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PHYLOGENETIC RELATIONSHIPS AMONG ISOLATES OF *CRYPTOSPORIDIUM*: EVIDENCE FOR SEVERAL NEW SPECIES

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ABSTRACT: Isolates of *Cryptosporidium* were characterized using nucleotide sequence analysis of the 18S rRNA and dihydrofolate reductase genes and also random-amplified polymorphic DNA analysis. Phylogenetic analysis confirmed the validity of the species of *Cryptosporidium* examined in this study such as *Cryptospordium muris* and *Cryptosporidium baileyi,* and also reinforced evidence from numerous researchers worldwide suggesting that *Cryptosporidium parvum* is not a single uniform species. The data obtained provided strong support for the validity of *Cryptosporidium felis.* Evidence suggests that the newly identified marsupial and pig genotypes may also be distinct and valid species, but biological studies are required for confirmation.

Cryptosporidium spp. are important enteric parasites that infect humans, domestic animals, wildlife, birds, reptiles, amphibians, and fish (Fayer et al., 1997). Although *Cryptosporidium* was first identified in 1907 by Tyzzer, inability to amplify the parasite in vitro (Flanigan et al., 1991) has limited our ability to identify species clearly because morphologic differentiation is insufficient. With the advent of the polymerase chain reaction (PCR), it became possible to characterize isolates of *Cryptosporidium* genetically, and in the last decade significant advances have been made in our knowledge of the genetics of this genus (cf. Fayer et al., 1997). Although a total of 22 different species of *Cryptosporidium* have been named on the basis of host occurrence, several species have been invalidated because oocysts originally described from rattlesnakes, foxes, king snakes, and lizards are clearly sporocysts of *Sarcocystis* spp. (Levine et al., 1980, 1984, 1986). At present, up to 8 species are regarded as valid on the basis of differences in oocyst morphology, site of infection, and vertebrate class specificity: *Cryptosporidium muris* that infects rodents and cattle; *Cryptosporidium parvum* that infects humans and other mammals; *Cryptosporidium meleagridis* and *Cryptosporidium baileyi* in birds; *Cryptosporidium serpentis* in reptiles; *Cryptosporidium nasorum* in fish; and *Cryptosporidium wrairi* from guinea pigs (Fayer et al., 1997). The species *Cryptopspridium felis* has been proposed for *Cryptosporidium* isolated from cats as it appears to be host specific (Iseki, 1979; Fayer et. al., 1997).

Cryptosporidium parvum appears to be the most frequently reported species and is the major cause of cryptosporidiosis in humans and livestock. However, recent genetic evidence indicates that *C. parvum* is not a genetically uniform species and encompasses a number of distinct genotypes, e.g., a human genotype, found only in humans; a cattle genotype found in domestic livestock and in some humans, indicating zoonotic transmission, and a number of additional distinct genotypes that appear to be host adapted, such as the pig, marsupial, cat, and mouse genotypes (Awad-El-Kariem et al., 1995; Bonnin et al., 1996; Carraway et al., 1997; Morgan et al., 1995, 1997; Morgan, Forbes, and Thompson, 1998; Morgan, Pallant et al., 1998; Morgan, Sargent, Deplazes et al., 1998; Morgan, Sargent, Elliot, and Thompson, 1998; Morgan, Buddle et al., 1999; Morgan, Sargent et al., 1999; Morgan, Sturdee et al., 1999; Peng et al., 1997; Sargent et al., 1998; Spano et al., 1997; Spano, Putignani, Crisati et al., 1998; Spano, Putignani, Guida, and Crisanti, 1998; Sulaiman et al., 1998, 1999; Vasquez et al., 1996; Widmer et al., 1998; Xiao et al., 1998). Because *C. parvum* is not a genetically uniform species and may in fact include several distinct species, a taxonomic re-examination is urgently required. The aim of the present study was to conduct a detailed phylogenetic analysis of the human, cattle pig, marsupial, cat, and mouse genotypes using nucleotide (nt) sequences from the genes encoding 18S rRNA and dihydrofolate reductase to determine their relationships to other *Cryptosporidium* species.

MATERIALS AND METHODS

Sources of parasite isolates, oocyst and DNA purification, and primer design

Sources of parasite isolates are listed in Table I. Isolates were derived directly from their host of origin without passaging in other animals. Oocysts were purified using Ficoll gradients as previously described (Morgan et al., 1995), incubated in 10% sodium hypochlorite on ice for 10 min, and washed 3 times in phosphate-buffered saline. DNA was purified as previously described (Morgan et al., 1997). Primers were designed using Amplify 2.1 (William Engels, University of Wisconsin), and oligonucleotides were synthesized by Gibco BRL (Gaithersburg, Maryland).

PCR amplification, cloning, and sequencing of the 18S rDNA gene

The complete 18S rRNA for each isolate was PCR amplified using the forward primer 5′AACCTGGTTGATCCTGCCAGTAGTC3′ and the reverse primer 5′GAATGATCCTTCTGCAGGTTCACCTAC3′. Reactions were performed on a Perkin-Elmer GeneAmp PCR System 2400 (Perkin-Elmer, Foster City, California). Samples were heated to 96 C for 2 min, followed by 50 cycles of 94 C for 30 sec, 58 C for 30 sec, and 72 C for 80 sec, and 1 cycle of 72 C for 7 min. TAQ Extender™ (Stratagene, La Jolla, California) was included in all reactions to minimize PCR error. PCR products were purified using Qiagen spin columns (Qiagen, Hilden, Germany), cloned into the PCR 2000™ T-vector (Invitrogen, Carlsbad, California), and transformants screened by PCR. Recombinant plasmids were sequenced using an ABI Prism™ Dye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, California) according to the manufacturer's instructions except that the annealing temperature was raised to 60 C. At least 3 clones of each PCR product were sequenced in both directions. Sequences were analyzed using SeqEd, version 1.0.3. (Applied Biosystems). Additional *Cryptosporidium* 18S rDNA sequences were obtained from GenBank: 2 bovine *C. parvum* isolates, AUCP-1 (L16996) and KSU-1 (AF040725), a hu-

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TABLE I. Isolates of *Cryptosporidium* used in study.

Code	Host	Genotype	Geographic origin	Source*
H1	Human	Human†‡	Perth, Western Australia	PMH
H7	Human	Human†‡	Perth, Western Australia	PMH
P18	Human	Human§	Central England	IDR
C1	Calf	Calf†‡	Millicent, South Australia	CVL
C13	Calf	Calf‖	U.S.A.	UND
sC33	Calf	Calf‖	Switzerland	IP
M11	Mouse	Mouse‖	Victoria, Australia	CSIRO
M24	Mouse	Mouse‖	Victoria, Australia	CSIRO
M26	Mouse	Mouse‖	Victoria, Australia	CSIRO
K1	Koala	Marsupial‖	South Australia	CVL
K2	Kangaroo	Marsupial#	Western Australia	MU
Pig 1	Pig	Pig‖	Switzerland	IP
Ct 1	Cat	*C. felis*#¶	Perth, WA	MU

* CSIRO = Commonwealth Scientific and Industrial Research Organisation, Victoria; CVL = Central Veterinary Laboratories, Adelaide; IDR = Institute of Parasitology, Rome, Italy; IP = Institute of Parasitology, Zurich, Switzerland; MU = Murdoch University, Western Australia; PMH = Princess Margaret Hospital, Perth; UND = University of North Dakota School of Medicine, Grand Forks, North Dakota.
† Morgan et al., 1995.
‡ Morgan et al., 1997.
§ Spano et al., 1997.
‖ Morgan, Sargent, Deplazes et al. 1998.
Morgan, Sargent et al., 1999.
¶ Sargent Putignani, Crisanti et al., 1998. ** 18S sequence analysis, unpublished.

man *C. parvum* isolate (L16997), as well as *C. parvum* (cattle genotype) (L25642), *C. wrairi* (U11440), *C. baileyi* (L19068), and *C. muris* (L19069). Sequences for *Cyclospora, Eimeria, Toxoplasma,* and *Neospora* species were also obtained from Genbank; *Cyclospora* sp. strain Gombe 22 (AF061566), *Cyclospora* sp. strain Gombe 34 (AF061567), *Cyclospora cayetanensis* (U40261), *Eimeria acervulina* (U67115), *Eimeria brunetti* (U67116), *Eimeria maxima* (U67117), *Eimeria mitis* (U67118), *Eimeria mivati* (U76748), *Eimeria necatrix* (U67119), *Eimeria praecox* (U67120), *Eimeria tenella* (U67121) (Barta et al., 1997), *Toxoplasma gondii* (L24381), and *Neospora caninum* (L24380).

PCR amplification and sequence analysis of the dihydrofolate reductase–thymidylate synthase gene

The dihydrofolate reductase (*dhfr*) locus was chosen for analysis as it is a single-copy gene and contains no introns (Vasquez et al., 1996). Degenerate *dhfr* primers were designed by aligning human and cattle-derived *C. parvum* nt sequences (Vasquez et al., 1996), with sequence information from the *Plasmodium falciparum* nt sequence (GenBank accession no. J03772). The resultant primers designated DihyF3 (5′AATGYAYTAGTTATGGGAAGAA3′) (positions 296–317) and DihyR2 (5′TAMKMATGTCTCAKKTATTTCTGG3′) (position 590–613) were designed to amplify a 318-bp fragment of *dhfr* at a 50 C annealing temperature. TAQ Extender™ (Stratagene, La Jolla, California) was included in all reactions to minimize PCR error. PCR products were purified using Qiagen spin columns (Qiagen, Hilden, Germany), sequenced, and analyzed as described above.

Phylogenetic analyses

Nucleotide sequences were aligned using Clustal X (Thompson et al., 1997). Sequence alignments can be obtained from the authors upon request. Phylogenetic analyses were performed using the following computer programs: MEGA (version 1.02, Kumar et al., 1994), PHYLIP (version 3.5c, 1993; J. Felsenstein, Department of Genetics, University of Washington, Seattle, Washington), and PAUP (version 3.1.1, 1993; D. L. Swofford, Illinois Natural History Survey, Champaign, Illinois). Distance-based analyses were conducted using Tamura–Nei distance estimates and trees were constructed using the neighbor-joining (NJ) algorithm. Parsimony analyses were conducted using the branch-and-bound search option of PAUP. Bootstrap analyses utilized 500 replicates. Phylograms were drawn using the TreeView program (Page, 1996). Split decomposition analyses were performed using SplitsTree (Dress et al., 1996). For *dhfr* sequences, distance-based analyses utilized all 3 codon base positions for the estimation of evolutionary distances.

Random-amplified polymorphic DNA (RAPD) analysis

RAPD analysis was performed using primers R-2817 and $[GACA]^5$ as previously described (Morgan et al., 1995) with the exception that reactions were performed on a Perkin-Elmer GeneAmp PCR System 2400 (Perkin-Elmer), and amplification cycles were increased to 50. A third primer ACoA F (Morgan, Sargent, Deplazes et al., 1998) was chosen at random and utilized under the same conditions as the previous primers. RAPD analysis was performed 3 times for each primer and only those bands that appeared reproducibly at each amplification were scored. For RAPD data, individual bands were scored as present or absent for each isolate, and the binary data were converted into a distance matrix using the inverse of Jaccard's coefficient as previously described (Morgan et al., 1993). Genetic relationships were inferred by NJ analysis and split decomposition.

RESULTS

Sequence analysis of the 18S rRNA gene

Complete sequences of the 18S rRNA gene were obtained from human (H7), mouse (M24), cattle (C1), pig (Pig 1), cat (Cat 1), and marsupial (K1) isolates of *C. parvum.* The GenBank accession numbers of the 18S rRNA genes are as follows: H7 (AF108865), M24 (AF108863), C1 (AF108864), Pig 1 (AF108861), Cat 1 (AF108862), and K1 (AF108860). Additional *C. parvum* isolates and other species of *Cryptosporidium* were obtained from GenBank as described above. The length of the gene varied from 1,735 bp for the *C. baileyi* isolate to 1,794 bp for the cat isolate (Cat 1).

Phylogenetic analysis of the 18S rRNA gene

The 18S rRNA gene sequences from *T. gondii, N. caninum,* and species of *Eimeria* were used as outgroups. Parsimony and distance analyses produced similar results, with both placing *C. muris* as the earliest branching species of *Cryptosporidium* examined herein, followed by *C. baileyi. Cryptosporidium wrairi* was clearly placed within *C. parvum* (NJ tree, Fig. 1). Isolates of *C. parvum* were placed into 1 of 4 clusters. Cat, marsupial,

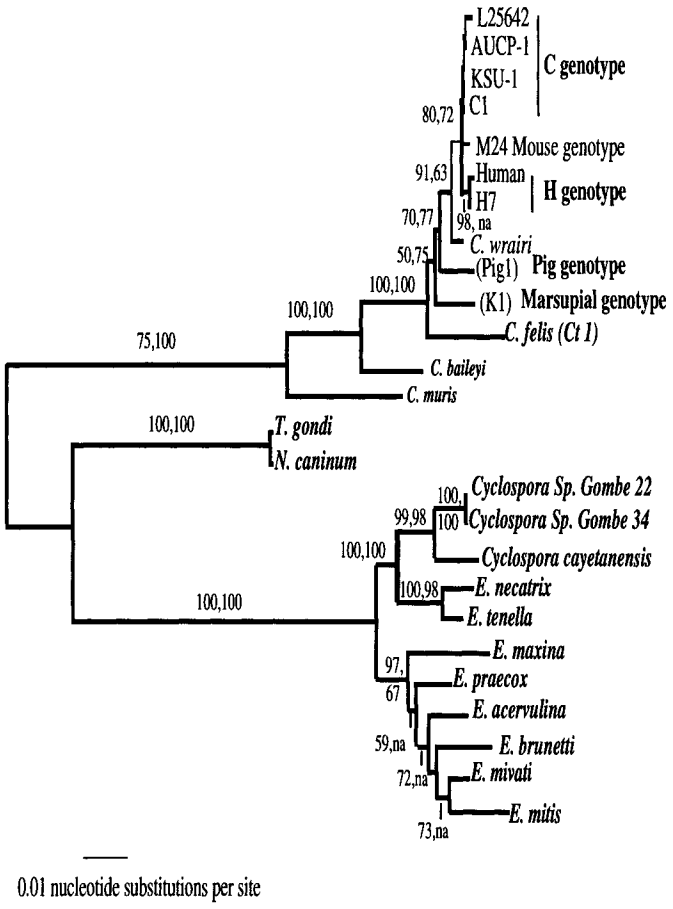

FIGURE 1. Phylogram depicting relationships inferred by NJ analysis of evolutionary distances inferred from 18S rRNA gene sequences. Bootstrap values (distance-based, parsimony analysis) of >50% are indicated at each node (H = human genotype; C = cattle genotype).

and pig isolates were each placed into a unique cluster, whereas the human, cattle, and mouse isolates were placed into a fourth cluster. The levels of interspecific variation between the recognized species of *Cryptosporidium* were greater than the levels of interspecific variation within avian species in the genus *Eimeria* and were also greater than the variation between *T. gondii* and *N. caninum* (Table II). The intraspecific variation within *C. parvum* was similar to the interspecific variation within *Eimeria* (Table II).

Bootstrap analysis of the distance data (Fig. 1) provided strong support for the placement of the cat, pig, and marsupial genotypes on the earliest branching lineages of *C. parvum* and for the clustering together of the human, mouse, and cattle genotypes. Strong support was also observed for the clustering of the human isolates with each other. Weaker support was found for the placement of *C. wrairi* as the sister group to the human/cattle/mouse group. The bootstrap values obtained by parsimony analysis (Fig. 1) provided some support for these nodes but were generally lower than the equivalent distance-based values. The cattle genotypes formed a single cluster that received low bootstrap support, possibly due to the low number of characters unique to this clade. Split decomposition of the distance data failed to identify any significant conflict within the data. None of the analysis procedures could reliably resolve the branch order of the 3 genotypes comprising the human/cattle/mouse group.

Phylogenetic analysis of the rRNA internal transcribed spacer (ITS) regions

Because phylogenetic analysis of the conserved 18S gene was unable to resolve the branch order of the human, cattle, and mouse cluster, a more variable region was examined. The region spanning the rRNA ITS1, 5.8S rRNA, and ITS2, previously sequenced (Morgan, Sargent et al., 1999), was therefore included in this analysis. For this region, the most closely related genotype of *C. parvum* was the marsupial genotype, and this was used as an outgroup. Cat and pig sequences were excluded from this analysis, because the high level of variability exhibited by these genotypes for this region adversely affected the sequence alignment and was likely to be too great to be informative phylogenetically. Analysis of this region by parsimony and distance-based methods produced a well resolved tree (Fig. 2) with all nodes supported by high (≥99%) bootstrap values. Isolates with the same genotypes all clustered within the same group. Both methods found strong support for the placement of the cattle and mouse genotypes as sister groups.

Sequence and phylogenetic analysis of the dihydrofolate reductase (*dhfr*) gene

Sequence analysis of the *dhfr* locus revealed that the human isolates from the U.K. and Australia (P18, H1, H7) were identical to the previously published sequence of a U.S. human-derived *Cryptosporidium* isolate (SFGH-1). Similarly, the Australian, European, and North American cattle isolates analyzed (C1, C2, sC33, S1, C13) were identical to the previously published sequence of a North American cattle-derived *Cryptosporidium* isolate (NINC-1). The mouse isolates (M11, M24, M26) and the marsupial isolates (K1, K2) were distinct from the human- and cattle-derived isolates analyzed. Repeated attempts to amplify cat and pig isolates using different reaction conditions and annealing temperature failed to produce amplicons, even though these isolates readily amplified using 18S rDNA primers.

For the phylogenetic analysis, *Plasmodium falciparum dhfr* was used as an outgroup sequence. Distance-based and parsimony analyses produced similar results (Fig. 3). In both cases, there was strong support for the placement of the human, cattle, and mouse genotypes into the same cluster. There was also strong support for the grouping of isolates with the same genotype. Exceptions to this were the cattle genotype isolates that, although identical at the nucleotide level for *dhfr,* failed to form a single cluster. The parsimony analysis and split decomposition both suggest that this is due to the absence of any character states that are unique for the cattle genotype and hence phylogenetically informative. The NJ tree was consistent with the 18S rRNA and ITS-based NJ tree, placing the human genotype external to the mouse/cattle genotype cluster, but the parsimony and split decomposition analysis of the *dhfr* nt data placed the human and mouse genotypes into the same clade. None of the analysis procedures found strong support for either of the 2 alternative branching patterns within the human/cattle/mouse group.

TABLE II. Similarity matrix of *Cryptosporidium* isolates and other genera at the 18S rRNA locus (Kimura's 2-parameter distance).

	KSU-1	C1	L25642	AUCP	Human	H7	M24	C. wr	Pig 1	K2	Ct 1	*C. bail*	*C. mu*	*E. ac*	*E. miv*	*E. brun*	*E. mit*	*E. pr*	*E. max*	*E. nec*	*E. ten*	Cyc22	Cyc34	Cycsp	*T. gon*
C1	100																								
C. parvum (L 25642)	99.9	99.9																							
AUCP-1 (L16996)	99.9	99.9	99.9																						
C. parvum (L16997)	99.7	99.7	99.5	99.7																					
H7	99.7	99.7	99.5	99.7	99.9																				
M24	99.8	99.8	99.7	99.8	99.5	99.5																			
C. wrairi (U11440)	99.5	99.5	99.3	99.5	99.3	99.2	99.4																		
Pig 1	98.8	98.8	98.5	98.7	98.8	98.9	98.7	98.7																	
K 1	98.5	98.5	98.1	98.4	98.3	98.3	98.5	98.6	98.3																
Ct 1	97.6	97.6	97.3	97.6	97.6	97.6	97.6	97.6	97.4	97.6															
C. baileyi (L19068)	96.6	96.6	96.3	96.6	96.4	96.4	96.4	96.5	95.8	96.2	95.9														
C. muris (L19069)	93.6	93.6	93.1	93.7	93.5	93.5	93.6	93.6	93.6	93.1	92.7	95.0													
Eimeria acervulina	80.0	90.0	81.3	80.0	79.9	79.9	79.9	80.3	80.3	79.8	78.9	80.4	81.4												
Eimeria mivati	79.9	79.9	91.4	79.9	79.8	79.8	79.8	80.2	80.2	79.7	78.8	80.3	81.5	98.7											
Eimeria brunetti	79.6	79.6	71.0	79.6	79.5	79.5	79.4	79.8	79.6	79.2	78.5	79.8	81.1	97.7	98.1										
Eimeria mitis	79.1	79.1	80.7	79.1	79.1	79.1	79.1	79.4	79.5	78.9	77.8	79.6	80.8	97.6	98.3	97.4									
Eimeria praecox	80.6	80.6	82.1	80.6	80.5	80.5	80.4	80.7	80.6	80.1	79.2	80.7	81.8	98.2	98.1	97.5	97.2								
Eimeria maxima	79.9	79.9	81.4	79.9	79.8	79.8	79.7	79.8	79.8	79.3	78.5	80.0	81.0	96.8	96.8	97.2	96.5	97.2							
Eimeria necatrix	80.0	80.0	81.6	80.0	80.0	80.0	79.9	80.2	80.1	79.8	78.6	80.5	81.2	96.8	96.7	95.9	96.0	96.5	95.5						
Eimeria tenella	80.1	80.1	81.8	80.1	80.1	80.1	80.0	80.4	80.2	79.9	78.9	80.7	81.5	97.2	97.0	96.2	96.2	97.0	96.0	99.0					
Cyclo sp. (Gombe 22)	80.1	80.1	81.3	80.1	80.0	80.0	80.0	80.3	80.2	79.9	79.0	80.6	81.6	96.6	96.6	96.2	96.0	95.9	95.3	97.0	97.2				
Cyclo sp. (Gombe 34)	80.1	80.1	81.3	80.1	80.0	80.0	80.0	80.3	80.2	79.9	79.0	80.6	81.6	96.6	96.6	96.2	96.0	95.9	95.3	97.0	97.2				
Cyclospora spp.	79.9	79.9	81.4	79.9	79.8	79.8	79.8	80.2	80.1	79.8	79.0	80.4	81.4	986.2	96.4	95.9	95.8	95.7	95.1	96.4	96.8	98.4	98.4		
Toxoplasma gondii	84.4	84.4	84.2	84.4	84.4	84.3	84.3	84.5	84.5	84.0	83.4	85.1	85.9	87.6	87.4	86.8	86.8	87.5	86.5	87.2	87.4	87.4	87.4	87.2	
Neospora caninum	84.4	84.4	84.2	84.4	84.5	84.4	84.3	84.5	84.5	84.0	83.4	85.0	85.8	87.6	87.4	86.8	86.9	87.5	86.6	87.4	87.6	87.6	87.6	87.4	99.8

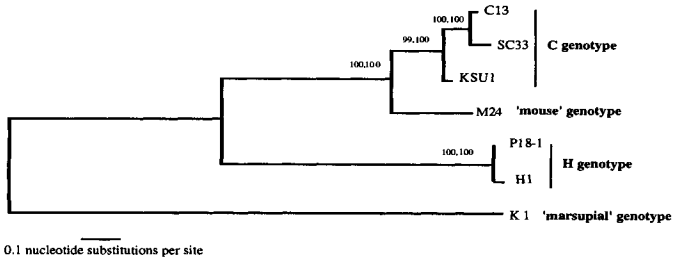

FIGURE 2. Phylogram depicting relationships inferred by NJ analysis of evolutionary distances inferred from ITS1, 5.8S rRNA, and ITS2. Bootstrap values (distance-based, parsimony analysis) of >50% are indicated at each node.

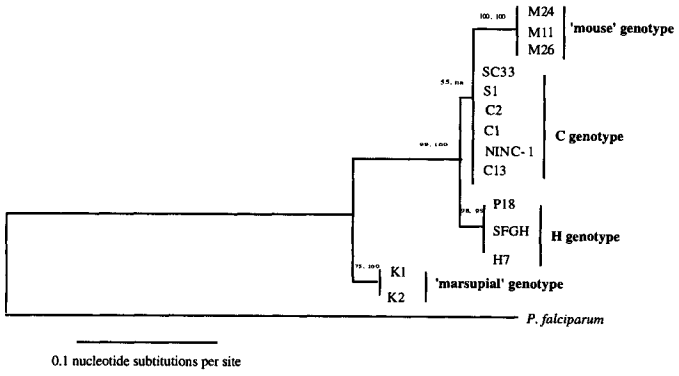

FIGURE 3. Phylogram depicting relationships inferred by NJ analysis of evolutionary distances inferred from *dhfr* gene sequences. Bootstrap values (distance-based, parsimony analysis) of >50% are indicated at each node.

RAPD analysis

Because a complete set of sequences could not be obtained using the *dhfr* locus, the relationships inferred by analysis of the 18S rDNA region were confirmed by subjecting representatives of the major genotypes of *C. parvum* to RAPD analysis. Human and cattle isolates each displayed identical profiles with all 3 primers, whereas pig, cat, marsupial, and mouse genotypes all consistently produced very different profiles (data not shown). There was insufficient material to perform RAPD analysis on multiple pig, marsupial, and cat isolates.

NJ analysis and split decomposition produced trees that clustered the mouse genotype with the human genotype (Fig. 4). Cat, pig, and marsupial genotypes grouped independently. The relative branching order of the cat, pig, and marsupial genotypes were different compared with the 18S rRNA-based tree (Fig. 1). Considering the limitations of the RAPD technique, especially when the banding patterns of 2 samples are highly dissimilar (as is the case herein), it is likely that the difference in the branching pattern is due to errors in the RAPD-based tree.

DISCUSSION

Phylogenetic analysis of *Cryptosporidium* isolates, using distance-based and parsimony analysis at a number of different loci, provided strong evidence that this genus is composed of several distinct and valid species. These findings contrast with those in which a pairwise comparison of 18S rDNA sequence data, obtained from GenBank for *C. wrairi, C. baileyi, C. muris,* and several bovine *C. parvum* isolates, suggested that interspecies and intraspecies values did not appear appreciably different (Tzipori and Griffiths, 1998). This suggestion was developed by comparing *Cryptosporidium* species variation to the considerable degree of variation within *Plasmodium* species (Tzipori and Griffiths, 1998). This comparison between *Cryptosporidium* and *Plasmodium* may be inappropriate for several reasons: (1) levels of interspecies divergence within *Plasmodium* is very large and greater in some cases than the intragenera differences between other apicomplexans, e.g., *Cryptosporidium* versus *Eimeria, Cryptosporidium* versus *Toxoplasma*; (2) the origin of the *Plasmodium* sequences indicating specific rRNA loci that are expressed at different stages of the *Plasmodium* life cycle were not considered; and (4) the levels of genetic divergence (based on sequences from GenBank) between some 18S rRNA loci within *Plasmodium vivax* are larger than divergences between *P. vivax,* and other *Plasmodium* species when compared at the same 18S rRNA locus (data not shown).

Data from the present study indicate that the levels of interspecific variation within *Cryptosporidium* are similar to or greater than those within avian *Eimeria* spp. and even between different genera. As shown in Table II, the percentage similarity between some of the recognized species of *Cryptosporidium,* e.g., 93.5% for *C. parvum* (H7) versus *C. muris,* is less than that observed between different avian species of *Eimeria* (99–95.3%) and between *E. tenella* and *C. cayetanensis* (96.8%). This provides good supporting evidence for the validity of the species of *Cryptosporidium* examined in this study. In addition, the levels of similarity between some of the genotypes of *C. parvum,* e.g., 97.6% for cat versus human, are within the range of interspecific variation observed for avian *Eimeria* spp. (99–95.3%). This supports the naming of the cat genotype as a distinct species (*C. felis*) (see below) and could also be used as evidence to support the recognition of the pig and marsupial genotypes as distinct species.

Phylogenetic analysis clearly demonstrated that *C. muris* was the most divergent species of *Cryptosporidium,* followed by *C. baileyi. Cryptosporidium serpentis* was not included in this analysis as a full 18S sequence was not obtained; however, recent analysis of a 713-bp region of the 18S gene from *C. muris* and *C. serpentis* isolates revealed these 2 species to be distinct but closely related (Morgan, Xiao et al., 1999). This is in agreement with biological data as *C. muris* and *C. serpentis*

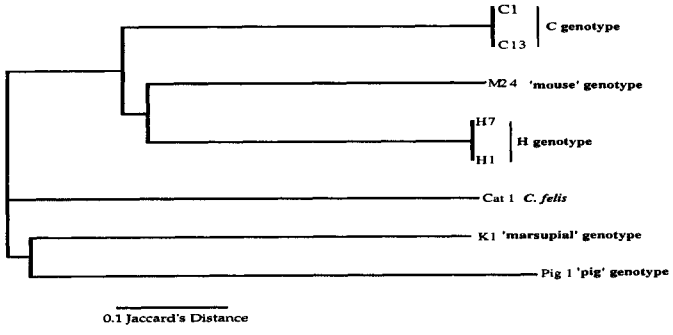

FIGURE 4. Phylogram depicting relationships inferred by NJ analysis of evolutionary distances inferred from RAPD data.

oocysts are the largest of all the *Cryptosporidium* species and mainly colonize the stomach, whereas *C. baileyi* mainly colonizes the respiratory tract and most other *Cryptosporidium* species infect the small intestine (Fayer et al., 1997).

Strong support was also provided for the description of *C. felis* as a distinct and valid species. To date, over 20 different feline isolates of *Cryptosporidium* from different continents have been characterized using a portion of the 18S rRNA gene (Morgan, Sargent, Elliott, and Thompson, 1998; Sargent et al., 1998; U. Morgan, unpubl. obs.). All feline isolates so far examined have been virtually identical at this locus. Considering the levels of genetic divergence between the cat genotype and other *C. parvum* isolates determined by 18S rRNA nt sequence analysis (in relation to the observed levels of interspecies divergence within avian spp. of *Eimeria* and between *Eimeria* and *Cyclospora,* as discussed above), coupled with their apparent host specificity and also morphological differences (Sargent et al., 1998), all confirm its placing as a valid species in the genus *Cryptosporidium.*

Using a similar rationale, evidence suggests that the pig and marsupial genotypes may be distinct species. Evolutionary distances between *C. parvum* (KSU-1) and more recently identified genotypes such as the marsupial and pig genotypes at the 18S rRNA locus were 0.015 and 0.0121 nt substitutions/site, respectively. This is larger than the observed distance of 0.0051 nt substitutions/site between *C. parvum* (KSU-1) and *C. wrairi. Cryptosporidium wrairi* is a valid species due to (1) genetic differences at a number of different loci (Chrisp and LeGendre, 1994; Spano et al., 1997; Spano, Putignani, Naitza et al., 1998); (2) its host specificity and unlike *C. parvum, C. wrairi* is not readily infective for mice (Vetterling et al., 1971); and (3) its strikingly different oocyst wall proteins to *C. parvum* (Tilley et al., 1991).

The *Cryptosporidium* pig genotype has so far been found only in pigs and is conserved across geographic areas, as pigs from Australia and Switzerland had identical genotypes at the loci examined (Morgan, Sargent, Deplazes et al., 1998; Morgan, Sargent et al., 1999). In addition, the pig genotype is not readily infectious to mice (Morgan, Buddle et al., 1999).

Little is known about the marsupial genotype and its prevalence in marsupials. *Cryptosporidium* infections have previously been reported in southern brown bandicoots (*Isoodon obesulus*), a hand-reared juvenile red kangaroo (*Macropus rufus*) from South Australia, and a wallaby from Tasmanian (*Thylogale billardierii*) (O'Donoghue, 1995). To date, only 3 marsupial isolates have been genotyped, e.g., a *Cryptosporidium* isolate from a koala (*Phascolarctos cincereus*) from South Australia, a second isolate from a red kangaroo from West Australia and, more recently, a koala from West Australia (Morgan et al., 1997; Morgan, Sargent et al., 1999; U. Morgan, unpubl. obs.). However, 18S rDNA, ITS, dhfr sequence analysis, and RAPD analysis have all confirmed their genetic distinctness. Indeed, at the ITS locus, the marsupial genotype exhibited only 64.13% and 58.55% similarity to the cattle and human *C. parvum* genotypes, respectively. Future genetic and biological studies on a wider range of isolates are required to confirm the validity of the pig and marsupial genotypes as distinct and valid species.

The human (H7) and cattle (KSU-1) *C. parvum* genotypes exhibited a similarity of 99.7%, whereas the mouse genotype and *C. parvum* (KSU-1) exhibited a similarity of 99.8% at the 18S rRNA locus. The similarity between *T. gondii* and *N. caninum* was 99.8% at this locus. For the ITS locus, the similarity between human and cattle genotypes and between mouse and cattle genotypes was much less at 82.23% and 93.14%, respectively. The similarity between *T. gondii* and *N. caninum* at this locus was 89.49%. There are also biological differences between the human and cattle genotypes, as the human genotype does not readily infect neonatal mice or cattle (Meloni and Thompson, 1996; Peng et al., 1997; Widmer et al., 1998). A recent report characterized a total of 28 human and animal isolates of *Cryptosporidium* originating from Europe, North and South America, and Australia using unlinked genetic polymorphisms (Spano, Putignani et al., 1998). All the isolates examined clustered into either the human or the cattle genotype. No recombinant genotypes were identified, which indicates reproductive isolation between the human and cattle genotypes (Spano, Putignani et al., 1998).

The mouse genotype is genetically distinct from human and cattle genotypes and was first identified in mice in Australia (Morgan, Putignani et al., 1998) but appears to be conserved across geographical areas. A larger study of mouse isolates of *Cryptosporidium* has revealed that this genotype is also present in mouse isolates from the U.K. and Spain (Morgan, Sturdee et al., 1999). Sequence analysis of the 18S rRNA, ITS, *dhfr* and acetylCoA loci as well as RAPD analysis have all confirmed the genetic distinctness of the mouse genotype (Morgan, Sargent, Deplazes et al., 1998; Morgan, Sargent et al., 1999; Morgan, Sturdee et al., 1999). Phylogenetic analysis of the ITS1 and 2 has revealed the mouse genotype to be related most closely to the cattle genotype.

Phylogenetic analyses of *Cryptosporidium* isolates confirmed the validity of the species examined in this study and provided strong evidence for the naming of several new species from within what is now named *C. parvum.* It is clear from these results that a re-examination of the taxonomy of the genus is urgently required and that future genetic studies should be carried out in conjunction with more extensive biological analysis in order to elucidate the true taxonomic status of these newly identified genotypes.

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