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Identification of Novel *Cryptosporidium* Genotypes from the Czech Republic

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Isolates of *Cryptosporidium* from the Czech Republic were characterized from a variety of different hosts using sequence and phylogenetic analysis of the 18S ribosomal DNA and the heat-shock (HSP-70) gene. Analysis expanded the host range of accepted species and identified several novel genotypes, including horse, Eurasian woodcock, rabbit, and cervid genotypes.

The protozoan parasite *Cryptosporidium* has been identified as the cause of numerous waterborne, food-borne, and day-care outbreaks of diarrheal disease worldwide (5, 6). Currently cryptosporidiosis represents the major public health concern of water utilities in developed nations (6).

At present, 13 species of *Cryptosporidium* are regarded as valid on the basis of differences in oocyst morphology, site of infection, vertebrate class specificity, and genetic differences: *Cryptosporidium muris*, which infects rodents; *Cryptosporidium andersoni*, which infects cattle; *Cryptosporidium parvum*, which infects cattle, humans, and other mammals; *Cryptosporidium hominis*, which infects humans; *Cryptosporidium meleagridis*, *Cryptosporidium baileyi*, and *Cryptosporidium galli* in birds; *Cryptosporidium serpentis* in snakes and lizards; *Cryptosporidium saurophilum* in snakes and lizards; *Cryptosporidium molnari* in fish; *Cryptosporidium wrairi* from guinea pigs; *Cryptosporidium felis* in cats; and *Cryptosporidium canis* in dogs (1, 6, 7, 8, 16, 24, 28).

C. parvum is the most widely studied species, and there is now strong evidence that there are numerous genetically distinct genotypes within the *C. parvum* group, which are likely to be cryptic species (12, 16, 26, 28). The *C. parvum* cattle genotype and *C. hominis* are responsible for the majority of human infections (16); however, increasingly, novel *Cryptosporidium* genotypes are being identified, and it appears that the transmission dynamics of *Cryptosporidium* is more complicated than previously thought (23, 27, 28).

There are currently no effective chemotherapeutics available for *Cryptosporidium* (6), and therefore, in order to adequately control outbreaks of the disease, a thorough understanding of the transmission dynamics of *Cryptosporidium* is required. We genetically typed 73 isolates of *Cryptosporidium* from different hosts from the Czech Republic. Results have identified several novel genotypes and expanded the host range of previously accepted species.

Sample collection and processing. All samples were collected from various host species from the Czech Republic (Table 1). The majority of isolates were obtained from large farms under intensive production conditions or from the Prague Zoo. Human-derived isolates were obtained from hospitalized patients. Fecal samples were examined using routine coprological methods (2, 3, 20). Oocyst identification was done at ×40 to ×100 magnification. Positive samples were concentrated and purified using Sheather's sugar flotation and stored in 2.5% potassium dichromate at +4°C until required for molecular analysis.

18S rDNA gene amplification and sequencing. DNA was purified using a QiAmp stool kit (Qiagen, Hilden, Germany). A two-step nested PCR protocol was used to amplify the 18S rDNA gene. For the primary PCR, a PCR product of 763 bp was amplified using the forward primer 18SiCF2 (5′-GAC ATA TCA TTC AAG TTT CTG ACC-3′) (base pair position 292) and the reverse primer 18SiCR2 (5′-CTG AAG GAG TAA GGA ACA ACC-3′) (base pair position 1007). The PCR mixture consisted of 200 μM (each) deoxynucleoside triphosphates, 1× PCR buffer (Fisher Biotech, Perth, Australia), 1.5 mM $MgCl_2$, 0.5 U of *Taq* polymerase (Fisher Biotech), and 12.5 pmol of forward and reverse primers in a total 25-μl reaction mixture. Forty-five PCR cycles (94°C for 30 s, 58°C for 30 s, 72°C for 30 s) were carried out in a Perkin Elmer Gene Amp PCR 2400 thermocycler with an initial hot start (94°C for 5 min) and a final extension (72°C for 10 min). For the secondary PCR, a fragment of ~587 bp was amplified using 1 μl of primary PCR product and nested forward 18SiCF1 (5′-CCT ATC AGC TTT AGA CGG TAG G-3′) (base pair position 289) and nested reverse 18SiCR1 (5′-TCT AAG AAT TTC ACC TCT GAC TG-3′) (base pair position 851) primers. The conditions for the secondary PCR were identical to those for the primary PCR. Secondary PCR products were sequenced directly in both directions. Each isolate was sequenced at least twice. TAQ Extender (Stratagene, La Jolla, Calif.) was included in all reactions to minimize PCR error.

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TABLE 1. Isolates of *Cryptosporidium* used in this study

Isolate code	Host species	Species, genotype (18S)	Species, genotype (HSP-70)
Czech-B1-1	Yellow rat snake, *Elaphe obsolete quadrivittata*	*C. serpentis*	*C. serpentis*
Czech-B1-2	*Elaphe obsolete quadrivittata*	*C. serpentis*	*C. serpentis*
Czech-B1-3	*Elaphe obsolete quadrivittata*	*C. serpentis*	*C. serpentis*
Czech-B1-4	*Elaphe obsolete quadrivittata*	*C. serpentis*	*C. serpentis*
Czech-B1-5	*Elaphe obsolete quadrivittata*	*C. serpentis*	*C. serpentis*
Czech-B1-6	Rosy boa, *Lichanura trivirgata*	*C. serpentis*	*C. serpentis*
Czech-B1-7	Colombian rainbow, *Epicrates cenchriamaurus*	*C. serpentis*	*C. serpentis*
Czech-B1-8	Crocodile monitor, *Varanus salvadori*	*C. muris*	ND[a]
Czech-B1-9	Nikolski viper, *Vipera nikolski*	*C. serpentis*	ND
Czech-B1-10	Desert monitor, *Varanus griseus*	*C. saurophilum*	ND
Czech-B1-11	Turkey chick, *Meleagris gallopavo*	*C. meleagridis*	*C. meleagridis*
Czech-B1-12	*Meleagris gallopavo*	*C. meleagridis*	*C. meleagridis*
Czech-B1-13	Chicken, *Gallus gallus* f. dom.	*C. baileyi*	*C. baileyi*
Czech-B1-14	*Gallus gallus* f. dom.	*C. baileyi*	*C. baileyi*
Czech-B1-15	*Gallus gallus* f. dom.	*C. baileyi*	*C. baileyi*
Czech-B1-16	*Gallus gallus* f. dom.	*C. baileyi*	*C. baileyi*
Czech-B1-17	Duck, *Anas plathyryncha* f. dom.	*C. baileyi*	*C. baileyi*
Czech-B1-18	*Anas plathyryncha* f. dom.	*C. baileyi*	*C. baileyi*
Czech-B1-19	*Anas plathyryncha* f. dom.	*C. baileyi*	*C. baileyi*
Czech-B1-20	Black-headed gull, *Larus ridibundus*	*C. baileyi*	*C. baileyi*
Czech-B1-21	Ostrich, *Struthio camelus*	*C. baileyi*	*C. baileyi*
Czech-B1-22	Channel-billed toucan, *Rhamphastus vitellinus*	*C. baileyi*	*C. baileyi*
Czech-B1-23	Red-rumped cocique, *Cacicus haemorrhous*	*C. baileyi*	*C. baileyi*
Czech-B1-24	Crested oropendola, *Psaracolius decumanus*	*C. baileyi*	*C. baileyi*
Czech-B1-29	Red-crowned amazon, *Amazona dufresniana*	*C. baileyi*	*C. baileyi*
Czech-B1-30	Rose-ringed parakeet, *Psittacula krameri*	*C. baileyi* + *C. meleagridis*	*C. baileyi* + *C. meleagridis*
Czech-B1-32	*Pullet*, *Gallus gallus* f. dom.	*C. meleagridis*	*C. meleagridis*
Czech-B1-33	Calf, *Bos taurus*	*C. parvum*, cattle genotype	*C. parvum*, cattle genotype
Czech-B1-34	Calf, *Bos taurus*	*C. parvum*, cattle genotype	*C. parvum*, cattle genotype
Czech-B1-35	Piglet, *Sus scrofa*	*Cryptosporidium*, pig genotype	*Cryptosporidium*, pig genotype
Czech-B1-36	Rabbit, *Oryctolagus cuniculus*	*C. parvum*, rabbit genotype	*C. parvum*, rabbit genotype
Czech-B1-37	*Oryctolagus cuniculus*	*C. parvum*, rabbit genotype	*C. parvum*, rabbit genotype
Czech-B1-38	Domestic cat, *Felis domestica*	*C. felis*	*C. felis*
Czech-B1-39	*Felis domestica*	*C. felis*	*C. felis*
Czech-B1-40	Nutria, *Myocastor coypus*	*C. parvum*, cattle genotype	*C. parvum*, cattle genotype
Czech-B1-41	Prezewalski's wild horse, *Equus przewalskii*	*C. parvum*, horse genotype	ND
Czech-B1-42	Blesbok, *Damaliscus dorcas philipsi*	Cervid genotype	Cervid genotype
Czech-B1-43	Boy (age 3), *Homo sapiens*	*C. parvum*, cattle genotype	*C. parvum*, cattle genotype
Czech-B1-44	Boy (age 6)	*C. parvum*, cattle genotype	*C. parvum*, cattle genotype
Czech-B1-45	Girl (age 17)	*C. parvum*, cattle genotype	*C. parvum*, cattle genotype
Czech-B1-46	Laboratory mouse, *Mus musculus*	*C. muris*	*C. muris*
Czech-B1-47	*Mus musculus*	*C. muris*	*C. muris*
Czech-B1-48	Golden hamster, *Mesocricetus auratus*	*C. muris*	*C. muris*
Czech-B1-49	*Mus musculus*	*C. muris*	*C. muris*
Czech-B1-50	Bactrian camel, *Camelus bactrianus*	*C. andersoni*	*C. andersoni*
Czech-B1-51	Dairy cow, *Bos taurus*	*C. andersoni*	*C. andersoni*
Czech-B1-52	Calf-1	*C. andersoni*	*C. andersoni*
Czech-B1-53	Calf-2	*C. andersoni*	*C. andersoni*
Czech-B1-54	Calf-3	*C. andersoni*	*C. andersoni*
Czech-B1-55	Young bull	*C. andersoni*	*C. andersoni*
Czech-B1-56	Young bull	*C. andersoni*	*C. andersoni*
Czech-B1-57	Young bull	*C. andersoni*	*C. andersoni*
Czech-B1-58	Young bull	*C. andersoni*	*C. andersoni*
Czech-B1-59	Young bull	*C. andersoni*	*C. andersoni*
Czech-B1-60	Camel, *Camelus bactrianus*	*C. andersoni*	*C. andersoni*
Czech-B2-1	Eurasian woodcock, *Scolopax rusticola*	New genotype/species	New genotype/species
Czech B2-2	Tawny frogmouth, *Podargus strigoides*	*C. muris*	ND
Czech-B2-4a	Bobak marmot, *Marmota bobac*	*C. andersoni*	*C. andersoni*
Czech-B2-4b	*Marmota bobac*	*C. andersoni*	*C. andersoni*
Czech-B2-4c	*Marmota bobac*	*C. andersoni*	*C. andersoni*
Czech-B2-5	European wisnet, *Bison bomasus*	*C. andersoni*	*C. andersoni*
Czech-B2-6	Mouflon sheep, *Ovis musimon*	Cervid genotype	Cervid genotype
Czech-B2-7	Nyala, *Tragelaphus angasi*	Cervid genotype	Cervid genotype
Czech-B2-8	Prezewalski's wild horse, *Equus przewalskii*	ND	*C. parvum*, cattle genotype
Czech-B2-9	Grey partridge, *Perdix perdix*	*C. baileyi*	*C. baileyi*
Czech-B2-10	Alpaca, *Lama quanico pacos*	*C. parvum*, cattle genotype	*C. parvum*, cattle genotype
Czech-B2-11	Laboratory mouse	*C. muris*	*C. muris*

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TABLE 1—*Continued*

Isolate code	Host species	Species, genotype (18S)	Species, genotype (HSP-70)
Czech-B2-12	Cornsnake, *Elaphe guttata*	*C. serpentis*	*C. serpentis*
Czech-B2-13	Pullet	*C. meleagridis*	*C. meleagridis*
Czech-B2-14	Human	*C. parvum*, cattle genotype	*C. parvum*, cattle genotype
Czech-B2-15	Human	*C. felis*	*C. felis*
Czech-B2-16	Human	*C. parvum*, cattle genotype	*C. parvum*, cattle genotype
Czech-B2-17	Yellow rat snake, *Elaphe obsolete quadrivittata*	*C. muris*	*C. muris*

[a] ND, not determined.

PCR products were purified using spin columns (Qiagen) and sequenced using an ABI Prism Dye Terminator Cycle sequencing kit (Applied Biosystems, Foster City, Calif.) according to the manufacturer's instructions except that the annealing temperature was raised to 60°C. Sequences were analyzed using SeqEd v1.0.3. (Applied Biosystems). Additional *Cryptosporidium* 18S rDNA sequences were obtained from GenBank.

HSP-70 gene amplification and sequencing. The HSP-70 gene was amplified and sequenced as previously described (15).

Phylogenetic analyses. Nucleotide sequences were aligned using Clustal X (25). (Sequence alignments can be obtained from the authors upon request). Phylogenetic analysis was performed using PAUP (D. L. Swofford, 1999). Distance-based analyses were conducted using Tamura-Nei distance estimates, and trees were constructed using the neighbor-joining algorithm. Bootstrap analyses were conducted using 1,000 replicates. Phylograms were drawn using the TreeView program (19).

Nucleotide sequence accession number. The nucleotide sequences of the 18S rRNA and HSP-70 sequences of *Cryptosporidium* isolates have been deposited in GenBank under the accession numbers AY273769 to AY273776.

Sequence and phylogenetic analysis of 18S rRNA gene. Partial sequences of the *Cryptosporidium* 18S rDNA gene were obtained from 12 reptile-derived isolates, 22 bird-derived isolates, 33 mammal-derived isolates, and 6 human-derived isolates (Table 1).

Analysis of the 18S rRNA nucleotide sequence data by distance-based methods identified two major clusters that grouped the gastric parasites (*C. serpentis*, *C. muris*, and *C. andersoni*) and an isolate from a Eurasian woodcock into one major group and placed all the remaining *Cryptosporidium* parasites in the second group (Fig. 1).

Sequence and phylogenetic analysis of the HSP-70 gene. Partial sequences of the *Cryptosporidium* HSP-70 gene were obtained from 9 reptile-derived isolates, 20 bird-derived isolates, 33 mammal-derived isolates, and 6 human-derived isolates (see Table 1). Analysis of the *hsp70* nucleotide sequence data by distance-based methods was largely consistent with the results of the 18S rDNA analysis (Fig. 2).

In this study a total of 13 species/genotypes of *Cryptosporidium* were found in birds, mammals, and humans. *C. baileyi* was identified in 15 out of the 22 avian-derived isolates examined, and *C. meleagridis* was identified in 5 avian-derived isolates. One isolate (B1-30), from a rose-ringed parakeet, exhibited a mixed infection of both *C. meleagridis* and *C. baileyi.* *Cryptosporidium* infections have been reported in more than 30 species of birds (5, 9, 13, 17); however, few studies have genetically characterized isolates from birds. A recent study genetically characterized avian isolates and reported the first finding of *C. baileyi* in quails (15). In the present study, we report the first finding of *C. baileyi* in a channel-billed Toucan (*Rhamphastus vitellinus*) (B1-22), a red-rumped Cocique (*Cacicus haemorrhous*) (B1-23), a crested oropendola (*Psaracolius decumanus*) (B1-24), and a red-crowned amazon (*Amazona dufresniana*) (B1-29), thus extending the host range of this species.

A novel *Cryptosporidium* genotype was identified in a Eurasian woodcock (*Scolopax rusticola*) (isolate B2-1). This bird had been obtained from the wild and transported to the Prague Zoo. During the quarantine period, parasitological examination of the feces identified *Cryptosporidium* oocysts, which corresponded in size to the upper-limit dimensions of *C. galli* (i.e., 8.5 by 6.4 μm) (22, 24). However, the inner structure, particularly the size and shape of the rest body and granules, was different. During the week the woodcock died, and at autopsy all endogenous developmental stages, including oocysts, were detected in the proventriculus only. At both the 18S and HSP-70 loci, this genotype was shown to be genetically distinct and grouped most closely with the gastric parasites (*C. serpentis, C. muris, and C. andersoni*). In a previous study by Morgan et al., two new avian genotypes/species of *Cryptosporidium* were identified: a black-duck genotype and a finch genotype, which has subsequently been confirmed as *C. galli* (15, 24). The Eurasian woodcock genotype identified as part of this study was genetically distinct from both the black-duck genotype and *C. galli* and shared only 95.5% similarity with *C. galli* and 96.6 to 97.4% similarity with the gastric parasites (*C. serpentis*, *C. muris*, *and C. andersoni*) at the 18S locus and 93 to 95.4% similarity at the HSP-70 locus. Further studies are required to confirm the species status of this new avian genotype.

C. andersoni was identified in 15 isolates from various hosts including cattle, camel, marmots, and a European wisnet. This is the first time that *C. andersoni* has been reported in the last two hosts. The marmots were captured wild overseas and imported into the Prague Zoo. During the quarantine period, large numbers of *C. andersoni*-like oocysts were detected in one male. Subsequent examinations showed that this animal was still positive 170 days after first shedding oocysts. During this time, one female also became naturally infected. Experimental transmission of oocysts from the marmots failed to produce infections in laboratory mice (I. Pavlasek, unpublished data). These data support cross-transmission studies,

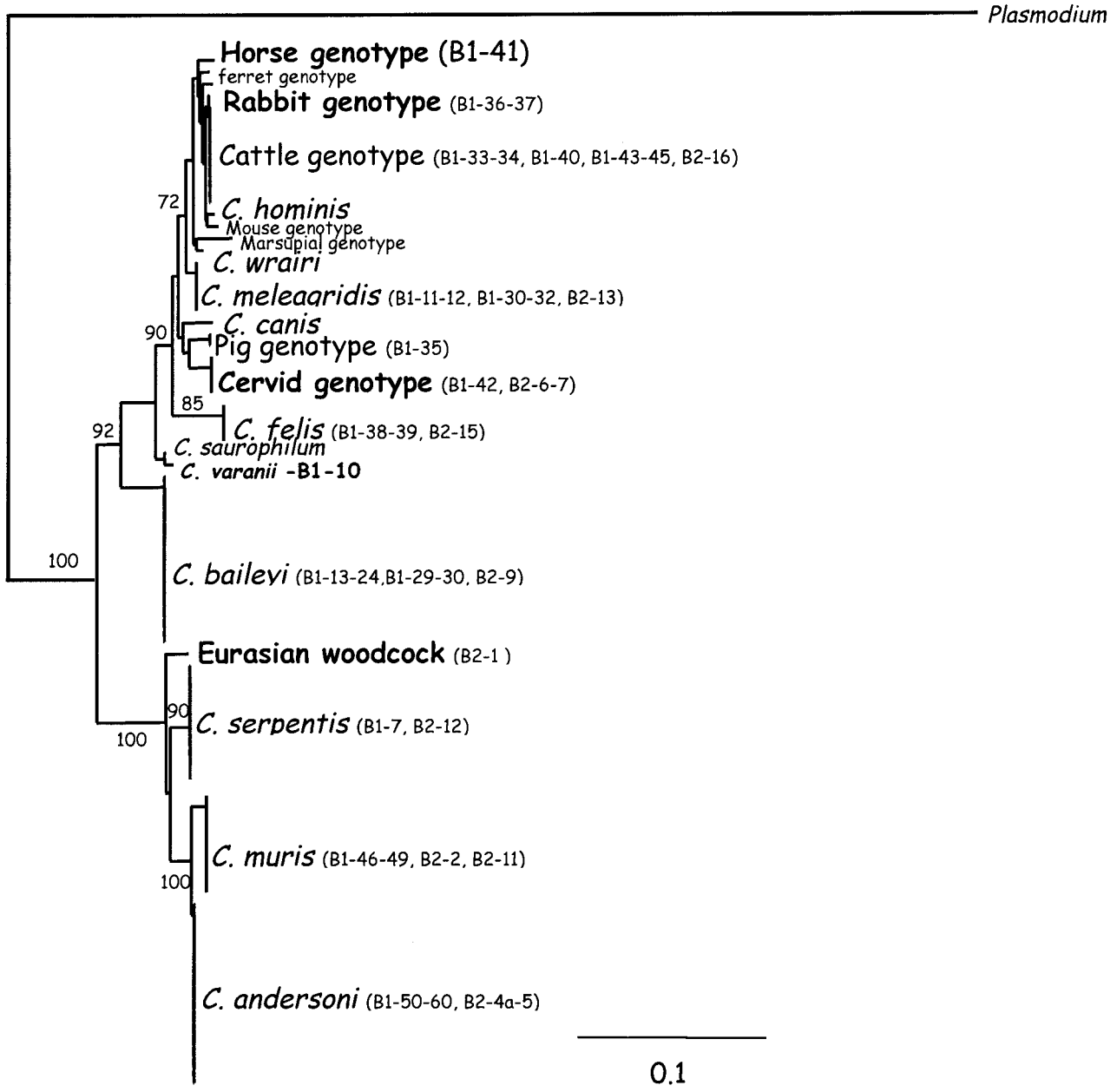

FIG. 1. Phylogenetic relationships of *Cryptosporidium* isolates inferred by neighbor-joining analysis of Tamura Nei distances calculated from pairwise comparisons of the 18S rDNA sequences. Percentage bootstrap support (>70%) from 1,000 replicate samples is indicated at the left of the supported node.

which have shown that *C. andersoni* is not transmissible to mice (10).

C. muris was identified in laboratory mice and a golden hamster (isolates B1-46-49 and B2-11). *C. muris* was also identified in two reptiles, a crocodile monitor (isolate B1-8) and a yellow rat snake (isolate B2-17), and an avian-derived isolate from a Tawny frogmouth (*Podargus strigoides*) (B2-2). *C. muris* has been reported previously in reptiles (11), and the most likely explanation is that the reptiles and the bird were passing oocysts from an infected rodent prey. However, the possibility that these hosts were infected with *C. muris* cannot be ruled out, particularly in the case of the Tawny frogmouth-derived isolate (B2-2), since large numbers of oocysts were detected in the feces.

C. serpentis was identified in nine reptiles, and *C. saurophilum* was identified in a desert monitor (*Varanus griseus*) (B1-10). *Cryptosporidium saurophilum* has been reported in lizards, Schneider's skink (*Eumeces schneideri*), and desert monitors (8). *C. saurophilum* differs from *C. serpentis* by having smaller oocysts, by developing in the intestine and not the gastric glands, and by the inability to infect snakes (8). However, prior to this publication, Pavlasek described endogenous developmental stages and oocysts which resembled *C. parvum* is size from the intestine of a monitor and proposed the name *Cryp-*

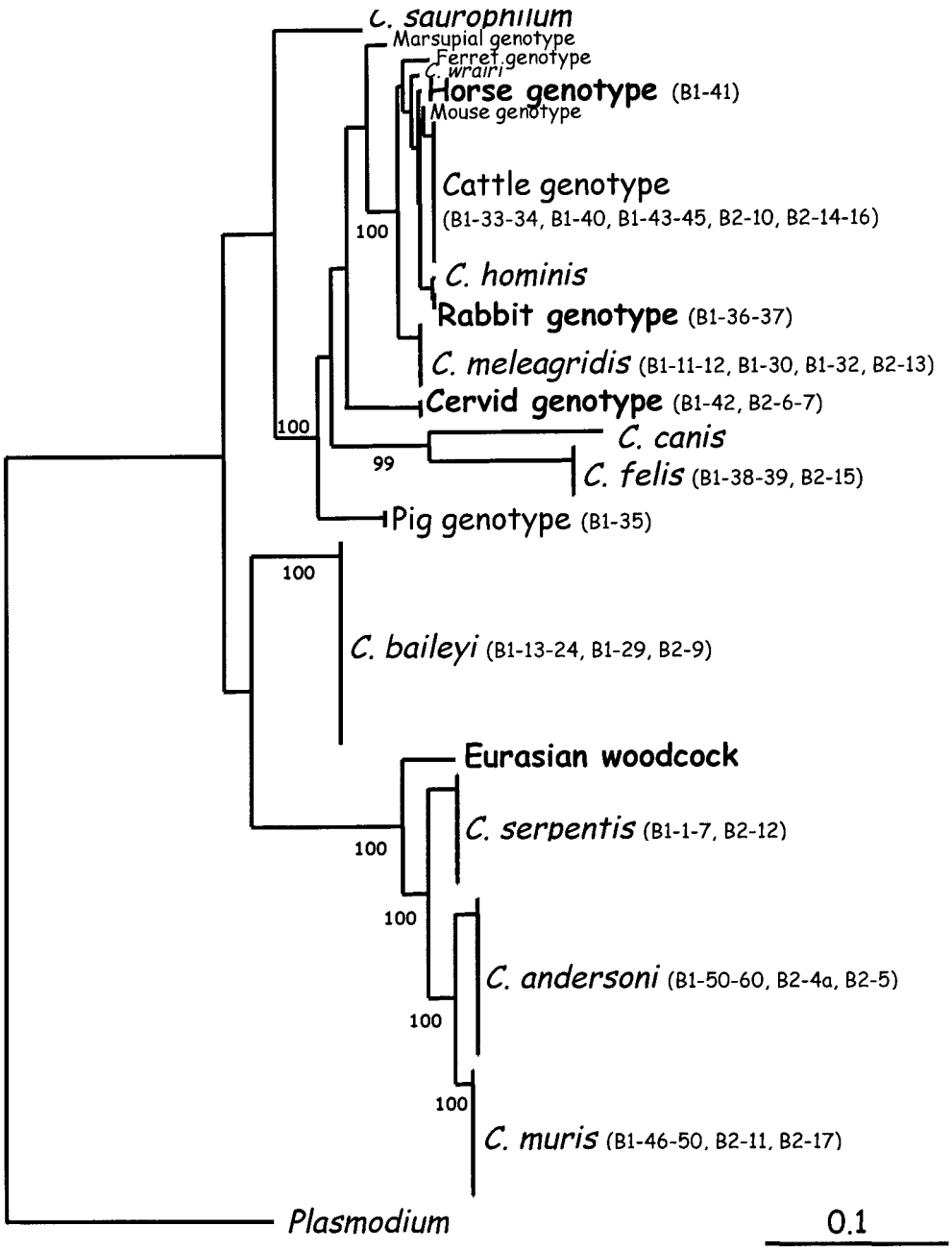

FIG. 2. Phylogenetic relationships of *Cryptosporidium* isolates inferred by neighbor-joining analysis of Tamura Nei distances calculated from pairwise comparisons of the HSP-70 DNA sequences. Percentage bootstrap support (>70%) from 1,000 replicate samples is indicated at the left of the supported node.

tosporidium varanii (21). The monitor-derived isolate (B1-10) which was examined as part of this study exhibited only three base pair differences from *C. saurophilum*, and thus, it would appear from sequence and phylogenetic analysis that *C. saurophilum* and *C. varanii* are synonyms of each other.

C. felis was identified in two domestic cat-derived isolates (B1-38 and B1-39), and the *C. parvum* pig genotype was identified in a pig-derived isolate (B1-25). All the human-derived isolates were the *C. parvum* cattle genotype with the exception of one human-derived isolate (B2-15), which was identified as *C. felis.* This isolate was from a 36-year-old HIV^+ male who was hospitalized for diarrhea. *C. felis* has previously been reported in HIV^+ patients (14), and it appears that immunocompromised individuals are susceptible to most *Cryptosporidium* species and genotypes.

A novel horse genotype was identified in a Prezwalski's wild horse (B1-41); however, in a second horse isolate (B2-8), the *C. parvum* cattle genotype was identified, indicating that horses can be infected with both genotypes. The horse-derived isolates (B1-41 and B2-8) were from a 161-day-old and a 12

day-old foal, respectively, born at the Prague Zoo. The novel horse genotype (B1-41) was most related to *C. wrairi* (98.7% similarity for 18S; 99% similarity for HSP-70) and the *C. parvum* mouse genotype (99.7% similarity for 18S; 97.7% for HSP-70).

The *C. parvum* rabbit genotype was identified in rabbit-derived isolates B1-36 and B1-37. This novel genotype has recently been reported in rabbit-derived isolates from China (28) and is genetically most closely related to *C. hominis*, sharing 99.2% similarity with *C. hominis* at the 18S locus and 99.7% similarity with *C. hominis* at the HSP-70 locus.

The novel *Cryptosporidium* cervid genotype was identified in isolate B1-42 from a blesbok, isolate B2-6 from a Mouflon sheep, and isolate B2-7 from a nyala. This is the first time that this genotype has been reported in these hosts (see Table 1). This genotype was first identified by Xiao et al. (27), from storm water samples in lower New York State (storm water isolate W4, GenBank accession no. AF262328). Subsequently, Perez and Le Blancq (23) identified this genotype in white-tailed deer-derived isolates from lower New York State and referred to it as genotype 3. The cervid genotype as also been identified in a lemur (*Propithecus verreauxi coquereli*) (4), in pig slurry in the United Kingdom (Xiao et al. unpublished data), and in humans in Canada (18).

It appears that the cervid genotype, like the *C. parvum* cattle genotype, has a wide host range and could possibly emerge as an important human pathogen with increasing contact between human and wildlife. Morphologically, the cervid genotype appears to be similar to *C. parvum*; however, genetically the cervid genotype is very distinct, sharing only 96.8 to 97.68% similarity to the *C. parvum* group at the 18S locus and 92.7 to 94.2% similarity at the HSP-70 locus. Further studies are required to confirm the species status of this novel genotype.

The present study has identified several novel genotypes/species of *Cryptosporidium* as well as expanding the host range of accepted species and highlights the importance of analyzing a wide range of *Cryptosporidium* isolates from different hosts in order to better understand the epidemiology and potential human health risks of this ubiquitous parasite.

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