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Mitochondrial genomes of Australian chicken *Eimeria* support the presence of ten species with low genetic diversity among strains

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ABSTRACT

Modern molecular approaches have vastly improved diagnostic capabilities for differentiating among species of chicken infecting *Eimeria*. Consolidating information from multiple genetic markers, adding additional poultry *Eimeria* species and increasing the size of available data-sets is improving the resolving power of the DNA, and consequently our understanding of the genus. This study adds information from 25 complete mitochondrial DNA genomes from Australian chicken *Eimeria* isolates representing all 10 species known to occur in Australia, including OTU-X, −Y and −Z. The resulting phylogeny provides a comprehensive view of species relatedness highlighting where the OTUs align with respect to others members of the genus. All three OTUs fall within the *Eimeria* clade that contains only chicken-infecting species with close affinities to *E. maxima*, *E. brunetti* and *E. mitis*. Mitochondrial genetic diversity was low among Australian isolates likely reflecting their recent introduction to the country post-European settlement. The lack of observed genetic diversity is a promising outcome as it suggests that the currently used live vaccines should continue to offer widespread protection against *Eimeria* outbreaks in all states and territories. Flocks were frequently found to host multiple strains of the same species, a factor that should be considered when studying disease epidemiology in the field.

1. Introduction

Protozoan parasites in the genus *Eimeria* cause the intestinal disease coccidiosis. This important global livestock disease has a significant economic impact on the poultry industry where high-density housing of large numbers of birds favours parasite transmission. Management of the disease is achieved by strict farm biosecurity as well as in-feed coccidiostats and or vaccination. Despite significant management efforts, coccidiosis outbreaks still occur due to widespread drug resistance and environmental persistence (Blake and Tomley, 2014). The disease has been further promoted by the expansion of floor reared, and free range husbandry techniques that increase flock exposure to infectious oocysts in the environment. Parasite elimination has not proved feasible; instead control is based on parasite suppression allowing birds to develop natural immunity. The annual global cost of coccidiosis, including production losses, prevention and treatment, has been estimated at over USD$3 billion (Dalloul and Lillehoj, 2006).

Seven species of *Eimeria* are recognized and found globally infecting chickens. In Australia a further three operational taxonomic units (OTU) were characterized by Cantacessi et al., 2008 (Cantacessi et al., 2008). Little is known about the prevalence or distribution of these OTU although all three cryptic species have now been identified in flocks from Africa (Clark et al., 2016; Fornace et al., 2013) and a recent global survey of *Eimeria* suggests they may be widespread in the southern hemisphere, but not the northern (Clark et al., 2016). It is unlikely that the OTU, or any of the chicken infecting *Eimeria* species, originated in Australia as chickens have only been present in the country since European settlement in the late 18th century.

Genetic characterisation of poultry *Eimeria* has historically been based on a limited number of relatively small (< 1000 bp) gene regions (Barta et al., 1997; Cantacessi et al., 2008; Lew et al., 2003; Ogedengbe et al., 2011). With the advent of genome sequencing, paired with a reduction in sequencing costs, it has now become possible to compare much larger regions of DNA. Phylogenetic comparisons of *Eimeria* based on entire mitochondrial genomes have now been published (Hikosaka et al., 2011; Lin et al., 2011; Liu et al., 2012; Ogedengbe et al., 2014; Ogedengbe et al., 2013) with the interesting discovery that *Eimeria necatrix* and *Eimeria tenella* are more closely related to turkey *Eimeria* than they are to the remaining chicken *Eimeria* (Miska et al., 2010; Ogedengbe et al., 2014).

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As with other apicomplexan parasites, the *Eimeria* mitochondrial genome has been reduced to roughly 6 kb in length and contains only 3 genes; cytochrome *c* oxidase subunit I (COI), cytochrome *c* oxidase subunit III (COIII) and cytochrome b (Cyt b) plus numerous short fragments of small and large subunit ribosomal DNA (SSU and LSU rDNA) (Lin et al., 2011).

An underlying question for all parasites is to understand how genetic diversity influences epidemiology and pathogenicity and its implication in therapeutic and vaccination strategies as well as disease control. Experimental outcomes can be profoundly influenced by the choice of *Eimeria* species and strain, and a lack of standardization among experiments currently hampers meaningful comparisons.

The aim of this study was to characterise the mitochondrial genomes of multiple strains of all Australian species of chicken infecting *Eimeria,* including OTU-X, −Y and −Z, to assess how they align to global isolates, and to determine if genetic markers capable of distinguishing among strains could be identified. Having this information could assist with improved vaccine monitoring and quality control and provide the tools for molecular epidemiology to characterise different strains of *Eimeria*.

2. Methods

2.1. Samples and DNA extraction

Animal Science Queensland, within the Queensland Department of Agriculture and Fisheries housed oocysts from pure Australian strains of each of the seven characterized *Eimeria* species and three OTU (Table 1). Prior to DNA extraction oocysts were washed with distilled water, then suspended in 80 µl of phosphate-buffered saline (PBS, pH 7.2) and mechanically homogenized using 0.1 g of 1 mm glass beads for 5 min in a MiniBeadbeater-96 (Biospec Products, Bartlesville, OK, USA). DNA was extracted from the cracked oocysts using a DNeasy Tissue Kit (Qiagen, Chadstone, VIC, Australia) following the manufacturer's guidelines into a final elution volume of 50 µl. DNA from two mammal infecting *Eimeria* species, *E. zuernii* and *E. falciformis* (Table 2), were sequenced as part of this study to help resolve basal lineages in the phylogeny.

All available online *Eimeria* spp. mitochondrial DNA genome sequences were included in preliminary phylogenetic analyses (Tables 1 and 2) including turkey-specific species which Ogedengbe et al. (2014) demonstrated were closely related to chicken *Eimeria*. In addition *Caryospora bigenetica*, *Cyclospora cayetanensis* and an avian *Isospora* sp. were included as these species have been reported paraphyletic with some representatives nested within the *Eimeria* clade (Barta et al., 2012; Chapman et al., 2013; Megía-Palma et al., 2015). A representative of the genus *Choleoeimeria* was used to root the phylogeny.

2.2. DNA amplification and sequencing

Complete mitochondrial genome sequence was obtained from three strains of each of the seven recognised species of *Eimeria* infecting chickens in Australia. Complete coverage of the genome was obtained with the use of seven primer pairs (Table 3). 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, 10 x HotMaster Taq buffer (Eppendorf, Macquarie Park NSW, Australia, containing 25 mM magnesium), 0.8 mM dNTP, and 0.05 units/µl of HotMaster Taq DNA polymerase (Eppendorf, Macquarie Park NSW, Australia). Thermal cycling conditions consisted of an initial denaturation (95 °C for 4 min) followed by 30 cycles of 95 °C for 30 s, 47–55 °C (refer to Ta column in primer tables) for 30 s and 72 °C for 1 min 30 s, with a final extension step of 72 °C for 7 min. Cycling was performed in a Biorad thermal cycler PTC-200 (DNA Engine Peltier). PCR products were viewed on 1.5% agarose and TBE gels stained with GelRed (Biotium, USA, distributed by Gene Target Solutions, Dural, New South Wales, Australia).

Table 1
Chicken infecting *Eimeria* included in study with new accessions underlined.

Species	Strain	Geographical Origin	Genbank Accession
E. acervulina	Newace	Queensland, Aus	KX094946
	Ponace	Queensland, Aus	KX094948
	Royace	Queensland, Aus	KX094947
	China	China	HQ702479[a]
	Emw-scaff1766[b]	UK	GeneDB, Sanger[b]
E. brunetti	Monbru	South Australia, Aus	KX094958
	Roybru	Queensland, Aus	KX094959
	Bowbru	New South Wales, Aus	KX094957
	China	China	HQ702480[a]
E. maxima	Ingmax	Victoria, Aus	KX094966
	ARI-M3	Victoria, Aus	KX094964
	Medmax	Victoria, Aus	KX094965
	China	China	HQ702481[a]
	Houghton	UK	MGI[c]
E. mitis	Kelmit	Queensland, Aus	KX094961
	Redmit	Queensland, Aus	KX094962
	Jormit	Queensland, Aus	KX094963
	China	China	JN864949[d]
	USDA 50	USA	KF501573[e]
E. necatrix	Gatnec	Queensland, Aus	KX094954
	Gronec	Queensland, Aus	KX094952
	Mednec	Victoria, Aus	KX094953
	China	China	HQ702482[a]
E. praecox	Jorpra	Queensland, Aus	KX094944
	Ingpra	New South Wales, Aus	KX094945
	Andpra	Queensland, Aus	KX094943
	China	China	HQ702483[a]
E. tenella	Darten	Queensland, Aus	KX094949
	Ingten	New South Wales, Aus	KX094951
	Redten	Queensland, Aus	KX094950
	China	China	HQ702484[a]
	NIAH	Japan	AB564272[f]
	Houghton	UK	GeneDB, Sanger[g]
E. sp. OTU X1	X1	Victoria, Aus	KX094967
E. sp. OTU Y1	Y1	Victoria, Aus	KX094960
E. sp. OTU Z1	Z1	Victoria, Aus	KX094955
E. sp. OTU Z2	Z2	New South Wales, Aus	KX094956

[a] (Lin et al., 2011).
[b] Contaminated *E. maxima* (Weybridge).
[c] Malaysia Genome Institute.
[d] (Liu et al., 2012).
[e] (Ogedengbe et al., 2013).
[f] (Hikosaka et al., 2011).
[g] (Logan-Klumpler et al., 2012).

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, Thermo-Fisher Scientific, Scoresby, Victoria, Australia) and were run on an Applied Biosystems 3130XL Genetic Analyser. Raw sequence chromatograms were aligned and edited with Sequencher (Vers 4.8 Gene Codes Corporation, Ann Arbor, MI, USA).

2.3. DNA sequence and amino acid alignment

Sequences were linearized to the same starting point as used by Lin et al. (2011). Complete sequences were aligned using ClustalW (Thompson et al., 1997). The ClustalW output was then eyeball edited prior to phylogenetic analysis to ensure codon reading frames were maintained across the alignment and gaps minimised. Coding sequence was determined by aligning files to annotated accessions on Genbank. Coding sequences were translated into amino acids using NCBI genetic code 4 for mold, protozoa and coelenterate mitochondrial DNA. Two