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VERSITA

Morphologic and genetic characterization of *Sarcocystis* sp. from the African grey parrot, *Psittacus erithacus*, from Costa Rica

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Abstract

A species of *Sarcocystis* is reported from a naturally infected African grey parrot, *Psittacus erithacus*, from Costa Rica. Only mature sarcocysts, measuring up to 2 mm in length and up to 750 µm in width, were observed. The sarcocyst wall was smooth. The villar protrusions on the sarcocyst wall were up to 5 µm long and up to 1.1 µm wide; they were folded over the sarcocyst wall giving a thin-walled appearance. The microtubules in villar protrusions were smooth and confined to villar protrusions. Bradyzoites in sections were 5.4–6.6 × 1.3–2.0 µm in size. Sequencing the small subunit and first internal transcribed spacer portions of ribosomal DNA related this parasite to, but distinguished it from, previously characterized species of *Sarcocystis* that encyst in the musculature of birds and complete their sexual development in New World opossums of the genus *Didelphis*. This evidence suggests that the parrot may have acquired its infection from an opossum from which it suffered a debilitating attack a year prior to the onset of depression, anorexia, and ultimately death.

Key words

African grey parrot, *Psittacus erithacus*, *Didelphis*, *sarcocysts, Sarcocystis*, coccidia, Costa Rica

Introduction

Species of *Sarcocystis* are apicomplexan parasites characterized by life cycles requiring 2 hosts, a predator species and its prey (Dubey *et al*. 1989). Herbivores (prey), carnivores (predator) serve, respectively, as the intermediate and definitive hosts. The definitive host becomes infected by ingesting the asexual stage (sarcocyst) encysted in the tissues (muscles) of the intermediate host. The sexual cycle occurs only in the carnivore host and it is restricted to the intestinal lamina propria. Typically, species of *Sarcocystis* exclusively parasitize a single intermediate host species. Although more than 100 species of *Sarcocystis* have been described, the life cycles of only a few are known completely (Dubey *et al*. 1989).

Little is known of the species of *Sarcocystis* in birds, but two avian species have been well characterized: *Sarcocystis rileyi* cycles between ducks (*Anas clypeata*, the intermediate host) and the skunk (*Mephitis mephitis*, the definitive host); *S. falcatula* employs passerine birds as intermediate hosts and the opossum (*Didelphis virginianus*) as the definitive host (Cawthorn *et al*. 1981; Box *et al*. 1984; Dubey *et al*. 1989, 2003). Recently, two new species, *S. ramphastosi* and *S. sulfuratusi* were reported from the keel-billed toucan (*Ramphastos sulfuratus*) from South Africa (Dubey *et al*. 2004). Here, we discuss our observations regarding the biology, morphology, phylogeny, and epidemiology of a species of *Sarcocystis* identified for the first time in an African grey parrot, *Psittacus erithacus*.

Materials and methods

Case history

A female parrot was bought as a chick from the Grand Caiman Islands and transported to Houston, Texas where it was housed for two months. In 1995, its final owner transported it to Costa Rica where it was housed outdoors with two other birds: An African grey parrot and an *Ara amazona*. This parrot was attacked by an opossum (*Didelphis* sp.) and suffered

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head and body trauma. One year after the attack, at ten years of age, the parrot became depressed, anorexic, and died. A necropsy was performed on the day of death.

Histopathology

Muscles containing macroscopic sarcocysts fixed in 10% buffered neutral formalin, 3% glutaraldehyde, 70% ethanol, and unfixed tissue were sent by air to the Animal Parasitic Diseases Laboratory (APDL), United States Department of Agriculture, Beltsville, MD. Routine histologic examination was performed on paraffin-embedded sections (5 µm), stained with haematoxylin and eosin (H & E) and periodic acid Schiff reaction (PAS).

Transmission electron microscopy

For transmission electron microscopy (TEM), glutaraldehyde-fixed muscles were postfixed in 1% osmium tetroxide in Millonig's phosphate buffer, rinsed in the same buffer, dehydrated in ethanol and embedded in epoxy resin. Semithin sections were stained with toluidine blue in 1% sodium tetraborate. The ultrathin sections were contrasted with uranyl acetate and lead citrate before examination in a transmission electron microscope.

Bioassay

Unfixed muscles heavily infected with sarcocysts were fed to a 3-month-old laboratory-raised cat (Dubey 1995). Faeces of the cat were examined microscopically by faecal flotation every day from 3–15 days after feeding sarcocysts.

Molecular characterization

Ten individual sarcocysts were dissected from the flesh and digested for DNA extraction, using the DNeasy tissue kit (Qiagen Corp.) for animal tissues (these procedures were also performed on two additional vials to which no tissue was added in order to confirm the absence of contaminating DNA in our reagents or equipment). All 10 specimens were subjected to PCR amplification assays targeting two portions of 18S ribosomal DNA. Five of these specimens were also subjected to assays using primers 18S1F/18S10R (a third assay targeting the 18S rRNA gene) and 69 with 70 (targeting the adjacent ITS-1 locus) (Table I). PCR products were directly sequenced on an ABI 3100 instrument after the removal of excess primers and unincorporated nucleotides using ExoSAP-IT (USB Corp.). Sequences were edited using Sequencher (GeneCodes Corp.) and compared to all available homologues identified by BLAST in the nonredundant nucleotide GenBank database. Multiple sequence alignments of all such sequences were constructed using CLUSTALW, and phylogenetic relationships inferred under the criterion of minimum evolution using Kimura 2-parameter distances using MEGA version 3.1 (Kumar *et al*. 2004).

Table I. PCR assays used to amplify parasite rDNA

Primers*	rDNA region
18S-1F/18S-11R	18S
18S-1F/18S-10R	18S
425-F/1125R	18S
69/70	ITS-1

*18S1F – GGATAACCGTGGTAATTCTATG, 18S10R – AGCATGACGTTTTCCTATCTCTA, 18S11R – TCCTATGTCTGGACCTGGTGAG, 425F– GGTGATTCATAGTAACCGAACG, 1125R – TTCACCTCTGACCAGTTAAA, 69 – CCTACCGATGTAGTGTTCCGGTGAAT, 70 – GCGTTCAGAAATCTGATGATTCCCTGA, 69IN374 – GCGTATGTTTCTGTAG (for sequencing only).

Results

Grossly visible sarcocysts measuring approximately 1–2 mm in length were evident in pectoral, wing and leg muscles (Fig. 1). These pale yellow sarcocysts had blunt ends. In 5-µm

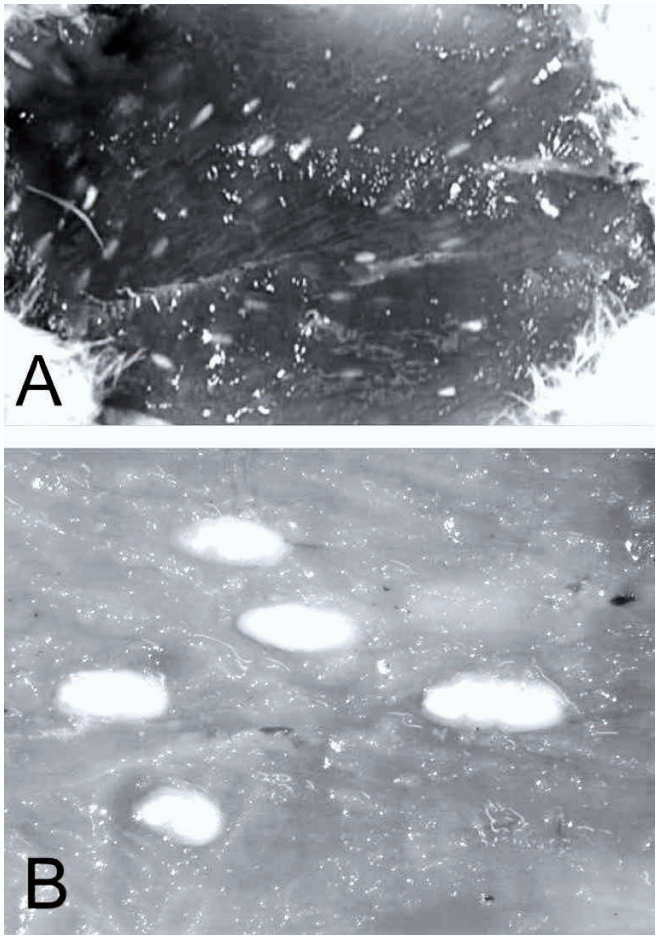

Fig. 1A and **B.** Sarcocysts of *Sarcocystis* sp. in skeletal muscles of the naturally-infected parrot. Unstained.

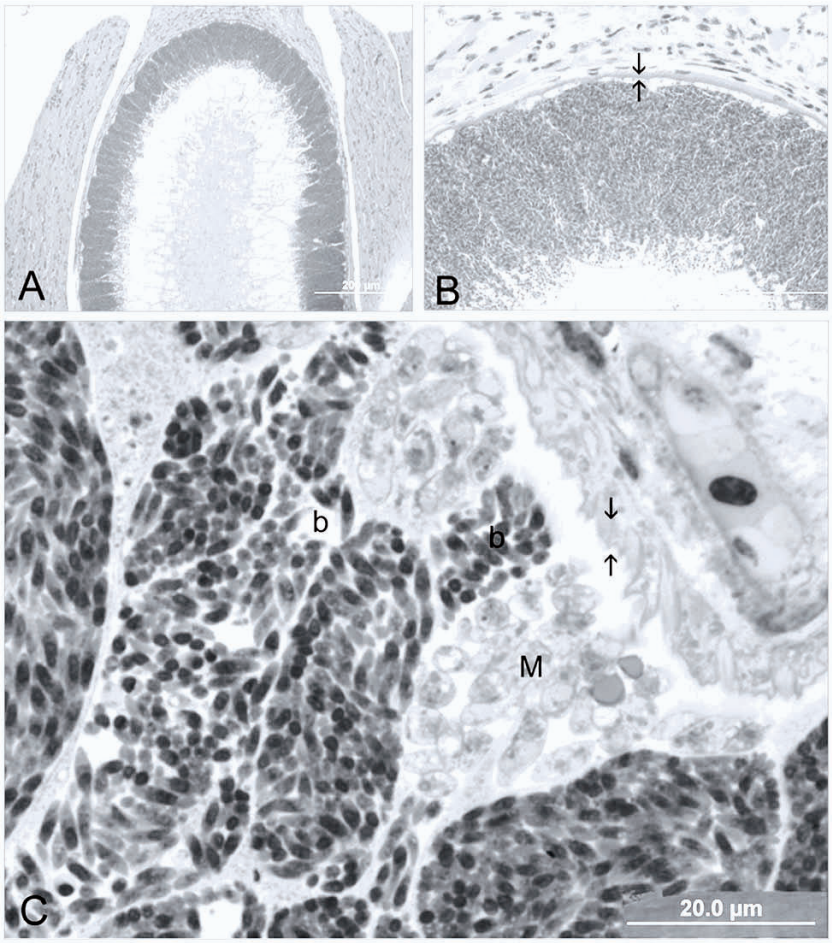

Fig. 2. Sarcocysts of *Sarcocystis* sp. in histologic section of skeletal muscle of the parrot. **A** and **B** – 5-µm section stained with haematoxylin and eosin. **C** – 1-µm section stained with toluidine blue. Note thin sarcocyst wall (opposing arrowheads), a peripheral area with intact bradyzoites and a central degenerating area. **C.** Note villar protrusions are indistinct (arrow), the metrocytes (M) are stained pale compared with darkly stained bradyzoites (b)

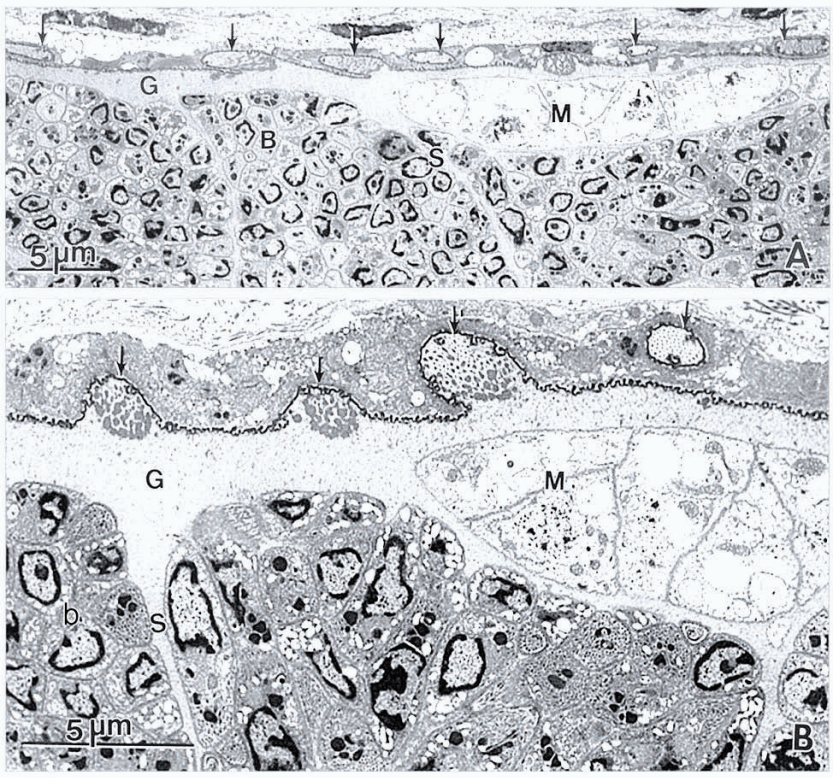

Fig. 3. TEM of two sarcocysts of *Sarcocystis* sp. **A.** Sarcocyst No. 1. **B.** Sarcocyst No. 3. Note variability in structure of the villar protrusions (arrows) on the sarcocyst wall. The ground substance (G) is smooth. Also note metrocytes (M), bradyzoites (b), and septa (S)

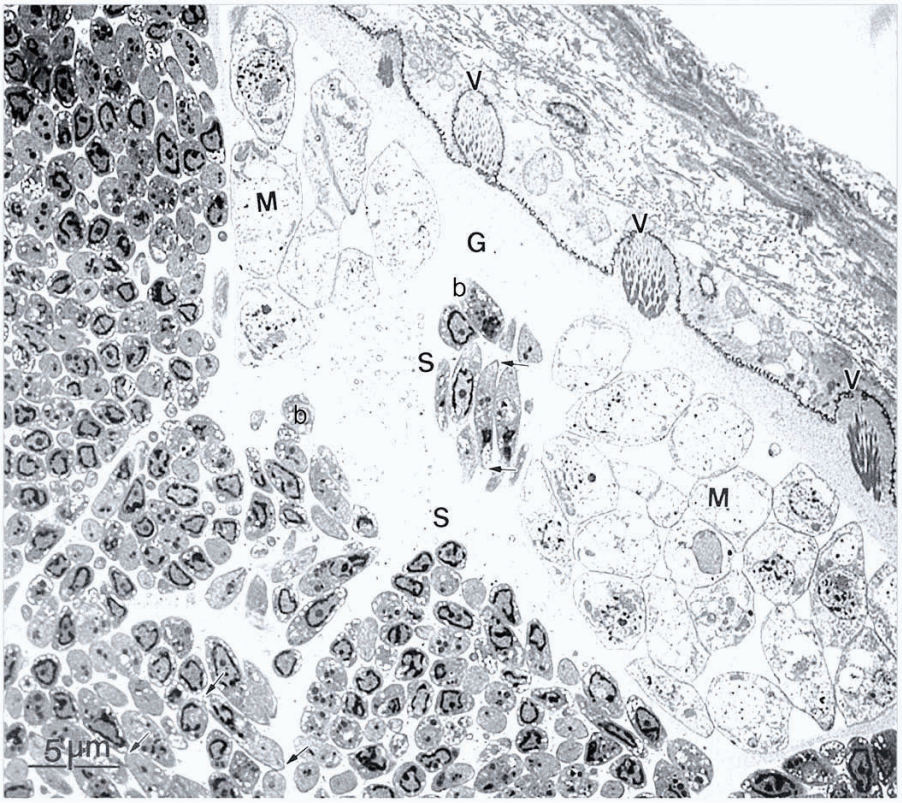

Fig. 4. TEM of sarcocyst walls of *Sarcocystis* sp. in sarcocyst No. 3. Note villar protrusions (V) located at irregular distances, smooth ground substance (G), metrocytes (M) and bradyzoites (b) located in groups, and septa (S). Arrows point to longitudinally cut bradyzoites

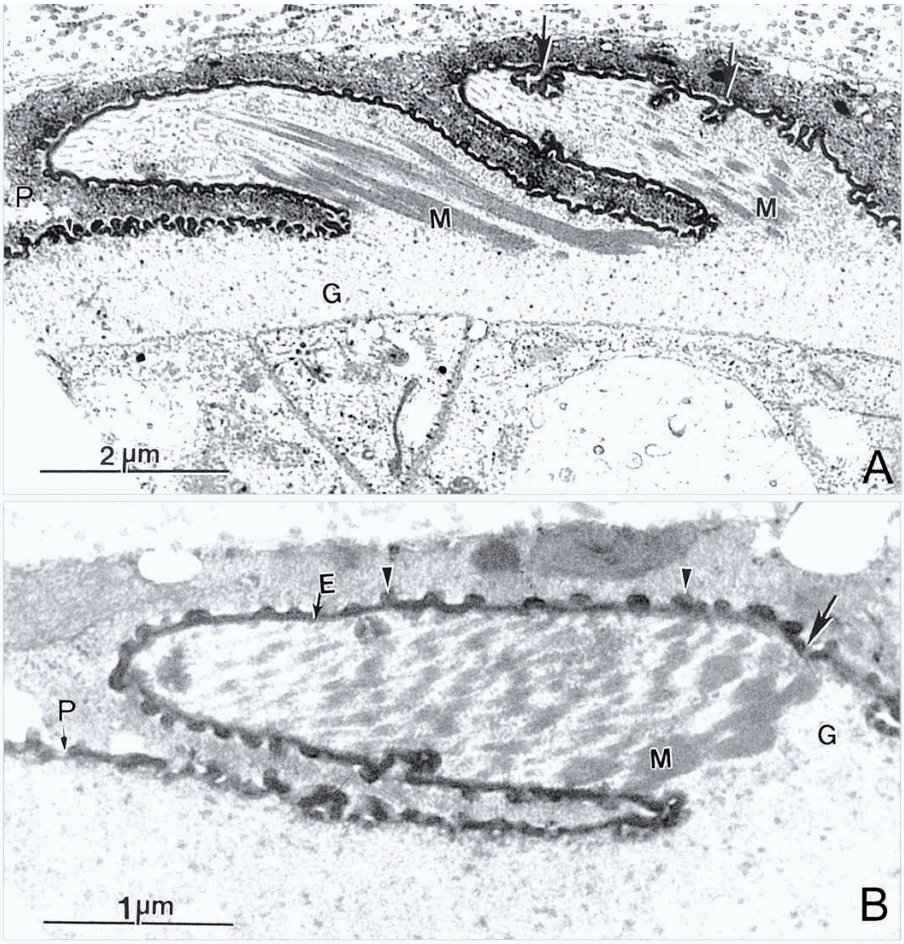

Fig. 5. TEM of sarcocyst walls of *Sarcocystis* sp. showing details of villar protrusions. The parasitophorous vacuolar membrane (P) is convoluted and has indentations (arrows) and protrusions (arrowheads). The parasitophorous vacuolar membrane is lined by an electron-dense layer (E). The villar protrusions contain microtubules that are sparse at the tip and more dense at the base. **A.** Sarcocyst No. 3. Two villi are cut at different angles. **B**. Sarcocyst No. 1. A villus protrusion lying parallel to the sarcocyst wall

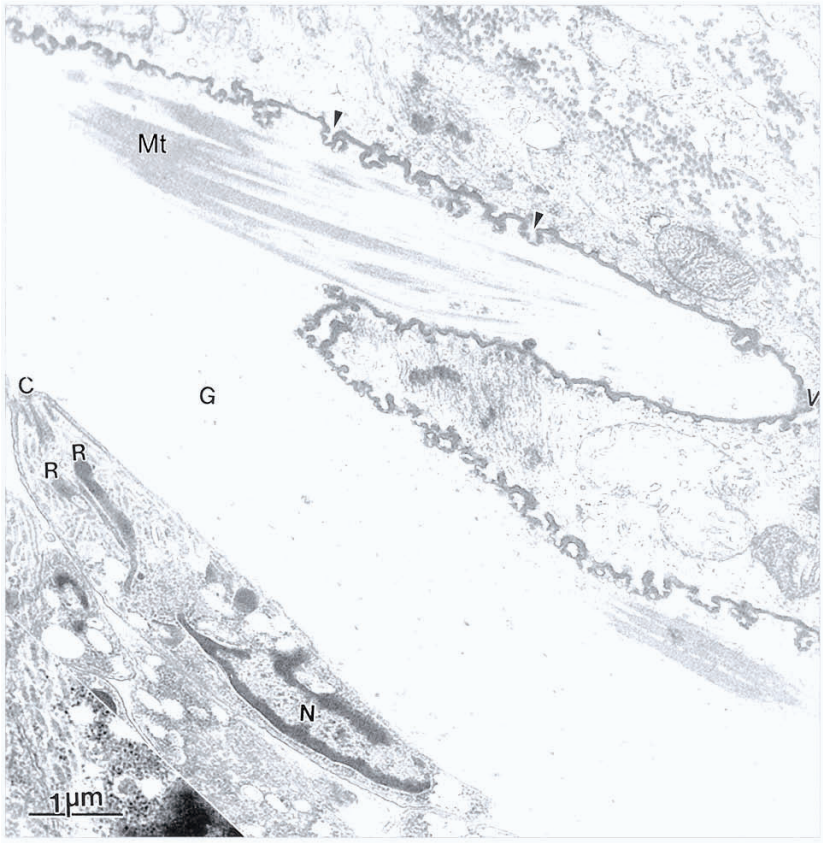

Fig. 6. TEM of sarcocyst wall of sarcocyst No. 2. Note a longitudinally cut villus protrusion (V) with involutions on the parasitophorous vacuolar membrane (arrowheads). The microtubules (Mt) do not extend into the ground substance (G). A longitudinally cut bradyzoite is present juxtaposed with the ground substance. Note the conoid (C), inverted rhoptry (R), and nucleus (N)

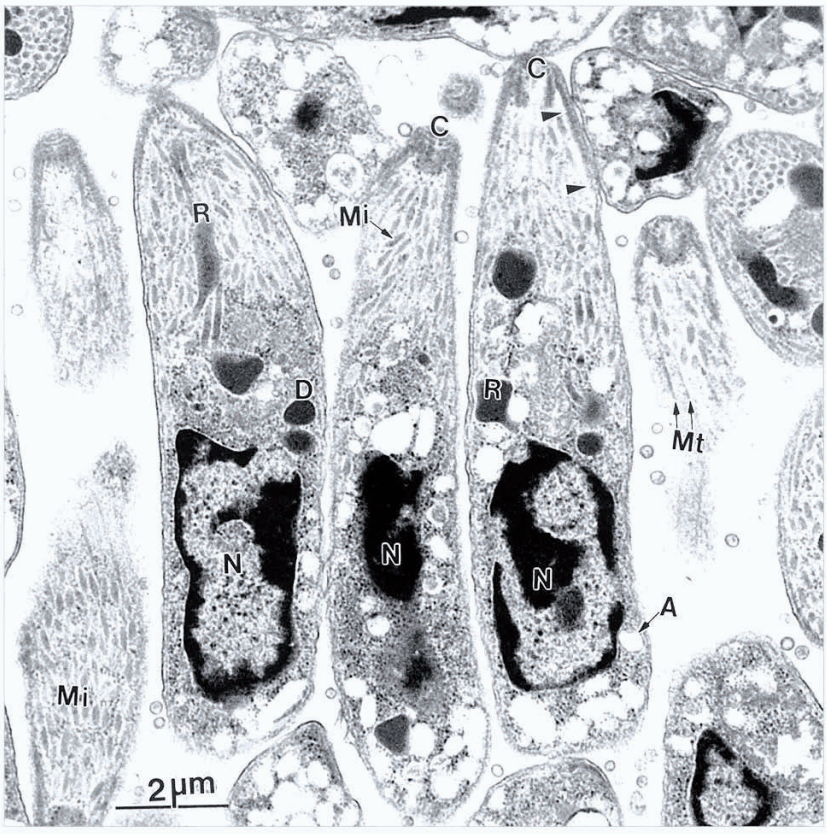

Fig. 7. Bradyzoites of *Sarcocystis* sp. in sarcocyst No. 2. Three longitudinally cut bradyzoites. Note conoid (C), pellicle with electron-dense thickening (arrowheads) at the conoidal ends, few rhoptries (R), numerous micronemes (Mi), subpellicular microtubules (Mt), amylopectin (A), and a terminal nucleus (N)

sections stained with H & E, the sarcocyst wall appeared thin (<1 µm) and smooth (Fig. 2A-C). The sarcocyst interior was divided into compartments by prominent septa. Two distinct zones were recognized, an outer zone containing intact bradyzoites and an inner central area with degenerated material (Fig. 2A). These zones were clearly demarcated in sarcocysts stained with PAS; the central core containing degenerated bradyzoites was intensely PAS-positive. In 1-µm sections stained with toluidine blue, the sarcocyst wall had indistinct villar protrusions folded over the sarcocyst surface (Fig. 2C). Sarcocysts measured up to 750 µm wide, containing bradyzoites 3–5 µm in length (Fig. 2C).

Sections of three sarcocysts were examined ultrastructurally (Figs 3–7). The sarcocyst wall contained conspicuous villar protrusions (V) which were sloping, one side being longer than the other (villar dimensions reported here refer to the shorter, closed side the villus). The outer layer of the sarcocyst, the parasitophorous vacuolar membrane (P), was wavy in outline and undulated (Figs 2–6). The P was lined by a 50–55 nm thick electron-dense layer (E), thickest at the undulated areas projecting from the P (Fig. 5). These undulations occurred at irregular distances and were present on both sides of the P (Fig. 5B). The V were spaced at irregular intervals (Figs 3–6). They appeared narrow at the tip and wider in the base. The inner (or closed) end of the V measured 2.7–5.0 µm long and 0.6-1.1 µm wide. They contained smooth microtubules (Mt) that converged towards the base of the V and that were more electron-dense at the base than at the tip of the V (Figs 5 and 6). Microtubules were restricted to V. The ground substance (G) appeared smooth and continued into the sarcocyst as septa. The ground substance layer measured up to 4 µm in width.

All sarcocysts were mature and contained fully formed bradyzoites. Longitudinally cut bradyzoites measured 5.3–6.6 × 1.3–2.2 µm (n = 10). Bradyzoites contained a conoid, micronemes, 1–3 rhoptries per section, subpellicular microtubules, and a posteriorly located nucleus (Fig. 7). The rhoptries had a long neck and were often looped so that the blunt end was directed towards the conoid. Micronemes were numerous and located anterior to the nucleus; most were arranged longitudinally. The pellicle was thickened towards the conoid (Fig. 7). The nucleus was located in the posterior half of the parasite. Amylopectin granules were present in the posterior two-thirds of the bradyzoites.

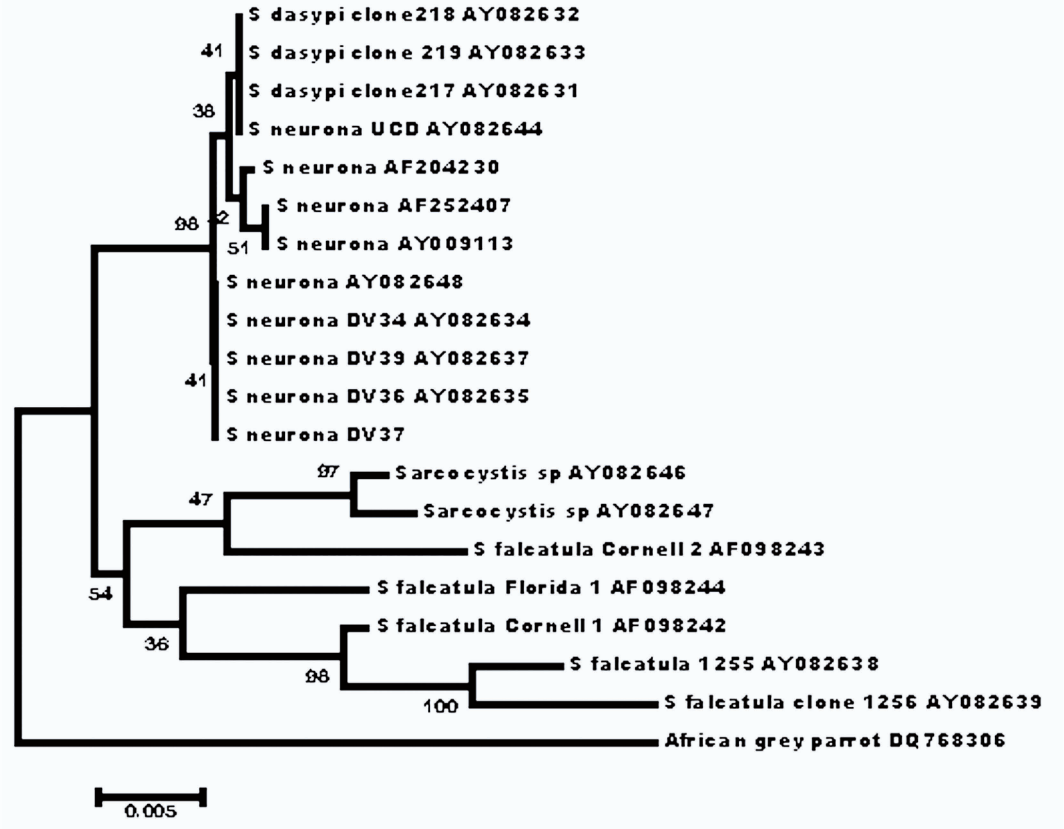

Fig. 8. Consensus of 500 bootstrap replicate midpoint-rooted phylogenetic trees reconstructed under the criterion of Minimum Evolution from Kimura 2-parameter distances assuming Gamma distributed variation among sites with parameter 0.25. The sequence derived from the African grey parrot is related to, but distinct from, isolates of *S. neurona* (which are nearly invariant) and from other, more disparate parasites employing avian intermediate hosts

The 974 bp of 18S rDNA sequenced from all 10 specimens were identical (GenBank Accession DQ768305). This nucleotide sequences closely resembled homologues previously characterized from other members of the Sarcocystidae, for example sharing over 98% identity with homologues from *Sarcocystis canis* and *Frenkelia* spp. (Dubey *et al*. 2006). Of all sequences available in the nonredundant nucleotide GenBank database, it most closely corresponded to one derived from the SN5 isolate of *S. neurona* (from which it differed only by a single "GG" dinucleotide insertion).

Greater diagnostic resolution was then sought by sequencing the adjacent ITS-1 portion of nuclear rDNA, which evolves much more rapidly and under far fewer functional constraints. In these assays, additional bands of high molecular weight were also observed, precluding full-length, dual-directional direct sequencing. Nonetheless, a total of 779 bp were sequenced from each of 5 specimens with the aid of an internal sequencing primer (GenBank Accession DQ768306). These sequences were in perfect agreement with one other, and were most similar to those species of *Sarcocystis* employing opossums (genus *Didelphis*) as their definitive hosts (including *S. neurona*, *S. falcatula*, *S. dasypi*, and *S. lindsayi*), to which it corresponded at over 90% of its residues. Excessive divergence from those of other species of *Sarcocystis* (i.e. *S. canis*) or from other tissue cyst-forming coccidia (i.e. *Toxoplasma gondii*, *Besnoitia tarandi*) precluded reliable alignment of homologous ITS-1 nucleotide positions across this broader sampling of coccidian taxa. Subsequent phylogenetic reconstruction excluded the parrot isolate from a group of markedly homogeneous isolates from *S. neurona* and *S. dasypi*, and differentiated it to some extent from those parasites previously isolated from the musculature of birds designated as *S. falcatula* (Fig. 8). Among those sequences presently available for phylogenetic comparison at the more discriminatory ITS-1 locus, the present isolate from the African grey parrot occupies a basal position in midpoint-rooted trees. The relationship of this taxon to other species employing opossum definitive hosts may ultimately be resolved only in the event that other taxa are identified, which may serve as appropriate outgroups.

The cat fed sarcocysts did not shed sporocysts.

Specimens deposited

One histological section stained with H & E and grossly visible sarcocysts in muscles in 70% ethanol were deposited in the United States National Parasite Collection (USNPC No. 098299.00), United States Department of Agriculture, Beltsville, Maryland 20705, U.S.A.

Discussion

Complete life cycles of *Sarcocystis* are known for only a few species of animals, mostly those in livestock (Dubey *et al*. 1989). Most *Sarcocystis* species have been named based on their intermediate host occurrence and their structure. Among all diagnostic morphological criteria, the structure of the sarcocyst wall is most valuable for differentiating those species that share a given host. Dubey *et al*. (1989) and Dubey and Odening (2001) recognized 35 types of sarcocyst walls based on their structure. The sarcocyst wall of the parasites occurring in this African grey parrot was structurally distinct from those of *S. rileyi*, *S. falcatula* and *S. sulfuratusi*. The sarcocyst wall in *S. rileyi* corresponds to type 21, characterized by cauliflower-like anastomosing villar protrusions (Dubey *et al*. 1989, 2003). By contrast, the sarcocyst wall in *S. falcatula* is thick, striated, with finger-like villar protrusions (Drouin and Mahrt 1980; Box *et al*. 1984; Dubey *et al*. 2000, 2001a, b, c; Luznar *et al*. 2001). Finally, the villar protrusions in *S. sulfuratusi* have distinctive microtubules that extend halfway into the granular layer and that are more electron-dense in the granular layer than in the V (Dubey *et al*. 2004). The parrot parasite most closely resembles *S. ramphastosi*. However, its villar protrusions are shorter than those of *S. ramphastosi*. Additionally, its bradyzoites appear longer than those in *S. ramphastosi* and the micronemes in the bradyzoites are more numerous in this parrot parasite than they are in *S. ramphastosi*. Whether these differences may have stemmed in part from fixation, artifacts cannot be known with certainty, underscoring the need for additional comparative analyses.

Significant portions of 18S and ITS-1 rDNA derived from extracts of several of these sarcocysts corresponded closely to those of New World species of *Sarcocystis* that employ opossums (genus *Didelphis*) as their definitive hosts, and most especially to those parasitizing avian intermediate hosts.

The cat fed infected muscles did not shed sporocysts, suggesting that felids play no role as this parasite's definitive host. The hawks and falcons that naturally prey upon the African grey parrot in its endemic West and Central African range could be considered as candidate definitive hosts for this newly recognized parasite. However, our analysis of molecular variation suggests, instead, that opossums of the New World genus *Didelphis* play that role. Indeed, this bird most likely contracted its infection in Costa Rica, a year prior to its death, when severely injured by an opossum.

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