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A NEW SPECIES OF *CRYPTOSPORIDIUM* (APICOMPLEXA: CRYPTOSPORIDIIDAE) FROM EASTERN GREY KANGAROOS (*MACROPUS GIGANTEUS*)

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ABSTRACT: *Cryptosporidium macropodum* n. sp is described. Oocysts of *C. macropodum* from the feces of kangaroos (*Macropus* spp.) are morphologically indistinguishable from other mammalian *Cryptosporidium* species, including *C. parvum*, *C. hominis*, *C. suis*, and *C. canis*. The oocysts are fully sporulated on excretion, lack sporocysts, and have an average width of 4.9 μm (4.5–6.0), a length of 5.4 μm (5.0–6.0), and a length:width ratio of 1.1. Phylogenetic analyses of the 18S ribosomal RNA, actin, and heat shock protein 70 (HSP70) loci demonstrate that *C. macropodum* is genetically distinct from all described *Cryptosporidium* species, including others found in marsupials. The parasite seems to be highly host-specific, because it has been found only in marsupials to date. Therefore, based on biological and molecular data, we consider *C. macropodum* a new species.

Species of *Cryptosporidium* have been identified in >150 vertebrate hosts (Fayer et al., 2000). There are currently 16 species of *Cryptosporidium* considered as valid; *C. serpentis* and *C. varanii* (syn. *C. saurophilum*) in reptiles; *C. molnari* and *C. scophthalmi* in fish; *C. baileyi* and *C. galli* in birds; and *C. meleagridis* in birds and humans; *C. suis* in pigs; *C. muris* and *C. wrairi* in rodents; *C. bovis* and *C. andersoni* in cattle; *C. felis* in cats; *C. canis* in dogs; *C. hominis* in humans; and *C. parvum* in multiple mammalian species, including humans (Fayer et al., 2000, 2001; Alvarez-Pellitero and Sitja-Bobadilla, 2002; Morgan-Ryan et al., 2002; Ryan et al., 2003, 2004; Alvarez-Pellitero et al., 2004).

Taxonomy of apicomplexan parasites is commonly based on morphological characters of the exogenous life cycle stage, the oocyst. Characters used for species delineation include oocyst size and shape, sporocyst number, sporozoite number, and sporulation times. However, for *Cryptosporidium* spp., morphological characteristics are unsuitable taxonomic measures, because *Cryptosporidium* spp. oocysts are fully sporulated when excreted, they contain naked sporozoites, and their exogenous stages are small in comparison with many other apicomplexans (Fayer et al., 2000). Additionally, the size of the oocysts is highly similar among many of the recognized *Cryptosporidium* species (Ryan et al., 2004). To overcome these limitations, *Cryptosporidium* taxonomy uses a combination of morphometrics, host specificity, and genetic characterization (Xiao et al., 2004).

Genetic studies of *Cryptosporidium* spp. of Australian marsupials have identified 2 host-adapted genotypes (Morgan et al., 1997; Power et al., 2004). The marsupial genotype I (*C. fayeri* n. sp., [Ryan et al., 2008]) was described after oocysts from a koala (*Phascolarctos cincereus*) were found to be genetically distinct from other described *Cryptosporidium* species. *Cryptosporidium fayeri* has since been characterized at 6 loci and described in a red kangaroo (*M. rufous*), eastern grey kangaroos (*M. giganteus*), a western barred bandicoot (*Perameles bougainville*), and a yellow footed rock wallaby (*Petrogale xanthopus*) (Morgan, Deplazes et al., 1999; Morgan, Monis et al., 1999; Xiao et al., 1999; Sulaiman et al., 2000; Power et al., 2003, 2004). More recently, the *Cryptosporidium* marsupial genotype II was described from eastern grey kangaroos (Power et al., 2004). Marsupial genotype II has since been identified in a swamp wallaby (*Wallabia bicolor*), red kangaroos, and western grey kangaroos (U. Ryan, unpubl. obs.). Marsupial genotype II has been characterized at 4 loci, the 18S rRNA, internal transcribed spacer 1 (ITS1), *Cryptosporidium* oocyst wall protein (COWP), and HSP70 loci, and each has confirmed the genetic distinctness of marsupial genotype II from described *Cryptosporidium* species and genotypes (Power et al., 2004).

In this study, we provide additional data to support the species status of marsupial genotype II and describe a new species of *Cryptosporidium*.

MATERIALS AND METHODS

Sources of oocysts

The oocysts of the new species used for morphometrics and genetic studies were obtained from a population of wild eastern grey kangaroos inhabiting the watershed of Lake Burragorang, Sydney, Australia.

Oocyst measurements and imaging

Oocysts were purified from eastern grey kangaroo feces by using immunomagnetic separation as described previously (Power et al., 2005). Measurements of length and width were obtained from oocysts (n = 50) purified from an individual sample that represents the type specimen. For imaging, oocysts were prepared as a wet mount and examined under oil immersion using ×100 Plan Achromat objective on an Olympus BH2 microscope with differential interference microscopy (Olympus, Hamburg, Germany). Images were captured using a Nikon DXM digital camera (Coherent Scientific, Hilton, South Australia, Australia).

DNA extraction

Oocysts of the new species were concentrated from eastern grey kangaroo feces using immunomagnetic separation that facilitated fluorescence-activated cell sorting of 10,000 oocysts onto polycarbonate membranes (Power et al., 2003). DNA was extracted from oocysts using prepGEM™ (Zygem Corporation Ltd., Hamilton, New Zealand) (Ferrari et al., 2007). The membrane containing oocysts was placed into prepGEM buffer 3 (98 μl) and the sample frozen at −80 C for 15 min. The oocysts were thawed, vortexed, and then 1 μl of lysozyme (5 mg/ml) (Sigma, Sydney, Australia) and 1 unit of prepGEM was added. Samples were then incubated at 37 C for 15 min, 75 C for 15 min, and 95 C for 15 min. After incubation, the oocysts were vortexed and centrifuged (6,500 *g*; 5 min), and the supernatant was transferred to a new tube containing 10 μl of EDTA (0.05 M). DNA was stored at −20 C until use.

Actin amplification and sequencing

Amplification and sequencing of an ~1,066-bp fragment of the actin gene was performed using a nested polymerase chain reaction (PCR)

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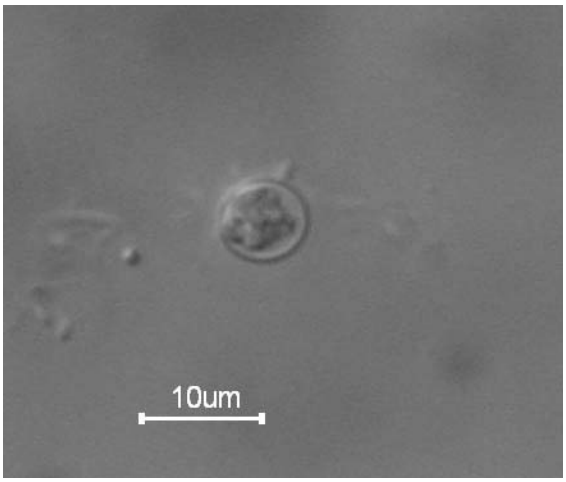

FIGURE 1. Photomicrograph of oocysts of *Cryptosporidium macropodum* from eastern grey kangaroo feces.

protocol as described previously (Sulaiman et al., 2002). PCR products were purified using the spin column PCR purification kit (QIAGEN GmbH, Hilden, Germany). Sequencing was performed using a capillary electrophoresis DNA Sequencer (Applied Biosystems, Foster City, California) using the BigDye™ terminator kit (Applied Biosystems). Sequence accuracy was determined by using products from 2 different animals, a minimum of 2 PCR reactions and by 2-directional sequencing.

Phylogenetic analysis

Nucleotide sequences for the 18S rRNA, HSP70, COWP, and ITS1 of the new species were retrieved from GenBank, accession numbers AF513227 and AY237630–AY237636. The nucleotide sequence for actin was generated for this study. Sequences were concatenated in the order of the 18s rRNA, actin, and HSP70 and aligned using ClustalW (Thompson et al., 1994) with manual adjustments where required. Neighbor-joining distance based analyses and parsimony analyses were conducted using MEGA 4 (Molecular Evolutionary Genetics Analysis software, Arizona State University, Tempe, Arizona) with *Plasmodium falciparum* as the outgroup. Tree reliability was determined using bootstrap analyses of 1,000 replicates. The actin nucleotide sequence generated in this study for the new species has been submitted to GenBank under the accession number EU124664.

DESCRIPTION

Cryptosporidium macropodum n. sp
(Fig. 1)

Diagnosis: Oocysts elliptical to slightly ovoid with an average width of 4.9 μm (4.5–6.0) and an average length of 5.4 μm (5.0–6.0). Length-to-width ratio 1.1. Oocysts sporulated when excreted with 4 sporozoites. Sporocyst is absent; oocyst residuum present.

Taxonomic summary

Type host: Macropus giganteus (eastern grey kangaroo).

Other hosts: Wallabia bicolor (swamp wallaby), *Macropus fuliginosis* (western grey kangaroo), and *Macropus rufus* (red kangaroo).

Type location: Sydney, Australia.

Additional locations: Throughout Australia.

Site of infection: Intestine.

Prepatent and patent periods: Unknown.

Specimen deposited: A photomicrograph of sporulated oocysts has been deposited at the Australian Registry of Wildlife Health, Taronga Zoo, Mosman, New South Wales, Australia, as ARWH reference 5966.1.

Etymology: C. macropodum is named after the Macropodidae, the family in which *C. macropodum* has been found.

TABLE I. Percentage of nucleotide similarities between *C. macropodum* and described *Cryptosporidium* species, and marsupial derived genotypes.

Species	18S rRNA	Actin	HSP70	COWP	ITS1
C. fayeri	96.17	87.34	91.42	86.3	64.2
Opossum I	95.77	87.47	91.76	NA	
Opossum II	96.37	79.98	83.19	NA	
C. suis	97.73	86.46	91.13	86.3	69.4
C. wrairi	96.95	86.98	91.44	87.4	
C. parvum	96.36	86.58	90.05	86.3	
Mouse genotype	96.56	86.33	90.74	85.3	
C. hominis	96.54	86.33	90.72	87.1	
C. meleagridis	96.35	87.22	89.4	86.6	
C. felis	95.36	76.06	82.4	83	
C. canis	96.35	79.82	78.09	84	
C. baileyi	94.77	85.12	82.17	82.9	
C. andersoni	89.65	81.5	75.13	73.5	
C. muris	90.31	83.18	73.67	74	
C. serpentis	91.8	82.45	75.13	73.8	
C. saurophilum	97.05	86.71	88.63	NA	
C. galli	89	83.18	76.47	NA	

Remarks

Epidemiology studies on marsupials have indicated that *C. macropodum* is host adapted to marsupial species as demonstrated by absence of clinical signs in marsupial hosts despite excretion of high oocyst numbers (Power et al., 2005; Thompson, 2007). There are no reports of experimental infections in any host species for *C. macropodum*.

Oocysts of *C. macropodum* are morphologically indistinguishable from *C. fayeri*, which is also specific to marsupials (Ryan et al., 2008), and from several described *Cryptosporidium* species, including *C. hominis*, *C. parvum*, *C. canis*, and *C. suis*, which are commonly found in habitats inhabited by marsupials.

The percentages of similarities at the 18S rRNA locus between *C. macropodum* and the marsupial-derived *C. fayeri*, opossum genotype I, and opossum genotype II were 96.2, 95.8, and 96.4, respectively. At the actin locus, *C. macropodum* was most similar to *C. fayeri* and the opossum genotype I, with similarities of 87.4 and 87.3%, respectively (Table I). At the HSP70 locus, *C. macropodum* was most similar to *C. fayeri* and the opossum genotype I, with similarities of 91.7 and 91.4%, respectively. The percentage of similarities between *C. macropodum* and opossum genotype II at the actin and HSP70 loci were 80.0 and 83.1%, respectively.

Cryptosporidium macropodum was most similar to *C. suis*, with a percentage of similarity of 97.7 at the 18S rRNA locus. These similarities are below those between *C. parvum* and *C. hominis* and *C. parvum* and *C. suis*.

The phylogenetic analyses supported the validity of *C. macropodum* (Fig. 2). The inferred phylogeny placed *C. macropodum* in a clade separate to the group making up the intestinal *Cryptosporidium* species that includes *C. fayeri* and opossum genotypes I and II, species and genotypes that are also found in marsupials. The topology for parsimony and neighbor joining had strong bootstrap support at all nodes. The only exception to this was the placement of *C. suis* within the intestinal clade.

DISCUSSION

The limitations associated with using morphological characteristics for classification of *Cryptosporidium* species have resulted in the establishment of guidelines to assist with defining new *Cryptosporidium* species (Xiao et al., 2004). These guidelines indicate a requirement for morphometric analyses, genetic characterization at several loci that includes 18S rRNA and functional loci, information on natural host specificity, and ex-

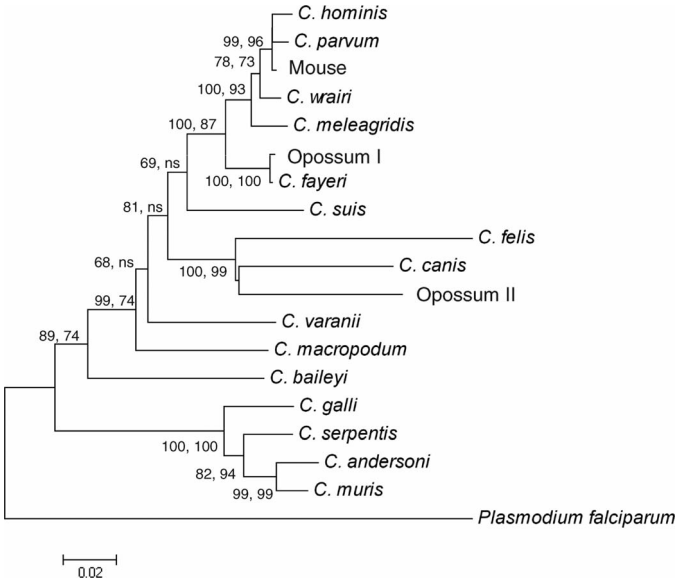

FIGURE 2. Phylogenetic relationships between *Cryptosporidium macropodum* and valid *Cryptosporidium* spp. inferred by neighbor joining. The tree was constructed by concatenating sequences of 18S rRNA, actin, and HSP70 loci. *Plasmodium falciparum* was used as the outgroup for each locus. Bootstrap values (>60%) for 1,000 replicates for neighbor joining (first value) and parsimony (second value) are indicated.

perimental host specificity where possible. Studies must concur with rules of the International Code of Zoological Nomenclature, which includes depositing a specimen or photograph and description in a culture collection cited by name and location [Art. 16.4].

Cryptosporodium macropodum oocysts measure 4.8 × 5.6 μm and have a shape index of 1.1. The oocyst measurements of *C. macropodum* overlap with those of *C. hominis* (Morgan-Ryan et al., 2002), *C. parvum* (O'Donoghue, 1995), *C. canis* (Fayer et al., 2001), and *C. suis* (Ryan et al., 2004). However, despite these morphological similarities, *C. macropodum* differs significantly from valid *Cryptosporidium* species at 5 loci. At the 18S rRNA locus, *C. macropodum* was most similar to *C. suis* (97.7% similarity) and *C. parvum* (96.3% similarity). This genetic variation is greater than that observed between recognized species, i.e., *C. parvum* and *C. suis* (98.3%), and *C. hominis* and *C. suis* (98.5%). Analyses of the actin and HSP70 loci indicate that *C. macropodum* was most similar to *C. fayeri* and the *Cryptosporidium* opossum genotype I, with nucleotide similarities of 87.3% (actin) and 91.4% (HSP70). At the COWP and ITS1 loci, *C. macropodum* and *C. fayeri* shared a nucleotide similarity of 86.3 and 64.2%, respectively. The genetic differences between the 2 *Cryptosporidium* species commonly found in marsupials suggest that although they are closely related and share the same host species, their genetic variation is greater than the genetic variation seen in valid species of *Cryptosporidium*.

There are no experimental data on host specificity of *C. macropodum*; however, this species has only been found in Australian wildlife to date, and it has never been reported in humans. Limited data are available for the prevalence of *C. macropodum* in marsupials, but a study of eastern grey kangaroos inhabiting an Australian watershed detected oocysts in 239 of 3,557 (6.7%) fecal samples screened and demonstrated that *C. macropodum* was present in 39% of the samples that were genotyped, with *C. fayeri* being present in the remaining 61% of samples (Power et al., 2005). In another recent study, *C. macropodum* was identified in 6 of 10 fecal samples from western grey kangaroos and 3 of 30 samples from red kangaroos (Thompson, 2007). The eastern grey kangaroo study demonstrated cyclic shedding patterns, with *C. macropodum* dominating during the cooler months and *C. fayeri* dominating during periods when the population contained high numbers of juveniles (Power et al., 2005). In contrast, *C. macropodum* was identified in captive western grey and red kangaroos during summer (Thompson, 2007). The pathogenicity of *C. macropodum* to marsupials is relatively unknown, but data indicate that this species is adapted to marsupials as oocyst shedding in eastern grey kangaroos ranged from 10 to 2.0×10^6 oocysts per gram and that samples with high oocyst loads were not associated with diarrhea (Power et al., 2005).

The genetic, morphologic, and host–parasite data presented here demonstrate that the differences between *C. macropodum* are comparable with those between *Cryptosporidium* species currently considered valid. Although *C. macropodum* and *C. fayeri* are found in the same host species, the data presented here confirm that they are 2 distinct species.

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