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DNA barcoding identifies *Eimeria* species and contributes to the phylogenetics of coccidian parasites (Eimeriorina, Apicomplexa, Alveolata) ☆

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ABSTRACT

Partial (~780 bp) mitochondrial cytochrome *c* oxidase subunit I (COI) and near complete nuclear 18S rDNA (~1,780 bp) sequences were directly compared to assess their relative usefulness as markers for species identification and phylogenetic analysis of coccidian parasites (phylum Apicomplexa). Fifteen new COI partial sequences were obtained using two pairs of new primers from rigorously characterised (sensu Reid and Long, 1979) laboratory strains of seven *Eimeria* spp. infecting chickens as well as three additional sequences from cloned laboratory strains of *Toxoplasma gondii* (ME49 and GT1) and *Neospora caninum* (NC1) that were used as outgroup taxa for phylogenetic analyses. Phylogenetic analyses based on COI sequences yielded robust support for the monophyly of individual *Eimeria* spp. infecting poultry except for the *Eimeria mitis/mivati* clade; however, the lack of a phenotypically characterised strain of *E. mivati* precludes drawing any firm conclusions regarding this observation. Unlike in the 18S rDNA-based phylogenetic reconstructions, *Eimeria necatrix* and *Eimeria tenella* formed monophyletic clades based on partial COI sequences. A species delimitation test was performed to determine the probability of making a correct identification of an unknown specimen (sequence) based on either complete 18S rDNA or partial COI sequences; in almost all cases, the partial COI sequences were more reliable as species-specific markers than complete 18S rDNA sequences. These observations demonstrate that partial COI sequences provide more synapomorphic characters at the species level than complete 18S rDNA sequences from the same taxa. We conclude that COI performs well as a marker for the identification of coccidian taxa (Eimeriorina) and will make an excellent DNA 'barcode' target for coccidia. The COI locus, in combination with an 18S rDNA sequence as an 'anchor', has sufficient phylogenetic signal to assist in the resolution of apparent paraphylies within the coccidia and likely more broadly within the Apicomplexa.

1. Introduction

The eimeriorinid coccidia (Eimeriorina, Minchin 1903) consist of several groups of apicomplexan parasites including pathogens of veterinary and medical importance. These include species in the Eimeriidae and Sarcocystidae such as *Eimeria* spp. and *Toxoplasma gondii*, respectively. Whether homoxenous or heteroxenous, parasites in these families undergo merogony, gamogony and formation of oocysts in the definitive host that are then shed, either sporulated or unsporulated, in the faeces. Unsporulated oocysts shed in the faeces sporulate after leaving the body, when exposed to the correct environmental conditions of temperature, moisture and oxygen. Members of the family Eimeriidae share distinct morphological features, such as a Stieda body in a variable number of sporocysts that varies with the genus, and refractile bodies within the enclosed sporozoites. This family possesses the largest number of described species within the phylum Apicomplexa (Perkins et al., 2000) that belong to many well described genera including *Eimeria*, *Caryospora*, *Cyclospora*, *Isospora* and *Tyzerria* among others. The number of sporocysts contained in a sporulated oocyst is typically used to assign coccidia to a particular genus within the family Eimeriidae (Perkins et al., 2000). The most speciose (over 1,000 species described) of these genera is *Eimeria*, where oocysts contain four sporocysts that each enclose two sporozoites. *Eimeria* spp. are an important burden on the health and productivity of domestic animals where the total cost of prophylaxis, chemotherapy and morbidity amounts to millions of dollars annually in the poultry industry alone (Dalloul and Lillehoj, 2006), hence there is a pressing need for rapid, accurate and cost effective identification tools. Oocysts containing four sporozoites in each of two sporocysts are typical of some parasites in both the Eimeriidae and the Sarcocystidae. However, parasites such as *Isospora robini* and *Isospora*

☆ *Note:* Nucleotide sequence data reported in this paper are available in the GenBank™, EMBL and DDBJ databases under Accession Nos. HM771673 through HM771690.

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gryphoni that have a Stieda body in each sporocyst and sporozoites containing refractile bodies have been shown by molecular phylogenetic analyses to belong to the family Eimeriidae (see Carreno and Barta, 1999) whereas morphologically similar monoxenous parasites of mammals that lack Stieda bodies have been transferred to the genus *Cystoisospora* within the Sarcocystidae (Barta et al., 2005). The identification and classification of eimeriid coccidia has been based largely on morphological and life cycle details (Levine, 1970, 1988). These classifications have been subjected to revisions, based on newly discovered features and more detailed biological studies. More recently, molecular data have been explored in an attempt to explain some rather confusing associations, not just within the Eimeriidae but within the phylum Apicomplexa generally.

Morrison et al. (2004) described the usefulness of 18S rDNA in classifying apicomplexan parasites, especially in support of higher taxonomic groupings within the phylum. Using 18S rDNA sequences from a variety of coccidian parasites, Morrison et al. (2004) noted that classical taxonomy of the monoxenous coccidia in the family Eimeriidae was not well supported by the molecular data; specifically, sequence data were unable to confirm the monophyly of all of the *Eimeria* spp. included in the analysis. Coccidia with morphologically distinct oocysts such as *Isospora* spp. infecting birds and *Cyclospora* spp. infecting mammals were interspersed among the *Eimeria* spp., making the latter genus paraphyletic. Similarly, Martynova-VanKley et al. (2008) found that near complete 18S rDNA sequences from chicken *Eimeria* spp. did not resolve into monophyletic clades.

Mitochondrial genes appear to give useful phylogenetic signals in many organisms that use oxidative phosphorylation as a means of respiration (Boore, 1999; Le et al., 2000). Cytochrome *c* oxidase subunit I (COI) in particular has shown to be useful in delineating recent speciation events (Lane, 2009) and may be a useful adjunct to nuclear genes for inferring recent evolutionary events. The use of genetic data to identify organisms has a long history and is gaining acceptance and popularity as an adjunct to morphology-based identifications. DNA barcoding has emerged as a standardised, sequence-based identification procedure that exploits genetic differences in specific gene regions of eukaryotic organisms, to aid species identification and document their diversity (Hebert et al., 2004a,b). Mitochondrial COI has been the most widely used genetic target in animal barcoding and has been found to be useful in species delineation on a large scale (Teletchea, 2010) with more than one million COI sequences having been deposited in the Barcode of Life Data System (BOLD; Ratnasingham and Hebert, 2007) to date. These developments have given rise to the International Barcode of Life Project (iBOL.org) which is calling for a barcode-based identification system for animals, including parasites. The barcoding technique involves the use of a short (~600–800 bp), standard "PCR friendly" gene region that is of universal occurrence and that can provide sufficient variability needed for distinguishing species efficiently (i.e. from a single read using a traditional Sanger sequencing approach). According to Ferri et al. (2009), a useful DNA barcode gene must exhibit variability between species, should be short enough to be sequenced in a single reaction and contain sufficiently conserved regions to facilitate the development of universal primers to enable PCR amplification of the target marker across as much taxonomic breadth as possible. The variation within species should be smaller than between species for the technique to work reliably. COI has been used alone or in conjunction with other genes in phylogenetic analysis or molecular identification of parasites including *Eimeria* spp. (e.g. Ros and Breeuwer, 2007; Huang et al., 2009; Schwarz et al., 2009).

The objectives of this study were to investigate the utility of COI partial sequences in identification and phylogenetic analysis of some parasites in the genus *Eimeria*, and to compare the phylogenetic informativeness (sensu Roe and Sperling, 2007) of partial COI sequences with the much longer 18S rDNA sequences traditionally used for molecular phylogenetic studies of coccidia. Similarly, the relative utility of mitochondrial COI gene sequences for species identification of coccidia was compared with that of more commonly used nuclear 18S rDNA sequences.

2. Materials and methods

2.1. Oocysts and DNA extraction

For all *Eimeria* spp. of chickens propagated in our laboratory, single oocyst or single sporocyst strains were isolated from field samples and amplified in specific pathogen-free birds. All animal usage was reviewed and approved by the Animal Care Committee of the University of Guelph, Canada and complied with the Canadian Council on Animal Care's "Guide to the Care and Use of Experimental Animals, vol. 1, 2nd ed.". Assignment of each strain to a particular *Eimeria* sp. was made based on oocyst measurements, location and histological appearance of endogenous stages, and clinical appearance of infection as in the original species descriptions summarised by Reid and Long (1979). A laboratory strain of the murine parasite *Eimeria falciformis* was kindly provided by Dr. Bill Chobotar (Andrews University, MI, USA). For DNA isolations, oocysts were later separated from faecal debris, washed and sporulated in 2.5% potassium dichromate. After sporulation, oocysts were purified and DNA extracted using DNAzol reagent (Invitrogen, USA) or a prepGEM™ Blood kit (ZyGEM, Hamilton, New Zealand) according to the manufacturer's protocol but with the addition of 500 μm glass beads (Ferro Microbeads; Cataphote Division, Jackson Mississippi, USA) to promote the release of sporozoites from sporocysts and oocysts. Samples were vortexed for 30–60 s to aid in oocyst disruption into the DNAzol or prepGEM reagent. DNA quantities were later estimated using a spectrophotometer (GeneQuant Pro, Amersham Biosciences; Golden Valley, MN, USA) and adjusted to a concentration of ~35 ng/μl in the final working dilution.

2.2. PCR reaction parameters

PCR was carried out in a MJ mini thermal cycler (Bio Rad, CA, USA) using the following parameters in a 50 μl reaction. After an initial denaturing temperature of 96 °C for 5 min, 40 cycles of 94 °C for 20 s, 55 °C for 30 s and 72 °C for 90 s were run, followed by a final extension at 72 °C for 10 min. Approximately 70 ng of parasite DNA were used as templates for each PCR with one unit of Platinum Taq (Invitrogen, Carlsbad CA, USA), 1 mM each of *Eimeria*-specific COI primers (Cocci_COI_For; GGTTCAGGTGTTGGTTGGAC and Cocci_COI_Rev; AATCCAATAACCGCACCAAG), 1× PCR buffer (Invitrogen) and 2.5 mM $MgCl_2$. A similar region of the COI gene was amplified from purified DNA samples obtained from *T. gondii* strain ME49, *T. gondii* Strain GT1 and *Neospora caninum* Strain NC-1 (kindly provided by Dr. J.P. Dubey, United States Department of Agriculture, Beltsville, MD, USA) using Toxoplasmatinae-specific primers (Toxo_COI_For; GGAGGAGGTGTAGGTTGGAC and Toxo_COI_Rev; CATTTTGTATTATCTCTGGG) with the same PCR conditions as above. Note that metazoan-specific 'universal' COI primers LCO1490 and HCO2198 (see Folmer et al., 1994) designed to amplify ~650 bp at the 5′ end of the mitochondrial COI gene (the BARCODE region) failed to produce amplicons from any of the coccidial DNA templates using the same amplification parameters, necessitating the development of new taxon-specific primers. Both negative and positive controls were included in all PCRs. PCR products were later electrophoresed on a 1.5% agarose submarine gel in 1× Tris–acetate–EDTA (TAE) buffer at 120 V for

45 min. The resulting gel was stained with ethidium bromide. PCR products (~800 bp – either in solution or excised from an agarose gel) were later purified using a Roche High Pure DNA purification kit (Roche Applied Science, Germany). Amplicons were sequenced in both directions with the forward and reverse amplification primers using an ABI Prism 7000 Sequence Detection System (Applied Biosystems Inc., Foster City CA, USA). All newly reported specimen provenance data, related images and electropherogram 'trace' files were deposited in the publicly accessible project ('Eimeria Barcodes' [EIMER]) within BOLD (Ratnasingham and Hebert, 2007). Data generated for this study generally complied with the BARCODE standards (Hanner, 2009). However, the new primers developed for *Eimeria* only exhibited a partial overlap (~328 bp) with the standard BARCODE region of COI.

2.3. Sequence alignment and phylogenetic analysis

COI sequences were obtained as optimised contigs using Geneious Bioinformatics software package (version 4.7.6 and subsequent versions, Biomatters Ltd., New Zealand available from www.geneious.com). Each new COI sequence was approximately 800 bp in length. Newly generated and existing COI sequences from GenBank were aligned using Clustal-X (Larkin et al., 2007) and translation-based alignment implemented from within Geneious. The resulting alignment used in these analyses was 782 bp in length including gaps but with primer regions trimmed. The protein translation of the sequences presented open reading frames without stop codons and there was no obvious codon bias when the sequences were analysed using MEGA version 4 (Tamura et al., 2007).

Phylogenetic analyses were performed on 109 mitochondrial COI sequences; 106 sequences representing nine *Eimeria* spp. from various hosts formed the ingroup for analyses and three sequences from members of the Toxoplasmatinae (*T. gondii* strains GT1 and ME49, and *N. caninum* strain NC-1) were used as taxonomic and functional outgroups. The newly generated mitochondrial COI sequences (followed by GenBank Accession Numbers) used in the analyses followed by pre-existing sequences downloaded from GenBank were: *Eimeria acervulina* strain NC3 (HM771673) and strain USDA 84 (HM771674) plus EF158855, FJ236428, FJ236427, FJ236420, FJ236419, FJ236443; *Eimeria brunetti* strain Guelph 80 (HM771675); *Eimeria mitis* strain USDA 50 (HM771681); *Eimeria mivati* FJ236433, FJ236441, FJ236434, EF174185; *Eimeria maxima* strain M6 (HM771684), strain Guelph 74 (HM771685) and strain USDA 68 (HM771686), plus EF174183, EF174184, EU025104–EU025106, FJ236380, FJ236386–FJ236394, FJ236401, FJ236402, FJ236406–FJ236418, FJ236429, FJ236432, FJ236435–FJ236440, FJ236442, FJ236448–FJ236452, FJ236454, FJ236456, FJ236457, FJ236459, EU025107,; *Eimeria necatrix* strain Guelph 84 (HM771680) plus EU025108; *Eimeria tenella* strain USDA80 (HM771676), strain MD1 (HM771677), strain Guelph 1D (HM771678) and strain Guelph 2D (HM771679) plus EF174186–EF174188, EU025109, FJ236381–FJ236384, FJ236396, FJ236395, FJ236397-FJ236399, FJ236385, FJ236400, FJ236403–FJ236405, FJ236421–FJ236426, FJ236430, FJ236444–FJ236447, FJ236453, FJ236455, FJ236458, *E. falciformis* strain Chob2 (HM771682); *Eimeria zuernii* strain Guelph 2007 (HM771687); *N. caninum* strain NC1 (HM771688); *T. gondii* strain ME49 (HM771690); *T. gondii* strain GT1 (HM771689).

Phylogenetic analyses were performed on 106 nuclear 18S rDNA sequences; 94 sequences representing eight *Eimeria* spp. from chickens formed the ingroup for analyses and 12 sequences from members of the Toxoplasmatinae were used as taxonomic and functional outgroups. Sequences were initially aligned using Clustal-X implemented from within Geneious, followed by refinement by eye using a staggered alignment method to maximise positional homology (Barta, 1997). The nuclear 18S rDNA sequences (followed by GenBank Accession Number) used in the analyses were: *E. acervulina* (EF210323.1, EF210324.1, EF175928.1, DQ538351.1, DQ136187.1, FJ236372.1, U67115.1); *E. brunetti* (U67116.1); *E. maxima* (FJ236329.1, FJ236333.1, DQ538350.1, DQ640012.1, DQ136186.1, EU025110.1, FJ236332.1, FJ236330.1, FJ236331.1, EU025111.1, FJ236334.1, EU025112.1, FJ236335.1, FJ263947.1, FJ236336.1, FJ236360.1, EF122251.2, DQ538349.1, FJ236339.1, FJ236355.1, FJ236353.1, FJ236346.1, FJ236347.1, FJ236348.1, FJ236344.1, FJ236354.1, FJ236345.1, FJ236358.1, FJ236359.1, FJ236338.1, FJ236343.1, FJ236350.1, FJ236352.1, FJ236342.1, FJ236351.1, EF210322.1, FJ236357.1, FJ236341.1, FJ236349.1, U67117.1, DQ538348.1, FJ236361.1, FJ236356.1, FJ236340.1, FJ236337.1); *E. mitis* (U40262.1, U67118.1, FJ236379.1); *E.* cf. *mivati* (FJ236373.1, FJ236376.1, FJ236375.1, FJ236378.1, FJ236374.1, FJ236377.1); *E. mivati* (U76748); *Eimeria praecox* (FJ236362.1, FJ236367.1, FJ236366.1, FJ236368.1, FJ236370.1), FJ236363.1, FJ236365.1, GQ421692.1, FJ236364.1, FJ236371.1, FJ236369.1, U67120.1; *E. necatrix* (DQ136185.1, U67119.1); *E. tenella* (U67121.1, EU025114.1, EF210325.1, DQ640011.1, EU025116.1, AF026388.1, DQ136180.1, DQ136176.1, U40264.1, DQ136182.1, EU025113.1, DQ136184.1, DQ136179.1, DQ136177.1, DQ136181.1, EU025115.1, DQ136178.1, DQ136183.1); *E. falciformis* (AF080614); *E. zuernii* (AY876932); *N. caninum* (U03069.1, AJ271354.1, U17346.1, L24380.1); *Neospora* sp. (U17345.1); *T. gondii*, (U00458.1; M97703.1; U03070.1; EF472967.1; U12138.1; L37415.1; L24381.1).

Nucleotide evolutionary models were evaluated using MrModeltest version 2.3 (Nylander, J.A.A., 2004. MrModeltest v2. Program distributed by the author. Evolutionary Biology Centre, Uppsala University) in PAUP 4.0b10 (Swofford, D.L., 2003. PAUP: Phylogenetic Analysis Using Parsimony (and other methods). Version 4.10b. Sinauer Associates, Sunderland, Massachusetts). The best-fit model, according to the Akaike Information Criterion evaluation of hierarchical likelihood ratio tests, suggested the general time reversible model with gamma (GTR+G) distribution of nucleotide substitution for all analyses. In all analyses, the two *T. gondii* strains and *N. caninum* were used as the taxonomic outgroup. Both maximum likelihood and maximum parsimony methods were carried out using nucleotide substitution parameters generated using MrModeltest version 2.3. The suggested Bayesian parameters for phylogenetic tree estimation were added to the dataset and the Markov Chain Monte Carlo performed at five million generations, with four chains, two runs and a sampling frequency of 1,000 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). The 'burnin' value was set at 100,000 and the resulting consensus tree was examined using Treeview-Win 32 (Page, 1996).

2.4. Species delimitation using mitochondrial COI or nuclear 18S rDNA sequences

The relative utility of mitochondrial COI or nuclear 18S rDNA sequences for the delimitation and identification of a number of *Eimeria* spp. was assessed using the Species Delimitation plug-in (Masters et al., 2011) within Geneious. This application measures the intraspecific and interspecific genetic differences and then uses the method of Ross et al. (2008) to estimate the probability that a new sequence would be correctly assigned to a putative species using the current multiple sequence alignment and tree as the reference dataset. Comparisons were made for seven of the *Eimeria* spp. that infect chickens for which both nuclear 18S rDNA and mitochondrial COI sequences were available. Because the BOLD 'identification engine' (Ratnasingham and Hebert, 2007) includes only 5′ COI sequences, it could not be used to perform a species identification analysis with the legacy data involving sequence data outside the barcode region.

3. Results

3.1. Phylogenetic analyses

Fig. 1 illustrates the consensus trees generated for COI and 18S rDNA genes using MrBayes and estimates of nucleotide substitution by MrModeltest (Nylander, 2004). Both maximum parsimony and maximum likelihood trees showed similar topologies and all three methods generated trees that showed support for the monophyly of each chicken *Eimeria* spp. (maximum parsimony and maximum likelihood trees not shown). The strict consensus tree generated by maximum parsimony from the mitochondrial COI sequences had a tree length of 718 steps with a consistency index of 0.708. Of the 782 aligned nucleotide positions, there were 386 constant characters, 116 variable characters that were parsimony uninformative and 280 variable characters that were parsimony informative. The maximum parsimony consensus tree for the nuclear 18S rDNA was 682 steps in length with a consistency index of 0.73; 1,347 characters were constant, 145 variable characters were parsimony uninformative and 287 variable characters were parsimony informative.

3.2. Species delimitation using mitochondrial COI or nuclear 18S rDNA sequences

Table 1 illustrates the pairwise estimates of sequence divergence between the parasites included in this study. Table 2 demonstrates intraspecific and interspecific sequence diversity of parasites for which more than one sequence of a particular genetic target was available. The genetic distances between the different taxa in both the mitochondrial COI and nuclear 18S rDNA sequences were computed using 109 sequences for COI and 106 sequences for the 18S rDNA. The aligned lengths (including alignment gaps) of the mitochondrial COI sequences was 782 bp compared with 1,778 bp for the 18S rDNA sequences. Nonetheless, the number of phylogenetically informative characters for each alignment was almost the same (280 for the COI alignment versus 287 for the 18S rDNA alignment). This was reflected in the pairwise distances of mitochondrial COI sequences; variation between *Eimeria* spp. ranged from 0.011 to 0.17 whereas variation within a single *Eimeria* sp. was limited (0.00023–0.004) (Tables 1 and 2). The largest variation between *Eimeria* spp. infecting birds was between *E. maxima* and *E. necatrix* (0.17), which was the same as between *E. maxima* and *E. zuernii* of cattle (data not shown). Mean pairwise genetic distances between species using nuclear 18S rDNA sequences indicated markedly lower genetic variation. For example, among the various *Eimeria* spp. infecting chickens, the greatest genetic distance between species was 0.042 ± 0.006 for nuclear 18S rDNA sequences compared with 0.171 ± 0.038 for mitochondrial COI sequences (Table 1).

Within the *Eimeria* spp. infecting chickens, intraspecific variation in the mitochondrial COI sequences (0.00023–0.004) and nuclear 18S rDNA sequences (0.002–0.013) was similar (Table 2). However, as noted above, interspecific genetic distances from an *Eimeria* spp. to its nearest genetic relative were generally greater for the mitochondrial COI sequences (0.020-0.098) compared with the nuclear 18S rDNA sequences (0.009-0.027). The similar intraspecific distances but larger interspecific distances with the mitochondrial COI sequences were reflected in the lower intraspecific/interspecific ratios for that genetic target. These greater genetic distances between mitochondrial COI sequences from *Eimeria* spp. of chickens produced higher P ID(Liberal) values (Table 2), compared with P ID(Liberal) values obtained for nuclear 18S rDNA sequences (Table 2). A higher P ID(Liberal) value serves as a prediction of the utility of a genetic target for species delimitation (Ross et al., 2008; Masters et al., 2011). As an example, with both mitochondrial COI and nuclear 18S rDNA sequences, *E. tenella* was the closest genetic relative of *E. necatrix*; however the P ID(Liberal) value (plus 95% confidence interval) for mitochondrial COI was 0.98 (0.82, 1.00) whereas the P ID(Liberal) value for nuclear 18S rDNA sequences was only 0.77 (0.62, 0.93).

4. Discussion

Eimeria tenella has recently been shown to possess a 6.2 kB concatenated mitochondrial genome (found at a 50-fold abundance relative to the nuclear genome of these parasites) that possesses three protein coding genes (COI and COIII, cytochrome *b* (cytB)) plus 19 lsrRNA and ssrRNAgene fragments (Hikosaka et al., 2011). The genetically similar mitochondrion of *Plasmodium* spp. (see Hikosaka et al., 2011) has been shown to be inherited uniparentally from macrogametes (Wilson and Williamson, 1997). Perkins and Schall (2002) demonstrated that mitochondrial cytB sequences were highly useful for inferring phylogenetic relationships among haemosporidian parasites, yet cytB has not been advanced as a standard marker for species identification for parasites. The utility of mitochondrial COI sequences for species identification and phylogeny reconstruction has been demonstrated for several parasite groups (Alcaide et al., 2009; Huang et al., 2009; Pagès et al., 2009). Martinsen et al. (2008) used mitochondrial COI sequences in addition to both plastid and nuclear genes to study the evolutionary relationships and events leading to host switching and diversification in *Plasmodium* spp. of birds, mammals and squamate reptiles. Cunha et al. (2009) have also used CO gene sequences (COI and COIII) in the molecular epidemiology of *Plasmodium* by developing highly sensitive (100%) and specific (88%) PCR-based methods for the diagnosis of *Plasmodium vivax* and *Plasmodium falciparum*. However, adoption of barcode meta data standards (Hanner, 2009) such as the reporting of electropherogram 'trace' files are still generally lacking. In the present study, we examined the utility of the COI gene for species identification (so-called 'DNA barcoding') and for phylogenetic reconstructions compared with 18S data of some coccidian parasites, particularly *Eimeria* sp. Importantly, we also deposited standard barcode meta data (trace files, images, catalogue numbers of voucher specimens) in BOLD to enhance the downstream utility of our data for subsequent molecular diagnostic applications.

The Barcode of Life (BOL) initiative is a global effort to facilitate species identification and discovery using PCR sequencing and analysis of short standardised DNA sequences known as "DNA barcodes" derived from the 5′ region of the mitochondrial COI gene. In the Apicomplexa, especially the coccidia, there are relatively few studies that have demonstrated that mitochondrial genes could act as useful tools in molecular systematics and/or parasite identification. There are, however, a growing number of workers exploring the mitochondrial genome as a potential tool to address problems in epidemiology, evolution or species identification that could not be solved by relying on the more commonly used nuclear genes alone. For example, Schwarz et al. (2009) used both the ribosomal small subunit gene and mitochondrial COI in assessing the population dynamics of different *Eimeria* spp. from various poultry farms. Ferri et al. (2009) applied barcodes in parasitic nematodes, showing high concordance between morphological and molecular data – and due to the ease of analysis stemming from the use of a coding region over a ribosomal marker, made the plea for using COI barcodes over 12S sequences. However, barcoding parasites requires development of new primers for reliable PCR amplification (Moszczynska et al., 2009).

In the present study, the utility of relatively short mitochondrial COI sequences was compared to the utility of complete nuclear 18S

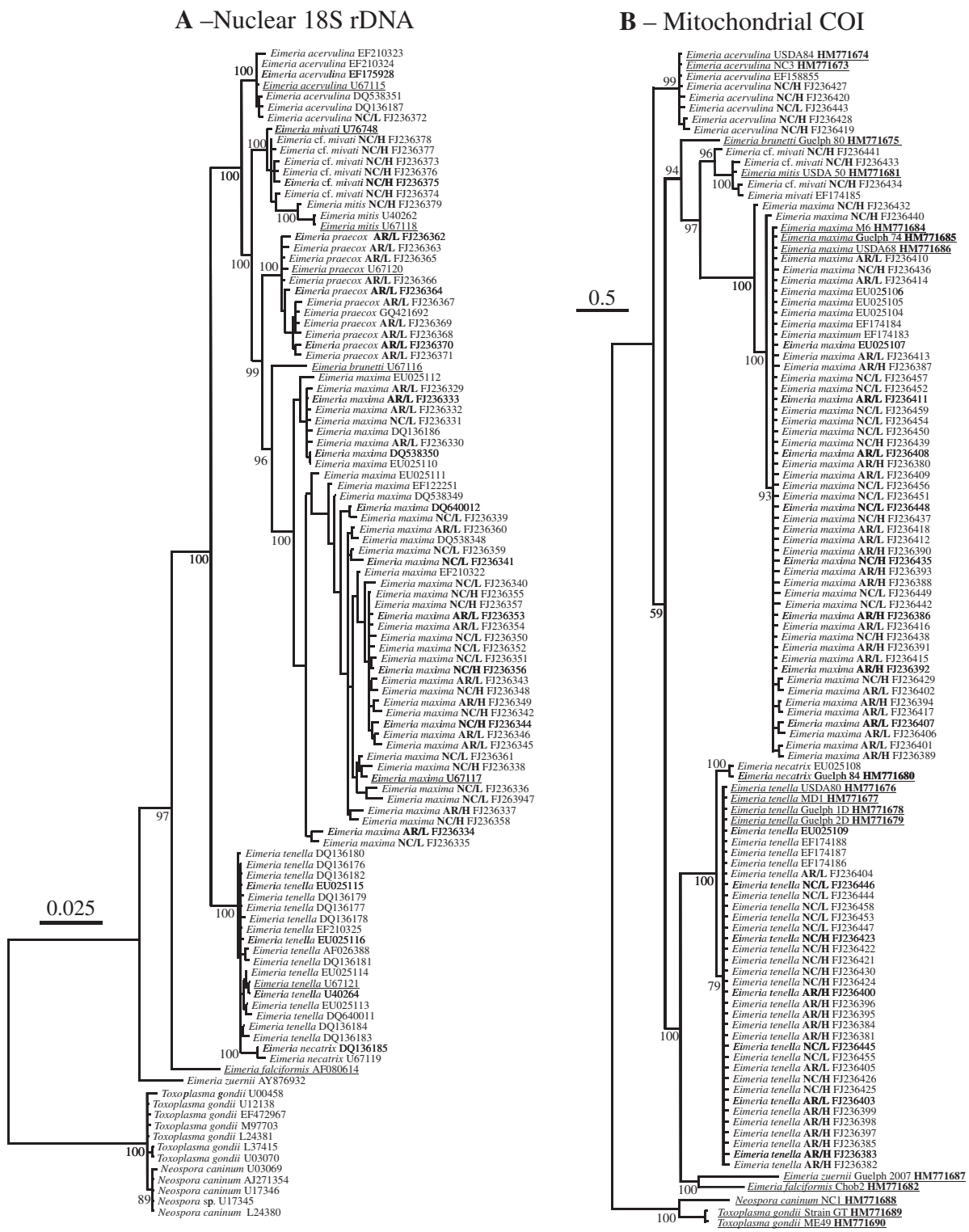

Fig. 1. Phylogenetic trees based on Bayesian analyses of nuclear 18S rDNA and mitochondrial cytochrome *c* oxidase subunit I (COI) sequences. Maximum likelihood and maximum parsimony trees were similar to the Bayesian trees for both genes (data not shown). The horizontal distance is proportional to hypothesized evolutionary change as indicated (scale bar). The posterior probability branch support is shown. In both analyses, support for the monophyly of individual *Eimeria* spp. infecting poultry was strong except for the *Eimeria mitis*/*mivati* clade. (A) Consensus tree for nuclear 18S rDNA sequences of *Eimeria* spp. infecting poultry using members of the Toxoplasmatinae as outgroup taxa. (B) Consensus tree for mitochondrial COI sequences of *Eimeria* spp. using members of the Toxoplasmatinae as outgroup taxa. *Note:* All sequences generated in our laboratory from phenotypically characterised laboratory strains of parasites are underlined; GenBank Accession Numbers are bolded for newly generated sequences; 18S and COI sequences generated by Schwarz et al. (2009) were obtained from four pooled DNA samples of oocysts (AL/L, AL/H, NC/L and NC/H as indicated, each containing four to five mixed *Eimeria* spp.) and assigned to species based on BLAST similarity (see Discussion for further details); all other sequences were obtained directly from GenBank and were assigned to species by the contributing authors.

Table 1
Comparison of pairwise differences (mean ± S.E.) of 18S nuclear rDNA sequences (upper block) and mitochondrial cytochrome oxidase *c* subunit 1 (COI) sequences (lower block) between selected coccidian taxa.

	Eimeria acervulina	*Eimeria brunetti*	*Eimeria mitis*	*Eimeria mivati*	*Eimeria maxima*	*Eimeria necatrix*	*Eimeria tenella*	*Toxoplasma gondii*
Eimeria acervulina		0.024 ± 0.004	0.023 ± 0.004	0.016 ± 0.003	0.029 ± 0.004	0.029 ± 0.004	0.027 ± 0.004	0.127 ± 0.013
Eimeria brunetti	0.099 ± 0.018		0.025 ± 0.004	0.021 ± 0.004	0.027 ± 0.004	0.040 ± 0.005	0.038 ± 0.005	0.136 ± 0.014
Eimeria mitis	0.106 ± 0.020	0.102 ± 0.020		0.014 ± 0.003	0.033 ± 0.004	0.039 ± 0.005	0.035 ± 0.005	0.138 ± 0.014
Eimeria mivati	0.109 ± 0.020	0.103 ± 0.019	0.011 ± 0.003		0.030 ± 0.004	0.032 ± 0.004	0.030 ± 0.004	0.134 ± 0.014
Eimeria maxima	0.139 ± 0.025	0.144 ± 0.026	0.129 ± 0.023	0.122 ± 0.021		0.042 ± 0.005	0.039 ± 0.005	0.125 ± 0.013
Eimeria necatrix	0.101 ± 0.018	0.124 ± 0.022	0.136 ± 0.024	0.140 ± 0.024	0.170 ± 0.030		0.008 ± 0.002	0.128 ± 0.013
Eimeria tenella	0.104 ± 0.019	0.124 ± 0.022	0.129 ± 0.023	0.131 ± 0.023	0.163 ± 0.028	0.018 ± 0.005		0.126 ± 0.013
Toxoplasma gondii	0.354 ± 0.086	0.342 ± 0.082	0.356 ± 0.088	0.365 ± 0.090	0.345 ± 0.083	0.336 ± 0.082	0.33 ± 0.081	

Table 2
Comparison of the genetic variation within species (intraspecific distance) and between closest species (interspecific distance) of seven *Eimeria* species of chickens using either mitochondrial cytochrome *c* oxidase – subunit I (COI) sequences or nuclear 18S rDNA sequences. The probability (plus 95% confidence interval) of making a correct identification of an unknown specimen based on either genetic target is indicated by P ID (Liberal).

Species	Closest species	Intraspecific distance	Interspecific distance (closest taxon)	Intra/InterRatio	P ID(liberal)[b]
Mitochondrial cytochrome c oxidase – subunit I (COI) sequences – species delimitation					
Eimeria acervulina	*Eimeria mivati*	0.004	0.095	0.04	0.99 (0.93, 1.0)
Eimeria brunetti	*Eimeria acervulina*	n/a[a]	0.098	0.00	0.96 (0.83, 1.0)
Eimeria maxima	*Eimeria mivati*	0.004	0.098	0.04	1.0 (0.97, 1.0)
Eimeria mitis	*Eimeria mivati*	0.004	0.033	0.13	0.95 (0.84, 1.0)
Eimeria mivati	*Eimeria mitis*	n/a[a]	0.033	0.00	0.96 (0.83, 1.0)
Eimeria necatrix	*Eimeria tenella*	0.00023	0.020	0.01	0.98 (0.82, 1.0)
Eimeria tenella	*Eimeria necatrix*	0.003	0.020	0.14	0.98 (0.95, 1.0)
Nuclear 18S rDNA sequences – species delimitation					
Eimeria acervulina	*Eimeria praecox*	0.002	0.021	0.11	0.97 (0.91, 1.0)
Eimeria brunetti	*Eimeria praecox*	n/a [a]	0.023	0.00	0.96 (0.83, 1.0)
Eimeria maxima	*Eimeria brunetti*	0.013	0.027	0.47	0.96 (0.93, 0.99)
Eimeria mitis	*Eimeria acervulina*	0.005	0.022	0.21	0.89 (0.74, 1.0)
Eimeria mivati	*Eimeria mitis*	0.003	0.014	0.22	0.95 (0.85, 1.0)
Eimeria necatrix	*Eimeria tenella*	0.003	0.009	0.34	0.77 (0.62, 0.93)
Eimeria tenella	*Eimeria necatrix*	0.002	0.009	0.23	0.97 (0.94, 1.0)

[a] *Not applicable:* Intraspecific variation is uninformative because there is only a single sequence for that species.
[b] *P ID(Liberal):* The mean probability, with the 95% confidence interval for the prediction, of making a correct identification of an unknown specimen of the focal species using BLAST (best sequence alignment), DNA barcoding (closest genetic distance) or placement on a tree, with the criterion that it falls sister to or within a monophyletic species clade (Ross et al., 2008; Masters et al., 2011).

rDNA sequences (the most widely available genetic marker for these parasites) for species identification and delimitation. Both newly generated and published sequences used for phylogenetic tree constructions indicate that mitochondrial COI sequences can differentiate common species of coccidia in chickens reliably; all resulting clusters of closely related sequences were monophyletic for a single *Eimeria* sp. Moreover, because COI is a protein coding gene it is more straightforward to align, increasing the likelihood that assertions of positional homology in the alignment are correct. All COI sequences generated in our study and many of the 18S and COI sequences from GenBank were obtained from laboratory strains that were purified and identified as a particular *Eimeria* sp. based on observed phenotype (Reid and Long, 1979) prior to DNA isolation, PCR and sequencing. In contrast, Schwarz et al. (2009) assigned mitochondrial COI and nuclear 18S sequences obtained from four pooled DNA samples of oocysts (each containing four to five mixed *Eimeria* spp.) to species based on BLAST (Altschul et al., 1990) similarity to existing 18S or COI sequences in GenBank; thus, the species assignments of the resulting sequences cannot be linked to a particular *Eimeria* sp. phenotype and must be considered tentative (see Fig. 1), as is often the case with GenBank data where sequences are reported without reference to specimens examined from reference collections (Ruedas et al., 2000). In all cases where the nuclear 18S or mitochondrial COI sequences were obtained from a particular *Eimeria* sp. that had been identified based on phenotype, the sequences were found within the same monophyletic clade in each analysis. All sequences obtained by Schwarz et al. (2009) clustered with the sequences from phenotypically characterised *Eimeria* spp. thus providing greater diversity in our subsequent species delimitation analysis using these data. The genetic distance matrix obtained from 18S rDNA sequences suggests a lower resolution of genetic differences between species compared with mitochondrial COI sequences. Except for sequences from *E. maxima* isolates, which showed less intraspecific genetic variability in 18S rDNA sequences, all other species used in this study showed appreciably greater genetic distances between species when mitochondrial COI sequences were analysed.

These observations suggest that mitochondrial COI sequences would be useful for molecular species identification and delimitation for coccidia and, based on similar observations for malaria parasites (Martinsen et al., 2008), perhaps apicomplexan parasites generally. Given sufficient sampling to generate a library of mitochondrial COI sequences from classically characterised coccidian species, it should be possible to develop primers and species-specific probes to generate quantitative PCR tools for studying the molecular epidemiology of *Eimeria* spp. and other apicomplexan parasites. This would be especially useful in animal hosts where this information could assist in the control of these economically important parasites. However, the importance of using expert-identified reference material is crucial and in this respect our data follow the enhanced annotation and reporting standards associated with BARCODE records in GenBank (as recorded in BOLD). Despite having only partial overlap (~328 bp) with the 5′ COI BARCODE region for animals, our newly contributed data exceed the minimum length specification (500 bp) and sequence quality score required for a BARCODE record. In sliding window analyses of a number of dipteran and lepidopteran COI sequences, Roe and Sperling (2007) demonstrated that the 5′ COI DNA barcoding

region was no better than other regions downstream in COI; the lengths of the mitochondrial COI sequences obtained were found to be most important for increasing the probability of sampling regions of high phylogenetic informativeness (Roe and Sperling, 2007). The enhanced meta data reporting standard is a key difference between properly conducted DNA barcoding studies and previous molecular assessments, which makes barcode data fit for use in molecular diagnostic applications (Teletchea, 2010).

Both the 18S and COI-based phylogenetic reconstructions reported here were in general agreement with earlier molecular analyses of the relationships among *Eimeria* spp. infecting the domestic fowl using 18S rDNA sequences (Barta et al., 1997). Furthermore, the 18S- and COI-based reconstructions were largely consistent with one another, demonstrating similar branching orders and phylogenetic relationships between and within individual clades. For example, in both analyses, *E. mivati* and *E. mitis* sequences clustered in a monophyletic clade with high bootstrap support, but the monophyly of *E. mivati* and *E. mitis* within that clade was not supported. In the COI analysis, *E. brunetti* was the sister taxon to a clade containing the *E. mitis*/*E. mivati* clade and *E. maxima*; in contrast, *E. brunetti* was the sister taxon to a large, monophyletic *E. maxima* clade based on 18S sequences. However, the lack of any sequences from *E. praecox* in the COI-based reconstruction may be a confounding factor contributing to the observed branching order differences. Both analyses showed that *E. tenella* and *E. necatrix* together formed a well-supported monophyletic clade but only the COI analysis was able to resolve monophyly of *E. tenella*. The various phylogenetic hypotheses erected based on nuclear 18S rDNA sequences of *Eimeria* spp. from chickens and other hosts suggest that nuclear 18S rDNA sequences are perhaps only useful in phylogenetic analyses to the genus level. Nuclear 18S rDNA sequences have been unable to resolve the monophyly of *Eimeria* spp. in this and other studies (e.g. Morrison et al., 2004). Whether this reflects a lack of resolving power of this molecular marker or reflects confused taxonomic assignment to a truly paraphyletic genus *Eimeria* is not known, however recent evidence suggests strongly that genus *Eimeria* itself is paraphyletic (e.g. Jirků et al., 2009). Our results support prior assertions (Besansky et al., 2003) that COI could be a widely useful molecular marker with potential for resolving evolutionary relationships among closely related parasites, such as members of the Apicomplexa, that possess a mitochondrion and, in particular, among the morphologically similar coccidia. This marker is likely to provide many species-level synapomorphies that would be highly complementary to analyses based on 18S sequences. Whether obtained for DNA barcoding, molecular identification or evolutionary studies, DNA sequence datasets should be subjected to robust phylogenetic analyses, such as Bayesian and maximum likelihood methods with well-aligned sequences and character-based evolutionary models. Phylogenetically appropriate analyses are particularly important for taxonomic groups in which there is only sparse taxon sampling. Such rigorous analyses will address misgivings expressed by DeSalle et al. (2005) that DNA barcode data had been analysed initially using only distance measures (e.g. Hebert et al., 2004a,b).

In summary, DNA barcoding as practiced using phenetic methods is a useful approach for clustering specimens and looking for patterns of genetic discontinuity suggestive of reproductive isolation (e.g. alpha taxonomy). When coupled with robust phylogenetic methods, and likely with additional molecular and/or morphological characters, barcode data can also contribute significantly toward an understanding of phylogeny. For barcoding, rRNA genes could be used to screen samples of completely unknown taxonomy, after which appropriate COI primers could be selected to obtain species-level identifications (Moszczynska et al., 2009). Ultimately, these analyses can provide diagnostic characters for subsequent identification of unknown specimens, thereby extending the application of taxonomy to epidemiology and diagnostics. Our results clearly suggest that rigorous phylogenetic analyses expand the utility of the COI sequence data beyond just DNA barcoding, at least for the coccidia. Mitochondrial COI sequences have been used successfully to delineate cryptic species of important disease vectors such as *Culicoides* spp. (Cunha et al., 2009; Pagès et al., 2009) and their parasites (e.g. *Haemoproteus* or *Plasmodium* spp.). This use of mitochondrial COI as a marker in the epidemiological study of vector–host interactions as well as our observations suggest a promising future for the mitochondrial COI locus as an integral component of DNA barcoding, species identification/delimitation and phylogenetic investigations in the phylum Apicomplexa.

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