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Genetic Diversity of *Cryptosporidium* spp. in Captive Reptiles

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The genetic diversity of *Cryptosporidium* in reptiles was analyzed by PCR-restriction fragment length polymorphism and sequence analysis of the small subunit rRNA gene. A total of 123 samples were analyzed, of which 48 snake samples, 24 lizard samples, and 3 tortoise samples were positive for *Cryptosporidium*. Nine different types of *Cryptosporidium* were found, including *Cryptosporidium serpentis*, *Cryptosporidium* desert monitor genotype, *Cryptosporidium muris*, *Cryptosporidium parvum* bovine and mouse genotypes, one *C. serpentis*-like parasite in a lizard, two new *Cryptosporidium* spp. in snakes, and one new *Cryptosporidium* sp. in tortoises. *C. serpentis* and the desert monitor genotype were the most common parasites and were found in both snakes and lizards, whereas the *C. muris* and *C. parvum* parasites detected were probably the result of ingestion of infected rodents. Sequence and biologic characterizations indicated that the desert monitor genotype was *Cryptosporidium saurophilum*. Two host-adapted *C. serpentis* genotypes were found in snakes and lizards.

Cryptosporidium infections are common in reptiles and have been reported in at least 57 reptilian species (10). Unlike in other animals in which *Cryptosporidium* infection is usually self-limiting in immunocompetent individuals, cryptosporidiosis in reptiles is frequently chronic and sometimes lethal in snakes. Two *Cryptosporidium* spp. are recognized in reptiles (2, 15): *Cryptosporidium serpentis* in snakes and *Cryptosporidium saurophilum* in lizards, which differ from each other in morphology (oocysts of *C. serpentis* are bigger than those of *C. saurophilum*) and predilection sites (*C. serpentis* is a gastric parasite, whereas *C. saurophilum* is an intestinal parasite). Morphometric studies on isolates recovered from wild snakes and lizards have suggested the occurrence of at least five different morphotypes (12), indicating that it is likely other *Cryptosporidium* spp. may also exist in reptiles.

Until recently there have been few molecular characterizations of *Cryptosporidium* spp. from reptiles. Morgan et al. characterized 15 isolates of *Cryptosporidium* from snakes and lizards and found that the majority of animals were infected with *C. serpentis*, with the rest of the isolates belonging to oocysts of the *Cryptosporidium parvum* bovine genotype (two cases) and *Cryptosporidium muris* (one case), probably from ingested prey or feeder mice (9). Thus, it is difficult to differentiate parasitic *Cryptosporidium* oocysts from those merely passing through the gastrointestinal tract, and some of the previously observed morphotypes may represent oocysts of *C. parvum* and *C. muris* resulting from the ingestion of infected rodents (4). The extent of genetic diversity within *C. serpentis* organisms is also not clear, but *C. serpentis* infection in lizards is usually asymptomatic, whereas the infection in snakes frequently causes clinical diseases (1, 3). Minor genetic differences have been observed between isolates from snakes and those from lizards (16). A *Cryptosporidium* isolate from a desert monitor has recently been shown to be genetically distinct and was related to the intestinal *Cryptosporidium* group (17). It is unclear, however, whether oocysts from the desert monitor belong to *C. saurophilum* from lizards.

In this study, we analyzed 123 samples from snakes, lizards, and tortoises and characterized the small subunit (SSU) rRNA gene of *Cryptosporidium*-positive samples by PCR-restriction fragment length polymorphism (PCR-RFLP) and DNA sequencing. Results of the analysis suggest the existence of extensive genetic diversity and some host adaptations in *Cryptosporidium* isolates from reptiles.

MATERIALS AND METHODS

Samples. A total of 123 diagnostic samples obtained from captive snakes, lizards, and tortoises from the United States, Switzerland, the Czech Republic, Ghana, and Australia were used in this study (Tables 1 through 3). These included 88 samples from snakes, 26 samples from lizards, and 9 samples from tortoises. Another 19 samples from cross-transmission studies (11 snake samples and 8 lizard samples) were also studied. With the exception of samples from the Saint Louis Zoo (78 snake samples, 7 lizard samples, and 3 tortoise samples), Louisville Zoo (6 tortoise samples), and transmission studies, all samples were previously diagnosed as *Cryptosporidium* positive by microscopy, and purified oocysts were used in molecular studies. Samples from the Saint Louis Zoo, Louisville Zoo, and National Zoological Park were mostly feces, with the exception of gastric washings from three snakes in the Saint Louis Zoo.

After initial diagnosis of cryptosporidiosis in snakes in the Saint Louis Zoo at the end of 1998, a cryptosporidiosis control program was initiated, which involved the diagnosis and differentiation of *Cryptosporidium* infection by PCR and euthanasia of *C. serpentis*- or *C. saurophilum*-infected snakes. To monitor the effectiveness of this cryptosporidiosis control measure, 8 to 17 snakes at the zoo were examined for *Cryptosporidium* infection periodically for 1 year from May 1999 to

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TABLE 1. Distribution of *Cryptosporidium* spp. and genotypes in snakes[a]

Snake		Source	Sample no.	Species and/or genotype identified by:	
Common name	Scientific name			RFLP	Sequence analysis
Amazon tree boa	*Corallus hortulanus*	Washington, D.C.	64	*C. serpentis*	*C. serpentis* type A
Ball python	*Python regius*	St. Louis Zoo	812	*C. serpentis*	*C. serpentis* type A
Ball python	*Python regius*	St. Louis Zoo	1999	*C. parvum* mouse genotype	ND
Black rat snake	*Elaphe obsoleta obsoleta*	St. Louis Zoo	762	*C. parvum* mouse genotype	ND
Black rat snake	*Elaphe obsoleta obsoleta*	St. Louis Zoo	756	*C. serpentis*	*C. serpentis* type A
Black rat snake	*Elaphe obsoleta obsoleta*	St. Louis Zoo	757	*C. parvum* mouse genotype	ND
Black rat snake	*Elaphe obsoleta obsoleta*	St. Louis Zoo	758	*C. muris*	*C. muris*
Black rat snake	*Elaphe obsoleta obsoleta*	St. Louis Zoo	825	*C. parvum* mouse genotype	ND
Boa constrictor	*Boa constrictor ortoni*	Maryland	694	*C. serpentis*	ND
Boa constrictor	*Boa constrictor ortoni*	St. Louis Zoo	446	*C. serpentis*	ND
Boa constrictor	*Boa constrictor ortoni*	St. Louis Zoo	819	*C. serpentis*	*C. serpentis* type A
Boa constrictor	*Boa constrictor ortoni*	St. Louis Zoo	821	*C. serpentis*	*C. serpentis* type A
Boa constrictor	*Boa constrictor ortoni*	St. Louis Zoo	899	*C. serpentis*	*C. serpentis* type A
Boa constrictor	*Boa constrictor ortoni*	St. Louis Zoo	1158	*C. serpentis*	*C. serpentis* type A
Boa constrictor	*Boa constrictor ortoni*	St. Louis Zoo	1993	*C. muris*	*C. muris*
Boa constrictor	*Boa constrictor ortoni*	St. Louis Zoo	1996	*C. parvum* mouse genotype	*C. parvum* mouse genotype
Boa constrictor	*Boa constrictor ortoni*	St. Louis Zoo	2162	*Cryptosporidium* sp.	*Cryptosporidium* sp.
Boelen's python	*Morelia boeleni*	St. Louis Zoo	7632	*C. serpentis*	ND
Boelen's python	*Morelia boeleni*	St. Louis Zoo	7634	*C. serpentis*	ND
Boelen's python	*Morelia boeleni*	St. Louis Zoo	7636	*C. serpentis*	ND
Bornmueller's viper	*Vipera bornmuelleri*	St. Louis Zoo	816	*C. serpentis*	*C. serpentis* type A
Bull snake	*Pituophis melanoleucuc sayi*	St. Louis Zoo	815	*C. saurophilum*	*C. saurophilum* type A
California king snake	*Lampropeltis getulus californiae*	St. Louis Zoo	753	*C. serpentis* and *C. parvum* mouse genotype	*C. serpentis* type A
California king snake	*Lampropeltis getulus californiae*	St. Louis Zoo	754	*C. serpentis*	*C. serpentis* type A
California king snake	*Lampropeltis getulus californiae*	St. Louis Zoo	828	*C. serpentis*	*C. serpentis* type A
Corn snake	*Elaphe guttata guttata*	Kansas	18	*C. serpentis*	*C. serpentis* type A
Corn snake	*Elaphe guttata guttata*	Maryland	695	*C. serpentis*	ND
Corn snake	*Elaphe guttata guttata*	St. Louis Zoo	759	*C. serpentis* and *C. parvum* mouse genotype	ND
Corn snake	*Elaphe guttata guttata*	St. Louis Zoo	820	*C. serpentis*	*C. serpentis* type A
Corn snake	*Elaphe guttata guttata*	St. Louis Zoo	760	*C. serpentis*	*C. serpentis* type A
Corn snake	*Elaphe guttata guttata*	St. Louis Zoo	1444	*C. parvum* mouse genotype	*C. parvum* mouse genotype
Corn snake	*Elaphe guttata guttata*	St. Louis Zoo	1446	*C. parvum* mouse genotype	ND
Corn snake	*Elaphe guttata guttata*	St. Louis Zoo	1545	*C. serpentis*	*C. serpentis* type A
Corn snake	*Elaphe guttata guttata*	St. Louis Zoo	1779	*C. muris* and *C. parvum* mouse genotype	*C. muris* and *C. parvum* mouse genotype
Corn snake	*Elaphe guttata guttata*	St. Louis Zoo	1998	*C. parvum* mouse genotype	ND
Corn snake	*Elaphe guttata guttata*	Switzerland	1430	*C. serpentis*	*C. serpentis* type A
Emerald tree boa	*Corallus caninus*	Maryland	693	*C. serpentis*	*C. serpentis* type A
Emerald tree boa	*Corallus caninus*	St. Louis Zoo	1159	*C. parvum* mouse genotype	ND
Fox snake	*Elaphe vulpina gloydi*	St. Louis Zoo	755	*C. serpentis*	*C. serpentis* type A
Fox snake	*Elaphe vulpina gloydi*	St. Louis Zoo	818	*C. parvum* mouse genotype	ND
Louisiana pine snake[b]	*Pituophis ruthveni*	St. Louis Zoo	691	*C. saurophilum*	*C. saurophilum* type A
Louisiana pine snake	*Pituophis ruthveni*	St. Louis Zoo	692	*C. saurophilum*	*C. saurophilum* type A
Milk snake	*Lampropeltis triangulum*	St. Louis Zoo	827	*C. parvum* mouse genotype	ND
Mountain viper	*Vipera wagneri*	St. Louis Zoo	822	*C. serpentis*	*C. serpentis* type A
Mountain viper	*Vipera wagneri*	St. Louis Zoo	2168	*C. serpentis*	*C. serpentis* type A
Mountain viper	*Vipera wagneri*	St. Louis Zoo	7010	*C. serpentis*	*C. serpentis* type A
Prairie king snake	*Lamproletis calligaster*	St. Louis Zoo	761	*C. parvum* mouse genotype	ND
Taipan	*Oxyuranus scutellatus*	Australia	1429	*C. serpentis*	ND

[a] *C. saurophilum*, *Cryptosporidium* desert monitor genotype; ND, not sequenced.
[b] Gastric washing from animal 692.

April 2000, with continuous euthanasia of infected animals (Table 4). To identify the source of isolates belonging to the *C. parvum* mouse genotype and *C. muris* in snakes, fecal samples were also collected from 11 of the feeder mice used in the Saint Louis Zoo and examined for *Cryptosporidium* species and genotypes.

Cross-transmission studies. To evaluate the infectivity of *C. saurophilum* to snakes and lizards, two corn snakes and leopard geckoes were inoculated with 10,000 oocysts originating from a bull snake (sample 815). Forty-five days after the experimental infection, animals were euthanized, and the stomach and intestine and their contents were examined for *Cryptosporidium* oocysts by PCR-RFLP and DNA sequence analysis of the SSU rRNA gene. The infectivity for snakes of *C. serpentis* isolated from lizards was assessed by experimental infection of a captive-born Burmese python (*Python mollurus*) with oocysts isolated from a wild imported juvenile Nile monitor (*Varanus niloticus*) from Togo. *Cryptosporidium* oocysts in the python's feces were genotyped by DNA sequencing of the SSU rRNA gene 88 days after the inoculation. The cross-transmission of *Cryptosporidium* spp. between snakes and lizards was further evaluated by the differentiation of *Cryptosporidium* spp. and genotypes in a group of six snakes and four lizards that were housed in the same room by the SSU rRNA PCR-RFLP analysis (Table 3).

Morphometric measurements. Oocysts of *C. serpentis* from a desert monitor (sample 806) and of *C. saurophilum* from a bull snake (sample 815) were measured under a differential interference contrast microscope at a magnification of ×1,000. Twenty oocysts were measured for *C. saurophilum* organisms, and 37 oocysts were measured for *C. serpentis* organisms. Mean length and width and the shape index were calculated along with the 95% confidence limits (CL) for each species.

DNA extraction. Purified oocysts or fecal samples containing oocysts were used in DNA extraction. DNA was extracted from stool samples by alkaline digestion and phenol-chloroform extraction, followed by DNA purification with a commercial kit. Briefly, 33.3 μl of 1 M KOH and 9.3 μl of 1 M dithiothreitol were added to a 1.5-ml microcentrifuge tube containing 100 μl of stool or oocyst sus-

TABLE 2. Distribution of *Cryptosporidium* spp. and genotypes in lizards and tortoises

Animal		Source	Sample no.	Species and/or genotype identified by[a]:	
Common name	Scientific name			RFLP	Sequence analysis
Gecko	*Gekkoninae* sp.	Switzerland	1433	*C. parvum* bovine genotype	*C. parvum* bovine genotype
Gecko	*Gekkoninae* sp.	Switzerland	1507	*C. saurophilum* and *C. parvum* bovine genotype	*C. saurophilum*
Gecko	*Gekkoninae* sp.	Switzerland	1508	*C. parvum* bovine genotype	*C. parvum* bovine genotype
Leopard gecko	*Eublepharis macularius*	Czech Republic	1665	*C. serpentis*	*C. serpentis* like
Leopard gecko	*Eublepharis macularius*	Czech Republic	7381	*C. saurophilum*	*C. saurophilum*
Green iguana	*Iguana iguana*	Switzerland	1431	*C. saurophilum*	*C. saurophilum*
Green iguana	*Iguana iguana*	Switzerland	1432	*C. parvum* bovine genotype	*C. parvum* bovine genotype
Green iguana	*Iguana iguana*	Switzerland	1506	*C. saurophilum* and *C. parvum* bovine genotype	ND
Desert monitor	*Varanus griseus*	Czech Republic	1667	*C. serpentis*	*C. serpentis* type B
Desert monitor	*Varanus griseus*	St. Louis Zoo	340	*C. saurophilum*	*C. saurophilum*
Desert monitor	*Varanus griseus*	St. Louis Zoo	600	*C. sepentis*	
Desert monitor	*Varanus griseus griseus*	St. Louis Zoo	806	*C. serpentis*	*C. serpentis* type A
Mangrove monitor	*Varanus indicus*	St. Louis Zoo	808	*C. parvum* mouse genotype	ND
Monitor	*Varanus* sp.	Switzerland	1434	*C. saurophilum*	*C. saurophilum*
Monitor	*Varanus* sp.	Switzerland	1504	*C. saurophilum* and *C. parvum* bovine genotype	*C. saurophilum*
Monitor	*Varanus* sp.	Switzerland	1505	*C. saurophilum*	*C. saurophilum*
Nile monitor	*Varanus niloticus*	Czech Republic	1172a	*C. serpentis*	*C. serpentis* type B
Savannah monitor	*Varanus exanthematicus*	Czech Republic	844	*C. serpentis*	*C. serpentis* type B
Savannah monitor	*Varanus exanthematicus*	Washington, D.C.	40	*C. serpentis*	*C. serpentis* type B
Savannah monitor	*Varanus exanthematicus*	Washington, D.C.	41	*C. serpentis*	ND
Savannah monitor	*Varanus exanthematicus*	Washington, D.C.	63	*C. serpentis*	*C. serpentis* type B
Savannah monitor	*Varanus exanthematicus*	Washington, D.C.	521	*C. serpentis* lizard type	*C. serpentis* type B
Plated lizard	*Gerrhosaurus* sp.	St. Louis Zoo	1786	*C. saurophilum*	*C. saurophilum*
Skink	*Mabuya perrotetii*	Ghana	956	*C. serpentis*	*C. serpentis* type B
Star tortoise	*Geochelone elegans*	Louisville Zoo	747	*Cryptosporidium* tortoise genotype	*Cryptosporidium* tortoise genotype
Star tortoise	*Geochelone elegans*	Louisville Zoo	750	*Cryptosporidium* tortoise genotype	*Cryptosporidium* tortoise genotype
Star tortoise	*Geochelone elegans*	Louisville Zoo	751	*Cryptosporidium* tortoise genotype	*Cryptosporidium* tortoise genotype

[a] *C. saurophilum*, *Cryptosporidium* desert monitor genotype; ND, not sequenced.

pension. After incubation at 65°C for 15 min, the solution was neutralized with 4.3 μl of 25% hydrochloric acid and buffered with 80 μl of 2 M Tris-HCl (pH 8.3). The DNA was extracted with 250 μl of phenol-chloroform-isoamyl alcohol (Invitrogen, Carlsbad, Calif.) after thorough mixing and centrifugation in an Eppendorf (Hamburg, Germany) microcentrifuge at 5,000 × *g* for 5 min. The supernatant was transferred to a 2.0-ml Eppendorf tube containing 1.0 ml of ASL buffer from the QIAamp DNA Stool Mini Kit (QIAGEN, Valencia, Calif.). The DNA was further purified following the manufacturer-suggested procedures. DNA was stored at −70°C before it was used in molecular analysis.

Species differentiation and genotyping. *Cryptosporidium* spp. and *C. parvum* genotypes present were diagnosed by a PCR-RFLP technique (13, 16, 17). In this method, a segment (~833 bp) of the *Cryptosporidium* SSU rRNA gene was amplified by nested PCR. Species and genotype diagnosis was made by restriction digestion of the secondary PCR product with *Ssp*I (New England BioLabs, Beverly, Mass.) and *Vsp*I (Promega, Madison, Wis.). Each sample was examined at least twice by independent PCR-RFLP analyses. To confirm the diagnosis of new *Cryptosporidium* spp. and to identify genetic heterogeneity within *C. serpentis* and *C. saurophilum*, secondary PCR products were sequenced in both directions on an ABI Prism 3100 analyzer (Applied Biosystems, Foster City, Calif.) by using forward and reverse primers, after PCR products had been purified with the Wizard PCR Prep Kit (Promega). Nucleotide sequences obtained from this study were aligned against each other by using the ClustalX (11) program and manual adjustment. A neighbor-joining tree was constructed from the aligned sequences as previously described by using the Treecon program, and genetic distances were calculated with the Kimura 2-parameter model (17).

Because of the presence of mixed *Cryptosporidium* species in samples from snakes and lizards that were housed together in cross-transmission studies, PCR products from one of the snakes (sample 938) and one of the lizards (sample 944) were cloned into a pGEM-T vector (Promega). Eight (for sample 944) or 15 (for sample 938) clones were sequenced for each PCR product to confirm the diagnosis.

Nucleotide sequence accession numbers. The nucleotide sequences of the partial SSU rRNA gene have been deposited in the GenBank database under accession numbers AF093499, AF093501, AF112573, AY120913 through AY120915, and AY268581 through AY268584.

RESULTS

***Cryptosporidium* spp. in reptiles.** SSU rRNA PCR confirmed that all snake and lizard samples from Maryland (three snake samples), Washington, D.C. (one snake sample and four lizard samples), Kansas (one snake sample), Ghana (one lizard sample), the Czech Republic (five lizard samples), Switzerland (one snake sample and nine lizard samples), and Australia (one snake sample and one lizard sample) were positive for *Cryptosporidium*. The samples were previously diagnosed as *Cryptosporidium* positive by microscopy. In contrast, 3 of the 6 tortoise samples from the Louisville Zoo, 36 of 81 snake samples, 4 of 7 lizard samples, and 0 of 3 tortoise samples from the Saint Louis Zoo were positive for *Cryptosporidium* by PCR. These animals had not been previously screened for *Cryptosporidium* oocysts by microscopy.

PCR-RFLP analysis of SSU rRNA PCR revealed banding patterns distinctive for *C. serpentis* and the *Cryptosporidium* desert monitor genotype (Tables 1 and 2). *Cryptosporidium serpentis* was found in 28 of 48 positive snake samples and 11 of 25 positive lizard samples, and the *Cryptosporidium* desert monitor genotype was identified in 3 of 48 positive snake samples and 9 of 24 positive lizard samples. Sequence analyses confirmed the results of RFLP analyses but also revealed genetic diversities within *C. serpentis* and the *Cryptosporidium* desert monitor genotype. Most desert monitor genotype isolates had identical SSU rRNA sequences, but isolates 1343 and 1786 had one single nucleotide polymorphism (SNP). Two genotypes (A and B) were seen in *C. serpentis* isolates, which

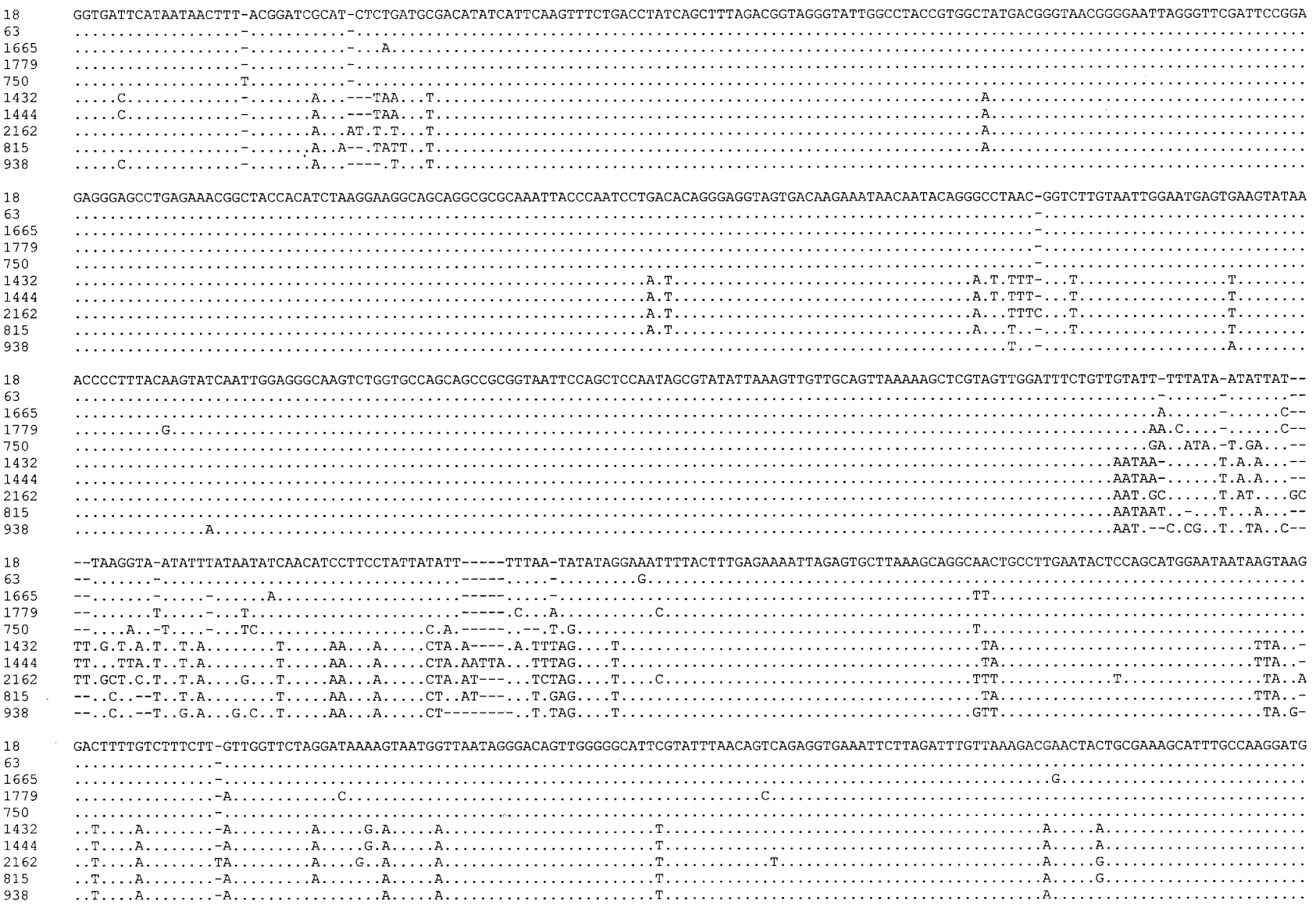

FIG. 1. Sequence diversity in the SSU rRNA gene among *Cryptosporidium* in reptiles. Dots denote sequence identity to isolate 18, dashes represent nucleotide deletions. Isolate designations: 18, *C. serpentis* genotype A; 63, *C. serpentis* genotype B; 1665, a *C. serpentis*-like *Cryptosporidium* in lizard 1665; 1779, *C. muris*; 750, *Cryptosporidium* tortoise genotype; 1432, *C. parvum* bovine genotype; 1444, *C. parvum* mouse genotype; 2162, another new *Cryptosporidium* genotype in snake 2162; 815, *C. saurophilum*; 938, a new *Cryptosporidium* genotype in snake 938.

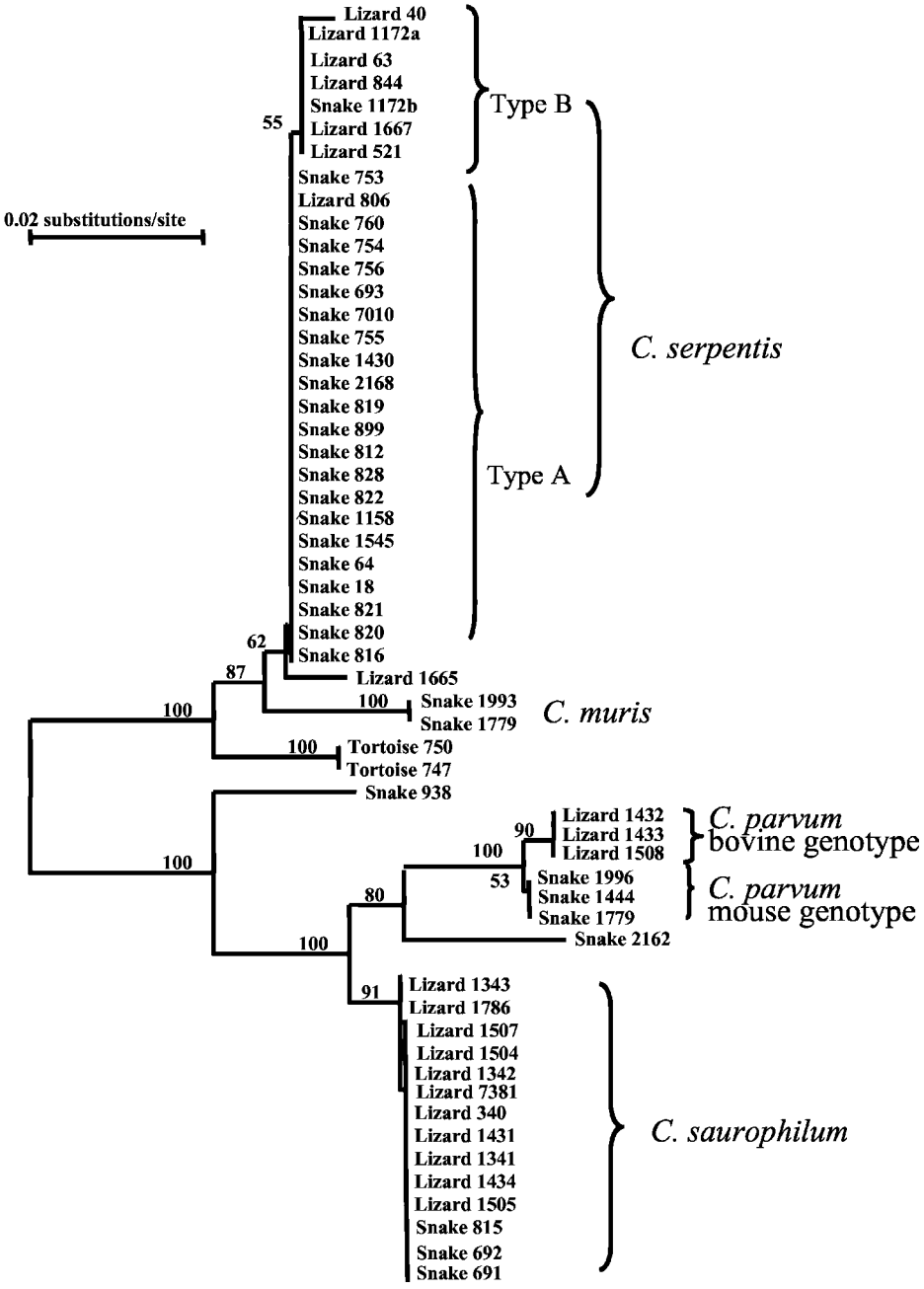

FIG. 2. Genetic relationship between *Cryptosporidium* spp. in reptiles inferred by a neighbor-joining analysis of the partial SSU rRNA gene sequences by using the Kimura two-parameter model and the Treecon program. Numbers on branches are percentage bootstrap values of 1,000 replicates. Only values above 50% are shown.

differed from each other by one SNP (Fig. 1). One isolate from a lizard (sample 1665) had the *C. serpentis* RFLP banding pattern, was related to the two *C. serpentis* genotypes, but had significant differences in nucleotide sequence (Fig. 1).

Several other *Cryptosporidium* spp. were also identified in reptiles. RFLP analysis showed banding patterns of the *C. parvum* mouse genotype in 12 snakes and 1 lizard, of the *C. parvum* bovine genotype in 6 lizards, and of *C. muris* in 3 snakes. DNA sequence analysis confirmed the identifications, as the sequences obtained were identical to previously reported sequences (16, 17). Two other new *Cryptosporidium* spp. were isolated in reptiles, as shown by both RFLP and sequence analyses. In RFLP analyses, one isolate from a snake (sample 2162) had an *Ssp*I band of over 800 bp but had a *Vsp*I band similar to the size of the band of the desert monitor genotype (between 600 to 700 bp). In contrast, three tortoise isolates had a slightly smaller *Ssp*I band size (near 800 bp) but had a *Vsp*I band similar to the band of *C. serpentis* (over 700 bp). DNA sequencing revealed that the sequences represented two new *Cryptosporidium* spp. A neighbor-joining analysis indicated

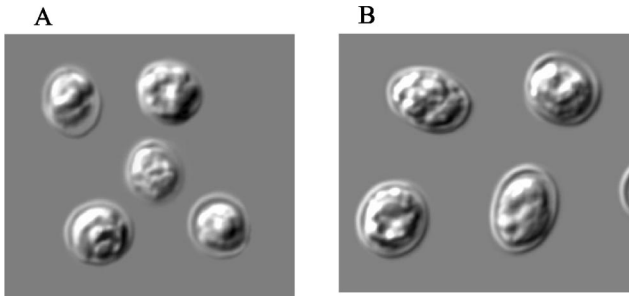

FIG. 3. Morphology of *C. saurophilum* (A) and *C. serpentis* (B) as seen under a differential interference contrast microscope (magnification, ×1,000).

that the tortoise genotype was related to *C. serpentis* and *C. muris*, whereas the new snake genotype was related to *C. parvum* (Fig. 2).

Because *C. muris* and the *C. parvum* mouse genotype were seen in high frequency in snakes and lizards from the Saint Louis Zoo, whose diet contained mice, fecal samples were taken from 11 feeder mice and analyzed for *Cryptosporidium*. Three such samples were positive for *Cryptosporidium* by PCR analysis of the SSU rRNA gene. RFLP and sequence analyses showed the presence of the *C. parvum* mouse genotype in two mice and a mixed infection of *C. muris* and the *C. parvum* mouse genotype in one mouse.

The identification of the desert monitor genotype as *C. saurophilum*. Previous characterization of the *Cryptosporidium* desert monitor genotype showed that the parasite is closely related to intestinal *Cryptosporidium* spp. Because the only known intestinal *Cryptosporidium* parasite in reptiles is *C. saurophilum*, morphometric measurements were done on the desert monitor genotype, and the data obtained were compared with those from *C. serpentis* and those previously reported for *C. saurophilum*. Oocysts of the desert monitor genotype were visibly smaller than those of *C. serpentis* (Fig. 3), with a mean length of 4.94 μm, a mean width of 4.49 μm, and a shape index (the length/width ratio) of 1.14. In comparison, oocysts of *C. serpentis* were 5.94 μm in length and 5.11 μm in width and had a shape index of 1.17 (Table 5). Thus, the morphometric measurements of the desert monitor genotype were similar to those previously reported for *C. saurophilum* (mean, 5.0 × 4.7 μm; range, 4.4 to 5.6 × 4.2 to 5.2 μm) (6).

Cross-transmission of *Cryptosporidium* between snakes and lizards. To assess the ability for cross-transmission of *Cryptosporidium* spp. between snakes and lizards, an isolate of the *Cryptosporidium* desert monitor genotype originating from a snake was used to inoculate two corn snakes and two leopard geckoes that were free of *Cryptosporidium* infection by microscopy. Both geckoes started to shed oocysts 21 days after inoculation, but both snakes remained negative through the observation period. All four inoculated animals were euthanized 45 days after the inoculation, and tissue sections were taken from the stomach and intestine for histology and PCR-RFLP analysis. *Cryptosporidium* was not found in any of the tissue sections from the snakes. In contrast, *Cryptosporidium* in developmental stages was seen in hematoxylin and eosin-stained gastric tissues. PCR analysis of DNA from gastric and intestinal fragments confirmed that both snakes were negative for *Cryptosporidium*. Positive PCR amplifications, however, were obtained with DNA from the intestine of one gecko and from both the stomach and intestine of the other gecko. RFLP analysis of the PCR products with *Ssp*I and *Vsp*I indicated that the parasites present belonged to the *Cryptosporidium* desert monitor genotype (Table 3). The python inoculated with *C. serpentis* oocysts from a Nile monitor started to shed *Cryptosporidium* oocysts 88 days after inoculation. PCR-RFLP analysis of oocysts isolated from the snake confirmed that it belonged to *C. serpentis* (Table 3). Sequence analysis produced an SSU rRNA sequence identical to that of the isolate from the monitor, *C. serpentis* type B (Fig. 2).

TABLE 3. Cross-transmission of *Cryptosporidium* spp. between lizards and snakes

Source of infection	Recipient host (tissues sampled)	Scientific name	Sample no.	*Cryptosporidium* parasite(s) identified[a]
Inoculated with *C. saurophilum* of snake origin (sample 815)	Corn snake S1 (stomach)	*Elaphe guttata guttata*	1336	Negative
	Corn snake S1 (intestine)	*Elaphe guttata guttata*	1337	Negative
	Corn snake S2 (stomach)	*Elaphe guttata guttata*	1338	Negative
	Corn snake S2 (intestine)	*Elaphe guttata guttata*	1339	Negative
	Leopard gecko, G1 (stomach)	*Eublepharis macularius*	1340	Negative
	Leopard gecko, G1 (intestine)	*Eublepharis macularius*	1341	*C. saurophilum*
	Leopard gecko G2 (stomach)	*Eublepharis macularius*	1342	*C. saurophilum*
	Leopard gecko G2 (intestine)	*Eublepharis macularius*	1343	*C. saurophilum*
Inoculated with *C. serpentis* of Nile monitor (sample 1172a)	Burmese python	*Python mollurus*	1172b	*C. serpentis* type B
Natural exposure via shared housing	Black rat snake	*Elaphe obsolete*	940	*C. serpentis*-*Cryptosporidium* sp.-*C. saurophilum*
	Green python	*Chondropython viridis*	941	*C. serpentis*-*Cryptosporidium* sp.-*C. saurophilum*
	Milk snake	*Lampropeltis triangulum*	939	*C. serpentis*-*Cryptosporidium* sp.-*C. saurophilum*
	New Guinea viper boa	*Candoia asper*	938	*C. serpentis*-*Cryptosporidium* sp.-*C. saurophilum*
	Pine snake	*Pituophis melanoleucus*	936	*C. serpentis*-*Cryptosporidium* sp.-*C. saurophilum*
	Pine snake	*Pituophis melanoleucus*	937	*C. serpentis*-*Cryptosporidium* sp.-*C. saurophilum*
	Bearded dragon	*Pogona vitticep*	944	*C. serpentis*-*C. saurophilum*
	Gargoyle gecko	*Rhodocodactylus auriculatus*	945	*C. serpentis*-*C. saurophilum*
	Mountain chameleon	*Chamaeleo montium*	942	*C. serpentis*-*C. saurophilum*
	Mountain chameleon	*Chamaeleo montium*	943	*C. serpentis*-*C. saurophilum*

[a] *C. saurophilum*, *Cryptosporidium* desert monitor genotype.

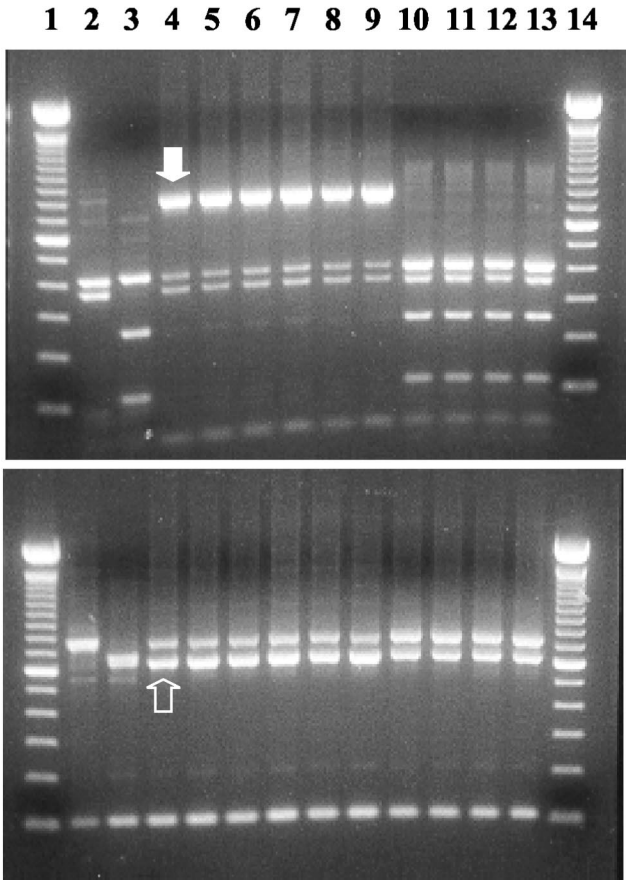

FIG. 4. Simultaneous presence of multiple *Cryptosporidium* spp. in a group of six snakes and four lizards housed together as revealed by PCR-RFLP analyses of the SSU rRNA gene. The upper panel shows the results of *Ssp*I digestion; the lower panel shows the results of *Vsp*I digestion. Lanes 1 and 14, 100-bp molecular markers; lane 2, positive control for *C. serpentis*; lane 3, positive control for *C. saurophilum*; lane 4, sample from pine snake 936; lane 5, sample from pine snake 937; lane 6, sample from a New Guinea viper boa (938); lane 7, sample from a milk snake (939); lane 8, sample from a black rat snake (940); lane 9, sample from a green python (941); lane 10, sample from mountain chameleon 942; lane 11, sample from mountain chameleon 943; lane 12, sample from a bearded dragon (944); lane 13, sample from a gargoyle gecko (945). Filled and open arrows are the *Ssp*I and *Vsp*I bands, respectively, for the new *Cryptosporidium* genotype in snakes. Three *Cryptosporidium* spp. (*C. serpentis*, a new *Cryptosporidium* genotype, and a trace of *C. saurophilum*) are seen in all six snakes (lanes 4 to 9), and two parasites (*C. serpentis* and *C. saurophilum*) are seen in all 4 lizards (lanes 10 to 13).

The cross-transmission of *Cryptosporidium* spp. between snakes and lizards was further assessed by the differentiation of *Cryptosporidium* spp. in a group of six snakes and four lizards that were housed in the same room. All snakes and lizards were positive for *Cryptosporidium*. PCR-RFLP analysis indicated that all animals were infected with multiple *Cryptosporidium* spp.; the six snakes were all infected with *C. serpentis*, a new *Cryptosporidium* genotype, and low levels of *C. saurophilum*, whereas the four lizards were all infected with *C. serpentis* and *C. saurophilum* (Fig. 4 and Table 3). Analysis of the cloned PCR products confirmed the diagnosis of *C. serpentis* and the new *Cryptosporidium* genotype in snakes and *C. serpentis* and *C. saurophilum* in lizards. Among the 15 clones of a PCR product from the snake isolate 938, 4 clones were identified as *C. serpentis*, and 11 clones were identified as the new *Cryptosporidium* genotype. Likewise, four of the eight clones of a PCR product from lizard isolate 944 belonged to *C. saurophilum*, and the remaining four clones belonged to *C. serpentis*. The new *Cryptosporidium* genotype was genetically related to the intestinal *Cryptosporidium* spp. and had a large *Ssp*I band (about 800 bp), similar to the tortoise genotype (Fig. 4, filled arrow), but had a *Vsp*I upper band (just over 600 bp) similar to that of *C. saurophilum* (Fig. 4, open arrow).

TABLE 4. Effectiveness of a diagnosis-euthanasia strategy on the occurrence of *Cryptosporidium* infection in snakes at the Saint Louis Zoo[a]

Sample date	No. of samples	Total no. of positives[b]	No. of isolates positive for the indicated parasite	
			C. serpentis	*C. saurophilum*
May 1999	10	5	5	
June 1999	17	8	7	1
September 1999	8	0		
November 1999	10	0		
December 1999	10	1	1	
March 2000	8	0		
April 2000	9	0		

[a] Euthanasia of infected animals started in March 1999 after the initial identification of cryptosporidiosis in snakes and lizards at the zoo between December 1998 and January 1999.

[b] *C. muris* and the *C. parvum* mouse genotype are excluded from the number of positives.

Effectiveness of the diagnosis-euthanasia control strategy. At the Saint Louis Zoo, a diagnosis-euthanasia program was initiated in March 1999 after the identification between December 1998 and January 1999 of chronic cryptosporidiosis in snakes. To monitor the effectiveness of the control measures, samples were periodically taken from snakes for 1 year. Right after the initiation of the control measure, 5 of 10 and 8 of 17 snakes sampled were positive for *C. serpentis* or *C. saurophilum* in May and June 1999, respectively. Afterwards, only 1 of 45 snake samples taken at five different time periods was positive for *C. serpentis* (Table 4).

DISCUSSION

A total of nine *Cryptosporidium* spp. were found in captive snakes, lizards, and tortoises in this study. The most common parasites were *C. serpentis* and the *Cryptosporidium* desert monitor genotype identified before (16, 17). Both parasites were detected in snakes as well as lizards. Two other *Cryptosporidium* spp. previously reported in captive snakes, *C. muris*

TABLE 5. Morphometric measurements (micrometers) of *Cryptosporidium* desert monitor genotype (*C. saurophilum*) in comparison with those of *C. serpentis*[a]

Parameter	Desert monitor genotype (n = 20)		*C. serpentis* (n = 37)	
	Mean	95% CL	Mean	95% CL
Length	4.94	4.81–5.07	5.94	5.82–6.06
Width	4.49	4.35–4.63	5.11	5.03–5.19
Shape index	1.14	1.11–1.17	1.17	1.14–1.20

[a] n, number of oocysts.

and the *C. parvum* mouse genotype, were also found in some snakes and one lizard. Another common *Cryptosporidium* parasite in mammals, the *C. parvum* bovine genotype, was also identified in six lizards from Switzerland. Four other *Cryptosporidium* spp. detected in this study, however, presented new *Cryptosporidium* spp.: a tortoise genotype identified in three tortoises, two new snake genotypes (one genotype identified in only one snake and the other genotype identified in six snakes), and another new *Cryptosporidium* genotype from a lizard (sample 1665), which was genetically distinct but was related to *C. serpentis*.

Molecular and biologic characterizations indicated that the *Cryptosporidium* desert monitor genotype was probably *C. saurophilum*. Phylogenetically, the desert monitor genotype belonged to the intestinal *Cryptosporidium* parasite group, indicating that it was most probably an intestinal *Cryptosporidium* parasite, which is in agreement with the initial description of *C. saurophilum* (6). Morphologically, oocysts of the desert monitor genotype were very similar to those of *C. saurophilum* in shape and size and were significantly smaller than oocysts of *C. serpentis* (6). Biologically, the desert monitor genotype preferentially infected lizards. Although the desert monitor genotype was found in a few snakes in this study, cross-transmission studies by oocyst inoculation or habitat sharing indicated that the infectivity to lizards was much higher than to snakes, which explains the failure of the establishment of the desert monitor genotype in two corn snakes inoculated with oocysts of this parasite. Infection with *C. saurophilum* may not be restricted to the intestine as previously suggested (6), because it was also found in gastric washings of several snakes infected with *C. saurophilum* and in the stomach tissue section of one experimentally infected lizard.

Oocysts of the *C. parvum* bovine and mouse genotypes and *C. muris* found in some of the snakes and lizards in this study probably do not represent true parasites of these animals. Instead, the oocysts were probably from rodents ingested by these carnivorous reptiles (4). This possibility was supported by the fact that none of the animals with these oocysts had clinical signs and by the presence of organisms belonging to *C. muris* and the *C. parvum* mouse genotype in some of the feeder mice which were fed to snakes and some lizards in the Saint Louis Zoo. *C. muris* and the *C. parvum* mouse genotype have previously been reported in captive snakes and lizards (9). Although the *C. parvum* bovine genotype has not been found in mice in the United States, it has been previously reported in mice in Australia (8). Thus, oocysts of the *C. parvum* bovine genotype seen in lizards in Switzerland could also be from ingested prey or feeder mice. Previously, it was shown that oocysts of the *C. parvum* bovine genotype were not infectious to snakes (4). Nevertheless, the possibility of organisms belonging to the *C. parvum* mouse and bovine genotypes and to *C. muris* infecting reptiles can only be totally ruled out by careful biologic and genetic studies.

Because the four new *Cryptosporidium* spp. found in this study have never been reported in other animals before, they probably were true parasites of these captive reptiles. The *Cryptosporidium* parasite in snake 1665 was clearly phylogenetically related to *C. serpentis*, even though significant differences between these two *Cryptosporidium* spp. (Fig. 1) were present. Likewise, the *Cryptosporidium* genotype found in three tortoises was related to *C. serpentis* and has also been found recently in a turtle in Portugal (L. Xiao and M. Alves, unpublished data). One of the two new *Cryptosporidium* spp. identified in snakes in this study was relatively common, because it was found in snake 938 and five other snakes in this study and was previously found in several storm water samples (genotype W11) in New York (13). The other snake genotype had only been found in one animal (snake 2162).

There were intraspecies genetic variations within *C. serpentis* and *C. saurophilum*. Two genotypes of *C. serpentis* were seen in the study, which differed from each other by one SNP. Likewise, most *C. saurophilum* isolates produced SSU rRNA sequences similar to the one for the desert monitor genotype reported previously (17). Two isolates, however, had one SNP. It is not clear whether the minor sequence difference in *C. saurophilum* was due to differences between copies of the SSU rRNA gene, as demonstrated in other *Cryptosporidium* spp. (7, 14). Even though *C. serpentis* was named for the *Cryptosporidium* parasite originally identified in snakes by Brownstein et al. (1) and *C. saurophilum* was named for a *Cryptosporidium* parasite in lizards (6), both parasites apparently have a host range broader than previously believed. Nevertheless, data from this study suggest the presence of host adaptation; most snakes (except for sample 1172, which was experimentally infected with an isolate from a lizard) had a *C. serpentis* genotype A sequence, whereas most lizards (except for sample 806) had a *C. serpentis* genotype B sequence.

Currently, there are no effective control strategies against cryptosporidiosis in reptiles. In a small-scale study, it was demonstrated that snakes with clinical and subclinical cryptosporidiosis could be effectively treated with hyperimmune bovine colostrum raised against *C. parvum* (5). A common control practice is to euthanize *Cryptosporidium*-infected snakes, which would prevent the spread of infection to other animals. This diagnosis-euthanasia strategy was apparently effective in the control of *Cryptosporidium* infection in snakes in the Saint Louis Zoo in this study. The effectiveness of the method was supported by the evident reduction of *C. serpentis* infection in snakes at the zoo. In addition to the premature death of infected animals, one problem with the control measure is the frequent presence of oocysts of *C. muris* and the *C. parvum* mouse genotype in snakes because of the use of feeder mice as part of the diet. Because it is difficult to differentiate oocysts of the pathogenic *C. serpentis* from those of nonpathogenic *Cryptosporidium* spp. that merely pass through the gastrointestinal tract, the diagnosis-euthanasia control strategy would lead to the killing of uninfected animals.

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