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, GT1, MAS, ME49, PTG, TgCatBr5, TgCat, Br64, TgCgCal, TgToucan); Neospora caninum (Strain NC1); Hammondia hammondi (Strain H.H–20); H. heydorni; H. ct. triffittae; Cystoisospora felis; C. suis; C. canis; C. rivolta; C. ct. oholensis; 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. ct. oholensis, 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 catenated 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].

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 disporocysts tetrazyotic oocysts without Stieda bodies (Carreno et al., 1998; Mugridge et al., 1999). Coprological identification of N. caninum, T. gondii, H. heydornii 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

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distinguish these two parasites (Dubey et al. 2002b). 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. trifittiae, 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. trifittiae 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 haemosporidian 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, 2013a, 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 Sarcocystidae 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. ohiensis, 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. ohiensis 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. ohiensis 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,
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 run 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 MgCl₂, 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
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 | Cox1_10F | Ogedengbe et al. (2011) | GGWDSWGGRWYGWGGWTGGAC | 50 | 517 |
|             | Cystoisospora felis | Cox1_500R | Ogedengbe et al. (2011) | CATRTGRTGDGCCCAWAC | 50 | 517 |
|             | Hammondia heydorni | Cox1_10F | Ogedengbe et al. (2011) | GGWDSWGGRWYGWGGWTGGAC | 55 | 795 |
|             | Neospora caninum | Cox1_10F | Ogedengbe et al. (2011) | GGWDSWGGRWYGWGGWTGGAC | 55 | 795 |
|             | Hammondia hammondi | Cox1_1202R | El-Sherry et al. (2013) | CCAKRAYHGCAACAAAGATA | 55 | 841 |
|             | Caryospora bigenetica | Sdae-Cox1_260F | Present study | GATCTTTATGTTYTTRATGCC | 50 | 875 |
|             | Hammondia heydorni | Sdae-Cox1_1147R | Present study | CATACCACATACACCC | 50 | 875 |
|             | Hammondia hammondi | Sarco -18S_123F | Present study | TATCAGCTTTTGAGCTGGTATATT | 875 | 1800 |

18SrDNA

| Parasites | Primer pairs | Reference | Primer sequence | \(T_a\) (°C) | Size (bp) |
|-----------|--------------|-----------|-----------------|----------------|----------|
| Hammondia heydorni | MEDLIN A | Medlin et al. (1988) | AACCTGGTTTGATCCCTGGCAGT | 50 | 1800 |
| Hammondia hammondi | MEDLIN B | Medlin et al. (1988) | GATCGGGCAGGGTCAGCCTAC | 50 | 1800 |
| Toxoplasma gondii | Cysto_18S_1711R | Present study | CGAATAATCGCAACAGCTCA | 50 | 1800 |
| Cystoisospora felis | Sarco -18S_123F | Present study | TATCAGCTTTTGAGCTGGTATATT | 875 | 1800 |
| Cystoisospora canis | ERIB1_FOR | Barta et al. (1997) | ACCTGGTTTGATCCCTGGCAG | 875 | 1800 |
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 oocytes 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: *Hepatozoon* sp.; *Plasmodium juxtamucosum*; *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 *Cystoisospora 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, 2013b). 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...
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).
hypervariable regions in the 18S rDNA 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 invariant 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 Haemoporida and Piroplasida. 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, Sarcozystis spp. has numerous (at least seven) expanded regions with no homology with members of the Toxoplasmatinae.
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 Nephropoispora 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.
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)
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 lieberkuhnii branched near the base of the Toxoplasmatinae. Nephrosoriasis petersiti 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.
Cystoisospora felis (MEO2010)  
Cystoisospora canis (MEO2015 – Milly)  
Cystoisospora cf. ohiensis (MEO2015a – Sedona)  
Cystoisospora suis (MEO2015-Friendship7)  
Cystoisospora rivolta (MEO2015-IDX443)  
Hammondia heydorni (Braun)  

| Parasite species (isolate or strain) | Host | 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. ohiensis (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                          |
| 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)        |

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

For the nuclear 18S rDNA dataset (3278 nucleotides), the ML analysis was performed with a gamma distribution shape parameter equal to 0·2041, the number of rate categories equal to 4 with the proportion of invariable sites (I) equal to 0·4366. Tree length in the MP analysis based on 904 parsimony-informative characters from 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. One n/a – sporulated oocysts were not available for this species.

Toxoplasma was found in the same clade with Hammondia/Neospora along with Besnoitia 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 2 and 3, 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 ssp. 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 ssp. formed a poorly resolved, polychotomous multispecies 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
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 polyphytomy 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. trifitae, 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. hammondii 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. trifitae and N. caninum) and parasites using felids as DHs (i.e. H. hammondii 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 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. 2002b) 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. 2002b) 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. trifitae at both the COI and 18S rDNA loci suggesting that the range of H. trifitae may include the entire temperate north. Although closely related, H. heydorni exhibits genetic and biological differences from H. trifitae
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 coccidias (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. ohiensis (KT184384) differed from the COI sequences of C. suis (KF854262–KF854265) and C. rivolta (KT184383) by 1 single nucleotide difference (SN), while differing from a second C. cf. ohiensis (KT184365) isolate by 3 SNs. It is possible that the pairwise differences in the COI sequences of the 2 C. cf. ohiensis isolates is detection of species level differences among parasites in the C. ohiensis 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. ohiensis, C. burroesi 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 SNs 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 SNs) 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. 2002a; 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. trifittiae, 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
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 and their sporulated oocysts are not infective to their DHs.

**NAME-BEARING TYPE:** *Heydornia heydorni* nov. comb. (Tadros and Laaman, 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 × 11 μm. Nassar et al. (1983) gives 11·9 × 11·1 (10·0–14·5 × 9·3–13·1) and Shankar et al. (1991) 11·0–14·0 × 10·5–13·0 μm.

**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 trifittae* nov. comb. (Nukerbaeva & Svanbaev, 1973)

Synonyms:  
*Isospora trifittae* Nukerbaeva & Svanbaev, 1973  
*Isospora trifittae* (Nukerbaeva & Svanbaev, 1973) Levine 1985  
*Hammondia trifittae* (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 12·5 ± 0·7 × 10·9 ± 0·5 μm (10·3–15·2 × 9·3–12·8 μm, n = 900) with L: W ratio 1·15 ± 0·07 (1·00–1·49, 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
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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