

Sarcocysts of an Unidentified Species of *Sarcocystis* in the Sea Otter (*Enhydra lutris*)

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ABSTRACT: The number of *Sarcocystis* species that infect sea otters (*Enhydra lutris*) is unknown. Sea otter tissues were recently shown to harbor sarcocysts of *S. neurona* and of unidentified species of *Sarcocystis*. Whereas sarcocysts of *S. neurona* have walls 1–3 μm thick with type 9 villar protrusions, ultrastructure of a distinct thin-walled sarcocyst (0.5–0.7 μm thick) lacking villar protrusions, but instead exhibiting minute type 1 undulations on the sarcocyst wall, is described in this report. Parasites characterized from a sea otter infection were inferred to be related to, but distinct from, other species belonging to *Sarcocystis*, based on sequencing and phylogenetic analysis of a portion of the beta subunit of the plastid-encoded RNA polymerase gene.

Disease in a group of sea otters (*Enhydra lutris*) was recently attributed to infection with *Sarcocystis neurona*, a parasite that causes fatal neurologic disease in horses and other mammals (Dubey, Lindsay, Saville et al., 2001). Sea otters from the coast of California and Washington died of encephalitis associated with *S. neurona* schizonts (Rosenke et al., 1999; Lindsay et al., 2000, 2001; Miller et al., 2001). In addition, *S. neurona* sarcocysts were found in 2 other sea otters (Rosenke et al., 1999; Dubey, Rosypal et al., 2001). Parasites lacking the thick sarcocyst walls and elongated villar protrusions characteristic of *S. neurona* also have been observed in sea otters (in sea otter no. 2 of Dubey, Rosypal et al., 2001). To characterize this unidentified parasite better, transmission electron microscopy (TEM) was used to define its ultrastructure. To further aid future efforts to identify and diagnose sea otter infections, the plastid-encoded beta subunit of RNA polymerase (*rpoB*) was amplified from infected sea otter tissue and compared with homologous sequences from *S. neurona*, *S. falcatula*, and *S. lindsayi* (Table I).

Two sarcocysts from a paraffin section of the skeletal muscle of sea otter were deparaffinized, postfixed in osmium tetroxide, and processed for TEM examination. In 1- μm toluidine blue-stained sections, the sarcocysts measured 95×60 and $110 \times 65 \mu\text{m}$. The sarcocyst wall was $<1 \mu\text{m}$ thick without visible villar protrusions (Fig. 1). Septa were indistinct.

Under TEM the sarcocyst wall was found to be 0.5–0.7 μm thick and bore minute, electron-dense undulations located at irregular intervals (Fig. 2A, B). The maximum observed width of the sarcocyst wall at the point of infolding and beginning of septa was 1.0 μm . Only bradyzoites were seen, and 3 longitudinally cut bradyzoites were $5.0\text{--}5.7 \times 1.6\text{--}1.9 \mu\text{m}$ in size. Rhoptries were prominent, and their bulbous blind end was sometimes turned toward the conoidal end (Fig. 2A). The micronemes were located in the anterior half of the bradyzoite. Thus, the thin-walled sarcocysts in the present report were ultrastructurally distinct from those of *S. neurona*, which typically bear walls 1–3 μm

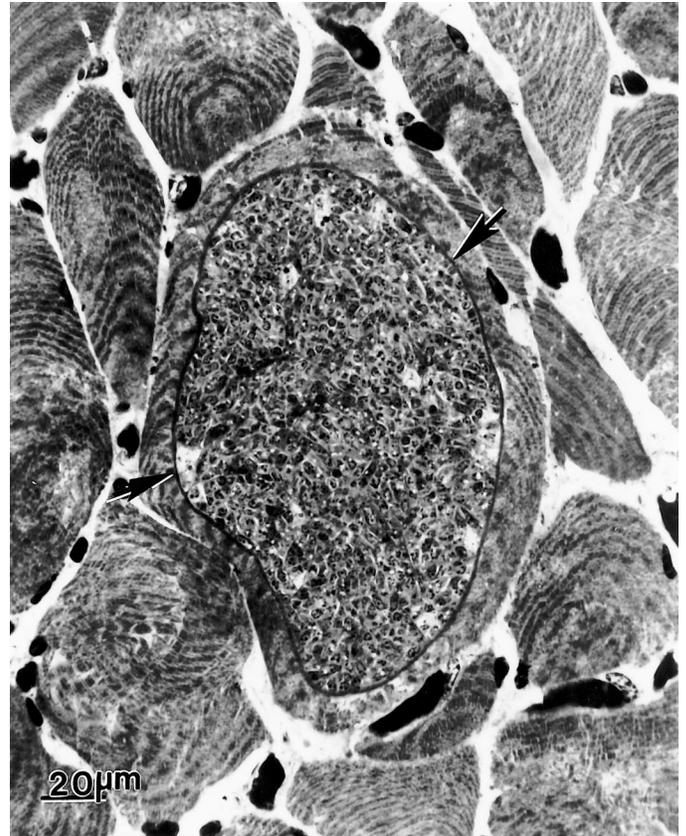


FIGURE 1. Section of sea otter skeletal muscles showing a *Sarcocystis* sp. sarcocyst. Note the thin sarcocyst wall (arrows). Toluidine blue stain.

in thickness featuring prominent villar type 9 protrusions (Dubey et al., 1989; Dubey, Lindsay, Fritz et al., 2001).

In the initial report of an unidentified sarcocyst in the musculature of an encephalitic sea otter, villi were present on the sarcocyst walls,

TABLE I. Sources of parasite isolates.

Name	Host species, locale, and reference	GenBank accession
<i>Sarcocystis lindsayi</i>	<i>Didelphis albiventris</i> ; Brazil; Dubey, Rosenthal, and Speer (2001).	AY164997
<i>Sarcocystis</i> sp.	<i>Enhydra lutris</i> ; Washington; Dubey, Rosypal, Rosenthal et al. (2001).	AY164998
<i>S. falcatula</i>	<i>Didelphis albiventris</i> ; Argentina; Dubey, Rosenthal, and Speer (2001).	AY164999
<i>S. neurona</i>	<i>Didelphis albiventris</i> ; Brazil, SN 35-OP; Dubey Lindsay, Kerber et al. (2001).	AY165000
<i>S. falcatula</i> -like	<i>Didelphis marsupialis</i> ; Argentina; Dubey, Lindsay, Rosenthal et al. (2001).	AY165001

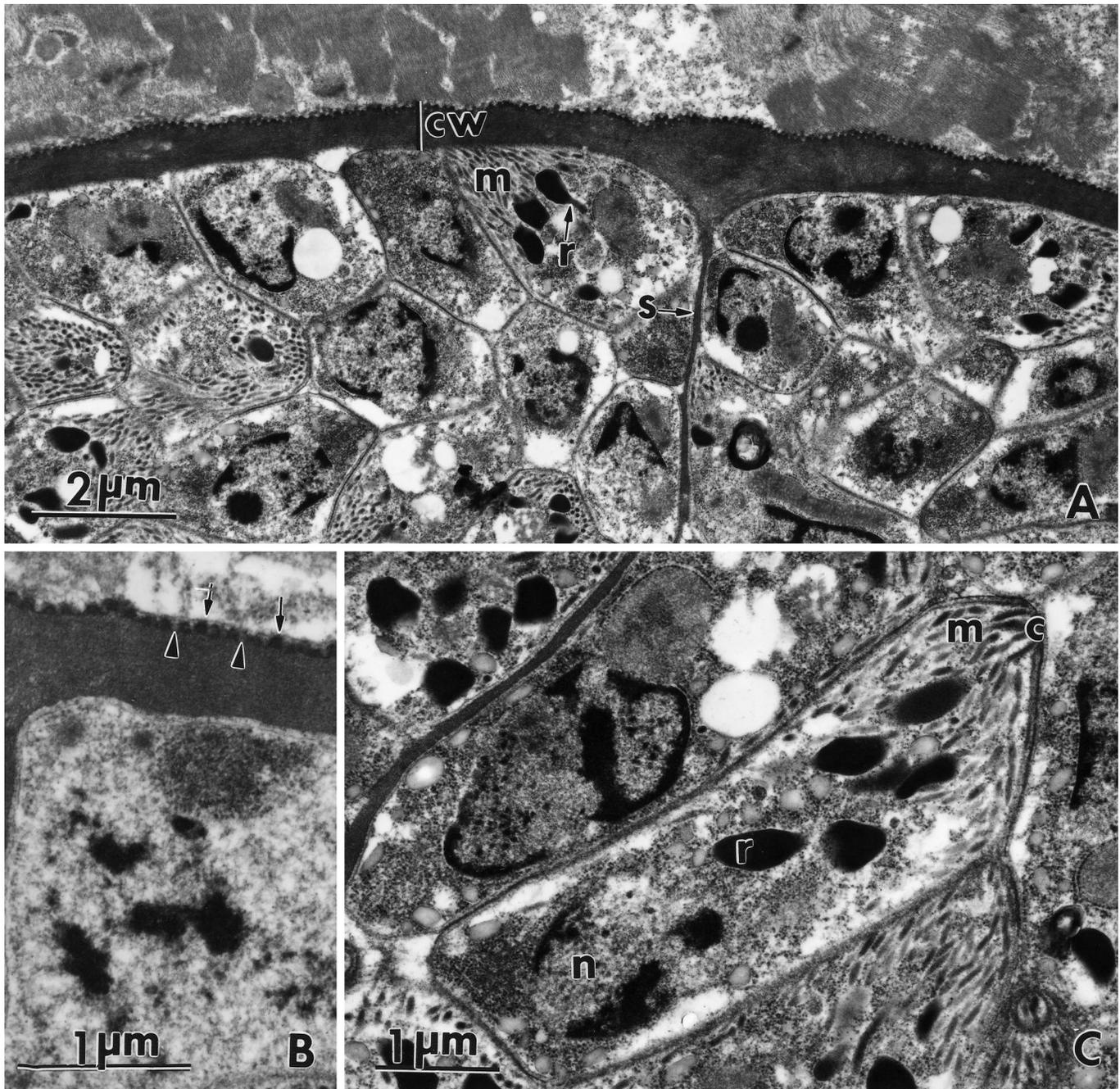


FIGURE 2. Transmission electron micrograph of a mature sarcocyst from the skeletal muscle of the sea otter. **A.** Note the thin cyst wall (cw) with minute protrusions. The ground substance is homogenous without microtubules and continues into the sarcocyst interior as septa (s). All organisms present are bradyzoites. A rhoptry (r) in 1 bradyzoite has its bulbous end turned toward the conoidal end. Also note numerous micronemes (m) toward the conoidal end. **B.** Higher magnification of the sarcocyst wall. Note the minute protrusions on the sarcocyst wall, interrupted at irregular intervals (arrow heads). **C.** Longitudinal section of a bradyzoite showing the conoid (c), micronemes (m), rhoptries (r), and terminal nucleus (n).

but autolysis obscured additional ultrastructural details (Rosonke et al., 1999). Dubey, Rosypal et al. (2001) described the ultrastructure of *S. neurona* sarcocysts in skeletal muscle of a sea otter that had died of *S. neurona*-associated encephalitis. Only sarcocysts resembling those of immature *S. neurona* were found in the encephalitic sea otter (sea otter no. 1 of Dubey, Rosypal et al., 2001). However, in the musculature of a second sea otter described by Dubey, Rosypal et al. (2001), light microscopy indicated that there were at least 2 additional types of sarcocysts; thin-walled sarcocysts, possessing septa but lacking villi, were

distinct from the third type of sarcocyst. The third type of sarcocysts were thick-walled, with 7- μ m villar protrusions, and were found in the tongue and not in the skeletal muscle. In the present report, using TEM, the presence of a structurally distinct, thin-walled sarcocyst was confirmed. Whether these sarcocysts correspond to those illustrated previously by Dubey, Rosypal et al. (2001) cannot be known with certainty because only 2 sarcocysts were examined ultrastructurally and because the true diversity of this mixed, natural infection is undefined.

DNA was extracted from sea otter isolate and used as a template in

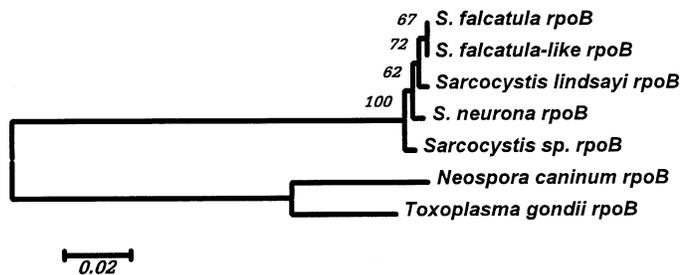


FIGURE 3. Midpoint-rooted neighbor-joining tree reconstructed from variation in the *rpoB* B gene. Kimura 2-parameter distances were calculated for each pair of sequences. The percentage of bootstrap replicates ($n = 1,000$) in which a given node was recovered is indicated. Five hundred base pairs of the *rpoB* gene were sequenced from *Sarcocystis* sp. and from representatives of *S. neurona*, *S. falcatula*, and *S. lindsayi* in the sea otter (see Table I for details on isolates). The *Neospora caninum* and *Toxoplasma gondii* homologs were obtained from GenBank (accession nos. AF095904 and AF138960, respectively).

a polymerase chain reaction (PCR) by using degenerate primers designed to amplify the *rpoB* gene, encoded by the plastid genome of apicomplexans—primers F1 (5'-gcg gtc cca aaa ggg tca gtg gat atg atw twt gaa gat gc) and R3 (5'-gcg gtc cca aaa ggg tca gtc ctt tat ktc cat rtc t). The resulting 504-bp PCR products were directly sequenced using BigDye chemistries and an ABI 3100 automated fluorescent sequencer. Homologous sequences were characterized from isolates of *S. neurona*, *S. falcatula*, and *S. lindsayi*, the origins of which are summarized in Table I. These were aligned to each other and to homologs from *Neospora caninum* and *Toxoplasma gondii* by using CLUSTAL W 1.8 (Thompson et al., 1994), available on the bioinformatics server of the Baylor College of Medicine. Relationships of these sequences were investigated by constructing a gene genealogy by calculating Kimura 2-parameter distances from 1,000 bootstrap replicates of the alignment and using the Neighbor-Joining algorithm using MEGA 2.1 (Kumar et al., 2001).

The *rpoB* sequence obtained from the otter isolate was placed as a basal member of a clade that also contained the other examined isolates belonging to *Sarcocystis* but that included neither *N. caninum* nor *T. gondii* (Fig. 3). Concordant topologies were obtained when the minimum evolution and maximum parsimony criteria were used (data not shown). Several nucleotide substitutions distinguish this otter specimen from the isolates representing other species of *Sarcocystis*. In contrast, the *rpoB* of isolates representing *S. falcatula* are comparatively homogeneous. Thus, morphological and genetic evidence indicates that sea otters, in addition to being at the risk of exposure to *S. neurona* parasites, serve as host to at least 1 other species of parasites belonging to the genus *Sarcocystis*.

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Morphology Is Not a Reliable Tool for Delineating Species Within *Cryptosporidium*

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ABSTRACT: Within the coccidia, morphological features of the oocyst stage at the light microscope level have been used more than any other single characteristic to designate genus and species. The aim of this study was to conduct morphometric analysis on a range of *Cryptosporidium*

sp. isolates and to compare morphological data between several genotypes of *C. parvum* and a second species *C. canis*, as well as a variation within a specific genotype (the human genotype), with genetic data at 2 unlinked loci (18S ribonucleic deoxyribonucleic acid and HSP

70) to evaluate the usefulness of morphometric data in delineating species within *Cryptosporidium*. Results indicate that morphology could not differentiate between oocysts from *C. parvum* genotypes and oocysts from *C. canis*, whereas genetic analysis clearly differentiated between the two. The small size of the *Cryptosporidium* spp. oocyst, combined with the very limited characters for analysis, suggests that more reliance should be placed on genetic differences, combined with biological variation, when delineating species within *Cryptosporidium*.

Taxonomic classification for parasitic protozoa is currently based on multiple phenotypic characters such as morphological features detected by light or electron microscopy, unique life cycles, and host specificity (Fayer et al., 2000). For coccidia, morphological features of the oocyst stage at the light microscope level have been used more than any other characteristic to designate genus and species (Morgan, Xiao et al., 1999).

There has been considerable confusion regarding the taxonomy of the genus *Cryptosporidium*, and currently 10 species are regarded as valid. These include *C. parvum* from many mammals, *C. muris* from mice, *C. andersoni* from ruminants, *C. felis* from cats, *C. canis* from dogs, *C. wrairi* from guinea pigs, *C. meleagridis* and *C. baileyi* from birds, *C. serpentis* from snakes and lizards, and *C. saurophilum* from lizards (Fayer et al., 2000, 2001).

Since Tyzzer's first description in 1912, oocysts corresponding in size and shape to *C. parvum* have been described in over 152 different host species (Fayer et al., 2000). Oocysts of *C. parvum* of bovine origin have been described as having a size range of 4.5–5.4 × 4.2–5.0 μm (mean 5.0 × 4.5 μm) and a shape index of 1.1 (Upton and Current, 1985). Few studies have conducted morphometric analysis on *C. parvum* oocysts from a range of hosts. However, molecular data at numerous unlinked loci have provided evidence for substantial genetic variation within what is termed as the *C. parvum* group. Currently, at least 8 different genotypes have been identified within the *C. parvum* group, including the human, cattle, mouse, pig, ferret, and marsupial genotypes, which are genotypically different but morphologically similar (Xiao et al., 2000). This raises the question of whether morphological analysis is a reliable tool for delineating species of *Cryptosporidium*. The aim of this study was to conduct morphometric analysis on a range of *Cryptosporidium* spp. isolates and to compare morphological data between several genotypes of *C. parvum* and a second species *C. canis*, as well as a variation within a specific genotype (the human genotype), with genetic data at 2 unlinked loci to evaluate the usefulness of morphometric data in delineating species within *Cryptosporidium*.

Cryptosporidium spp. oocysts from fresh stool samples from humans, cattle, marsupials, and dogs were purified using phosphate-buffered saline (PBS)-ether and Ficoll® density centrifugation (Meloni and Thompson, 1996). Isolates were genotyped by sequence analysis of the 18S rDNA and HSP 70 loci, using previously described methods (Morgan et al., 1997, 2001). Nucleotide sequences were aligned using Clustal X (Thompson et al., 1997). Distance-based analysis was performed us-

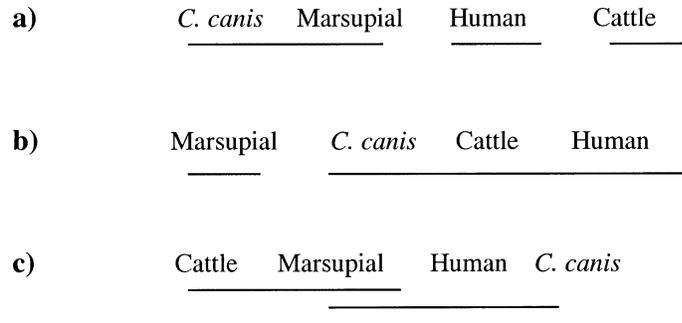


FIGURE 1. Groupings of *Cryptosporidium* species and genotypes determined by Scheffé test. **a.** Groupings based on mean length. **b.** Groupings based on mean width. **c.** Groupings based on mean shape ratio. Underlining joins isolates that are similar, and those significantly different are not joined.

ing Kimura's distance. Trees were constructed using the neighbor-joining algorithm. Bootstrap analyses were conducted using 1,000 replicates. Phylograms were drawn using the TreeView program (Page, 1996).

Cryptosporidium oocysts were measured using the Optimus Image Analysis Package version 5.2 at ×1,000 magnification. The area of analysis was set to enclose each individual oocyst in order to minimize computational time. Threshold was optimized by eye to differentiate the oocyst background. Once the optimal threshold was reached, the software was set to recognize the oocyst as an area object. All measurements (length, width, area, and circularity) were transferred to Microsoft Excel. Statview version 4.0 (Abacus Concepts Inc., Berkeley, California) was used to perform an analysis of variance (ANOVA) with these data. A multiple range test (Scheffé test) at a significance level of 0.05 was then carried out to group the isolates based on differences in their means from the ANOVA.

Significant differences were identified in oocyst length ($P < 0.001$), width ($P < 0.001$), and shape ratio ($P < 0.001$) for human, cattle, and marsupial genotypes of *C. parvum* and *C. canis* in the stub column of Table I. However, a Scheffé test failed to group *C. canis* separately from the *C. parvum* genotypes (Fig. 1).

Smaller, yet significant differences, also were noted in oocyst length ($P < 0.005$), width ($P < 0.001$), and shape ratio ($P < 0.001$) from different isolates within the *C. parvum* human genotype (Table I). The Scheffé test depicts the differences between the human genotype isolates as shown in Figure 2.

Phylogenetic analysis at the 18S rDNA and HSP 70 loci grouped *C. canis* separately and clearly demonstrated that the *C. parvum* group is polyphyletic because *C. wrairi* and *C. meleagridis* were placed within

TABLE I. Morphometric analysis of *Cryptosporidium* oocysts.

Code	Species, genotype	Oocyst length (mean) (μm)	Oocyst width (mean) (μm)	Oocyst shape index (mean) (L/W)*	Oocyst count
AusDog1	<i>C. canis</i>	5.1–4.6 (4.8)	4.6–3.8 (4.2)	1.10–1.21 (1.14)	50
S26	<i>C. parvum</i> , cattle	5.5–5.0 (5.2)	5.0–3.7 (4.3)	1.10–1.35 (1.20)	40
K2	<i>C. parvum</i> , marsupial	5.1–4.5 (4.9)	5.0–3.8 (4.3)	1.02–1.18 (1.14)	40
H22	<i>C. parvum</i> , human	5.5–4.6 (5.0)	4.7–3.8 (4.2)	1.17–1.21 (1.19)	50
H54	<i>C. parvum</i> , human	5.7–4.5 (5.1)	4.7–3.7 (4.2)	1.21–1.21 (1.21)	50
H65	<i>C. parvum</i> , human	5.4–4.3 (4.8)	4.6–3.8 (4.2)	1.17–1.13 (1.14)	50
H79	<i>C. parvum</i> , human	5.5–4.4 (4.9)	4.7–3.8 (4.3)	1.17–1.16 (1.14)	50
H136	<i>C. parvum</i> , human	5.6–4.5 (5.0)	4.6–3.7 (4.2)	1.20–1.20 (1.20)	50
H139	<i>C. parvum</i> , human	5.4–4.6 (5.0)	4.7–3.8 (4.2)	1.15–1.21 (1.19)	50
H156	<i>C. parvum</i> , human	5.6–4.5 (5.0)	4.7–3.9 (4.3)	1.19–1.15 (1.16)	50
H41625	<i>C. parvum</i> , human	5.3–4.7 (5.0)	4.8–3.9 (4.4)	1.10–1.2 (1.13)	50

* L, length, W, width.

a)	H65	H79	H41625	H139	H136	H22	H54	H156
b)	H65	H136	H54	H139	H79	H22	H156	H41625
c)	H54	H136	H139	H22	H65	H156	H79	H41625

FIGURE 2. Groupings of *Cryptosporidium parvum* human genotypes determined by Scheffé test. **a.** Groupings based on mean length. **b.** Groupings based on mean width. **c.** Groupings based on mean shape ratio. Underlining joins isolates that are similar, and those significantly different are not joined.

this group, providing strong support that the *C. parvum* marsupial genotype is also a distinct species. The *C. parvum* cattle genotype grouped separately from the *C. parvum* human genotype isolates, which were all genetically identical at both loci (Fig. 3). Bootstrap analysis of the data provided strong support for these groupings (Fig. 3).

Cryptosporidium canis is a recognized species because of its apparent host specificity and genetic distinction at numerous loci (Fayer et al., 2001). The *C. canis* isolate used in this study was genetically identical at the loci examined to the isolates used in the study by Morgan, Xiao et al. (2000) and Fayer et al. (2001). As seen in the study by Fayer et al. (2001), this isolate could not be distinguished from the oocysts of different *C. parvum* genotypes by morphological analysis. This overlap in size range between valid species of *Cryptosporidium* is not confined to *C. canis*. *Cryptosporidium meleagridis* and *C. wrairi* also overlap in size with *C. parvum* of bovine origin and are morphologically very similar (Vetterling et al., 1971; Fayer et al., 2000), but they are genetically and biologically distinct and have different host ranges (Fayer et al., 2000).

There is also biological evidence that marsupial, cattle, and human genotypes may be separate species. The marsupial genotype appears to be host specific and is not infectious to nude mice (Morgan, Xiao et al., 1999; Xiao et al., 2000). The human and cattle genotypes have been shown to be genetically distinct at a wide range of loci, and they also exhibit differences in host specificity because the human genotype appears to be confined largely to humans, whereas the cattle genotype infects a wide host range (Morgan, Xiao et al., 1999; Xiao et al., 2000). Differences in growth rates in *in vitro* culture (Hijawi et al., 2001) as well as fundamental differences in ribosomal gene expression (Le Blancq et al., 1997) also have been reported. In addition, genetic recombinants between the human and cattle genotypes have never been detected despite the fact that both genotypes can infect humans and coinfections have been reported to occur (Patel et al., 1988).

In this study the *C. parvum* cattle genotype oocysts (isolate S26) had a size range of $5.5\text{--}5.0 \times 5.0\text{--}3.7 \mu\text{m}$ (mean $5.2 \times 4.3 \mu\text{m}$) and a shape index of 1.20. Other authors have reported a size range of $4.7\text{--}6.0 \times 4.4\text{--}5.0 \mu\text{m}$ (mean $5.0 \times 4.7 \mu\text{m}$) and a shape index of 1.06 (Fayer et al., 2001). Upton and Current (1985) have reported a size range of $4.5\text{--}5.4 \times 4.2\text{--}5.0 \mu\text{m}$ (mean $5.0 \times 4.5 \mu\text{m}$) and a shape index of 1.1. Similarly, in this study there was significant variation between different isolates of the *C. parvum* human genotype (see Table I; Fig. 2), yet genetic analysis at 2 loci grouped all the human genotype isolates as identical. Genetic analysis at the hypervariable GP-60 locus also did not reveal any differences between these isolates (U. Morgan-Ryan, unpubl. obs.).

Oocysts of *Cryptosporidium* are among the smallest exogenous stages of the apicomplexans; therefore, all morphological differences may not be clear at light microscope levels. Oocyst measurements are frequently conducted using different types of microscopes, different objectives, and different measuring systems (Morgan, Xiao et al., 1999). Any inaccurate measurement is of great significance because of the small size of the oocyst. The age and storage conditions of the oocyst and the isolation techniques also can affect the shape of the oocyst and its measurement. This makes interlaboratory comparisons very problematic. Morphometric analysis of *Cryptosporidium* is further limited by the lack of distinguishing morphological characters, because there are only 2 characters that can be analyzed (length or width, and shape index). Electron mi-

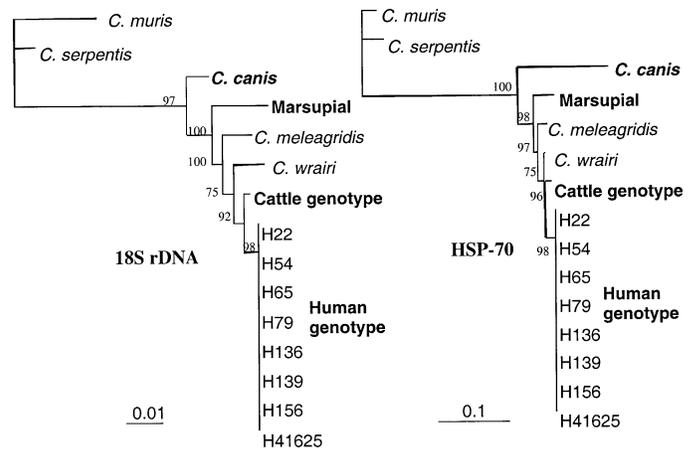


FIGURE 3. Phylogram of *Cryptosporidium* species and genotypes inferred by neighbor-joining analysis of Kimura's distances at the 18S rDNA and HSP 70 gene loci. Percent bootstrap support from 1,000 replicate samples is indicated at the left of the supported node.

croscopy study to distinguish ultrastructural differences is usually not possible because in most cases oocysts are recovered from fecal samples, and tissue sections are not available. Genetic analysis, however, is much more informative than morphological data in the case of *Cryptosporidium* spp. because the number of characters available for analysis is limited only by the size of the genome.

The difficulties of using morphology as a means of delineating species within the Apicomplexa are well known. For example, oocysts of *Toxoplasma gondii* (average size $10 \times 11 \mu\text{m}$) are disporic with tetra-zoic sporocysts and overlap in size and shape with *Hammondia hammondi* and *Neospora caninum*, all of which belong to separate genera (Levine, 1982). A similar problem with the identity of the exogenous stage arose within the species of *Sarcocystis*. When excreted, the weak-walled sporulated oocysts ruptured and released sporocysts that appeared to be identical to sporocysts of other species (Morgan, Monis et al., 1999). Some *Frenkelia* species appear to be identical to *Sarcocystis*, and both genera are differentiated by host specificity and cyst tissue characteristics.

Morphology is an invaluable tool for delineating species in many organisms. However, in the case of *Cryptosporidium* spp. the problems outlined above suggest that it is not a reliable means of delineating species in this genus and that more reliance should be placed on genetic and biological data such as host occurrence when delineating species of *Cryptosporidium*.

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Molecular Cloning of a Novel Multidomain Kunitz-Type Proteinase Inhibitor From the Hookworm *Ancylostoma caninum*

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ABSTRACT: Degenerate oligonucleotide primers derived from conserved serine protease inhibitors were used to amplify a 90–base pair (bp) amplicon from an *Ancylostoma caninum* adult-stage complementary deoxyribonucleic acid (cDNA) library by polymerase chain reaction (PCR). The amplicon was labeled and used as a probe to screen the library, and a 2,300-bp cDNA clone was identified. The 5′ end of the molecule was obtained from adult cDNA by 5′-RACE. The complete sequence named *A. caninum* Kunitz-type protease inhibitor (*Ac-kpi-1*) was 2,371 bp and encoded a 759–amino acid open reading frame. The deduced amino acid sequence had a calculated molecular weight of 84,886 Da and contained an amino terminal signal peptide, suggesting that the protein is secreted. Analysis of the predicted protein sequence indicates 12 highly conserved Kunitz-type serine protease inhibitor domains connected by short, conserved spacers. On the basis of sequence analysis, the first 11 domains are predicted to be active serine protease inhibitors based on the P1 amino acid. Domains 5–8 have identical amino acid sequences, and the remaining domains are 38–88% identical. Domain 12 lacks several of the conserved cysteine residues and has an atypical amino acid in the P1 position, suggesting that it is nonfunctional. Reverse transcriptase-PCR indicated that the *Ac-kpi-1* messenger ribonucleic acid is present in egg, L₁, L₃, and adult stages but is most abundant in the adult stage. *Ac-KPI-1* is most similar in domain architecture to several extracellular matrix proteins involved in cellular remodeling during insect development. In addition, there are 44 nematode proteins containing one or more Kunitz domains in GenBank, including several with multiple domains.

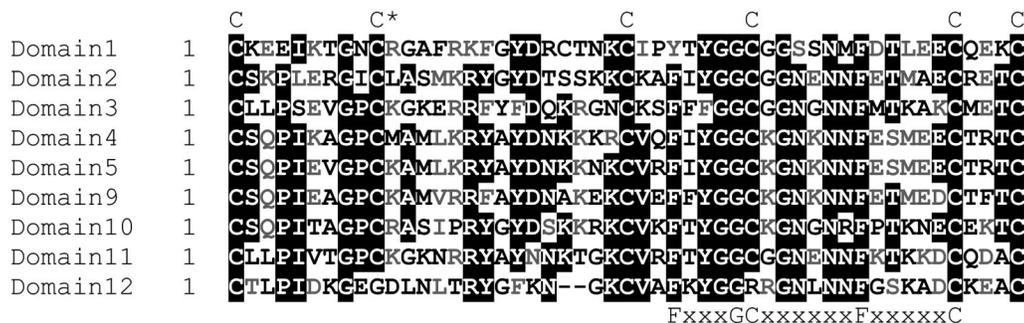
Many parasitic nematodes release molecules that interfere with host proteases, presumably enabling the parasite to avoid the detrimental effects of proteolysis. For example, hookworms, and possibly other hematophagous nematodes, release anticoagulant peptides, which inhibit the proteases of the host coagulation pathways (Eff, 1966; Cappello et al., 1996), and *Ascaris* releases serine protease inhibitors, which interfere with host digestive enzymes (Martzen et al., 1990).

Kunitz inhibitors (KI) are a class of serine protease inhibitors that include basic pancreatic trypsin inhibitor (Fioretti et al., 1985), tissue factor pathway inhibitor (TFPI) (Wun et al., 1988; Sprecher et al.,

1994), and numerous dendrotoxins and venoms (Dufton, 1985). Kunitz inhibitor domains are characterized by a conserved spacing between cysteine residues (C{8x}C{15x}C{7x}C{12x}C{3x}C) and a characteristic disulfide bonding pattern. The inhibitors may comprise a single Kunitz domain or “head” or have several domains separated by variable spacer regions. Recently, a small, single-domain KI has been described from adults of the hookworm *Ancylostoma ceylanicum* (Milstone et al., 2000). In this study, we describe the cloning of a novel KI from adults of the related hookworm *A. caninum*. The *A. caninum* Kunitz-type protease inhibitor (*Ac-KPI-1*) complementary deoxyribonucleic acid (cDNA) encodes a large multiheaded protein containing 12 tandem Kunitz domains.

An adult *A. caninum* cDNA library was constructed in vector λ-ZAP II (Stratagene, La Jolla, California) according to standard methods (Hawdon et al., 1995). Phage DNA was isolated from infected bacteria and used as a template in a polymerase chain reaction (PCR), using degenerate primers derived from the conserved serine protease inhibitor sequences (SPI-5′-PstI, 5′-GGTACTGTCAGTACGGY CDDTGYAARG-3′ and SPI2-3′-HindIII, 5′-GGTCAAGCTTGTTRCCRCARCCRCGTA-3′, where Y = C or T, D = AGT, and R = A or G). Each primer was designed by eye from back-translated Kunitz domain protein sequence and contained a 5′ restriction enzyme sequence (underlined) to facilitate amplicon cloning. A hemi-nested strategy was used in the PCR. In round 1, the forward primer SPI-5′-P was used together with the opposite-flanking λ-ZAP II vector primer (T7 promoter) in a “hot-start” PCR (Arnheim and Erlich, 1992). Ten μl reaction mixtures containing the template and 100 ng of each primer were subjected to a temperature of 94 C for 5 min followed by 85 C for 5 min, after which 10 μl containing buffer, 1.5 mM MgCl₂, 0.2 mM of each deoxynucleotide triphosphate, and 1 U of *Taq* polymerase (Promega, Madison, Wisconsin) was added. The reactions were subjected to 30 cycles of denaturation at 94 C for 1 min, annealing at 37 C for 1 min, and extension at 72 C for 1 min. In second-round PCR, 1 μl of a one-tenth dilution of the first-round reaction was used as template with the forward primer and the nested reverse primer SPI2-3′-HindIII. The reactions were subjected to the same cycles as above, except that the annealing temperature was increased to 42 C.

The hemi-nested PCR strategy yielded a 90–base pair (bp) amplicon,



b.

	D1	D2	D3	D4	D5	D9	D10	D11	D12
D1	1.000	0.400	0.360	0.360	0.380	0.400	0.420	0.460	0.269
D2	---	1.000	0.480	0.580	0.600	0.540	0.540	0.460	0.403
D3	---	---	1.000	0.400	0.460	0.500	0.440	0.560	0.384
D4	---	---	---	1.000	0.880	0.740	0.620	0.500	0.403
D5	---	---	---	---	1.000	0.780	0.620	0.560	0.423
D9	---	---	---	---	---	1.000	0.600	0.560	0.384
D10	---	---	---	---	---	---	1.000	0.520	0.384
D11	---	---	---	---	---	---	---	1.000	0.480
D12	---	---	---	---	---	---	---	---	1.000

FIGURE 1. Similarity between the Kunitz domains of *Ac*-KPI-1. **a.** Alignment of individual domains of *Ac*-KPI-1. Shaded residues are shared by 50% or more of the domains, and gray residues indicate similarity. The conserved cysteines are noted, and the active site P1 residue is marked with an asterisk. The residues involved in PROSITE signature PS00280 (F-3x-G-C-6x-[FY]-5x-C) are shown below the alignment. Domains 6, 7, and 8 are identical to domain 5 and are therefore not included. **b.** Proportion of identical amino acids between the individual domains of *Ac*-KPI-1.

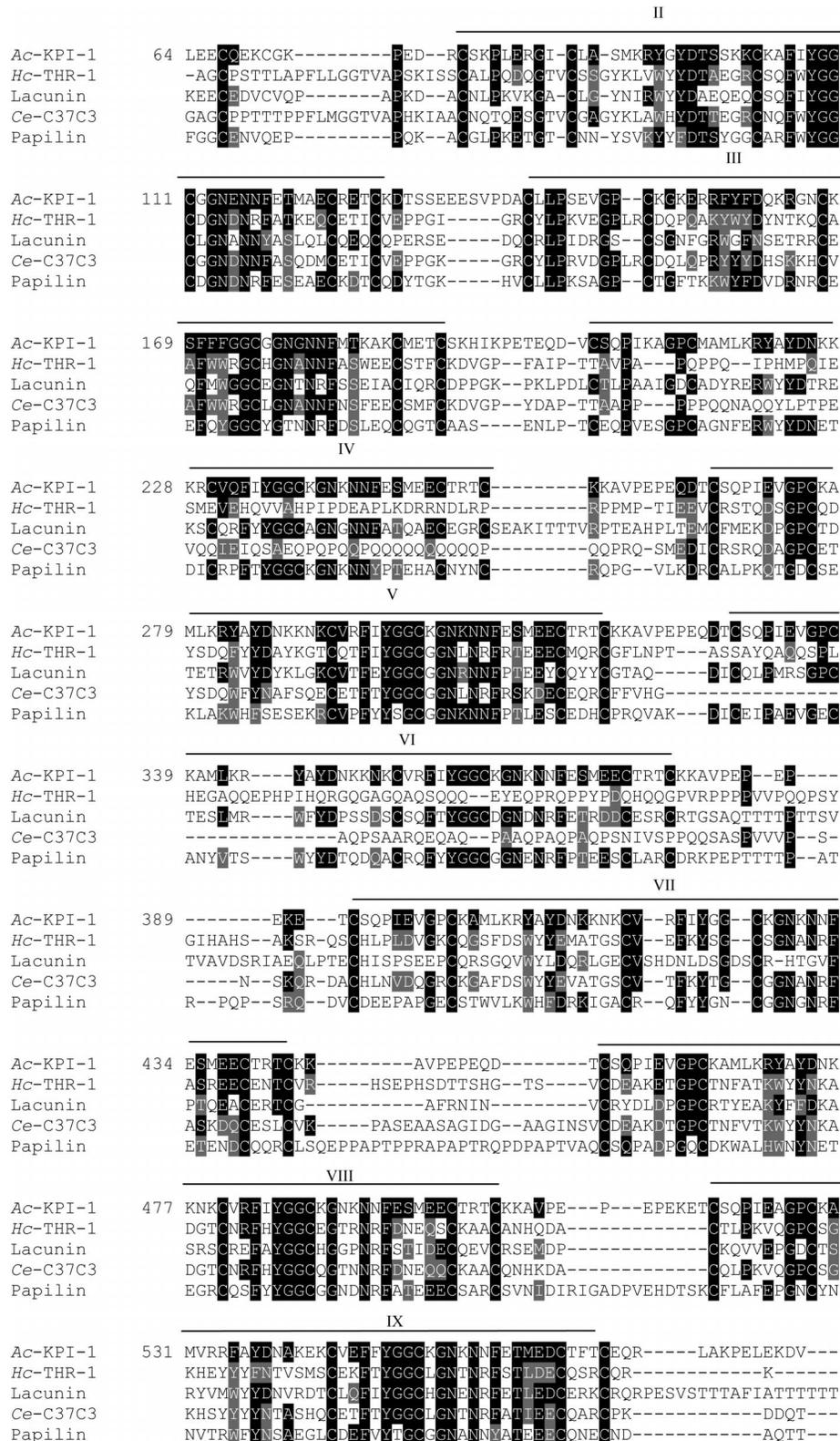
which was used to isolate a near-full length clone from the adult *A. caninum* cDNA library. The probe was radiolabeled by random priming and hybridized to duplicate plaque lifts at 42 C for 12–16 hr in hybridization buffer (5× standard saline citrate [SSC], 5× Denhardt's, 100 μg/ml denatured salmon sperm DNA, 1.0% sodium dodecyl sulfate [SDS]) containing 50% formamide. The filters were washed for 45 min in 2× SSC and 0.01% SDS once at 55 C and once at 60 C. A screen of 1 × 10⁶ phage plaques yielded 7 independent clones after 2 rounds of plaque purification. Plasmid DNA was produced from the positive clones by *in vivo* excision (Short and Sorge, 1992), and 4 of the clones were sequenced manually by the dideoxy method (Sanger et al., 1977; Hattori and Sakaki, 1986) using Sequenase 2 (United States Biochemical, Cleveland, Ohio). All the clones had identical 3' ends and contained intact poly d(A) tails. The largest clone (2.3 kb) was renamed *Ac-kpi-1* and sequenced completely. Initially, approximately 800 bp of the sense strand were sequenced by "walking" with sequential primers. However, spurious results caused by multiple priming and extension from repeated sequences were seen. To circumvent this problem, 1.7-kb *EcoRI/NcoI* (KPI-RN) and 680-bp *NcoI/XhoI* (KPI-NX) fragments, covering the entire cDNA, were subcloned into pBluescript. The remaining approximately 1 kb of KPI-RN, containing 4 identical Kunitz domains, was sequenced by constructing overlapping deletions in both directions, using the Exonuclease III/Mung bean nuclease kit (Stratagene). The nested deletions were sequenced using flanking vector primers, and the resulting overlapping sequences were compiled to form the complete sequence. Fragment KPI-NX was sequenced by primer walking. Both strands of the molecule were sequenced completely. The 5' end was obtained by a modified 5'-RACE described previously (Hawdon et al., 1996). Sequences were analyzed using the Wisconsin Genetics Group software package version 8 (Genetics Computer Group, Madison, Wisconsin).

The complete *Ac-kpi-1* cDNA (GenBank AF533590) is 2,371 bp and encodes a 759-amino acid open reading frame (ORF) with a calculated molecular weight of 84,886 Da and a calculated pI of 8.92. The original 90-bp amplicon used to screen the library is encoded by bases 1,976–2,059 of the *Ac-kpi-1* sequence. The 5' end of the cDNA contains an

11-bp untranslated region (UTR) and lacks the conserved nematode spliced leader sequence (Bektesh et al., 1988; Hawdon et al., 1995). The 3' end of the cDNA contains an 83-bp UTR and a canonical polyadenylation signal (AATAAA) (Blumenthal and Steward, 1997) 20 bp upstream of the poly d(A) tail (nucleotides 2,352–2,357). The N-terminus of the *Ac*-KPI-1 predicted ORF contains a 16 amino acid hydrophobic leader peptide with a predicted cleavage site between ala₁₆ and leu₁₇ (Nielsen et al., 1997). Processing at this site would result in a protein of 83,232 Da. There are 2 potential N-linked glycosylation sites at thr₆₇₂ and thr₇₂₃ predicted by PROSITE (release 17.21; Falquet et al., 2002).

A search of the protein domains using the simple modular architecture research tool (Schultz et al., 1998) revealed that the *Ac*-KPI-1 deduced protein sequence contains 11 Kunitz-type acid serine protease inhibitory domains of the general structure C{8x}C{15x}C{7x}C{12x}C{3x}C and contains the PROSITE signature pattern F-3x-G-C-6x-[FY]-5x-C (PS00280; Falquet et al., 2002). A C-terminal 12th domain contains the signature pattern but lacks several of the conserved cysteines found in the other domains (Fig. 1a). The domains are linked by short, 5- to 16-amino acid conserved spacer regions. The first 11 of the 51-amino acid Kunitz domains contain the classic spacing of the highly conserved cysteines. Domains 5–8 are identical, whereas the others are 36–88% identical (Fig. 1b). Examination of the P₁ reactive site residues indicates that all domains except the 12th domain are probably active inhibitors (Fig. 1a). Domains 2 and 4 have amino acids (leucine and methionine, respectively) consistent with activity against chymotrypsin-like enzymes, whereas the remaining domains contain either lysine or arginine at the P₁ site, typical of inhibitors of trypsin-like enzymes (Laskowski and Kato, 1980). Domain 12 lacks the second (at P₂) and fourth conserved cysteines and has an aspartic acid at the P₁ position, which is not considered a typical reactive site residue. These differences suggest that domain 12 is inactive as a protease inhibitor (Laskowski and Kato, 1980).

The individual domains of *Ac*-KPI-1 show high levels of homology to other members of the Kunitz family of inhibitors, including TFPI, bovine pancreatic trypsin inhibitor, α-1-microglobulin, and inter-α-tryp-



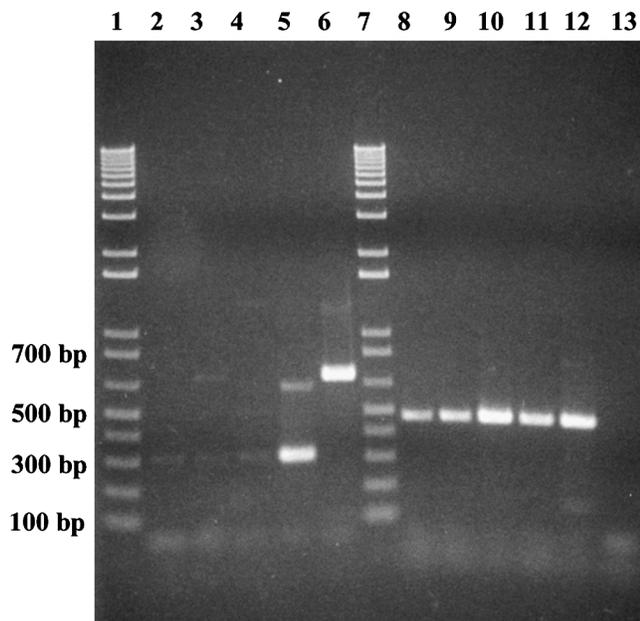


FIGURE 3. Stage specificity of *Ac-KPI-1* expression. First-strand cDNA was synthesized from total RNA isolated from *Ancylostoma caninum* eggs, L₁, L₃, and adults and used as template in a PCR using *Ac-kpi-1*-specific primers (FOR, 5'-ACCGGCCCATGCAAAGG; REV, 5'-AGGCTTCTTTGCAATC) designed by eye from the 3' end of the cDNA sequence (lanes 2–5). Control primers from the 3' UTR of the *A. caninum* protein kinase A subunit were used on the same cDNA preparations and amplified at the same time (lanes 9–12). Lanes 1 and 7, DNA markers; lanes 2 and 8, egg cDNA; lanes 3 and 9, L₁ cDNA; lanes 4 and 10, L₃ cDNA; lanes 5 and 11, adult cDNA; lanes 6 and 12, genomic DNA; lane 13, negative control (no template).

sin inhibitor, although none has as many domains or similar organization. Although there is no obvious *Ac-KPI-1* ortholog with the identical domain organization in GenBank, there is a family of molecules that contain multiple, tandemly arranged C-terminal Kunitz domains. These large, multidomain molecules are characterized by the presence of 6–7 thrombospondin domains in the N-terminus, together with the Kunitz and several other domain types. They include 2 related (possibly alternately spliced) proteins of unknown function from *Drosophila melanogaster* (AAF56794 and AAF56795), similar molecules from the nematodes *Caenorhabditis elegans* (cosmid C37C3.6; NP_505017) and *Haemonchus contortus* (GenBank AAB99830), and the extracellular matrix proteins papilin from *Drosophila* (XP_081921) and lacunin from *Manduca sexta* (AAF04457.1) (Fig. 2). Lacunin is a large protein (3,198 amino acids) involved in morphogenesis, which contains 9 different domains, including 7 thrombospondin domains near the N-terminus and 11 tandem Kunitz domains near the C-terminus (Nardi et al., 1999, 2001).

There are a total of 44 nematode proteins containing one or more Kunitz domains in GenBank, 42 of which are from *C. elegans*. Other than C37C3.6, no protein has 11 tandemly arranged Kunitz domains like *Ac-KPI-1*, although several proteins contain multiple Kunitz domains interspersed with other domains. Finally, a single-domain KI has been reported from the congener hookworm *A. ceylanicum*, which inhibits chymotrypsin, trypsin, and elastases (Milstone et al., 2000). This molecule shows the highest homology with domain 4 of *Ac-KPI-1* (53% identity).

To determine the expression pattern of *Ac-KPI-1*, total ribonucleic acid (RNA) was isolated from *A. caninum* eggs, L₁, L₃, and adult stages, and first-strand cDNA synthesized using oligo dT and reverse transcriptase (Hawdon et al., 1999). Polymerase chain reaction primers specific to the 3' end were used to amplify a portion of the *Ac-kpi-1* gene from the stage-specific first-strand cDNA. Primers that amplified at least 1 intron from genomic DNA (Fig. 3, lane 6) were chosen to ensure that the products were from cDNA and not from contaminating genomic

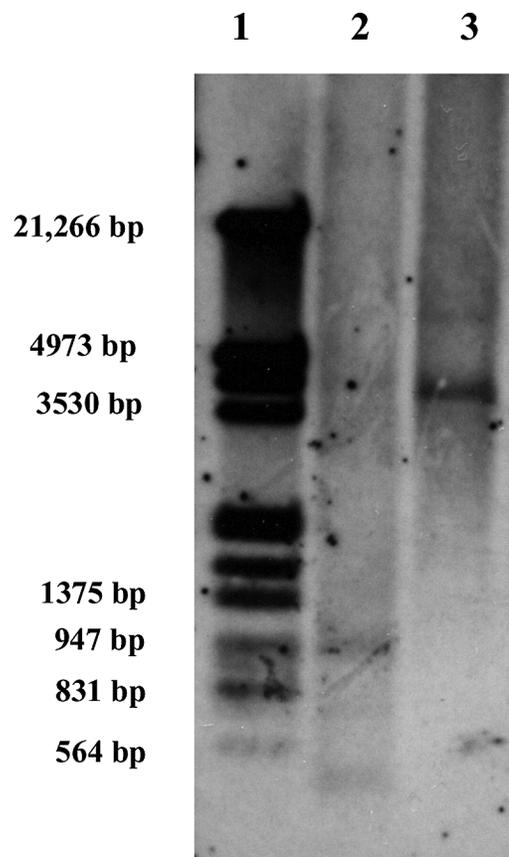


FIGURE 4. Southern blot of *Ancylostoma caninum* genomic DNA with *Ac-kpi-1*. Approximately 5 μ g of *A. caninum* genomic DNA was digested and transferred to a nylon membrane by capillary transfer. A 489-bp probe corresponding to a 3' portion of the sequence (1,791–2,280 bp) was synthesized by PCR in the presence of digoxigenin-labeled deoxyuridine triphosphate and hybridized at 42 C for 18 hr. The signal was detected according to the manufacturer's instructions (Roche, Indianapolis, Indiana). Lane 1, digoxigenin-labeled markers (Roche 1218603); lane 2, DpnII-digested DNA; lane 3, EcoRI-digested DNA.

DNA. As shown in Fig. 3, *Ac-KPI-1*-specific messenger RNA (mRNA) of the expected size is expressed at low levels in all stages but at high levels in the adult stage (lane 5). Efficient amplification from all stages using control primers specific to the 3' UTR of the catalytic subunit of protein kinase A (Hawdon et al., 1995) suggests that the low levels of product seen with the *Ac-kpi-1*-specific primers are the result of less *Ac-kpi-1* mRNA template in the preadult stages rather than an amplification artifact. The additional faint bands are probably due to nonspecific priming and amplification, perhaps from other Kunitz-type inhibitors. The simple pattern seen on a Southern blot using a digoxigenin-labeled probe hybridized to genomic *A. caninum* DNA suggests that *Ac-kpi-1* is a single-copy gene (Fig. 4).

The function of *Ac-KPI-1* is not known. The presence of a hydrophobic leader peptide, together with the absence of a transmembrane domain, suggests that the molecule is secreted (Briggs and Gierasch, 1986). *Ac-KPI-1* might be an extracellular matrix protein involved in morphogenesis, similar to lacunin in insects (Nardi et al., 1999, 2001). Hookworms undergo extensive tissue reorganization during maturation from the L₃ to the adult stage. This would suggest that the protein would be expressed in the parasitic L₃ and L₄ stages as well as in the adult. There is no evidence that *Ac-KPI-1* is expressed in these stages because of the difficulty in obtaining these life-history stages from infected hosts. Alternatively, *Ac-KPI-1* might be released or located on the external surface of the adult hookworm and function directly in parasitism. One possibility is as an additional anticoagulant. Many of the molecules in the coagulation pathway are serine proteases, and hookworms are

known to secrete inhibitors of both factor Xa and the VIIa tissue factor complex (Cappello et al., 1996; Stanssens et al., 1996). Ac-KPI-1 might inhibit host digestive enzymes such as trypsin, chymotrypsin, and pancreatic elastase (Hawley and Peanasky, 1992). Inhibitors of these enzymes have been reported from other parasitic nematodes, including *Ascaris suum* (Bernard and Peanasky, 1993), *Trichuris suis* (Rhoads et al., 2000), *Anisakis simplex* (Lu et al., 1998), and *A. ceylanicum* (Milstone et al., 2000). A multiheaded molecule might effectively inhibit multiple digestive enzyme molecules simultaneously, thereby preventing damage to the parasite. Finally, modulation of the host immune effector cell function by inhibition of serine proteases has been suggested as a parasite defense mechanism. A serine protease inhibitor from *Taenia taeniaeformis* inhibits complement activation (Hammerberg and Williams, 1978), lymphocyte proliferation (Leid et al., 1986), and leukocyte function (Leid et al., 1986; Potter and Leid, 1986), and a similar function has been suggested for a secretory serine protease inhibitor from *T. suis* (Rhoads et al., 2000). Immunomodulation has been suggested as a possible explanation for the apparent lack of significant immunity to reinfection characteristic of hookworm infections (Riffkin et al., 1996; Sun et al., 1998; Liu et al., 1999; Yong et al., 1999; Lili et al., 2000). By neutralizing mast cell or neutrophil serine proteases, which function in inflammation, Ac-KPI-1 might help hookworms survive in previously infected hosts.

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A Human Case of Gnathostomiasis Nipponica Confirmed Indirectly by Finding Infective Larvae in Leftover Largemouth Bass Meat

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ABSTRACT: A human case of creeping eruption due to *Gnathostoma nipponicum* was confirmed indirectly by finding infective advanced third-stage larvae in leftover largemouth bass meat. This is the first report indicating that the largemouth bass (*Micropterus salmoides*) serves as a source of *G. nipponicum* infection in humans.

Human gnathostomiasis nipponica is a well-known zoonotic disease in Japan since the first discovery of the human infection in Mie Prefecture in 1986 (Ando et al., 1988). Since then, confirmed and suspected patients have been found frequently, especially in the northern part of Japan, e.g., Akita and Aomori Prefectures (Sato et al., 1992; Kubota et al., 1997). Nevertheless, the source of human infection in these areas is still obscure.

A 60-yr-old woman from Akita City in Akita Prefecture, northern Honshu, Japan, was admitted to Akita Kumiai General Hospital on 11 June 2001 with skin complaints. She had eaten raw largemouth bass on 20 May, caught in a reservoir in a suburban area of Akita City. Two weeks later, she noticed erythematous patches with a linear extension on her abdomen (Fig. 1). With the informed consent of the patient, we excised the most recent erythematous patches at 3 sites on her abdomen, which were then sectioned and stained with hematoxylin and eosin. We failed to detect any fragments of parasite in these sections. However, we found ellipsoidal spaces in the upper dermis, which were sometimes located beneath blisters in the epidermis and were infiltrated densely with inflammatory cells consisting mostly of lymphocytes and eosinophils. These lesions are suggestive of the migratory paths of the worms. Upon readmission on 18 June, 2 tracks on the lower right abdomen were found that extended slightly beyond the excised portion. Thiabendazole (30 mg/kg body weight in 3 divided doses) was given consecutively for 17 days, and the linear eruptions disappeared.

Laboratory studies on 11 June revealed slight blood eosinophilia (6.9%) without other abnormalities. Using dot-ELISA, serum antibodies against 12 species of helminth antigens, i.e., *Dirofilaria immitis*, *Toxocara canis*, *Ascaris suum*, *Anisakis simplex*, *G. doloresi*, *Strongyloides stercoralis*, *Paragonimus westermani*, *P. miyazakii*, *Fasciola* sp., *Clonorchis sinensis*, *Spriometra erinacei*, and *Cysticercus cellulosae*, were undetectable. The nonspecific IgE level was 170 IU/ml.

We examined 170 g of largemouth bass tissue, which the patient had kept frozen in a deep freezer. In an attempt to find larvae, the thawed tissue was chopped with a pair of scissors and digested with artificial gastric juice. Seven nematode larvae were found. Morphological examination revealed that they were all the advanced infective larvae (L3)

of *G. nipponicum*. Measurements are given in Table I. The larvae were colorless except for the brown-colored intestine (Fig. 2). Two pairs of cervical sacs were present in the region of the club-shaped esophagus. The head bulb had 3 transverse rows of hooklets, each hooklet appearing rectangular at its base (Fig. 3). The whole body was encircled by more than 200 transverse rows of minute, cuticular spines. The spines gradually decreased in size and density toward the posterior regions of the body and finally disappeared near the tip of the tail.

We performed an additional study on natural infections of some freshwater fish species, i.e., largemouth bass (9 individuals), *Carassius auratus cuvieri* (3), and *Tribolodon hakonensis* (5), all of which were captured in the same reservoir located in Kamiotomo, Akita City, where the largemouth bass eaten by the patient had been caught. The survey revealed that only largemouth bass, ranging from 19.5 to 32.8 cm in body length, were positive (prevalence = 66.7%). A total of 9 larvae were recovered from 6 individuals, each of which harbored 1–4 larvae. The larvae were found in the head (a single L3 from 1 fish), the viscera (1 L3 from each of 3 fish) and muscles of the abdominal wall (1 and



FIGURE 1. Creeping eruptions of the patient. Arrowheads denote the sites of the biopsy.

2 L3 from 2 individuals), and other sites (2 larvae from 1 individual). The morphology and measurements of these advanced L3 were identical to those found in the leftover fish (Table I).

Gnathostoma nipponicum requires 2 intermediate hosts to complete its life cycle, i.e., cyclopoid copepods as the first intermediate host and fishes, amphibians, reptiles, birds, or mammals as the second intermediate host (Ando et al., 1992). Some of the latter also serve as paratenic hosts.

The human case showed typical creeping eruptions suggestive of gnathostomiasis or larval spiruriniasis. The migration of at least 3 individual worms was implicated (Fig. 1). However, the serum from the patient was negative by a dot-ELISA test with *G. doloresi* antigen, and worm fragments were absent in histological sections made from excised tissues of the putative worm track. However, detection of *Gnathostoma* worm fragments in skin sections is not always successful (Takahashi et al., 1993). The discovery of advanced *G. nipponicum* L3 (Figs. 2, 3; Table I) in the leftover frozen fish made it possible to diagnose the current case indirectly. The largemouth bass eaten by the patient must have been heavily infected with *G. nipponicum* because worms were detected in only 170 g of the leftover fish. The morphological features and measurements of the advanced L3 obtained from the bass are similar to those of the larvae obtained from loach (Oyamada, Kudo et al., 1995) and catfish (Oyamada, Kawagoe et al., 1995) (Table I), indicating the larvae to be *G. nipponicum*.

The largemouth bass is one of the most popular game fish species in Japan and is frequently eaten raw. The prevalence of *G. nipponicum* in the largemouth bass is high (66.7%) when compared with other fishes. For instance, the prevalence of advanced *G. nipponicum* L3 has been shown to be 1–1.3% in loaches (*Misgurnus anguillicaudatus*) (Oyamada, Kudo et al., 1995), 46.7% in catfish (*Silurus asotus*) (Oyamada, Kawagoe et al., 1995), 2.8% in *Oncorhynchus masou* and 0.2% in *T. hakonensis* (Oyamada, Esaka, Kudo, Oyamada et al., 1996), and 3.4% in *Chaenogobius urotaenia* (Oyamada et al., 1997). Some of these species may serve as a second intermediate host, a paratenic host, or both. The largemouth bass is well known to be a voracious carnivore, and hence this species may be both an important second intermediate host and a paratenic host for *G. nipponicum*.

The loach (Ando et al., 1988) and catfish (Ando et al., 1991) have also been incriminated as sources of human gnathostomiasis nipponica. Moreover, it has been reported that gnathostomiasis patients frequently report that they had eaten raw common ice-fish (*Salangichthys microdon*) before developing creeping eruptions (Sato et al., 1992; Takahashi et al., 1993). Nevertheless, natural infection of common ice-fish with *G. nipponicum* has never been reported, although an extensive survey



FIGURE 2. A *Gnathostoma nipponicum* larva recovered from leftover largemouth bass meat. Bar = 300 μ m.

TABLE I. Measurements (μ m) of larval *Gnathostoma nipponicum* recovered from leftover largemouth bass and comparison with those from other fresh-water fishes.

Host	Largemouth bass*	Loach†	Catfish‡
No. of specimens	7	34–38	72–79
Body length	970–1,685	745–1,684	508–1,675
Body width	110–155	98–186	90–170
Headbulb height	38–63	28–60	32–66
Headbulb width	88–105	54–108	78–108
Esophagus length	470–555	321–594	334–600
Cervical sac length	190–295	156–363	113–360
Tail length	30–73	15–62	21–63
No. of transverse striations	201–237	188–267	212–246
No. of hooklets on headbulb			
First row	26–35	28–39	26–41
Second row	35–39	30–42	25–40
Third row	36–47	25–46	34–45

* Present authors.

† Oyamada, Kudo et al. (1995).

‡ Oyamada, Kawagoe et al. (1995).

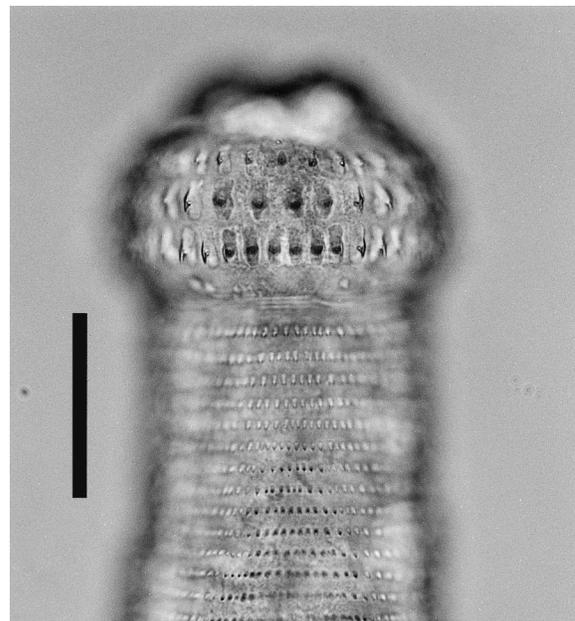


FIGURE 3. Head bulb showing 3 rows of hooklets on the surface. Note the minute, cuticular body spines. Bar = 100 μ m.

was conducted (Takahashi et al., 1993; Oyamada, Esaka, Kudo, and Yoshikawa 1996).

Our study suggests that the specific serological diagnosis of gnathostomiasis nipponica with *G. doloresi* antigen is difficult. The dot-ELISA test using *G. nipponicum* antigen and other serological methods remain to be developed. In the present case we failed to extirpate migratory worms because new creeping eruptions appeared after the biopsy. However, the treatment with thiabendazole seemed to be effective, although it is not clear whether the cure was due to the drug or to the spontaneous death of the migrating larvae.

We thank Tetsuo Takahashi for collecting freshwater fishes. The current case was detected when T.K. was working for Akita Kumiiai General Hospital.

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Two Species of Canine *Babesia* in Australia: Detection and Characterization by PCR

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ABSTRACT: The haemoprotozoan *Babesia canis* has been recognized in Australia for many years, and a second, smaller species has recently been discovered. Amplification and sequencing of a partial region of the 18S small subunit ribosomal RNA (rRNA) gene enabled detection and characterization of the large and small canine babesiae of Australia for the first time. Isolates from northern Australia were genetically characterized to be 99% homologous to *Babesia canis vogeli*, confirming previous speculation about the subspecies of *B. canis* endemic to Australia. The partial 18S rRNA gene sequence amplified from isolates obtained in southeastern Australia was genetically identical to *Babesia gibsoni*, a species not previously known in Australia. The polymerase chain reaction (PCR) used was shown to be specific to *Babesia* and had a high sensitivity, detecting DNA at a parasitemia of approximately 0.0000027%. This study also reports the first known detection and characterization of *B. canis* DNA in *Rhipicephalus sanguineus* ticks using PCR.

Babesiosis is an emerging tick-transmitted disease of both veterinary and medical significance, resulting from intraerythrocytic infection by species of *Babesia* (Homer et al., 2000; Kjemtrup and Conrad, 2000). Clinical signs and pathogenesis of the disease are variable and depend on the species or strain of the parasite and the immune status of the infected animal. The canine *Babesia* have traditionally been recognized as belonging to 2 morphologically distinct species, the large *B. canis* and the small *B. gibsoni*. Recently, the phylogenetic classification of these canine piroplasms has received renewed attention, and it is now

believed that multiple species exist worldwide (Carret et al., 1999; Zahler, Rinder, Zwegarth et al., 2000).

At least 3 species of small canine piroplasm have been described (Kjemtrup et al., 2000). Those genetically characterized as *B. gibsoni* have been reported throughout Asia (Patton, 1910; Fowler et al., 1971; Farwell et al., 1982; Zahler, Rinder, Zwegarth et al., 2000) and parts of the United States (Anderson et al., 1979; Birkenheuer et al., 1999). Genotypically distinct, yet morphologically indistinguishable, from these are the small piroplasm species found in dogs from northern Spain, which have been given the name *Theileria annae* (Zahler, Rinder, Schein et al., 2000; Camacho et al., 2001). A third species has been found in the blood of dogs in California and remains unnamed (Kjemtrup et al., 2000). Genetically uncharacterized small piroplasms also have been found in dogs in Italy (Casapulla et al., 1998) and northern Africa (Botros et al., 1975).

Three subspecies of *B. canis* are now recognized, *B. canis canis*, *B. canis rossi*, and *B. canis vogeli* (Uilenberg et al., 1989; Zahler et al., 1998; Carret et al., 1999). Furthermore, it has been suggested that each of these is distinct enough to assume species status on the basis of genetic characterization (Carret et al., 1999). *Babesia canis canis* is transmitted by *Dermacentor reticulatus* ticks in Europe, and *B. canis rossi* is transmitted by *Haemaphysalis leachi* in southern Africa (Uilenberg et al., 1989). *Babesia canis vogeli* is transmitted by *R. sanguineus* and is distributed throughout various tropical and subtropical countries, including Australia.

Within Australia, it was originally believed that *B. canis* was the only endemic species reported to cause disease throughout northern and

semitemperate regions (Hill and Bolton, 1966; Irwin and Hutchinson, 1991). These large piroplasms were shown to be experimentally transmitted by *R. sanguineus* ticks (Hill, 1966), and using information regarding *B. canis* subspecies vector specificity (Uilenberg et al., 1989), it was later inferred that the Australian strain was likely to be *B. canis vogeli* (Irwin and Hutchinson, 1991). *Babesia canis* also has been reported in dingoes (*Canis familiaris dingo*) (Callow, 1984); these wild canids may represent a potential reservoir of infection for domestic dogs. Recently, *B. gibsoni* was identified in the blood of 3 dogs in southeastern Australia (Muhlnickel et al., 2002).

Various diagnostic techniques have been used to detect the presence of *Babesia* sp. infection, with differing levels of success. Detection of *Babesia* sp. parasites is usually achieved using microscopic examination of blood smears, but this technique is limited because of low sensitivity and the difficulty of distinguishing morphologically similar strains and species (Krause et al., 1996). The immunofluorescent antibody test also is used to assess exposure to *Babesia* sp. infections in dogs; however, it has poor specificity as a result of antigen cross-reactivity (Yamane et al., 1994). It also fails to identify current infection. The use of alternative techniques, such as polymerase chain reaction (PCR), has become necessary to detect and identify *Babesia* sp. infections effectively and has been reported in numerous recent studies (Carret et al., 1999; Ano et al., 2001; Fukumoto et al., 2001). It is hoped that these molecular techniques will provide a solution to the issues of low sensitivity and specificity exhibited by the more traditional diagnostic methods. Furthermore, DNA sequence analysis can provide taxonomic information when morphological characteristics of different parasites are identical. In this study, we used a genus-specific PCR to detect and characterize *Babesia* sp. in canine blood and *R. sanguineus* ticks by amplifying and sequencing a portion of the 18S ribosomal RNA (rRNA) gene. This is the first report of molecular characterization of the Australian canine *Babesia* sp. parasites and the first time PCR was used to detect canine *Babesia* sp. in Australia.

Sixty-four samples of venous blood were collected from 34 dogs of various breeds and ages with anemia or thrombocytopenia (or both) that were presented to veterinary clinics in northern Australia and from 3 dogs in Victoria (southeastern Australia). Twelve dogs were known to be infected with *Babesia* sp. through microscopic observation of the parasites in blood smears made from peripheral blood samples. Blood was prevented from clotting by addition of ethylenediamine-tetraacetic acid (EDTA) and was frozen at -20°C until analysis. An isolate of sonicated whole *B. canis* parasite originating from north Queensland was used as a positive control sample (Irwin, 1989).

DNA was isolated from 200- μl aliquots of EDTA blood using a QIAamp[®] DNA mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The primers PIRO A1 (5' AGGGAGCC-TGAGAGACGGCTACC 3') and PIRO-B (5'TTAAATACGAATGCCCAAC 3') were used to amplify an approximately 450-bp region of the 18S rRNA gene. The reverse primer PIRO B was used previously by Carret et al. (1999) and the forward primer PIRO A1 was developed to amplify most *Babesia* species using sequence information from GenBank. One microliter of extracted DNA was added to a 24- μl reaction mixture comprising 0.625 units of HotStarTaq[™] DNA Polymerase (QIAGEN), 200 μM of each deoxynucleoside triphosphate, 12.5 pmol of each primer, and 2.5 μl of $10\times$ PCR buffer (containing 15 mM MgCl_2) (QIAGEN). Amplification was performed using a GeneAmp PCR System 2400 Thermal Cycler (Perkin-Elmer, Foster City, California). An initial activation step at 95 $^{\circ}\text{C}$ for 15 min, 62 $^{\circ}\text{C}$ for 1 min, and 72 $^{\circ}\text{C}$ for 2 min was followed by 35 cycles of amplification (94 $^{\circ}\text{C}$ for 30 sec, 62 $^{\circ}\text{C}$ for 20 sec, and 72 $^{\circ}\text{C}$ for 30 sec) and a final extension step of 7 min at 72 $^{\circ}\text{C}$. The PCR products were electrophoresed on a 1% agarose gel and purified using an UltraClean[™] Gelspin DNA Purification Kit (MO Bio Laboratories, Inc., Sohlana Beach, California) according to manufacturer's instructions.

Products of PCR were sequenced using an ABI Prism[™] Dye Terminator Cycle Sequencing Kit (Applied Biosystems [ABI], Foster City, California). The sequenced products were analyzed using SeqEd v.1.0.3 (ABI), compared with sequence data available from GenBank[™] using the BLAST 2.1 program (<http://www.ncbi.nlm.nih.gov/BLAST/>), and aligned using CLUSTAL X (Thompson et al., 1997).

Semiengorged *R. sanguineus* ticks were collected from 2 dogs in northern Australia from which blood samples were concurrently obtained. Ticks were macerated using a scalpel blade, and DNA was ex-

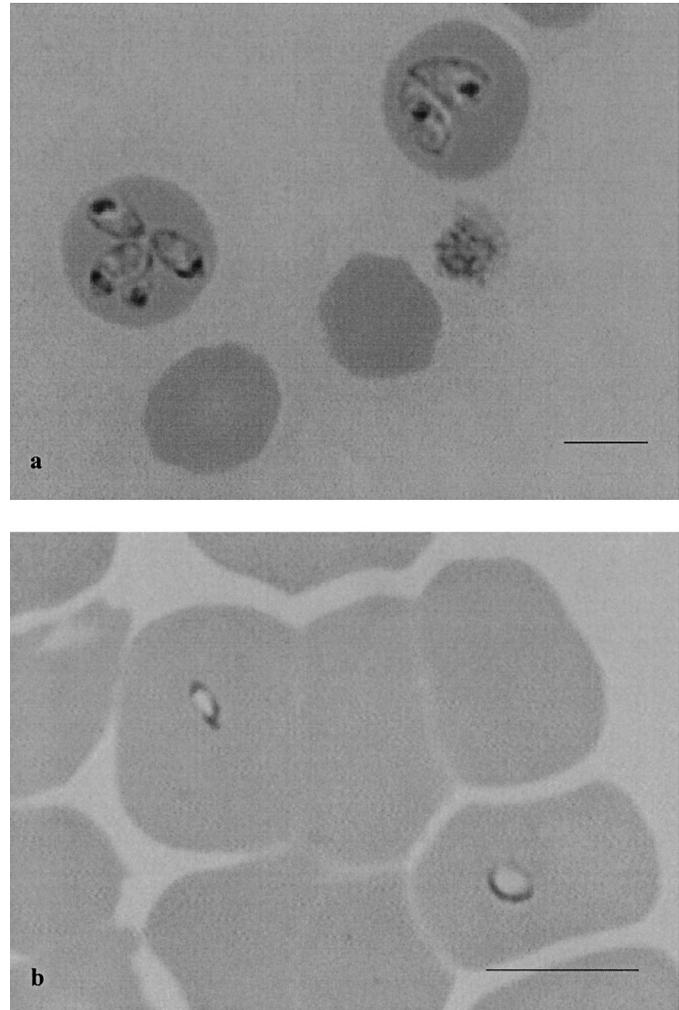


FIGURE 1. Light microscope view of canine erythrocytes infected with *Babesia canis vogeli* (a) and *Babesia gibsoni* (b). Bar = 5 μm .

tracted using a QIAamp[®] DNA Mini Kit (QIAGEN) according to the manufacturer's instructions. The extracted DNA was amplified and sequenced according to the protocol previously described for blood.

The detection limit of the PCR was estimated using a system of serial dilutions of parasitized blood in normal, uninfected canine blood. A venous blood sample with a *Babesia* sp. parasitemia of 2.72% (calculated according to Read and Hyde, 1993) was diluted 1:10 in canine blood known to be free from *Babesia* infection by microscopy and by its lack of exposure to tick vectors. This process was repeated until a 1×10^{-7} dilution was obtained. DNA was extracted and amplified from each dilution according to the protocol previously described.

Babesia specificity was confirmed by BLAST searching of the PIRO A1 and B primers (<http://www.ncbi.nlm.nih.gov/BLAST/>). This also was used to ensure that the primers would not amplify human DNA or DNA from other blood-borne canine parasites and bacteria. The primers also were tested against a variety of other pathogens potentially found in canine blood, including *Ehrlichia canis*, *E. platys*, *Rickettsia rickettsia*, *Bartonella vinsonii*, *Neospora caninum*, *Toxoplasma gondii*, *Trypanosoma cruzi*, and *Dirofilaria immitis*. Sixteen blood samples also were obtained from clinically normal dogs in New Zealand, a country reportedly free from canine babesiosis, as an added negative control.

Babesia sp. trophozoites in the blood of dogs from northern Australia were observed using light microscopy as intraerythrocytic piroplasms, the morphology of which was consistent with previous descriptions (Hill and Bolton, 1966; Irwin and Hutchinson, 1991) (Fig. 1a). The protozoans were polymorphic; however, many were pyriform-shaped.

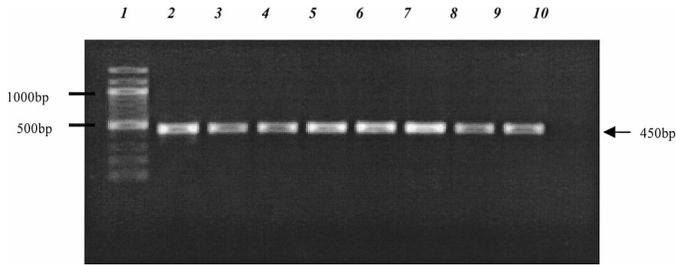


FIGURE 2. Ethidium bromide-stained 1% agarose gel showing amplification of a 450-bp product for *Babesia*-positive samples (lane 1, molecular marker; lane 2, positive control; lanes 3–9, *Babesia*-positive samples; lane 10, negative control).

Trophozoites occurred singularly, paired, or as multiple organisms within individual red blood cells. The piroplasms ranged in size from 3.0 to 5.0 μm . *Babesia* sp. from the 3 dogs from Victoria were identified as singular or paired intraerythrocytic parasites. Free merozoites were occasionally observed. The parasites were polymorphic and ranged in diameter from 1 to 3 μm (Fig. 1b).

An approximately 450-bp product was amplified from the blood of 14 of the 34 dogs from northern Australia (Fig. 2). A similar-sized product was obtained from the blood of all 3 dogs from Victoria. Four of the 14 positive *Babesia* samples, in addition to the positive control sample from Queensland, were sequenced, and the partial 18S rDNA sequences showed 100% homology to one another (sequences submitted to GenBank, accession numbers AY102162 and AY102163). The sequences were 99% homologous to an Egyptian isolate sequence of *B. canis vogeli* obtained from GenBank (accession number AJ009769). These Australian isolates differed by 2 nucleotides within the Egyptian isolate and in both cases involved a pyrimidine substitution. The partial 18S rRNA gene of all three Victorian isolates were sequenced (submitted to GenBank, accession number AY102164) and showed an identical alignment with *B. gibsoni* (accession number AF271082).

Babesia sp. DNA was amplified from 1 of the semiengorged ticks originating from a dog from northern Australia that was also PCR positive for *Babesia* sp. The amplified product was sequenced and was identical to the *B. canis vogeli* genotype found in each of the canine blood samples from northern Australia.

The PCR used within this study was able to detect an estimated parasitemia of 0.0000027% (1 infected red blood cell in 4 million). None of the 16 blood samples from New Zealand contained amplifiable *Babesia* sp. DNA. The primers amplified DNA of *T. gondii* and *N. caninum*; however, the amplified products for these species could be differentiated based on size and were approximately 60 bp larger than the *Babesia* sp. products.

This study documents the first reported genetic characterization of the Australian strain of *B. canis*. On the basis of an amplified region of the 18S rRNA gene of the northern Australian isolates, it can be assumed that the Australian strain is *B. canis vogeli*. This confirms previous speculation by Irwin and Hutchinson (1991), who tentatively defined the Australian strain on the basis of pathogenicity and vector specificity. The amplified products from each of the large canine *Babesia* sp. isolates from northern Australia were found to be identical to one another and were 99% homologous with the Egyptian genotype of the same parasite. This suggests that there may be different genotypes of the subspecies *B. canis vogeli*; however, a paucity of available sequences of isolates from other geographic locations has prevented assessment of the degree of diversity among this subspecies. Additional research is required to assess the extent of differences between the various isolates of *B. canis vogeli*.

Sequencing of the amplified region of the 18S rRNA gene also allowed for genetic confirmation of a small canine *Babesia* sp. recently reported in Australia by Muhlnickel et al. (2002). Based on the 18S rRNA gene, all 3 Victorian isolates were found to be identical to isolates of *B. gibsoni* from Oklahoma and North Carolina in the United States and from Okinawa in Japan. Zahler, Rinder, Zweygarth et al. (2000) have proposed that the isolates from Asia retain the name *B. gibsoni* on the basis of the description of these piroplasms in dogs in India by Patton (1910). It is implied that these Indian parasites are genetically

similar to those found throughout other regions of Asia. It can then be assumed that all small canine piroplasms that are genetically similar to the Asian genotypes also are allocated to the species *B. gibsoni*. This includes those from Oklahoma and North Carolina in the United States (Kjemtrup et al., 2000) and the Australian isolates described in this study. Further research is necessary to identify the genotype of isolates from other countries in order to allocate the correct name to each geographic isolate and help avoid further confusion.

Babesia gibsoni is a more pathogenic species than *B. canis vogeli* and is therefore of greater concern from a clinical perspective. Its discovery provides support for canine babesiosis being considered an emerging disease; however, this may have only been a result of increased awareness and better diagnostic techniques. The clinical signs and laboratory findings of *B. gibsoni* have a history of being inadvertently diagnosed as another form of anaemia such as idiopathic or immune-mediated haemolytic anaemia (Birkenheuer et al., 1999). Despite this it seems unlikely that this relatively virulent form of canine babesiosis would have remained undetected in Australia for a long period of time.

This is also the first report of the use of PCR to detect the presence of canine *Babesia* sp. DNA in *R. sanguineus* ticks. Molecular detection of pathogen DNA within ticks has been reviewed extensively by Spargano et al. (1999); however, there are no reports of canine *Babesia* sp. DNA being extracted and amplified from ticks. The ticks used in this study were semiengorged, which made it difficult to assess whether they were themselves infected with *Babesia* parasites or whether the parasites were only present within the blood meal of the vector.

Ticks were not available from the dogs infected with *B. gibsoni* from Victoria, and it is therefore difficult to assess the vector responsible for transmission of these parasites in Australia. It has been reported that *B. gibsoni* can be transmitted by the ticks *Haemophysalis bipinosa*, *Haemophysalis longicornis*, and, possibly, *R. sanguineus* (Wozniak et al., 1997). Both *R. sanguineus* and *H. longicornis* are endemic in certain regions of Australia, thus increasing the transmission and distribution potential of *B. gibsoni*.

The use of PCR to detect the presence of canine *Babesia* sp. in Australia confirms that this technique is a highly sensitive and specific method of parasite detection. The advantage of using a genus-specific PCR is that multiple species, including novel genotypes, can be detected with the same set of primers. Although the PCR used in this study only detected *B. canis* and *B. gibsoni*, it has the potential to detect other *Babesia* species. Genetic sequencing can then be used to characterize each sample and can be further used to assess phylogenetic relationships between isolates. The PCR assay used in our study had the ability to detect a *Babesia* sp. infection with a blood parasitemia of 0.0000027%, which is comparable with previous reports (Fukumoto et al., 2001). It also exceeds the sensitivity of previously developed PCR assays for detection of *Babesia* species, which could amplify DNA from a sample with a parasitemia of 0.0001% (Ano et al., 2001) and 0.00008% (Roy et al., 2000). Despite a possible limitation in subclinical detection, PCR shows great promise as an effective means of detecting and characterizing canine *Babesia* sp. parasites and has the potential to be implemented in a clinical diagnostic laboratory situation.

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Cutaneous Trematode *Collyriclum faba* in Wild Birds in the Central European Carpathians

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ABSTRACT: The occurrence of cutaneous trematode *Collyriclum faba* in wild birds was monitored in the central European Carpathians from 1996 to 2001. A total of 5,414 birds, representing 86 species, was examined. *Collyriclum faba* was found at 7 sites (5 in Slovakia, 1 in Poland, and 1 in the Czech Republic), and prevalences at the sites

varied from 1 to 16%. Ten species of passerine birds were infected: blackcap (*Sylvia atricapilla*) (16 positive/622 tested, 2% prevalence), black redstart (*Phoenicurus ochruros*) (2/25, 8%), chaffinch (*Fringilla coelebs*) (7/113, 6%), common blackbird (*Turdus merula*) (1/143, 1%), common redstart (*Phoenicurus phoenicurus*) (1/30, 3%), dipper

(*Cinclus cinclus*) (1/9, 11%), European robin (*Erithacus rubecula*) (103/838, 12%), goldcrest (*Regulus regulus*) (1/76, 1%), grey wagtail (*Motacilla cinerea*) (5/25, 20%), and yellowhammer (*Emberiza citrinella*) (1/73, 1%). Cutaneous cysts of *C. faba* were found in the birds from the end of May to mid-September, with the prevalence peaking in July and August. One to 21 cysts per bird were found. In black redstart, chaffinch, common redstart, European robin, and yellowhammer, cysts were most frequently observed on the legs, particularly in the crural region. In blackcap, common blackbird, dipper, and grey wagtail, almost all the cysts were found around the vent and on the abdomen. In goldcrest, the cyst was located above the coccygeal gland. *Collyriclum faba* is a common parasite in birds during summer in central European Carpathians. *Collyriclum faba* was observed for the first time in Poland. It appears that *C. faba* may be fatal for some of the bird hosts.

Adult *Collyriclum faba* (Bremser in Schmalz, 1831) infects a number of bird species in vast areas of Eurasia and North, Central, and South America (Farner and Morgan, 1944; Sharpilo and Loskot, 1967; Speich, 1971; Stunkard, 1971; Blankespoor et al., 1985; Kirmse, 1987). They are usually found in pairs in subcutaneous cysts located most often around the vent. Subcutaneous cysts also may be found in the abdominal region, from the vent to the sternum, under the wings, on the thorax, shanks, and the head. Reports of *C. faba* in Eurasia have been sporadic, and this fluke has been considered a rare bird trematode (Bychovskaya-Pavlovskaya, 1974; Denzler and Lobsiger-Mollet, 1991). There, however, are a few reports from the early 19th century about a more frequent occurrence of *C. faba* in Switzerland (Miescher, 1838).

Until recently, there have been no reports of the occurrence of *C. faba* in mountain areas of the central European Carpathians of the Czech Republic, Slovakia, and Poland. In 1996, however, the first endemic focus of *C. faba* in Bukovské Vrchy Mountains in Slovakia, the westernmost tip of the eastern Carpathians, was discovered by Literák and Sitko (1997). These authors found *C. faba* at that location and at other sites in the Carpathians in Slovakia, Poland, and the Czech Republic from 1996 to 2001. The aim of the study was to document the occurrence of *C. faba* at individual sites in the Carpathians, to ascertain the range of wild bird species infected, to determine the prevalence of the infection, to characterize the infection intensity and the distribution of subcutaneous cysts of *C. faba* on the body, and to describe the dynamics of *C. faba* occurrence at 2 monitored sites.

Mist nets were used to trap wild birds at various locations in central European Carpathians. Trapped birds were visually examined for the presence of cutaneous cysts of *C. faba*. Directly, or after winnowing the feathers, their legs (especially the shanks), the abdominal region from the vent to the sternum, the neck, the region under the wings, the upper body part around the coccygeal gland, and the head were examined. Small scissors were used to cut the top of any cutaneous cysts, their content was carefully squeezed out to determine whether they contained *C. faba*. In infected birds, the number and location of cutaneous cysts containing *C. faba* were recorded. After the examination and the collection of trematodes and preservation in 70% ethyl alcohol, the birds were released.

Ten collecting sites were used in Slovakia, Poland, and the Czech Republic (Fig. 1). In Slovakia, these included Blatnica (48°56'N, 18°56'E; 152 birds of 20 species were examined from 2 August 1999 to 8 August 1999) (study site 1 in Fig. 1), Necpaly (48°59'N, 18°58'E; 101 birds of 20 species were examined from 25 July 2000 to 6 August 2000) (study site 2 in Fig. 1), Runina (49°04'N, 22°25'E; 46 birds of 14 species were examined in several days in June 1999 and June 2000) (study site 3 in Fig. 1), Ruské (49°07'N, 22°21'E; birds were examined in August and September 1996 through 1999 at 2-wk intervals and for the period of 2 mo from 28 July 2001 to 29 September 2001; 3,454 birds of 68 species were examined) (study site 4 in Fig. 1), Socovce (48°57'N, 18°52'E; 514 birds of 41 species were examined from 25 July 1999 to 1 August 1999) (study site 5 in Fig. 1), Svätý Jur (48°15'N, 17°13'E; 76 birds of 23 species were examined for 4 days at the end of June 2001) (study site 6 in Fig. 1), and Tatranská Javorina (49°16'N, 20°09'E; 252 birds of 25 species were examined between 8 August 2000 and 19 August 2000) (study site 7 in Fig. 1). In Poland, birds were examined in Krempana (49°30'N, 21°29'E; 248 birds of 31 species were examined between 21 August 2000 and 26 August 2000) (study site 8 in Fig. 1) and in Roztoki Górne (49°09'N, 22°19'E; 227 birds of 33

species were examined from 30 July 2001 to 9 August 2001) (study site 9 in Fig. 1). In the Czech Republic, birds were examined in Horní Němč (48°56'N, 17°38'E; 345 birds of 30 species were examined in September 1999 and from April 2000 to September 2000) (study site 10 in Fig. 1).

Collyriclum faba was found at 7 of 10 sites (Table I). Total prevalence of *C. faba* differed among the sites. Highest prevalences were observed in Blatnica and Necpaly, where 16 and 15% of the birds were infected, respectively. Species range of infected birds was quite narrow. *Collyriclum faba* was found in only 10 species, all of them from the passerines. In almost all cases, hatch-year birds were infected. Of 133 infected birds, infection of adults was observed in only 5 European robins (*Erithacus rubecula*) and 1 chaffinch (*Fringilla coelebs*).

Seasonal dynamics of *C. faba* was studied at 2 sites. In Horní Němč, the first *C. faba* cysts were observed in the birds trapped in late May (Table II). The number of infected birds increased until the end of July. At the beginning of September, the number of infected birds decreased, and no *C. faba* cyst was found in late September. In Ruské, where the trapping and examinations of birds were conducted for 2 mo in 2001, the time was divided into approximately 10-day periods, and the prevalence of *C. faba* in birds was examined in those periods. In the first, second, and third 10-day periods of August, prevalences were 3, 3, and 2%, respectively (Table III). In September, *C. faba* was found only during the first and second 10-day periods, with prevalences of 0.3 and 0.6%, respectively. In Ruské, a significant decrease in the *C. faba* prevalence in September also was observed.

In 4 cases, no *C. faba* cyst was found when birds were first trapped and examined. When the same bird was reexamined later, *C. faba* cysts were found that must have developed in the period between the 2 examinations. In European robins, the cysts developed in 13, 14, 17, and 19 days. The number of *C. faba* cysts per bird varied from 1 to 21. Most often, only 1 cyst was found; 10 or more cysts was rare (Table IV). A total of 21 cysts was found in 1 blackcap (*Sylvia atricapilla*) in Blatnica on 3 August 1999.

The localization of cutaneous cysts showed marked differences among individual bird species (Table V). In black redstart (*Phoenicurus ochruros*), chaffinch, common redstart (*Phoenicurus phoenicurus*), European robin, and yellowhammer (*Emberiza citrinella*), the cysts were most often located on the legs, especially in the shank region. In blackcap, common blackbird (*Turdus merula*), dipper (*Cinclus cinclus*), and grey wagtail (*Motacilla cinerea*), almost all cysts were found around the vent and on the abdominal surface. One goldcrest (*Regulus regulus*) had a cyst above the coccygeal gland. Exceptionally, cysts were found on the thorax, the wing, near the bill, and near the eye.

In most cases, no apparent alteration of the health status of infected birds was found. In several birds, however, negative consequences of the *C. faba* infection were obvious. One grey wagtail with 8 cysts around the vent was extremely emaciated (the bird examined in Blatnica on 3 August 1999). Another grey wagtail with 14 cysts around the vent and on the abdomen had its vent caked with excrement and cyst fluid to such an extent that it made defe-

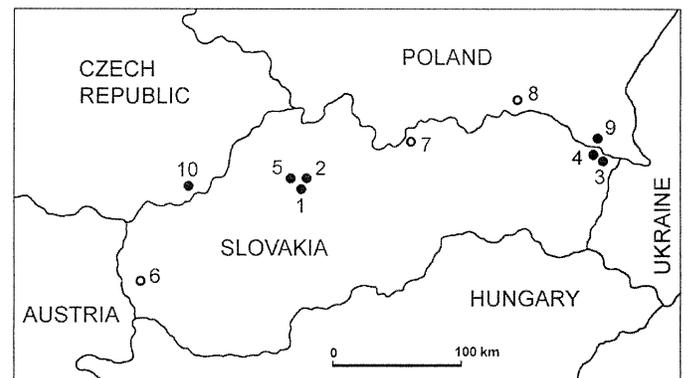


FIGURE 1. Study sites 1–10 in the Central European Carpathians. Full circles—sites with *Collyriclum faba* in wild birds; empty circles—*C. faba* was not found in these sites.

TABLE I. Prevalence of *Collyriclum faba* in wild birds at 10 localities in the central European Carpathians (1–7, Slovakia; 8 and 9, Poland; and 10, the Czech Republic).

Species	1	2	3	4	5	6	7	8	9	10	Total	% Prevalence
Blackcap (<i>Sylvia atricapilla</i>)	9/28*	7/20	—	346	81	12	15	34	19	67	16/622	2
Black redstart (<i>Phoenicurus ochruros</i>)	—	1	3	2	—	—	3	1	2/15	—	2/25	8
Chaffinch (<i>Fringilla coelebs</i>)	2/6	—	2/6	54	1/3	4	3	27	2	2/8	7/113	6
Common blackbird (<i>Turdus merula</i>)	—	8	—	79	22	8	—	1	—	1/25	1/143	1
Common redstart (<i>Phoenicurus phoenicurus</i>)	—	—	—	1/30	—	—	—	—	—	—	1/30	3
Dipper (<i>Cinclus cinclus</i>)	1/3	—	—	5	—	—	—	—	1	—	1/9	11
European robin (<i>Erithacus rubecula</i>)	8/26	7/38	1	66/595	4/5	8	27	27	4/36	14/75	103/838	12
Goldcrest (<i>Regulus regulus</i>)	—	1/1	—	10	—	—	64	—	—	1	1/76	1
Grey wagtail (<i>Motacilla cinerea</i>)	5/11	2	1	5	—	—	—	—	6	—	5/25	20
Yellowhammer (<i>Emberiza citrinella</i>)	—	—	4	1/32	7	3	—	13	—	14	1/73	1
Total (all 86 species)	25/152	15/101	2/46	68/3454	5/514	0/75	0/252	0/248	6/227	17/345	138/5414	3
Prevalence (%)	16	15	5	2	1	0	0	0	3	5	3	

* Infected/examined.

cation impossible (the bird examined in Blatnica on 4 August 1999). If the plug had not been removed, the bird would have likely died. A young European robin was found dead on a trail about 8 km from the Ruské on 14 August 2001. An examination of this specimen revealed 14 *C. faba* cysts of various age. Except for extreme emaciation and presence of cysts, it showed no other pathological condition. It was clear that the death of the bird was related to the high intensity of *C. faba* infection.

In this study, the occurrence of *C. faba* is described for the first time in blackcap, black redstart, common blackbird, dipper, and yellowhammer. The occurrence of *C. faba* has been reported in the literature in chaffinch, common redstart, European robin, goldcrest, and grey wagtail, but the number of reports of *C. faba* occurrence in these species is very small. In chaffinch, the first occurrence of *C. faba* was observed in eastern Prussia (today the Kaliningrad region in Russia) (Mühling, 1898) and later in Basel, Switzerland (Jegen, 1917). Chaffinch with a high intensity of *C. faba* infection was trapped in autumn in Germany (Groth, 1964). *Collyriclum faba* infection in common redstart was reported in Basel, Switzerland (Jegen, 1917), and in Kurskaya Peninsula in Russia (Bychovskaya-Pavlovskaya and Khotenovsky, 1964; Bychovskaya-Pavlovskaya, 1974). Outside the Carpathians, *C. faba* was described only in 1 European robin trapped during its spring passage over the Kurskaya Peninsula (Bychovskaya-Pavlovskaya, 1974). The unique findings of *C. faba* in this species in the Slovakian Carpathians in 1996 (Literák and Sitko, 1997) were a stimulus for the monitoring of the *C. faba* occurrence in wild birds in this region until 2001. Infected goldcrests have so far been found in the foothills of the Swiss Alps and in Brdy Mts. in the Czech Republic (part of the Czech Massif) (Denzler and Lobsiger-Molliet, 1991; Beaud, 1993; Brinke and Literák, 2001). In 2 cases (Beaud, 1993; Brinke and Literák, 2001), trematodes were found in birds trapped as late as October, at the time when their occurrence, according to present research into its seasonal character, is not very probable. In October, European populations of goldcrest usually migrate and they may have become infected elsewhere than at the trapping site, and the central European Carpathians may be one of such places. One of the sources responsible for the fact that the *C. faba* trematode was originally described as *Monostoma faba* was the description of trematodes collected from grey wagtail around Vienna by Professor Fischer (Bremser in Schmalz, 1831). In the same species, *C. faba* was also found in the 19th century in Vienna (Diesing, 1850).

The first findings of *C. faba* in Slovakia are from 1996 (Literák and Sitko, 1997). The first report for *C. faba* in Poland is in the present article. In the Czech Republic, *C. faba* was found for the first time in 2 common starlings (*Sturnus vulgaris*) (Kopřiva and Tenora, 1961). The first endemic focus of *C. faba* in the Czech Republic is reported in the present article.

Collyriclum faba occurrence seems to be focal in character and is probably dependent on the density or availability of *C. faba* intermediate hosts. The scale on which *C. faba* was found in the central European Carpathians was surprising. The authors believe that this is a consequence of increased attention given to the parasite in the regions monitored rather than a case of a discovery of some new foci. Prevalence of *C. faba* infection and the spectrum of infected species differed between individual sites. Chaffinch and European robin were regularly infected. It was very interesting to note high prevalences of *C. faba* in blackcap at Blatnica and Nespaly. Birds of this species were also frequently trapped at other sites, but no infected bird was found there. It can be only surmised that the reason may be a different diet, not containing the parasite's intermediate host species (that is still unknown), of blackcap at some sites or a difference in the life cycle of another (cryptic) species of *Collyriclum*.

The highest *C. faba* prevalence was observed in Horní Němčí at the end of July; the *C. faba* prevalence in Ruské was higher in August than in September. The *C. faba* prevalence in birds at these 2 sites was markedly seasonal. The seasonal dynamics of the *C. faba* prevalence in birds is probably related to the dynamics of the intermediate hosts, which, however, have not yet been identified. Because most of the infected birds were young that must have been born at the site in spring or summer, the assumed period between the entry of metacercaria into the bird (probably with food) and development to the adult stage is several weeks. The fact that mostly young birds are infected was previously noted by Farmer and Morgan (1944), who assumed that the birds

TABLE II. Seasonal dynamics of *Collyriclum faba* occurrence in wild birds in Horní Němčí, Bílé Karpaty Mts., Czech Republic.

Terms of examinations	28 March 2000 to 1 May 2000	27 May 2000 to 28 May 2000	24 June 2000 to 25 June 2000	21 July 2000 to 23 July 2000	3 September 1999 to 5 September 1999	29 September 2000 to 30 September 2000
Number of birds examined	60	51	51	57	68	58
Number of birds with <i>C. faba</i>	0	1	4	7	5	0
Prevalence (%)	0	2	8	12	7	0

TABLE III. Seasonal dynamics of *Collyriclum faba* occurrence in wild birds in 2001 in Ruské, Bukovské Vrchy Mts., Slovakia.

Terms of examinations	29 July to 10 August	11 August to 20 August	21 August to 31 August	1 September to 10 September	11 September to 20 September	21 September to 29 September
Number of birds examined	306	275	324	384	499	424
Number of birds with <i>C. faba</i>	9	9	6	1	3	0
Prevalences (%)	3	3	2	0.3	0.6	0

TABLE IV. Numbers of cutaneous cysts of *Collyriclum faba* per bird infected.

Species and number of cysts	1	2	3	4	5	6	7	8	9	10	>10
Dipper (n = 1)	—	—	—	—	—	—	—	1*	—	—	—
Yellowhammer (n = 1)	1	—	—	—	—	—	—	—	—	—	—
European robin (n = 100)	53	22	13	3	1	4	1	—	—	—	3
Chaffinch (n = 5)	2	1	1	—	—	—	—	—	1	—	—
Gray wagtail (n = 5)	—	1	1	1	—	—	—	1	—	—	1
Black redstart (n = 2)	—	1	—	1	—	—	—	—	—	—	—
Common redstart (n = 1)	—	1	—	—	—	—	—	—	—	—	—
Goldcrest (n = 1)	1	—	—	—	—	—	—	—	—	—	—
Blackcap (n = 16)	4	1	4	—	—	1	2	—	—	1	3
Common blackbird (n = 1)	1	—	—	—	—	—	—	—	—	—	—
Total (n = 133)	62	27	19	5	1	5	3	2	1	1	6

* Number of birds infected.

TABLE V. Distribution of cutaneous cysts of *Collyriclum faba* on the body of infected birds.

Species and number of cysts	Shank, thigh	Thorax	Vent, abdomen	Wing	Near the bill	Rump	Near the eye
Dipper (n = 1)	—	—	8*	—	—	—	—
Yellowhammer (n = 1)	1	—	—	—	—	—	—
European robin (n = 100)	204	5	3	4	6	2	1
Chaffinch (n = 5)	13	—	1	1	1	—	—
Grey wagtail (n = 5)	—	—	31	—	—	—	—
Black redstart (n = 2)	4	—	2	—	—	—	—
Common redstars (n = 1)	2	—	—	—	—	—	—
Goldcrest (n = 1)	—	—	—	—	—	1	—
Blackcap (n = 16)	—	—	88	—	—	5	—
Common blackbird (n = 1)	—	—	1	—	—	—	—

* Total number of cysts in body area.

became infected when they were fed in the nest. The decrease of *C. faba* prevalence in September is probably caused by 2 factors. The sites are visited by birds migrating from other areas that may be free of *C. faba*, and the decline in prevalence is due to a "dilution" effect. Alternatively, it may be that the infected birds use their natural defense system to fight against the parasitic infection. If the infection is not very severe, the trematodes eventually die, the content of the cysts suppurates, and the cysts heal.

The number of cysts on infected birds probably depends on the number of intermediate hosts ingested. *Collyriclum faba* cysts were localized principally in 3 different body regions in different hosts. If this is one and the same trematode species, it is quite difficult to explain why. The differences would be easier to explain if we were dealing with 3 different (cryptic) species of *Collyriclum*. The answer to the question of species-intraspecific variability of *Collyriclum* raised by the finding of different final hosts at different sites with the occurrence of identical bird species and different types of localization of trematode cysts in various bird species might in the future be provided by a detailed morphometric study of adult trematodes and especially by methods of DNA analysis.

In spite of frequent findings of *C. faba* cysts in various bird species, there are only very few reports of the pathogenic effect of this parasite on the health of their hosts (Cole, 1911; Riley and Kernkamp, 1924; Prosl and Loupal, 1985). In the present study, a markedly negative effect of the *C. faba* infection on the host was recorded only in 2 grey wagtails and 1 European robin; the *C. faba* infection seems to have been the cause of death of the latter. It, however, can be assumed that the infection in some birds was so severe that those birds did not survive it and were not even among the birds examined.

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First Report of a Natural Hybrid Between *Schistosoma mansoni* and *S. rodhaini*

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ABSTRACT: Experimental crosses between *Schistosoma mansoni* and *S. rodhaini* have shown that hybrid offspring are viable, yet, until now, no naturally occurring hybrid has been identified. A collection of freshwater snails from Nyamlebi-Ngoma, Ukerewe Island, Lake Victoria,

Tanzania, yielded a mixed infection within a single *Biomphalaria sudanica* of *S. mansoni* females and *S. mansoni*–*S. rodhaini* hybrid males. The hybrids were identified using deoxyribonucleic acid (DNA) sequences. Mitochondrial DNA 16S and 12S sequences of the hybrids

match those of *S. mansoni*, whereas their nuclear ribosomal DNA ITS1 and ITS2 sequences match those of *S. rodhaini*. The identification of hybrids in Tanzania highlights the possibility that the genetic identity of either parasite species might be modified by introgression.

Species of *Schistosoma* infect a range of mammals, including humans. The genus has been divided into species groups based on egg morphology and specificity for intermediate snail hosts responsible for their transmission. *Schistosoma mansoni* and *S. rodhaini* belong to the lateral-spined egg group, and both are transmitted through freshwater planorbid snails (*Biomphalaria* spp.) (Rollinson and Simpson, 1987). Although predominantly found in humans, *S. mansoni* isolates have also been found in rodents (Théron and Pointier, 1995; D'Andrea et al., 2000) and wild primates, particularly baboons (Ghandour et al., 1995; Müller-Graf et al., 1997; Munene et al., 1998). *Schistosoma rodhaini* infects rodents, carnivores, and, very occasionally, humans (D'Haenens and Santele, 1955; Rollinson and Simpson, 1987). The distribution of *S. rodhaini* is patchy and limited to Africa, whereas that of *S. mansoni* includes Africa and adjacent regions, plus South America and some islands of the Caribbean.

Under laboratory conditions, it is known that worms of the 2 species will form heterospecific pairs that can produce viable hybrid offspring (LeRoux, 1954; Taylor, 1970; Théron, 1989). Given the overlap in distributions in Africa, and that both parasite species can infect the same species of snail host concurrently (Schwetz, 1953), it seems likely that mixed infections in mammalian hosts would be frequent. Despite the coexistence of both species at some foci, for example the township of Kisumu on the eastern shore of Lake Victoria in Kenya (Nelson et al., 1962; Saoud et al., 1966), so far there has been no report of natural hybrids.

The dioecious condition of *Schistosoma* makes detection of hybrids using molecular techniques relatively easy. Biparental mitochondrial inheritance in *S. mansoni* was suggested by Jannotti-Passos et al. (2001). This was shown not to be the case by Bieberich and Minchella (2001), who demonstrated that the high mutation rate in the selected mitochondrial marker produced the false appearance of partial paternal inheritance. These authors reconfirmed that *S. mansoni* mitochondrial deoxyribonucleic acid (mtDNA) is maternally inherited (Bieberich and Minchella, 2001). The parentage of hybrids can be tested if their mitochondrial genome is maternally inherited (Avisé et al., 1987).

A substantial number of sequences are available from GenBank for *S. mansoni* (59,353 at the time of writing this manuscript). In contrast, only 3 markers, the nuclear ribosomal DNA (rDNA) ITS2, partial rDNA 18S, and a 283-bp portion of the mtDNA 16S gene, have been sequenced for *S. rodhaini*.

Our laboratory is currently collecting natural African isolates of *S. mansoni* for a broad-scale population study of the species. *Schistosoma rodhaini* was collected and sequenced to use as the outgroup taxon for phylogenetic analyses. During the course of sequencing and aligning isolates, a male specimen from Tanzania was found whose mtDNA sequence matched the mtDNA sequence of *S. mansoni*, but its nuclear sequence matched the *S. rodhaini* nuclear sequence. Sequencing of 2 additional male worms from the same infection confirmed the initial results, indicating that a natural hybrid had been found.

Cercariae of pure *S. rodhaini* were obtained from field *B. sudanica* collected from Luanda River, Homa Bay, Kenya (00°28'39"S, 34°17'38"E, altitude 1,140 m). Cercariae of pure *S. mansoni* were obtained from field *B. sudanica* collected from Mnazi Mmoja Beach, Nansio, Ukerewe Island, Lake Victoria, Tanzania (02°06'27"S, 33°05'01"E, altitude 1,156 m). Laboratory mice were infected with the cercariae to obtain adult worms. The identity of the worms was confirmed by extracting, sequencing, and comparing their DNA with published sequences for *S. rodhaini* and *S. mansoni* (rDNA ITS2 and mtDNA 16S). Cercariae from a natural *B. sudanica* snail found at Nyamlebi-Ngoma, Ukerewe Island, Lake Victoria in Tanzania (02°07'49.2"S, 33°10'06.6"E, altitude 1,139 m) produced the hybrid worms. A mouse was infected with these cercariae, and both male and female worms were recovered, indicating that the snail hosted a mixed-sex infection.

Deoxyribonucleic acid was extracted from individual worms (1 pure *S. rodhaini* male, 1 pure *S. mansoni* male, and 3 males plus 1 female from the Nyamlebi-Ngoma, field-collected snail). Specimens stored in ethanol were placed in 1× TE buffer (10 mM Tris-Cl, 1 mM ethylenediaminetetraacetic acid, pH 8.0) for 2 days before extraction. Deoxyri-

bonucleic acid was extracted in a total final volume of 200 µl, using the HotShot method described by Truett et al. (2000). The polymerase chain reaction (PCR) was used to amplify complete rDNA ITS1, 5.8S, and ITS2, plus 662 bases of partial mtDNA 16S leading into 12S. Primers used for PCR and sequencing were ITS, forward primers BD1 5'-GTC-GTAAACAAGGTTTCCGTA, and 3S 5'-GGTACCGGTGGATCACGTGGCTAGTG and reverse primers 4SR, 5'-GCAATATCCGTTCAAGATGTCGATG and ITS2.2, 5'-CCTGGTTAGTTTCTTTTCTCCCGC; 16S forward 16SF2 5'-GTGCTAAGGTAGCATAAATAT, and reverse 12SR2, 5'-AACC GCGACTGCTGGCACTG. Each reaction mixture contained 0.5 µM of each primer pair, combined with 10–100 ng of template DNA, 10× Taq buffer, 0.8 mM deoxyribonucleoside triphosphate, 3.75 mM magnesium, and 0.05 units/µl of Taq polymerase (Promega, Madison, Wisconsin). This mixture was thermocycled in a Whatman Biometra T Gradient thermocycler for 30 cycles, programmed to ramp between temperatures at 1 C per second. Cycle 1 was 95 C for 60 sec, 50 C for 45 sec, and 72 C for 90 sec. This was followed by 29 shorter cycles at 95 C for 30 sec, 50 C for 30 sec, and 72 C for 90 sec. The mixture was kept at 72 C for 7 min to complete extension, and then the temperature was dropped to 4 C. Products were viewed on an ethidium bromide-stained 1% agarose and TAE gel.

Polymerase chain reaction products were concentrated and desalted before sequencing using a Microcon centrifugal filter device (Millipore, Bedford, Massachusetts). Polymerase chain reaction products were sequenced using an ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Foster City, California), and the products were run on an ABI 377 automated sequencer. Forward and reverse sequences were aligned and edited using Sequencher[®] version 4.0.5 (Gene Codes Corp., Ann Arbor, Michigan). These sequences were then compared with published *S. mansoni* and *S. rodhaini* rDNA ITS and mtDNA 16S sequences for identification.

A total of 966 bases of nuclear rDNA spanning the 3' end of 18S, complete ITS1, 5.8S, ITS2 and the 5' end of 28S were sequenced from *S. rodhaini*, *S. mansoni*, and the male Nyamlebi-Ngoma worms (GenBank[®] AF531312–AF531314). Sequences from the 3 male Nyamlebi-Ngoma worms were 100% identical. The published *S. mansoni* (Puerto Rican isolate AF029309) and *S. rodhaini* (Burundi isolate L03659) ITS sequences differed from the sequences we obtained for these species at 6 and 7 sites, respectively. With the exception of 1 base change in the *S. rodhaini* sequence, all these differences were gaps often associated with repeated bases or gaps close to the priming sites. These gaps in the GenBank sequences are probably spurious results from old radiolabeled sequencing methods that were frequently hampered by band compression in GC-rich areas (Beck et al., 1993).

In addition, 662 bases of the mtDNA 16S and 12S were sequenced from each sample (GenBank[®] AF531309–AF531311). The 3 sequences from male Nyamlebi-Ngoma worms were again 100% identical. No differences were detected between the GenBank *S. rodhaini* (Burundi isolate L03648) and our Kenyan *S. rodhaini* sequences. The 9 differences observed between the sequence of GenBank *S. mansoni* (Puerto Rican isolate AF216698) and that of our Tanzanian *S. mansoni* reflects population differences. On the basis of our sequences, Kenyan *S. rodhaini* differs from Tanzanian *S. mansoni* at 6 nucleotide positions in the rDNA ITS and at 74 positions in the mtDNA 16S-12S. The Puerto Rican *S. mansoni* differs from *S. rodhaini* at 77 positions in the mtDNA 16S-12S.

The percent identity matrix in Table I clearly shows that the Nyamlebi-Ngoma hybrid worms have sequence nearly identical to that of our Tanzanian *S. mansoni* through the mtDNA 16S-12S marker (1 base difference) but match that of our Kenyan *S. rodhaini* through the nuclear rDNA ITS. The 3 male hybrids were extracted and sequenced independently of one another, reducing the likelihood of mislabeling or contamination. The adult female, taken from the same mixed infection, was 100% identical to Tanzanian *S. mansoni* through both mtDNA and nuclear markers.

This project has increased the available sequence data for *S. rodhaini* to now include nuclear rDNA ITS1 and 5.8S plus a further 379 bases of mtDNA. This additional sequence has almost tripled the number of variable sites available to distinguish *S. rodhaini* from *S. mansoni*. Direct fluorescent sequencing of fresh samples of *S. rodhaini* and *S. mansoni* has also identified several sites in the older, published rDNA ITS sequences that are probably misread, resulting from GC compressions.

Deoxyribonucleic acid sequence alignments confirm the identity of a

TABLE I. Percent identity of pairwise comparisons of *Schistosoma* spp. samples for nuclear (above diagonal), and mitochondrial DNA (below diagonal), gaps are included.

		rDNA ITS (966 bases)		
		<i>S. rodhaini</i> , Kenya	Hybrid, Tanzania	<i>S. mansoni</i> , Tanzania
mtDNA 16S-12S (662) bases	<i>S. rodhaini</i> , Kenya	—	100%	99.4%
	Hybrid, Tanzania	88.8%	—	99.4%
	<i>S. mansoni</i> , Tanzania	88.8%	99.8%	—

natural hybrid between *S. mansoni* and *S. rodhaini* from the freshwater snail, *B. sudanica*, in Lake Victoria. Mitochondrial 16S-12S sequence from the hybrid matches that of *S. mansoni*, whereas nuclear rDNA ITS sequence matches that of *S. rodhaini*. Several attempts were made to find *S. mansoni* nuclear rDNA ITS sequence, including repeated PCRs using different primer combinations and different worms as template. Despite these attempts, only rDNA ITS from *S. rodhaini* was identified. An F1 hybrid between the 2 species should carry both maternal and paternal copies of the nuclear genes (Rollinson et al., 1990). One explanation for the observed result is that the hybrid is the product of past introgression. That is, a hybridization event took place in the past, followed by backcrossing with male *S. rodhaini*. This has resulted in the homogenization of the *S. rodhaini* rDNA but in the maintenance of the *S. mansoni* mtDNA. The male hybrid we have identified is likely the result of a cross between a hybrid female and a male *S. rodhaini*.

Schistosoma mansoni and *S. rodhaini* display distinct cercariae emergence rhythms, diurnal versus nocturnal, to maximize contact with their respective definitive hosts (Combes et al., 1994). The identification of a natural hybrid suggests that the timing of cercariae emergence alone is not sufficient to eliminate gene flow between the 2 species.

A natural hybrid has been found in Lake Victoria, East Africa, where the ranges of *S. mansoni* and *S. rodhaini* overlap. Both species are common in this region, and hybrids might be expected. Other locations where mixed infections have been reported (predominantly from water and swamp rats but also from a dog), and thus hybrids might occur, include Kenya and the Democratic Republic of the Congo (Pitchford, 1977). The influence that hybrid worms have on the maintenance of the 2 species and the degree of mixing that naturally occurs are yet to be determined.

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