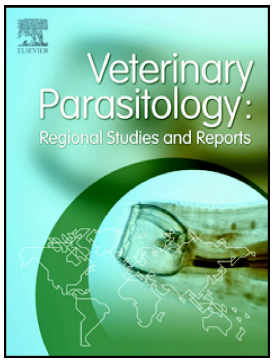

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Morphological and molecular characterization of three *Eimeria* species from captured rangeland goats in Western Australia

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ABSTRACT

Faecal shedding of *Eimeria* by captured rangeland goats (*Capra hircus*) was investigated using a longitudinal observational study. Faecal samples were collected from 125 male goats on four occasions. The first sampling occurred following capture and transport, immediately after arrival at a commercial goat depot (feedlot) in Western Australia, with subsequent 3 sample collections occurring at one month intervals thereafter. Goats were composite breed and aged approximately 9–12 months on arrival at the feedlot. Prevalence and shedding intensity (faecal oocyst concentration) for *Eimeria* were determined using qPCR. Species were identified from individual oocysts (isolated using micromanipulation) using molecular analysis at two loci, specifically 18S rRNA and mitochondrial cytochrome oxidase gene (COI), and confirmed by microscopy. Longitudinal prevalence (animals positive at least once) for *Eimeria* spp. by qPCR was 90.4%, with 60% goats shedding *Eimeria* spp. on more than one occasion. Point prevalence (prevalence at a single sampling occasion) ranged from 2.4% (fourth sampling) to 70.4% (second sampling). Three species were identified at the 18S rRNA locus and confirmed by microscopy: *E. christenseni* (longitudinal prevalence for single infection 34.4%), *E. hirci* (17.6%) and *E. arloingi* (8.8%) over the four sample collections. Mixed infections were identified in 56.8% goats (longitudinal prevalence). 18S rRNA sequences from *E. christenseni* and *E. hirci* and were 100% homologous with ovine *E. ahsata* and *E. crandallis* respectively, and *E. arloingi* was 100% similar to caprine *E. arloingi.* At the COI locus, *E. christenseni, E. hirci* and *E. arloingi* grouped separately, and were closely related to ovine *E. ahsata*, with genetic similarities of 96.5%, 92.6% and 91.4% respectively. This is the first report for molecular characteristics of caprine-derived *Eimeria* spp. using a combination of 18S rRNA and COI. Molecular techniques can be used to identify *Eimeria*

spp. in goat faecal samples, specifically through characterization at 18S locus and other gene loci when used in parallel. Molecular techniques offer some advantages over microscopy for identification of *Eimeria* species, particularly with respect to precision.

Keywords: *Eimeria*; 18S rRNA; COI; Rangeland goats

1. Introduction

Strong growth in the Australian goat meat industry has been largely based on rangeland goats (MLA, 2015). Rangeland goats are trapped, transported and may be managed under intensive management in feedlots (goat depots) for variable periods prior to slaughter. Diarrhoea and ill-thrift are cited as major issues in captured rangeland goats (MLA, 2016), yet relatively little is known about the underlying causes. Coccidiosis caused by *Eimeria* spp. and associated with stress of capture, transport, overcrowding and domestication of rangeland goats has been suggested as a cause of diarrhea and ill thrift in captured goats (MLA 2016), but the epidemiology of *Eimeria* spp. in rangeland goats is not well described.

Ten *Eimeria* species have been reported from domestic and wild goats in Australia, based on microscopic examination of faeces; *E. ninakohlyakimovae, E. arloingi* (considered homologous with ovine *E. bakuensis*), *E. hirci* (considered homologous with ovine *E. crandallis*), *E. christenseni* (considered homologous with ovine *E. ahsata*), *E. alijevi*, *E. caprina*, *E. caprovina*, *E. jolchijevi*, *E. apsheronica* and *E. paltida* have been identified (Kanyari, 1988; O'Callaghan, 1989). Many *Eimeria* infections in goats are asymptomatic; however, some species have been associated with diarrhoea and stunted growth (Chartier and Paraud, 2012; Ruiz et al., 2012). Of the 16 *Eimeria* species described in goats worldwide, *E. ninakohlyakimovae* and *E. arloingi* are considered the most pathogenic (Koudela and Boková, 1998; Cavalcante et al., 2012; Chartier and Paraud, 2012; Khodakaram-Tafti et al., 2013).

Identification of *Eimeria* spp. has traditionally been made on the basis of the morphological characteristics of the sporulated oocysts and host specificity. However, morphological techniques have been described as having relatively low sensitivity, and practical limitations associated with the time (Carvalho et al., 2011a; 2011b), labor, and

training required for microscopy (Khodakaram-Tafti et al., 2013). Furthermore, the morphological similarity of oocysts of some *Eimeria* spp. is a limitation in supporting (or refuting) identification based on microscopy alone (Tenter et al., 2002; Haug et al., 2007; Kawahara et al., 2010; Hatam-Nahavandi et al., 2016). For example, *Eimeria* spp. from goats and sheep may be morphologically identical, but cross infection studies (demonstrating patent infections in naïve animals following infection) are required to confirm host specificity. Molecular tools can address limitations with respect to sensitivity and unambiguous identification of *Eimeria* spp., and have been used to describe the epidemiology of *Eimeria* spp. in Australian sheep (Yang et al., 2014). The 18S rRNA locus has been used extensively as a molecular marker in phylogenetic analysis. As it is a conservative gene, the 18S may not be the most suitable gene for differentiating closely related *Eimeria* species and therefore this locus should be used in parallel with other gene loci for characterisation of *Eimeria* spp. (Ogedengbe et al., 2011).

The aim of this study was to describe the species of *Eimeria* from captured rangeland goats managed under typical conditions for meat production in Western Australia using molecular and morphological tools. The hypothesis tested was that molecular techniques can be used to identify *Eimeria* spp. in goat faecal samples.

2. Materials and methods

2.1. Animals and faecal sample collection

This was a longitudinal observational study with 125 male rangeland goats (composite breed) sampled once monthly for four months (S1 to S4) commencing February 2014. Goats were captured from a sheep and cattle extensive rangeland grazing

property, North Wooramel station, located 78 km east of Denham and 113 km south east of Carnarvon in the Gascoyne region of Western Australia. The first sample collection (S1) occurred immediately after transport and arrival at a commercial goat depot (feedlot) near Geraldton, Western Australia, where goats were housed for the duration of the study. On arrival at the feedlot (S1), goats weighed 30.7 ± 0.3 kg (mean ± standard error) with an estimated age of 9–12 months based on dentition. Goats were housed in four group pens (approximately 30 goats per pen). Grain-based pellets, hay and water were supplied *ad libitum*. Straw-bedding was provided with bare dirt covering the majority of available pen space. No pasture was available for the duration of the study. Goats were consigned for slaughter after conclusion of the experiment when they reached acceptable slaughter weight.

Faecal samples were collected directly from the rectum and stored on ice or in a refrigerator (4.0°C) until DNA extraction or sporulation for microscopy were performed. Sample collection methods were approved by Murdoch University Animal Ethics Committee (approval number R2617/13).

2.2. Treatments

All goats were treated with an anthelmintic, 5 mg/kg moxidectin (Cydectin oral plus selenium®, Virbac Australia), and an anti-coccidial treatment (20 mg/kg toltrazuril, Baycox®, Bayer Australia) immediately after the first (S1) and second (S2) sampling as part of the standard management practice for goats being introduced to the feedlot.

2.3. DNA isolation

For each faecal sample (n=500), four freeze–thaw cycles were employed followed by genomic DNA extraction for 200 mg faeces from each faecal sample (n=500). Extractions were performed using a Power Soil DNA Kit (MolBio, Carlsbad, California), which included a mechanical bead disruption step using glass beads to increase the efficiency of DNA extraction. A negative control (no faecal sample) was used in each extraction group.

2.4. qPCR screening and quantification

All faecal samples (n=500) were screened by qPCR at the 18S ribosomal RNA (rRNA) locus, and oocyst concentrations in faecal samples (oocyst per gram of faeces) were determined by qPCR as previously described (Yang et al., 2014).

2.5. PCR amplification and sequencing at the 18S rRNA locus

All qPCR *Eimeria* positive samples (n=210) were subjected to a two-step PCR at the 18S locus which was used for the molecular genotyping of *Eimeria* species using the primers EiGTF1 and EiGTR1 (Yang et al., 2016) for the external PCR and the primers EiGTF2 and EiGTR2 (Yang et al., 2015) for the internal reaction. The expected PCR product was ~1,510 bp. However, this process yielded mixed chromatograms for some samples (n=100).

2.6. Isolation of morphologically similar Eimeria *spp. oocysts using a micromanipulator*

Morphologically identical sporulated *Eimeria* oocysts were isolated from all qPCR positive faecal samples, including single infections (n=110) and samples that produced mixed chromatograms (n=100) via sequencing of nested 18S PCR amplicons as described above. The process used for sporulation is described in more detail below (section 2.9). Sporulated

oocysts were examined by microscopy, and a 3 axis hydraulic micromanipulator (MO-102, Nirashige, Japan) was used to select four morphologically similar *Eimeria* spp. oocysts from each faecal sample. Where multiple morphotypes were observed (i.e. mixed infections), four oocysts of each morphotype were selected and transferred to separate slides as described above.

The morphologically similar oocysts (n=4 per morphotype) isolated from each qPCR positive faecal sample were transferred to a new slide, examined and photographed using microscopy (Olympus DP71 digital micro-imaging camera) to confirm morphological similarity. Measurements were recorded for species identification based on morphological characteristics (section 2.9).

2.7. DNA extraction from isolated oocysts

Morphologically similar *Eimeria* spp. oocysts (n=4 oocysts per morphotype), isolated from each qPCR positive faecal sample were transferred into a PCR tube containing 10 µl of lysis buffer (0.005% SDS in TE solution) by washing the coverslip with 100 µl saline. After a brief centrifugation, the tube was frozen in liquid nitrogen and thawed in a 95 °C water bath for four rounds to disrupt the oocyst walls. After the addition of 0.5 µl proteinase K (20 mM), the tube was incubated at 56 °C for 2 h and then at 95 °C for 15 min. The entire lysate of the morphologically similar oocysts was used for two separate PCRs (18S rRNA and COI) as described below (section 2.8).

2.8. PCR amplification and sequencing of isolated oocysts at the 18S and COI loci

PCR amplification at the 18S rRNA locus was conducted as previously described

(section 2.5) on the DNA extracted from the morphologically similar *Eimeria* spp. isolated oocysts (section 2.6) of the faecal samples initially screened positive by qPCR.

A partial mitochondrial cytochrome oxidase gene (COI) gene sequence (723 bp) was amplified using a nested PCR with the following primers COIF1 (Ogedengbe et al., 2011) and COXR1 (Dolnik et al., 2009) for the external reaction and COIF2 (Yang et al., 2013b) and COXR2 (Dolnik et al., 2009), for the internal reaction.

The amplified DNA fragments from the secondary 18S rRNA and COI PCR products were separated by gel electrophoresis and purified using an in-house filter tip method and used for sequencing without any further purification as previously described (Yang et al., 2013a). The results of the sequence reactions were analysed and edited using FinchTV (Version 1.4), compared to existing *Eimeria* spp. 18S and COI sequences on GenBank using BLAST searches and aligned with reference genotypes from GenBank using ClustalW in BioEdit (V7.2.5) (www.mbio.ncsu.edu/bioedit/).

2.9. Phylogenetic analysis of Eimeria spp.

Phylogenetic trees were constructed for *Eimeria* spp. at the 18S rRNA and COI loci with additional isolates from GenBank. Distance estimation was conducted using TREECON (Van de Peer and De Wachter, 1994), based on evolutionary distances calculated with the Tamura–Nei model. Parsimony and Maximum Likelihood (ML) analyses were conducted using Molecular Evolutionary Genetics Analysis software (MEGA version 6) (Tamura et al., 2013). Bootstrap analyses were conducted using 1,000 replicates to assess the reliability of inferred tree topologies.

2.9. Speciation based on morphological characteristics

Morphological characteristics for sporulated oocysts were determined for faecal samples that were qPCR positive (section 2.4). Approximately 2g faeces were placed in 2% (w/v) potassium dichromate solution ($K_2Cr_2O_7$), mixed well and poured into petri dishes to a depth of less than 1 cm and kept under close observation at room temperature in the dark to facilitate sporulation. Faecal flotation was conducted using a saturated sodium chloride and 50% sucrose (w/v) solution (Soulsby, 1982). A sample from the supernatant layer was transferred to a slide.

Sporulated oocysts were observed using an Olympus DP71 digital micro-imaging camera and images were taken using Nomarski contrast imaging system with a 100× oil immersion objective. Morphological features were recorded and measurements were performed on oocysts (n=35) of each identified *Eimeria* species based on morphological similarity. All measurements are given in micrometres (μm) as the mean followed by the range in parentheses. Minor shape variations were observed. The number of oocyst cell wall layers was confirmed by crushing individual oocysts with gentle coverslip pressure.

Species were differentiated by reference to the descriptions given by Honess (1942), Levine et al., (1962a) and Shah and Joshi (1963).

2.11. Statistical analyses

Goats were classified as positive (parasite DNA detected) or negative (no parasite DNA detected) separately for *Eimeria* (all species), *E. arloingi*, *E. christenseni*, *E. hirci* or mixed infection (more than one *Eimeria* spp.). Point prevalence was determined by proportion of positive goats for each sample occasion. Longitudinal prevalence was

calculated as the proportion of goats with *Eimeria* DNA detected on at least one occasion. Prevalence 95% confidence intervals were calculated using Jeffrey's interval method (Brown et al., 2001). Goats were categorised for frequency of *Eimeria* detection across the four sampling occasions (ie positive on 0, 1, 2, 3 or 4 occasions).

Two-tailed Z tests (Sergeant, 2016) were used to compare point prevalence between sampling occasions, and proportion of goats for each frequency of *Eimeria* detection across the four sampling occasions (ie positive on 0, 1, 2, 3 or 4 occasions). P-values of 0.05 were used to declare statistical significance.

Faecal shedding intensity was log transformed for analysis using LOG10 (OPG+1). Differences in *Eimeria* spp. shedding intensity between time points were assessed using a univariate general linear model (SPSS Statistics for Mac version 21, IBM) with timepoint included as a fixed factor and least squares difference post hoc test. P-values of 0.05 were used to declare statistical significance.

3. Results

3.1. Observed prevalence and shedding intensity of Eimeria *spp. using qPCR and genotyping at 18S rRNA locus*

Overall, 210/500 faecal samples were qPCR positive for *Eimeria* spp., and 191/210 qPCR positive samples were successfully sequenced. Single infections with three *Eimeria* species were identified in samples successfully sequenced at 18S rRNA locus; *E. christenseni* (53/191), *E. hirci* (23/191) and *E. arloingi* (15/191). Infections with mixed *Eimeria* spp. were identified in 100/191 successfully sequenced samples at 18S rRNA locus. The prevalence and shedding intensity for single and mixed *Eimeria* spp. infections are shown in Table 1.

Frequencies of *Eimeria* detection over the four sampling occasions are shown in Table 2. Over the four sample collections, 60% (75/125) of goats were shedding *Eimeria* spp. on more than one occasion. More goats were shedding on two of the four sampling occasions (53/125) than either one occasion (38/125) or three occasions (22/125; Table 2). No goats were identified as shedding *Eimeria* spp. on more than three occasions. Mixed *Eimeria* spp. infections, single *E. christenseni* infections, single *E. hirci* infections were all more commonly identified on one occasion compared with two or three occasions.

[Insert Table 1]

[Insert Table 2]

3.2. Phylogenetic analysis of three Eimeria *spp. at the 18S rRNA locus*

A 1,229 bp PCR product of *E. christenseni* and *E. hirci* and *E. arloingi*, was successfully amplified and sequenced. Phylogenetic analyses of these species at the 18S locus using Distance, Parsimony and ML analyses produced similar results (Fig. 1, Distance tree shown). *Eimeria christenseni* from rangeland goats grouped in a clade with *E. ahsata* (AF338350) with 100% homology, and *E. hirci* from rangeland goats grouped in a clade with *E. crandallis* (AF336339) with 100% homology (Fig. 1), both of which were from domestic sheep (*Ovis aries*) in Turkey (Kaya et al., 2007). The single available goat-derived *E. arloingi* 18S sequence on GenBank (KC507792) was only 637 bp in length and therefore an insert tree (Fig. 1a) was generated to compare the 18S rRNA sequences from *E. arloingi*, *E. hirci* and *E. christenseni*. This analysis revealed that *E. arloingi* from rangeland goats in the present study was 100% identical to *E. arloingi* (KC507792) from Iranian native goat kids

(*Capra hircus*) (Khodakaram-Tafti et al., 2013).

The 18S rRNA nucleotide sequences of the three *Eimeria* spp. from the rangeland goats in the present study were deposited in GenBank under the accession numbers KX845684 (*E. christenseni*), KX845685 (*E. hirci*) and KX845686 (*E. arloingi*).

[Insert Figure 1]

3.3. Phylogenetic analysis of the three Eimeria *spp. at the COI locus*

Phylogenetic analysis of the 670 bp COI sequence placed *E. christenseni* from rangeland goats in the same clade with ovine *E. ahsata* (KT184373) from Canada (Ogedengbe et al., 2016) (Fig. 2). *Eimeria christenseni*, *E. hirci* and *E. arloingi* from rangeland goats exhibited 96.5%, 92.6% and 91.4% genetic similarity respectively with ovine *E. ahsata* (KT184373). The partial COI nucleotide sequences from these three *Eimeria* spp. from rangeland goats were deposited in GenBank under the accession numbers KX857468 (*E. christenseni*), KX857469 (*E. hirci*) and KX857470 (*E. arloingi*).

[Insert Figure 2]

3.4. Morphology of three Eimeria *spp. by microscopy*

Morphological characteristics of oocysts and sporocysts are shown in Tables 3a and 3b. Oocyst sporulation was achieved within 48-72 hr at room temperature. Except for the absence of the polar granules in the examined sporulated oocysts, the morphological features (shape and size) of the oocysts and sporocysts from the three identified *Eimeria* spp. were

consistent ranges previously reported for the respective species (Tables 3a and 3b), and morphological species identification was consistent with the species identification by sequencing. All *Eimeria* oocysts examined in the present study had bi-layered oocyst walls and possessed micropolar caps, which were more prominent in *E. arloingi* compared with those of *E. christenseni* and *E. hirci* (Figure 3a). No polar granules (Table 3a) or Stieda bodies (Table 3b) were observed. A sporocyst residuum was present in all three species (Table 3b), with more obvious shattered granules for *E. arloingi* and *E. christenseni* (Figure 3). Minor shape variations were observed.

[Insert Table 3a and 3b]

[Insert Figure 3]

4. Discussion

This study is the first to describe a combination of morphological and molecular characteristics for *Eimeria* species from Australian rangeland goats. Three *Eimeria* spp. were identified at the 18S and COI loci (*E. christenseni*, *E. hirci* and *E. arloingi)*. Morphological characteristics of oocysts were consistent with previous reports, and confirmed sequencing results. Observations supported the hypothesis tested that molecular techniques can be used to identify *Eimeria* spp. in goat faecal samples, specifically, through characterisation at 18S locus and other gene loci when used in parallel. Molecular techniques offer some advantages over microscopy for identification of *Eimeria* species, particularly with respect to precision for species identification. The molecular techniques confirmed that *Eimeria* shedding was common in the captured goats, with over 90% of goats at the feedlot shedding *Eimeria* spp. on at least one sampling occasion. Mixed infections were identified in 57% of goats.

The three *Eimeria* spp. identified in this study have all been previously reported in Australian rangeland goats (O'Callaghan, 1989). All three *Eimeria* spp. are considered pathogenic in goats (Andrews, 2013), although to a lesser extent compared to *E. ninakohlyakimovae*, which was not identified in the present study. *Eimeria christenseni* has been associated with severe diarrhoea, anorexia, polydipsia, poor hair coat, and extreme weakness in neonatal goat kids (Lima, 1981). *Eimeria hirci* (ovine homologous *E. crandallis*) is considered pathogenic in goats, but the lesions and pathology caused have not been completely delineated (Taylor et al., 2016). *Eimeria crandallis* (caprine homologous *E. hirci*) resulted in loss of surface epithelial cells, villous atrophy, crypt destruction and severe diarrhoea when experimentally inoculated into lambs up to 3 months of age (Gregory and Catchpole, 1990; Taylor et al., 2003). *Eimeria arloingi* has been associated with both

subclinical and clinical coccidiosis and subsequent production losses (Jalila et al., 1998; Koudela and Boková, 1998).

Mixed *Eimeria* spp. infections were commonly identified at the first three sampling occasions. The pathogenicity of mixed infection in rangeland goats has yet to be tested, but previous studies in sheep have shown that mixed *Eimeria* infections extend patency and increase oocyst production, which may aggravate the overall effect of infection (Catchpole et al., 1976). Similarly, co-infections with other parasites and bacteria may exacerbate clinical outcome of infection in sheep and goats (Foreyt, 1990; Glastonbury, 1990; Rahman, 1994; Navarre and Pugh, 2002; Andrews, 2013), therefore the role of *Eimeria* spp. in conjunction with other infectious agents on diarrhoea and ill thrift in captured rangeland goats should be addressed in future studies.

Eimeria prevalence and shedding intensity determined by qPCR were highest at the second sampling occasion, approximately one month after capture, transport and arrival at the feedlot. It was not possible to determine specific factors that contributed to the rise in prevalence and shedding intensity observed between arrival (S1) and S2, but stress associated with transport, mixing of animals and confinement of undomesticated goats are possible causes (Main and Creeper, 1998). This observational study likely failed to identify peak shedding intensity for *Eimeria* as a consequence of the one month interval between S1 (immediately after capture and transport) and S2. Kommuru et al. (2014) reported increased oocyst counts in goat kids (16 weeks of age) one week after weaning, transport and change of housing from pasture to pens, but oocyst counts then steadily decreased for the following four weeks (Kommuru et al., 2014). Severe (often fatal) coccidiosis following mustering and transport has been reported in Western Australian rangeland goats, with onset of clinical

signs 5-7 days after entering feedlot (Main and Creeper, 1998).

It is possible that the two anti-coccidial treatments given immediately after the first (S1) and second (S2) sampling impacted the prevalence and shedding intensity of *Eimeria* spp., particularly for those species with longer pre-patent period. These treatments were given as part of the normal husbandry at the feedlot. Despite treatment, both prevalence and intensity of *Eimeria* shedding increased between S1 and S2 (i.e. one month after the first treatment) and declined between S2 and S3 (i.e. one month after the second treatment). Coccidiosis in goats is generally self-limiting. Given the increase in prevalence and shedding intensity of *Eimeria* observed at S2 (one month after the first toltrazuril treatment), the fall in prevalence and shedding intensity of *Eimeria* observed between S2 and S3 (i.e. one month after the second treatment) was more likely attributable to goats acquiring immunity following exposure to infection, than to any effect of treatment. Variable effects of toltrazuril on *Eimeria* shedding by goats have been reported. For example, Iqbal et al. (2013) reported 100% reduction in oocyst shedding 28 days following a single toltrazuril treatment (20mg/kg) in 1-3 month-old goats housed in group pens, whereas Chartier et al. (1992) reported a reduction in faecal oocyst counts for only 14 days following single treatment (20mg/kg) in 4–6 month old goats housed in group pens. Further investigation into whether toltrazuril is effective in reducing *Eimeria* shedding, the duration of response to treatment, and whether treatment is effective in reducing clinical coccidiosis is warranted before recommendations can be made with respect to use of toltrazuril in captured rangeland goats.

Except for the absence of the polar granules in the examined sporulated oocysts from rangeland goats, the other morphological characteristics of oocysts and sporocysts of the identified species were consistent with previous reports. Polar granules were reported in *E.*

christensni (syn. *E. ahsata*) (Honess, 1942) and *E. arloingi* (syn. *E. bakuensis*) (Levine et al., 1962a; Shah and Joshi, 1963), however none of the authors reported whether their observations of the polar granules were made on fully sporulated oocycts or not. Levine and Ivens (1981) reported disappearance of polar granules of some *Eimeria* spp. after sporulation.

Identification of *Eimeria* species based on morphological characteristics was consistent with identification by sequencing. The combined use of morphological and molecular tools offers advantages in confirming *Eimeria* spp. identification (Kawahara et al., 2010; Hatam-Nahavandi et al., 2016), particularly with respect to speciation for morphologically similar *Eimeria* spp. oocysts in faecal samples. For example, *E. crandallis* (described in goats) and *E. weybridgensis* (described in sheep) share similar morphological characteristics, with only minor differences evident following sporulation. Molecular tools can be used to confirm identification of morphologically similar oocysts from different hosts (for example sheep and goats) to determine if they are genetically identical (same species), or different species that are morphologically similar, without the need for cross infection studies.

This is the first study to report characterization of *Eimeria* spp. from goats using both 18S and COI loci. Using the 18S locus and other gene loci in parallel improves precision for molecular characterization. The 18S locus has been extensively used as a molecular marker in a plethora of phylogenetic analysis; however, as the 18S gene is highly conserved, the COI locus was also included as it has been shown to have higher resolving power for *Eimeria* spp., especially with respect to recent speciation events (Ogedengbe et al., 2011).

The phylogenetic analysis at the 18S locus revealed that *E. christenseni* and *E. hirci* from rangeland goats were identical to ovine homologous *E. ahsata* (AF336339) and *E. crandallis* (AF338350) from domestic sheep in Turkey (Kaya et al., 2007), and clustered

together on a single clade. This was consistent with reports that some *Eimeria* species, including *E. ahsata* and *E. crandallis*, may infect both goats and sheep (Vercruysse, 1982; More at al., 2015). Both *E. ahsata* and *E. crandallis* have been reported in Australian sheep using molecular identification (Yang et al., 2014).

The present study is the first to produce a longer (1,229 bp) 18S rRNA sequence of *E. arloingi*. Sequence comparison of this species was not possible due to the non-availability of longer 18S sequences in GeneBank. Therefore, only 673 bp of common sequence overlap was used to conduct the comparison with *E. arloingi* (KC507792). The sequence for *E. arloingi* from rangeland goats in the present study was identical to an 18S sequence from *E. arloingi* reported in goats in Iran (Khodakaram-Tafti et al., 2013).

Goat-derived sequences were not available in GenBank at the COI locus except for one *E. ahsata* sequence from domestic sheep. Sequences from *E. christenseni, E. hirci* and *E. arloingi* exhibited 96.5%, 92.6% and 91.4% genetic similarities respectively with ovine *E. ahsata* (KT184373), which is within the range of accepted species and suggests that the COI gene is a suitable locus for differentiating closely *Eimeria* related species in small ruminants. As more sequences from small ruminants become available in GenBank, phylogenetic analysis of the COI locus will be able to provide more meaningful information on relationships between caprine and ovine *Eimeria* species. Analyzing the isolates at multiple loci will provide a more in-depth analysis of the evolution of caprine-derived *Eimeria* spp.

In conclusion, three *Eimeria* spp. (*E. christenseni, E. hirci* and *E. arloingi*) were identified from captured rangeland goats at the 18S and COI loci, and this was confirmed with morphological characteristics of oocysts. To our knowledge this study reports the first combination of 18S and COI genes for molecular characterization of *Eimeria* species in goats.

Molecular methods offer improved precision for species identification for oocysts, and may be used to determine whether morphologically identical *Eimeria* spp. from different hosts are genetically similar (same species) or different species with similar morphological characteristics, without the need for cross infection studies. Molecular tools have application in investigations to improve understanding of coccidiosis epidemiology, treatment and control studies, and diagnostic investigations of outbreaks. In this study, *Eimeria* spp. shedding was common in captured rangeland goats under typical feedlot management conditions, with 90% of goats shedding at least once over the four sampling occasions. Mixed infections were identified in 57% of goats. Additionally, this study serves as a prelude for future epidemiological studies pertaining to the clinical relevancy of coccidiosis in goats and interventions that may ameliorate the impact of coccidiosis for captured rangeland goats and small ruminants more generally.

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Fig. 1 Evolutionary relationships of *Eimeria* spp. inferred by distance analysis of using 18S rRNA gene. Accession numbers of samples follow the species name. Percentage support (>50%) from 1000 pseudoreplicates from distance, ML and parsimony analysis, respectively, is indicated at the left of the support node (dash (−) = value was <50%).

Fig. 2 Evolutionary relationships of *Eimeria* spp. inferred by distance analysis of the cytochrome c oxidase subunit I (COI) gene. Accession numbers of samples follow the species name. Percentage support (>50%) from 1000 pseudoreplicates from distance, ML and parsimony analysis, respectively, is indicated at the left of the support node (dash (−) = value was <50%).

Fig. 3 Nomarski interference-contrast photomicrographs of the *Eimeria* oocysts from rangeland goats; *E. arloingi* (A), *E. christenseni* (B) and *E. hirci* (C) showing oocyst wall (OW), micropolar cap (MC), sporozoites (SP), and sporocyst residuum (SR). Scale bar = 20 μm.

Table 1 *Eimeria* spp. prevalence and shedding intensity observed for captured rangeland goats (n=125) sampled on 4 occasions (S1-S4).

	Sampling occasion				**Longitudinal prevalence**
	S1	S2	S3	S4	
Prevalence (% (95% confidence interval))					
Eimeria spp. [#]	50.4 (41.7, 59.1)[a]	70.4 (62.0, 77.9)[b]	44.8 (36.3, 53.6)[a]	2.4 (0.7, 6.3)[c]	90.4 (84.3, 94.6)
Single infections[##]	20.8 (14.4, 28.5)[a]	34.4 (26.5, 43.0)[b]	16.8 (11.0, 24.1)[a]	0.8 (0.1, 3.7)[c]	54.4 (45.7, 62.9)
E. christenseni	12.0 (7.2, 18.5)[a]	19.2 (13.0, 26.8)[b]	11.2 (6.6, 17.6)[a]	0 (0, 2.0)[c]	34.4 (26.5, 43.0)
E. hirci	6.4 (3.1, 11.7)[a]	8.8 (4.8, 14.7)[a]	2.4 (0.7, 6.3)[ac]	0.8 (0.1, 3.7)[c]	17.6 (11.7, 25.0)
E. arloingi	2.4 (0.7, 6.3)[ac]	6.4 (3.1, 11.7)[a]	3.2 (1.1, 7.4)[a]	0 (0, 2.0)[c]	8.8 (4.8, 14.7)
Mixed infections[##]	24.0 (17.2, 32.0)[a]	30.4 (22.9, 38.8)[a]	24.0 (17.2, 32.0)[a]	1.6 (0.3, 5.0)[b]	56.8 (48.0, 65.2)
E. christenseni + *E. hirci*	3.2 (1.1, 7.4)[a]	6.4 (3.1, 11.7)[a]	1.6 (0.3, 5.0)[ab]	0 (0, 2.0)[b]	10.4 (5.96, 16.6)
E. christenseni + *E. arloingi*	2.4 (0.7, 6.3)[a]	1.6 (0.3, 5.0)[a]	2.4 (0.7, 6.3)[a]	0.8 (0.1, 3.7)[a]	7.2 (3.6, 12.7)
E. hirci + *E. arloingi*	3.2 (1.1, 7.4)[a]	0.8 (0.1, 3.7)[ab]	0.8 (0.1, 3.7)[ab]	0 (0, 2.0)[b]	3.2 (1.1, 7.4)
E. christenseni + *E. hirci* + *E. arloingi*	15.2 (9.7, 22.3)[a]	21.6 (15.1, 29.4)[a]	19.2 (13.0, 26.8)[a]	0.8 (0.1, 3.7)[b]	44.0 (35.5, 52.8)
Species prevalence (single + mixed infections)					
E. christenseni	32.8 (25.0, 41.4)[a]	48.8 (40.1, 57.5)[b]	34.4 (26.5, 43.0)[a]	1.6 (0.3, 5.0)[c]	74.4 (66.3, 81.4)
E. hirci	28.0 (20.7, 36.3)[ab]	37.6 (29.5, 46.3)[a]	24.0 (17.2, 32.0)[b]	1.6 (0.3, 5.0)[c]	63.2 (54.5, 71.3)
E. arloingi	23.2 (16.5, 31.2)[a]	30.4 (22.9, 38.8)[a]	25.6 (18.6, 33.7)[a]	1.6 (0.3, 5.0)[b]	54.4 (45.7, 62.9)
Not sequenced*	5.6	5.6	4.0	0	–
Faecal shedding intensity (oocysts per gram)					
All samples (mean ± SE)	530±183[a]	10525±2496[b]	350±88[a]	1±1[c]	
Positive samples only (mean ± SE)	1051±352[a]	15,851±3627[b]	841±193[a]	31±21[a]	
All samples (range)	0–15,908	0–191,821	0–5375	0–73	

[abc] Point prevalence (z test) and mean shedding intensity (LSD post hoc test, faecal oocyst count log transformed for analysis) values in rows with different superscripts are significantly different ($P<0.05$).

[#] all *Eimeria* species.

[##] Based on sequencing at 18S rRNA locus.

*Samples qPCR positive but not successfully sequenced at 18S rRNA locus.

SE: standard error.

Table 2 Frequency of detection of *Eimeria* spp. shedding in 125 rangeland goats.

	Frequency of detection of *Eimeria* (n)				
	0	1	2	3	4
Eimeria spp.	12[a]	38[b]	53[c]	22[a]	0[d]
Single infections					
E. christenseni	82[a]	35[b]	6[c]	2[cd]	0[d]
E. hirci	103[a]	21[b]	1[c]	0[c]	0[c]
E. arloingi	114[a]	8[b]	2[bc]	1[c]	0[c]
Mixed infections	54[a]	46[a]	21[b]	4[c]	0[d]

[abc] Frequency values in rows with different superscripts are significantly different (two tailed, $P<0.05$).

Table 3a Oocyst morphological features for *Eimeria* spp. from rangeland goats compared with previous reports.

		Oocyst							
			Size mean (range) µm						
Species (synonymous names)	**Host**	**Shape**	Height	Width	**Shape index** mean (range)	**Wall**	**Micropolar cap**	**Polar granule**	**Reference**
E. christenseni [a]									
Observed	Goat	ellipsoid –ovoid*	34.5 (28.9–35.8)	23.3 (16.4-25.8)	1.5 (1.2–1.8)	bi-layered	present	absent	
Previously reported	Sheep	ellipsoid	33.4 (29–37)	22.6 (17-28)	1.48 (1.2–1.8)	bi-layered	n/a	present	Honess, 1942
E. hirci [b]									
Observed	Goat	ellipsoid –ovoid*	20.7 (17.4–23.4)	18.2 (16.8-22.2)	1.14 (1.04–1.2)	bi-layered	present	absent	
Previously reported	Sheep	broadly ellipsoid –ovoid	21.9 (17–23)	19.4 (17-22)	1.11 (1.00–1.35)	bi-layered	present	n/a	Honess, 1942
E. arloingi [c]									
Observed	Goat	ellipsoid –ovoid*	28.3 (23.4–29.2)	20.1 (18.4–21.2)	1.41 (1.23–1.59)	bi-layered	prominent	absent	
Previously reported	Goat	ellipsoid - slightly ovoid	28 (22–31)	20 (17-22)	1.4 (1.3–1.6)	bi-layered	present	present	Levine et al., 1962a
	Goat	ellipsoid – ovoid	28 (22–35)	21 (18-26)	1.4 (1.3–1.6)	bi-layered	present	present	Shah and Joshi, 1963

[a] *E. ahsata* has been considered synonymous with *E. christenseni*, and has been redescribed in domestic sheep with slightly larger measurements (Levine et al., 1962b).
[b] *E. crandallis* has been considered synonymous with *E. hirci* (Chevalier, 1966).
[c] *E. bakuensis* has been considered synonymous with *E. arloingi* (Chevalier, 1966).
*although majority of *E. arloingi* were ellipsoid and *E. christenseni* and *E. hirci* were ovoid, minor shape variation occurred within species.

Table 3b Sporocyst morphological features for *Eimeria* spp. from rangeland goats compared with previous reports.

		Sporocysts					
			Size mean (range) µm				
Species (synonymous names)	**Host**	**Shape**	Height	Width	**Stieda body**	**Residuum**	**Reference**
E. christenseni [a]							
Observed	Goat	broadly elongate–ovoid	15.4 (13.9–17.7)	8.6 (8.1-10.5)	absent	present**	
Previously reported	Sheep	elongate– ovoid	15.4 (n/a)	7.81 (n/a)	n/a	present	Honess, 1942
E. hirci [b]							
Observed	Goat	slightly ovoid-round	9.2 (8.1–10.8)	6.6 (5.9-8.3)	absent	present***	
Previously reported	Sheep	ovoid	9. 5 (8–11)	6.4 (5-8)	n/a	n/a	Honess, 1942
E. arloingi [c]							
Observed	Goat	elongate ovoid	13.8 (11.7–15.7)	8.2 (7.0–9.8)	absent	present**	
Previously reported	Goat	elongate ovoid	14 (12–16)	8 (6-8)	absent	present	Levine et al., 1962a
	Goat	elongate ovoid	13 (11–17)	8 (6-10)	absent	present	Shah and Joshi, 1963

[a]*E. ahsata* has been considered synonymous with *E. christenseni*, and has been redescribed in domestic sheep with slightly larger measurements (Levine et al., 1962b).
[b] *E. crandallis* has been considered synonymous with *E. hirci* (Chevalier, 1966).
[c]*E. bakuensis* has been considered synonymous with *E. arloingi* (Chevalier, 1966).

**numerous granules in a spherical mass.
***few granules in a spherical mass.

Fig. 1

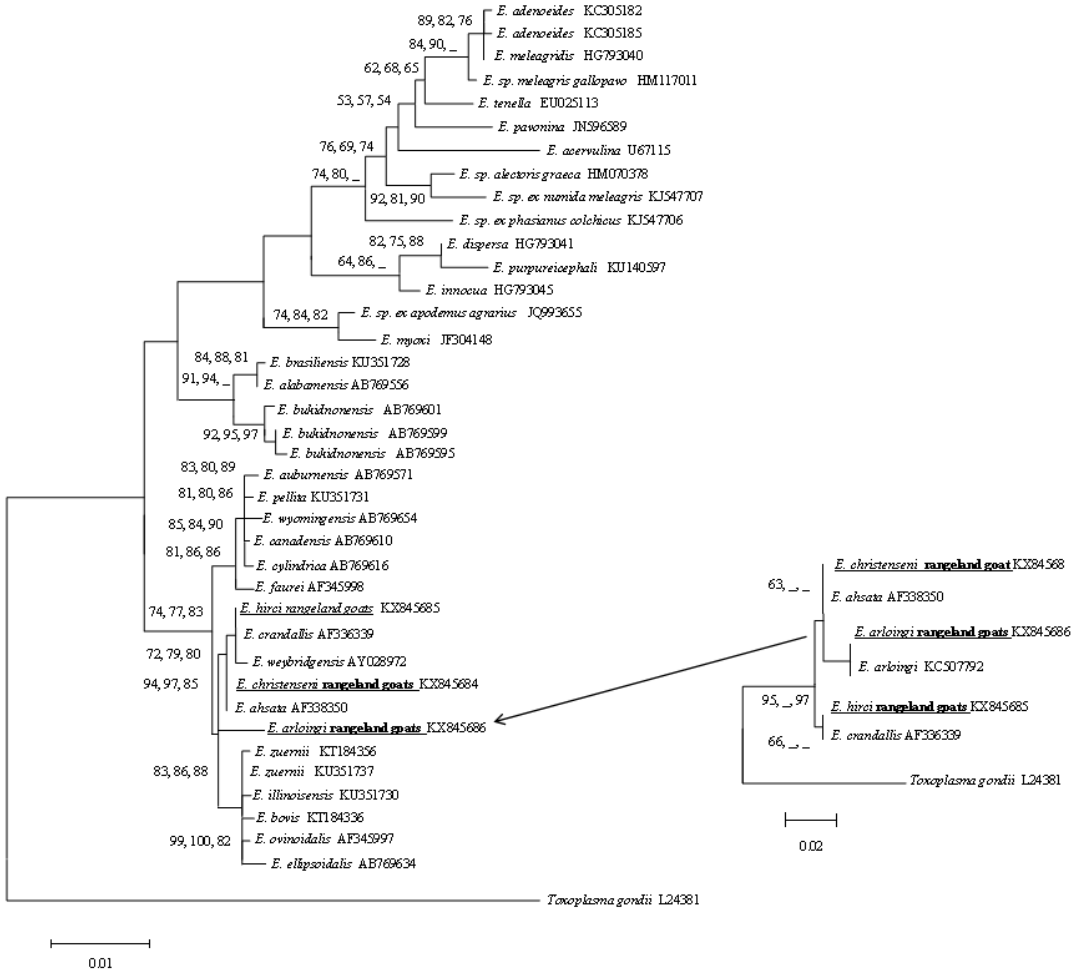

Fig. 2

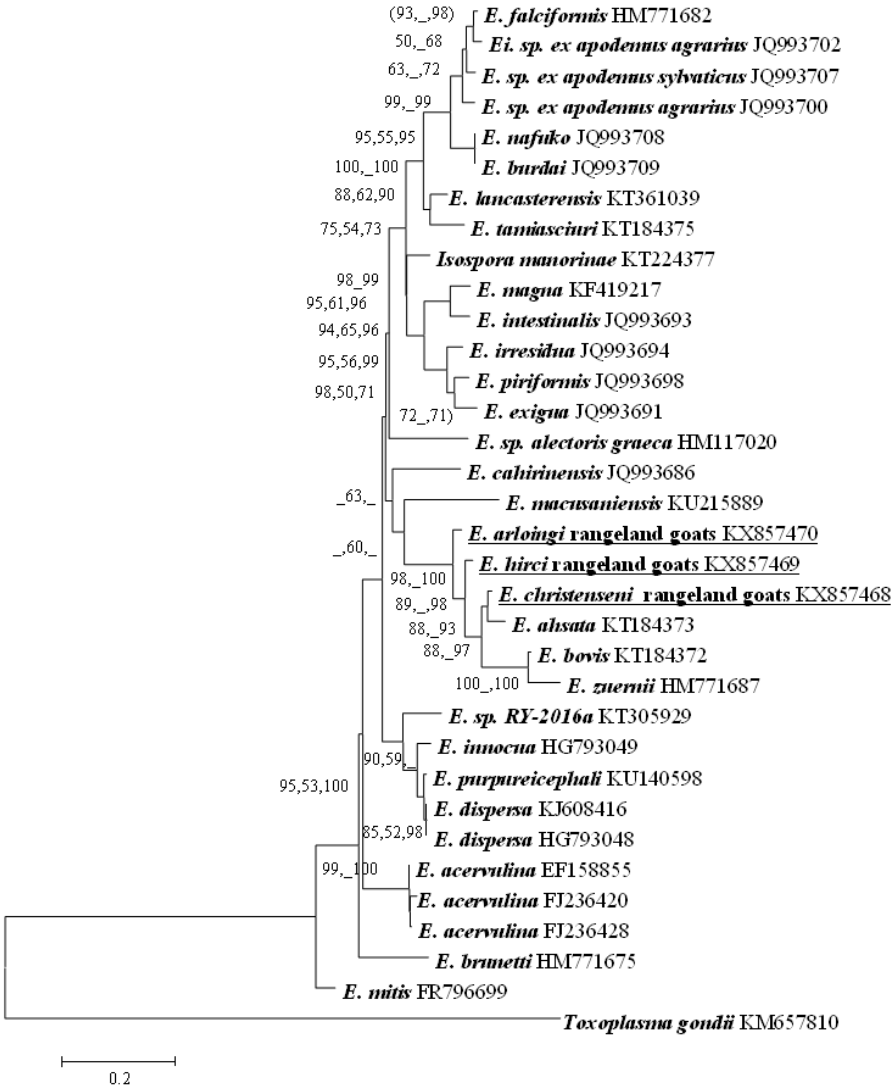

Fig. 3