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Divergent nuclear 18S rDNA paralogs in a turkey coccidium, *Eimeria meleagrimitis*, complicate molecular systematics and identification ☆

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ABSTRACT

Multiple 18S rDNA sequences were obtained from two single-oocyst-derived lines of each of *Eimeria meleagrimitis* and *Eimeria adenoeides*. After analysing the 15 new 18S rDNA sequences from two lines of *E. meleagrimitis* and 17 new sequences from two lines of *E. adenoeides*, there were clear indications that divergent, paralogous 18S rDNA copies existed within the nuclear genome of *E. meleagrimitis*. In contrast, mitochondrial cytochrome *c* oxidase subunit I (COI) partial sequences from all lines of a particular *Eimeria* sp. were identical and, in phylogenetic analyses, COI sequences clustered unambiguously in monophyletic and highly-supported clades specific to individual *Eimeria* sp. Phylogenetic analysis of the new 18S rDNA sequences from *E. meleagrimitis* showed that they formed two distinct clades: Type A with four new sequences; and Type B with nine new sequences; both Types A and B sequences were obtained from each of the single-oocyst-derived lines of *E. meleagrimitis*. Together these rDNA types formed a well-supported *E. meleagrimitis* clade. Types A and B 18S rDNA sequences from *E. meleagrimitis* had a mean sequence identity of only 97.4% whereas mean sequence identity within types was 99.1–99.3%. The observed intraspecific sequence divergence among *E. meleagrimitis* 18S rDNA sequence types was even higher (approximately 2.6%) than the interspecific sequence divergence present between some well-recognized species such as *Eimeria tenella* and *Eimeria necatrix* (1.1%). Our observations suggest that, unlike COI sequences, 18S rDNA sequences are not reliable molecular markers to be used alone for species identification with coccidia, although 18S rDNA sequences have clear utility for phylogenetic reconstruction of apicomplexan parasites at the genus and higher taxonomic ranks.

1. Introduction

Coccidiosis is worldwide disease affecting turkeys and other galliform birds. Seven *Eimeria* spp. that infect turkeys have been described: *Eimeria adenoeides*; *Eimeria dispersa*; *Eimeria gallopavonis*; *Eimeria innocua*; *Eimeria meleagridis*; *Eimeria meleagrimitis;* and, *Eimeria subrotunda* (see Chapman, 2008). Description of these species was based primarily on morphometric and biological characteristics. However, these parameters are insufficiently precise to reliably differentiate between species because morphometric and biological features frequently overlap and variations in these features were reported among strains and isolates in a single species (Long et al., 1977). Molecular data have become invaluable for the differentiation and classification of *Eimeria* spp.

The ssrRNA (18S rRNA) gene has been used extensively for classification of apicomplexan parasites (Morrison et al., 2004). Nuclear rDNA sequences have been used successfully in defining many taxonomic groups and proved to be good targets for species differentiation in the case of chicken *Eimeria* spp. (see Barta et al., 1997). Recently, however, two distinct types of 18S rDNA were reported for the chicken parasite, *Eimeria mitis*, in which sequence divergence between the two types was 1.3–1.7% whereas the sequence divergence within types (0.3–0.6%) was much more limited (Vrba et al., 2011). To put these levels of sequence divergence between the two rDNA types of *E. mitis* in perspective, the sequence variability between the rDNA sequences of *Eimeria tenella* and *Eimeria necatrix* is approximately 1.1%, less than the variability between rDNA types within a single *Eimeria* sp. Finding multiple, distinct rDNA sequences was previously reported for *Plasmodium* spp. (McCutchan et al., 1988; Nishimoto et al., 2008) in which up to three paralogous rDNA types may occur. However, Vrba et al. (2011) were the first to describe such divergent 18S rDNA loci within single oocyst lines of *Eimeria*.

The gene encoding mitochondrial cytochrome *c* oxidase subunit I (COI) has been widely used for phylogenetic analysis of many

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organisms due to its near universal presence in organisms that use oxidative phosphorylation as an energy source (Hebert et al., 2003, 2004). Partial COI sequences have proven to be effective species-specific markers for *Eimeria* spp. and related coccidia (Ogedengbe et al., 2011).

In the present work, multiple single-oocyst-derived lines of the turkey parasites, *E. adenoeides* and *E. meleagrimitis*, were generated and then both mitochondrial COI and nuclear 18S DNA genes were sequenced from each of the generated lines of parasites. Surprisingly high sequence diversity was found among nuclear 18S rDNA copies, suggesting that this genetic target may be unreliable as a species-specific marker; these observations are contrasted with the utility of the mitochondrial COI for the same purpose.

2. Materials and methods

2.1. Parasites – strains and generation of single-oocyst-derived lines

Eimeria meleagrimitis USMN08-01 was isolated by Dr. H.D. Chapman (University of Arkansas, Fayetteville AR, USA) from a litter sample originating on a turkey farm in Minnesota, USA in 2008. Specific identity was established by demonstrating the presence of macrogamonts in histological sections of the jejunum and by the size of the oocysts (~20 by 17 μm) (H.D. Chapman, personal observation).

An isolate of *E. adenoeides* was originally obtained from a commercial turkey flock in Ontario, Canada, in approximately 1985. This isolate has been propagated periodically in specific parasite-free poults in the Campus Animal Facility, University of Guelph, Guelph, Ontario, Canada (CAF), since that time. Oocyst dimensions (~18.7 by 14.2 μm) and sequence data (18S rDNA and mitochondrial COI partial sequences – data not shown) agree with the description of the KCH strain of *E. adenoeides* as defined by Poplstein and Vrba (2011).

Freshly passaged *E. adenoeides* KCH and *E. meleagrimitis* USMN08-01 were prepared using single-oocyst isolations in vivo (four mono-specific lines for each species) and propagated in specific parasite-free poults in CAF. Turkey poults were provided feed and water ad libitum; all experimental manipulations were reviewed and approved by the University of Guelph's Animal Care Committee and complied with the Canadian Council on Animal Care's 'Guide to the Care and Use of Experimental Animals' (2nd edition). Single-oocyst derived lines of these coccidia were obtained essentially as described by Remmler and McGregor (1964) with the modification that agar plugs carrying a single oocyst were delivered individually to the crop of birds within gelatin capsules. Oocysts were partially purified from collected feces by salt flotation (Reid and Long, 1979). The oocysts were suspended in 2.5% potassium dichromate and incubated at 26 °C on a rotary shaker to sporulate. Hereafter, the progeny of these single-oocyst-derived lines will be referred to as: *E. adenoeides* KCH-Line 3, KCH-Line 4, KCH-Line 5 and KCH-Line 7; and *E. meleagrimitis* USMN-08-01-Line 1, USMN-08-01-Line 4, USMN-08-01-Line 5 and USMN-08-01-Line 8. Oocysts were pelleted by centrifugation in 15 ml centrifuge tubes for 10 min at 2500*g*, and then suspended in household bleach (4.25% sodium hypochlorite, w/v aqueous) on ice for approximately 10 min. Oocysts were diluted with 10× volumes of distilled water and then washed three times with distilled water by centrifugation for 10 min at 1500*g*. After decanting the final water wash, the pelleted oocysts were resuspended in 200 μl of DNAzol (Invitrogen, Burlington, ON, Canada) according to the manufacturer's protocol; disruption of the oocysts was assisted by adding approximately 1× volume of 0.5 mm sterile glass beads (Ferro Micro beads; Cataphote Division, Jackson Mississippi, USA). An additional 800 μl of DNAzol were added and the microfuge tube was incubated at room temperature for 30 min with gentle manual agitation every 2 min. The microfuge tube was then centrifuged for 15 min at 13,000*g* and the supernatant transferred to a clean tube. The resulting DNA was precipitated using 100% ethanol and then washed with 70% cold ethanol before the precipitated DNA was pelleted by centrifugation for 5 min at 13,000*g*. The resulting pellet was air-dried briefly before being resuspended in nuclease-free water; the tubes containing water and pelleted DNA were incubated in a water bath for 5–10 min at 50 °C to assist solubilisation. Yield and purity were determined spectrophotometrically using a NanoDrop 2000 instrument (NanoDrop, Wilmington DE, USA); DNA was stored at −20 °C.

2.2. PCR

Nuclear ssrDNA was amplified using universal eukaryote-specific primers (Medlin et al., 1988): Medlin A (5′-AACCTGGTTGATCC TGCCAGT-3′) and Medlin B (5′-GATCCTTCTGCAGGTTCACCTAC-3′). The mitochondrial COI gene of *E. adenoeides* was amplified using primers 400F (5′-GGDTCAGGTRTTGGTTGGAC-3′) and 1202R (5′-C CAAKRAYHGCACCAAGAGATA-3′). More degenerate primers 10F (5′-GGWDSWGGWRYWGGWTGGAC-3′) and 500R (5′-CAT-RTGRTGDGCCCAWAC-3′) were used to amplify a portion of the COI gene for *E. meleagrimitis*. PCRs were carried out in an MJ Mini thermal cycler (Bio Rad, CA, USA). PCRs contained ~100 ng of genomic DNA from each *Eimeria* sp., 50 mM $MgCl_2$, 1 mM dNTPs, 10 × PCR buffer and 0.4 U Platinum Taq (Invitrogen). The PCR thermal profile was as follows: initial heat activation of polymerase at 96 °C for 10 min; 35 cycles of denaturation at 94 °C for 30 s, annealing at 48 °C for 30 s (for both 18S rDNA and COI reactions), extension at 72 °C for 1 min; 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 1× Tris–Acetate–EDTA (TAE) buffer at 109 V for 45 min. The resulting gel was stained with ethidium bromide and the size of products estimated by comparison with a 100 bp to 10 kb DNA ladder (Bio Basic. Inc., Mississauga ON, Canada).

A QIA quick gel extraction kit (Qiagen, Toronto ON, Canada) was used to purify excised bands. The resulting DNA was then 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) available in the Molecular Biology Unit of the Laboratory Services Division, University of Guelph (Guelph ON, Canada). For the COI products, DNA sequencing was accomplished exclusively using PCR product templates. PCR-generated DNA template resulting from the Medlin A/B primer pair could not be successfully sequenced directly for any of the four *E. adenoeides* or *E. meleagrimitis* lines. Therefore, the 18S rDNA products from *E. adenoeides* KCH-Line 4 and KCH-Line-5 as well as *E. meleagrimitis* USMN-08-01-Line 4 and USMN-08-01-Line 5 were cloned individually using the TOPO TA cloning kit (Invitrogen). Recombinant clones from each of these four parasite lines were selected and the size of each cloned insert was confirmed using M13 Forward/M13 Reverse PCR amplification from putative positive colonies (data not shown). Clones containing inserts of approximately 1800 bp were cultured overnight in Luria broth (LB) and plasmid DNA isolated using Quick plasmid miniprep Kit (Invitrogen). The inserts in the resulting plasmids were sequenced completely in both directions using M13 Forward and M13 Reverse primers as well as four internal sequencing primers: 676F (5′-GTTG CAGTTARAARGCTCGTA-3′); 1139R (5′-CAATTCCTTTAAGTTTC-3′); 1125F (5′-GAAACTTAAAGGAATTG-3′); and 696R (5′-TACGAGCYTT YTAACTGCAAC-3′).