Parasitol Res (2015) 114:1761–1768
DOI 10.1007/s00436-015-4361-y

ORIGINAL PAPER

Simultaneous identification and DNA barcoding of six *Eimeria* species infecting turkeys using PCR primers targeting the mitochondrial cytochrome *c* oxidase subunit I (mtCOI) locus

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Received: 12 September 2014 / Accepted: 30 January 2015 / Published online: 14 February 2015

Abstract Species-specific PCR primers targeting the mitochondrial cytochrome *c* oxidase subunit I (mtCOI) locus were generated that allow for the specific identification of the most common *Eimeria* species infecting turkeys (i.e., *Eimeria adenoeides*, *Eimeria meleagrimitis*, *Eimeria gallopavonis*, *Eimeria meleagridis*, *Eimeria dispersa*, and *Eimeria innocua*). PCR reaction chemistries were optimized with respect to divalent cation ($MgCl_2$) and dNTP concentrations, as well as PCR cycling conditions (particularly anneal temperature for primers). Genomic DNA samples from single oocyst-derived lines of six *Eimeria* species were tested to establish specificity and sensitivity of these newly designed primer pairs. A mixed 60-ng total DNA sample containing 10 ng of each of the six *Eimeria* species was used as DNA template to demonstrate specific amplification of the correct product using each of the species-specific primer pairs. Ten nanograms of each of the five non-target *Eimeria* species was pooled to provide a non-target, control DNA sample suitable to test the specificity of each primer pair. The amplifications of the COI region with species-specific primer pairs from pooled samples yielded products of expected sizes (209 to 1,012 bp) and no amplification of non-target *Eimeria* sp. DNA was detected using the non-target, control DNA samples. These primer pairs specific for *Eimeria* spp. of turkeys did not amplify any of the seven *Eimeria* species infecting chickens. The newly developed PCR primers can be used as a diagnostic tool capable of specifically identifying six turkey *Eimeria* species; additionally, sequencing of the PCR amplification products yields sequence-based genotyping data suitable for identification and molecular phylogenetics.

Keywords Molecular characterization · Turkey coccidia · Diagnostics · Phylogenetic analysis · Species identification

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Introduction

Coccidiosis is a ubiquitous and economically important threat to the poultry industry (Long et al. 1977). Coccidiosis may cost the broiler chicken industry alone over USD$ 3 billion annually with almost 70 % of this estimated cost is due to subclinical coccidiosis (Allen and Fetterer 2002; Long 1973; Dezfoulian et al. 2010). The disease is caused by protozoan parasites belonging to the genus *Eimeria*. In the domestic turkey (*Meleagris gallopavo*), seven species have been described: *Eimeria adenoeides*, *Eimeria meleagrimitis*, *Eimeria gallopavonis*, *Eimeria meleagridis*, *Eimeria dispersa*, *Eimeria innocua*, and *Eimeria subrotunda* (McDougald 2003; Chapman 2008). Four species (*E. adenoeides*, *E. meleagrimitis*, *E. gallopavonis*, and *E. dispersa*) are of primary economic concern due to morbidity and mortality experienced in commercial turkeys associated with these species (Cook et al. 2010). Coccidiosis is a disease complex with

simultaneous infections common and with each *Eimeria* species making separate, distinct contributions to the pathogenicity in the host. Specific diagnosis of the agent(s) responsible for coccidiosis in turkeys can be challenging due to similar oocyst dimensions, nonspecific lesion development, and overlapping biological features among the potential causative *Eimeria* species (Long et al. 1977; Chapman 2008; El-Sherry et al. 2014a, 2015).

Species identification has been based on morphological features of oocysts (shape, size, and refractile granules), biological features (e.g., pre-patent period or sporulation time), or pathological changes induced by the parasites (e.g., intestinal site and shape of lesion) similar to those criteria used to differentiate *Eimeria* species infecting chickens (Joyner and Long 1974). Mixed infections can make precise identification based on lesions in an infected intestinal tract nearly impossible (Jeffers 1975; McDougald and Jeffers 1976). Control of coccidiosis is based on prophylactic use of anticoccidial drugs in the feed but live vaccines are being used increasingly in the field (Chapman et al. 2002; Allen and Fetterer 2002).

Reliable species discrimination is imperative for vaccine development and production as well as epizootiological and population biology studies. In the past, different tests have been used to identify the pathogenic *Eimeria* species infecting turkeys. The earliest methods of species differentiation were based on species-specific immune responses.

Molecular differentiation of species was first attempted using isoenzyme analysis for species identification through molecular polymorphism (Shirley 1975; Johnston and Fernando 1997). Thereafter, PCR assays were described for identification of *Eimeria* species using different regions of the ribosomal cistrons including 5S rDNA, small subunit (18S) rDNA, and the ribosomal internal transcribed spacer regions 1 and 2 (ITS-1 and ITS-2, respectively) (Stucki et al. 1993; Tsuji et al. 1997; Schnitzler et al. 1998; Woods et al. 2000; Gasser et al. 2001; Su et al. 2003; Morrison et al. 2004; Rampin et al. 2006; Cook et al. 2010; Poplstein and Vrba 2011; Ogedengbe et al. 2011; El-Sherry et al. 2013). SCAR-based, species-specific PCR assays were developed for *Eimeria* spp. infecting the domestic fowl (Fernandez et al. 2003a, b). However, only a single PCR-based method for the specific identification of various *Eimeria* species in turkeys has been published (Cook et al. 2010). This assay targeted the ITS-1 region of the ribosomal gene array and could identify only four species specifically. The nuclear rDNA is subject to recombination events and, in at least some coccidia, 18S rDNA (and presumably the adjoining ITS-1 region) has been duplicated within the genome and the paralogs are highly divergent (e.g., Vrba et al. 2011; El-Sherry et al. 2013).

The mitochondrial cytochrome *c* oxidase subunit I (mtCOI) locus is present in many apicomplexan parasites and has been used extensively for sequence-based genotyping and species identification (so-called DNA barcoding) of numerous organisms that exploit oxidative phosphorylation as an energy source (Hebert et al. 2003). The mitochondrial genome has the advantage of replicating mitotically with strict maternal inheritance making recombination events less likely. For these reasons, genes encoded in the mitochondrial genome have also been used extensively in molecular phylogenetics of many organisms, including haemosporinid parasites (Perkins and Schall 2002), but these loci have not been exploited widely for inferring the evolutionary history of coccidian parasites (Traversa et al. 2007). Sequences from mtCOI of a number of *Eimeria* species have been demonstrated to be much more robust than nu 18S rDNA for identification and differentiation of closely related parasites (e.g., Ogedengbe et al. 2011). The mtCOI locus appears to lack the paralog issues demonstrated in the rDNA of these parasites (El-Sherry et al. 2013).

The nature of the mtCOI locus of coccidia has been exploited in this paper to generate a set of species-specific PCR primers based on this locus that permit the specific identification of six common *Eimeria* species infecting the domestic turkey and, by optional direct sequencing of the resulting diagnostic PCR fragments, DNA barcode data that can be used for molecular phylogenetics.

Materials and methods

Parasites

Numerous, single oocyst-derived lines of five *Eimeria* species infecting domestic turkeys were available for this study. Each line with the origin of the original isolates from which each was derived is summarized by El-Sherry et al. (2015). The parasites available for study are as follows: *E. adenoeides* Guelph strain (El-Sherry et al. 2014b, 2015), *E. meleagrimitis* USMN08-01 strain (see Long et al. 1977; El-Sherry et al. 2014a, 2015 for biological features), *E. gallopavonis* Weybridge strain (see Hein, 1969 for biological features), *E. gallopavonis* USKS06-01, *E. meleagridis* USAR97-01 strain (see Matsler and Chapman 2006 for biological features), and *E. dispersa* Briston strain.

All animal experimentation was conducted in coccidia-free birds in the Animal Isolation Unit of the Campus Animal Facility (University of Guelph, Guelph ON, Canada); all experimental procedures were reviewed and approved by the University Animal care Committee and complied with the Canadian Council on Use of Experimental Animals (2nd edition).

DNA extraction from oocysts

Isolation of DNA was performed after purification and surface treatment to reduce fecal contamination. Partially purified oocysts were suspended in saturated salt solution (sat. NaCl,

aqueous) and then floated free of fecal debris by centrifugation at 1,500×*g* (10 min). The supernatant was diluted in 10× vol. of water and then collected by centrifugation (2,500×*g* for 10 min). Oocysts were then suspended in 4× ice-cold household bleach (4.25 % sodium hypochlorite, aqueous) and placed on ice for 10 min. After surface treatment, oocysts were diluted with 10× vol. of double-distilled water and collected by centrifugation (2,500×*g* for 10 min). The pelleted oocysts were washed with double-distilled water at least three more times prior to extraction. DNA extraction using DNAZol® (Life Technologies Inc., Burlington ON, Canada) assisted by vortexing of the sample with 0.5 mm glass beads (Ferro Micro beads: Cataphote Division, Jackson Mississippi, USA) to enhance oocyst breakage was done essentially as described by El-Sherry et al. (2013). Yield and purity were determined spectrophotometrically using NanoDrop 2000 instrument (NanoDrop, Wilmington DE, USA). Bulk cellular parasite DNA from a European isolate of *E. innocua* was kindly provided by Dr. V. Vrba (BIOPHARM, Research Institute of Biopharmacy and Veterinary Drugs, Pohori-Chotoun, Jilove u Prahy 254 49, Czech Republic) who isolated and characterized this parasite recently (see Vrba and Pakandl 2014).

To confirm that the resulting bulk cellular parasite DNA isolated locally or provided was suitable for PCR amplification, primers ITS-1 and ITS-2 of White et al. (1990) targeting the internal transcribed spacer 1 (ITS-1) region of the nuclear ribosomal RNA gene arrays were used to amplify the ITS-1 region of each parasite species (data not shown). Likewise, presence of suitable parasite mtDNA in the DNA isolated from each of the *Eimeria* spp. was confirmed by using each DNA sample as template for PCR amplification with primers Eim_COI_366F-M13F and Eim_COI_879R-M13R as described by El-Sherry et al. (2015); the latter primer pair amplifies a portion of the mtCOI gene from all *Eimeria* species described to date from turkeys or chickens.

PCR primer design

The complete mtCOI CDS from five of the six *Eimeria* species listed above were extracted from complete mt genome sequences obtained for these parasites (Ogedengbe et al. 2014). A partial COI sequence for *E. innocua* was obtained from GenBank (1,257 bp, GenBank HG793049, see Vrba and Pakandl 2014). All available mtCOI sequences were aligned using a translation-based multiple alignment function within the bioinformatics package Geneious (V. 6.0 and later versions). The resulting alignment contained neither indels nor gaps; the complete mtCOI was 1,443 bp for the five *Eimeria* species for which complete CDS was available and translation products from all *Eimeria* species were well conserved. Setting each mtCOI sequence as a reference sequence sequentially permitted the identification of regions of low sequence identity that could be exploited for manual generation of primers.

PCR amplification was accomplished using six newly designed species-specific primer pairs for *Eimeria* spp. infecting turkeys. These primer pairs, recommended PCR annealing temperatures, and expected PCR product sizes are found in Table 1.

PCR reactions were carried out in an MJ Mini® thermal cycler (Bio-Rad Laboratories (Canada) Ltd., Mississauga, ON, Canada) using 50 ng of template DNA, 50 mM $MgCl_2$, 1 mM dNTPs, 1× PCR buffer, and 0.4 U Platinum® Taq polymerase (Life Technologies Inc., Burlington ON, Canada). The PCR thermal profile was as follows: initial heat activation of polymerase at 95 °C for 10 min; 35 cycles of denaturation at 94 °C for 30 s, annealing at 50–62 °C for 45 s (anneal temperatures vary depending on the species-specific primer pair) and extension at 72 °C for 70 s; and a final extension at 72 °C for 10 min. Both negative and positive template control reactions were included with each PCR run. PCR products were electrophoresed on a 1.5 % agarose submarine gel in Tris-acetate-EDTA (TAE) buffer (40 mM Tris, 20 mM acetic acid, and 1 mM EDTA) at ~100 V for ~45 min. The resulting gel was stained with ethidium bromide and the product size estimated by comparison with a 100-bp to 10-kb DNA ladder (Bio Basic Inc., Mississauga ON, Canada) visualized using UV transillumination.

Optimization of PCR for specificity and sensitivity

Various equimolar mixtures of genomic DNA samples obtained from single oocyst-derived lines of six *Eimeria* species infecting domestic turkeys were prepared to permit testing of the specificity and sensitivity of these newly designed primer pairs. A mixed DNA sample containing equivalent amounts of each of the six *Eimeria* species was used as the DNA template to demonstrate that PCR could amplify specifically the correct product using each of the species-specific primer pairs from a mixed *Eimeria* spp. template. In such reactions, 60 ng total DNA (10 ng per *Eimeria* sp.) was used as template for each of the PCR primer pairs individually. To test for the specificity of each of the primer pairs, equivalent amounts of each of the five non-target *Eimeria* spp. were mixed to provide a negative control DNA sample suitable for each primer pair. For example, the mixed species negative template DNA to test the specificity of the *E. adenoeides*-specific primers would contain 10 ng of genomic DNA from each of *E. meleagrimitis*, *E. dispersa*, *E. meleagridis*, *E. gallopavonis*, and *E. innocua*. A total of six "non-target" negative control DNA samples were made. Similarly, a mixture containing equal amounts of genomic DNA from the seven *Eimeria* species infecting the domestic fowl (*Eimeria acervulina*, *Eimeria brunetti*, *Eimeria maxima*, *Eimeria mitis*, *Eimeria necatrix*, *Eimeria praecox*, and *Eimeria tenella*) was generated and also used as DNA template in PCR reactions with each of the turkey *Eimeria* species-specific primer pairs to determine if any of these *Eimeria* spp. of chickens could be amplified by the new primer pairs.

Table 1 Primer pairs, recommended PCR annealing temperatures, and expected PCR product sizes for the specific amplification of mitochondrial cytochrome c oxidase subunit I (COI) from 6 *Eimeria* species that infect turkeys

Primer name	Required anneal temp.	Sequence (5′ to 3′)	Size (bp)
E.ad.CO1_427F	62 °C	5′-CCAACCTCAGTAGATCTAATTGTA-3′	713
E.ad.CO1_1186R		5′-GTGGAAGTGAGCAATGACA-3′	
E.disp.CO1_577F	55 °C	5′-ACAGCTATTATGTTAATTGGT-3′	451
E.disp.CO1_1028R		5′-GCATACCAAGTATCTAATGAA-3′	
E.gal.CO1_292F	62 °C	5′-AGAGTGAATTGTGTATCACTATTAT-3′	861
E.gal.CO1_1153R		5′-GAGATAATACGAAATGGAAGTGG-3′	
E.md.CO1_431F	58 °C	5′-CCTCAGTAGATTTAATTGTC-3′	1,012
E.md.CO1_1443R		5′-TTAGAAGATTAGGGAATATAA-3′	
E.mel.CO1_474F	52 °C	5′-CTCAAGTTTCCTATCCTCAG-3′	554
E.mel.CO1_1028R		5′-GCGTACCAGATATCTAAGGAG-3′	
E.inn.COI.396 F	50 °C	5′-TCCATTAAGTACATCCCTG-3′	209
E.inn.COI.604R		5′-GAAGTGTACCAATTAACATAATG-3′	

Results

DNA samples Isolated DNA from each of the six *Eimeria* spp. infecting turkeys was successfully amplified using primers ITS-1 and ITS-2 of White et al. (1990) targeting the internal transcribed spacer 1 (ITS-1) region of the nuclear ribosomal RNA gene arrays. The amplifications of ITS region with ITS-1 and ITS-2 primer pair yielded a product of approximately 400 bp from each species. Primers Eim_COI_366F-M13F and Eim_COI_879R-M13R of El-Sherry et al. (2015) that target a portion of the mtCOI gene produced a PCR product of ~550 bp from all *Eimeria* species (data not shown).

PCR primer selection Six pairs of PCR primers designed to be species-specific with respect to the five *Eimeria* species of turkeys available for study were generated as follows: (1) *E. adenoeides*-specific primers "E.ad.COI.427 F" (5′ CAAC CTCAGTAGATCTAATTGTA 3′) and "E.ad.COI.1140R" (5′ GTGGAAGTGAGCAATGACA 3′) generate a 713-bp product; (2) *E. dispersa*-specific primers "E.disp.COI.577 F" (5′ A C A G C T A T T A T G T T A A T T G G T 3′) and "E.disp.COI.1028R" (5′ GCATACCAAGTATCTAATGAA 3′) generate a 451-bp product; (3) *E. gallopavonis*-specific primers "E.gal.COI.292 F" (5′ AGAGTGAATTGTGTATCA CTATTAT 3′) and "E.gal.COI.1153R" (5′ GAGATAATAC GAAATGGAAGTGG 3′) generate a 861-bp product; (4) *E. meleagridis*-specific primers "E.md.COI.431 F" (5′ CCTCAGTAGATTTAATTGTC 3′) and "E.md.COI.1443R" (5′ TTAGAAGATTAGGGAATATAA 3′) generate a 1,012-bp product; and (5) *E. meleagrimitis*-specific primers "E.mel.COI.474 F" (5′ CTCAAGTTTCCTATCCTCAG 3′) and "E.mel.COI.1028R" (5′ GCGTACCAGATATCTAAG GAG 3′) generate a 554-bp product; *E. innocua*-specific primers "E.inn.COI.396 F" (5′ TCCATTAAGTACATCC CTG 3′) and "E.inn.COI.604R" (5′ GAAGTGTACCAATT AACATAATG 3′) generate a 209-bp product as summarized in Table 1.

PCR optimizations The mtCOI species-specific primer sets successfully amplified single species template DNA of each of the six species at a wide range of annealing temperatures. The PCR reaction chemistries were optimized by adjusting magnesium chloride ($MgCl_2$) and dNTP concentrations. Extension, annealing temperatures, and cycle parameters were optimized using combinations of mixed DNA samples containing all six *Eimeria* spp. infecting turkeys or only the five non-target *Eimeria* species as PCR templates (data not shown). Ultimately, target-specific PCR reactions were obtained for primer pairs specific for *E. adenoeides*, *E. dispersa*, *E. gallopavonis*, *E. meleagridis*, *E. meleagrimitis*, and *E. innocua* with primer annealing temperatures of 62, 55, 62, 58, 52, and 50 °C, respectively (see Fig. 1 and Table 1). No inter-species cross-reactivity was observed at the optimized anneal temperatures; use of lower anneal temperatures or more permissive PCR reaction chemistries (e.g., higher divalent cation concentrations) may permit slight amplification of one or more non-target species (data not shown).

The amplifications of the COI region with species-specific primer pairs yielded the expected product sizes for *E. adenoeides* (713 bp), *E. meleagrimitis* (554 bp), *E. gallopavonis* (861 bp), *E. meleagridis* (1,012 bp), *E. dispersa* (451 bp), and *E. innocua* (209 bp) (see Table 1). The amplification bands of expected sizes were obtained only using the appropriate species-specific primer pair even when the target DNA (10 ng) was mixed with fivefold of mixed genomic DNA from the five other non-target species. No amplification was produced using any of the five primer pairs when 70 ng of mixed chicken *Eimeria* spp. (10 ng per *Eimeria* species) was used as the template DNA (Fig. 2).

Discussion

Molecular markers from the nuclear genome such as ribosomal ITS-1, ITS-2, and 18S rDNA have been tested previously

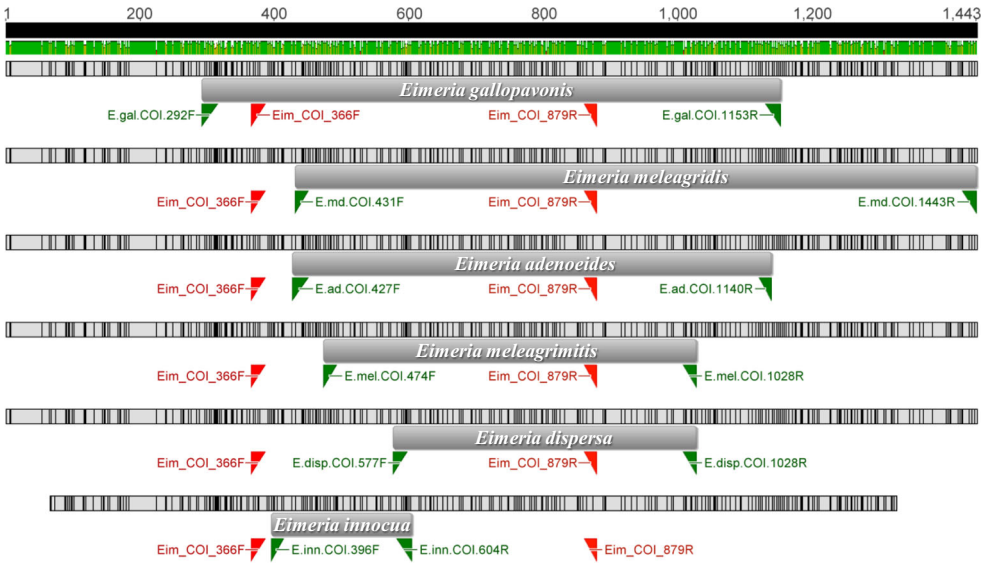

Fig. 1 Aligned complete or near complete mitochondrial COI sequences from six *Eimeria* species infecting turkeys (from Ogedengbe et al. 2014 and Vrba and Pakandl 2014) with the locations of the various species-specific primers and resultant PCR products mapped onto each sequence

for use in identifying *Eimeria* species infecting domestic turkeys and chickens (e.g., Stucki et al. 1993; Tsuji et al. 1997; Schnitzler et al. 1998; Woods et al. 2000; Gasser et al. 2001; Su et al. 2003; Rampin et al. 2006; Cook et al. 2010; Poplstein

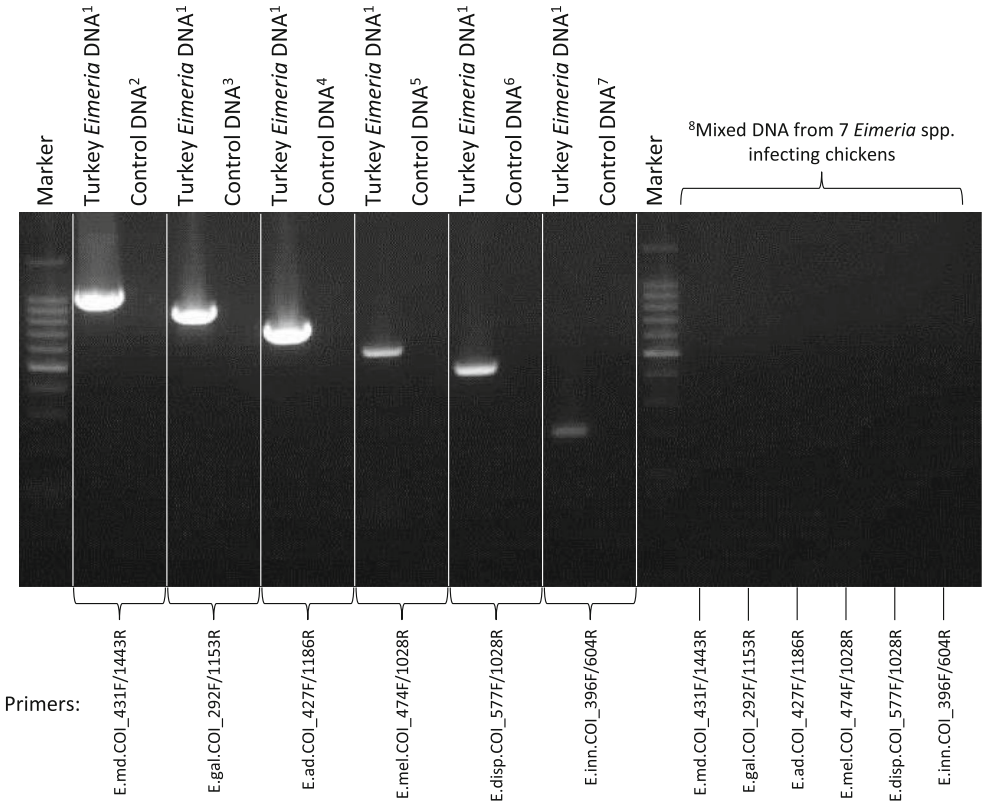

Fig. 2 Specificity of species-specific PCR reactions targeting the mitochondrial cytochrome c oxidase subunit I (COI) loci of six *Eimeria* species that infect turkeys. Each species-specific primer pair was tested (using the anneal temperatures found in Table 1) for its ability to amplify the target *Eimeria* species in a mixed DNA template containing 10 ng total genomic DNA from each of *E. meleagridis*, *E. gallopavonis*, *E. adenoeides*, *E. meleagrimitis*, *E. dispersa*, and *E. innocua* ("Turkey Eimeria DNA"[1]). Absence of amplification of non-target template DNA was tested using a mixture containing 10 ng total genomic DNA from the five *Eimeria* species that infect turkeys not target by the primer pair being tested ("Control DNA"[2–7]). Additionally, each species-specific primer pair was also tested for absence of amplification when a mixture containing 10 ng total genomic DNA from each of seven *Eimeria* species that infect chickens (i.e., *E. acervulina*, *E. brunetti*, *E. maxima*, *E. mitis*, *E. necatrix*, *E. praecox*, and *E. tenella*; "Mixed DNA from seven *Eimeria* spp. from chickens"[8]). Specific amplification of PCR products of the expected amplicon sizes (see Table 1) were only detected in PCR reactions that contained the target DNA

and Vrba 2011; Ogedengbe et al. 2011; El-Sherry et al. 2013); likewise, RAPD/SCAR-based species-specific PCR assays have been described by Fernandez et al. (2003a, b) and Vrba and Pakandl (2014) for chickens and turkey *Eimeria* spp., respectively. The comparatively conserved nature of the nuclear 18 s rDNA locus has permitted evolutionary relationships among many taxonomic groups in the Apicomplexa, including chicken *Eimeria* spp., to be inferred successfully using 18 s rDNA sequences (e.g., Barta et al. 1997 and many others). In contrast, nuclear ribosomal ITS sequences varied distinctly among strains of a single species, as well as within a single parasite, due to polymorphisms among copies within the nuclear ribosomal gene arrays (e.g., Barta et al. 1998; Cook et al. 2010; Vrba and Pakandl 2014). The high rate of sequence divergence at the ITS loci makes the ITS regions suitable for work at the level of strains but this renders these loci as unreliable markers for use at the species level (Cruickshank 2002; Lew et al. 2003). Although nuclear 18S rDNA has clear utility for use at the genus level and higher taxonomic ranks, 18S rDNA is not highly suited for species identification within coccidia (El-Sherry et al. 2013); like the ITS loci, the nuclear 18S rDNA loci can have highly divergent paralogous copies within an individual coccidium that can confuse sequence-based genotyping of these parasites (e.g., Vrba et al. 2011; El-Sherry et al. 2013).

The mtCOI locus, in contrast, has been shown to be more effective as a species-level genetic marker for *Eimeria* spp. infecting turkeys and other galliform birds than nuclear 18S rDNA (Ogedengbe et al. 2011; Vrba et al. 2011; El-Sherry et al. 2013) due to comparatively faster genetic divergence within the former locus and lack of paralogous gene duplication, at least within the eimeriid coccidia as far as is known (Ogedengbe et al. 2014). The current study describes a useful PCR-based diagnostic method for the detection of commonly documented species of *Eimeria* infecting turkeys. The described PCR targets overlapping regions of the mtCOI gene of each species. Despite the comparatively wide range of recommended anneal temperatures used for the primer pairs described in the present paper, it is possible to run PCR reactions using all six primer pairs in a single gradient thermocycler simultaneously.

The specificity of the primers developed in the present work was confirmed using a mixed species DNA template suggesting that these primer sets could be useful for conducting field surveys to describe the range of *Eimeria* species commonly encountered in the commercial poultry industry. Several attempts have been made to use genus-specific nuclear 18S rDNA or mtCOI primers in field samples containing multiple unidentified *Eimeria* species (e.g., Cook et al. 2010; Miska et al. 2010). In these cases, the resulting PCR products were cloned and sequenced as a means of estimating the species that were present in each sample. Unfortunately, a considerable portion of the resulting clones demonstrated chimeric sequences that apparently reflect PCR artefacts (see El-Sherry et al. 2013 for a more detailed explanation). RAPD/SCAR-based PCR assays for six *Eimeria* species infecting turkeys has been described recently (Vrba and Pakandl 2014) that depends on amplification and quantification of single copy, divergent loci within the nuclear genomes of these *Eimeria* species. A potential drawback of any RAPD/SCAR-based species-specific PCR assay is that they may be less sensitive than assays based on nuclear ribosomal DNA loci (e.g., 18S, ITS-1, ITS-2) that have many gene copies (likely more than 100 copies per nuclear genome in *Eimeria* species; see Logan-Klumpler et al. 2012) within the nuclear genome (Vrba et al. 2010) or those assays, including the present work, based on mitochondrial loci. In the case of mitochondrial genes, these single copy genes are found in multiple mt genome copies in each life cycle stage of *Eimeria* species; for example, there are approximately 50-fold mt genome copies of *E. tenella* compared to the nuclear genome (Hikosaka et al. 2011).

In the case of RAPD-SCAR-based multiplex PCR for seven *Eimeria* species infecting chickens (Fernandez et al. 2003a, 2003b), the species-level stability of the sequence target is unknown for RAPD/SCAR-based assays (Vrba et al. 2010). Testing of the assay developed by Fernandez et al. (2003b) demonstrated that genomic DNA from a single species produced a species-specific product from DNA representing one or a few oocysts, but this sensitivity dropped to 20 or more oocysts when applied to field samples (Frölich et al. 2013). Further, Frölich et al. (2013) found that a mixed species template containing 1 % of a contaminating species could not be detected by the assay when run as a multiplexed PCR reaction.

The PCR products generated with the COI species-specific primers described in the present work can be sequenced successfully to give DNA sequence that can be useful in a number of ways. Sequencing of any PCR product generated by this assay can provide data with utility for sequence-based genotyping as well as for phylogenetic analyses. This is not possible with the nu ITS loci because of copy to copy variations in template sequences, including widely divergent product lengths (e.g., Cook et al. 2010). The mtCOI locus is a protein-coding sequence without introns making amplification products of stable lengths that can be readily checked using translation to confirm that the sequenced product was of the correct genetic target.

The amplicons generated by the PCR assay described herein generate a minimum of 166 bp (in the case of the shortest product generated by the *E. innocua*-specific primer pair) found within a region of the mtCOI gene for which considerable comparative sequence information is available (amplified by primers 400 F/1202R, see El-Sherry et al. 2013). Thus, the PCR products can contribute to molecular phylogenetic analyses as well. In contrast, RAPD/SCAR-based assays (e.g., Fernandez et al. 2003a, b for *Eimeria* spp. of chickens or Vrba

and Pakandl 2014 for *Eimeria* spp. of turkeys) do not amplify homologous loci thus sequence data cannot be compared among species. In the case of the present COI-based assay, a PCR failure with the species-specific primers can be backed up with genus-specific mtCOI primers (e.g., primers Eim_COI_366F-M13F and Eim_COI_879R-M13R of El-Sherry et al. 2014b) that amplify successfully a portion of the mtCOI gene from all turkey and chicken *Eimeria* species tested to date (see El-Sherry et al. 2015). Use of such genus-specific primers provides a useful positive control to establish the suitability of DNA templates being used for any of the species-specific PCR reactions and will permit characterization of *Eimeria* species of turkeys (e.g., *E. subrotunda*) and other hosts for which species-specific primers have yet to be developed. As the publically available dataset of partial and complete mtCOI sequences for various *Eimeria* species grows, the value of PCR-based identification, combined with sequence-based genotyping on the species-specific PCR amplicons from these parasites, will be enhanced.

Acknowledgments This work was supported by a Discovery Grant from the Natural Science and Engineering Council of Canada (NSERC) and an Applied Research and Commercialization (ARC) Grant from FedDev (Southern Ontario). In vivo work was supported, in part, by the Ontario Ministry of Food and Rural Affairs (OMAFRA). Special thanks to Dr. V. Vrba (BIOPHARM, Research Institute of Biopharmacy and Veterinary Drugs, Pohori-Chotoun, Jilove u Prahy 254 49, Czech Republic) for providing DNA from a European isolate of *E. innocua*.

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