting was necessary to optimize PCR template DNA concentration for the field samples.

Microsatellite analysis

For each locus, a haplotype results table was constructed in ABI Genemapper 3.7 software (Applied Biosystems, Foster City, CA, USA). This data was then exported and concatenated into a genotype table in Microsoft Excel 2010 (Microsoft Corporation, Redmond, Washington, USA). Because samples from flocks could contain more than 2 alleles, the microsatellites were treated as dominant markers following Cidade *et al.* (2013). Accordingly, the data was converted to binary code for analysis (1 = presence / 0 = absence of each allele). The quality of the microsatellite loci was measured by their Heterozygosity (H) and polymorphism information content (PIC); loci with bigger values being more informative for differentiating among strains. Descriptive statistics for the microsatellite loci including H and PIC were calculated in PICcalc (Nagy *et al*. 2012).

A pairwise similarity matrix was constructed for each species using Jaccard's similarity index (Jaccard, 1908), which calculates the proportion of matches over all non-blanks. Then a principal component analysis was conducted using GenStat 15.3 (VSN International, 2011) to depict how genetic diversity within each species varied across geographic space. Samples with incomplete genotypes were included in the analysis with loci scored as "missing data".

Temporal sampling

Faeces from twenty-seven flocks were temporally sampled (2 to 5 times over the course of the project between 2010 and 2013) and screened for *Eimeria*. Ten flocks (nine back-yard, one commercial) were specifically targeted as samples were available in the DAFF genetic

library that had been collected prior to 2010. The longest temporal sampling period for a continuous flock was 1995-2012.

DNA was extracted from faecal samples and screened using either real-time PCR or the mtDNA-CE assay as outlined above under “Species Diagnostics”. Where sufficient oocysts were present to amplify, strain typing was also completed using species-specific mtDNA and nDNA markers.

Results

Sampling

Objective 1 – Nationwide sampling 5 strains per state & territory

Over the course of the study, 260 samples were collected and screened for *Eimeria* (Table 12). On average, 33 samples were collected per state and territory. Only backyard samples were collected from the ACT and NT as there are no commercial broiler flocks in these territories. Seventy-five percent of samples were extracted directly from faeces, 11% were from gut tissues and the remaining 14% were directly from oocysts (separated from faeces).

Table 12 Distribution and number of samples collected in nationwide survey.

	ACT	NSW	NT	QLD	SA	TAS	VIC	WA	Total
Backyard	18	14	26	18	13	16	11	19	135
Commercial	0	23	0	15	32	17	26	12	125
Total	18	37	26	33	45	33	37	31	260

Species diagnostics

Real-time PCR assay

During the first year of sampling, inconsistencies were identified between screening for oocysts using a microscopic visual survey (crypto) and a genetic, DNA based survey using species-specific real-time PCR assays. The genetic survey was underestimating infection levels (quantification of oocysts present using the genetic markers didn't correlate with oocysts counted) and obvious visual infections of large numbers of oocysts were not being detected using the genetic assays. The "missing" DNA was thought likely to belong to OTUs X, Y and Z which were not targeted by the real-time PCR assays. Developing new real-time PCR assays to detect the three OTUs would have added considerable time and cost to the project and risked missing as yet undiscovered OTUs. Instead a new genetic assay was developed for one tube diagnostic screening of all *Eimeria* species, including the OTUs. The assay uses capillary electrophoresis to differentiate PCR fragments of diagnostic length. Following validation, the new CE assay was used to screen all incoming samples.

Capillary-electrophoresis assay (CE-assay)

CE-assay species detection

Profiles of diagnostic peaks resulting from CE of PCR amplicons for each of the known species of *Eimeria*, including two strains of *E. praecox*, and OTUs X, Y and Z are shown in Figure 5. The size of each of these fragments and their corresponding bin positions in Genemapper (v3.7, Applied Biosystems USA) are shown in Table 4. Although some samples migrated slightly faster (falling into a bin smaller than predicted) their peak positions remained unchanged throughout the project. Electrophoresis was reliably able to resolve fragments differing by as little as one base pair even when the DNA of all 11 samples was

multiplexed into a single tube for PCR amplification (last row of Figure 5). All species present in the 11 sample mix were distinct with easily discernible peaks making this assay easy to interpret. The signals obtained for each species in the mix ranged between 341 rfu for *E. brunetti* to 3351 rfu for *E. mitis* with the average signal for each of the 11 species being 1630 rfu.

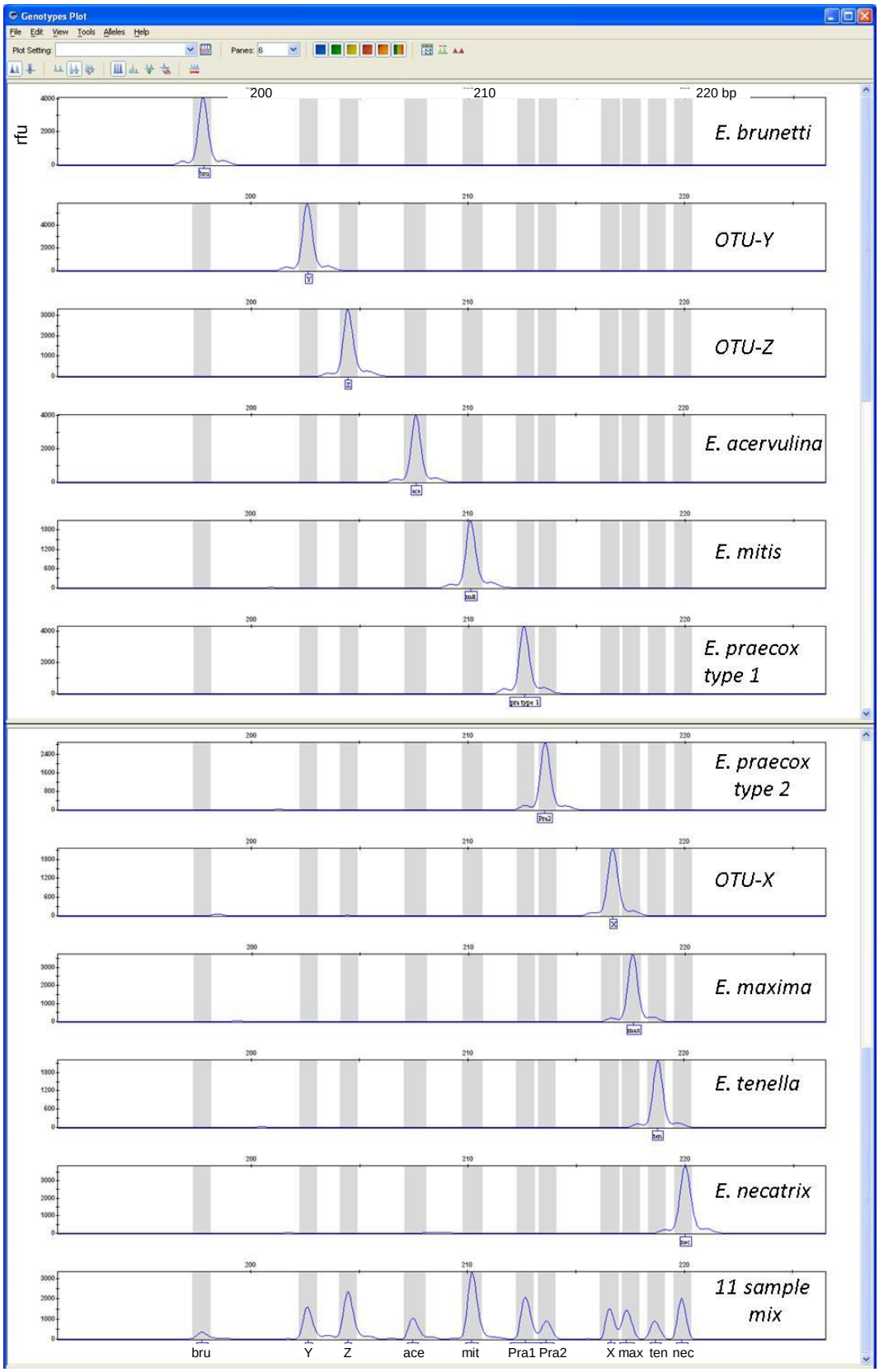


Figure 5 Genotype plot profiles of diagnostic peaks resulting from mtDNA CE assay for all *Eimeria* species found in Australia (including two variants of *E. praecox* and OTU X, Y and Z). Amplicon size (base pairs) is indicated at the top of the figure and peak height is measured in relative fluorescence units (rfu).

CE-assay specificity and sensitivity

The CE assay was designed off DNA sequences conserved among Australian and International strains (Genbank sequences from Chinese and English strains of characterized species where available) and was tested on at least two Australian strains of each of the seven recognized *Eimeria* species plus three OTUs. The assay was found to be specific with no evidence of cross reactivity among species. Two genetic length variants of *E. praecox* exist and these were also reliably distinguished. When DNA was extracted from a large number (10^5) of oocysts from each species and progressively diluted prior to PCR, the assay was sensitive enough to detect DNA from 0.1 oocyst equivalents for all species except *E. tenella* and OTU-X which were detected at 1 oocyst equivalent. When DNA was extracted from diminishing numbers of oocysts such as those found in field samples, the assay was sensitive enough for positive detection of 10 oocysts equivalents from a 1000 oocyst extraction (Table 13).

Table 13 Sensitivity of the capillary electrophoresis (CE) assay for detecting DNA extracted from diminishing numbers of *E. maxima* oocysts. Each DNA extraction was eluted in 100µL and 1µL was used for PCR amplification.

Oocysts/ DNA Extraction	Oocyst equivalents used as PCR template	Detection Peak Height (rfu)
10^5	1000	off scale >8000
10^4	100	7900
10^3	10	200
10^2	1	-
10^1	0.1	-
10^0	0.01	-

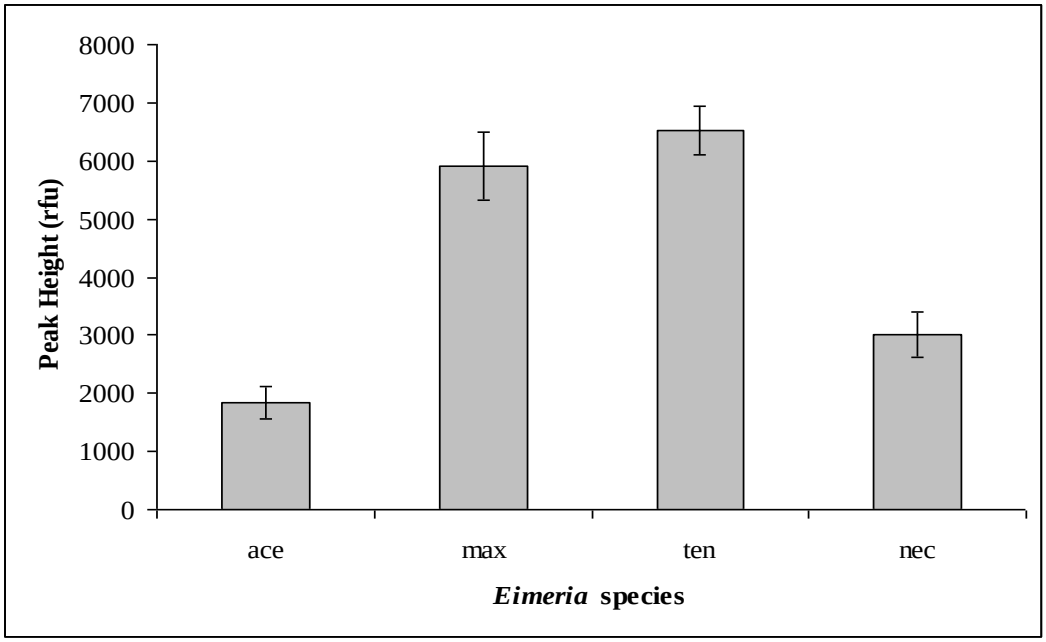
CE-assay reproducibility

The reproducibility of the CE assay was first assessed by testing the 11 sample mixed-template control on 20 different occasions. On all occasions Genemapper was able to automatically and correctly call the diagnostic fragments for each of the 11 samples from the mixture of amplicons generated during the PCR.

Second, the assay was conducted in triplicate on three replicate DNA extractions of the commercial live vaccines Eimeriavax 4m (Bioproperties Pty Ltd, Victoria, Australia) and in triplicate on two replicate DNA extractions of Paracox 8 (MSD Animal Health, UK) (Figure 6). All species present in each vaccine were clearly detected on each occasion the assay was performed, with diagnostic peaks being automatically assigned to bins by Genemapper. Minor variation in fluorescence, as shown by the standard error bars about each species mean, was observed between extractions and replicates (Figure 6). Strandard error bars for *E. mitis* are not shown for Paracox 8 because although a diagnostic peak was obtained for this species, the florescence was saturated (>8000 rfu) and therefore no estimate of error could be made for this species.

Both Eimeriavax 4m and Paracox 8 are mixed-species vaccines containing attenuated strains. Eimeriavax 4m contains *E. acervulina* (ace), *E. maxima* (max), *E. tenella* (ten) and *E. necatrix* (nec) in a 1:2:3:2 ratio while Paracox 8 contains *E. brunetti* (bru), *E. acervulina* (ace), *E. mitis* (mit), *E. maxima* (max), *E. tenella* (ten), *E. necatrix* (nec), and *E. praecox* (pra) in a 1:5:10:3:5:5:1 ratio. In (b) no standard error bar is shown for *E. mitis* as fluorescence signal was saturated each time the assay was run and therefore no estimate of error can be made for this species.

(a)



(b)

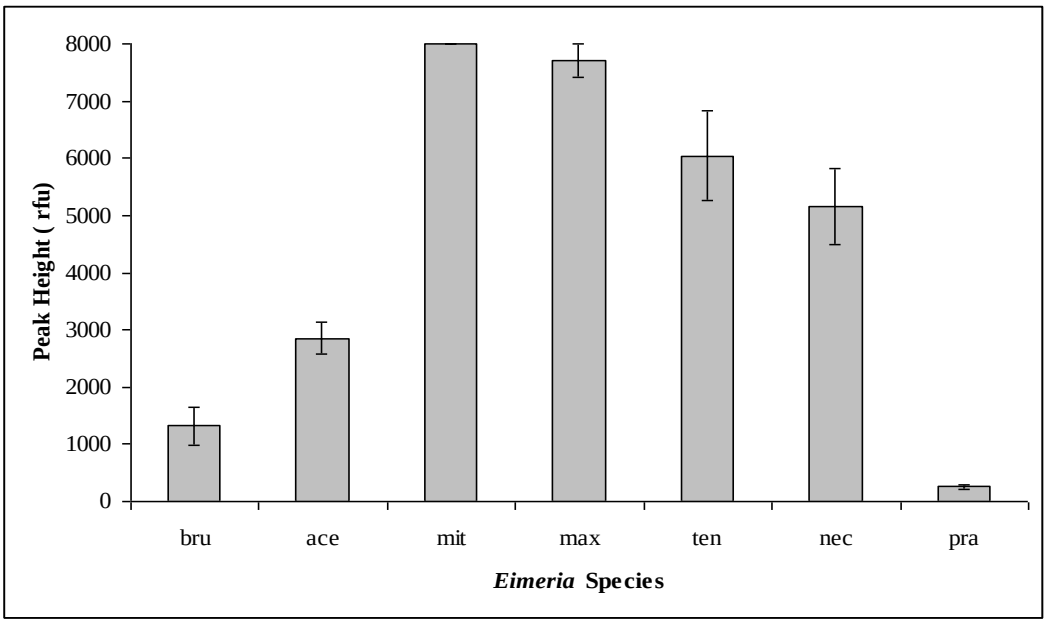
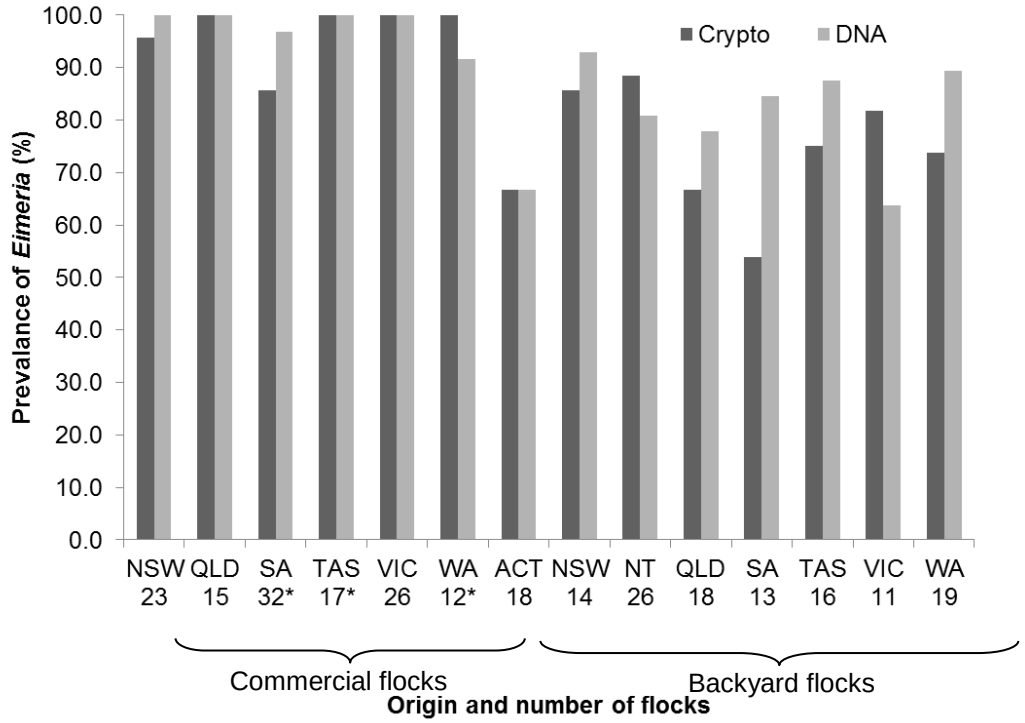


Figure 6 Mean peak heights and standard errors from the CE assay when tested on triplicate vaccine samples of (a) three separate Eimeriavax 4m (Bioproperties Pty Ltd Victoria, Australia) DNA extractions and (b) two separate Paracox 8 (MSD Animal Health, UK) DNA extractions.

Both Eimeriavax 4m and Paracox 8 are mixed-species vaccines containing attenuated strains. Eimeriavax 4m contains *E. acervulina* (ace), *E. maxima* (max), *E. tenella* (ten) and *E. necatrix* (nec) in a 1:2:3:2 ratio while Paracox 8 contains *E. brunetti* (bru), *E. acervulina* (ace), *E. mitis* (mit), *E. maxima* (max), *E. tenella* (ten), *E. necatrix* (nec), and *E. praecox* (pra) in a 1:5:10:3:5:5:1 ratio. In (b) no standard error bar is shown for *E. mitis* as fluorescence signal was saturated each time the assay was run and therefore no estimate of error can be made for this species.

Flock screening

The infection status of flocks was determined independently by microscopic examination (crypto) and genetic screening (DNA, initially using RT-PCR then switching to the CE assay) (Figure 7). Overall genetic screening was more sensitive than microscopic examination, particularly in backyard flocks where infections were typically characterised by low numbers of oocysts. DNA screening failed to detect oocysts in only four flocks that tested visually positive for *Eimeria* (two from NT and two from VIC). Nationally, oocysts were found in almost every commercial broiler flock (98% positive with a range among states of 92-100%) while infection rates were more variable in backyard flocks (81% positive with a range among states of 64-93%).



* Number includes caecal samples (SA = 11, TAS = 7, WA = 10) which were only screened using DNA assays.

Figure 7 Prevalence of *Eimeria* infections (%) in commercial and backyard flocks over the 2010-2012 sampling period determined by microscopic examination (crypto) versus DNA screening.

In backyard flocks (n=135) the three most prevalent species were *E. mitis* (54%), OTU-Y (45%) and *E. acervulina* (28%) while in commercial broiler flocks (n=125) the most prevalent species were *E. acervulina* (67%), *E. maxima* (58%) and *E. mitis* (46%), see Figure 8. The least common species in both backyard and commercial flocks was *E. brunetti*. Screening faecal samples alone identified only nine *E. tenella* infections (9%) from commercial flocks. In contrast the prevalence of *E. tenella* increased to 48% when caecal samples from commercial broiler flocks were screened (n=29).

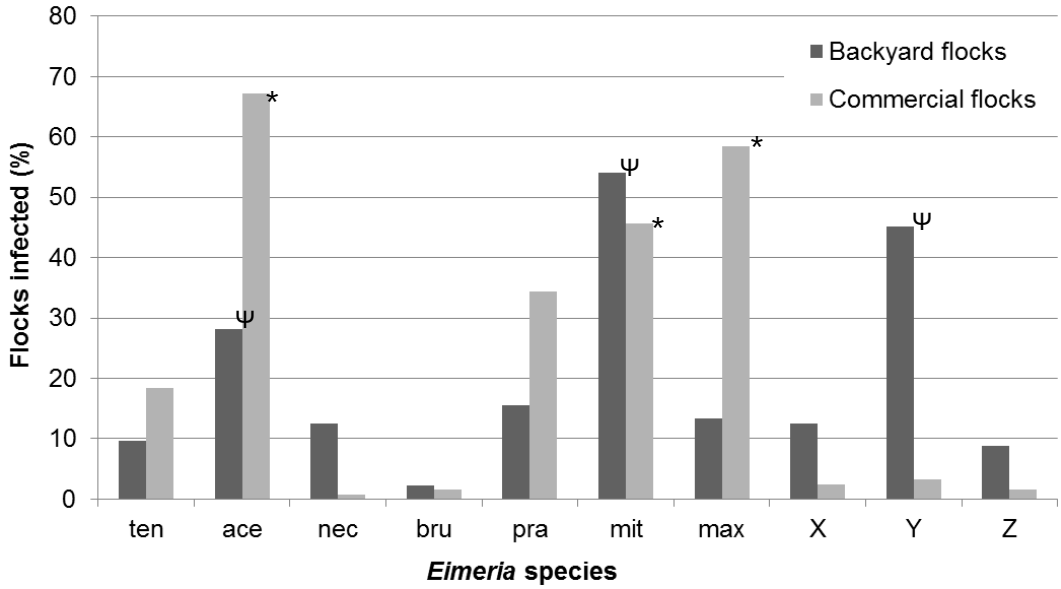


Figure 8 The prevalence of *Eimeria* species (%) in commercial and backyard flocks over the 2010-2012 sampling period. *E. tenella* (ten), *E. acervulina* (ace), *E. necatrix* (nec), *E. brunetti* (bru), *E. praecox* (pra), *E. mitis* (mit), *E. maxima* (max), OTU-X, OTU-Y and OTU-Z. The three most common species in each flock type are marked with * for commercial and Ψ for backyard flocks.

Chi squared homogeneity tests were performed to determine if the relative abundance of *Eimeria* species in faecal samples, from backyard (Table 14) and commercial flocks (Table 15), differed among Australian states and territories. No significant difference was found in backyard flocks ($p = 0.385$) indicating that collection location had no effect on the *Eimeria* species present. Caecal samples (known to be biased toward *E. tenella*) were removed from the commercial sample dataset prior to analysis which resulted in the exclusion of WA from the comparison. A significant difference was detected for commercial broiler flocks ($p = 0.0004$) indicating collection location did effect which *Eimeria* species were present. A plot of the percentage of each species present in the infected flocks suggests that the difference was driven by the lack of *Eimeria* diversity in commercial flocks from Tasmania (Figure 9). Removing Tasmanian samples from the analysis resulted in no significant difference ($p=0.184$) in the relative abundance of *Eimeria* species among the remaining commercial flocks.

Table 14 Contingency table of observed (Obs) and expected (Exp) frequencies of *Eimeria* species in backyard flocks collected from different Australian states and territories.

	ACT		NSW		NT		QLD		SA		TAS		VIC		WA		
	Obs	Exp	Obs	Exp	Obs	Exp	Obs	Exp	Obs	Exp	Obs	Exp	Obs	Exp	Obs	Exp	Tot.
ten	1	1.3	3	1.3	1	2.9	1	1.5	1	1.1	0	1.9	1	0.8	5	2.3	13
ace	3	3.9	4	3.9	10	8.4	6	4.3	4	3.1	4	5.5	1	2.3	6	6.6	38
nec	3	1.7	5	1.7	4	3.7	0	1.9	1	1.4	2	2.5	0	1.0	2	3.0	17
bru	0	0.3	0	0.3	1	0.7	0	0.3	1	0.2	0	0.4	0	0.2	1	0.5	3
pra1	0	1.4	3	1.4	4	3.1	1	1.6	0	1.1	3	2.0	0	0.8	3	2.4	14
pra2	2	1.3	0	1.3	7	2.9	0	1.5	0	1.1	3	1.9	1	0.8	0	2.3	13
mit	10	7.5	7	7.5	14	16.1	9	8.4	6	5.9	12	10.6	3	4.4	12	12.8	73
max	1	1.8	2	1.8	3	4.0	3	2.1	3	1.5	0	2.6	2	1.1	4	3.1	18
X	1	1.7	0	1.7	2	3.7	1	1.9	2	1.4	5	2.5	4	1.0	2	3.0	17
Y	7	6.2	6	6.2	15	13.4	8	7.0	5	5.0	8	8.8	4	3.7	8	10.7	61
Z	1	1.2	0	1.2	2	2.6	1	1.4	0	1.0	3	1.7	0	0.7	5	2.1	12
W	5	5.4	4	5.4	10	11.7	8	6.1	4	4.3	8	7.7	4	3.2	10	9.3	53
total	34		34		73		38		27		48		20		58		332

Chi-squared homogeneity test
H_0: The relative proportion of *Eimeria* species in backyard flocks does not change among collection locations.
$X^2 = 80$, df = 77
p = 0.385
Therefore fail to reject H_0

Table 15 Contingency table of observed (Obs) and expected (Exp) frequencies of *Eimeria* species in commercial flocks (excluding caecal samples) collected from different Australian states and territories.

	NSW		QLD		SA		TAS		VIC		
	Obs	Exp	Obs	Exp	Obs	Exp	Obs	Exp	Obs	Exp	Total
ten	3	2.2	0	1.5	0	1.8	5	0.8	1	2.6	9
ace	21	18.4	10	12.6	14	14.9	6	6.4	23	21.6	74
nec	1	0.2	0	0.2	0	0.2	0	0.1	0	0.3	1
bru	1	0.5	0	0.3	1	0.4	0	0.2	0	0.6	2
pra1	4	5.0	7	3.4	2	4.0	5	1.7	2	5.8	20
pra2	2	6.2	4	4.2	9	5.0	0	2.2	10	7.3	25
mit	11	12.0	7	8.2	9	9.7	6	4.2	15	14.0	48
max	18	16.2	12	11.0	12	13.1	0	5.7	23	19.0	65
X	0	0.7	1	0.5	2	0.6	0	0.3	0	0.9	3
Y	1	1.0	2	0.7	1	0.8	0	0.3	0	1.2	4
Z	1	0.5	0	0.3	1	0.4	0	0.2	0	0.6	2
total	63		43		51		22		74		253

Chi-squared homogeneity test
H_0: The relative proportion of *Eimeria* species in commercial flocks does not change among collection locations.
$X^2 = 76.9$, df = 40
p = 0.0004
Therefore reject H_0, the relative proportion of *Eimeria* species in commercial flocks does change among collection locations.

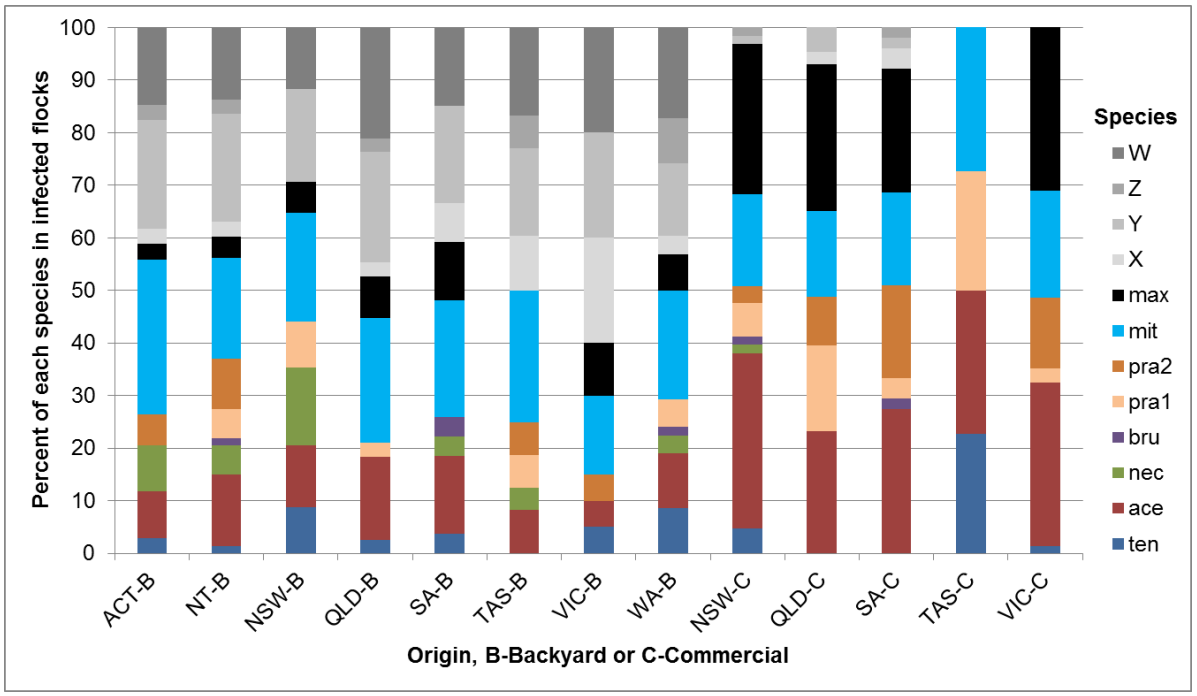


Figure 9 Percentage of each *Eimeria* species present in infected faecal samples from backyard (B) and commercial (C) flocks collected from each state and territory.

Mixed species infections were common in both backyard and commercial broiler flocks (Figure 10). Backyard management displayed the greatest variability with a higher proportion of uninfected flocks, but also the worst mixed infection found (7 species). The average number of species found in a flock, irrespective of whether they were backyard or commercial, was 2.

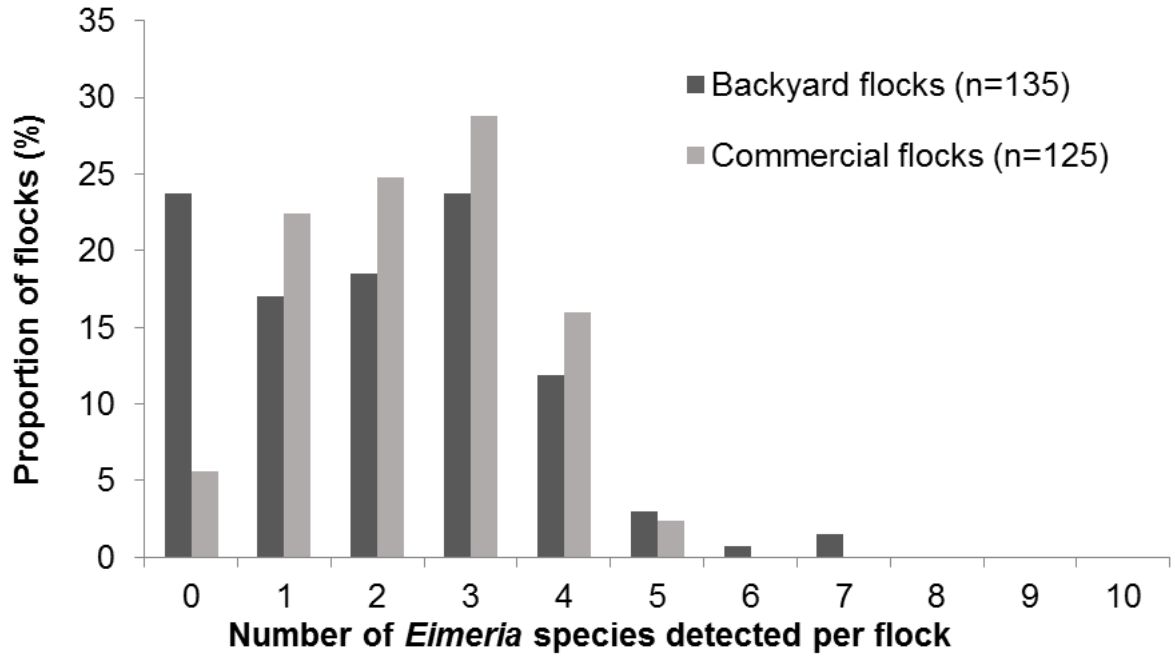


Figure 10 Prevalence of mixed-species *Eimeria* infections as determined by the capillary electrophoresis (CE) assay in the 260 flocks sampled.

Laboratory propagation of oocysts and storage

Oocyst propagation

Following each of bi-annual field collections (5 in total), purified oocysts were selected for further propagation under laboratory conditions. Two birds per strain were infected and inoculum doses ranged from 10 to 1 million oocysts per bird (Table 16). Doses of 1,000,000 oocysts of 10 month old samples proved too high and unfortunately caused mortality in two treatments. Samples were selected based on origin (states and territories poorly represented in strain collections were given preference) and level of infection (samples with higher infection levels were given preference). Some isolates required more than one round of propagation to obtain sufficient numbers of oocysts to cryopreserve. For some of the samples (generally low level infections isolated from backyard flocks) propagation was necessary to boost the numbers of oocysts to obtain sufficient DNA for the population genetic screen.

Table 16 Field isolates selected for laboratory propagation of oocysts.

Trial information	Submission number	State of origin	Inoculum dose (oocysts)	Total oocysts recovered (million)
Trial 1	S167	NSW	500	0.02
Start 25/10/2010	S180	NSW	125	0.33
Location	S185	NSW	15	6.50
ARI, Yeerongpilly	S166	QLD	500	0.001
	S171	SA	55	0.002
	S173	SA	10	2.00
	S176	SA	25	0.36
	S178	TAS	10	0.29
	S179	TAS	10	1.80
	S196	NT	10	0.001
	S197	NT	10	0.02
	S200	NT	25	0.14
	S192	WA	10	0.03
	S193	WA	10	0.001
	S194	WA	25	0.04
Trial 2	S167	NSW	5,000	4.80
Start 3/03/2011	S185	NSW	10,000	85.60
Location	S166	QLD	400	4.00
ARI, Yeerongpilly	S173	SA	10,000	4.80
	S176	SA	10,000	4.00
	S178	TAS	10,000	1.30
	S179	TAS	10,000	2.60
	S228	NT	50	0.001
	S229	NT	280	7.74
	S224	SA	40	0.01
	S239	TAS	4,000	35.00
	S250	VIC	15,000	1.54
	S214	WA	50	1.80
	S217	WA	80	26.10
	S220	WA	200	0.01

Trial information	Submission number	State of origin	Inoculum dose (oocysts)	Total oocysts recovered (million)
Trial 3	S293	ACT	1,000	0.10
Start 27/09/2011	S276	NSW	10,000	9.60
Location	S279	NSW	10,000	14.70
CAAS, Gatton	S271	NT	4,000	0.13
	S289	NT	2,000	1.04
	S295	NT	10,000	26.40
	S274	VIC	5,000	0.24
	S283	VIC	1,500	2.32
	S300	ACT	3,000	0.003
	S290	NT	300	0.001
	S301	NT	10,000	20.40
	S285	TAS	10,000	0.84
	S298	TAS	2,000	0.26
	S303	WA	500	4.22
Trial 4	S293	ACT	10,000	0.10
Start 6/02/2012	S300	ACT	10,000	0.11
Location	S311	NSW	5,000	0.09
CAAS, Gatton	S318	NSW	400	0.03
	S271	NT	10,000	3.62
	S290	NT	10,000	0.78
	S324	NT	800	0.64
	S326	SA	1,200	1.02
	S327	SA	1,500	0.70
	S310	QLD	300	1.16
	S335	QLD	200	0.34
	S331	TAS	500	0.54
	S347	TAS	5,000	5.20
	S313	VIC	10,000	3.20
	S325	WA	300	0.14
	S340	WA	1,000	0.33
Trial 5	S295	NT	1,000,000	0.78
Start 1/10/2012	S324	NT	1,000,000	3.16
Location	S310	QLD	1,000,000	2.70
UQ, Pinjarra Hills	S358	QLD	150	0.23
	S326	SA	1,000,000	3.58
	S239	Tas	1,000,000	2.18
	S384	Tas	3,000	2.50
	S340	WA	1,000,000	2.30
	S271	NT	50,000	3.62
	S290	NT	50,000	1.94
	S335	QLD	50,000	3.40
	S331	TAS	50,000	3.54
	S388	TAS	5,000	2.46
	S374	VIC	150	1.36
	S325	WA	50,000	2.04
	S340	WA	10,000	2.30

Oocyst cryopreservation

Over the duration of the project forty-two isolates amplified in sufficient numbers to cryopreserve (Table 17). Oocyst concentration for cryopreserving was preferably 1 million oocysts per tube but where total oocysts numbers were low, smaller concentrations were used (down to 0.25 million). Unfortunately the OTUs (X, Y and Z) were frequently lost during propagation so relatively few strains were cryopreserved (Table 17). In a number of instances species absent from preliminary faecal screens were gained following propagation (italics in Table 17). In total, oocysts from 123 different infections of *Eimeria* species, originating from every Australian state and territory were cryopreserved (Figure 11). Unfortunately no OTU-X and only one isolate of *E. brunetti* and OTU-Y amplified sufficient numbers to be cryopreserved.

Table 17 Details of strains cryopreserved following propagation trials. Species labels correspond to *E. tenella* (ten), *E. acervulina* (ace), *E. necatrix* (nec), *E. brunetti* (bru), *E. praecox* (pra), *E. mitis* (mit), *E. maxima* (max), OTU-X, OTU-Y and OTU-Z. Species gained during propagation are in italics.

Stabilate reference	Submission number	State of origin	Number of tubes	Oocysts per tube (million)	*Eimeria* species	Species lost
W01	S167	NSW	8	0.5	ace, pra1,mit, max	
W02	S185	NSW	23	1	ace, pra1, max	
W03	S229	NT	7	1	ace,pra1	pra2
W04	S166	QLD	6	0.5	mit,max	
W05	S173	SA	8	0.5	ten,ace,mit,max	
W06	S176	SA	6	0.5	bru	mit, Y
W07	S179	TAS	8	0.25	ace,pra1,mit	pra2
W08	S239	TAS	25	1	ten,nec,pra1,mit,Z	
W09	S178	TAS	2	0.5	mit	
W10	S250	VIC	25	1	nec,pra2,mit,max,Z	
W11	S214	WA	2	0.5	ten,ace,mit	
W12	S217	WA	18	1	ten,ace,pra1,mit,max,Y	
W13	S279	NSW	24	1	ten,ace,mit,max	bru
W14	S276	NSW	24	1	ten,ace,pra2,mit,max	
W15	S347	TAS	25	1	ace,pra1,mit	
W16	S303	WA	24	1	ace	
W17	S283	VIC	25	1	ten,ace,mit,max	
W18	S295	NT	20	1	ace,nec,mit	pra2, Z
W19	S301	NT	15	1	ace,pra1,mit,Z	Y
W20	S285	TAS	10	1	ace,pra1,mit	
W21	S298	TAS	8	0.5	ace,max	
W22	S274	VIC	3	1	pra1,pra2	
W23	S271	NT	25	1	pra1,pra2,mit	Y
W24	S300	ACT	2	0.5	ace,nec,mit,Z	Y
W25	S293	ACT	2	0.5	pra1,mit,Z	Y
W26	S290	NT	25	1	pra2,mit	Y
W27	S289	NT	8	1	ace,mit	ten, Y
W29	S358	QLD	4	0.5	mit,Z	Y
W30	S395	NSW	6	0.5	ace,pra2,mit	max
W31	S327	SA	10	1	pra1,pra2,mit	Z
W32	S310	QLD	24	1	ten,mit	
W33	S313	VIC	24	1	ten,ace,pra2,mit,max	
W34	S324	NT	24	1	ace,pra1,mit	X, Y, Z
W35	S325	WA	23	1	ace,mit,max	Y
W36	S326	SA	24	1	ace,max	pra2, X
W37	S331	TAS	24	1	ace,pra1,pra2,mit	Y, Z
W38	S335	QLD	24	1	ace,pra1,mit,max	X
W39	S340	WA	24	1	ten,pra1,mit	nec, Y
W40	S374	VIC	24	1	ten,pra1,max	
W41	S384	Tas	24	1	ten,ace	
W42	S388	TAS	24	1	ten,ace,mit	

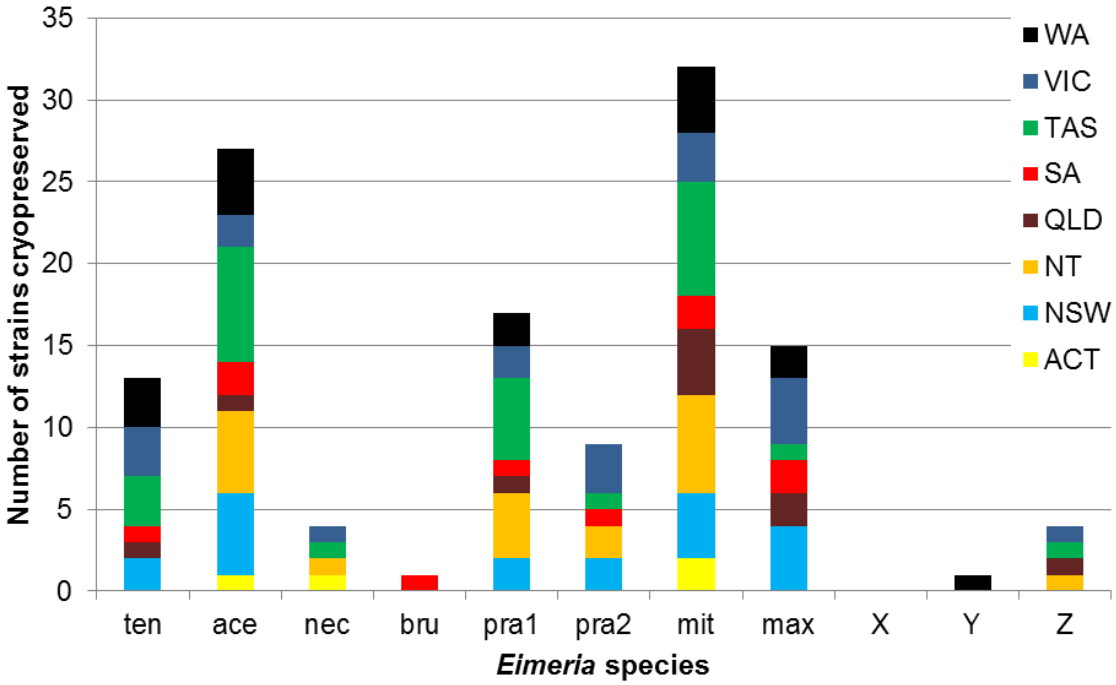


Figure 11 Summary of the *Eimeria* species cryopreserved and their origin

Genetic marker screening for population genetic analysis

Mitochondrial DNA sequencing

Objective 2 – sequence 500 bp of strains of 7 Eimeria species

Complete mitochondrial genomes have been sequenced for 25 isolates representing the 7 described species of *Eimeria* plus the three OTU's described by Cantacessi *et al.* (2008). The genomes range in length from 6166bp (*E. brunetti*) to 6419bp (*E.mitis*) and contain three coding genes; cytochrome oxidase 1 (*Cox*I), cytochrome oxidase III (*Cox*III) and cytochrome b plus several small ribosomal RNA coding regions. A pairwise distance matrix comparing International species and strains (Table 18) indicates species within the genus diverge by 2 to 11.5% (130 to 750 SNPs) while maximum strain diversity within a species was 0.6% (42 SNPs) among *E. tenella* isolates. The three OTU's fall well within the range observed for species status, 3.9 – 10.4% divergence from the other *Eimeria* species. A maximum likelihood phylogenetic tree (Figure 12) clearly positions the three OTU within the chicken *Eimeria* clade. The tree also highlights the close within species, versus between species distances.

Single nucleotide polymorphisms (SNPs) distinguishing strains within each species, based on complete mitochondrial genome alignments, are displayed in Tables 19 - 26 (excluding OTU-X and OTU-Y as at time of sequencing only one pure strain was available). The most informative SNPs were targeted in the mtDNA strain differentiation assays (boxed regions in the Tables 19-26).

Table 18 Maximum number of base differences (SNPs) below the diagonal and percent divergence above the diagonal, for pairwise species comparisons of complete mtDNA genome sequences (based on a 6506 bp alignment). Within species SNP differences, including a comparison among just Australian strains (in brackets), lies along the diagonal.

	ten	*nec*	*bru*	*mit*	*ace*	*pra*	*max*	X	Y	Z
E. tenella	42 (2)	2.0	9.1	8.4	7.4	7.6	11.5	11.5	9.1	8.5
E. necatrix	130	11 (1)	8.7	8.0	7.0	7.2	11.2	11.3	8.5	8.2
E. brunetti	590	563	12 (2)	7.1	6.5	6.6	10.4	10.3	4.7	7.2
E. mitis	544	518	460	29 (8)	5.8	6.2	9.9	10.2	7.1	6.5
E. acervulina	481	453	421	380	4 (1)	4.7	9.2	9.5	6.7	6.0
E. praecox	494	469	432	403	309	8 (8)	9.4	9.7	6.8	6.2
E. maxima	750	727	675	644	600	609	9 (7)	3.9	10.2	10.2
OTU-X	750	733	671	661	617	629	254	0 (0)	10.4	10.3
OYU-Y	590	552	307	463	433	445	663	676	0 (0)	7.5
OTU-Z	556	533	467	425	389	403	666	668	487	3 (3)

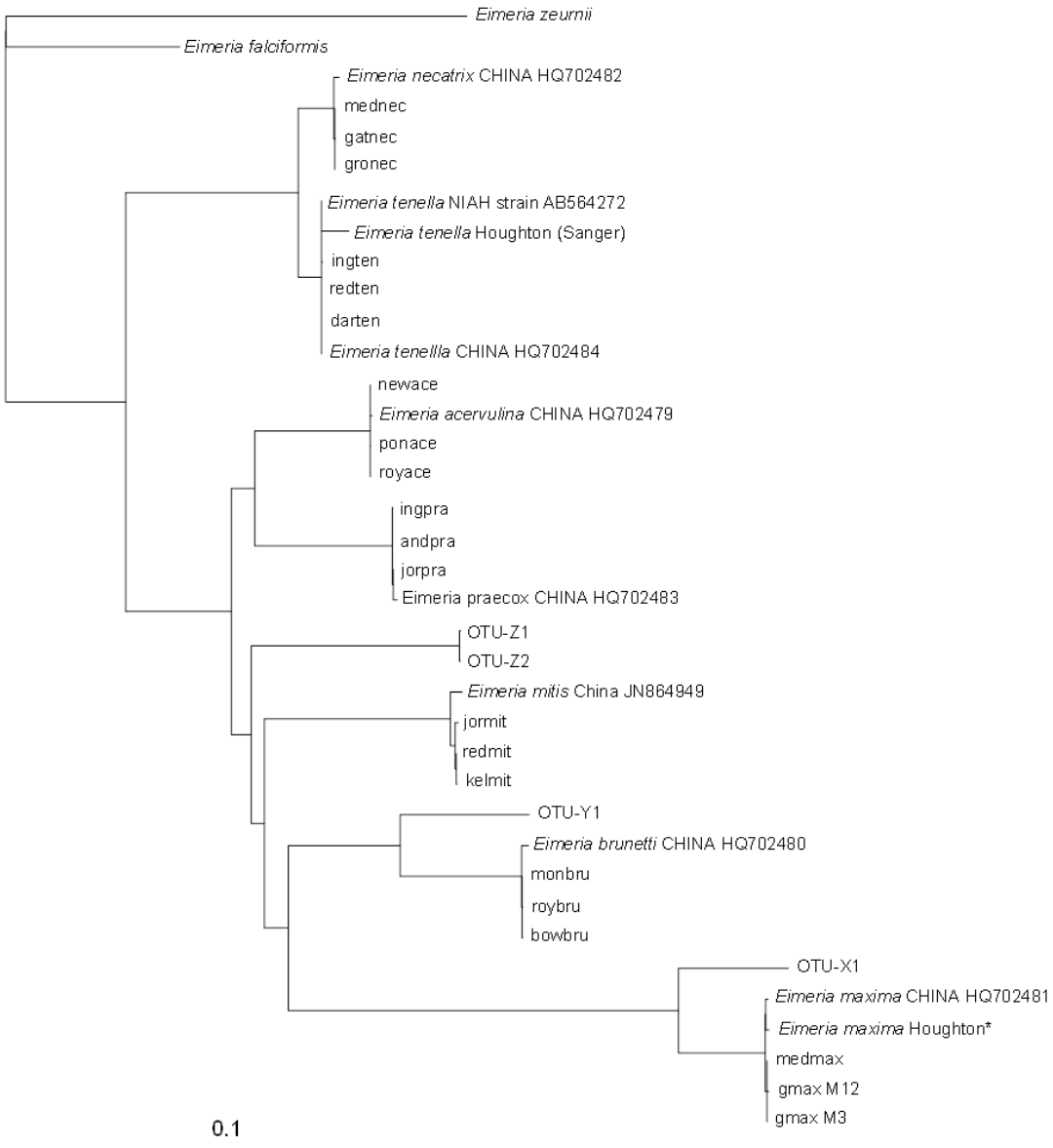


Figure 12 Maximum likelihood phylogenetic tree based on complete *Eimeria* mitochondrial genomes.

Table 19 Mitochondrial genome strain differences for *Eimeria tenella*. Boxes indicate the position of SNPs targeted in species-specific PCR assays (sequence gap = -). Nucleotides in bold italics correspond to informative SNPs in Australian isolates.

Species	*E. tenella*					
Strain	Houghton	China	NIAH	darten	redten	ingten
Origin	UK	China	Japan	QLD	QLD	NSW
224	C	A	A	A	A	A
274	C	A	A	A	A	A
328	C	A	A	A	A	A
330	C	A	A	A	A	A
350	C	A	A	A	A	A
714	C	G	G	G	G	G
802	G	T	T	T	T	T
834	G	T	T	T	T	T
882	G	T	T	T	T	T
990	G	T	T	T	T	T
1051	G	T	T	T	T	T
1085	G	T	T	T	T	T
1114	G	T	T	T	T	T
1124	G	T	T	T	T	T
1210	G	T	T	T	T	T
1254	G	T	T	T	T	T
1269	G	T	T	T	T	T
1274	G	T	T	T	T	T
1436	G	T	T	T	T	T
1469	G	T	T	T	T	T
3468	***C***	***C***	***C***	***C***	***C***	***A***
4274	G	T	T	T	T	T
4284	***T***	***T***	***T***	***T***	***T***	***C***
4324	G	T	T	T	T	T
4432	G	T	T	T	T	T
4472	G	T	T	T	T	T
4524	G	T	T	T	T	T
4619	G	T	T	T	T	T
4641	G	T	T	T	T	T
4645	G	T	T	T	T	T
4839	G	T	T	T	T	T
4976	G	T	T	T	T	T
5774	C	A	A	A	A	A
5798	C	A	A	A	A	A
5858	C	A	A	A	A	A
5940	C	A	A	A	A	A
5942	C	A	A	A	A	A
6013	C	A	A	A	A	A
6038	C	A	A	A	A	A
6054	C	A	A	A	A	A
6092	C	A	A	A	A	A
6200	C	A	A	A	A	A

Table 20 Mitochondrial genome strain differences for *Eimeria maxima*. Boxes indicate the position of SNPs targeted in species-specific PCR assays (sequence gap = -). Nucleotides in bold italics correspond to informative SNPs in Australian isolates.

Species		*E. maxima*		
Strain	China	medmax	gmaxM3Ing	gmaxM12redlea
Origin	China	VIC	VIC	NSW
651	-	A	A	A
654	-	A	A	A
1321	A	-	-	-
1322	T	-	-	-
1538	G	A	A	A
2086	A	C	C	C
2311	G	T	T	T
3422	***A***	***C***	***C***	***C***
3560	***T***	***T***	***T***	***A***
3645	***T***	***T***	***T***	***T***
4038	***C***	***C***	***C***	***A***
4052	***T***	***T***	***T***	***T***
4238	***C***	***T***	***T***	***C***
4240	***T***	***T***	***T***	***T***
4384	***C***	***C***	***C***	***C***
4486	G	-	-	-
4487	T	-	-	-
5255	A	G	G	G

Table 21 Mitochondrial genome strain differences for *Eimeria necatrix*. Boxes indicate the position of SNPs targeted in species-specific PCR assays (sequence gap = -). Nucleotides in bold italics correspond to informative SNPs in Australian isolates.

Species		*E. necatrix*		
Strain	China	gronec	mednec	gatnec
Origin	China	NSW	VIC	QLD
377	T	C	C	C
946	-	C	C	C
950	A	T	T	T
955	A	T	T	T
2005	T	C	C	C
3281	-	A	A	A
3291	A	-	-	-
3293	G	-	-	-
4538	T	-	-	-
5364	***A***	***C***	***C***	***C***
5410	***C***	***C***	***C***	***T***
6072	A	G	G	G
6079	A	-	-	-

Table 22 Mitochondrial genome strain differences for *Eimeria acervulina*. Boxes indicate the position of SNPs targeted in species-specific PCR assays (sequence gap = -). Nucleotides in bold italics correspond to informative SNPs in Australian isolates**.**

Species		*E. acervulina*		
Strain	China	newace	royace	ponace
Origin	China	QLD	QLD	QLD
197	***A***	***A***	***A***	***T***
3430	***A***	***C***	***C***	***C***
3457	G	A	A	A
4893	G	A	A	A

Table 23 Mitochondrial genome strain differences for *Eimeria brunetti*. Boxes indicate the position of SNPs targeted in species-specific PCR assays (sequence gap = -). Nucleotides in bold italics correspond to informative SNPs in Australian isolates.

Species		*E. brunetti*		
Strain	China	bowbru	monbru	roybru
Origin	China	NSW	SA	QLD
79	T	A	A	A
621	-	C	C	C
622	-	A	A	A
623	-	G	G	G
624	-	G	G	G
625	-	C	C	C
626	-	T	T	T
627	-	G	G	G
653	***G***	***A***	***A***	***A***
1428	***C***	***C***	***C***	***T***
1667	T	G	G	G
2700	C	T	T	T
3062	C	T	T	T
3149	T	A	A	A
3635	C	T	T	T
3958	A	T	T	T
4628	C	T	T	T
4892	C	T	T	T
5751	***C***	***A***	***C***	***C***
6076	C	T	T	T
6120	-	G	G	G

Table 24 Mitochondrial genome strain differences for *Eimeria mitis*. Boxes indicate the position of SNPs targeted in species-specific PCR assays (sequence gap = -). Nucleotides in bold italics correspond to informative SNPs in Australian isolates.

Species	*E. mitis*			
Strain	China	jormit	kelmit	redmit
Origin	China	QLD	QLD	QLD
139	-	A	-	-
454	T	C	C	C
491	T	G	G	G
822	G	T	T	T
1210	T	-	T	T
1407	-	A	A	A
1623	***G***	***G***	***A***	***A***
1737	***T***	***T***	***T***	***T***
1968	***A***	***A***	***A***	***A***
1989	***T***	***A***	***T***	***T***
2170	***A***	***T***	***A***	***T***
2329	G	A	A	A
2473	C	T	T	T
2814	***A***	***A***	***-***	***-***
2815	***T***	***T***	***-***	***-***
2816	***T***	***T***	***-***	***-***
2817	***A***	***A***	***A***	***A***
3219	T	A	A	A
3260	***G***	***T***	***G***	***G***
3289	T	A	A	A
3290	A	T	T	T
3291	T	A	A	A
3352	***A***	***T***	***A***	***A***
3362	***-***	***A***	***-***	***-***
3492	C	T	T	T
3494	T	T	A	A
3656	A	T	T	T
3693	G	A	A	A
3916	A	G	G	G
4292	C	T	T	T
4725	T	A	A	A
5048	G	A	A	A
5445	C	T	T	T
5843	T	G	T	T
6264	T	G	T	T

Table 25 Mitochondrial genome strain differences for *Eimeria praecox*. Boxes indicate the position of SNPs targeted in species-specific PCR assays (sequence gap = -). Nucleotides in bold italics correspond to informative SNPs in Australian isolates.

Species		*E. praecox*		
Strain	China	jorpra	ingpra	andpra
Origin	China	QLD	NSW	QLD
67	G	T	T	T
412	***C***	***C***	***C***	***C***
507	***T***	***T***	***G***	***T***
635	A	-	-	-
1316	A	T	T	T
2137*	***C***	***C***	***-***	***-***
4009	T	A	A	A
4645	T	C	C	C
4647	T	C	C	C
4840	***G***	***G***	***A***	***A***
5482	A	T	T	T

* Position 2137 corresponds to the diagnostic SNP which separates pra1 and pra2 in the mtDNA CE species diagnostic assay.

Table 26 Mitochondrial genome strain differences for *Eimeria* OTU-Z. Boxes indicate the position of SNPs targeted in species-specific PCR assays (sequence gap = -). Nucleotides in bold italics correspond to informative SNPs in Australian isolates.

Species	OTU-Z	
Strain	Z strain 1	Z strain 2
Origin	NSW	?
1660	***G***	***A***
2496	***C***	***T***
2940	***A***	***C***

Screening field isolates with mitochondrial SNP-based assays

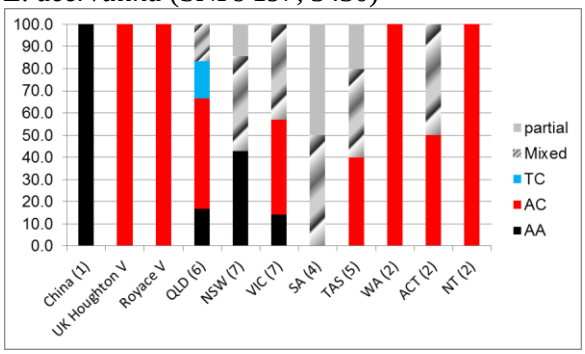
Using DNA sequencing, and species-specific primers (Table 6), all available *Eimeria* strains belonging to the seven recognised species and OTU-Z were screened for the mtDNA SNPs outlined above. Where sequences were available for International strains they were included for comparison.

Including international strains, a total 38 strains of *E. acervulina*, 36 strains of *E. maxima*, 17 strains of *E. necatrix*, 35 strains of *E. tenella*, 11 strains of *E. brunetti*, 36 strains of *E. mitis*, 30 strains of *E. praecox* and 13 strains of OTU-Z were screened. Within Australian populations the greatest mitochondrial diversity was detected among *E. mitis* strains (11 SNPs and 5 unique genotypes) with two distinct but widespread lineages (differentiated by a 3 base deletion at position 2814) (Figure 13). Compared to the remaining species Australian strains of *E. maxima* were also quite diverse (8 SNPs and 5 genotypes) (Table 27). In contrast, little genetic diversity was found among strains of *E. acervulina* (2 SNPs and 3 genotypes) and *E. tenella* (2 SNPs and 4 genotypes) despite screening a similar number of flocks (Figure 13, Table 27). Chinese strains of every species were genetically distinct with

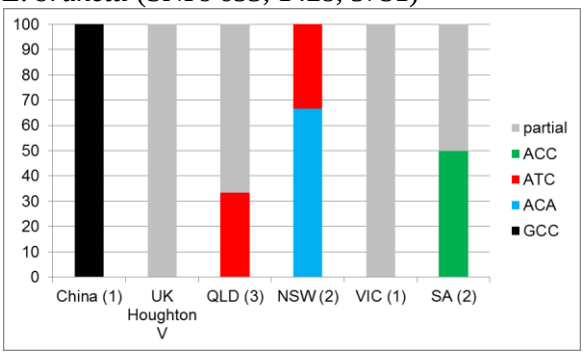
the exception of *E. tenella*. The Houghton strains of most of the species (originating from the United Kingdom) were generally more similar to Australian isolates than their Asian counterparts (Figure 13). Mixed strain infections of *E. acervulina*, *E. mitis*, *E. praecox* and OTU-Z were common in flocks (Figure 13). The direct sequence chromatograms of these samples showed mixed peak signals signifying multiple, different *Eimeria* stains, belonging to the same species, were concurrently infecting the flocks.

The Paracox 8 vaccine strain of *E. mitis* (UK Houghton) had a unique mtDNA genetic signature (genotype). All other vaccine strains (marked with V's on Figure 13) shared genotypes (or alleles where only partial genotypes could be determined) with Australian strains.

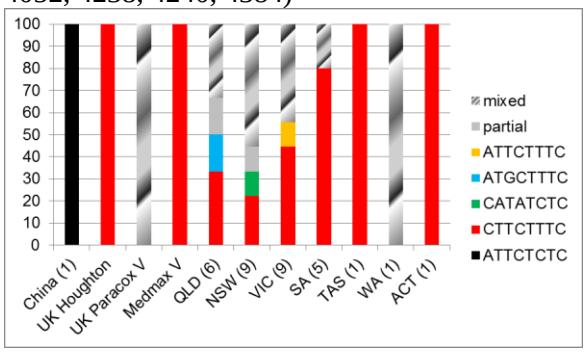
E. acervulina (SNPs 197, 3430)



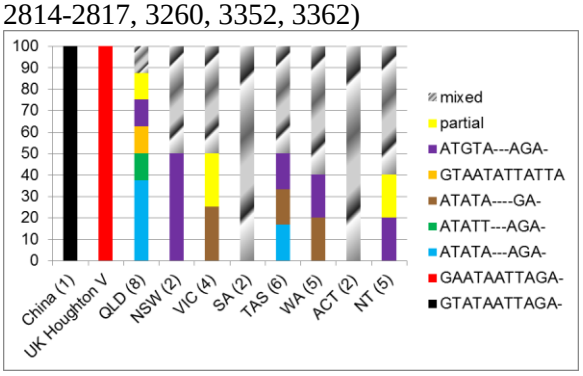
E. brunetti (SNPs 653, 1428, 5751)



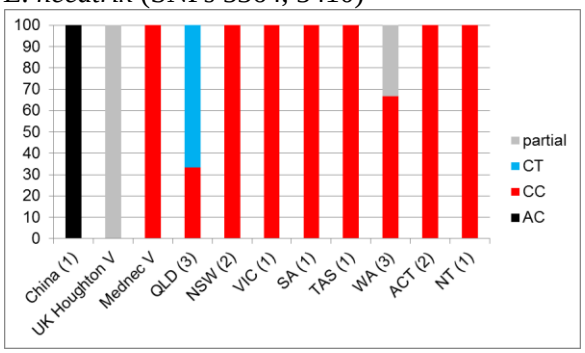
E. maxima (SNPs 3422, 3560, 3645, 4038, 4052, 4238, 4240, 4384)



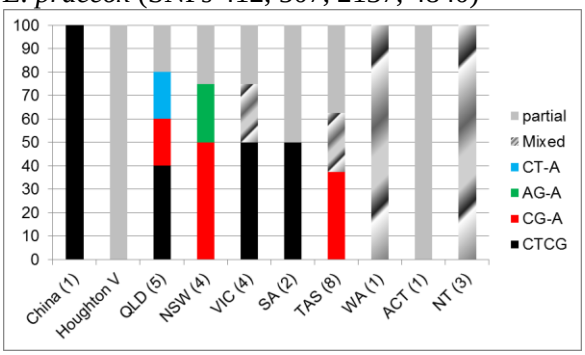
E. mitis (SNPs 1623, 1737, 1968, 1989, 2170, 2814-2817, 3260, 3352, 3362)



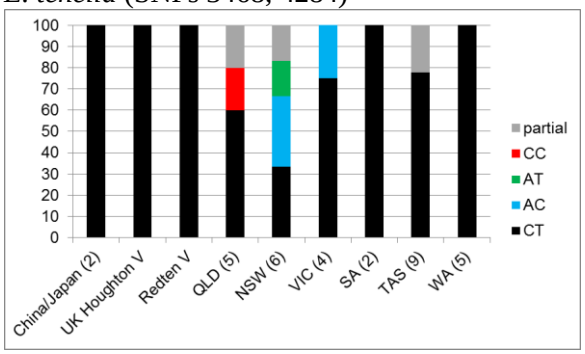
E. necatrix (SNPs 5364, 5410)



E. praecox (SNPs 412, 507, 2137, 4840)



E. tenella (SNPs 3468, 4284)



OTU-Z (SNPs 1660, 2496, 2940)

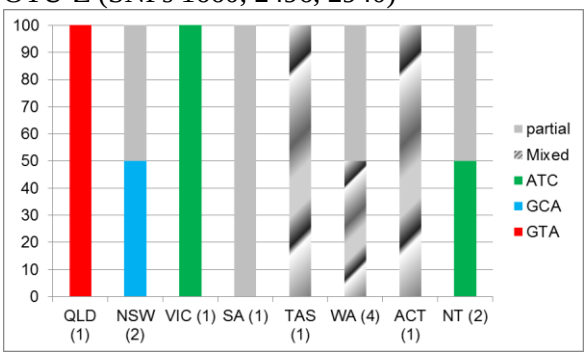


Figure 13 Mitochondrial DNA diversity of *Eimeria* species (% haplotype abundance) for vaccine (V) and Australian field isolates, plus international isolates where available.
Different colours represent different haplotypes in the legend (SNP positions follow species in title). Sample sizes (flocks) are given in brackets following strain origin.

Table 27 Mitochondrial DNA molecular diversity indices for Australian populations of different *Eimeria* species including the number of flocks sequenced (n), the number of unique genotypes and the number of polymorphic sites (SNPs).

Species	Origin	Flocks (n)	Genotypes	SNPs
E. acervulina	QLD	7	3	2
	NSW	7	3	2
	VIC	7	2	1
	SA	4	2	1
	TAS	5	2	1
	WA	2	1	0
	ACT	2	2	1
	NT	2	1	0
	total	36	3	2
E. maxima	QLD	6	5	3
	NSW	9	5	5
	VIC	10	4	3
	SA	5	2	1
	total	30	5	8
E. necatrix	QLD	3	2	1
	NSW	2	1	0
	VIC	2	1	0
	WA	3	2	1
	ACT	2	1	0
	total	12	2	2
E. tenella	QLD	6	2	1
	NSW	6	3	2
	VIC	4	2	2
	SA	2	1	0
	TAS	9	1	0
	WA	5	1	0
	total	32	4	2
E. brunetti	QLD	3	1	0
	NSW	2	2	2
	SA	2	2	1
	total	7	3	3
E. mitis	QLD	8	5	10
	NSW	2	2	5
	VIC	4	3	3
	SA	2	2	7
	TAS	6	5	8
	WA	5	5	5
	ACT	2	3	8
	NT	5	3	6
	total	34	5	11
E. praecox	QLD	5	3	3
	NSW	4	3	2
	VIC	4	2	3
	SA	2	1	0
	TAS	8	2	1
	NT	3	3	3
	total	26	3	4
OTU-Z	NSW	2	2	2
	WA	4	2	1
	NT	2	2	1
	total	8	3	3

Apicoplast DNA sequencing

Objective 3 – sequence 500 bp of strains of 7 *Eimeria* species

PCR amplification and DNA sequencing of *Eimeria* apicoplast DNA proved difficult. After much troubleshooting with primer design (longer primers proved better), annealing temperatures (changing had little impact on assays) and extension temperatures (lowering to 60°C with a 3 minute hold had a large impact on assay success) a 630 bp sequence was obtained spanning partial RpoB to ABC transporter genes. A preliminary screen of two to three strains per species identified no intraspecific variability among *E. acervulina*, *E. maxima*, *E. necatrix*, *E. brunetti* or OTU-Z (Table 28). Although the marker was not informative for strain comparisons within Australia, differences were observed among international strains of *E. tenella* (Australia, China and UK). Attempts to PCR amplify the RpoC2 gene produced inconsistent results for *E. tenella* and *E. necatrix* although a small number of SNPs were identified. Species-specific primers designed to amplify the RpoC2 SNPs in mixed species field samples failed to amplify products. Apicoplast screening was not progressed past this point due to poor amplification success and lack of intraspecific variability.

Table 28 Summary of results of apicoplast sequencing.

	RpoC2	RpoB to ABC transporter
E. acervulina	no difference	no difference
E. maxima	failed to amplify	no difference
E. necatrix	4 SNPs AUS strains but poor amplification	no difference
E. tenella	6 SNPs Int'l, 2 SNPs AUS strains	10 SNPs Int'l, no difference AUS strains
E. brunetti	failed to amplify	no difference
E. mitis	failed to amplify	1 SNP AUS strains
E. praecox	failed to amplify	2 SNPs AUS strains
OTU-Z	failed to amplify	no difference

Nuclear DNA microsatellites

Objective 4 – (i) develop variable microsatellite loci for ace and max and (ii) genotype nec and ten with existing microsatellite loci

Thirty eight percent of the 101 *Eimeria* microsatellite loci tested successfully amplified products that were heterozygous among Australian strains of *E. acervulina* (10/22 loci), *E. maxima* (10/30 loci), *E. tenella* (10/26 loci) and *E. necatrix* (8/23 loci). The most diverse species was *E. maxima* (mean number of alleles 9.2 ± 1.6 SE) followed by *E. acervulina* (mean number of alleles 5.7 ± 0.56 SE), then *E. necatrix* (mean number of alleles 3.75 ± 0.62SE), and *E. tenella* (mean number of alleles 3.5 ± 0.4 SE).

Amplification results and descriptive statistics for the successful microsatellites of each of the four species are detailed in Table 29 *E. acervulina*, Table 30 *E. maxima*, Table 31 *E. necatrix*

and Table 32 *E. tenella*. Combining all of the loci together, the best 10 loci by allele number, Heterozygosity (H) and polymorphism information content (PIC) contained 10 to 64 repeat elements (mean 23), had 4 to 20 alleles (mean 8), H of 0.64 to 0.91 (mean 0.74) and PIC 0.61 to 0.9 (mean 0.71).

Table 29 Descriptive statistics for *E. acervulina* microsatellite loci.

Locus	Repeat and number of copies	Predicted size (bp)	Size range (bp)	Number of alleles	Heterozygosity (H)	Polymorphism Information Content (PIC)
Medace_2532	AT_{12}	379	367-379	8	0.78	0.74
Medace_2566	AGC_{12}	248	242-251	4	0.66	0.60
Medace_2826	AGC_{9}	191	189-201	3	0.20	0.19
Medace_3375	AGC_{11}	128	118-133	6	0.45	0.43
Medace_3808	AGC_{13}	219	196-214	5	0.52	0.49
Medace_3820	AGC_{12}	225	206-218	5	0.65	0.61
Medace_3837	AGC_{14}	143	123-156	8	0.80	0.78
Medace_4572	AGC_{10}	158	144-156	4	0.72	0.67
Royace_0884	AT_{11}	142	122-158	7	0.71	0.67
Royace_1399	AT_{13}	259	237-265	7	0.71	0.68
Medace_1406	AGC_{9}	136	125-149	6	mispriming	
Medace_2854	AGC_{9}	252	239-253	4	mispriming	
Medace_4514	AT_{15}	196	104	1	mispriming	
Medace_5458	AT_{11}	248	52	1	mispriming	
Medace_6013	AGC_{15}	145	61	1	mispriming	
Medace_4611	AGC_{9}	165	163-166	2	poor amplification	
Medace_0681	AGC_{9}	148	149	1	too similar	
Medace_0994	AGC_{12}	126	122-123	2	too similar	
Medace_3286	AGC_{10}	217	218-221	2	too similar	
Medace_3825	AGC_{13}	213	211-214	2	too similar	
Medace_5509	AGC_{9}	147	148-154	2	too similar	
Royace_1567	AGC_{12}	152	147-150	2	too similar	

Table 30 Descriptive statistics for *E. maxima* microsatellite loci

Locus	Repeat and number of copies	Predicted size (bp)	Size range (bp)	Number of alleles	Heterozygosity (H)	Polymorphism Information Content (PIC)
max01638	ACT_{64}	279	231-291	20	0.91	0.90
max02223	AC_{50}	304	221-341	16	0.89	0.88
max02230	AGC_{19}	265	238-268	7	0.65	0.61
max03565A	AAC_{20}	199	198-245	7	0.68	0.63
max04369	AGC_{21}	277	246-276	4	0.26	0.25
max05657	AGC_{26}	193	192-240	7	0.64	0.61
max09866	GCT_{34}	200	172-199	7	0.76	0.72
max10581	AGC_{19}	233	218-239	6	0.67	0.63
max15327	AGC_{21}	160	159-213	10	0.65	0.62
max25273	$ATKT_{23}$	194	140-193	8	0.78	0.74
max02306	AC_{61}	337	-	-	failed to amplify	
max02511	GYT_{26}	191	-	-	failed to amplify	
max03351	$AATG_{53}$	395	-	-	failed to amplify	
max07989	GGA_{23}	203	-	-	failed to amplify	
max09657	CTT_{20}	176	-	-	failed to amplify	
max19109	ACT_{31}	313	-	-	failed to amplify	
max02036	AAC_{28}	284	246-283	8	mispriming	
max02095	CAG_{23}	172	171-213	4	mispriming	
max02384	AC_{43}	290	252-298	6	mispriming	
max02425	AGC_{19}	255	240-258	6	mispriming	
max02830	AGC_{21}	160	159-209	6	mispriming	
max04435	GCT_{21}	213	212-243	4	mispriming	
max08489	GCT_{21}	230	229-273	4	mispriming	
max17013	AAT_{19}	238	206-243	6	mispriming	
max03565B	AAC_{20}	171	128-131	3	poor amplification	
max14162	AGC_{23}	272	226-271	4	poor amplification	
max18007	GCA_{21}	171	170-218	3	poor amplification	
max03437	WGC_{22}	195	194-218	3	too similar	
max11590	AAC_{46}	188	107-187	2	too similar	
max13231	AGC_{19}	278	257	1	too similar	

Table 31 Descriptive statistics for *E. necatrix* microsatellite loci.

Locus	Repeat and number of copies	Predicted size (bp)	Size range (bp)	Number of alleles	Heterozygosity (H)	Polymorphism Information Content (PIC)
Etm13n	TGC_{12}	287	284-305	6	0.76	0.73
Etm24n	GCY_{25}	168	141-171	6	0.75	0.71
Gronec_0358	AGC_{14}	124	105-120	3	0.55	0.46
Gronec_0479	AGC_{11}	334	327-342	5	0.72	0.69
Gronec_0997	AGC_{9}	171	156-168	4	0.69	0.64
Gronec_2028	AGC_{13}	129	125-140	2	0.47	0.36
Mednec_0647	AGC_{12}	138	133-148	2	0.47	0.36
Mednec_2437	AGC_{12}	162	155-158	2	0.49	0.37
Gronec_0937	AGC_{11}	176	-	-	failed to amplify	
Gronec_1497	AGC_{20}	154	-	-	failed to amplify	
Gronec_1591	AC_{8}	304	-	-	failed to amplify	
Gronec_1824	AGC_{13}	149	-	-	failed to amplify	
Gronec_1507	AGC_{10}	208	208-223	6	mispriming	
Gronec_1880	AGC_{9}	127	123-135	3	mispriming	
Mednec_2338	AC_{12}	144	126-154	11	mispriming	
Mednec_2703	AGC_{8}	151	150-215	-	mispriming	
Gronec_0931	AGC_{11}	162	159-397	2	poor amplification	
Mednec_1532	AGC_{8}	131	129	-	poor amplification	
Etm27n	$AAACCCT_{11}$	185	185-192	2	too similar	
Gronec_0378	AGC_{10}	144	146	1	too similar	
Gronec_1710	AGC_{7}	159	159-172	2	too similar	
Gronec_1909	AGC_{6}	144	141-158	2	too similar	
Mednec_1399	AGC_{6}	129	128-179	2	too similar	

Table 32 Descriptive statistics for *E. tenella* microsatellite loci.

Locus	Repeat and number of copies	Predicted size (bp)	Size range (bp)	Number of alleles	Heterozygosity (H)	Polymorphism Information Content (PIC)
Etm 09t	TGC_{17}	396	390-401	5	0.61	0.57
Etm 13t	TGC_{12}	280	280-283	2	0.48	0.36
Etm 14t	GCA_{12}	441	438-441	2	0.48	0.36
Etm 18	WSB_{21}	196	183-196	5	0.62	0.56
Etm 19	GCT_{12}	159	159-162	2	0.48	0.36
ten03997	TGC_{54}	214	188-206	4	0.42	0.39
ten05039	GCT_{63}	228	225-234	3	0.28	0.26
ten05838	TGC_{54}	218	207-262	5	0.68	0.63
ten08884	AGC_{54}	230	217-226	4	0.66	0.60
ten09245	GCA_{54}	219	210-219	3	0.54	0.47
ten02393	GCA_{72}	183	-	-	failed to amplify	
ten04556	GCT_{63}	202	-	-	failed to amplify	
ten06683	GCA_{54}	238	-	-	failed to amplify	
ten11885	GCT_{54}	193	-	-	failed to amplify	
ten12461	CTG_{54}	201	-	-	failed to amplify	
ten02737	GCA_{81}	289	204-367	3	mispriming	
ten02895	AGC_{72}	234	121-229	4	mispriming	
ten05645	CAG_{72}	218	210-230	3	mispriming	
ten01680	GCA_{54}	235	226	2	poor amplification	
ten02664	TGC_{45}	243	218-232	3	poor amplification	
ten03153	TGC_{63}	274	355-368	3	poor amplification	
ten09728	CAG_{63}	241	223-239	3	poor amplification	
ten00040	GCT_{72}	177	176	1	too similar	
ten02303	GCT_{54}	181	180	1	too similar	
ten03434	GCA_{81}	200	193	1	too similar	
ten03438	CTG_{72}	259	255	1	too similar	

Although the loci were variable, and DNA samples were extracted from hundreds to thousands of diploid oocysts, most samples returned a homozygous phenotype for the majority of loci. Eight *E. acervulina* samples, 9 *E. necatrix* samples, 6 *E. maxima* samples and 18 *E. tenella* samples were homozygous at all loci. While many of these samples were purified laboratory strains, over half were collected from the field.

In samples from flocks that were known to contain mixed strain infections (based on mtDNA results) generally two and, for *E. maxima* only, up to 5 alleles were found in one or more of the microsatellite loci.

A few rare, and potentially diagnostic, haplotypes were found but most alleles were shared among samples and across regions. Rare alleles were most common in the international strains. The combination of haplotypes across all loci produced many unique genotypes. For *E. acervulina* strains, only one duplicate genotype found. The field sample S390-TAS genotype was also found in a field sample with a mixed strain infection S385-TAS. Only one duplicate genotype was found among the *E. necatrix* strains; mixed strain field sample S200-

NT shared at least one allele at all loci with mixed strain field sample S340-WA. None of the *E. maxima* strains shared genotypes. Five duplicated genotypes were identified among the *E. tenella* strains, although some were based on partial genotypes (samples with missing data if a locus failed to amplify). Three lab strains contained identical genotypes (Redten-QLD, Macten-QLD and Darten-QLD) and this strain was also found in a field sample with a mixed strain infection S421-WA. Three field isolates had identical genotypes (S384-TAS, S388-TAS and S426-NSW). Field sample S063-VIC genotype was also found in a field sample with a mixed strain infection S420-WA. Although based on partial genotypes, S310-QLD could not be differentiated from S173-SA, and S399-SA could not be differentiated from S403-SA.

Differentiating vaccine strains using microsatellites

The most informative microsatellite loci for differentiating *E. acervulina*, *E. maxima*, *E. necatrix* and *E. tenella* strains in the two commercially available live vaccines used in Australia (Eimeriavax 4M and Paracox 8) are detailed below.

Eimeriavax 4M (Bioproperties, Victoria, Australia) vaccine strains

E. acervulina Royace strain had a unique genotype which was diagnostic using a minimum of two loci (Medace_2532 = 377 and Medace_3375 = 130).

E. maxima Medmax strain had a unique genotype containing two diagnostic haplotypes (max15327 = 193 and/or max25273 = 160).

E. necatrix Mednec strain had a unique genotype which was diagnostic using a minimum of two loci (Gronec_0479 = 333 and Etm13n= 293 or Gronec_0997 = 159).

E. tenella Redten strain could not be differentiated from Macten-QLD, Darten-QLD or a mixed strain infection S421-WA. The *E. tenella* Redten genotype (present in 31% of the flocks screened) can be identified using a minimum of 2 loci (Etm18 = 187 and ten8884 = 226, or ten9245 = 213, or ten05838 = 259).

Paracox 8 (MSD Animal Health, UK) vaccine strains

E. acervulina Houghton strain had a unique genotype containing two diagnostic haplotypes (Medace_3375 = 121 and/or Royace_0884 = 158).

E. necatrix Houghton strain had a unique genotype containing two diagnostic haplotypes (Etm24n = 144 and/or Gronec_0358 = 105).

E. maxima mixed strains (Chichester and MFP, a mix of 5 UK lines) had a unique genotype containing five diagnostic haplotypes (max01638 = 264 & 291, or max02230 = 259 & 262, or max04369 = 246 & 251, or max05657 = 240, or max15327 = 196 & 202; where more than one allele is present the diagnostic alleles are underlined)

E. tenella Houghton strain had a unique genotype containing two diagnostic haplotypes (Etm18 = 196 and/or ten05039 = 225).

Eimeria population structure

The multivariate analyses of the microsatellite data for *E. necatrix*, *E. acervulina*, *E. maxima* and *E. tenella* are shown in Figure 14. Each point in the principal coordinate analysis (PCoA) represents a single sampling time point and can be either a single or a mixed strain infection. Temporal samples are connected with solid lines and samples sourced through the same commercial provider are linked with dashed lines. Flocks infected with common genotypes are circled with a dashed line.

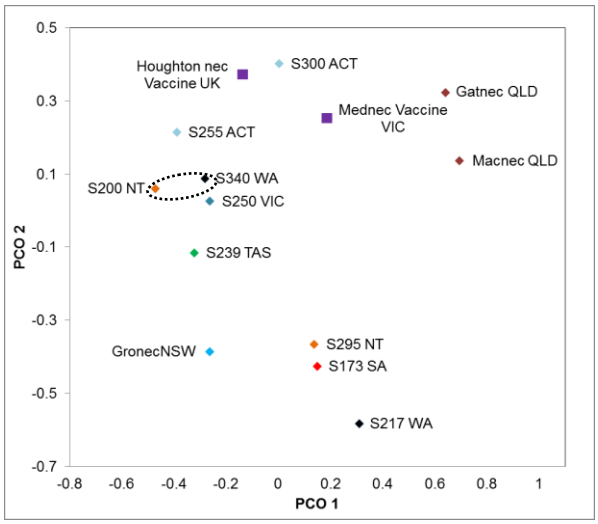
For *E. necatrix* (Figure 14a), the majority of infections were genetically distinct including the two vaccine strains (Paracox, Houghten nec; Eimeriavax, Mednec). No geographic structuring was visible in the plot, although sample numbers were low.

High genetic diversity was also observed in the largest data-set, *E. acervulina* (35 flocks) (Figure 14b). The one temporally sampled flock from TAS was interesting. Sample S347 was collected in March 2012 and contained a mixed strain infection. Two sheds were subsequently sampled 7 months later in October 2012 (S388 and S390). The sheds were different to each other but both contained *E. acervulina* strains with alleles in common with the mixed strain sample collected in March. Flocks sourced through the same commercial provider (samples connected by dashed lines in Figure 14) were never identical, but the farms were seldom neighbouring. As with *E. necatrix*, the genetic diversity of *E. acervulina* strains could not be explained by geographic structuring, however, between state differences in allele frequencies did occur at locus medace_2532 and locus medace_3837.

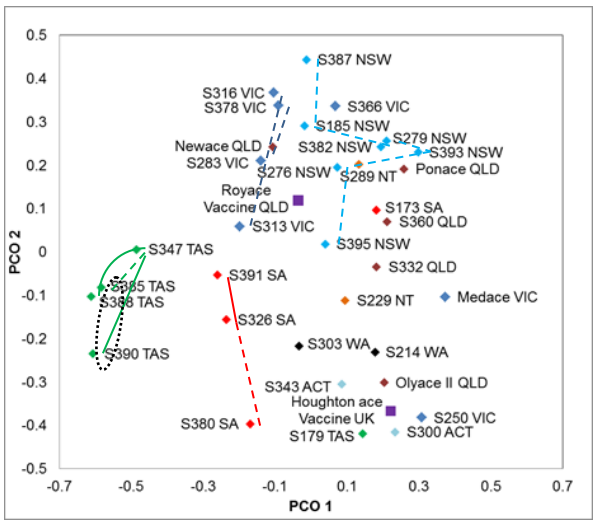
The *E. maxima* samples (Figure 14c) were also genetically widespread. A few states displayed geographic clustering for at least some of their samples (NSW, SA and VIC). As with *E. acervulina*, flocks sourced through the same commercial provider did not carry the same strains.

The lowest genetic diversity was recorded for *E. tenella*. Indistinguishable samples are circled in Figure 14d (not all points overlay each other because many of the samples are missing alleles due to poor amplification). Unlike any of the other *Eimeria* species, common widespread haplotypes of *E. tenella* were found. Interestingly one of these common haplotypes was originally isolated in 2004 in Victoria (S063) and was collected again in 2012 from WA (S420).

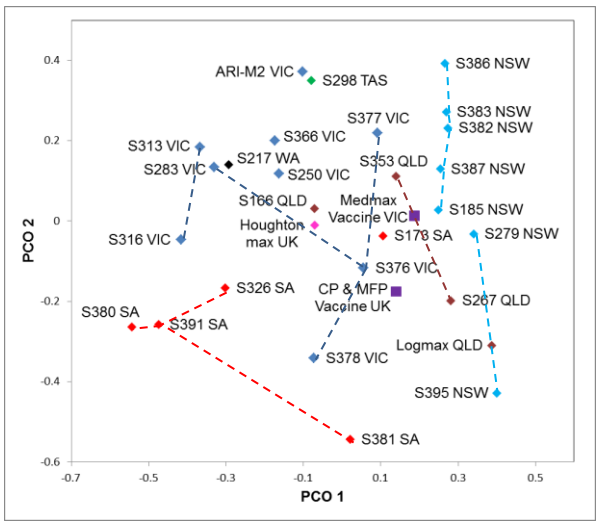
a. *E. necatrix*



b. *E. acervulina*



c. *E. maxima*



d. *E. tenella*

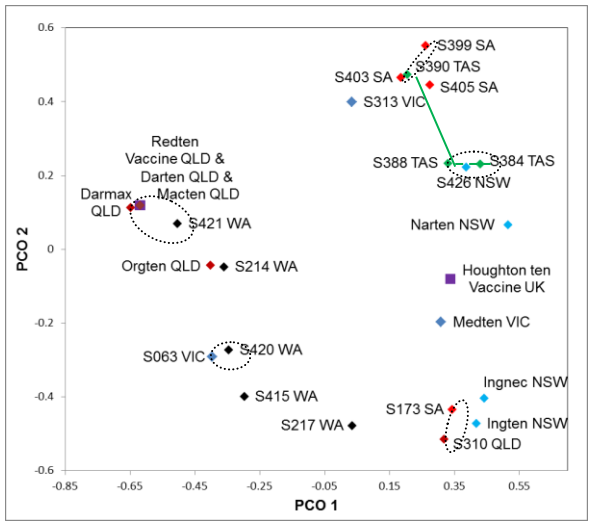


Figure 14 Results of principal coordinate analyses (PCoA) for *E. necatrix*, *E. acervulina*, *E. maxima* and *E. tenella*.

Temporal samples (same flock) are marked with solid lines. Samples from flocks sourced through a single commercial provider are linked with dashed lines. Dashed circles contain flocks infected with a shared genotype.

Temporal sampling

Objective 5 – strain stability through time

Ten commercial (Table 33) and 17 backyard (Table 34) flocks were sampled multiple times over the course of the project. DNA from historical samples provided additional resolution for some locations with the earliest collection dating back to 1995. Species level screening of these samples clearly demonstrates that *Eimeria* species are dynamic. Even within species only one strain was found at the same shed over two sampling periods, (Figure 14). Different species and strains appeared and disappeared in flocks within a 4 month sampling period. In commercial broiler flocks different species and strains were observed in different sheds, at the same location, within the same sampling period.

Although an ongoing persistent infection of *E. mitis* was detected in the same backyard flock in 1995, 2010, 2011 and 2012, a closer inspection using mitochondrial DNA markers diagnosed the infections as belonging to three different strains.

Table 33 Results of temporal sampling from commercial flocks.

Strain ID	Date of Collection	Farm ID and origin	Flock size	Bird age	Species detected (CE-assay or RT-PCR prior to S166) with strain typing in superscript					
					ten	ace	pra	mit	max	X
S278	26/09/2011	C1-NSW	22,000	31 days		ace		mit	max	
S395	24/10/2012	C1-NSW	?	26 days		ace	pra2		max	
S081	18/11/2005	C2-NSW	?	?	ten	ace		mit	max	
S275	26/09/2011	C2-NSW	42,000	24 days		ace		mit	max	
S276	26/09/2011	C2-NSW	42,000	21 days		ace[a]				
S277	26/09/2011	C2-NSW	42,000	27 days		ace		mit	max	
S393	24/10/2012	C2-NSW	40000	22 days		ace[b]	pra1&2	mit	max	
S227	7/03/2011	C3-SA	?	?						
S379	10/10/2012	C3-SA	43120	42 days		ace			max	
S381	10/10/2012	C3-SA	47360	36 days				mit	max	
S273	21/09/2011	C4-SA	30,000	47 days		ace	pra2		max	
S355	8/10/2012	C4-SA	48,000	27 days					max	
S402	8/02/2010	C5-SA	?	53 days		ace		mit		
S268	21/09/2011	C5-SA	24,000	?						
S356	8/10/2012	C5-SA	45,000	35 days		ace		mit	max	
S401	8/02/2010	C6-SA	?	56 days			pra1&2			
S357	8/10/2012	C6-SA	40,600	33 days		ace		mit	max	
S406	30/07/2010	C7-SA	?	52 days						
S323	7/02/2012	C7-SA	18,400	22 days		ace	pra1&2		max	
S404	21/06/2010	C8-SA	?	53 days				mit	max	
S326	8/02/2012	C8-SA	?	?		ace[a]	pra1&2		max[a]	X
S391	22/10/2012	C8-SA	?	?		ace[b]		mit	max[b]	
S392	22/10/2012	C8-SA	?	?		ace		mit	max	
S347	8/03/2012	C9-TAS	12,460	27 days		ace[ab]	pra1	mit		
S388	22/10/2012	C9-TAS	11,200	33 days	ten[a]	ace[a]				
S389	22/10/2012	C9-TAS	17,600	26 days	ten	ace		mit		
S390	22/10/2012	C9-TAS	18,000	22 days	ten[b]	ace[b]	pra1	mit		
S282	27/09/2011	C10-VIC	?	?		ace	pra2	mit	max	
S284	27/09/2011	C10-VIC	?	?		ace		mit	max	
S374	10/10/2012	C10-VIC	?	?					max	
S375	10/10/2012	C10-VIC	?	?		ace	pra2	mit	max	
S376	10/10/2012	C10-VIC	?	?		ace		mit	max	
S378	10/10/2012	C10-VIC	?	?		ace		mit	max	

[a] & [b] Different strains are marked with superscript letters in samples where sufficient oocysts were present for strain-typing.

Table 34 Results of temporal sampling from backyard flocks

Strain ID	Date of Collection	Farm ID and origin	Flock size	Bird age (min)	Species detected (CE-assay or RT-PCR prior to S166) with strain typing in superscript						
					ten	ace	nec	pra	mit	max	XYZ
S164	14/06/2009	B1-ACT	3	4 yrs			nec		mit		
S202	11/11/2010	B1-ACT	2	1 yr					mit		
S300	13/10/2011	B2-ACT	12	4 mths		ace[a]	nec		mit		YZ
S342	22/02/2012	B2-ACT	4	6 mths		ace[b]		pra2	mit	max	
S204	22/11/2010	B3-NSW	50	8 wks		ace	nec		mit	max	
S212	9/02/2011	B3-NSW	1	8 wks		ace					
S317	3/02/2012	B3-NSW	68	10 wks	ten		nec	pra1	mit	max	Y
S165	23/06/2009	B4-NSW	10	6 mths		ace			mit		
S187	10/11/2010	B4-NSW	10	1 yr					mit		X
S154	3/06/2009	B5-NT	30	1 yr							
S199	12/11/2010	B5-NT	20	3 mths		ace		pra2	mit		Y
S336	14/02/2012	B5-NT	3	7 mths							Y
S155	2/06/2009	B6-NT	12	1 yr							
S197	12/11/2010	B6-NT	6	3 yrs					mit		Y
S334	14/02/2012	B6-NT	10	2 yrs				pra2	mit		Y
S156	2/06/2009	B7-NT	5	1 yr							
S290	28/09/2011	B7-NT	6	?							Y
S196	12/11/2010	B8-NT	12	12 mths		ace			mit		Y
S295	29/09/2011	B8-NT	12	18 wks			nec	pra2	mit		Z
S228	14/03/2011	B9-NT	14	?							
S324	6/02/2012	B9-NT	15	12 mths		ace		pra1	mit	max	XYZ
S207	22/11/2010	B10-QLD	?	?		ace			mit		Y
S307	24/10/2011	B10-QLD	8	2 yrs							
jormit	1995	B11-QLD	?	?					mit[a]		
jorpra	1995	B11-QLD	?	?				pra2			
S166	27/10/2010	B11-QLD	?	?					mit[b]	max	
S253	25/03/2011	B11-QLD	?	?						max	
S262	20/09/2011	B11-QLD	4	?		ace			mit	max	Y
S310	1/02/2012	B11-QLD	5	3 mths	ten						
S358	10/10/2012	B11-QLD	4	1 yrs					mit[bc]		YZ
S161	8/06/2009	B12-SA	3	1 yr							
S174	8/11/2010	B12-SA	3	3 yrs			nec		mit		X
S238	15/03/2011	B13-TAS	4	6 mths					mit		X
S333	12/02/2012	B13-TAS	4	14 mths					mit		XY
S240	15/03/2011	B14-TAS	2	2 yrs					mit		X
S320	7/02/2012	B14-TAS	2	2 1/2 yrs							Z
S158	3/06/2009	B15-TAS	4	?							
S177	8/11/2010	B15-TAS	5	1 yr							
S157	15/06/2009	B16-TAS	?	?		ace		pra1			
S203	17/11/2010	B16-TAS	?	?		ace			mit		Y
S331	1/02/2012	B16-TAS	12	4 wks		ace		pra1&2	mit		YZ
S220	3/03/2011	B17-WA	17	12 wks					mit		YZ
S338	17/02/2012	B17-WA	?	?		ace					YZ

[a] & [b] Different strains are marked with superscript letters in samples where sufficient oocysts were present for strain-typing.

Discussion

Nationwide sampling

Objective 1 - Obtain 40 strains of each of the seven species of *Eimeria* from every Australian state and territory

Of the 260 samples collected overall, 85% tested positive to infection with *Eimeria*. Coccidiosis was more prevalent in commercial broiler flocks (98%) compared to backyard flocks (81%). Initial screening using species-specific real-time PCR assays underestimated infection levels. The new CE-assay determined the cause of the underestimate to be the higher than expected prevalence of OTU's X, Y and Z plus an uncharacterised OTU-W identified with the new CE-assay (but not reported on here). Considerable progress was made towards meeting Objective 1. The target of 40 strains per species was easily met for *E. acervulina* (122 strains), *E. mitis* (130 strains), *E. maxima* (91 strains), *E. praecox* (77 strains) and OTU-Y (65 strains). The target was nearly met for *E. tenella* (36 strains) but fell short for the rarer species *E. necatrix* (18 strains), *E. brunetti* (5 strains), OTU-X (20 strains) and OTU-Z (14 strains).

CE species diagnostic assay

The CE assay successfully differentiated among the 11 unique PCR products corresponding to the 7 characterized *Eimeria* species, including 2 strains of *E. praecox*, plus three OTUs.

The bin positions in the CE assay vary slightly from the known product size because mobility patterns of PCR products through the capillary polymer may differ slightly due to sequence-specific conformational characteristics that are thought to result from incomplete denaturation of the synthesized DNA (Applied Biosystems, 2009).

Although every effort was made to mix equal quantities of DNA for each species in the 11 sample mix, the differences in peak height are more likely a consequence of variations in initial template concentration rather than primer amplification preference towards particular species. Two sources of error may contribute to variations in initial template concentration. The first is that oocyst count is a rough proxy for DNA extracted. The proportion of sporulated oocysts varies among samples. Sporulated oocysts contain four times more DNA than unsporulated oocysts. Unsporulated oocysts are often not counted because they are nonviable yet they still contribute DNA. Second, there may be errors in DNA quantification using a spectrophotometer. The linear range of detection for the Nanodrop 1000 (Thermo Scientific, USA) is 1.5-3700 ng uL^{-1} with SD of +/- 1.5 ng uL^{-1}. The range of DNA concentrations of the single species used in the 11 sample mix was 1- 20 ng uL^{-1}. This is at the lower end of the range for the instrument and therefore the error margin for some

species, was almost as great as the measured concentration. This could lead to more variable amplification in PCR. The presence of host DNA can also lead to an imprecise measures of *Eimeria* DNA in any sample. Pure DNA has OD260/280 reading of approximately 1.8 whereas genomic DNA obtained from pure oocysts in this study had OD260/280 reading ranging from 1.4-2.0. Though a QIAGEN kit was used to extract DNA the presence of contaminating compounds may have influenced the accuracy of readings. Despite variations in peak height caused by template concentration, efficient amplification of target fragments of all species occurred from both single species templates as well as an 11 sample mixed template.

DNA concentrations may need adjustment to optimise peak heights each time a new 11 sample positive control is developed. For testing field samples the 11 sample mix can be treated as a worst case infection scenario. Even when all species are present in a single tube, the CE assay successfully detects all samples. If competition among the species is occurring for reagents, this will have a lesser impact when screening field samples because flocks, on average, are infected with fewer than 3 species. To be conservative, if peaks appear below the threshold line on a first screen then it may be prudent to run the sample at a different dilution.

Unlike real-time PCR this assay does not rely on the use of fluorescent probes and therefore the reagents have no greater susceptibility to degradation than conventional PCR reagents. However the M13 primer has a 5' fluorescent FAM tag. Labelled M13 primers are commonly used in microsatellite studies and frozen stocks remain stable for years. Being a primer it is more robust than a real-time PCR probe (no quencher and less impacted by 3' degradation). The reagents in this study were used over a three year period (stored at -20ºC) and showed no loss in sensitivity.

The CE assay was specific, and in terms of oocyst detection, it was as sensitive as published assays based on PCR (Gasser *et al.*, 2005), quantitative PCR (Vrba *et al.*, 2010) and loop-mediated isothermal amplification (LAMP) (Barkway *et al.*, 2011) technology which reportedly detect between one and ten nuclear genome copies (approximately 0.1-1 oocysts). When DNA was extracted from diminishing numbers of oocysts the CE assay was less sensitive but could still detect 10 oocyst equivalents from a 1000 oocyst extraction (Table 13). This result was expected since significant losses of DNA occur during genomic DNA extraction and the transfer of small numbers of oocysts can be highly variable.

The assay produced highly reproducible results for both the testing of the 11 sample mixed-template control and for the testing of mixed species commercial vaccine samples. Though the relationship between fluorescence and amplicon concentration is not linear, for both vaccines, the most abundant species gave the highest florescence signal and similarly the least abundant species gave the weakest florescence signal (Figure 6). The CE assay worked well on Paracox 8 vaccine, which is comprised of UK strains, which suggests that the assay may have application internationally as well as in Australia.

Species distribution and abundance

The high prevalence of *Eimeria* in field samples (commercial broiler flocks 98%, backyard flocks 81%) reflects a similar finding by Morris *et al*. (2007) who screened 7 commercial broiler farms in NSW and found *Eimeria* species in all. Australia's moderate climate is likely the reason for the observed high prevalence of *Eimeria* in commercial and backyard flocks. Moisture and temperature affect oocyst sporulation success with ambient temperature 25°C and high humidity (>60%) favouring the disease (Anderson *et al.*, 1976; Fayer, 1980). Average autumn, summer and spring temperatures in Australia are 22°C, 27.5°C and 22.5°C respectively (based on 1961-1990 data, Australian Bureau of Meteorology, Australian climate variability & change time-series graphs, mean temperature, www.bom.gov.au). A similar high prevalence (90%) of *Eimeria* has been reported from subtropical regions of Argentina (Mattiello *et al.*, 2000).

Morris *et al*. (2007) made a pertinent observation that holds true for the majority of samples in this study. They commented that the widespread prevalence of *Eimeria* species did not necessarily suggest that coccidiosis was a welfare concern for the chickens or that it was limiting the efficiency of the poultry operation studied. "*These chickens were asymptomatic. The correct, judicious use of anticoccidial drugs, in combination with good animal husbandry practices, appears to have enabled the producer to maintain the low level of infection necessary to ensure that a protective level of specific immunity develops in the flocks without the occurrence of clinical coccidiosis*."

The individual prevalence of the 7 characterized *Eimeria* species in commercial flocks were slightly lower here than those reported by Morris *et al*. (2007) from 7 flocks, but the relative abundance of the species mirrored that study with the exception of *E. brunetti* (*E. acervulina* 67% here versus 89% in Morris *et al*., *E. maxima* 58% versus 87%, *E. mitis* 46% versus 64%, *E. praecox* 34.4% versus 44%, *E. tenella* 18.4% versus 26%, *E. brunetti* 1.6% versus 36%, and *E. necatrix* 0.8% versus 10%). The relative abundance of *Eimeria* species in faecal samples from backyard flocks did not differ among Australian states or territories. However,

collection location was found to have a significant impact on the relative abundance of *Eimeria* species in faecal samples from commercial broiler flocks. Less *Eimeria* species diversity was found in Tasmanian commercial flocks. This result is probably a seasonal sampling artefact, rather than a true difference in distribution, as all species (with the exception of rare *E. brunetti*) were found to occur in Tasmania.

The national prevalence of *E. tenella* in both backyard and commercial flocks has been grossly underestimated in this study (and the study by Morris *et al.* (2007)) due to both studies screening for oocysts predominantly from faecal samples. Unlike other *Eimeria* species, *E. tenella* is most concentrated in the caeca of chickens (Conway and McKenzie, 2007). For this species, oocysts are largely released with caecal excrement rather than faeces (Clarke, 1979). When caecal samples, collected from commercial broiler flocks, were screened (at the very end of the project) the prevalence of *E. tenella* increased from 9% to 48%. This higher prevalence of *E. tenella* is a better reflection of overseas findings using gut lesion scoring for *Eimeria* diagnostics (Kucera, 1990; Jordan and Pattison, 1996; Mattiello *et al.*, 2000). More research is needed to assess the true prevalence of *E. tenella* in Australian flocks. The under-representation of *E. tenella* in this study, and the inclusion of caecal samples that under-represent the intestinal species, has downwardly biased estimates of mean species abundance in flocks. On average, two species were found per flock, both backyard and commercial. Morris *et al.* (2007) reported a mean infection of 3.6 species in their study of commercial flocks which excluded the OTU's. Mean infection values will likely vary seasonally and with bird age.

The low prevalence of *E. necatrix* in commercial broiler flocks (0.8% in this study) is usually attributed to its low reproductive potential (Williams, 1998; Mattiello *et al.*, 2000; Morris *et al.*, 2007) such that it is generally not a problem until flocks are past harvesting age. This study supports that finding with the prevalence of *E. necatrix* in backyard flocks (largely older layers) increasing to 12.6%.

The shift in dominant species between backyard (*E. mitis*, OTU-Y then *E. acervulina*) and commercial (*E. acervulina*, *E. maxima* then *E. mitis*) flocks likely reflects bird age and the use of in-feed chemical coccidiostat shuttle programs in commercial broiler flocks. The majority of backyard flocks were older (in many cases by years) and had no coccidiosis control in place. This discovery highlights the importance that backyard flocks are acting as reservoirs for *Eimeria* species. The second most prevalent species in backyard flocks was OTU-Y which was found in 45% of the flocks tested. The high abundance of this species explains why our initial genetic screening results, using species-specific real-time PCR assays that were

unable to detect the OTUs, were so confounded. Although less prevalent in commercial broiler flocks, the OTUs were still found. These results flag the need for more research into these poorly understood *Eimeria* species to determine their impact on the poultry industry.

It appears that coccidiostat shuttle programs are not supressing all species equally in commercial broiler flocks. The coccidiostats seem to have lowered the prevalence of *E. brunetti*, *E. necatrix* (although prevalence may be more age related for this species) and the three OTUs, but *E. acervulina, E. maxima* and *E. praecox* were commonly found. Although not eliminating infection, the coccidiostats are suppressing the severity of disease enabling the flocks to develop immunity.

Strain diagnostics

Mitochondrial DNA assays

Objective 2 – Sequence 500 base pairs of the mitochondrial genome for strains of each of the seven species of chicken *Eimeria* from every Australian state and territory

Complete mitochondrial genomes have been published for six *Eimeria* species (Lin *et al.*, 2011; Liu *et al.*, 2012; Ogedengbe *et al.*, 2013). This study has sequenced the complete mitochondrial genomes for 25 Australian isolates representing the 7 described species of *Eimeria* plus the three OTU's described by Cantacessi *et al.* (2008). A phylogenetic tree of the Australian *Eimeria* confirms their identity against other characterised species and highlights the genetic differences of the three OTU. Based on strain genome alignments two molecular assays per species, targeting regions containing variable SNPs were designed. These assays were species-specific and were used to screen DNA from field collected samples. Strain diagnostic assays were not developed for OTU-X or OTU-Y because at the time of genome sequencing, only one strain was available for these taxa. Based on their mitochondrial DNA, the most genetically diverse Australian species are *E. mitis*, *E. praecox* and *E. maxima*. Although international strains of *E. tenella* displayed high levels of mitochondrial DNA diversity this was not reflected among Australian isolates.

Multiple mitochondrial genomes are located in the cytoplasm of a single cell. The genomes within a cell are highly conserved in sequence and they are maternally inherited. Although it is possible that the genome copies might contain sequence variants, this was not observed in any of the laboratory strains of *Eimeria* that were sequenced. Thus samples from flocks that returned signals indicating the presence of mixed mitochondrial sequences were assumed to represent mixed strain infections. Although flocks may suffer from mixed strain infections, it is likely that only one strain occurs per bird due to cross protective immunity.

For *E. acervulina* 1096 bases were sequenced and 36 flocks were screened with strains originating from all Australian state and territories. Two SNPs were variable and three genotypes found, two of which were extremely widespread. A third of the flocks were infected with more than one strain of *E. acervulina*.

For *E. maxima* 1627 bases were sequenced and 30 flocks were screened with strains sourced largely from commercial flocks from QLD, NSW, VIC and SA. Eight SNPs were variable and five genotypes found, one of which was extremely widespread. A third of the flocks were infected with more than one strain of *E. maxima*.

For *E. necatrix* 1929 bases were sequenced and 12 flocks were screened with strains sourced largely from laboratory strains and backyard flocks from QLD, NSW, VIC, WA and ACT. Two SNPs were variable and two genotypes found, one of which was extremely widespread. No mixed strain infections were observed but nationwide prevalence of *E. necatrix* was low.

For *E. tenella* 2254 bases were sequenced and 32 flocks were screened with strains sourced flocks from QLD, NSW, VIC, SA, TAS and WA. Two SNPs were variable and four genotypes found, one of which was extremely widespread. A rarer genotype was found only in NSW and VIC. No mixed strain infections were observed but many of the samples were sourced from caeca and representing a single bird, not a flock.

For *E. brunetti* 1647 bases were sequenced and 7 flocks were screened with strains sourced flocks from QLD, NSW and SA. Three SNPs were variable and three genotypes found. Sequence quality was poor for a number of samples and only partial genotypes could be obtained. More samples are needed to investigate the genotypic diversity of *E. brunetti*.

For *E. mitis* 2246 bases were sequenced and 34 flocks were screened with strains originating from all Australian state and territories. Eleven SNPs were variable and five genotypes found. The genotypes fell into two distinct variants that differed by a 3-4 nucleotide deletion. Both variants were common throughout Australia but only the longer variant was observed in China and the UK vaccine strain. Half of the flocks were infected with more than one strain of *E. mitis*. The Houghton strain of *E. mitis* (present in the Paracox vaccine) had a diagnostic SNP that distinguished it from all other *E. mitis* strains. The mitochondrial DNA of *E. mitis* strains was the most variable of all the *Eimeria* species sequenced.

For *E. praecox* 2115 bases were sequenced and 26 flocks were screened with strains sourced flocks from QLD, NSW, VIC, SA, TAS and NT. Four SNPs were variable and three genotypes found, two of which were widespread. One quarter of the flocks were infected with more than one strain of *E. praecox*.

For OTU-Z 1144 bases were sequenced and 8 flocks were screened with strains sourced flocks from NSW, WA and NT. Three SNPs were variable and three genotypes found. Half of the flocks were infected with more than one strain of OTU-Z. Sequence quality was poor for a number of samples due to low oocysts numbers and only partial genotypes could be obtained. More samples are needed to investigate the genotypic diversity of OTU-Z.

Overall mitochondrial diversity was lower than expected for all *Eimeria* species, however, this genetic marker proved useful for identifying the high occurrence of mixed infections in flocks. For all species, except *E. tenella*, the Chinese strains of *Eimeria* could be differentiated from the Australian strains. Interestingly, this was not the case with the English Houghton strains which frequently carried a common Australian genotype. This result suggests the mitochondrial assays may prove to be more informative for international comparisons of strains of different *Eimeria* species. Screening international strains could perhaps identify the origins of Australian *Eimeria* which can only have a recent history in Australia since British colonization in 1788, and subsequent introduction of chickens.

Apicoplast assays

Objective 3 - Sequence 500 base pairs of the apicoplast genome for strains of each of the seven species of chicken *Eimeria* from every Australian state and territory

The A+T richness of the *Eimeria* apicoplast genome hampered progress to identify strain diagnostic mutations. For the majority of samples, PCR amplification was only achieved when the extension temperature was lowered from 72°C to 60°C. Even after considerable troubleshooting, amplification success remained inconsistent. When a 630 base region of the RpoB to ABC transporter gene was finally amplified, for two strains of each species, only two species displayed intraspecific variability (*E. mitis* 1 SNP and *E. praecox* 2 SNPs). A decision was made at this point to suspend further work targeting this marker. In the future, as genome sequences become available for more *Eimeria* species, it will be possible to align their apicoplast gene sequences to identify variable hotspots for targeted strain-diagnostic assays.

Microsatellite assays

Objective 4 - Develop at least 5 microsatellite markers to distinguish among strains of *E. acervulina* and *E. maxima* and genotype these, and *E. necatrix* and *E. tenella* strains using existing microsatellites, from every Australian state and territory

Ten variable microsatellite loci were developed for each of *E. acervulina*, *E. maxima*, and *E. tenella* and eight variable loci were developed for *E. necatrix* to distinguish among Australian strains. A further 18 microsatellite loci varied among international strains but were not useful for differentiating among Australian isolates. Improved bioinformatics pipelines for mining of *Eimeria* genomes for microsatellite loci in the future should narrow the search range to target roughly 23 repeat elements, rather than targeting loci with the highest number of the repeats. Although longer repeat elements are more prone to slippage, they are also more susceptible to allele dropout, and increased stutter, making them more difficult to amplify and consistently score (Guichoux *et al.*, 2011).

A striking finding of this study was that despite being diploid organisms, and inheriting two alleles at each locus, all of the species, across all of the loci, were predominantly homozygous. This finding was not limited to inbred laboratory lines but also included field isolates. Null alleles occur in microsatellite loci either from poor DNA quality, or when mutations in primer annealing sequences result in alleles failing to amplify during PCR. They complicate the analysis of microsatellite data because they alter allele frequencies making estimates of relatedness faulty. Null alleles alone cannot explain the observed deficit seen across all species and all loci. A huge and highly variable heterozygote deficit was also found in the tick *Ixodes ricinus* (de Meeûs *et al.*, 2002; de Meeûs *et al.*, 2004). The authors were unable to completely explain the deficit. A partial explanation may be that combining datasets from several small subpopulations into one large population from each state and territory has resulted in a deficiency of heterozygotes via the Wahlund effect (Wahlund, 1928). Again, this effect alone cannot explain the scale of homozygous excess observed in this study.

The population genetics of parasites with complex life-cycles are poorly understood (Prugnolle *et al.*, 2005). *Eimeria* are highly infective, highly mobile and have both asexual and sexual reproductive modes within the parasitic phase of their life-cycle. Substantial heterozygous deficit indicates high levels of inbreeding (Rougeron *et al.*, 2009). Chickens develop protective immunity from further infection by the same species (Williams, 1998) thus they likely only carry a single strain of each species. This suggests that sexual reproduction of the parasite is probably predominantly through selfing. A high level of inbreeding in *Eimeria* is the best explanation for the observed deficit of heterozygotes across all loci. Not all *E. maxima* strains cross-protect equally (Danforth, 1998; Smith *et al*., 2002). This species would be a good target to investigate the recombination potential of *Eimeria*.

For diagnostic purposes, excess homozygocity was an advantage. The majority of strains had unique genotypes. Every *E. maxima* strain was unique, and only one duplicate strain of

E. acervulina and *E. necatrix* was found. This result is an extremely promising outcome for vaccine strain diagnostics. Strains of *E. maxima*, *E. acervulina* and *E. necatrix* for both the Australian vaccine (Eimeriavax) and UK vaccine (Paracox), and the Paracox Houghton strain of *E. tenella* could be differentiated from all wild strains using at most two microsatellite loci. The results for Eimeriavax Redten strain of *E. tenella* were not as promising. Five duplicate genotypes were found including the vaccine strain which matched two other lab strains and a field isolate. Laboratory cross contamination cannot be excluded as all of the lab strains were isolated and purified in the same laboratory. However, this seems an unlikely explanation given the apparent absence of cross contamination from the other species that were handled in the same laboratory, over the same time period. Although not unique, the Eimeriavax Redten *E. tenella* could still be distinguished from two thirds of the wild isolates.

Of the four species investigated *E. maxima* displayed the greatest microsatellite variability, a finding congruent with the mitochondrial DNA results. Microsatellite diversity was also high for *E. acervulina*, while *E. tenella* and *E. necatrix* were more conserved. Schwarz *et al.* (2009b) found greater genetic diversity among *E. maxima* strains compared to *E. tenella* and *E. acervulina* strains in their DNA sequence-based study of broiler farms in North Carolina, USA. It will be interesting to determine if the global diversity or *Eimeria* follows the pattern observed in Australia or whether this is an artefact of the relatively recent introduction of chickens and their associated *Eimeria* to the country.

Another striking finding of this population genetic study was that mixed strain infections in flocks were common for *E. acervulina* and *E. maxima*. A flock may be exposed to multiple strains of the same species but infection in a single bird is likely to be limited to a single strain due to the development of protective immunity. Finding largely homozygous haplotypes supports this theory. This will likely also be the case for *E. tenella* but was not recorded here due to caecal sampling. To determine if this also occurs with *E. necatrix*, more targeted sampling of older birds would be needed. The discovery of mixed strain infections helps to explain why coccidiosis outbreaks are so variable in flocks. Virulent strains are probably cycling through sheds concurrently with less virulent strains, thus only a portion of the flock succumbs. Based on limited flock sampling the most common number of infections was two, however, for one unfortunate Victorian flock (S313-VIC) five alleles, suggesting five strains of *E. maxima* were detected. So it appears that flocks are not only carrying multiple species of *Eimeria*, they are also carrying multiple strains.

The different microsatellite loci were not equally informative. Polymorphism content ranged from 0.19 to 0.9. Future screening studies could reduce costs, without losing much resolving

power, by screening a panel of only the most informative loci. Further cost reductions could also be made if these loci could be multiplexed so that multiple alleles could be scored simultaneously in a single reaction tube. Another important consideration for future research is sampling methodology. The resolving power of different sampling and screening methodologies should be understood before embarking on a project. Screening a pooled sample of flock faeces provides a good indicator of flock health in a single tube reaction with minimal cost and flock disruption. This approach, however, underestimates the prevalence of *E. tenella* and complicates strain diagnostics due to mixed strain genotypes. A study interested in diagnosing *Eimeria* strains should consider sampling individual birds. Similarly, if investigating a coccidiosis outbreak in a vaccinated flock, collecting a range of samples including mixed-bird faeces, single-bird faeces and single bird gut and caecal contents will greatly assist in differentiating between vaccine failure versus vaccine application failure.

Eimeria population genetics

In diploid organisms microsatellites are assumed to be in Hardy-Weinberg equilibrium (HWE). An excessive frequency of homozygotes causes deviations from HWE expectations and can lead to significantly increased estimates of population differentiation (Chapuis and Estoup, 2007). Using F statistics to apportion genetic diversity within and between populations, and to predict effective population size, is beyond the scope of this study and may not ever be possible for *Eimeria* using classical methods. However, the high diversity of microsatellites in *Eimeria* makes them extremely useful markers for characterising different strains.

Coccidiosis in Australian flocks does not correlate with a panmictic sweep of one genetic variant of each species. A high level of genetic diversity was observed among strains with very few duplicate genotypes found. Although extensive genetic diversity exists, multivariate analyses of strain diversity within *E. acervulina E. necatrix*, *E. maxima* and *E. tenella* displayed little, if any geographic grouping of strains. Genetic diversity appears to be both high and widespread. Haplotype frequency may prove to vary among states for some loci, however, more samples are needed from single bird infections to test if this pattern represents true genetic structure or chance. Sampling strategies must be carefully considered to test this question. Commercial flocks are easier to screen because they are almost always infected, and typically have higher oocyst counts than backyard flocks. However, samples from commercial flocks are not strictly independent. There is a greater chance of *Eimeria* transfer among flocks owned by the same commercial company than

among flocks belonging to different companies. Thus care needs to be taken to ensure sampling bias doesn't influence study results.

Now that genetic markers are in hand that can differentiate among strains a range of studies and questions can be tested over a number of spatial scales. By simultaneously inoculating a bird with two strains it might be possible to create a heterozygous genotype from two homozygous lines. The scope for mixed strain challenge studies have broadened because strains within a single infection can now be differentiated. Temporal fluctuations in the relative abundance of different strains in a flock can be recorded to determine better management practices. The spread of outbreaks can be traced and the effectiveness of biosecurity measures and vaccines tested. If virulent strains can be genetically differentiated then it might be possible to identify virulence genes and map chromosome markers.

Temporal stability of *Eimeria*

Objective 5 - Temporal sampling from locations where historical strains were isolated
Twenty-seven flocks were temporally sampled over the course of the project with historical samples dating back to 1995. This study provides strong evidence that *Eimeria* species are dynamic. Within as little as four months changes in species and strains were apparent. This high turnover may reflect parasite cycling. The first colonizers dominate the flock while other species and strains remain at background levels. This could explain the Tasmanian farm where over 7 months two strains from an initial mixed infection separated into different sheds. It is not clear how effective on farm biosecurity measures are at limiting transmission. These parasites are extremely widespread and for intensively reared flocks, the only way to prevent outbreaks is to suppress oocyst numbers and allow protective immunity to develop via coccidiostats or vaccination.

Showing less genetic diversity, *E. tenella* may display greater stability through time. Field sample S063-VIC, collected in 2004, had the same *E. tenella* microsatellite profile as field sample S420-WA, a mixed strain infection collected in 2012. Interestingly S063 carried a different mitochondrial genotype to S420. The apparent overlap in microsatellite genotypes observed for this species may simply reflect a lack of markers variable enough to capture the full story.

Oocyst propagation and cryopreserved species and strains

Eimeria were successfully propagated from inoculum containing as few as 10 oocysts. For two treatments, a 1,000,000 oocyst dose of ten month old oocysts proved fatal. It is not known if the oocysts in these treatments had a higher than expected survival rate (Jeston *et*

al. (2002) reported a 1% or lower survival rate of *E. tenella* after 10 months), or if the treatment species and strains were particularly virulent. Unfortunately the OTUs (X, Y and Z) were frequently lost during propagation so relatively few strains were cryopreserved. In some instances species appeared during the process of propagation. Either between cage contamination occurred, or low abundance species were not detected in the preliminary genetic screen but amplified in the birds. Starting oocyst numbers were frequently very low, particularly those isolated from backyard flocks, thus it is highly likely that the DNA sample and subsequent CE assay may have missed oocysts of species present in low number. Every effort was made to avoid cross contamination during trials but this cannot be excluded as a contributing factor. By isolating cages by state of origin, possible contamination was hopefully minimised to within state.

Forty-two stabilates, representing 123 different infections of *Eimeria* species, were cryopreserved over the course of this project. These stabilates are a significant addition to the valuable cryopreserved *Eimeria* resource that has been developed by DAFF and more recently added to by QAAFI.

Implications

The overall conclusion that can be drawn from this study is that coccidiosis in Australia is widespread and that species are genetically diverse. Over 50 new genetic assays have been developed for improved species and strain diagnostics of *Eimeria*. Australia wide screening has found that species are ubiquitous and that strain genetic diversity does not appear to be geographically structured. Thus national measures to control the spread of coccidiosis do not appear to be necessary. The immediate outcomes of the project are listed below.

Outcomes

1. Developed a new CE assay for species diagnostics
2. Developed 15 new species-specific assays targeting variable SNPs in the mitochondrial genome for Australian strain diagnostics (7 characterised species plus OTU-Z)
3. Developed 38 new species-specific assays targeting variable microsatellite loci in the nuclear genome for Australian strain diagnostics (4 species)
4. Expanded the existing library of cryopreserved *Eimeria* species and strains with the addition of 42 new stabilates containing 123 infections

The impact of the first outcome on industry is that a cheaper and more comprehensive diagnostic assay for all known Australian *Eimeria* species is now available. As a comparison

with real-time PCR (which can currently detect 7 species), the CE assay can detect 10 species and is roughly one fifth the price per sample.

The impact of outcomes 2 and 3 is that Australian strains of *Eimeria* can, for the first time, be differentiated from one another. This includes vaccine strains. It will now be possible to differentiate a failed vaccine from a vaccination failure. Training and education can readily fix problems with vaccination technique, developing a new vaccine or importing an overseas product would be extremely costly to industry.

The expansion of the *Eimeria* collection in Outcome 4 will hopefully reduce future sampling expenditure and increase the capacity of downstream projects by providing an invaluable collection of species and strains sourced from all over Australia. This rare collection also has a temporal component that adds to its value.

Recommendations

- A manuscript detailing the CE assay is in press in *Electrophoresis* and manuscripts detailing mitochondrial DNA variability and the new strain diagnostic assays will be prepared after the completion of this final report.
- Over the course of the project our results have been disseminated via two international conference presentations (World Association for the Advancement of Veterinary Parasitology, 2013), two national conference presentations (Australian Veterinary Parasitology Association, 2010 & 2012), two national conference posters (Australian Society for Parasitology, 2011) and two Poultry CRC Ideas Exchange presentations (2011 & 2012).
- Our research results flag the need for more research into the poorly understood *Eimeria* OTU's to determine their impact on the poultry industry. OTU-Y was the second most prevalent species in backyard flocks with 45% prevalence. Although the species appear to have less impact on broilers, they may have a low reproductive potential like *E. necatrix* and have a more significant impact on layers and breeders. A preliminary research proposal to RIRDC in 2012 to work on the OTU was unfortunately unsuccessful.
- More research is needed to assess the true prevalence of *E. tenella* in commercial flocks. A significant Australia-wide collection of caecae exists from a now complete campylobacter study (RIRDC project PRJ-003801). A preliminary research proposal was submitted to RIRDC in 2013 to fund a collaborative project to screen those samples with the *E. tenella* strain diagnostic assays developed in this project.

- Further testing of the strain diagnostic assays for vaccine differentiation is needed on vaccinated commercial flocks. Vaccinated flocks were purposely excluded from this study in an effort to target wild strains of *Eimeria*. Additional work is also needed to develop multiplexed strain-diagnostic assays for the most informative loci to reduce sample, reagent and labour costs.

Acknowledgements

The authors would like to thank Ms Anthea Bruyeres and Ms Catherine Minchen, for their very capable technical support. We thank Ms Aniqah Mahamudin and Mr Elmabruk Gamag for their contribution to *Eimeria* microsatellite development while studying for an Honour's degree and Master's degree respectively through The University of Queensland. We would like to thank David Mayer and Marco Kienzle for their assistance with the microsatellite analyses. We also thank Rima Youil and Wayne Woods at Eimeria Pty Ltd for providing *Eimeria* strains and Tom Grimes at Schering-Plough for a sample of the Paracox vaccine. Finally we would like to thank friends and collaborators around Australia at the various poultry farms and backyard flocks who assisted us with sample collection for *Eimeria* screening.

Glossary and abbreviations

apicDNA: apicoplast DNA.
ARI: Animal Research Institute.
CE: Capillary-electrophoresis, procedure for separating DNA fragments based on size with resolution to a single nucleotide.
Coccidiosis: disease caused by infection with species of *Eimeria.*
Cryopreservation: Process of freezing and storing live, viable cells.
Ct score: Cycle threshold number, real-time PCR cycle where amplification curve crosses threshold line.
DAFF: Department of Agriculture Fisheries and Forestry (formerly DPI&F) Queensland Government.
DEEDI: Department of Employment, Economic Development and Innovation (formerly DPI&F) Queensland Government.
DNA: Deoxyribonucleic acid.
DPI&F: Department of Primary Industries and Fisheries, Queensland Government.
Genome: the entire DNA of an organism. Each *Eimeria* cell contains a nuclear genome in the nucleus and many mitochondrial and apicoplast genomes in the cytoplasm.
Genotype: The combined haplotype information across a number of loci.
Haplotype: A mutation (or set of mutations that are inherited together) at a single locus.
H: Heterozygosity. The H score is the probability of heterozygosity and is dependent on the number of alleles and their frequency in a given population. The score ranges in value between 0 (no variability) and 1 (high variability).
HWE: Hardy Weinberg Equilibrium, a principle stating that the genetic variation in a population will remain constant from one generation to the next assuming random mating and no migration, mutation or selection ($p^2+2pq+q^2=1$).
ITS2: Internal Transcribed Spacer 2 region of ribosomal DNA.
$K_2Cr_2O_7$: Potassium dichromate.
Locus/Loci: a particular position on a chromosome, in a diploid organism each locus is represented by two alleles.
Missing data: incomplete haplotype information due to a locus failing to amplify.
Msat: Microsatellite: short (2-6 bases long) repetitive element of DNA.
mtDNA: mitochondrial DNA.
nDNA: nuclear DNA.
Null allele: microsatellite alleles that fail to amplify during PCR due to mutations in the priming annealing sequence.
Oocyst: resistant life stage of *Eimeria* that is excreted into the environment.
OTU: Operational Taxonomic Unit, phylogenetic grouping that represents a genetically distinct, but as yet uncharacterised lineage (in this case probably new species).
Partial genotype: incomplete genotype missing haplotype information due to a locus failing to amplify.
PBS: phosphate buffered saline.
PCR: Polymerase Chain Reaction, a procedure for amplifying DNA.
PIC: Polymorphism information content. PIC is the probability that a locus will be informative in one given mating in a random breeding population. It is closely related, but always less than or equal to the Heterozygosity score (H), and values range between 0 (no allelic variation) and 1 (all alleles are different) for co-dominant markers.
Primer: Short string of DNA (around 20 bases long) that matches a target sequence and is used to prime PCR and sequencing reactions.
QAAFI: Queensland Alliance for Agriculture and Food Innovation, The University of Queensland.
rDNA: ribosomal DNA.
RIRDC: Rural Industries Research and Development Corporation.
RT: Room temperature.

RT-PCR: Real-time PCR: a PCR incorporating fluorescence dyes so that DNA amplification can be measured in real time.
SE: standard error.
SNP: single nucleotide polymorphism.
PCA: Principal Coordinate Analysis, method of exploring and visualising similarities and differences in data.
Ta: predicted maximum annealing temperature for primers.
Wahlund effect: reduction in heterozygosity in a population caused by subpopulation structure.

References

Anderson, B.C., 1981. Patterns of shedding of cryptosporidial oocysts in Idaho calves. *Journal of the American Veterinary Medical Association* 178, 982-984.

Anderson, W.I., Reid, W.M., Johnson, J.K., 1976. Effects of high environmental temperatures on cecal coccidiosis. *Poultry Science* 55, 1429-1435.

Applied Biosystems, 2009. DNA sequencing by capillary electrophoresis. Applied Biosystems Chemistry Guide. In, Applied Biosystems, USA (http://www3.appliedbiosystems.com/cms/groups/mcb_support/documents/generaldocuments/cms_041003.pdf).

Barkway, C.P., Pocock, R.L., Vrba, V., Blake, D.P., 2011. Loop-mediated isothermal amplification (LAMP) assays for the species-specific detection of *Eimeria* that infect chickens. *BMC Veterinary Research* 7, 67.

Cai, X.M., Fuller, A.L., McDougald, L.R., Zhu, G., 2003. Apicoplast genome of the coccidian *Eimeria tenella*. *Gene* 321, 39-46.

Callow, L.L., 1984. Animal Health in Australia: Volume 5 Protozoal and rickettsial diseases. Australian Government Publishing Service, Canberra.

Cantacessi, C., Riddell, S., Morris, G.M., Doran, T., Woods, W.G., Otranto, D., Gasser, R.B., 2008. Genetic characterization of three unique operational taxonomic units of *Eimeria* from chickens in Australia based on nuclear spacer ribosomal DNA. *Veterinary Parasitology* 152, 226-234.

Chapuis, M.P., Estoup, A., 2007. Microsatellite null alleles and estimation of population differentiation. *Molecular Biology and Evolution* 24, 621-631.

Cidade, F.W., Vigna, B.B., de Souza, F.H., Valls, J.F., Dall'Agnol, M., Zucchi, M.I., de Souza-Chies, T.T., Souza, A.P., 2013. Genetic variation in polyploid forage grass: assessing the molecular genetic variability in the Paspalum genus. *BMC Genetics* 14, 50.

Clarke, P.L., 1979. Coccidial infection with *Eimeria tenella* and caecal defaecation in chicks. *British Poultry Science* 20, 317-322.

Conway, D.P., McKenzie, M.E., 2007. *Poultry coccidiosis diagnosis and testing procedures.* Blackwell Publishers, Ames, Iowa, U.S.A.

Danforth, H.D., 1998. Use of live oocyst vaccines in the control of avian coccidiosis: experimental studies and field trials. *International Journal for Parasitology* 28, 1099-1109.

de Meeûs, T., Béati, L., Delaye, C., Aeschlimann, A., Renaud, F., 2002. Sex-biased genetic structure in the vector of lyme disease, *Ixodes ricinus*. *Evolution* 56, 1802-1807.

de Meeûs, T., Humair, P.-F., Grunau, C., Delaye, C., Renaud, F., 2004. Non-Mendelian transmission of alleles at microsatellite loci: an example in *Ixodes ricinus*, the vector of Lyme disease. *International Journal for Parasitology* 34, 943-950.

Fayer, R., 1980. Epidemiology of protozoan infections: The coccidia. *Veterinary Parasitology* 6, 75-103.

Gasser, R.B., Skinner, R., Fadavi, R., Richards, G., Morris, G., 2005. High-throughput capillary electrophoresis for the identification and differentiation of seven species of *Eimeria* from chickens. *Electrophoresis* 26, 3479-3485.

Guichoux, E., Lagache, L., Wagner, S., Chaumeil, P., LÉGer, P., Lepais, O., Lepoittevin, C., Malausa, T., Revardel, E., Salin, F., Petit, R.J., 2011. Current trends in microsatellite genotyping. *Molecular Ecology Resources* 11, 591-611.

Hodgson, J.N., 1970. Coccidiosis: Oocyst counting technique for coccidiostat evaluation. *Experimental Parasitology* 28, 99-102.

Jaccard, P., 1908. Nouvelles recherches sur la distribution florale. *Bulletin de la Société vaudoise des sciences naturelles* 44, 223-270.

Jeffers, T.K., 1975. Attenuation of *Eimeria tenella* through selection for precociousness. *The Journal of Parasitology* 61, 1083-1090.

Jeston, P.J., Blight, G.W., Anderson, G.R., Molloy, J.B., Jorgensen, W.K., 2002. Comparison of infectivity of *Eimeria tenella* oocysts maintained at 4, 12 or 28°C for up to 10 months. *Australian Veterinary Journal* 80, 91-92.

Jordan, F.T.W., Pattison M. (Editors), 1996. Poultry Diseases. W.B. Saunders, London.

Jorgensen, W.K., Stewart, N.P., Jeston, P.J., Molloy, J.B., Blight, G.W., Dalgliesh, R.J., 1997. Isolation and pathogenicity of Australian strains of *Eimeria praecox* and *Eimeria mitis*. *Australian Veterinary Journal* 75, 592-595.

Kucera, J., 1990. Identification of *Eimeria* species in Czechoslovakia. *Avian Pathology* 19, 59-66.

Lin, R.-Q., Qiu, L.-L., Liu, G.-H., Wu, X.-Y., Weng, Y.-B., Xie, W.-Q., Hou, J., Pan, H., Yuan, Z.-G., Zou, F.-C., Hu, M., Zhu, X.-Q., 2011. Characterization of the complete mitochondrial genomes of five Eimeria species from domestic chickens. *Gene* 480, 28-33.

Liu, G.H., Hou, J., Weng, Y.B., Song, H.Q., Li, S., Yuan, Z.G., Lin, R.Q., Zhu, X.Q., 2012. The complete mitochondrial genome sequence of *Eimeria mitis* (Apicomplexa: Coccidia). *Mitochondrial DNA* 23, 341-343.

Mattiello, R., Boviez, J.D., McDougald, L.R., 2000. *Eimeria brunetti* and *Eimeria necatrix* in chickens of Argentina and confirmation of seven species of *Eimeria*. *Avian Diseases* 44, 711-714.

Morgan, J.A.T., Constantinoiu, C.C., Jorgensen, W.K., 2009a. New diagnostic assays to improve control of coccidiosis in poultry. In, Vol. 09/038, Rural Industries Research and Development Corporation, Canberra.

Morgan, J.A.T., Morris, G.M., Wlodek, B.M., Byrnes, R., Jenner, M., Constantinoiu, C.C., Anderson, G.R., Lew-Tabor, A.E., Molloy, J.B., Gasser, R.B., Jorgensen, W.K., 2009b. Real-time polymerase chain reaction (PCR) assays for the specific detection and quantification of seven *Eimeria* species that cause coccidiosis in chickens. *Molecular and Cellular Probes* 23, 83-89.

Morris, G.M., Woods, W.G., Grant Richards, D., Gasser, R.B., 2007. The application of a polymerase chain reaction (PCR)-based capillary electrophoretic technique provides detailed insights into Eimeria populations in intensive poultry establishments. *Molecular and Cellular Probes* 21, 288-294.

Nagy, S., Poczai, P., Cernák, I., Gorji, A.M., Hegedűs, G., Taller, J., 2012. PICcalc: An online program to calculate polymorphic information content for molecular genetic studies. *Biochemical Genetics* 50, 670-672.

Ogedengbe, M.E., Hafeez, M.A., Barta, J.R., 2013. Sequencing the complete mitochondrial genome of *Eimeria mitis* strain USDA 50 (Apicomplexa: Eimeriidae) suggests conserved start positions for mtCOI- and mtCOIII-coding regions. *Parasitology Research*.

Posada, D., Crandall, K.A., 1998. MODELTEST: testing the model of DNA substitution. *Bioinformatics* 14, 817-818.

Prugnolle, F., Liu, H., de Meeûs, T., Balloux, F., 2005. Population genetics of complex life-cycle parasites: an illustration with trematodes. *International Journal for Parasitology* 35, 255-263.

Rougeron, V., de Meeûs, T., Hide, M., Waleckx, E., Bermudez, H., Arevalo, J., Llanos-Cuentas, A., Dujardin, J.-C., De Doncker, S., Le Ray, D., Ayala, F.J., Bañuls, A.-L., 2009. Extreme inbreeding in *Leishmania braziliensis*. *Proceedings of the National Academy of Sciences* 106, 10224-10229.

Rozen, S., Skaletsky, H., 2000. primer 3 on the WWW for general users and for biologist programmers. In: Krawetz, S.,Misener, S. (Eds.), Bioinformatics methods and protocols: Methods in molecular Biology, Humana Press, Totowa, New Jersey, pp. 365–386. Source Code: http://bioinfo.ut.ee/primer3/

Schuelke, M., 2000. An economic method for the fluorescent labeling of PCR fragments. *Nature Biotechnology* 18, 233-234.

Schwarz, R.S., Jenkins, M.C., Klopp, S., Miska, K.B., 2009a. Genomic analysis of *Eimeria spp*. populations in relation to performance levels of broiler chicken farms in Arkansas and North Carolina. *The Journal of Parasitology* 95, 871-880.
Schwarz, R.S., Jenkins, M.C., Klopp, S., Miska, K.B., 2009b. Genomic analysis of *Eimeria* spp. populations in relation to performance levels of broiler chicken farms in Arkansas and North Carolina. *Journal of Parasitology* 95, 871-880.
Smith, A.L., Hesketh, P., Archer, A., Shirley, M.W., 2002. Antigenic diversity in *Eimeria maxima* and the influence of host genetics and immunization schedule on cross-protective immunity. *Infection and Immunity* 70, 2472-2479.
Shirley, M.W., Smith, A.L., Tomley, F.M., 2005. The biology of avian *Eimeria* with an emphasis on their control by vaccination. In: Baker, J.R., Muller, R.,Rollinson, D. (Eds.). *Advances in Parasitology* 60, 285-330.
Swofford, D.L., 2002. PAUP* Phylogenetic analysis using parsimony (*and Other Methods). In, Sinauer Associates, Sunderland, Massachusetts.
Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G., 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* 25, 4876-4882.
Vrba, V., Blake, D.P., Poplstein, M., 2010. Quantitative real-time PCR assays for detection and quantification of all seven *Eimeria* species that infect the chicken. *Veterinary Parasitology* 174, 183-190.
VSN International, 2011. GenStat for Windows Release 15.3. Web page: GenStat.co.uk. In, Hemel Hempstead, UK.
Wahlund, S., 1928. The combination of populations and the appearance of correlation examined from the standpoint of the study of heredity. *Hereditas* 11, 65-106.
Williams, R.B., 1998. Epidemiological aspects of the use of live anticoccidial vaccines for chickens. *International Journal for Parasitology* 28, 1089-1098.