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A newly described strain of *Eimeria arloingi* (strain A) belongs to the phylogenetic group of ruminant-infecting pathogenic species, which replicate in host endothelial cells in vivo

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Abstract

Coccidiosis caused by *Eimeria* species is an important disease worldwide, particularly in ruminants and poultry. *Eimeria* infection can result in significant economic losses due to costs associated with treatment and slower growth rates, or even with mortality of heavily infected individuals. In goat production, a growing industry due to increasing demand for caprine products worldwide, coccidiosis is caused by several *Eimeria* species with *E. arloingi* and *E. ninakohlyakimovae* the most pathogenic. The aims of this study were genetic characterization of a newly isolated European *E. arloingi* strain (A) and determination of phylogenetic relationships with *Eimeria* species from other ruminants. Therefore, a DNA sequence of *E. arloingi* strain (A) containing 2290 consensus nucleotides (the majority of 18S rDNA, complete ITS-1 and 5.8S sequences, and the partial ITS-2) was amplified and phylogenetic relationship determined with the most similar sequences available on GenBank. The phylogenetic tree presented a branch constituted by bovine *Eimeria* species plus *E. arloingi*, and another one exclusively populated by ovine *Eimeria* species. Moreover, *E. arloingi*, *E. bovis* and *E. zuernii*, which all replicate in host intestinal endothelial cells of the lacteals, were found within the same cluster. This study gives new insights into the evolutionary phylogenetic relationships of this newly described caprine *Eimeria* strain and confirmed its close relationship to other highly pathogenic ruminant *Eimeria* species characterized by macromeront formation in host endothelial cells of the central lymph capillaries of the small intestine.

Keywords: *Eimeria*, Coccidiosis, *Eimeria arloingi*, host, Phylogenetic relationship, ITS-1 and 18S

Introduction

Currently, more than 1200 *Eimeria* species are known (Chapman et al., 2013) and it is assumed that many more remain to be discovered (Blake, 2015). The great majority of these species are monoxenous enteropathogens of vertebrates which usually induce only mild pathology and mild or non-clinical disease. Nonetheless, certain species such as *E. bovis*, *E. zuernii*, *E. alabamensis* (cattle), *E. ovinoidalis*, *E. bakuensis* (sheep), *E. cameli, E. dromedari* (camels), *E. ninakohlyakimovae* and *E. arloingi* (goat) are considered highly pathogenic, defined by the formation of macromeronts, and severe intestinal lesions.

Worldwide, coccidiosis is particularly relevance to ruminant and poultry production (Chapman et al., 2013; Daugschies and Najdrowski, 2005). The economic impact in both industries is enormous and was recently valued as a 6-9% reduction in gross margin for ruminants, and to exceed US$3 billion for poultry (Blake and Tomley, 2014; Lassen and Ostergaard, 2012). Costs of prevention and treatment, combined with the morbidity and mortality of heavily infected individuals, are the main factors influencing economic losses. Every year, more than one billion goats are reared worldwide (FAOSTAT, 2014) and coccidiosis constitutes a major concern for the caprine industry. Historically, morphology of sporulated oocysts has been largely used for identification of distinct *Eimeria* species (Levine, 1985), but recently molecular characterization has been widely used to clarify precise species classification, particularly where morphological differentiation is difficult due to similarities in shape and size. (Kokuzawa et al., 2013; Ogedengbe et al., 2011). Therefore, the aim of this study was to analyse a newly described European *E. arloingi* strain (A) isolated from Portugal (Silva et al., 2015) and investigate its phylogenetic relationship to other *Eimeria* species which infect ruminants.

Material and methods

Parasites

E. arloingi (strain A) oocysts were isolated from naturally infected goat kids and passaged in male White German goat kids as previously described (Silva et al., 2015). Isolated oocysts were allowed to sporulate at RT in a 2% (*w*/*v*) potassium dichromate solution (Hermosilla et al., 2002) and then stored at 4 °C until further use.

Purification of oocysts

Sporulated *E. arloingi* oocysts were washed to remove all traces of potassium dichromate. Three million oocysts were pelleted (750 × *g*, 10 min) and re-suspended in 5% sodium hypochlorite, swirling intermittently. After 10 min of treatment oocysts were washed with tap water (750 × *g*, 10 min). The supernatant was removed and the pellet was re-suspended in saturated salt. The 100 ml vessel was filled up to 2 cm from the top, overlaid with Milli-Q water and centrifuged as before. Oocysts present at the interface between the salt and water phases were collected and washed three times. After the final wash, the *E. arloingi* purified oocysts were re-suspended in Milli-Q water and stored at 4 °C.

DNA extraction

Approximately three million purified sporulated *E. arlongi* oocysts were chilled on ice and homogenized using a Mini Beadbeater-8 (Biospec Products, Bartlesville, USA)with an equal volume of sterile glass beads (0.4-0.6 mm, Sigma, Gillingham, UK), at 3,000 oscillations/min. Subsequently, genomic DNA was extracted with TRIzol® Reagent (VWR, Carlsbad, USA)

according to the manufacturer´s instructions, re-suspended in 20 µl MQ water and stored at -20°C until further use.

Polymerase chain reaction (PCR), molecular cloning and sequencing

PCR amplification was performed using Taq DNA Polymerase (Invitrogen, California, USA), as previously described (Marugan-Hernandez et al., 2016), with the primers ERIB1, ERIB10, EITSF2 and EITSR2 [sequences as described elsewhere (Honma et al., 2011; Schwarz et al., 2009); synthesized by Sigma-Aldrich, Gillingham, UK]. PCR products were evaluated by agarose gel electrophoresis and cloned into pGEM®-T Easy (Promega, Southampton, UK). Plasmids were propagated in *Escherichia coli* Fast-Media® (InvivoGen) and colonies were picked in triplicate for purification using a QIAprep® Spin Miniprep Kit (Qiagen, Hilden, Germany) and sequenced (GATC Biotech, Konstanz, Germany) as described by the respective manufacturers. The consensus nucleotide data reported in this paper are available from the GenBank™ database under the accession number: **MF356556**.

Sequence analysis

Sequence reads were assembled and manually curated using CLC Main Workbench (v6.0.2; Qiagen). Sequences showing similarity to the consensus sequence of interest were searched in the National Center for Biotechnology Information database using the BLASTn system. The top 100 sequences most similar to the 2290 nucleotides (nt) sequence (*E. arloingi* European strain A) available on GenBank™ on 9th May 2016 (see supplementary data) were used to performed an alignment in Clustal W (Larkin et al., 2007). The aligned sequences were used to construct a phylogenetic tree using Neighbor-Joining, Maximum Likelihood and Minimum Evolution methods with 1,000 bootstrap replication using MEGA 6 software (Tamura et al., 2013). Additionally, the

104 genetic distances among groups of species (previously constructed) were determined by the
105 Kimura´s 2 parameter method (Kimura, 1980).

Results

The amplified *E. arloingi* sequence consisted of 2290 nt that contained the majority of the 18S rDNA (1704 nt of ~1777 nt), complete internal transcribed spacer 1 (ITS-1) and 5.8S sequences, and the partial ITS-2 (61 nt of ~580 nt). The Neighbor-Joining phylogenetic tree presented two distinct branches, one primarily constituted by bovine *Eimeria* species (e. g. *E. bovis* and *E. zuernii*) plus *E. arloingi*; the second constituted exclusively by ovine *Eimeria* species (*E. ovinoidalis*, *E. faurei*, *E. crandallis*, *E. ahsata* and *E. weybridgensis*; Fig. 1). Maximum Likelihood and Minimum Evolution methods achieved comparable topologies. Resolution was improved for the bovine/caprine branch by repeating the analysis without the ovine *Eimeria* sequences, revealing three clades (Fig. 2). The first clade (A) was populated by bovine *Eimeria* species that replicate in host intestinal epithelial cells: *E. auburnensis*, *E. cylindrica*, *E. wyomingensis* and *E. canadensis*. The second clade (B) included bovine (*E. bovis* and *E. zuernii*) and caprine (*E. arloingi*) species which replicate within host intestinal endothelial cells of the lacteals, and a bovine species which replicates in host epithelial cells (*E. ellipsoidalis*). Lastly, clade (C) included the bovine *Eimeria* species *E. alabamensis*, *E. bukidnonensis*, both of which also replicate exclusively in host epithelial cells. Moreover, the most pathogenic ruminant species included in this study *E. arloingi* (goat), *E. bovis* and *E. zuernii* (cattle) were found to be closely related phylogenetically, clustering within the same clade.

The 2290 nt sequence corresponding to *E. arloingi* was most closely related to sequences from *E. zuernii*, with a genetic distance of 0.003, followed by *E. bovis*, with a genetic distance of 0.005 and *E. ellipsoidalis* with a genetic distance of 0.006 (Table 1).

Discussion

Ruminant coccidiosis caused by parasites of the genus *Eimeria* is still one of the most widespread infections of livestock worldwide (Daugschies and Najdrowski, 2005; Witcombe and Smith, 2014). *Eimeria* species are monoxenous parasites with complex life cycles. After sporogony (environment), merogony and gamogony take place within specific-host cells and -sites of the intestinal mucosa. Whilst most ruminant *Eimeria* species replicate in intestinal epithelial host cells, other species [e. g. *E. bovis*, *E. zuernii* (cattle), *E. arloingi, E. ninakohlyakimovae, E. christenseni* (goats)*, E. cameli, E. dromedari* (camels), *E. ovinoidallis* (sheep)] replicate in endothelial host cells of the lymph capillaries of the lacteals of the small intestine, where they form macromeronts of up to 400 µm in size. These first generation macromeronts can release up to 170,000 merozoites I that invade new host epithelial cells resulting in severe destruction of the gut mucosa (Daugschies and Najdrowski, 2005; Hermosilla et al., 2012). Consistent with these common replication features, the phylogenetic tree generated in this study suggests a shared evolutionary history for *E. arloingi*, *E. bovis* and *E. zuernii*, all pathogenic species, which replicate in highly immunocompetent host endothelial cells and form huge first-generation macromeronts. A similar association has been observed in avian *Eimeria* phylogenetic analysis, where the highly pathogenic species *E. tenella* and *E. necatrix* which replicate deep within the lamina propria of the lower intestinal tract (Levine, 1985), also constitute a monophyletic group independent from other chicken-infecting *Eimeria* species (Barta et al., 1997). A similar feature has also been suggested for the pathogenic species *E. bovis* and *E. zuernii* (Kawahara et al., 2010). It is intriguing to verify that despite the different host origin and morphological features, *E. bovis*, *E. zuernii* and *E. arloingi* may have evolved from one common ancestor species capable of colonizing a new niche within the ruminant small intestine. It is hypothesised that the sporozoites of this ancestor species might have been able to migrate

through the epithelia and infect endothelium cells to fulfil the nutritional requirements of macromeront formation or that the ability to develop macromeronts may have been permitted by access to greater nutritional resources.

The phylogenetic proximity of *E. arloingi* and *E. bovis* may be in accordance with other common features of parasite-host cell interactions, such as the modulation of host cell-apoptosis (Lang et al., 2009), -cytoskeleton (Hermosilla et al., 2008) and -metabolism (Hamid et al., 2014; Hamid et al., 2015; Silva et al., 2015; Taubert et al., 2010) to guarantee successful macromeront formation. For example, *E. bovis* depends on the host endothelial cell supply of energy and cellular building blocks for its massive offspring formation (Hamid et al., 2014; Hamid et al., 2015). Most notably for cholesterol and given that apicomplexan protozoa are considered as defective in *de novo* cholesterol biosynthesis (Coppens et al., 2000; Ehrenman et al., 2013; Taubert et al., 2010), this parasite appears to scavenge cholesterol via different pathways during macromeront formation (Hamid et al., 2015) for successful replication. Additionally, the downregulation of early host endothelial cell immune reactions in presence of *E. arloingi* (Silva et al., 2015), *E. bovis* (Taubert et al., 2006; Taubert et al., 2010) and also *E. ninakohlyakimovae* (Perez et al., 2015) was reported. In this study, we obtained similar but not identical outcomes to those obtained by Khodakaram-Tafti et al. (2013) that compared the ITS1 sequence of an Iranian *E. arloingi* isolate with other *Eimeria* sequences and found it to be most similar to *E. bovis*, with a more distant relationship to *E. zuernii*. Comparison of a partial 18S rDNA sequence (637 nt) was also performed, however the phylogeny obtained differed considerably from our own, primarily due to the higher resolution achieved here using a sequence of 2290 nt, while Khodakaram-Tafti et al. (2013) used much shorter sequences: 392 nt of ITS1 and 637nt of 18S, studied independently. More recently, a phylogenetic analysis of *Eimeria* from local infections in Australia have been reported (Al-Habsi et al., 2017)

with a slightly different result, mainly explained by the different genes and the length of the sequence analysed. ITS sequences have been found to vary significantly between isolates of the same species recovered from different continents in avian *Eimeria* species such as *E. maxima* and *E. mitis* (Clark et al., 2016; Kawahara et al., 2010). Therefore, further analysis is required to assess the extent of genetic diversity influenced by the geographical distribution of each *E. arloingi* isolate. Additionally, Nahavandi et al. (2016) characterized the molecular-typing of *E. ahsata* and *E. crandallis* isolated from slaughterhouses wastewater samples (suburban area of Tehran, Iran). Authors inferred a close genetic relation between these two species and the *E. arloingi* isolate from Iran using 18S rDNA (KC507792), while in our comparison *E. ahsata* and *E. crandallis* branch in a different clade to the European *E. arloingi* strain (A). Again, the differences obtained here might be due to the shorter KC507792 sequence used in the former study. Single 18S rDNA sequences have been described as the basis for several phylogenetic studies (Hillman et al., 2016; Hofmannova et al., 2016; Kokuzawa et al., 2013; Nahavandi et al., 2016), although other authors have questioned the usefulness of 18S rDNA sequences for classifying apicomplexan parasites since classical taxonomy of the monoxenous coccidia in the family Eimeriidae was not well supported by such molecular data (Martynova-Vankley et al., 2008; Morrison et al., 2004). Specifically, sequence data were unable to confirm the monophyly of all *Eimeria* species analysed so far (Ogedengbe et al., 2011).

Expanding knowledge of the phylogenetic evolutionary relatedness among *Eimeria* species can provide invaluable insights into coccidian biology, immunology and metabolic requirements, as well as underpin improvement in the development of anticoccidial control using existing and novel drugs or even vaccines (Blake, 2015). Therefore, we call for more sequences from clearly neglected

195 *Eimeria* species of caprine species to be produced and available on GenBank in order to develop a
196 more robust molecular taxonomy.
197 Here, we obtained new insights into evolutionary phylogenetic relationships of this newly
198 described caprine *E. arloingi* strain. Furthermore confirming its close relationship to other highly
199 pathogenic ruminant *Eimeria* species characterized by macromeront formation in host endothelial
200 cells of the lymph capillaries of the small intestine.
201

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List of tables

Table 1: Kimura 2-parameter distances. Sequences were grouped according to *Eimeria* species. The shortest distance is the one between the sequence of interest and the group of sequences representing *E. zuernii*.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	E. arloingi															
2	E. ellipsoidalis	0.006														
3	E. bovis	0.005	0.005													
4	E. zuernii	0.003	0.005	0.004												
5	E. auburnensis	0.011	0.011	0.01	0.01											
6	E. cylindrica	0.012	0.011	0.01	0.011	0.003										
7	E. ovinoidalis	1.129	1.138	1.129	1.125	1.13	1.138									
8	E. wyomingensis	0.013	0.012	0.01	0.011	0.004	0.004	1.132								
9	E. ahsata	1.136	1.144	1.136	1.132	1.136	1.144	0.003	1.139							
10	E. crandallis	1.135	1.143	1.135	1.131	1.135	1.143	0.004	1.138	0.001						
11	E. weybridgensis	1.136	1.144	1.136	1.132	1.136	1.144	0.004	1.139	0.001	0.001					
12	E. canadensis	0.018	0.018	0.017	0.017	0.012	0.013	1.138	0.013	1.144	1.143	1.144				
13	E. faurei	1.135	1.143	1.135	1.131	1.135	1.143	0.007	1.138	0.006	0.007	0.007	1.143			
14	E. alabamensis	0.032	0.032	0.031	0.031	0.028	0.028	1.139	0.031	1.146	1.145	1.146	0.034	1.145		
15	E. bukidnonensis	0.031	0.032	0.031	0.03	0.027	0.027	1.134	0.03	1.14	1.139	1.14	0.034	1.139	0.01	

List of figures

Figure 1: Neighbor-Joining phylogenetic tree generated using an *E. arloingi* partial 18S-ITS1-5.8S- partial ITS2 sequence and the 100 most similar sequences available in GenBank as of 9th May 2016 (see supplementary data). The consensus of 1,000 bootstrap replicates is shown. The sequence of interest *E. arloingi* is marked with a red square. The number at the end of each node indicates how many sequences constitute each of the collapsed branches. The host species of each parasite is shown: bovine (cow drawing); ovine (sheep drawing); caprine (goat drawing). Maximum Likelihood and Minimum Evolution methods achieved comparable topologies.

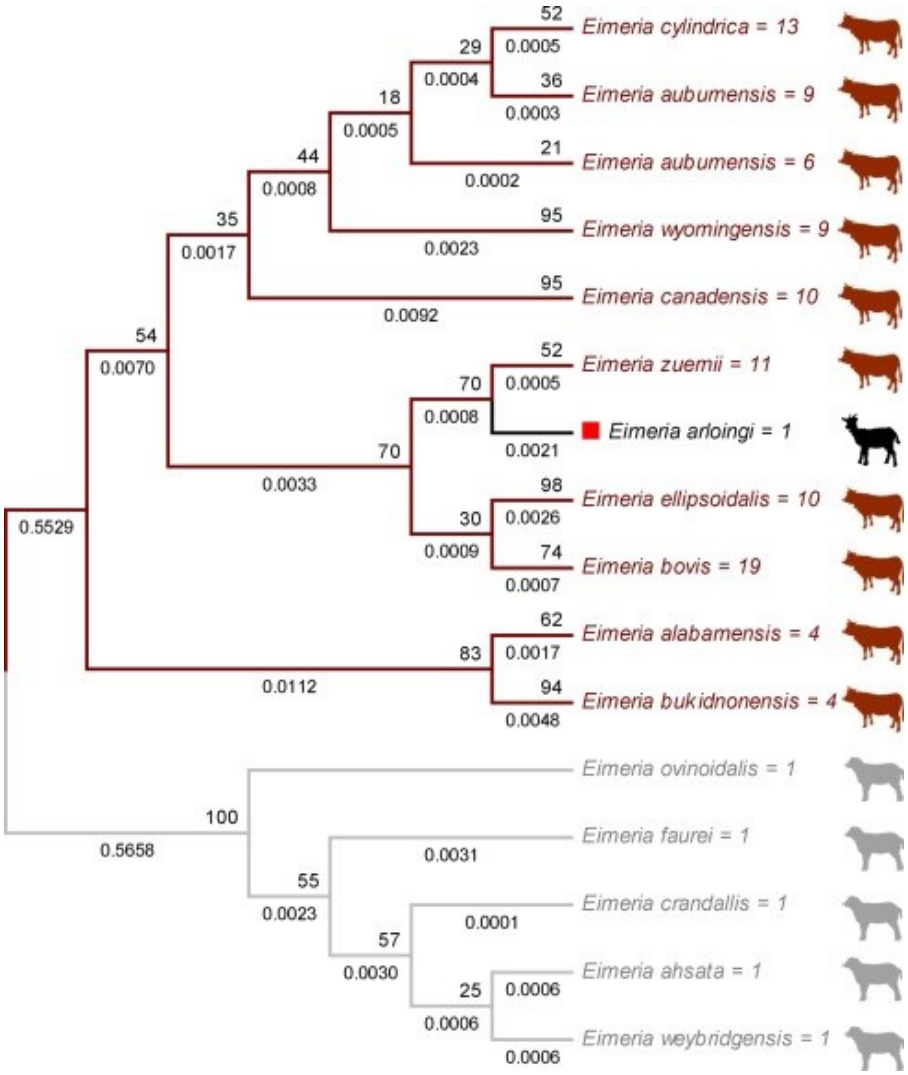

Figure 2: Higher resolution Neighbor-Joining phylogenetic tree representing the bovine/caprine *Eimeria* species branch presented in Figure 1. The sequence of interest (*E. arloingi*, marked with a red square) and the 95 most similar sequences (see supplementary data) available in GenBank are observed. In each of the nodes the values of 1,000 replicates are observed. The triangles indicate that the branch was collapsed because it consisted of sequences belonging to the same species of *Eimeria* (and were closely related to each other) and the number of sequences constituting each branch is indicated at the end of the annotation for each node. The first clade (A) shows bovine *Eimeria* species that replicate in host intestinal epithelial cells (blue). The second clade (B) include bovine (*E. bovis* and *E. zuernii*) and caprine (*E. arloingi*) species which replicate within host intestinal endothelial cells of the lacteals (brown), and a bovine species which replicates in host epithelial cells (*E. ellipsoidalis*). Lastly, clade C include bovine *Eimeria* species, which replicate exclusively in host epithelial cells (blue). Maximum Likelihood and Minimum Evolution methods achieved comparable topologies.