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ORIGINAL PAPER

Ancient, globally distributed lineage of *Sarcocystis* from sporocysts of the Eastern rat snake (*Pantherophis alleghaniensis*) and its relation to neurological sequalae in intermediate hosts

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Abstract There is an emerging concern that snakes are definitive hosts of certain species of *Sarcocystis* that cause muscular sarcocystosis in human and non-human primates. Other species of *Sarcocystis* are known to cycle among snakes and rodents, but have been poorly characterized in the USA and elsewhere. Although neurological sequalae are known for certain species of *Sarcocystis,* no such neurological symptoms are known to typify parasites that naturally cycle in rodents. Here, sporocysts of a species of *Sarcocystis* were found in the intestinal contents of a rat snake (*Pantherophis alleghaniensis*) from Maryland, USA. The sporocysts were orally infective for interferon gamma gene knockout (KO) mice, but not to Swiss Webster outbred mice. The KO mice developed neurological signs, and were necropsied between 33 and 52 days post-inoculation. Only schizonts/merozoites were found, and they were confined to the brain. The predominant lesion was meningoencephalitis characterized by perivascular cuffs, granulomas, and necrosis of the neuropil. The schizonts and merozoites were located in neuropil, and apparently extravascular. Brain homogenates from infected KO mice were infective to KO mice and CV-1 cell line. DNA extracted from the infected mouse brain, and infected cell cultures revealed the highest identity with *Sarcocystis* species that employ snakes as definitive hosts. This is the first report of *Sarcocystis* infection in the endangered rat snake (*P. alleghaniensis*) and the first report of neurological sarcocystosis in mice induced by feeding sporocysts from a snake. These data underscore the likelihood that parasites in this genus that employ snakes as their definitive hosts constitute an ancient, globally distributed monophyletic group. These data also raise the possibility that neurological sequalae may be more common in intermediate hosts of *Sarcocystis* spp. than has previously been appreciated.

Keywords *Sarcocystis* · Rat snake · Mice · Host · Bioassay

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Introduction

Sarcocystis species are cosmopolitan protozoan parasites of homoeothermic and poikilothermic animals (Dubey et al. 2016). *Sarcocystis* species have a two-host life cycle, with herbivores as intermediate hosts and carnivores as definitive hosts. Clinically, sarcocystosis is typically a mild intestinal illness of humans with occasional incidental findings of sarcocysts on histopathological examination of the muscle tissue. Recently, mysterious and diagnostically challenging outbreaks of human illness associated with muscular sarcocyst infection have been reported in persons that had a history of travel to Malaysian regions; these human infections have been attributed to *Sarcocystis* species that have snakes as definitive hosts (Abubakar et al. 2013; Arness et al. 1999; Esposito et al. 2012; Italiano et al. 2014; Lau et al. 2014; Yang et al. 2005).

Little is known of the *Sarcocystis* infection in snakes in the USA. In the present study, we isolated *Sarcocystis* sporocysts from the intestinal contents of a road-killed rat snake (*Pantherophis alleghaniensis*) in the USA and investigated the parasite's development in vivo and in vitro. Additionally, we conducted multilocus sequencing and phylogenetic analysis

to provide genetic information for future investigations in epidemiology and deferential diagnosis.

Materials and methods

Naturally infected snake

A deceased adult Eastern rat snake was found on the property of the Beltsville Agricultural Research Center, Maryland, in May 2015. The snake was evaluated of *Sarcocystis* infection. For this, the intestinal contents were homogenized in a blender, filtered through two-layered gauze, and subjected to sucrose floatation for examination of *Sarcocystis* sporocysts. After microscopic examination, the floats were mixed with 2 % sulfuric acid, and stored at 4 °C as described by Dubey et al. (2016).

Attempted transmission to mice

Sporocysts were bioassayed in mice. For this, *Sarcocystis* sporocysts in 2 % sulfuric acid were neutralized with 3.3 % NaOH, washed with phosphate buffered saline (PBS), diluted serially, and inoculated orally by feeding needle into outbred Swiss Webster (SW) and interferon gamma gene knockout (KO) mice (Table 1). The recipient mice were observed daily for signs of illness. Because the inoculated KO mice developed neurological signs, we specifically examined these mice for suspected *Sarcocystis neurona* infection. Small pieces of the brain, and the cerebellum in particular, were squashed on glass slides, mixed with saline, and examined microscopically for merozoites. Muscle squashes were examined microscopically for sarcocysts (Dubey et al. 2016).

After collecting samples for histology (see below) the whole mouse carcass was homogenized in saline (100 ml) in blender and incubated in acid pepsin solution at 37 °C for 1 h to release bradyzoites from sarcocysts. After centrifugation, the sediment was suspended in about 10 ml saline (0.85 % NaCl) and ~25 μl of each digest was screened for the presence of bradyzoites at 40× magnification using a light microscope.

Histological examination

Portions of the heart, lung, spleen, tongue, eye, brain, kidney, liver, intestine, and muscles were fixed in 10 % buffered neutral formalin. Fixed tissue samples were cut into sections (2.5 × 0.7 cm) placed in cassettes, embedded in paraffin, and sectioned 5 μm thick. Tissue sections were stained with hematoxylin and eosin and observed using light microscopy.

For immunohistochemical examination, paraffin sections were reacted with anti *S. neurona* serum prepared from a rabbit injected with culture-derived *S. neurona* merozoites, as described by Dubey et al. (1999). The specificity of the *S. neurona* antiserum has been described by Dubey and Hamir (2000).

Table 1 Bioassay of sporocysts collected from rat snake in interferon gamma gene knockout (KO) mice and Swiss Webster (SW) outbred mice

Dose	Mouse	Necropsy (d.p.i.)	Diagnosis		Clinical signs[a]	Sub passage in KO mouse[b]	Cell culture
			Brain	Muscle			
NC	KO	39	Positive	Negative	Yes	ND	ND
	KO	33	Positive	Negative	Yes		
8×10^4	KO	46	Positive	Negative	Yes	Yes, positive	Yes, positive
	KO	44	ND	ND	Yes	ND	ND
	SW	155	Negative	Negative	No		
	SW	155	Negative	Negative	No		
8×10^3	KO	36	Positive	Negative	Yes	Yes, positive	Yes, positive
	KO	46	Positive	Negative	Yes	Yes, positive	Yes, positive
	SW	155	ND	ND	No	ND	ND
	SW	155	ND	ND	No		
8×10^2	KO	52	Positive	Negative	Yes	ND	Yes, positive
	KO	46	Positive	Negative	Yes	Yes, positive	Yes, positive
	SW	155	Negative	Negative	No	ND	ND
	SW	155	Negative	Negative	No		
8×10^1	KO	155	Negative	Negative	No	ND	ND
	SW	155	Negative	Negative	No		

NC not counted, *ND* not done, *d.p.i.* day post-inoculation

[a] Weak, neurological signs

[b] Necropsy was done on 38 or more days post-inoculation

In vitro culture

We examined the development of the parasite from KO mice in African green monkey kidney (CV-1) cells (ATCC CCL-70, Manassas, Virginia, USA). Host cells were grown in 25-cm^2 cell culture flasks or on 22-mm^2 glass coverslips in six-welled cell culture plates in RPMI 1640 cell culture medium (Mediatech, Inc., Manassas, VA, USA) containing 100 IU penicillin/milliliter, 100 μg/ml streptomycin/milliliter and 10 % (*v*/*v*) fetal bovine serum (FBS). Cells were maintained in the same medium except the concentration of FBS was 2 %.

The infected mouse brain was placed in 3 ml of HBSS and passed through a sterile 10-ml syringe into a 15-ml screw cap centrifuge tube. Sterile 2-mm glass beads were added up to about 0.5 ml in the test tube and the sample vortexed for about 10 s. The samples were examined for the presence of merozoites using light microscopy on the unstained sample. The sample (about 0.5 ml/flask) was inoculated onto each of five monolayers of CV-1 cells in 25 cm^2 flasks. The inocula were removed 2 h later, when 5 ml of maintenance medium was added. Flasks were examined daily for 21 days and then every other day thereafter.

Once merozoites were observed in inoculated cultures, the media was used to infect other flasks of CV-1 cells to keep the cultures growing. This was done at various times to obtain parasites for developmental studies. Parasite development was examined using CV-1 cells grown on sterile 22-mm^2 glass coverslips. Coverslips containing CV-1 cells each inoculated with merozoites in maintenance medium. The numbers of merozoites was not determined. The inoculum was removed 24 h post-inoculation (p.i) and replaced with maintenance medium. One or two coverslips were removed at 1–8 days p.i and processed for light microscopic examination after staining with Giemsa. The 22-mm^2 coverslips were fixed in 10 % (*v*/*v*) buffered formalin solution for 10 min then post-fixed in 100 % methanol for 10 min, air dried, stained with Giemsa for 1 h, and mounted on glass slides using Permount™ (Fisher Scientific Company, Fair Lawn, NJ, USA) for microscopic examination.

DNA extractions and PCR amplification

The merozoites/schizonts derived from the brain of infected KO mice and in vitro cultures were used for molecular characterization. Genomic DNA was extracted using the DNeasy® Blood and Tissue Kit (Qiagen, Valencia, CA, USA) according manufacturer's instructions. DNA quantity and quality were determined by the NanoDrop Lite Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). Molecular characterization was done by amplification and sequencing of three regions of the nuclear ribosomal DNA unit: *18S rRNA*, internal transcribed spacer-1 (*ITS-1*), and *28S rRNA*, and a mitochondrial cytochrome c oxidase subunit 1 (*cox1*) locus following the procedures as described previously by Gjerde and Josefsen (2015). The PCR amplifications were performed in 50 μl total reaction volume containing 10 pmol of each primer pair and 1× Taq PCR Master Mix Kit (Qiagen, Inc., Valencia, USA). The thermal cycler (Veriti® Thermal Cycler, Applied Biosystems, Foster City, USA) conditions were set at initial denaturation at 95 °C for 10 min, 40 cycles of amplification (95 °C for 45 s, 52–56 °C for 45 s, and 72 °C for 1 min) and final extension at 72 °C for 10 min. Appropriate positive (DNA of *S. neurona* merozoites) and negative (H_2O) controls were included in all analyses. The amplified PCR products were run on 2.5 % (*w*/*v*) agarose gel with ethidium bromide staining, and visualized by using the Gel Logic 212 Imaging Systems (Eastman Kodak Company, Rochester, NY, USA).

DNA sequencing and phylogenetic analysis

The PCR amplicons of *18S rRNA*, *28S rRNA*, *ITS-1*, and *cox1* were excised from the gel, and purified using the QIAquick Gel Extraction (Qiagen, Inc., Valencia, USA) according to the manufacturer's recommendation. The purified PCR products were sent to Macrogen Corporation (Rockville, MD, USA) for direct sequencing using the same primer pair used in amplification to obtain both reads. The resulting sequences were imported, read, edited manually if necessary, and analyzed using the software Geneious version 9.0.4 (Biomatters Ltd. Auckland, NZ). New sequences were compared with other sequences deposited in NCBI GenBank by BLASTn analysis to detect intraspecies and interspecies variation on these DNA regions.

A phylogenetic tree based on *18S rRNA* sequences was constructed using sequences obtained from the rat snake *Sarcocystis* sp. and sequences of various species of *Sarcocystis* deposited in the NCBI GenBank. One hundred bootstrap replicates of the phylogenetic relationships among these sequences were reconstructed under the criterion of maximum likelihood using PhyML as implemented in the Geneious version 9.0.4 (Guindon et al. 2010). The HKY85 model of nucleotide substitution was used, estimating the transition/transversion ratio and the gamma distribution parameter to model rate variation among sites. No sites were presumed to be invariant. The complete deletion method was used for ambiguous/missing data, and topologies were searched using the nearest neighbor interchange method. The final dataset incorporated 54 sequences corresponding to region of 1357 nucleotides, and *Besnoitia bennetti* (AY665399) as an out group.

Results

Numerous sporocysts were found in the intestinal contents of the rat snake. Fully sporulated oocysts/sporocysts were seen in fresh samples before being placed into sulfuric acid. The oocysts measured approximately 16 × 10.5 μm ($n = 20$) and

sporocysts 18 × 11 μm ($n = 20$). Oocysts/sporocysts were infective to KO mice but not to SW mice (Table 1). Depending on the dose, the inoculated KO mice developed neurological signs resulting in death or the need for euthanasia when they became moribund between 33 and 52 days p.i. *Sarcocystis* schizonts and merozoites were found in the brains, particularly the cerebellum (Fig. 1). Sub inoculation of brain homogenate of infected KO mice to naive KO mice was found to produce infections (Table 1).

Histological examination revealed meningoencephalitis, characterized by infiltration of mononuclear cells around the blood vessels, focal necrosis of the neuropil, particularly in the cerebellum. Immature and mature schizonts, free and intracellular merozoites were scattered in the brain. Isolated inflammatory foci contained degenerating and intact merozoites among neutrophils. The schizonts were up to 45 μm in size and contained up to 34 merozoites. The merozoites were up to 12 μm long and had a terminal nucleus (Fig. 1A–D). Neither sarcocysts nor bradyzoites were detected by any of the methods employed. The organisms in KO mice did not react with *S. neurona* antibody. Three of the five cultures were positive for merozoites at 6 days p.i. The merozoites in the inocula were infectious for other CV-1 cell cultures (Table 1). Parasite density was low and most stages were schizonts in various stages of development. Developing schizonts varied in size, varying with the maturity of the nucleus. They increased in size as the nucleus developed numerous nucleoli and became lobed. The largest multilobed schizont observed was 54 × 26 μm. Mature schizonts could contain over 50 merozoites (Fig. 1E, F). Merozoites were approximately 7.2 × 1.9 μm ($n = 20$) and were produced on blastophores that developed on the surface of schizonts.

PCR analysis with schizont/merozoite DNA as the template yielded amplicons of the expected size for the *18S rRNA* (in three fragments), *28S rRNA* (in two fragments), *ITS-1*, and *cox1* loci. PCR-DNA sequencing of amplicons resulted in the unambiguous sequences of three nuclear DNA regions: *18S rRNA* (1790 bp), *28S rRNA* (1530 bp), and *ITS-1* (900 bp), and a mitochondrial DNA locus, *cox1* (1015 bp). These sequences were submitted to the NCBI GenBank with accession numbers KU891600 (*18S rRNA*), KU891601 (*28S rRNA*), KU891602 (*ITS-1*), and KU891603 (*cox1*) and designated as originating from *Sarcocystis* sp., ex., *Pantherophis alleghaniensis*.

Analysis of *18S rRNA* sequence of *Sarcocystis* sp., ex., *P. alleghaniensis*, confirmed its membership among species of *Sarcocystis* and indicated an especially close relationship to other parasites in this genus that employ snakes as their definitive hosts and also to other *Sarcocystis* which use rodents as intermediate hosts but for which the definitive hosts are yet unknown (Fig. 2). The *18S rRNA* sequence of *Sarcocystis* sp., ex., *P. alleghaniensis*, shared 98 % identity with sequences of *Sarcocystis* sp. 1 JJH clone 1 (KF309698) and 97 % with *Sarcocystis* sp. 1 JJH-2013 clone 2 (KF309699) followed by 94 % identity with other sequences such as *Sarcocystis nesbitti* (HF544323), *Sarcocystis* sp., ex., *Morelia viridis* GM (KC201639), *Sarcocystis singaporensis* clone GN8-5 (AF434054), and many other species of *Sarcocystis*.

In phylogenetic tree based on *18S rRNA* sequences, *Sarcocystis* sp., ex., *P. alleghaniensis*, clustered consistently with *Sarcocystis eothenomysi* (from a vole in China, and suspected to have a snake definitive host), and with less bootstrap support to *S. nesbitti*; these three were most typically grouped with the following other species: *Sarcocystis atheridis*, *S. singaporensis, Sarcocystis* sp., ex., *Morelia viridis* GM-2013, *Sarcocystis* cf. *clethrionomyelaphis* JJH-2013, *Sarcocystis zuoi*, and *Sarcocystis* sp. MA#347 (Fig. 2); all these employ snakes (python, cobra, rat snake, viper) as their definitive hosts (Lau et al. 2013; More et al. 2014; Hu et al. 2014, 2015; Dubey et al. 2016). Relationship of *Sarcocystis* sp., ex., *P. alleghaniensis*, to the *S. eothenomysi*, and *S. nesbitti* was stable when substituting various out group taxa (*B. bennetti*, *Toxoplasma gondii*, *Eimeria anguillae*). The partial *28S rRNA* sequence of *Sarcocystis* sp., ex., *P. alleghaniensis*, shared the highest identity (94 %) with sequence of *S. singaporensis* (AF237617), and 93 % identity with *Sarcocystis zamani* (AF237616) followed by ≤92 % identity with other sequences of *Sarcocystis* spp. The new *ITS-1* sequence of *Sarcocystis* sp., ex., *P. alleghaniensis*, shared poor nucleotide sequence similarities with other *Sarcocystis* species, but no *ITS-1* sequences have yet been reported from any *Sarcocystis* species that use snakes as definitive hosts. The partial *cox1* sequence of *Sarcocystis* sp., ex., *P. alleghaniensis*, shared the highest identity (98–97 %) with *Sarcocystis speeri* (KT207461), *Sarcocystis lutrae* (KM657808, KF601326), *Sarcocystis arctica* (KF601318-KF601321), *Sarcocystis rileyi* (KJ396582), and *S. neurona* strain BR2012 (KF854272). The uses of *cox1* gene as a genetic marker for *Sarcocystis* species discrimination has been proposed only recently, and so sequences of limited species are available for comparative use (Gjerde 2013).

Discussion

In the present study, natural infections of *Sarcocystis* were detected in the intestines of Eastern rat snake (*P. alleghaniensis*) for the first time from the USA. The Eastern rat snake is an endangered, nonvenomous colubrid species, endemic to North America. It occupies a diverse habit including farmlands, hardwood forests, forested wetlands, thickets and fields adjacent to forests, isolated urban woodlots, and backyards. They prey on rodents, lizards, frogs, birds, bird's eggs, young chickens, and chicks (Conant and Collins 1998). We noted the presence of 3–4 partial digested house

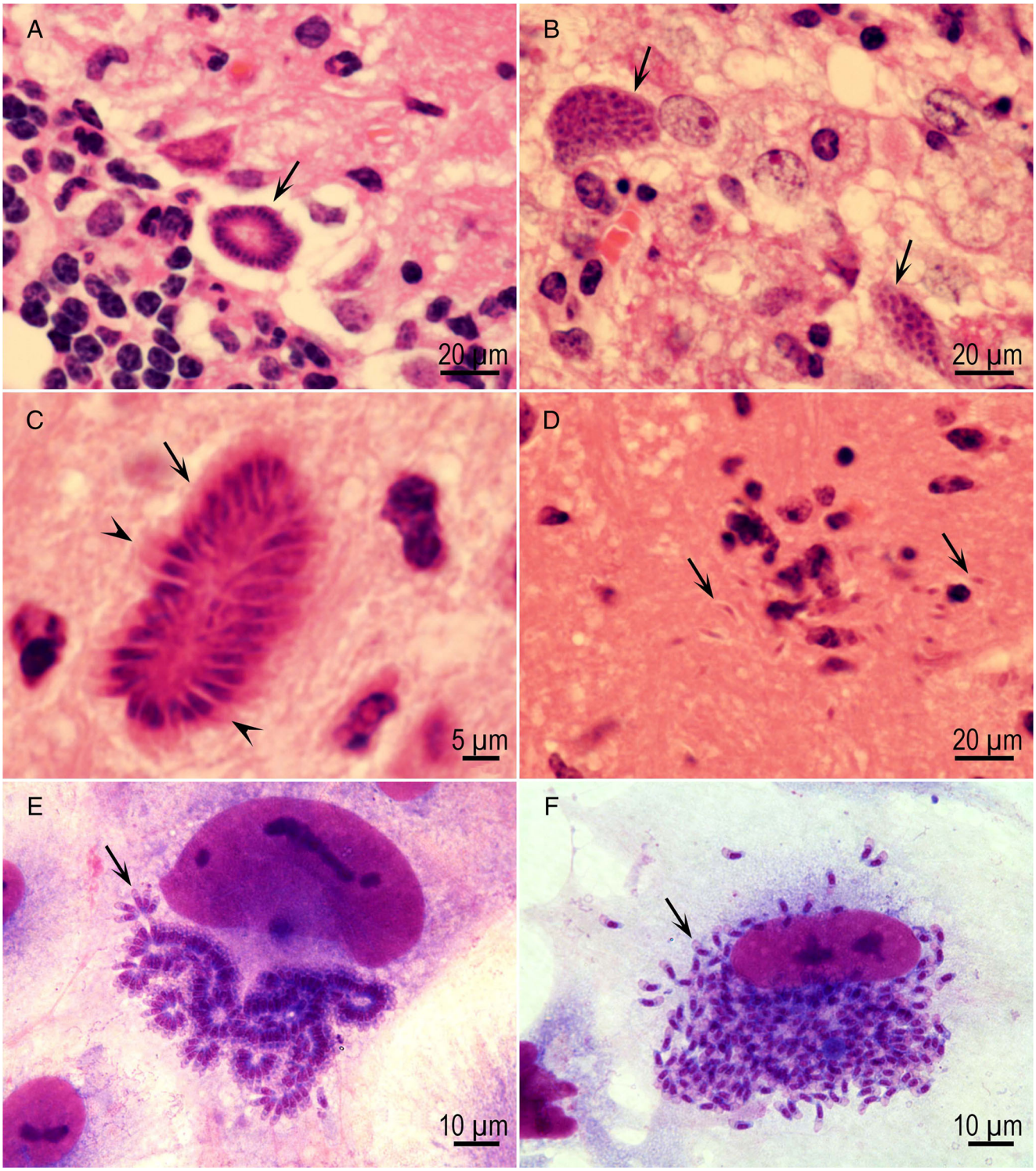

Fig. 1 *Sarcocystis* schizonts and merozoites. **A**–**D** Histological sections of the brain of KO mice inoculated with *Sarcocystis* sporocysts. Hematoxylin and eosin stain. **E**, **F** Stages in CV-1 cells, Giemsa stain. **A** Schizont (*arrow*) at the periphery of mononuclear cell infiltrations around a blood vessel. **B** Two immature schizonts (*arrows*). **C** Mature schizont with a rosette of merozoites (*arrow*) and individual merozoites (*arrowheads*). **D** Focal encephalitis and necrosis around free merozoites (*arrows*). **E** Schizont with lobulated nucleus with merozoites formation (*arrow*). **F** Schizont with more than 50 free merozoites (*arrow*)

mice in the gut of the snake at the time of necropsy. However, we were unable to transmit the infection to SW mice.

The development of schizonts in the brain and of neurological signs in the inoculated KO mice suggested a *S. neurona-*

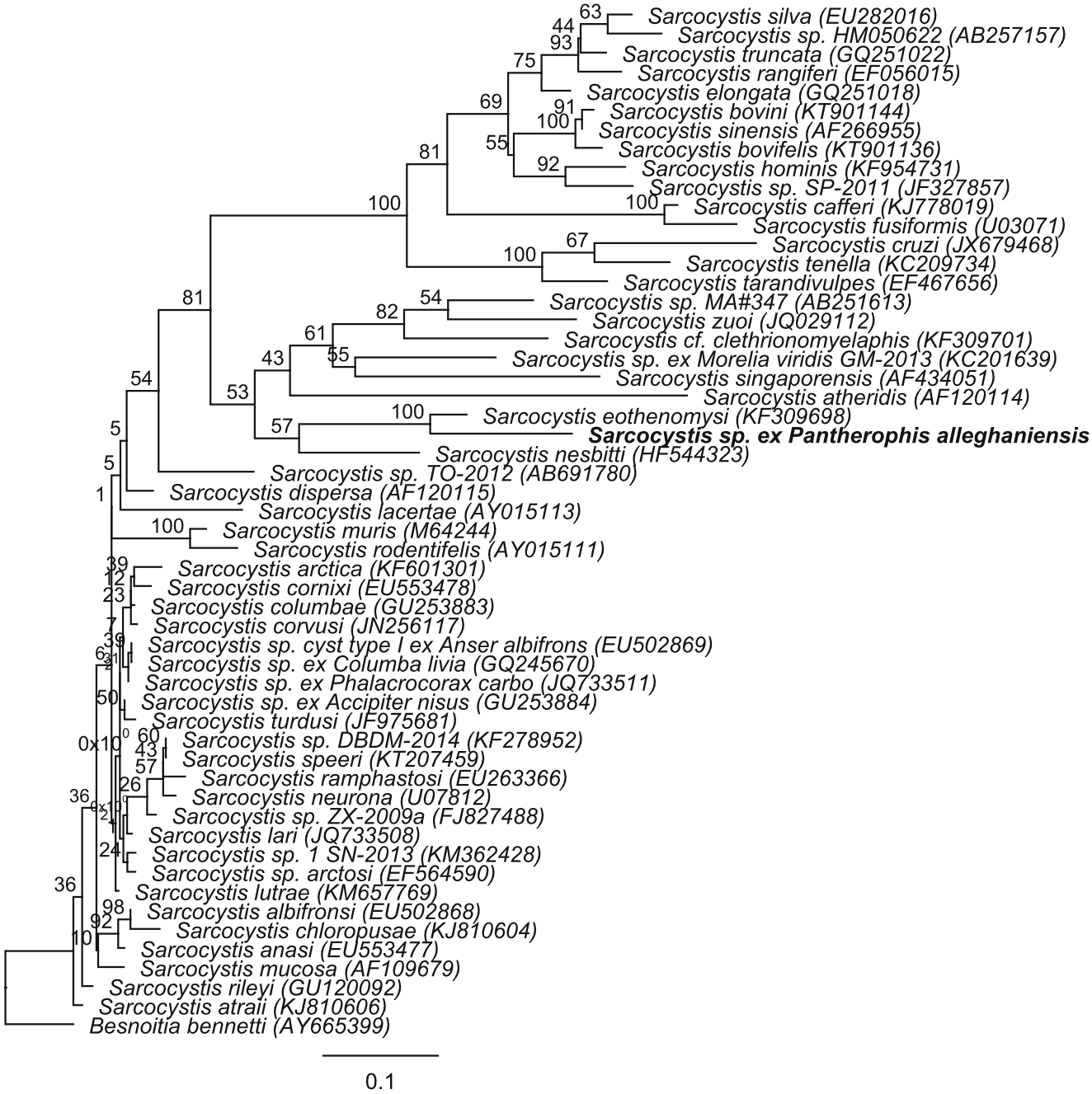

Fig. 2 Phylogenetic tree based on *18S rRNA* sequences. Input sequences were the *18S rRNA* regions of various species of *Sarcocystis* retrieved from NCBI GenBank, and new *18S rRNA* sequence obtained from *Sarcocystis* sp. from rat snake (*Sarcocystis* sp., ex., *Pantherophis alleghaniensis*). Accession numbers of sequences were given in parenthesis following the species name. One hundred bootstrap replicates of the phylogenetic relationships among these sequences were reconstructed under the criterion of maximum likelihood using PhyML as implemented in the Geneious version 9.0.4. *Sarcocystis* sp., ex., *Pantherophis alleghaniensis*, showed close relationship with those employ snakes (python, cobra, rat snake, and viper) as their definitive hosts, and rodents as intermediate hosts or which the natural hosts are yet unknown

like infection, but the parasite did not react to *S. neurona* antibodies and was genetically distinct from *S. neurona*. In the absence of sarcocysts, it was not possible to determine the *Sarcocystis* species or better classify the parasite. Molecular characterization of the parasite from the KO mice revealed a closer relationship with *Sarcocystis* species that use snakes as definitive hosts (Dubey et al. 2016). *ITS-1* sequences are a more variable portion of ribosomal DNA, and show higher intraspecies and interspecies divergence in genus *Sarcocystis*. We were unable to perform phylogenetic analyses based on hyper-variable *ITS-1* sequences due to lack of *ITS-1* sequences of snake-derived *Sarcocystis* species in database.

However, the *18S rRNA* provided a good basis to compare the current isolates to previously sampled species of *Sarcocystis*. The *18S rRNA* sequences have been used frequently to show phylogenetic relationship and diagnosis of *Sarcocystis* species (Dubey et al. 2016).

Sarcocystis atheridis sporocysts were isolated from a Nitsche's bush viper (*Atheris nitschei nitschei*), from Uganda, and experimental transmission resulted in sarcocyst development in the skeletal muscles of laboratory mice (CD1 and Barbary striped mice) not in rats (Wistar H) (Dolezel et al. 1999; Slapeta et al. 1999). The sequences of *Sarcocystis* sp. 1 JJH-2013 clone 1 and clone 2 were submitted from *Sarcocystis eothenomysi* infecting the large oriental vole (*Eothenomys miletus*) from Anning, China, and suspected to have a snake definitive host (Hu et al. 2014). *Sarcocystis* sp. MA347 was reported in raccoons (intermediate hosts) introduced to Japan from North America (Jinnai et al. 2009). Definitive hosts are unknown, possibly carnivorous snakes by phylogenetic analysis. *Sarcocystis zuoi* was described from wild-caught Norway rats (*Rattus norvegicus*) in China, and king rat snakes (*Elaphe carinata*) were experimentally defined as definitive host (Hu et al. 2005, 2012). *Sarcocystis singaporensis* is a well described species that uses python snakes as definitive hosts and rodents as intermediate hosts (Dubey et al. 2016). Recently, *Sarcocystis* sp., ex., *Morelia viridis*, was described from *Sarcocystis* spp. sporocysts from pooled feces from four green pythons (*Morelia viridis*) from Germany (More et al. 2014). Review of the literature and results of the present study point to a high diversity of undefined *Sarcocystis* species infecting to snakes.

In conclusion, the present study described the natural infection of *Sarcocystis* species in rat snake. We believe the parasites we observed have not previously been reported from rat snake, and provisionally refer to them as *Sarcocystis* sp., ex., *Pantherophis alleghaniensis*. Molecular and parasitological characteristics suggest the parasite cycle between snakes as definitive hosts and possibly rodents as intermediate hosts. These data underscore the likelihood that parasites in this genus that employ snakes as their definitive hosts constitute an ancient, globally distributed monophyletic group. These data also raise the possibility that neurological sequalae may be more common in intermediate hosts of *Sarcocistis* spp. than has previously been appreciated.

Compliance with ethical standards All investigations reported here were approved by the institutional animal care and use protocol committee of the US Department of Agriculture.

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