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PHYLOGENETIC ANALYSIS OF *CRYPTOSPORIDIUM* ISOLATES FROM CAPTIVE REPTILES USING 18S rDNA SEQUENCE DATA AND RANDOM AMPLIFIED POLYMORPHIC DNA ANALYSIS

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ABSTRACT: Sequence alignment of a polymerase chain reaction-amplified 713-base pair region of the *Cryptosporidium* 18S rDNA gene was carried out on 15 captive reptile isolates from different geographic locations and compared to both *Cryptosporidium parvum* and *Cryptosporidium muris* isolates. Random amplified polymorphic DNA (RAPD) analysis was also performed on a smaller number of these samples. The data generated by both techniques were significantly correlated ($P < 0.002$), providing additional evidence to support the clonal population structure hypothesis for *Cryptosporidium.* Phylogenetic analysis of both 18S sequence information and RAPD analysis grouped the majority of reptile isolates together into 1 main group attributed to *Cryptosporidium serpentis,* which was genetically distinct but closely related to *C. muris.* A second genotype exhibited by 1 reptile isolate (S6) appeared to be intermediate between *C. serpentis* and *C. muris* but grouped most closely with *C. muris,* as it exhibited 99.15% similarity with *C. muris* and only 97.13% similarity with *C. serpentis.* The third genotype identified in 2 reptile isolates was a previously characterized 'mouse' genotype that grouped closely with bovine and human *C. parvum* isolates.

Oocysts resembling those of *Cryptosporidium* were found in large numbers in the feces of *Crotalus confluentus* and were described as *Cryptosporidium crotali* (Triffit, 1925). However, the oocysts, which measured 10–11 μm by 10.8–12.5 μm, were much larger than any confirmed species of *Cryptosporidium* and have not been seen since. Therefore, this species is considered invalid. *Cryptosporidium lampropeltis* was described based on oocysts recovered from kingsnakes (Anderson et al., 1968). However, morphologically these resemble sporocysts of *Sarcocystis* and the species is also considered invalid. What appears to be the first valid finding of *Cryptosporidium* spp. in a reptile was the report of 14 captive snakes with severe hypertrophic gastritis (Brownstein et al., 1977). Since then, *Cryptosporidium* spp. have been reported in more than 57 different reptilian species, including 40 species of snakes, 15 species of lizards, and 2 species of tortoises (O'Donoghue, 1995). In 1980, Levine assigned *Cryptosporidium serpentis* to snake isolates on the basis of host occurrence and descriptions provided by Brownstein et al. (1977).

However, few characterization studies have been done on reptilian isolates of *Cryptosporidium,* and there appears to be some confusion as to the identity of the species of *Cryptosporidium* infecting reptiles. Tilley et al. (1990) differentiated reptilian isolates of *Cryptosporidium* spp. from *Cryptosporidium parvum* on the basis of oocyst size and electrophoretic protein patterns. In their study, oocysts from snakes measured approximately 6.2 × 5.3 μm and were not infective to mice. However, the oocyst dimensions described by Brownstein et al. (1977) were small (3.0–3.8 μm). It is possible that they were a rare species not seen subsequently, that they were developmental stages misidentified as oocysts, or that processing of paraffin-embedded tissues had caused the oocysts to shrink. The oocyst dimensions described by Tilley et al. (1990) are now the accepted dimensions for *C. serpentis,* as the majority of captive snake isolates measured conform to these dimensions (Fayer et al., 1995; Graczyk et al., 1995). Morphometric studies on isolates recovered from wild snakes and lizards have indicated the occurrence of at least 5 different morphotypes (Upton et al., 1989).

More recently, genetic characterization of 3 snake isolates based on sequence analysis of a portion of the 18S rDNA gene revealed 2 distinct types (Morgan et al., 1998), 1 of which exhibited a genotype most closely related to *Cryptosporidium muris.* These results raise questions as to the identity of the species of *Cryptosporidium* that infect reptiles. Therefore, the aim of the present study was to genetically characterize a larger number of reptile isolates from different geographic locations in order to gain a better understanding of which genotypes of *Cryptosporidium* most commonly infect reptiles.

MATERIALS AND METHODS

Sources of isolates, morphometrics, and DNA purification

A total of 15 *Cryptosporidium* isolates from reptiles and 1 *C. muris* isolate from a bovine host was obtained from different geographic locations (Table I). Three additional *C. parvum* isolates previously sequenced were also included for comparison; these were a human isolate (H1) displaying the 'human' genotype; a representative 'human' genotype (H7) is available for Genbank (AF108865), a bovine isolate (C1) exhibiting the 'cattle' genotype (Genbank acc. AF108864), and a mouse isolate (M24), exhibiting the 'mouse' genotype (Genbank acc. AF108863) (Morgan, Constantine, Forbes, and Thompson, 1997; Morgan et al., 1998). *Cryptosporidium muris* (Genbank acc. L19069), *Cryptosporidium wrairi* (Genbank acc. U11440), and *Toxoplasma gondii* (Genbank acc. M97703) were included as a comparison. Where possible, morphometric analysis was performed on isolates using an Optimas v5.2 system at ×100 magnification (Table I). DNA was purified as previously described (Morgan, Constantine, Forbes, and Thompson, 1997).

Polymerase chain reaction (PCR) amplification and sequencing

The primers and their sequences used to amplify a 713-base pair (bp) product from the 18S rDNA gene were 18S-SF, forward primer (5′-

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

Isolate code	Host	Species	Oocyst dimensions (μm)	Geographical location	Source
S1	Taipan (*Oxyuranus scutellatus*)	*C. serpentis*	6.2 × 5.3	Tanunda, South Australia	CVL
S2	Taipan (*O. scutellatus*)	*C. serpentis*	6.3 × 5.5	Tanunda, South Australia	CVL
S3	Woma python (*Aspidites ramsayi*)	*C. parvum*	4.5 × 4.0	Perth Zoo, Western Australia	MU
S4	Taipan (*O. scutellatus*)	*C. serpentis*	6.2 × 5.4	South Australia	CVL
S5	Red-bellied black snake (*Pseudechis porphyriacus*)	*C. parvum*	4.5 × 4.3	Armadale Reptile Park, Perth, Western Australia	MU
S6	Mulga/king brown snake (*Pseudechis australia*)	*C. muris*?	5.9 × 5.2	Armadale Reptile Park, Perth, Western Australia	MU
S7	Black rat snake (*Elaphe obsoleta*)	*C. serpentis*	6.0 × 5.3	Maryland, U.S.A.	BZ
S8	Bull snake (*Pituophis melanoleucus melanoleucus*)	*C. serpentis*	ND	Maryland, U.S.A.	BZ
S9	Corn snake (*Elaphe guttata guttata*)	*C. serpentis*	ND	Maryland, U.S.A	BZ
S10	Corn snake (*E. g. guttata*)	*C. serpentis*	6.5 × 5.8	Zurich, Switzerland	IP
S11	Common death adder (*Acanthophis antarticus*)	*C. serpentis*	6.2 × 5.4	South Australia	CVL
S12	Eastern/mainland tiger snake (*Notechis scutatus*)	*C. serpentis*	6.3 × 5.5	Victoria, South Australia	CVL
41	Savanna monitor (*Varanus exanthematicus*)	*C. serpentis*	ND	Washington, DC, U.S.A.	CDC
63	Savanna monitor (*V. exanthematicus*)	*C. serpentis*	ND	Washington, DC, U.S.A.	CDC
64	Amazon tree boa (*Corallus hortulanus*)	*C. serpentis*	ND	Washington, DC, U.S.A.	CDC
20	Cattle	*C. muris*	ND	Idaho, U.S.A.	CDC
H1	Human	*C. parvum*	5.1 × 4.4	Perth, Western Australia	PMH
C1	Cattle	*C. parvum*	5.2 × 4.5	Millicent, South Australia	CVL
M24	Mouse (*Mus musculus*)	*C. parvum*	4.5 × 4.0	Walpeup, Victoria, Australia	CSIRO

* BZ = Baltimore Zoo, Baltimore, Maryland, U.S.A.; CDC = Centers for Disease Control, Atlanta, Georgia, U.S.A.; CSIRO = Commonwealth Scientific and Industrial Research Organisation, Victoria, Australia; CVL = Central Veterinary Laboratories, Adelaide, South Australia; IP = Institute of Parasitology, Zurich, Switzerland; MU = Murdoch University, Perth, Western Australia; PMH = Princess Margaret Hospital, Perth, Western Australia; ND = not determined.

AGTCATAGTCTTGTCTCAAAGATT-3′) and 18SiR, reverse primer (5′-CCTGCTTTAAGCACTCTAATTTTC-3′). Reactions were performed on a PE 2,400 thermal cycler (Perkin Elmer, Foster City, California). Samples were heated to 96 C for 2 min, followed by 52 cycles of 94 C for 30 sec, 55 C for 30 sec, and 72 C for 45 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), and 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 55 C. PCR products were sequenced in both directions. Sequences were analyzed using SeqEd v1.0.3. (Applied Biosystems) and aligned using the Clustal V sequence alignment program (Higgins et al., 1991).

Random Amplified polymorphic DNA (RAPD) analysis

RAPD analysis was performed using primers R-2817 and $[GAA]^7$ as previously described (Morgan et al., 1995) with the exception that reactions were performed on a PE 2,400 thermal cycler and amplification cycles were increased to 50.

Phylogenetic analysis

Phylogenetic analysis of 18S rDNA sequence data was conducted using PHYLIP 3.5p (Felsenstein, 1989), using *T. gondii* as an outgroup. Distance-based analyses were conducted using the formula for Kimura's distance and also using the Jukes and Cantor method. Trees were constructed using the Neighbor Joining and Unweighted Pair-Group Method (UPGMA) algorithms available in PHYLIP 3.5p (Felsenstein, 1989). Parsimony analysis was conducted using the DNAPARS program also available in PHYLIP 3.5p (Felsenstein, 1989). Phylograms were drawn using the TreeView program (Page, 1996).

For RAPD data, individual bands were scored as present or absent for each isolate and the inverse of Jaccards coefficient was calculated from the resulting data matrices as previously described (Morgan et al., 1993). RAPD analysis was performed 3 times for each primer and only those bands that appeared reproducibly at each amplification were scored. Phylogenetic relationships were determined by the UPGMA using a modified Fortran computer program (UPGAMA2) (Constantine et al., 1994). The dissimilarity matrices obtained from both the 18S sequence information and the RAPD analysis were tested for statistical correlation using Manly's (1985) version of Mantel's (1967) nonparametric test. The test is based upon determining whether the statistic Z is significant when compared to the distribution of Z obtained by random permutations of the rows and columns of the second matrix compared to the first matrix. One thousand random permutations were carried out.

Experimental infection of nude mice

Approximately 10^4 purified oocysts from the *Cryptosporidium* snake isolate S3 were inoculated orally into 6-day-old nude mice, which were returned to their dams for 7 days and then killed. The whole intestine was removed and processed as previously described (Meloni and Thompson, 1996). Fecal pellets were monitored for the presence of *Cryptosporidium* spp. oocysts before inoculation and each day postinoculation. It was not possible to infect mice with oocysts from other snakes as the oocysts were either no longer viable or were supplied as DNA.

RESULTS

Sequence analysis of 18S rDNA

Sequence analysis of the 18S rDNA gene of *Cryptosporidium* isolates from various snakes from Australia, Switzerland, and North America revealed that the reptile genotype is conserved across geographic locations. All of of the reptile isolates, which conformed morphologically to *C. serpentis,* were genetically identical at this locus, with the exception of isolates 63 and 41, which differed at only 1 point along the sequence (see Fig. 1). A representative *C. serpentis* sequence (S1) has been submitted to Genbank (AF108866). Snake isolates 3 and 5 were virtually identical to a new genotype referred to as the 'mouse' genotype

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S1       AGTCATATGCTTGTCTCAAAGATTAAGCCATGCATGTCTAAGTATAAGCTTTTATACGGCGAAACTGCGAATGGCTCATTAAAACAGTTATAGTTTACTT
S6       AGTCATATGCTTGTCTCAAAGATTAAGCCATGCATGTCTAAGTATAAGCTTTTATACGGCGAAACTGCGAATGGCTCATTAAAACAGTTATAGTTTACTT
C.muris  AGTCATATGCTTGTCTCAAAGATTAAGCCATGCATGTCTAAGTATAAGCTTTTATACGGCGAAACTGCGAATGGCTCATTAAAACAGTTATAGTTTACTT
S3       AGTCATATGCTTGTCTCAAAGATTAAGCCATGCATGTCTAAGTATAAACTTTTATACGGTTAAACTGCGAATGGCTCATTATAACAGTTATAGTTTACTT
         ********************************************** ***********  ******************** ******************

S1       GATAATCAAAACT-ACATGGATAACCGTGGTAATTCTAGAGCTAATACATGCGAAAAGGCCCGACTTTTTGGAAGGGTTGTATTTATTAGATAAAGAACC
S6       GATAATCAAAACT-ACATGGATAACCGTGGTAATTCTAGAGCTAATACATGCGAAAAAACCCAACTTTGCGGAAGGGTTGTATTTATTAGATAAAGAACC
C.muris  GATAATCAAAACT-ACATGGATAACCGTGGTAATTCTAGAGCTAATACATGCGAAAAAACCCAACTTCGCGGAAGGGTTGTATTTATTAGATAAAGAACC
S3       GATAATCTTTACTTACATGGATAACCGTGGTAATTCTAGAGCTAATACATGCGAAAAAACTCGACTTTATGGAAGGGTTGTATTTATTAGATAAAGAACC
         *******   *** ********************************************  * * ****   ******************************

S1       AATATTTTTGGTGATTCATAATAACTTTACGGATCGCATCTCTGATGCGACATATCATTCAAGTTTCTGACCTATCAGCTTTAGACGGTAGGGTATTGGC
S6       AATGAGCTTGGTGATTCATAATAACTTTACGGATCGCATCTCTGATGCGACATATCATTCAAGTTTCTGACCTATCAGCTTTAGACGGTAGGGTATTGGC
C.muris  AATGAGCTTGGTGATTCATAATAACTTTACGGATCGCATCTCTGATGCGACATATCATTCAAGTTTCTGACCTATCAGCTTTAGACGGTAGGGTATTGGC
S3       AATATAATTGGTGACTCATAATAACTTTACGGATCACATT--AAATGTGACATATCATTCAAGTTTCTGACCTATCAGCTTTAGACGGTAGGGTATTGGC
         ***    ******* ******************** ***     *** ***************************************************

S1       CTACCGTGGCTATGACGGGTAACGGGGAATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCTAAGGAAGGCAGCAGGCGCGCAAA
S6       CTACCGTGGCTATGACGGGTAACGGGGAATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCTAAGGAAGGCAGCAGGCGCGCAAA
C.muris  CTACCGTGGCTATGACGGGTAACGGGGAATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCTAAGGAAGGCAGCAGGCGCGCAAA
S3       CTACCGTGGCAATGACGGGTAACGGGGAATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCTAAGGAAGGCAGCAGGCGCGCAAA
         ********** ***************************************************************************************

S1       TTACCCAATCCTGACACAGGGAGGTAGTGACAAGAAATAACAATACAGGGCCTAACGGTCTTGTAATTGGAATGAGTGAAGTATAAACCCCTTTACAAGT
S6       TTACCCAATCCTGACACAGGGAGGTAGTGACAAGAAATAACAATACAGGGCCTAACGGTCTTGTAATTGGAATGAGTGAAGTATAAACCCCTTTACGAGT
C.muris  TTACCCAATCCTGACACAGGGAGGTAGTGACAAGAAATAACAATACAGGGCCTAACGGTCTTGTAATTGGAATGAGTGAAGTATAAACCCCTTTACGAGT
S3       TTACCCAATCCTAATACAGGGAGGTAGTGACAAGAAATAACAATACAGGACTTTTTGGTTTTGTAATTGGAATGAGTTAAGTATAAACCCCTTTACAAGT
         ************ * ********************************** * *   *** ***************** ****************** ***

S1       ATCAATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAAAGTTGTTGCAGTTAAAAAGCTCGTAGTTGGATTTC
S6       ATCAATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAAAGTTGTTGCAGTTAAAAAGCTCGTAGTTGGATTTC
C.muris  ATCAATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAAAGTTGTTGCAGTTAAAAAGCTCGTAGTTGGATTTC
S3       ATCAATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAAAGTTGTTGCAGTTAAAAAGCTCGTAGTTGGATTTC
         ****************************************************************************************************
                                                                                                      +
S1       TGTTGTATT-TTTATA-ATATTATTA-AGGTAATATTTATAA--TATCAACATCCTTCCTATTA-TATTTTTAA-T----ATATAGGAAATTTTACTTTG
S6       TGTTGTATAATCTATA-ATATTACTA-AGGTATATATTATAT--TATCAACATCCTTCCTATTA-TATTTCTAAAT----ATATAGGAAACTTTACTTTG
C.muris  TGTTGTATAATTTATA-ATATTACCA-AGGTAATTATTATAT--TATCAACATCCTTCCTATTA-TATT-CTAAAT----ATATAGGAAATTTTACTTTG
S3       TGTTA-ATAATTTATATAAAATATTTTAATTAATATTTATATAATATTAACATAATTCATATTACTATAATTATTTTTTAGTATATGAAATTTTACTTTG
         ****  **  * **** * * **    *  **    *****   *** *****  *** ***** ***   **  *     **** **** *********

S1       AGAAAATTAGAGTGCTTAAAGCAGG
S6       AGAAAATTAGAGTGCTTAAAGCAGG
C.muris  AGAAAATTAGAGTGCTTAAAGCAGG
S3       AGAAAATTAGAGTGCTTAAAGCAGG
         *************************
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FIGURE 1. Sequence alignments of the rDNA PCR fragment from representative reptile (S1, S3, and S6), and *Cryptosporidium muris* isolates. (+ = isolates 41 and 63 exhibited a G at this position).

previously identified (Morgan et al., 1998a, 1999a; Morgan et al., 1999b). Snake isolate 6, while sharing some similarities with the majority of reptile isolates, was genetically more similar to *C. muris* than all the other reptile isolates. Representative sequences from the main reptile genotype (S1), the 'mouse' genotype (S3), snake 6, and *C. muris* are listed in Figure 1.

RAPD analysis

RAPD analysis was performed on 11 *Cryptosporidium* isolates, 8 of which were reptile isolates. The remaining isolates were either not sufficiently purified or had insufficient template. RAPD analysis using primers R-2817 and $[GAA]^7$ revealed the 8 reptile isolates analyzed to be genetically very similar with the exception of S3, which shared little or no homology with the other reptile isolates (Fig. 2). Faint banding differences were observed among the reptile isolates but, because these differences were not amplified consistently, they were not included in the analysis. Isolate S3 shared a number of bands with the 'calf' (C1) and 'human' (H1) *C. parvum* genotypes using both primers. The *C. muris* isolate (20), although sharing all the same banding patterns as the reptile isolates using primer R-2817, also had a number of additional bands not present in the reptile isolates. Using primer $[GAA]^7$, the *C. muris* isolate (20) shared only 1 band with the majority of reptile isolates (Fig. 2).

Phylogenetic analysis of 18S rDNA sequence data

Kimura's distance and Jukes and Cantor analysis both produced identical trees (data not shown). UPGMA and Neighbor Joining also produced similar trees and a representative tree generated using Kimura's distance and UPGMA is depicted in

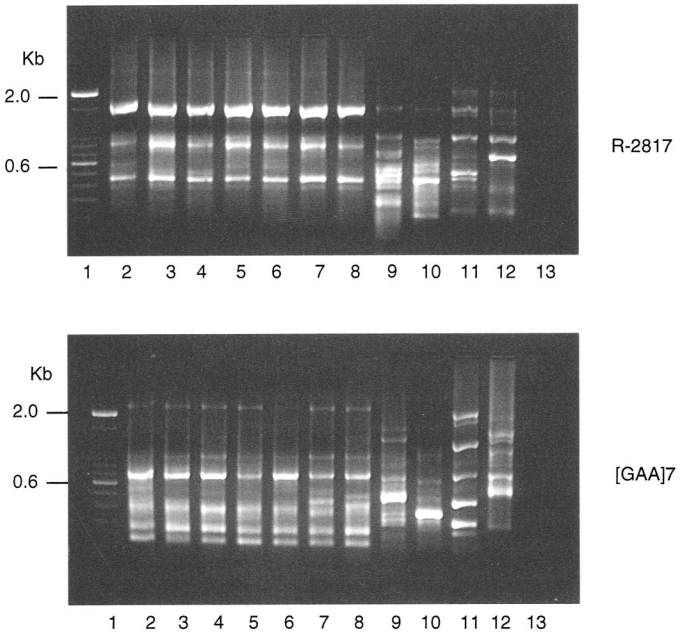

FIGURE 2. Random amplified polymorphic DNA profiles generated using 2 different primers, R-2817 and $[GAA]^7$, respectively. Lane 1 = molecular weight marker (100-base pair ladder; Gibco-BRL, Gaithersburg, MD); 2 = S1; 3 = S2; 4 = S4; 5 = S11; 6 = S12; 7 = 63; 8 = 64; 9 = S3; 10 = 20; 11 = C1; 12 = H1; 13 = negative control. kb = kilobases.

Figure 3a. Parsimony analysis is depicted in Figure 3b. Three major groups were evident from phylogenetic analysis of aligned 18S sequence information; a *C. serpentis* group, which contained all the reptilian isolates with the exception of S3 and S5 (which conformed morphologically to oocyst dimensions for the 'mouse' genotype); a *C. muris* group, which contained the 2 *C. muris* isolates and the S6 isolate; and a third broad group, which contained *C. wrairi,* both 'human' and 'cattle' *C. parvum* isolates, and M24, S3, and S5 (Fig. 3a, b). A similarity index of the *Cryptosporidium* isolates examined generated using Kimura's distance revealed that a representative *C. serpentis* isolate (S1) and *C. muris* exhibited 97.42% similarity compared to 93.38% similarity between *C. serpentis* and *C. parvum* (Table II).

Phylogenetic analysis of RAPD data

As with the phylogenetic analysis of the 18S sequence data, a phylogram based on Jaccard's distance constructed from the RAPD data also produced 2 major groups (Fig. 4). All the reptilian isolates with the exception of S3 grouped with *C. muris.* The human, cattle, and reptile isolate (S3) formed the second major group (Fig. 4). Isolates S5 or S6 were not included in this analysis because of insufficient template purity. The results generated by both 18S sequence analysis and RAPD analysis were significantly correlated ($P < 0.002$).

Mouse infectivity study

Experimental inoculations of the snake S3 isolate that exhibited the 'mouse' genotype (S3) produced an infection in nude mice. Eight mice were inoculated and approximately 2 million oocysts were recovered per mouse. The genotype of the oocysts

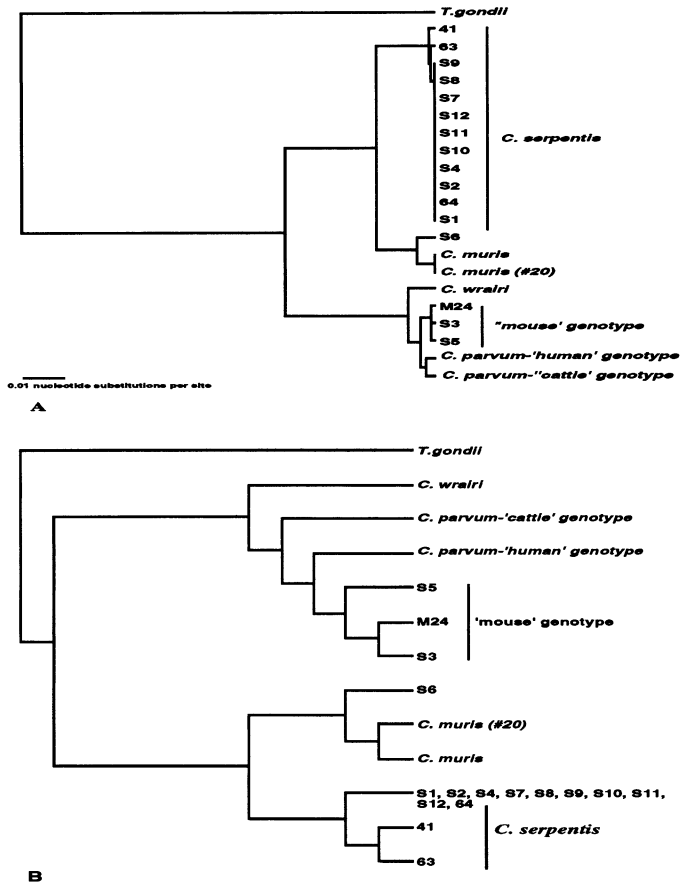

FIGURE 3. **a.** Phylogram of Kimura's distance generated from 18S rDNA sequence information among isolates of *Cryptosporidium* clustered using the Unweighted Pair Group Method of Analysis (UPGMA). **b.** Parsimony analysis of 18S rDNA sequence data from *Cryptosporidium* isolates.

recovered was confirmed as 'mouse' genotype using 18S sequence analysis as previously described (data not shown).

DISCUSSION

In the present study, reptile isolates were analyzed using both 18S rDNA sequence analysis and RAPD analysis and compared to *C. muris* as well as 'human', 'cattle,' and 'mouse' genotypes of *C. parvum.* The majority of reptile isolates conformed morphologically to the oocyst dimensions assigned to *C. serpentis*

TABLE II. Similarity values for selected species of *Cryptosporidium* generated using Kimura's distance analysis of 18S rDNA sequence alignments.

Species comparison	% Similarity
C. parvum 'human' vs. 'cattle' genotype	98.58
C. parvum (C1) vs. S5	99.44
C. parvum (C1) vs. *C. wrairi*	98.87
C. parvum (C1) vs. *C. muris*	92.14
C. parvum (C1) vs. *C. serpentis*	93.38
C. muris vs. *C. serpentis*	97.42
C. muris vs. S6	99.15
C. serpentis vs. S6	97.13

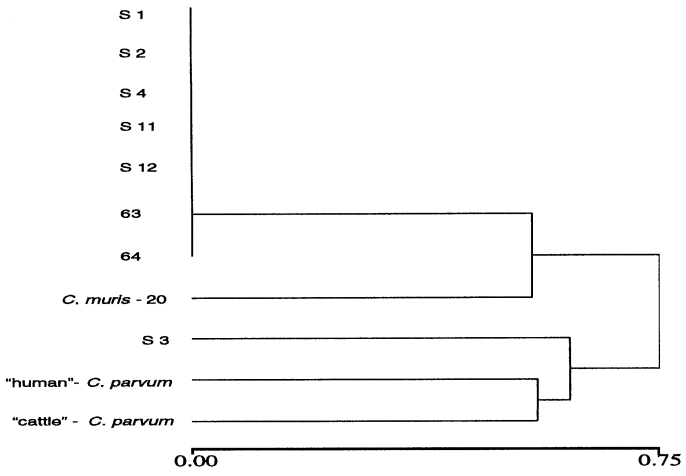

FIGURE 4. Phylogram of Jaccard's distance among rapdemes of *Cryptosporidium,* clustered using the Unweighted Pair Group Method of Analysis (UPGMA.)

(Tilley et al., 1990), and exhibited a common genotype that we will refer to here as *C. serpentis.* This genotype is conserved across geographical areas, as identical genotypes were found in *Cryptosporidium* snake isolates from Australia, Switzerland, and North America. The occurrence of widespread genotypes and the correlation between the RAPD and 18S sequence analyses provides additional evidence to support the clonal population structure hypothesis for *Cryptosporidium* (Morgan, Constantine, and Thompson, 1997), because parity between 2 sets of independent genetic markers suggests that recombination might be biologically restricted.

Cryptosporidium serpentis was also found to be genetically very close to *C. muris,* which is complemented by their morphologic similarity. *Cryptosporidium muris* oocysts can vary in length from 6.6 to 7.9 μm and in width from 5.3 to 6.5 μm (Upton and Current, 1985). The dimensions of *C. serpentis* are similar and can range from 5.8 to 6.6 μm in length and 4.8 to 5.9 μm in width (Upton et al., 1989; Graczyk et al., 1995). Previous genetic characterization of a number of snake isolates based on sequence analysis of a 298-bp region of the 18S rDNA gene grouped *C. serpentis* isolates with *C. muris* (Morgan et al., 1998a). However analysis of a larger (713-bp) region of the 18S gene and also RAPD analysis has revealed that, although closely related, *C. serpentis* is genetically distinct from *C. muris.* The validity of *C. serpentis* as a separate species is supported by its host specificity and the fact that the similarity between 2 recognized species (*C. parvum* and *C. wrairi*) is 98.87%, which is greater than the similarity between *C. serpentis* and *C. muris* (97.42%).

The second genotype exhibited by 1 reptile isolate (S6) appeared to be intermediate between *C. serpentis* and *C. muris,* but grouped most closely with *C. muris,* as it exhibited 99.15% similarity with *C. muris* and only 97.13% similarity with *C. serpentis.* The genetic similarity of isolate S6 to *C. muris* suggests that reptiles may be susceptible to *C. muris*; however, it is also possible that the snake was passing oocysts from infected prey. Funk (1987) suggested that snakes could become infected with *Cryptosporidium* by ingestion of infected mice or other prey. However, attempts by 2 groups of investigators to infect suckling mice with oocysts of numerous isolates of *C. serpentis* have failed (Tilley et al., 1990, Fayer et al., 1995). Snakes were reported to have been successfully infected experimentally with *C. parvum* oocysts of human origin (Arcay et al., 1995) but, because uninfected controls were not utilized it is difficult to confirm this finding. Another group was unable to infect corn snakes with *C. parvum* oocysts of bovine origin, which were infectious for suckling mice (Graczyk et al., 1996). A more recent study revealed that although *C. serpentis* isolates were transmissible to other snakes, *C. muris, C. wrairi, C. baileyi,* and *C. meleagridis* did not produce infections in captive snakes (Graczyk and Cranfield, 1998). However, additional studies are required before the infectivity of various *Cryptosporidium* species, and *C. muris* in particular, to snakes can be determined.

A third *Cryptosporidium* spp. genotype designated the 'mouse' genotype was isolated in 2 of the *Cryptosporidium* spp. reptile isolates examined (S3 and S5). This is a newly identified genotype recently discovered in mice that is more similar morphologically to *C. parvum,* but that is genetically distinct from the 'human' and 'cattle' *C. parvum* genotypes (Morgan et al., 1998a, 1998b). This 'mouse' genotype was first reported in mice from Australia, but appears to be conserved across geographical areas, because a larger study of mice isolates of *Cryptosporidium* spp. has revealed that this genotype is also present in mice from the U.K and Spain (Morgan et al., 1999). Because the oocysts from isolates S3 and S5 were obtained from fecal pellets alone and autopsy was not possible, it is impossible to be certain whether the snakes were actually infected or were simply passing oocysts from infected prey. However, the numbers of oocysts obtained from the fecal samples suggest that the snakes may actually have been infected. Experimental transmission of the 'mouse' genotype to snakes is required in order to confirm this assertion. The 'mouse' genotype identified in snake isolate S3 was successfully transmitted experimentally to nude mice. RAPD analysis using primer R-2817 revealed that the isolate S3 shared some bands with the 'cattle' genotype. Sequence analysis of the rDNA internal transcribed spacers (ITS1, ITS2) has also shown this genotype to be closely related to the 'cattle' genotype (Morgan et al., 1998b).

Upton et al. (1989) suggested the existence of up to 5 *Cryptosporidium* species in wild reptiles based on morphologic differences between isolates. In this study of captive snake isolates of *Cryptosporidium,* 3 distinct genotypes were identified. Future studies should include a wider range of isolates from both captive and wild reptiles and, where possible, necropsies should be performed in order to confirm that the snakes were actually infected and not simply passing oocysts from infected prey.

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