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Toxoplasma gondii infection in Amami spiny rat on Amami-Oshima Island, Japan

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ABSTRACT

The Amami spiny rat (*Tokudaia osimensis*) is an endangered rodent species that is endemic to the forests of Amami-Oshima Island, Kagoshima, Japan. In July 2018, a deceased adult male Amami spiny rat was found on the Yuwandake Mountain Trail on the south-central coast of Amami-Oshima Island. Histopathological observations revealed protozoan infections in the liver, lungs, and heart. Nested or semi-nested PCRs targeting the *B1*, *SAG3*, *GRA6*, and *ROP18* genes successfully detected the genomic DNA of *Toxoplasma gondii* in the formalin-fixed and paraffin-embedded specimen. Sequence analyses of the *SAG3*, *GRA6*, and *ROP18* genes suggested that the strain detected in the study specimen was related to the type II strain of *T. gondii*. This is the first confirmed case of *T. gondii* infection in an Amami spiny rat.

1. Introduction

Amami-Oshima Island is a subtropical island located ~400 km south-west of Kyusyu, Japan. Reflecting the natural environment and geographical history of the island, this area is home to several endemic mammals, including the Amami spiny rat (*Tokudaia osimensis*), Ryukyu long-furred rat (*Diplothrix legata*), and Amami rabbit (*Pentalagus furnessi*). The Amami spiny rat is now categorized as endangered by the International Union for Conservation of Nature Red List of Threatened Species due to a rapid population decline as a result of diminishing habitats and predation by the non-native small Indian mongoose (*Herpestes auropunctatus*), and outdoor cats (*Felis silvestris catus*) and dogs (*Canis lupus familiaris*) (Ishii, 2016). For these above-mentioned reason, feral and stray cats are recognized as direct threats to the integrity of endemic mammals and natural ecocystems (Mameno et al., 2017).

Toxoplasma gondii (Apicomplexa: Sarcocystidae) is an intestinal coccidian parasite that targets members of the family Felidae as the final hosts, but also infects a wide range of warm-blooded animals as intermediate/paratenic hosts (Dubey and Odening, 2001). The prevalence of *T. gondii* in wildlife is correlated with the presence of final hosts since they contribute to environmental contamination via the excretion of oocysts in their feces (Lehrer et al., 2010; Fredebaugh et al., 2011). Recently, *T. gondii* antibodies were detected in 9.0% of feral and stray cats in Aammi-Oshima Islands (Matsuu et al., 2017), which indicated a potential risk of *T. gondii* transmission to endemic mammals.

In the present study, we report the first case of disseminated *T. gondii* infection in a deceased free-ranging Amami spiny rat.

2. Materials and methods

2.1. History

On July 8, 2018, a deceased adult male Amami spiny rat was found on a trail route on the Yuwandake Mountain on the south-central coast of the Amami-Oshima Island (28°29′ 19.59"N, 129°31′80.6"N) (Fig. 1A). After collection, the specimen was preserved in a refrigerator for 3 days until a necropsy was performed at the Amami Wildlife Conservation Center. A death notification of the animal was submitted to the Agency

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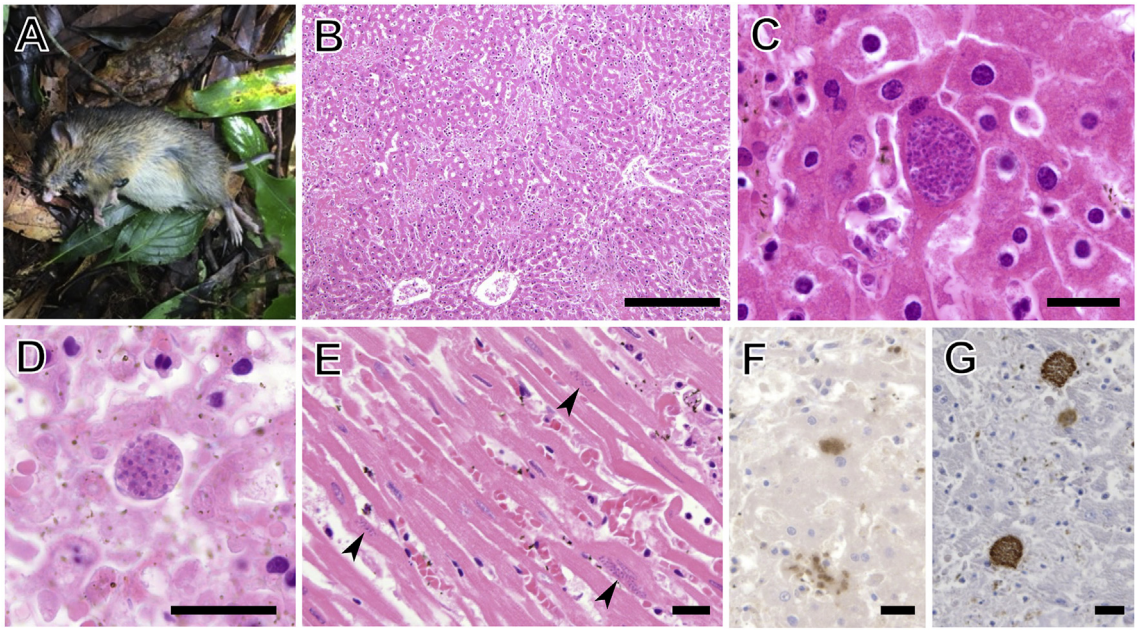

Fig. 1. Gross morphology, histopathology, and immunochemistry of the Amami spiny rat. (A) Lateral view of the specimen at the time of discovery, showing loss of tissues from lips to cheeks. (B) Histopathology of the liver showing focal necrosis. H&E, scale bar = 200 μm. (C to E) Intracellular protozoa in the tissue of the liver (C), lung (D), and myocardium (E, arrowheads indicate the protozoa). H&E. (F and G) Immunostaining of anti-SAG1 (F) and anti-BAG1 (G). Scale bar = 20 μm.

Table 1
List of primers and conditions for nested or semi-nested PCR.

Genes	Reaction	Primers (5′to 3′)	PCR conditions	
			Cycles	Annealing
B1	First	B1_Fext (GGAACTGCATCCGTTCATGAG) and B1_Rext (TCTTTAAAGCGTTCGTGGTC) (Burg et al., 1989)	40	57 °C, 10 sec
	Second	B1_Fint (TGCATAGGTTGCAGTCACTG) and B1_Rint (GGCGACCAATCTGCGAATACACC) (Burg et al., 1989)	40	62.5 °C, 10 sec
SAG3	First	SAG3_Fext (CAACTCTCACCATTCCACCC) and SAG3_Rext (GCGCGTTGTTAGACAAGACA) (Grigg et al., 2001)	35	60 °C, 30 sec
	Second	SAG3_Fint (TCTTGTCGGGTGTTCACTCA) and SAG3_Rint (CACAAGGAGACCGAGAAGGA) (Grigg et al., 2001)	25	65 °C, 30 sec
GRA6	First	GRA6_Fext (ACACGGTGGCATCCATCTGA) and GRA6_R (TCCGAAGGGGTCTGCTTAAC)	40	58 °C, 30 sec
	Second	GRA6_Fint (CCATCTGAGGCAGAAGCGTA) and GRA6_R	25	55 °C, 30 sec
ROP18	First	ROP18_Fext (TCGTCCGAATGGGTTTAGCG) and ROP18_R (TCGAGTGCTTTCTGTCGCTC)	40	60 °C, 30 sec
	Second	ROP18_Fint (TGTCTTGCGGKGTTAAMTGT) and ROP18_R	25	50 °C, 30 sec

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2.2. Gross and histopathological examination

Tissue samples from the liver, spleen, lungs, kidneys, adrenal grands, heart, and digestive tracts were fixed in 10% neutral buffered formalin and processed into paraffin blocks for routine histopathologic processing. Paraffin-embedded tissues were sectioned at 5 μm and stained with hematoxylin and eosin (H&E) for microscopic examination. Selected liver sections were subjected to immunohistochemical staining using the mouse *T. gondii* SAG1 (anti-SAG1) and BAG1 (anti-BAG1) antibodies diluted to 1:1,000. These antibodies were made (by author K. Ike) following previously described methods for *Neospora caninum* (Uchida et al., 2004; Kobayashi et al., 2013), except for use of recombinant *T. gondii* SAG1 and BAG1 proteins expressed with *Escherichia coli*. These antibodies were revealed using the EnVision Detection System (DaKo, USA). For the controls, we used sections without the primary antibodies, known *T. gondii*-negative tissues from a squirrel monkey (*Saimiri sciureus*) and mouse (*Mus musculus*), and positive liver tissues from a squirrel monkey that was spontaneously infected with *T. gondii*.

2.3. DNA extraction, PCR amplification, and sequence analyses

Total genomic DNA was extracted from the formalin-fixed and paraffin-embedded samples. Three 7 μm sections were collected from the liver and transferred to 1.5 ml tubes. Each tissue sample was deparaffinized with xylene three times, and genomic DNA was extracted with the DEXPAT Easy kit (TaKaRa, Japan) in accordance with the manufacturer's instructions. For molecular confirmation, *B1* and *SAG3* genes were amplified using nested PCR primers, as previously described (Burg et al., 1989; Grigg et al., 2001). To further reveal the extent of genetic diversity of the *T. gondii* isolate, two polymorphic loci, *GRA6* (Fazaeli et al., 2000; Kyan et al., 2012) and *ROP18* genes (Khan et al., 2009), were selected using the following analyses. *GRA6* and *ROP18* sequences were collected from the International Nucleotide Sequence Database (INSD) and were separately aligned using ClustalW in MEGA software version 7.0 (Kumar et al., 2016). From the multiple alignments, the conserved sequences were selected to design novel primers for semi-nested PCRs and the sequencing of *GRA6* and *ROP18* (Table 1).

The PCR solution (50 μl) was prepared following a standard procedure using Takara Ex Taq (TaKaRa, Japan). For the subsequent amplifications, 1 μl of the first PCR product was used as the template. The reaction consisted of an initial denaturation at 94 °C for 3 min, followed by 25–40 cycles of denaturation at 94 °C for 30 s, annealing at 55–65 °C for 10–30 s, and extension at 72 °C for 30 s. A final extension cycle was

performed at 72 °C for 7 min. Detailed information regarding the primers and amplification cycling conditions are shown in Table 1.

The amplicons from the second amplification of four loci were analyzed by agarose gel electrophoresis, and *SAG3, GRA6,* and *ROP18* amplicons were sent to Macrogen Company (Kyoto, Japan) where the DNA sequencing was conducted using an ABI DNA sequencing system. Phylogenetic analyses were performed with the following reference strains that are available in INSD: archetypal type I (GT1, RH), type II (BEVERLEY, DEG, PTG, Pruginaud, ME49), type III (CEP, CTG, NED, VEG), atypical strains (BOF, CAST, CASTELLS, COUG, GUYDOS, GUYKOE, GUYMAT, FOU, MAS, TgCatBr5, VAND), and strains isolated from domestic animals in Okinawa Island, Japan (Ok24–Ok129) (Kyan et al., 2012). Phylogenetic analyses were performed using MEGA software, using a maximum-likelihood algorithm, with distances calculated by the Tamura-Nei model for *SAG3* and *GRA6,* and the kimura 2-parameter model for *ROP18*. The stability of the topology was evaluated by bootstrapping with 1,500 replicates.

3. Results

3.1. Gross and histopathological examination

The animal weighed 88.7 g and measured 19.5 cm in total length and 6.1 cm in tail length. There were no external injuries except for the mouth part, which showed loss of tissues from the lips to the cheeks. Gross examination of internal organs showed no significant changes other than bile imbibition. Histologic postmortem changes were observed in almost all tissues and were especially severe in the lungs. Within the liver, multiple focal necrosis (Fig. 1B) and mild infiltration of inflammatory cells around the Glisson's capsule and central veins, and a number of intracellular protozoa, similar to *T. gondii* (Fig. 1C), were observed. The lungs showed congestion of the alveolar capillary, pulmonary edema, infiltration of inflammatory cells around large vessels, and intracellular protozoa (Fig. 1D). The myocardium had a few intracellular protozoa (Fig. 1E), and as a result, the section that was immunostained with anti-SAG1 to reveal a large number of positive brown-staining parasites and aggregates of numerous parasites in the cytoplasm (Fig. 1F). In the section that was immune-stained with anti-BAG1, strongly brown-staining cyst-like structures were observed, which enclosed hundreds of parasites (Fig. 1G).

3.2. Molecular analyses

Four genetic loci (*B1, SAG3, GRA6,* and *ROP18*) were successfully amplified, of which 3 were successfully sequenced. The length of the obtained sequences (excluding primer sequences) and the DNA Data Bank of Japan accession numbers were: *SAG3*, 186 bp (LC474391); *GRA6*, 107 bp (LC474392); and *ROP18,* 155 bp (LC474393), respectively. In all phylogenetic trees, the sequences in the present study were clustered with type II strains (Fig. 2). Although several atypical strains (BOF, COUG, GUYKOE, FOU) also fell within the same clades, the phylogenetic position of these atypical strains were inconsistent in the trees that used *GRA6* (Fig. 2B) and *ROP18* (Fig. 2C) sequences.

4. Discussion

Based on the histopathological findings and molecular analyses, the deceased Amami spiny rat was diagnosed with disseminated toxoplasmosis. Since the present case was a well-padded individual and without any other lesions, the cause of death was presumably a result of acute disseminated *T. gondii* infection.

In the Amami-Oshima Islands, there are no known endemic felid species, and domestic cats serve as the only final hosts of *T. gondii.* An estimated 600–1,200 feral cats roam the forested areas of the Amami-Oshima Island (Shionosaki, 2016). In an epidemiological study in the Amami-Oshima Island, seroprevalence of *T. gondii* among outdoor cats in these mountain areas was shown to be higher than that in urban areas (Matsuu et al., 2017). Furthermore, in such natural areas, the Amami spiny rat, Ryukyu long-furred rat, and Amami rabbit are known to be important prey species for feral cats (Shionosaki et al., 2015). These data suggest that non-native cats and endemic mammals play specific roles in the maintenance of *T. gondii* infection in these areas. The prevalence of *T. gondii* in endemic mammals remains unknown, and only a single suspected case of disseminated infection was previously reported in an Amami rabbit (Kubo et al., 2013). A larger epidemiological study on the prevalence of *T. gondii* in endemic mammals, including endangered species, is urgently needed.

The source of *T. gondii* infection can vary among animal species, feeding behaviors, and habitats. The main source of infection is from the oocysts shed in the feces of a host felid or from tissue cysts of intermediate hosts (Dubey and Odening, 2001). Since the Amami spiny rat is omnivorous and consumes seeds and insects (Iwasa, 2009), environmental contamination by oocysts from outdoor cats is considered to be the most probable source of infection.

In general, the pathogenicity of *T. gondii* is determined by the strain and host factors (Dubey and Odening, 2001). With regards to the strain, type I strains are lethal in mice, whereas type II and III strains are avirulent or have low virulence in the same animal (Sibley and Boothroyd, 1992). Molecular analysis suggested that the sequences determined in this study differed from the type I and III strains, but were closely related to the type II strain. Unfortunately, there are no other published reports on the molecular characterization of *T. gondii* from the Amami-Oshima Island and, therefore, the strain could not be compared with the present database of sequences. With regards to the host factor, mice of any age are susceptible to *T. gondii* infection, and

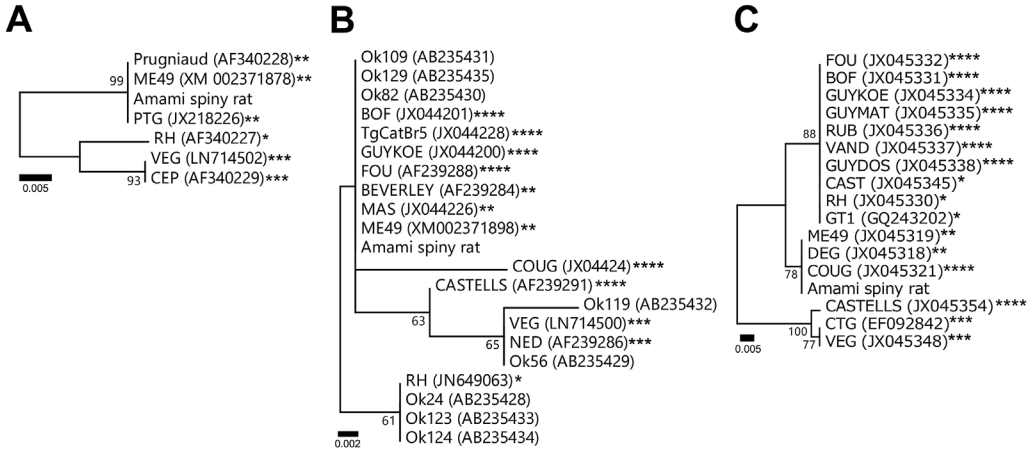

Fig. 2. Mid-point phylogenetic trees using *SAG3* (A), *GRA6* (B), and *ROP18* (C) sequences of *Toxoplasma gondii* detected from the Amami spiny rat specimen and the references available in the public databases. *, type I strain; **, type II strain; ***, type III strain; ****, atypical strain. Bars represents the number of nucleotide substitutions per site.

more severe infections have been observed in pregnant or lactating mice than in nonlactating mice (Dubey and Odening, 2001). Adult rats are usually resistant, whereas some juveniles have shown clinical symptoms (Dubey and Odening, 2001). Furthermore, the consequences of infection vary between different host species. For example, hares (*Lapus* spp.) are considered to be more susceptible to *T. gondii* infection than domestic rabbits (*Oryctolagus cybuculus*), despite both belonging to the order Lagomorpha. An experimental infection study revealed that hares showed a high mortality rate at very low oocyst doses, whereas domestic rabbits were able to tolerate higher oocyst doses (Gustafsson et al., 1997; Sedlák et al., 2000). The different responses to varying levels of exposure to *T. gondii* infection among these lagomorphs is believed to be due to differences in the natural susceptibility to *T. gondii* infection or the negative impact of stress to the immune status of hares (Sedlák et al., 2000). The evolutionary history of hosts may be related to the susceptibility to *T. gondii* infection. *Toxoplasma gondii* can cause fatal infections in Australian marsupials and New World monkeys, and is thus these groups are considered to be the most susceptible groups of species to *T. gondii* infection. These animals have largely evolved separately from the felids, and hence also separately from exposure to *T. gondii*, and have thus not developed resistance to *T. gondii* infection (Innes, 1997; Carme et al., 2009). Such a hypothesis seems applicable to the Spiny rats which have a unique and ancient origin. Spiny rats that belong to the genus *Tokudaia* are distributed only in the three neighboring islands: Amami-Oshima Island for Amami spiny rat, Tokunoshima Island for Tokunoshima spiny rat (*T. tokunoshimensis*), and Okinawa Island for Okinawa spiny rat (*T. muenninki*). The divergence event for the genus *Tokudaia* and other Murinae (*Apodemus, Micromys, Mus,* and *Rattus*), distributed in the surrounding areas, is estimated to have occurred several million years ago (Suzuki et al., 2000; Sato and Suzuki, 2004). Since there are no endemic felids species in these islands, the Amami spiny rat is a certainly accidental intermediate host for *T. gondii* and plays no part in its natural history and evolution.

In conclusion, this is the first case report of *T. gondii* infection in the Amami spiny rat. Islands are of particular importance for the conservation of global rodent diversity (Amori et al., 2008). However, invasive predators, particularly cats, have directly contributed to population declines or the extinction of many native rodent species since these historically isolated, island animals lack evolved defenses against such predators (Medina et al., 2011). The present case demonstrates an example of the negative indirect impact of non-native predators. To reduce *T. gondii* infection in local wildlife, the populations of feral and stray cats should be controlled in these regions.

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Declarations of interest

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijppaw.2019.06.001.

References

Amori, G., Gippoliti, S., Helgen, H.M., 2008. Diversity, distribution, and conservation of endemic island rodents. Quat. Int. 182, 6–15.

Burg, J.L., Grover, C.M., Pouletty, P., Boothroyd, J.C., 1989. Direct and sensitive detection of a pathogenic protozoan, *Toxoplasma gondii,* by polymerase chain reaction. J. Clin. Microbiol. 27, 1787–1792.

Carme, B., Ajzenberg, D., Demar, M., Simon, S., Dardé, M.L., Maubert, B., de Thoisy, B., 2009. Outbreaks of toxoplasmosis in captive breeding colony of squirrel monkeys. Vet. Parasitol. 163, 132–135.

Dubey, J.P., Odening, K., 2001. Toxoplasmosis and related infections. In: Samuel, W.M., Pybus, M.J., Kocan, A.A. (Eds.), Parasitic Diseases of Wild Mammals, second ed. Iowa State University Press, Ames, pp. 478–519.

Fazaeli, A., Carter, P.E., Darde, M.L., Pennington, T.H., 2000. Molecular typing of *Toxoplasma gondii* strains by *GRA6* gene sequence analysis. Int. J. Parasitol. 30, 637–642.

Fredebaugh, S.L., Mateus-Pinilla, N.E., McAllister, M., Warner, R.E., Weng, H.Y., 2011. Prevalence of antibody to *Toxoplasma gondii* terrestrial wildlife in a natural area. J. Wildl. Dis. 47, 381–392.

Grigg, M.E., Ganatra, J., Boothroyd, J.C., Margolis, T.P., 2001. Unusual abundance of atypical strains associated with human ocular toxoplasmosis. J. Infect. Dis. 184, 633–639.

Gustafsson, K., Uggla, A., Järplid, B., 1997. *Toxoplasma gondii* infection in the mountain hare (*Lepus timidus*) and domestic rabbit (*Oryctolagus cuniculus*). I. Pathology. J. Comp. Pathol. 177, 351–360.

Innes, E.A., 1997. Toxoplasmosis: comparative species susceptibility and host immune response. Comp. Immnol. Infect. Dis. 20, 131–138.

Ishii, N., 2016. *Tokudaia osimensis.* The IUCN Red List of Threatened Species 2016: e e.T21973A22409638. (Downloaded on 2019 April).

Iwasa, M.A., 2009. *Tokudaia osimensis* (abe, 1933). In: Ohdachi, Y., Ishibashi, Y., Iwasa, M.A., Saitoh, T. (Eds.), The Wild Mammals of Japan. Shoukado Book Sellers and the Mammalogical Society of Japan, pp. 159–160.

Khan, A., Taylor, S., Ajioka, J.W., Rosenthal, B.M., Sibley, D., 2009. Selection of a single locus leads to widespread expansion of *Toxoplasma gondii* lineages that are virulent in mice. PLoS Genet. 5 e1000404.

Kobayashi, T., Narabu, S., Yanai, Y., Hatano, Y., Ito, A., Imai, S., Ike, K., 2013. Gene cloning and characterization of protein encoded by the *Neospora caninum* bradyzoite-specific antigen gene *BAG1*. J. Parasitol. 99, 453–458.

Kubo, M., Nakashima, T., Honda, T., Kochi, Y., Ito, Y., Hattori, S., Kuraishi, T., 2013. Histopathological examination of spontaneous lesions in Amami rabbits (*Pentalagus furnessi*): a preliminary study using formalin-fixed archival specimens. Jpn. J. Zoo Wildl. Med. 18, 65–70 (Article in Japanese).

Kumar, S., Stecher, G., Tamura, K., 2016. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol. Biol. Evol. 33, 1870–1874.

Kyan, H., Taira, M., Yamamoto, A., Inaba, C., Zakimi, S., 2012. Isolation and characterization of *Toxoplasma gondii* genotypes from goats at an abattoir in Okinawa. Jpn. J. Infect. Dis. 65, 167–170.

Lehrer, E.W., Fredebaugh, S.L., Schooley, R.L., Mateus-Pinilla, N.E., 2010. Prevalence of antibody to *Toxoplasma gondii* in woodchucks across an urban-rural gradient. J. Wildl. Dis. 46, 977–980.

Mameno, K., Kubo, T., Suzuki, M., 2017. Social challenges of spatial planning for outdoor cat management in Amami Oshima Island, Japan. Glob. Ecol. Conserv. 10, 184–193.

Matsuu, A., Yokota, S., Ito, K., Masatani, T., 2017. Seroprevalence of *Toxoplasma gondii* in free-ranging and feral cats on Amami Oshima Island, Japan. J. Vet. Med. Sci. 79, 1853–1856.

Medina, F.M., Bonnaud, E., Vidal, E., Tershy, B.R., Zavaleta, E.S., Donlan, C.J., Keitt, B.S., Matthieu Le Corre, M., Horwath, S.V., Nogales, M., 2011. A global review of the impacts of invasive cats on island endangered vertebrates. Glob. Chang. Biol. 17, 3503–3510.

Sato, J.J., Suzuki, H., 2004. Phylogenetic relationships and divergence times of the genus *Tokudaia* within Murinae (Muridae; Rodentia) inferred from the nucleotide sequences encoding the Cyt*b* gene, RAG1, and IRBP. Can. J. Zool. 82, 1343–1351.

Sedlák, K., Literák, I., Faldyna, M., Toman, M., Benák, J., 2000. Fatal toxoplasmosis in brown hares (*Lepus europaeus*): possible reasons of their high susceptibility to the infection. Vet. Parasitol. 93, 13–28.

Shionosaki, K., 2016. The Study of Impacts of Invasive Domestic Cat (*Felis silvestris Catus*) on Endemic Endangered Mammals and Measures for Protecting These Endangered Species in Amami-Oshima Islands, Japan. Kyoto University, Japan. Thesis.

Shionosaki, K., Yamada, F., Ishikawa, T., Shibata, T., 2015. Feral cat diet and predation on endangered endemic mammals on a biodiversity host spot (Amami-Ohshima Island, Japan). Wildl. Res. 42, 343–352.

Sibley, L.D., Boothroyd, J.C., 1992. Virulent strains of *Toxoplasma gondii* comprise a single clonal lineage. Nature 359, 82–85.

Suzuki, H., Tsuschiya, K., Takezaki, N., 2000. A molecular phylogenetic framework for the Ryukyu endemic rodents *Tokudaia osimensis* and *Diplothrix legata*. Mol. Phylogenetics Evol. 15, 15–24.

Uchida, Y., Ike, K., Kurotaki, T., Imai, S., 2004. Monoclonal antibodies preventing invasion of *Neospora caninum* tachyzoites into host cells. J. Vet. Med. Sci. 66, 1355–1358.