Molecular phylogenetic analyses of tissue coccidia (sarcocystidae; apicomplexa) based on nuclear 18s RDNA and mitochondrial COI sequences confirms the paraphyly of the genus *Hammondia*

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SUMMARY

Partial mitochondrial cytochrome *c* oxidase subunit I (mt COI) sequences were generated from: *Toxoplasma gondii* (strains CTG, GTI, MAS, ME49, PTG, TgCatBr5, TgCat, Br64, TgCgCal, TgToucan); *Neospora caninum* (Strain NC1); *Hammondia hammondi* (Strain H.H–20); *H. heydorni; H.* cf. *triffittae; Cystoisospora felis; C. suis; C. canis; C. rivolta; C.* cf. *ohioensis; Caryospora bigenetica; Sarcocystis rileyi*; and *S. neurona*. Nuclear 18S rDNA sequences were generated for *H. heydorni, H. hammondi, C. suis, C. canis, C. felis, C. rivolta, C.* cf. *ohioensis, S. neurona*, and *S. rileyi*. Aligned, concatenated 18S rDNA and COI sequences were Bayesian analysed using partitioned nucleotide substitution models [HKY + I + G for 18S; GTR + I + G codon (code = metmt) for COI]. Phylogenetic hypotheses supported a monophyletic Sarcocystidae and its subfamilie with two major clades within the Toxoplasmatinae: (1) a monophyletic clade of *Cystoisospora* spp. with *Nephroisospora eptesici*; and (2) a clade of *Toxoplasma, Neospora* and *Hammondia*. Within the latter, *Hammondia* was shown to be paraphyletic; *H. heydorni* and *H. triffittae* were monophyletic with *N. caninum* [canine definitive hosts (DHs)], whereas *H. hammondi* was monophyletic with *T. gondii* (feline DHs). A new genus is erected to resolve the paraphyly of the genus *Hammondia* confirmed using mt COI and combined 18S/COI sequence datasets.

Key words: Sarcocystidae, Toxoplasmatinae, mitochondrial COI, nuclear 18S rDNA, molecular phylogenetics, *Hammondia*, *Heydornia* n. gen., paraphyly, monophyly.

INTRODUCTION

Coccidia in the family Sarcocystidae, the tissue coccidia, are important pathogens of many vertebrates, including humans (Velmurugan and Dubey, 2008). Many species within the subfamily Toxoplasmatinae (e.g. *Toxoplasma gondii, Neospora caninum, Hammondia heydorni* and *Hammondia hammondi*) exhibit facultative or obligatory heteroxenous life cycles. Exogenous sporulation produces disporocystic tetrazoic oocysts without Stieda bodies (Carreno *et al*. 1998; Mugridge *et al*. 1999). Coprological identification of *N. caninum, T. gondii, H. heydorni* and *H. hammondi* is difficult because their unsporulated oocysts are morphologically indistinguishable for all practical purposes (Frenkel and Dubey, 1975; Dubey and Lindsay, 1996; Lindsay *et al*. 1997). Only some *Cystoisospora* spp. (e.g. *C. canis* and *C. felis*) possess distinctly larger oocysts that can be diagnosed reliably using microscopy. There are only two genera in the Sarcocystinae (*Sarcocystis* Lankester 1882 and *Frenkelia* Biocca 1968) distinguished by the location of tissue cysts within intermediate hosts; the monophyly of these genera has been questioned repeatedly based on phylogenetic hypotheses erected using nuclear rDNA sequences (e.g. Mugridge *et al*. 2000; LSU rDNA, Morrison *et al*. 2004). The number of valid genera and species within the coccidian subfamily Toxoplasmatinae has been the subject of ongoing debate (e.g. Dubey, 1977; Carreno *et al*. 1998; Ellis *et al*. 1999; Mehlhorn and Heydorn, 2000; Dubey and Sreekumar, 2003; Morrison, 2009). Currently, there are seven described genera recognized in the subfamily: *Hyaloklossia* Labbé 1896; *Toxoplasma* Nicolle & Manceaux 1908; *Besnoitia* Henry 1913; *Cystoisospora* Frenkel 1977; *Hammondia* Frenkel and Dubey 1975; *Neospora* Dubey *et al*. 1988; and *Nephroisospora* Wünschmann *et al*. 2010. *Toxoplasma gondii* was described from tissue cysts (bradyzoites) observed in rodents in 1908. Dubey *et al*. (1988) proposed the name *N. caninum* to reflect the distinctiveness of *N. caninum* from *T. gondii* and summarized the biological, morphological, molecular and antigenic differences that

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distinguish these two parasites (Dubey *et al*. 2002*b*). The genus *Besnoitia* Henry 1913 was named for tissue coccidia that form thick-walled polyzoic cysts within connective tissue of their intermediate hosts (e.g. *Besnoitia wallacei* Frenkel 1977). Frenkel and Dubey (1975) erected the genus *Hammondia* to accommodate a feline coccidium, *H. hammondi*, with an obligatory two host life cycle. *Hammondia heydorni* Dubey, 1977, a parasite of canids, was characterized by Dubey (1977) and Blagburn *et al*. (1988) from oocysts retrieved from feces of dogs. A second *Hammondia* sp. of canids, *H. triffittae*, has been recognized in European foxes by Gjerde and Dahlgren (2011) who distinguished this parasite from other isosporoid coccidia of dogs (i.e. *H. heydorni* or *N. caninum*) by subtle differences in oocyst dimensions, genetic differences and the inability of *H. triffittae* to infect dogs. The separation of these two *Hammondia* spp. of canids was supported by later molecular comparisons (Schares *et al*. 2003; Dahlgren and Gjerde, 2010; Gjerde and Dahlgren, 2011).

Most molecular phylogenetic studies of apicomplexan parasites in the family Sarcocystidae relied on small subunit (18S) rDNA sequences (e.g. Tenter and Johnson, 1997; Mugridge *et al*. 2000). The nuclear 18S rDNA locus is a useful genetic target from which to obtain sequence data due to its high copy number and conserved terminal sequences that are convenient for PCR primer design and amplification (Morrison and Ellis, 1997; Mugridge *et al*. 2000; Ouvrard *et al*. 2000). These attributes and the relatively conserved nature of the 18S rDNA within the nuclear genome have made 18S rDNA an important and widely exploited genetic target for biodiversity and species identification studies. In most eukaryotes, rDNA copies undergo concerted evolution that homogenizes copy-to-copy variability in the nuclear genome (Hillis and Dixon, 1991). However, this is not always true for members of the Apicomplexa. Highly divergent paralogous copies of nuclear rDNA have been demonstrated within haemosporinid parasites (McCutchan *et al*. 1988, 1995; Li *et al*. 1997), cryptosporidia (Le Blancq *et al*. 1997), piroplasms (Goethert *et al*. 2006) and, more recently, eimeriid coccidia (Vrba *et al*. 2011; El-Sherry *et al*. 2013). The possibility of highly divergent rRNA genes (e.g. A, S and O 18S rRNA genes that vary in both primary and secondary structures) could make determining homology at the gene-level problematic. Morrison *et al*. (2004) noted that complete 18S rDNA sequences were inadequate to resolve relationships among closely related tissue coccidia. Phylogenetic reconstructions based on 18S rDNA sequences support a monophyletic Sarcocystidae consistently (Ellis and Morrison, 1995; Carreno *et al*. 1998; Mugridge *et al*. 2000; Morrison *et al*. 2004); however, monophyly of individual genera within the Sarcocystidae is not supported in these analyses. Unlike nuclear 18S rDNA sequences, comparatively short sequences obtained from the mitochondrial cytochrome *c* oxidase subunit I (mt COI) gene provide sufficient sequence divergence to clearly differentiate closely related coccidia. Ogedengbe *et al*. (2011) demonstrated that ~500–800 bp partial mt COI sequences could better distinguish closely related coccidia (Eimeriidae) compared with complete or near-complete nuclear 18S rDNA sequences. Until recently (Gjerde, 2013*a*, *b*), lack of primers capable of amplifying the mt COI locus from sarcocystid coccidia restricted the use of mt COI sequences to only eimeriid coccidia (e.g. Eimeriidae) and a few members of the Toxoplasmatinae (see Ogedengbe *et al*. 2011). Based on the ability to better resolve relationships among closely related coccidia using mt COI sequences (e.g. Ogedengbe *et al*. 2011; El-Sherry *et al*. 2013), the monophyly of the described genera in the Sarcocystinae might best be tested using this genetic locus.

In this study, we used existing and newly designed PCR primers to amplify and sequence portions of the mt COI gene from a variety of tissue coccidia (i.e. parasites belonging to the genera *Toxoplasma, Neospora, Hammondia, Cystoisospora, Nephroisospora* and *Sarcocystis* in the Sarcocystidae). Partial mt COI sequences, nuclear 18S rDNA sequences or a concatenation of sequences from both loci were utilized to infer evolutionary relationships among these tissue coccidia and confirm the monophyly of some named genera in the family Sarcocystidae.

MATERIALS AND METHODS

Parasite sources

Parasites and parasite genomic DNA were obtained from a variety of sources. Fecal specimens containing oocysts of *C. felis, C. suis, C.* cf. *ohioensis, C. canis* and *H. heydorni* were obtained from diagnostic fecal specimens submitted to the Animal Health Laboratory, Laboratory Services Division, University of Guelph (Guelph, ON, Canada). Additional fecal specimens (containing *C. felis, C. canis, C. suis, C.* cf. *ohioensis* or *C. rivolta*) from a variety of hosts were kindly provided by Dr Donald Martin (IDEXX Laboratories, Markham, ON). Some fecal samples containing *C.* cf. *ohioensis* were provided by Dr Scott Weese (Department of Pathobiology, University of Guelph, ON). DNA samples of *Sarcocystis* spp. from experimentally infected hosts (i.e. *S. rileyi* 908131_Duck2 #4) or from tissue culture (i.e., *S. neurona* MIH2) were kindly provided by Dr Ben Rosenthal (USDA, Beltsville, MD, USA). Purified parasite genomic DNA was obtained for *H. hammondi* strain H.H-20 and *T. gondii* strains GT1, MAS, PTG, TgCat,

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CalBr64, CalBr5, TgCal and TgToucan prepared from scrapes of tachyzoite-infected cell cultures were provided by Dr Chunlei Su (Department of Microbiology, University of Tennessee, TN); the origins of these lines have been described previously (Su *et al*. 2012).

Identification of oocysts

Sporulated *Cystoisospora* oocysts collected and measured for this study were from dogs, cats, and pigs, and were identified as *C. canis, C. felis, C. rivolta* and *C. suis*. Small subspherical oocysts shed by dogs were considered part of the *C.* cf. *ohioensis* species complex and assigned the name *C.* cf. *ohioensis* to reflect the uncertainty in their species identification. In all cases prior to DNA extraction, initial assignment to species was on the basis of oocyst morphometrics. Oocysts images were captured using a Provis AX 70 photomicroscope (Olympus 95 Canada, Richmond Hill, ON) fitted with a digital imaging device (Infinity3–1C, Lumenera Corporation Ottawa, ON) controlled using iSolution Lite image analysis software (Hoskin Scientific, Burlington, ON). Measurements are reported as means ± standard deviation in μm with the range in parenthesis.

Oocyst purification and DNA extraction

Oocysts were isolated from fecal samples using saturated salt (NaCl) flotation followed by a bleach treatment to remove exogenous DNA prior to parasite DNA extraction (Ogedengbe *et al*. 2013, 2014). DNA isolation was accomplished using glass bead disruption and DNAzol nucleic acid extraction (Invitrogen, Carlsbad, CA) as previously described (Ogedengbe *et al*. 2013). DNA concentration was estimated using a Nanodrop 2000 spectrophotometer (NanoDrop Products, Wilmington, DE) and stored at 4 °C for immediate use or −20 °C for later use.

Primers for PCR amplification of mt COI

Initial PCR reactions attempted with 'universal' COI LCO1490 and HCO2198 primers that amplify many metazoan mt COI (Folmer *et al*. 1994) failed to amplify the COI locus from any species within the Toxoplasmatinae. Previously described coccidian-specific primers (Ogedengbe *et al*. 2011) were able to amplify some, but not all, members of the Sarcocystidae. Consequently, a pair of new Sarcocystidae-specific primers was designed using an alignment of all available COI sequences from tissue coccidia (data not shown). A pair of Sarcocystidae group specific degenerate primers (Sdae–COI 260F, degeneracy 4; Sdae–COI 1147R, degeneracy 2) was designed from two comparatively conserved regions with the aid of Primer3 (Untergrasser *et al*. 2012) executed from within the bioinformatics package Geneious (Version 6.1·8; http://www.geneious.com, Kearse *et al*. 2012). The previously published and newly designed primers were then used in various combinations to amplify ~500–900 bp fragments of the mt COI locus (Table 1). PCR reactions were ran with the anneal Tms determined with the Primer3 program for the primer pairs. Beginning with the lowest possible Tms for a pair of degenerate primers, anneal Tms were move up if multiple product bands were obtained to eliminate amplification of spurious DNA products. Extension times were usually set at the standard 1 min kb^{-1} expected product length.

PCR – partial mt COI and near-complete nuclear 18S rDNA

PCR amplification was carried out in an MJ Mini thermal cycler (Bio Rad, CA) with reactions consisting of 1 × PCR buffer (Invitrogen, Carlsbad, CA) supplemented with 4 mM $MgCl_2$, 200 μM dNTPs, 0·5 μM of each of the amplification primers (Table 1), 2·5 U of Platinum® *Taq* Polymerase (Invitrogen) and 50–100 ng DNA template. Cycling conditions were initial denaturation at 96 °C for 5 min followed by 35 cycles of 94 °C for 30 s, annealing at 50–55 °C (see Table 1 for anneal conditions required for specific primer combinations) for 30 s and extension at 72 °C for 30–90 s (depending on expected product size). The PCR reaction was completed with a final extension step at 72 °C for 7–10 min. PCR reaction products were electrophoresed using a 1·5% submarine agarose gel, stained with ethidium bromide and visualized using UV trans-illumination (Spectronics Corporation, New York, NY). The apparent size of DNA bands were determined by comparison with a 100 bp DNA ladder (Bio Basic Inc., Markham, ON). Bands were excised using a fresh scalpel blade and gel purified using a QIAGEN Gel Extraction Kit (Valencia, CA) according to the manufacturer's instructions. The purified PCR products were cycle sequenced using an ABI Prism 7000 Sequence Detection System (Applied Biosystems Inc., Foster City, CA) by the Molecular Biology Unit of the Laboratory Services Division, University of Guelph (Guelph, ON) using the amplification primers to obtain sequences in both directions. For the longer nuclear 18S rDNA fragments, internal sequencing primers were used so that complete, double-stranded sequencing was obtained.

Sequence assembly

The sequencing reads were assembled using *de novo* assembly within the Geneious bioinformatics software package. For both loci, the strict consensus sequence, less the amplification primers, was

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Table 1. Amplification primers for mitochondrial COI and nuclear 18S rDNA loci for various tissue coccidian parasites including anneal temperatures (T_a) and expected PCR product sizes.

Gene target	Parasites	Primer pairs	Reference	Primer sequence	T_a (°C)	Size (bp)
mt COI	*Cystoisospora suis* *Cystoisospora felis* *Cystoisospora canis* *Hammondia heydorni* *Hammondia hammondi* *Toxoplasma gondii* *Neospora caninum*	Cox1_10F	Ogedengbe *et al.* (2011)	GGWDSWGGWRYWGGWTGGAC	50	517
		Cox1_500R	Ogedengbe *et al.* (2011)	CATRTGRTGDGCCCAWAC		
	Toxoplasma gondii *Cystoisospora suis* *Cystoisospora felis* *Cystoisospora canis* *Caryospora bigenetica* *Hammondia heydorni* *Hammondia hammondi* *Neospora caninum*	Cox1_10F	Ogedengbe *et al.* (2011)	GGWDSWGGWRYWGGWTGGAC	55	795
		Cox1_780R	Ogedengbe *et al.* (2011)	CCCAGAGATAATACAAAATGG		
	Hammondia heydorni *Toxoplasma gondii* *Hammondia heydorni* *Hammondia hammondi* *Toxoplasma gondii* *Cystoisospora felis* *Cystoisospora canis* *Cystoisospora rivolta*	Cox1_10F	Ogedengbe *et al.* (2011)	GGWDSWGGWRYWGGWTGGAC	55	841
		Cox1_1202R	El-Sherry *et al.* (2013)	CCAAKRAYHGCACCAAGAGATA		
		Sdae-Cox1_260F	Present study	GATCTTTATGTTYTTRATGCC	50	875
		Sdae-Cox1_1147R	Present study	CATTACCCATAACYACACC		
18SrDNA	*Hammondia heydorni* *Hammondia hammondi* *Toxoplasma gondii* *Cystoisospora felis* *Cystoisospora canis* *Cystoisospora rivolta*	MEDLIN A	Medlin *et al.* (1988)	AACCTGGTTGATCCTGCCAGT	50	1800
		MEDLIN B	Medlin *et al.* (1988)	GATCCTTCTGCAGGTTCACCTAC		
		Cysto_18S_1711R	Present study	CGAATAATTCACCGGAACACTCA		
	Sarcocystis neurona	Sarco -18S _123F	Present study	TATCAGCTTTCGACGGTAGTGTATT		875
		ERIB1_FOR	Barta *et al.* (1997)	ACCTGGTTGATCCTGCCAG		

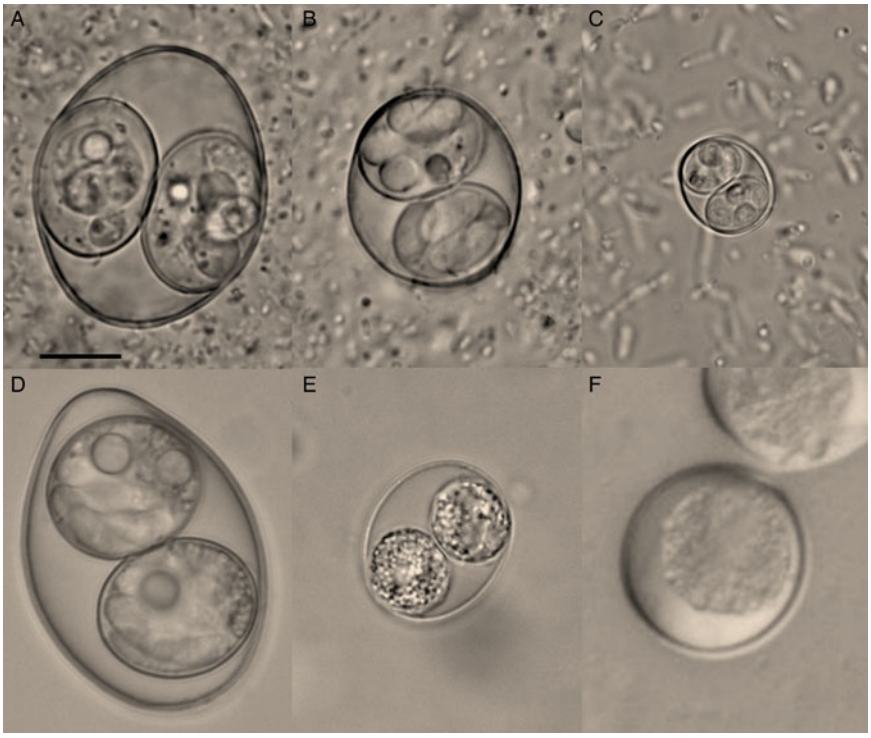

Fig. 1. Sporulated oocysts of some tissue coccidia: *Cystoisospora* species that infect dogs (A, *Cystoisospora canis*; B, *Cystoisospora ohioensis*); *Cystoisospora* species that infect cats (D, *Cystoisospora felis*; F, unsporulated *Cystoisospora rivolta*); *Cystoisospora suis*, infecting pigs (E); and *Hammondia heydorni*, a parasite of dogs (C). All photographs are at the same scale (scale bar represents 10 μm).

submitted to GenBank to obtain accession numbers reported in Supplementary Table 1.

Added to newly generated sequences, existing publically available COI and corresponding complete or near-complete 18S rDNA sequences generated from the same strain or isolate, when available, were used preferentially for phylogenetic analyses (see Supplementary Table 1 for sequences and strains used in this study). GenBank sequence FJ357797, previously identified as an *Isospora* sp. (strain Harbin/01/08) retrieved from the Siberian tiger (*Panthera tigris altaica*), was renamed as *Cystoisospora* sp. (strain Harbin/01/08) in this study on the basis of sporulated oocysts possessing sporocysts without Stieda bodies and with sporozoites lacking refractile bodies (see Fig. 2A and B of Zhijun *et al*. 2011); these morphological features are consistent with the genus *Cystoisospora* rather than *Isospora* (see Barta *et al*. 2005). In addition, the following selected mt COI and 18S rDNA sequences from apicomplexan parasites outside of the Eimeriorina were used as the taxonomic outgroup taxa for rooting the resulting trees: *Hepatocystis* sp.; *Plasmodium juxtanucleare; P. vivax; P. falciparum; P. malariae; Babesia caballi; B. bovis; B. bigemina; B. rodhaini; Theileria annulata* and *T. parva*. Accession numbers for all sequences included in the analyses are indicated on the resultant trees and in Supplementary Table 1.

Where possible, sequences obtained following bacterial cloning were excluded in the analyses to avoid sequence variations resulting from nucleotide misincorporation during PCR amplification (Olivieri *et al*. 2010; Ogedengbe *et al*. 2013). The 18S rDNA sequence from *Isospora rivolta* (AY618554) was removed from all analyses because a BLAST search determined that this sequence was identified incorrectly; AY618554 is likely derived from a basidiomycete fungus (see Whipps *et al*. 2012). In this study, only the newly generated nuclear 18S rDNA sequences from *Cystoisopora canis* were obtained from cloned PCR products.

Multiple sequence alignment

Multiple sequence alignments based on the primary structure were generated for the partial mt COI sequences using the 'Translation Align' algorithm within Geneious Ver. 6·1·8 bioinformatics software (Cost Matrix = Blosum 62; Gap open penalty = 12; Gap extension penalty = 3) (http://www.geneious.com, Kearse *et al*. 2012).

Nuclear 18S rDNA sequences were trimmed at both ends to exclude primer regions with sequences starting at the identical homologous nucleotide position 14 and ending at position 1778 if compared with the GenBank sequence DQ060683 of *C. belli* (Gjerde, 2013*b*). Alignments were performed first with MAFFT (Katoh *et al*. 2009; Katoh and Toh, 2010) and ClustalW (Larkin *et al*. 2007) executed from within Geneious. The occurrence of

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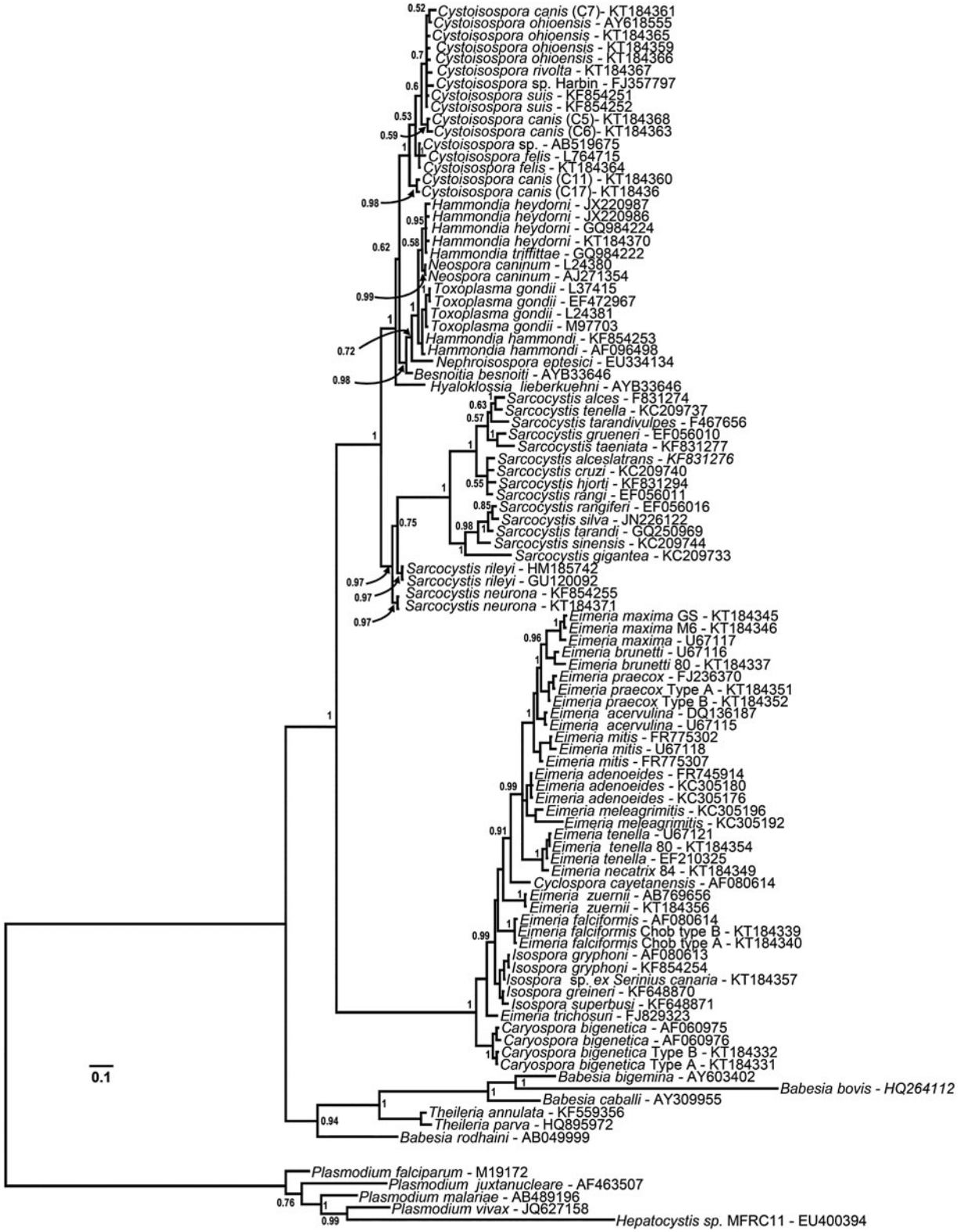

Fig. 2. Phylogeny of eimeriid coccidia (Eimeriidae and close relatives) and isosporoid coccidia (Sarcocystidae) based on the 18S rDNA dataset generated by BI using the HKY (nst = 2 gamma categories) model of nucleotide substitution with a discrete gamma distribution rate variation among sites (G) and accounting for proportion of invariant sites (I) estimated base frequencies. The coccidia were rooted using a number of haemosporinid and piroplasmid parasites. The monophyly of the coccidia (eimeriid and isosporoid coccidia) was strongly supported. The 18S rDNA dataset supported monophyly of the eimeriid coccidia as well as the family Sarcocystidae and its subfamilies Toxoplasmatinae (PP = 1·00) and Sarcocystinae (PP = 0·97).

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hypervariable regions in the 18S rRNA gene warranted that the MAFFT alignment was further staggered to address regions for which positional homology was uncertain (Barta, 1997). The sequence alignments were analysed as three separate sequence datasets: (1) an '18S rDNA dataset'; (2) a 'COI dataset'; and (3) a 'concatenated dataset'. The 18S rDNA dataset consisted of 100 aligned nucleotide sequences obtained from 61 spp. There were a total of 3273 character positions in the final 18S rDNA dataset of which 1689 characters were constant, 904 informative characters and 685 variable but uninformative characters. The COI dataset consisted of 91 aligned nucleotide sequences obtained from 59 spp. There were a total of 756 character positions in the final dataset of which 226 characters were constant, 501 informative characters and 29 variable but uninformative characters. The concatenated dataset combined the nuclear 18S rDNA and mt COI datasets into a single dataset without modifying the pre-existing sequence alignments for each genetic locus. The concatenated dataset had 4061 total characters of which 1943 characters were constant, 1408 informative characters and 710 variable but uninformative characters. All positions containing gaps were treated as unknown.

Phylogenetic analyses

Phylogenetic analyses for all datasets (i.e., 18S rDNA, COI or concatenated) were performed using three tree building methods: Bayesian Inference (BI) executed from within MrBayes Version 3.1.2. (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003); Maximum Likelihood (ML) (Guindon *et al*. 2010) executed from within PAUP version 4.0 Beta (Swofford, 2002); and Maximum Parsimony (MP) (Felsenstein, 1985) executed from within PAUP version 4.0 Beta. For MP analyses, all characters were treated as unordered and equal weight with gaps treated as missing.

For the likelihood-based analyses, best fit models and parameters were based on the Akaike Information Criterion (AIC) for 24 models of DNA substitution using the hierarchical likelihood ratio test performed within MrModeltest v2·3 (Nylander J. A. A. 2004. MrModeltest v2 Program; distributed by the author, Evolutionary Biology Centre, Uppsala University). Based on AIC, the Hasegawa, Kishino and Yano (HKY, nst = 2 gamma categories) model with a discrete gamma distribution rate variation among sites (G) and accounting for proportion of invariant sites (I) estimated base frequencies was recommended for the 18S rDNA dataset (Hasegawa *et al*. 1985). The general time reversible model with discrete gamma distribution, including invariable site (GTR + I + G, nst = 6 gamma categories) (Tavaré, 1986) was recommended for the COI data; a codon nucleotide model (i.e. Nucmodel = Codon) was implemented for all COI data using metazoan mitochondrial translation (i.e. Code = metmt). The concatenated dataset was partitioned in the ML and BI analyses. The COI portion of the concatenated dataset was analysed using a GTR + I + G (nst = 6, nucmodel = codon, code = metmt) substitution model and the 18S rDNA portion of the concatenated dataset was analysed using the HKY + I + G (nst = 2, nucmodel = 4by4) substitution model.

For all BI analyses, 1 000 000 generations of Markov Chain Monte Carlo (heated chains = 4, chain temperature = 0·2, unconstrained branch length with exponential = 10) were executed with a sampling frequency of 1000; burn-in length was set at 10% of the number of generations (i.e. 100 000). For MP and ML analyses, bootstrap consensus trees were constructed from 500 replicates of each analysis. Branches with less than 50% bootstrap support were collapsed. In all analyses, trees were rooted using members of the Haemosporida and Piroplasmida. Only BI trees (Figs 2–5) are shown. Trees were drawn such that horizontal branch lengths are proportional to hypothesized genetic divergence.

RESULTS

Oocyst dimensions

Representative oocysts collected from pigs, cats or dogs are illustrated in Fig. 1. The mean dimensions for oocysts and sporocysts of parasites isolated from fecal material are summarized for these parasites in Table 2.

Sequences obtained

Pairs of degenerate coccidia- and Sarcocystidae-specific primers designed to amplify a portion of the mt COI gene (see Table 1) produced PCR products that ranged from 418 to 848 bp in length (excluding primer regions); translations of all sequences indicated that no indels existed within the region amplified by any of these primers from the sarcocystid parasites. New sequences from nuclear 18S rDNA ranged from 573 bp (partial sequence from *C. rivolta*) to near full-length sequences of ~1800 bp. A total of 31 new COI sequences and 17 new 18S rDNA sequences were generated from 11 different species (see Supplementary Table 1 for the GenBank accession numbers for all sequences used in this study). Among the Sarcocystidae, no shared indels were found within 18S rDNA sequences from parasites in the Toxoplasmatinae; in contrast, *Sarcocystis* spp. has numerous (at least seven) expanded regions with no homology with members of the Toxoplasmatinae.

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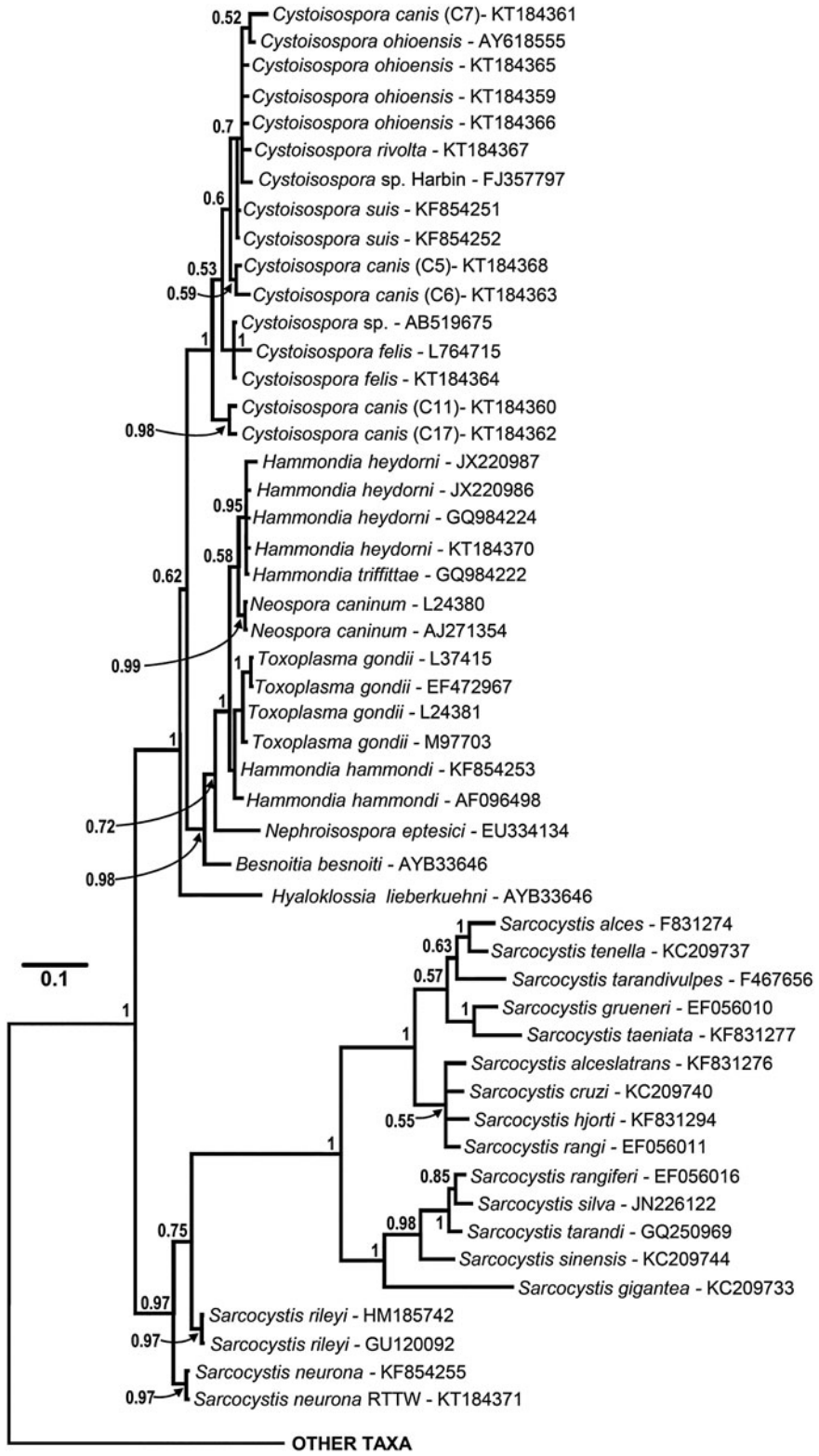

Fig. 3. Enlarged portion of the 18S rDNA sequence based tree (see Fig. 2 for complete tree) illustrating relationships among isosporoid coccidia. *Hyaloklossia lieberkuehni* branched basally to two, well supported clades that contained all remaining members of the Toxoplasmatinae (all *Cystoisospora* spp. in one; all remaining taxa in the second). Monophyly of individual *Cystoisospora* species within the first clade was supported rarely; as an example, a single polytomy contained four *Cystoisospora* spp. from four different host species. In the second clade, *Besnoitia besnoiti* and *Nephroisospora eptesici* branched basally to a clade of poorly resolved taxa belonging to the genera *Hammondia, Toxoplasma* and *Neospora*. Although monophyly of *N. caninum* and *T. gondii* was each supported (PP = 0·99 and 0·71, respectively), sequences from individual *Hammondia* spp. did not form monophyletic clades and the genus *Hammondia* itself was paraphyletic as well.

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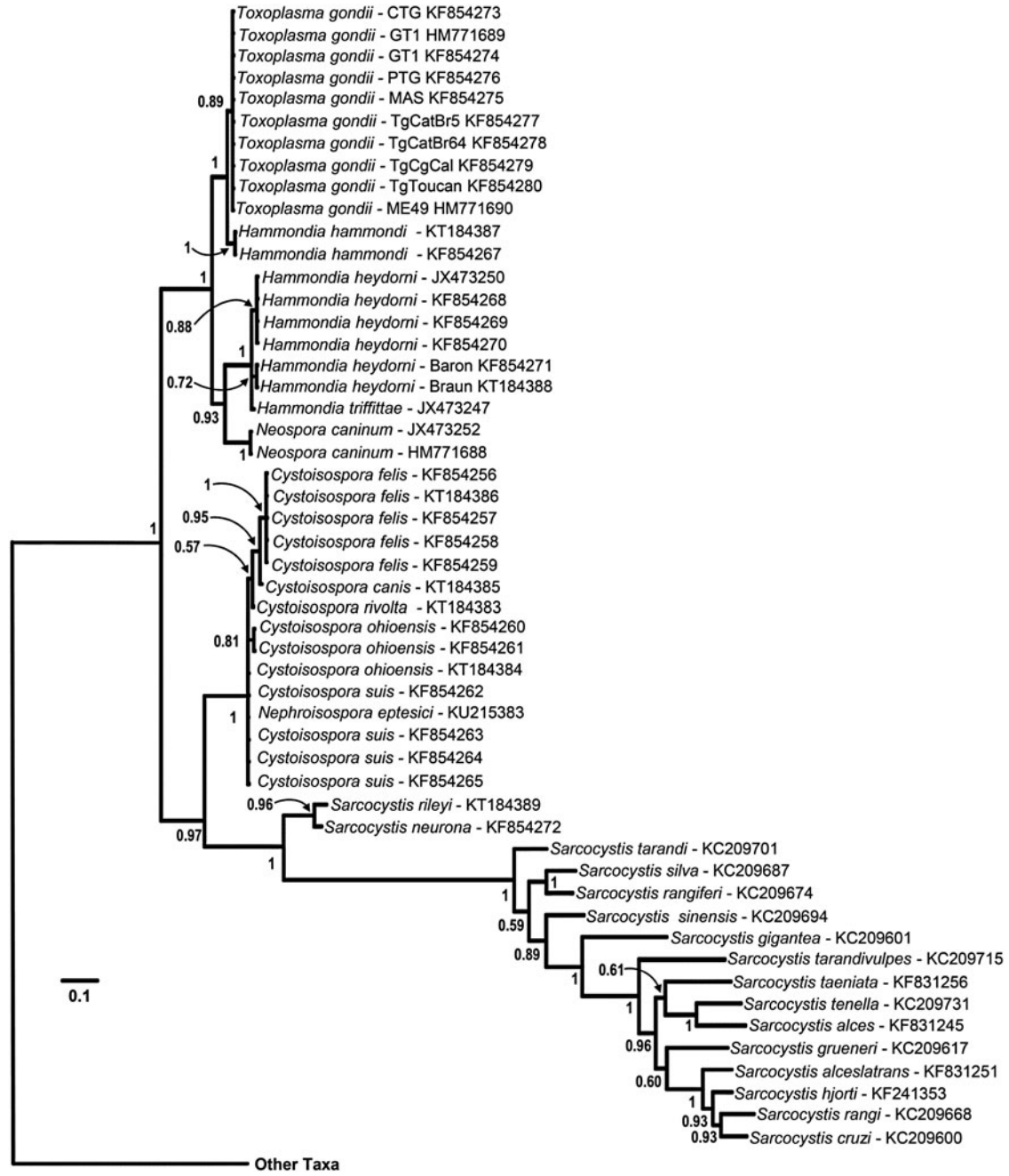

Fig. 4. Phylogeny of isosporoid coccidia based on the COI dataset using the same outgroups as in Fig. 2; only the clade containing members of the family Sarcocystidae are illustrated with the branch leading to the other taxa indicated. Trees were generated using BI and the general time reversible model with discrete gamma distribution including invariable site (GTR + I + G, nst = 6 gamma categories) using a codon nucleotide model (i.e. Nucmodel = Codon) implemented using metazoan mitochondrial translation (i.e. Code = metmt). Three major clades were supported within a monophyletic family Sarcocystidae: (1) a clade of *Sarcocystis* spp.; (2) *Cystoisospora* spp. plus *Nephroisospora eptesici*; and (3) a clade of *Neospora*, *Toxoplasma* and *Hammondia* species. Monophyly of the subfamily Toxoplasmatinae was not supported in this tree. *Hammondia* species do not form a monophyletic group; *Hammondia* species using canids as their definitive hosts grouped with *N. caninum*, whereas *Hammondia* spp. using felids as their definitive hosts grouped with *T. gondii*.

Phylogenetic analyses based on COI and 18S rDNA sequences

All phylogenetic analyses using BI, ML or MP methods with COI, 18S rDNA or concatenated sequences produced topologically similar trees that supported the monophyly of the family Sarcocystidae (BI trees illustrated in Figs 2–5).

The ML analysis on the COI dataset (756 nucleotides) was performed with a gamma distribution shape parameter of 1·2495, a rate category of 4, and proportion of invariable sites (I) of 0·2621; the number of distinct patterns under this model was 561. The MP analysis on the COI dataset generated at tree length of 3641 with a consistency index (CI)

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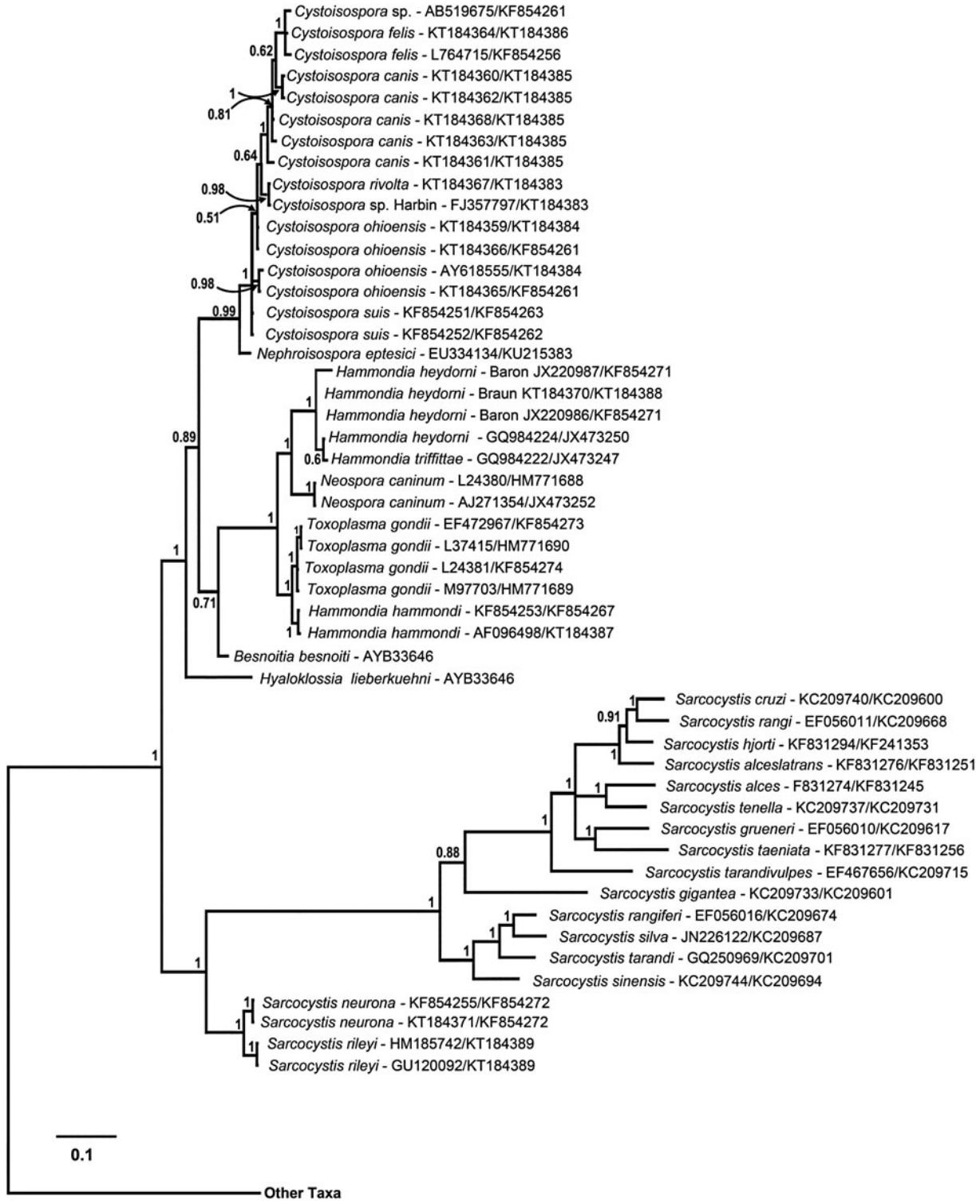

Fig. 5. Phylogeny of isosporoid coccidia based on the concatenated dataset (18S rDNA and COI sequences) using the same outgroups as in Fig. 2. The concatenated dataset was partitioned with the COI portion of the dataset analysed using a GTR + I + G (nst = 6, nucmodel = codon, code = metmt) substitution model and the 18S rDNA portion analysed using the HKY + I + G (nst = 2, nucmodel = 4by4) substitution model. Only the clade containing members of the family Sarcocystidae are illustrated with the branch leading to the other taxa indicated. Monophyly of the Sarcocystidae and its subfamilies Sarcocystinae and Toxoplasmatinae was supported strongly (all PP = 1·00). *Besnoitia besnoiti* and *Hyaloklossia lieberkuehni* branched near the base of the Toxoplasmatinae. *Nephroisospora eptesici* formed a well-supported sister clade to the *Cystoisospora* spp. Individual species within the clade containing *Toxoplasma, Neospora* and *Hammondia* spp. were well supported with the exception of *Hammondia* spp. infecting canids. As in the tree based on the COI dataset alone, *Hammondia* species using dogs as their definitive host grouped with *N. caninum* whereas *Hammondia* spp. using cats as their definitive host grouped with *T. gondii*.

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Table 2. Oocyst and sporocyst dimensions for some parasites obtained from fecal samples.

Parasite species (isolate or strain)	Host	Oocysts			Sporocysts	
		n	Length × width (range) (μm)	Shape index (range)	*n*	Length × width (range) (μm)
Cystoisospora felis (MEO2010)	*Cat*	25	41·1 (34–45) × 30·9 (28–34)	1·3 (1·5–1·1)	25	21·5 (18–25) × 18·3 (17–23)
Cystoisospora canis (MEO2015 – Milly)	*Dog*	19	35·1 (27–42) × 29·5 (22–32)	1·2 (1·4–1·0)	8	19·2 (15–22) × 14·4(14–16)
Cystoisospora cf. *ohioensis* (MEO2015a – Sedona)	*Dog*	25	24·2(21–27) × 21·2 (17–24)	1·1 (1·3–1·0)	25	15·9 (13–26) × 12·3 (10–24)
Cystoisospora suis (MEO2015-Friendship7)	*Pig*	24	20·8 (19–23) × 17·6 (16–20)	1·16 (1·2–1·1)	25	11·4 (10–14) × 9·6 (8–11)
Cystoisospora rivolta (MEO2015-IDX443)	*Cat*	25	22·4 (20–24) × 20·8(18–24)	1·1 (1·3–1·0)	0	n/a[a]
Hammondia heydorni (Braun)	*Dog*	25	12·2 (11–13) × 10·5 (9–12)	1·4 (1·5–1·2)	25	8·3 (8–9) × 6·1 (5–7)

[a] n/a – sporulated oocysts were not available for this species.

of 0·3087 based on 501 parsimony-informative characters.

For the nuclear 18S rDNA dataset (3278 nucleotides), the ML analysis was performed with a gamma shape parameter was 0·2769, and a rate category of 4. The proportion of invariable sites (I) was 0·1791 and the number of distinct data patterns under this model was 1580. The MP analysis on the 18S rDNA dataset generated a tree length of 3100 based on 904 parsimony-informative characters with a CI of 0·7200.

For the concatenated sequence dataset (4061 nucleotides), the ML analysis had 2140 distinct data patterns under the selected nucleotide modes with the proportion of invariable sites (I) equal to 0·2041, the number of rate categories equal to 4 and gamma distribution shape parameter equal to 0·4366. Tree length in the MP analysis based on 1408 parsimony-informative characters was 6766 with CI 0·4957.

In all analyses, three clades were consistently supported within the family Sarcocystidae: (1) a clade containing all *Sarcocystis* spp. (Sarcocystinae) with PP ≥ 0·97 and bootstrap support of ≥99%; (2) a clade containing all *Cystoisospora* spp. (including *Nephroisospora eptesici* in the COI-based tree only) with PP ≥ 0·99 and bootstrap support of ≥99%; and (3) a clade containing all of the included parasites in the genera *Hammondia, Neospora* and *Toxoplasma*.

Besnoitia besnoiti and *Hyaloklossia lieberkuehni* were determined to branch near the base of the Toxoplasmatinae in the 18S rDNA and concatenated dataset trees but there were no COI sequences available for these parasites. The position of *N. eptesici* varied depending on the genetic loci. In the tree generated from the COI dataset, the *N. eptesici* clustered within a clade with all *Cystoisospora* spp. In the tree generated from the 18S rDNA dataset, *N. eptesici* was found in the same clade with *Toxoplasma/Hammondia/Neospora* along with *B. besnoiti; H. lieberkuehni* branched earliest amongst all taxa in the Toxoplasmatinae. However, in the concatenated sequence dataset, *N. eptesici* formed a well-supported sister clade to the *Cystoisospora* spp.

In the analyses based on 18S rDNA and concatenated datasets (Figs 3 and 5, respectively) monophyly of both the subfamilies Toxoplasmatinae and Sarcocystidae was well-supported. However, monophyly of the Toxoplasmatinae was not supported in trees generated from COI sequences alone (Fig. 4) because the rooting position within the family Sarcocystidae was between *Cystoisospora/Nephroisospora* clade and the remaining members of the Toxoplasmatinae rather than between the *Sarcocystis* spp. and other members of the Sarcocystidae as was found in trees based on the other datasets (Figs 2, 3 and 5).

The relationships among closely related species within the Toxoplasmatinae were not resolved fully, particularly in trees based on the 18S rDNA (Figs 2 and 3) or concatenated datasets (Fig. 5). For example, trees based on the 18S rDNA dataset (Figs 2 and 3) did not support monophyly of *Hammondia* spp.; 18S rDNA sequences from *Hammondia* spp. formed a polytomy with those of *T. gondii* and *N. caninum*. Similarly, monophyly of individual *Cystoisospora* spp. was not supported with the exception of the *C. felis* sequences. The remaining 18S sequences from *Cystoisospora* spp. formed a poorly resolved, polychotomous multi-species cluster in which sequences from individual species did not form monophyletic clades. Five *C. canis* cloned 18S rDNA sequences failed to resolve into a monophyletic group in the phylogenetic analyses based on this locus. Based on pairwise sequence comparisons of alignments of these five sequences, the clones clustered into at least two distinct sets of paralogous 18S rDNA sequences. One

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pair of sequences ('Type A' – clones 11 and 17) had pairwise identity of 99·8% and clustered into a well-supported (PP = 0·98) monophyletic clade. The second set of three sequences ('Type B' – clones 5–7) had mean pairwise identity of 99·6% but did not resolve into a monophyletic clade in the 18S rDNA analyses.

In the trees based on COI sequences (Fig. 4), monophyletic clades of sequences corresponding to recognized species within the Toxoplasmatinae were supported in most cases. However, sequences from *C. suis*, *C.* cf. *ohioensis* and *N. eptesici* were found in an unresolved polychotomy basal to the remaining *Cystoisospora* species. The *Cystoisospora* spp. that possess large, egg-shaped oocysts (i.e. *C. felis* and *C. canis*) formed a well-supported monophyletic clade (PP = 0·95). Similarly, species of typically heteroxenous parasites infecting dogs and cats (species in the genera *Toxoplasma*, *Neospora* and *Hammondia*) were all found in a well-supported monophyletic clade in trees based on COI sequences (Fig. 4). However, species in the genus *Hammondia* remained paraphyletic. The canid-infecting *Hammondia* spp. [i.e. *H. triffittae*, *H. heydorni* and an unnamed *Hammondia* sp. from North America (i.e. strains 'Baron' and 'Braun')] were each monophyletic and formed a sister clade to *N. caninum* whereas the felid-infecting *H. hammondi* was sister to *T. gondii*.

In the concatenated dataset analyses, sequences from canid-infecting *Hammondia* species were not resolved into monophyletic clades representing named species although they all formed a well-supported monophyletic clade (Fig. 5). Again, as observed in the COI sequence-based analyses (Fig. 4), parasites using canids as definitive hosts (DHs, i.e. *H. heydorni*, *H. triffitae* and *N. caninum*) and parasites using felids as DHs (i.e. *H. hammondi* and *T. gondii*) each formed monophyletic clades; but the monophyly of *Hammondia* spp. was not supported.

DISCUSSION

Monophyly of genera in the Toxoplasmatinae remains uncertain despite numerous phylogenetic analyses based on nuclear rDNA sequences and other loci (e.g. Mugridge *et al.* 2000; Morrison *et al.* 2004; Morrison, 2009). In the present study, Toxoplasmatinae-specific PCR primers were used to obtain partial mt COI sequences with the goal of resolving evolutionary relationships among these tissue coccidia that could not be resolved using nuclear 18S rDNA sequences (e.g. Morrison, 2009). Culture-derived parasites of known identity as well as parasites obtained from naturally infected hosts were used to obtain mt COI and nuclear 18S rDNA sequences (if such sequences were unavailable in public databases).

Nuclear 18S rDNA possesses numerous indels (most commonly within the single-stranded loops between the helices) and some hypervariable regions (e.g. the E21–1, E21–3 and E21–5 helices in the Sarcocystinae) that can make alignments problematic for these parasites (Morrison *et al.* 2004). Morrison *et al.* (2004) concluded that nuclear 18S rRNA gene sequences provide sufficient phylogenetic signal for deep relationships but have insufficient signal to discern species-level relationships within the Eimeriidae and Sarcocystidae. Deleting the 'problematic' regions from phylogenetic analysis may compromise the phylogenetic support for the various taxonomic groups (Morrison *et al.* 2004). Barta (1997) suggested a staggered alignment approach as a solution for retaining hypervariable regions while still maintaining positional homologies among those taxa for which positional homology can be reasonably assumed; this staggered alignment approach was used in the present work to maximize the information content of the 18S rDNA sequences in both the single locus and concatenated datasets. Even when such strategies are employed, 18S rDNA sequences from a single species (e.g. *Cystoisospora canis* in the present study) can be highly divergent. It is possible that the two paraphyletic clades *C. canis* identified using 18S rDNA sequences represent paralogous rDNA loci within the nuclear genome of this parasite as demonstrated for eimeriid coccidia previously (e.g. El-Sherry *et al.* 2013).

Hammondia species were paraphyletic in all analyses containing COI sequences and paraphyly was not refuted by the 18S rDNA dataset (see also Morrison *et al.* 2004). Phylogenetic analyses based on LSU rDNA and ITS1 sequence data (Ellis *et al.* 1999), similarities in G + C content of DNA sequences (Johnson *et al.* 1987), as well as oocyst structure and antigenic responses (Dubey *et al.* 2002*b*) have consistently indicated that species in the genus *Hammondia* do not form a monophyletic group. We confirmed the lack of monophyly of species currently placed in the genus *Hammondia* as reported previously (Johnson *et al.* 1987; Ellis *et al.* 1999; Dubey *et al.* 2002*b*) using a mitochondrial genetic marker (COI) alone or combined with nuclear 18S rDNA sequences.

In the present analyses, *Hammondia* spp. formed a polytomy with *T. gondii* and *N. caninum* based on 18S rDNA sequences. All canid-infecting *Hammondia* species formed a monophyletic clade that was the sister group to *N. caninum* as reported by Ellis *et al.* (1999). Sequences obtained from a canine isolate of a *Hammondia* spp. from central Canada were nearly identical with those of *H. triffittae* at both the COI and 18S rDNA loci suggesting that the range of *H. triffittae* may include the entire temperate north. Although closely related, *H. heydorni* exhibits genetic and biological differences from *H. triffittae*

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(Schares *et al*. 2002, 2003; Abel *et al*. 2006; Gjerde and Dahlgren, 2011).

Hammondia hammondi clustered with *T. gondii* in all analyses. Partial mt COI sequences from all nine strains of *T. gondii* were identical. The mt COI sequence from the H.H-20 strain of *H. hammondi* shared 98·5% identity to the *T. gondii* sequences. The 1·5% divergence between these two species is identical to the divergence between *C. felis* and *C. rivolta* (1·5%) and similar to divergence between two *Isospora* spp. infecting the same avian host (1·3%, Hafeez *et al*. 2014) or between the chicken parasites *E. tenella* and *E. necatrix* (1·7%, Ogedengbe *et al*. 2011). The genetic divergence supports species-level separation of *H. hammondi* from *T. gondii* in contrast to Mehlhorn and Heydorn's (2000) and Heydorn and Mehlhorn's (2001) arguments against the recognition of *H. hammondi* as a distinct species. These latter authors had suggested that *T. gondii* and *H. hammondi* may be strains of a single species based on oocyst wall structure, tachyzoite ultrastructure and molecular similarities (Mehlhorn and Heydorn, 2000) and cross-immunity (Frenkel and Dubey, 2000). Although partial COI sequences appeared well suited for species delimitation (Ogedengbe *et al*. 2011) and inferring phylogenetic relationships among the eimeriid coccidia (Ogedengbe *et al*. 2011 and current study) as well as members of the *Hammondia*/*Neospora*/*Toxoplasma* clade, the COI sequences from *Cystoisospora* spp. demonstrated remarkably limited sequence variation. For example, the partial COI sequence of *C.* cf. *ohioensis* (KT184384) differed from the COI sequences of *C. suis* (KF854262–KF854265) and *C. rivolta* (KT184383) by 1 single nucleotide difference (SND), while differing from a second *C.* cf. *ohioensis* (KT184365) isolate by 3 SNDs. It is possible that the pairwise differences in the COI sequences of the 2 *C.* cf. *ohioensis* isolates is detection of species level differences among parasites in the *C. ohioensis* spp. complex. Only isolation of a pure line of each parasite and observations on the endogenous development of these parasites would be able to assign these COI sequences unequivocally to *C. ohioensis*, *C. burrowsi* or *C. neorivolta*.

Phylogenetic trees based on COI or concatenated datasets strongly support *H. hammondi* as a sister species to *T gondii*; the support for the close relationship of the other *Hammondia* spp. to *N. caninum* is equally strong. The COI– and the concatenated sequence-based trees show that relationships among species within the *Hammondia*/*Neospora*/*Toxoplasma* clade reflect the DHs infected by these parasites.

Mehlhorn and Heydorn (2000) proposed a radically simplified taxonomic scheme for parasites in the Toxoplasmatinae. They suggested that *T. gondii* and *H. hammondi* should be synonymized under a single species, *T. gondii*. The two described *Neospora* species, *N. caninum* and *N. hughesi*, were to be synonymized under the single species *H. heydorni*, and then *H. heydorni* was to be transferred to the genus *Toxoplasma* to give *T. heydorni* as the name for these parasites of dogs. Synonymizing *N. caninum*, *N. hughesi* and *H. heydorni* under *T. heydorni* by Mehlhorn and Heydorn (2000) would appear to be a 'taxonomic lumping' too far. Would this taxonomic arrangement reflect both biological features and COI genotypes? Synonymizing *T. gondii* and *H. hammondi* (and, presumably, *Hammondia pardalis* Hendricks, Ernst, Courtney and Speer, 1979 that also infects a felid DH) under *T. gondii* may reflect their biological similarities but does not reflect the COI genotypes of these distinct species because of the 10 SNDs between the H.H-20 strain of *H. hammondi* and the various strains of *T. gondii* over 751 bp of the COI gene (1·3% genetic difference); to put this in perspective, *C. felis* and *C. canis* have only 0·9% sequence divergence (6 SNDs) in the same region of the COI gene and they do not share the same DH.

To re-establish monophyly of species currently recognized to belong to the genus *Hammondia*, taxonomic revision will be necessary. The closely related tissue coccidian currently classified in the genera *Toxoplasma*, *Neospora* and *Hammondia* have biological and genetic affinities that directly conflict (Dubey, 1977; Carreno *et al*. 1998; Ellis *et al*. 1999; Mehlhorn and Heydorn, 2000; Dubey *et al*. 2002*a*; Dubey and Sreekumar, 2003; Morrison, 2009). For the tissue coccidia infecting felines as DHs, *T. gondii* (facultatively heteroxenous exhibiting horizontal transmission among intermediate hosts) is closely related genetically to *H. hammondi* (and presumably *H. pardalis*; both obligately heteroxenous, lacking horizontal transmission among intermediate hosts). However, *H. hammondi* and *H. pardalis* are more similar biologically to canid-infecting *Hammondia* species, namely *H. heydorni* and *H. triffittae*, both of which are obligately heteroxenous and lack horizontal transmission among intermediate hosts. Genetically, the latter two *Hammondia* species are related more closely to *Neospora* species (facultatively heteroxenous, horizontal transmission among intermediate hosts).

Transferring *H. hammondi* and *H. pardalis* into the genus *Toxoplasma* would appear to be a taxonomic change that reflects molecular features of these parasites but would group a facultatively monoxenous parasite (*T. gondii*) with an obligately heteroxenous parasite (*H. hammondi*). By the same logic, the *Hammondia* species infecting canine DHs would then be most suitably placed into a single genus with *Neospora* species. The only remaining biological distinction between the two genera that remain, *Toxoplasma* and *Hammondia*, would be their use of feline *vs* canine DHs; clearly this is not a distinguishing feature that warrants separation at

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the level of genus (c.f. *Sarcocystis* species). So there are only two choices for revising generic assignments of species in the genera *Toxoplasma, Hammondia* and *Neospora*: (1) re-assign all of these species to the genus *Toxoplasma* and redefine a broader definition for this genus; or (2) retain all three genera but, to maintain monophyly of species within each genus, name a new genus for the obligately heteroxenous coccidia closely related genetically to *Neospora* spp. (i.e. described *Hammondia* species that utilize canines as DHs).

The lack of comparative molecular data from representatives of all currently recognized genera in the Toxoplasmatinae (i.e. *Besnoitia* species are not represented) makes the lumping of the parasites examined in this study into single genus premature. To maintain taxonomic stability and to reflect the common usage of many of these genus names in the literature, the most conservative approach is to erect a fourth genus to address the paraphyly of *Hammondia* species as confirmed in the present study. We therefore propose the erection of a new genus to contain those *Hammondia* species that utilize canines as DHs.

TAXONOMIC SUMMARY
Apicomplexa Levine, 1980
Conoidasida Levine, 1988
Coccidiasina Leuckart, 1879
Eucoccidiorida Léger, 1911
Eimeriorina Léger & Duboscq, 1911
Sarcocystidae Poche, 1913
Toxoplasmatinae Biocca, 1957
Heydornia n. gen.

DEFINITION: With features of the family Sarcocystidae (heteroxenous or facultatively heteroxenous coccidia; oocysts with two sporocysts lacking Stieda bodies, each with four sporozoites; tissue cysts polyzoic; in vertebrates) and subfamily Toxoplasmatinae (metrocytes not formed in tissue cysts; oocysts sporulate exogenously). Members of the genus *Heydornia* n. gen. are obligately heteroxenous coccidia of vertebrates using canid DHs (where known); lack horizontal transmission among intermediate hosts; about two named species.

NAME-BEARING TYPE:
Heydornia heydorni nov. comb. (Tadros and Laarman, 1976)

Synonym: *Isospora bigemina* (Stiles, 1891) Luhe, 1906 'small form' of various authors, pro parte

Synonym: *Isospora bigemina* of Dubey and Fayer, 1976

Synonym: *Isospora wallacei* Dubey, 1976

ETYMOLOGY: The genus is named in honour of Professor Dr Alfred Otto Heydorn (Institute for Parasitology and Tropical Veterinary Medicine, Free University Berlin) who worked extensively on this 'small form' of *I. bigemina* in canids.

TYPE HOST: *Canis lupus familiaris*

Oocysts: Dubey and Fayer (1976) reported oocysts as spherical, $12 \times 11\ \mu$m. Nassar *et al.* (1983) gives $11{\cdot}9 \times 11{\cdot}1$ ($10{\cdot}0$–$14{\cdot}5 \times 9{\cdot}3$–$13{\cdot}1$) and Shankar *et al.* (1991) $11{\cdot}0$–$14{\cdot}0 \times 10{\cdot}5$–$13{\cdot}0\ \mu$m.

REMARKS: This coccidian is obligatorily heteroxenous and uses a wide range of intermediate hosts, including ruminants, cervids and rodents, in which muscle tissue cysts are formed. Commonly referred to as the 'small form' of *I. bigemina* (Stiles, 1891) Lühe, 1906 in the older literature. Oocysts are passed unsporulated. Members of the genus *Heydornia* n. gen. can be distinguished from *Neospora* species because the former lack horizontal transmission among intermediate hosts and their sporulated oocysts are not infective to their DHs.

OTHER NAMED SPECIES:
Heydornia triffittae nov. comb. (Nukerbaeva & Svanbaev, 1973)

Synonyms:

Isospora triffitti Nukerbaeva & Svanbaev, 1973

Isospora triffittae (Nukerbaeva & Svanbaev, 1973) Levine 1985

Hammondia triffittae (Nukerbaeva & Svanbaev, 1973) Gjerde & Dahlgren, 2011

TYPE HOSTS: Red fox (*Vulpes vulpes*) and arctic fox *(Vulpes lagopus)*.

REMARKS: See Gjerde and Dahlgren (2011) for a comprehensive description of this species and its differentiation from *Heydornia heydorni* n. comb.

Oocysts: Gjerde and Dahlgren (2011) reported oocysts as $12{\cdot}5 \pm 0{\cdot}7 \times 10{\cdot}9 \pm 0{\cdot}5\ \mu$m ($10{\cdot}3$–$15{\cdot}2 \times 9{\cdot}3$–$12{\cdot}8\ \mu$m, $n = 900$) with L: W ratio $1{\cdot}15 \pm 0{\cdot}07$ ($1{\cdot}00$–$1{\cdot}49$, $n = 900$)

In summary, *Heydornia* n. gen. is proposed for *Hammondia* species infecting canid DHs to resolve the well documented paraphyly of coccidia in the genus; the name-bearing type for the genus *Hammondia, H. hammondi* and *H. pardalis* both infect felid DHs and remain in the genus. The use of both nuclear and mitochondrial genetic loci was demonstrated to provide good resolution of most species in the Sarcocystidae; inclusion of additional, biologically diverse members of the subfamily Toxoplasmatinae that were not included in the current COI dataset, such as *Besnoitia* or *Hyaloklossia* spp., might help stabilise the rooting of the phylogenetic hypothesis for these parasites. Addition of some 'primitive' eimeriid coccidia, such as *Goussia* or *Choleoeimeria* spp., to the taxonomic outgroup might also promote stability of the rooting point within the Sarcocystidae for this genetic locus. The observations reported herein support the use of combined nu 18S rDNA and mt

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COI sequences for generating phylogenetic hypotheses that are likely to demonstrate stability of deeper nodes combined with fine resolution of terminal taxa.

SUPPLEMENTARY MATERIAL

To view supplementary material for this article, please visit http://dx.doi.org/10.1017/pao.2015.7

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CONFLICT OF INTEREST

None.

ETHICAL STANDARDS

The work is in no violation of ethical standards. Human or laboratory animals were not used in this work.

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