Molecular genetic markers for parasite species identification and epidemiological study with special reference to *Gongylonema pulchrum* and *Oesophagostomum stephanostomum*

寄生虫の種同定と疫学研究において有用な遺伝子マーカーの研究―特に *Gongylonema pulchrum* と *Oesophagostomum stephanostomum* に注目して―

DISSERTATION

Presented in partial fulfillment of the requirement for the degree of
Doctor of Philosophy in the United Graduate School of Veterinary Science,
Yamaguchi University

The United Graduate School of Veterinary Science,
Yamaguchi University

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September 2014

Dedication

To

The Almighty

Acknowledgments

The present study was conducted in the Laboratory of Parasitology, Joint Faculty of Veterinary Medicine, Yamaguchi University, Japan during 2010 – 2014.

First of all, I am thankful to the Ministry of Education, Culture, Sports, Science and Technology (Monbukagakusho) of Japan, and SATREPS project by Japan Science and Technology Agency (JST) and Japan International Cooperation Agence (JICA) for the financial support during my four years Ph.D study in Japan.

I am sincerely grateful to my supervisor, Prof. Dr. Hiroshi SATO for accepting me and guiding my study until graduation. I am indebted to him for guidance, patience and direction during my graduated studies.

I would also like to extend my gratitude to my co-supervisors Prof. Dr. Yoshimi YAMAMOTO and Assoc. Prof. Dr. Tetsuya TANAKA for their advice and suggestion during my study.

My sincere thanks are forwarded to Assoc. Prof. Dr. Tetsuya YANAGIDA for his advice and for reviewing this thesis.

I am indebted to Assoc. Prof. Dr. Shiho FUJITA for helping me in all the process to join Yamaguchi University.

I am grateful to all members of Laboratory of Parasitology, Department of Veterinary Medicine, Yamaguchi University for their kind support and cooperation. I am also thankful to all the friends for their kindness, support and encouragement during my study.

Finally, I would like to express my deepest appreciation and love to my wife for her encouragement and kindness. The thesis is dedicated to all my family members.

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ABSTRACT

Human-wildlife interactions have reached unprecedented levels recently because of expanding anthropogenic activity on the earth's ecosystem, giving rise to serious negative effects such as mutual disease transmissions between humans and wildlife, or those between domestic animals and wildlife animals. Reliable identification of a causative pathogen(s) of zoonotic diseases found synchronously in humans and animals or domestic animals and wildlife animals is critically important for understanding the disease transmission dynamics in the confined environment or nature. For species identification of parasitic helminths, morphological characterization of adult worms play a pivotal role, and recently molecular-based approaches define more exactly or finely the species or cryptic species, or further 'units' for understanding the disease transmission in nature. In the present study, I have tried to know the disease transmission dynamics among multiple animal species in the field.

In Chapter I, I studied the gullet worm, *Gongylonema pulchrum* Molin, 1857, found worldwide from a variety of mammals such as cattle, sheep, goats, camels, pigs, equines, cervids, rodents, bears and primates including humans. Due to its wide host range, it has been suggested that the worm may be transmitted locally to any mammalian host by chance. To investigate this notion, the ribosomal RNA gene (rDNA), mainly regions of the internal transcribed spacers (ITS) 1 and 2, and a cytochrome *c* oxidase subunit I (COI) region of mitochondrial DNA (mtDNA) of *G. pulchrum* were characterized using parasites from the following hosts located in Japan: cattle, sika deer, wild boars, Japanese macaques, a feral Reeves's muntjac and captive squirrel monkeys. The rDNA nucleotide sequences of *G. pulchrum* were generally well conserved regardless of their host origin. The COI sequences of *G. pulchrum* were divided into multiple haplotypes, and two groups of haplotypes, i.e. those from a majority of sika deer, wild boars and Japanese macaques and those from cattle and zoo animals, were clearly differentiated. Our findings indicate that domestic and sylvatic transmission cycles of the gullet worm are currently present, at least in Japan.

In Chapter II, I characterized morphologically and genetically the gullet worms collected from Murrah cross water buffaloes (*Bubalus bubalis*) in the Kathmandu and Chitwan districts of Nepal. The morphology and measurements of collected worms were identical to those of *G. pulchrum*, except for the length of the left spicules relative to the body length. The ITS regions exhibited higher variations between the buffalo-collected worms and *G. pulchrum* from the other host mammals (85-88% identity for ITS1 and 56-80% identity for ITS2). The COI also showed lower identities (89.2-90.2%), although only a single amino acid

substitution was noted compared with the majority of *G. pulchrum* samples collected in Japan. Based on these molecular genetic characters in the rDNA and COI mitochondrial DNA, together with a shorter left spicule length relative to body length, the gullet worms isolated from buffaloes in Nepal might belong to a distinct local or buffalo-preferring population of *G. pulchrum*, although its geographical distribution on the continent and host specificity remain to be clarified.

In Chaper III, I studied a local mange epizootic, which was caused by *Sarcoptes scabiei* (Acari: Sarcoptidae), in wild mammals such as raccoon dogs, wild boars, feral racoons, badgers, martens, and serows in Japan. The ITS2 region of the rDNA and the partial 16S and COI genes of mtDNA were characterized in the mangy skin lesions of 128 animals. The majority of *S. scabiei* mites had almost identical ITS2 nucleotide sequences to those recorded in a variety of mammals worldwide. Partial 16S and COI fragments of mtDNA showed an identical nucleotide sequence except for one site ('C' vs. 'T') for the former and four sites ('G', 'C', 'C', 'C' vs. 'A', 'T', 'T', 'T', respectively) for the latter fragment. Furthermore, these substitutions were always synchronized, with the two mtDNA haplotypes, i.e. 'C/GCCC' and 'T/ATTT', appearing to separately colonize in small geographical units. Moreover, the 'T/ATTT' haplotype was cladded into a branch where animal-derived mites worldwide dominated, whereas the 'C/GCCC' haplotype formed a geographical branch unique to Japanese isolates. These results suggest that heterologous populations of monospecific *S. scabiei* are expanding their populations and distributions regardless of host species in an apparently local mange epizootic of wild mammals in Japan.

In Chapter IV, the prevalence of the nodular worm *Oesophagostomum stephanostomum* (Nematoda: Strongylida) in western lowland gorillas at Moukalaba-Doudou National Park (MDNP), Gabon, was determined in fecal samples, along with their coprocultures. Genetic analyses of the parasites suggest that at MDNP, Gabon, only a single population of *O. stephanostomum* with a degree of genetic diversity is prevalent in western lowland gorillas, without zoonotic complication in local inhabitants.

Through the studies shown above, I have clarified the presence of previously unnoticed 'transmission units' of *G. pulchrum*, and involvement of different 'parasite units' of distinct origins in a local epidemic of the parasitic disease. Appropriately selected genetic markers of parasites may allow us to predict the potential risk(s) of the epizootic disease of the wildlife animals to humans and domestic animals having a possible contact with them, and vice versa.

General introduction

1. Zoonoses

Zoonoses are diseases and infections naturally transmitted from animals to humans (W.H.O., Expert Committee on Zoonoses, WHO tech rep., 1951, Ser No. 40), and constitute a sizeable proportion of the new, emerging and re-emerging diseases (Schwabe, 1984; Meslin, 1995). Recently, the issue of zoonosis has gained increased attention because many pathogens are crossing species barriers and generating severe health problems to human beings. There are 1,415 species of infectious agents known to be pathogenic to human beings, of which approximately 60% of them are identified as zoonotic. Moreover, over the past two decades, 75% of the emerging diseases had wildlife origins (Taylor et al., 2001; Woolhouse, 2002).

In the preceding studies carried on primates and the ecology of their infectious diseases, Chapman et al. (2005) identified six factors that help to understand the possibility of mutual transmission of pathogens: 1) changes in human demographics and behavior; 2) changes in technology and industry; 3) international travel and commerce; 4) microbial adaptation; 5) breakdown of public health measures; and 6) environmental changes and land use. It is sustaining a previous opinion by Brack (1998) on zoonoses from non-human primates to humans that the close contact between them facilitates the parasitic infections to disseminate across human and other primates. Parasitic species exchanged between non-human primates and human beings include *Plasmodium* spp., *Trypanosoma cruzi*, *Giardia* spp., *Cryptosporidium* spp. and a variety of gastrointestinal helminths (Chapman et al., 2005).

Until recently, it was thought that the transfer of the simian malaria to human beings was an extremely rare event (Warren et al., 1970; Antinori et al., 2013). However, studies have disclosed many human cases of naturally acquired infection with *Plasmodium knowlesi*, for which macaques are believe to be the

primary host (Jongwutiwes al., 2004; Singh et al., 2004; Ng et al., 2008; Tang et al., 2010; Orth et al., 2013). Nonetheless, in most of the human cases, *P. knowlesi* is morphologically misidentified as *Plasmodium malariae*, and others as *Plasmodium falciparum* or *Plasmodium vivax*. Therefore molecular identification is necessary for accurate diagnosis. For instance, Singh et al. (2004) could identify *P. knowlesi* only by PCR in 120/208 (58%) of human malaria cases, whereas morphological diagnosis in stained blood films misidentified as *P. malariae* in most of the cases.

Although humans and non-human primates historically have shared habitats, recently radical changes have emerged in the dynamics of human-primate interactions (Gillespie et al., 2008). Especially since anthropogenic habitat change forces humans and primates into closer and more frequent contact, the risk of interspecific disease exchange is increasing (Chapman et al., 2005). A variety of routes have been identified for the parasite flow from the wildlife to humans: arthropod vectors (e.g. *Leishmania*), contaminated foods (e.g. *Trichinella* in meat) or contaminated water (e.g. *Cryptosporidium*), and infested wildlife hosts (e.g. *Sarcoptes*). The accelerating development and integration of new diagnostic methods may lead in the future to the discovery of emerging cause of human diseases similarly to human malaria caused by *P. knowlesi*.

2. Context of study

Gabon, a nation of Central Africa, bestrides the equator and abuts between the Gulf Guinea to west, Cameroon and Equatorial Guinea to the north, and Congo to the east. It locates in the Congo Basin, the world's great wilderness containing the second largest rainforest block on earth next to the Amazon Basin (Sanderson et al., 2002), noticeably rich in an outstanding biological diversity including great apes and the forest elephants (*Loxodonta africana cyclotis*) (Kamdem-Toham et al., 2003). Gabon is considered to have the richest wildlife and plant faunas in Africa, with up to 20% of its species are endemic to the

country (Quammen, 2003). Moreover, approximately 40% of the world's gorillas *Gorilla gorilla* are thought to live in Gabon (Walsh, 2006). In order to fulfil one of the commitments of the "Congo Basin Forest Partnership", the Gabonese Government established 13 national parks that encompass 11% of the country's land area (about 30,000 km^2) in 2002, and include most of the important terrestrial, costal, and marine ecosystem in the country. Recently, Gabon introduced ecotourism into the management system of the national parks to promote sustainable use of natural resources and contribute financially to the conservation activities. The great apes, such as gorillas and chimpanzees (*Pan troglodytes*) that inhabit in the rainforest are the key species for this project, because they draw a lot of tourists from the worldwide countries. Along with this project, it is supposed that greater human-wildlife contacts are inevitable, resulting in higher possibilities of mutual pathogen transmission. To evaluate this point, it is needed to conduct an assessment of the potential risk of wildlife (especially, great apes)-human contacts through ecotourism in view of pathogen transmission. This assessment enables us to plan appropriate measures to protect health of tourists and the human residents as well as the great apes through adequate management plans of ecotourism.

(a) (b)

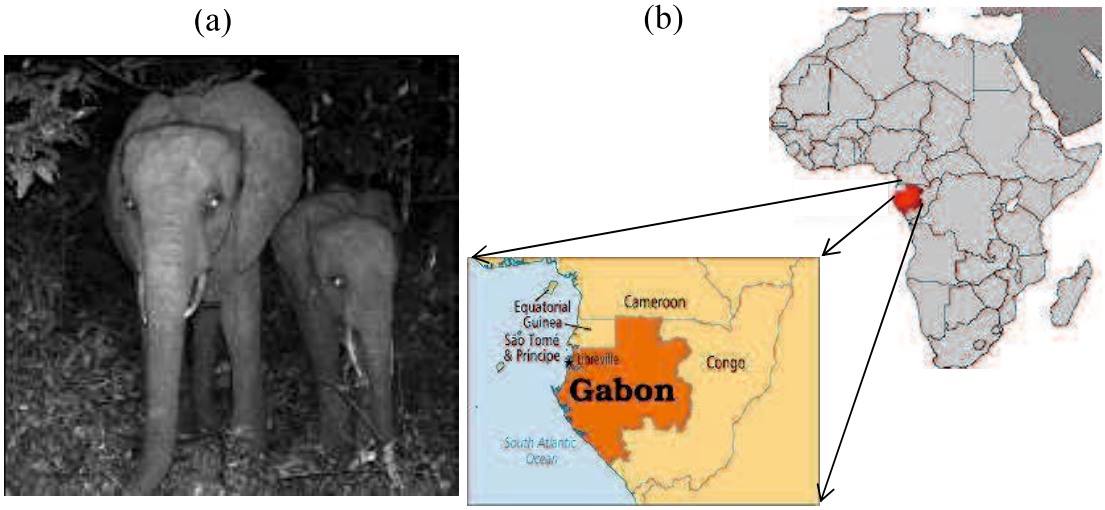

Figure 1. Photographs of (a) Gabon supports spectacular wildlife such as forest elephants (Photo by W.F Laurence) and (b) map of Gabon

3. Objectives of the study

Ecotourism has become the most universally accepted powerful tool to promote wildlife conservation because it is increasing public awareness, empowering community members to understand their natural heritage and take action against habitat degradation, as well as raising funds for protecting endangered species (particularly great apes) (Filion et al., 1994; Homsy, 1999; Muehlenbein & Ancrenaz, 2009). However, with expanding ecotourism, human contact with wildlife, mainly great apes, is increasing, and human-ape contacts are raised as another important threat to the survival of these endangered species because great apes are extremely susceptible to human diseases. Along with poaching and habitat loss known as major threats to African apes (Walsh et al. 2003), Ryan & Walsh (2011) described that infectious disease is now a threat of similar magnitude. Recently, due to the emergence of Ebola virus, the decline of great apes populations in Gabon and the Republic of Congo was reported (Walsh et al., 2003; Leroy et al., 2004). Leroy et al. (2004) noticed that between 2001 and 2003, 80% of great ape populations in the border region of these two countries were devastated. However, all the dead apes were not positive for Ebola, suggesting the involvement of other pathogens. The identification of the pathogen(s) responsible of the observed death can clarify both the involved agent(s) and the transmission dynamics of the pathogen, mainly the mutual pathogen transmission between humans (researchers, tourists, and others) and apes (Hirsch et al., 1995; Homsy, 1999; Wallis, 2000; Rouquet et al., 2005). Many pathogens such as bacterial, mycoplasmal, spirochetal, fungal, parasitic and viral ones may be involved. Thus, continuing to collect information on the ecology of natural diseases of primates is paramount to the sustainable use of wildlife biodiversity and human health.

In future when I go back to Gabon, I would like to focus on parasitic infection of wildlife, especially the great apes, and the human residents, because parasitic infection has great impacts on animal and human survivals and health

conditions, and the infection could be examined by collecting samples by a noninvasive method. Humans and great apes, including gorillas, shared parasite diseases such as malaria, schistosomiasis, giardiasis, filariasis, and infections with *Strongyloides* spp., *Entamoeba* spp., *Oesophagostomum* spp., *Acanthocephala* spp., *Cyclospora cayetanensis* and *Sarcoptes scabiei* (Benirschke & Adams, 1980; Kalter, 1980, 1986; Smith et al., 1982; Toft, 1986; Ashford et al., 1990, 1996; Wolfe et al., 1998, 2001; Homsy, 1999; Sleeman et al., 2000). Until recently, human infection with the nematode parasite *Oesophagostomum bifurcum* was considered as a rare zoonosis. However, the parasite was commonly found in human residents at high level in Northern Togo and Ghana. (Blotkamp et al., 1993; Polderman & Blotkamp, 1995; Pit et al., 1999; Yelifari et al., 2005; Gasser et al., 2006). In spite of the medical significance of *O. bifurcum* in these regions of Africa, little is known about its epidemiology. However, recently, multifaceted genetic analyses have demonstrated that different genotypes of *O. bifurcum* are prevalent in human patients and local non-human primates (Mona monkeys, Patas monkeys, Green monkeys, and Olive baboons) in Ghana and Togo (de Gruijter et al., 2004, 2005; reviewed by Gasser et al., 2006, 2009). The results of these studies support parallel assumptions based on epidemiological and morphological analyses (de Gruijter et al., 2006; van Lieshout et al., 2005). Indeed, de Gruijter et al. (2006) demonstrated clear differences in morphological characters (suh as parasite length, width, length of the oesophagus and length of spicules) between *O. bifurcum* worms from humans, the Mona, Patas or Green monkey and/or Olive baboons. Whereas, van Lieshout et al. (2005) noticed high prevalence of *O. bifurcum* (75-99%) in olive baboons and Mona monkeys, and no human infection despite the behavior of non-human primates regarding defecation, food consumption and sharing of habitat with the local human population indicated favourable conditions for zoonotic transmission. Moreover, in the recent work study in western Uganda, three genotypes of *Oesophagostomum* were isolated in

the people and primates (Ghai et al., 2014). Found nematodes included *O. bifurcum*, *O. stephanostomum* and a possibly new and zoonotic *Oesophagostomum* species infecting humans and various monkeys. In the present study, I challenged to disclose the transmission dynamics of two representative parasitic infections caused by agents believed to have a wide spectrum of hosts including wildlife and domestic mammals and humans, i.e. *Gongylonema pulchrum* and *Sarcoptes scabiei*. I characterized the ribosomal RNA gene (rDNA) and the cytochrome *c* oxidase subunit I (COI) gene of mitochondrial DNA (mtDNA) of them. Using these genetic markers, I disclose previously unnoticed 'transmission units' of *G. pulchrum*, and involvement of different 'parasite units' of distinct origins in a local epidemic of the parasitic disease. Appropriately selected genetic markers of parasites may allow us to predict the potential risk(s) of the epizootic disease of the wildlive animals to humans and domestic animals having a possible contact with them, and vice versa. At last, I employed these genetic markers to samples from great apes of Moukalaba-Doudou National Park (MDNP), Gabon, and human residents near the park, where the habituation of western gorillas has been conducted for the research and ecotourism purposes, in order to define the genetic diversity of the parasites.

CHAPTER I

Genetic variation of *Gongylonema pulchrum* from wild animals and cattle in Japan based on ribosomal RNA and mitochondrial cytochrome *c* oxidase subunit I genes

The work described in the chapter has been published as follows;

Makouloutou, P., Setsuda, A.,Yokoyama, M., Tsuji, T., Saita, E., Torii, H., Kaneshiro, Y., Sasaki, M., Maeda, K., Une, Y., Hasegawa, H. and Sato, H. (2013) : Genetic variation of *Gongylonema pulchrum* from wild animals and cattle in Japan based on ribosomal RNA and mitochondrial cytochrome *c* oxidase subunit I genes. *J. Helminthol.* 87: 326-335.

1. 1 Abstract

The gullet worm (*Gongylonema pulchrum*) has been recorded from a variety of mammals worldwide, including monkeys and humans. Due to its wide host range, it has been suggested that the worm may be transmitted locally to any mammalian host by chance. To investigate this notion, the ribosomal RNA gene (rDNA), mainly regions of the internal transcribed spacers (ITS) 1 and 2, and a cytochrome *c* oxidase subunit I (COI) region of mitochondrial DNA of *G. pulchrum* were characterized using parasites from the following hosts located in Japan: cattle, sika deer, wild boars, Japanese macaques, a feral Reeves's muntjac and captive squirrel monkeys. The rDNA nucleotide sequences of *G. pulchrum* were generally well conserved regardless of their host origin. However, a few insertions/deletions of nucleotides along with a few base substitutions in the ITS1 and ITS2 regions were observed in *G. pulchrum* from sika deer, wild boars and Japanese macaques, and those differed from *G. pulchrum* in cattle, the feral Reeves's muntjac and captive squirrel monkeys. The COI sequences of *G. pulchrum* were further divided into multiple haplotypes and two groups of haplotypes, i.e. those from a majority of sika deer, wild boars and Japanese macaques and those from cattle and zoo animals, were clearly differentiated. Our findings indicate that domestic and sylvatic transmission cycles of the gullet worm are currently present, at least in Japan.

1. 2 Introduction

The gullet worm, *Gongylonema pulchrum* Molin, 1875, is a thread-like spirurid nematode found worldwide. A variety of mammals, such as cattle, sheep, goats, camels, pigs, equines, cervids, rodents, bears and primates, serves as the definitive host and dung beetles plays a role as the intermediate host (Lichtenfels, 1971; Kirkpatrick et al., 1986; Anderson, 1992). In Japan, this nematode has been found in cattle (Kudo et al., 1992; Suzuki et al., 1992; Sato,

2009), sika deer (*Cervus nippon*) (Yokohata & Suzuki, 1993; Kitamura et al., 1997; Sato, 2009), macaques (*Macaca fuscata*) (Uni et al., 1992, 1994), zoo-kept squirrel monkeys (*Saimiri boliviensis*) (Sato et al., 2005) and humans (Haruki et al., 2005; Hara et al., 2010).

The gullet worm occurs in a variety of mammals and the host animals appear to live sympatrically (e.g. deer graze in fields for cattle in some localities). The transmission cycle of the worm may be shared by both domestic and wild animals through accidental ingestion of infected dung beetles or water contaminated with the third-stage larvae (L3) having emerged from dead dung beetles (Kudo et al., 1996). Furthermore, a high prevalence of the gullet worm in local populations of sika deer in localities where stock farming is prosperous seems to support this view (Yokohata & Suzuki, 1993)

Here, I characterize the 18S to 28S ribosomal RNA gene (rDNA) and the cytochrome *c* oxidase subunit I (COI) of mitochondrial DNA (mtDNA) using specimen from cattle, sika deer, wild boars (*Sus scrofa leucomystax*), Japanese macaques, a feral Reeves' muntjac (*Muntiacus reevesi*) and captive squirrel monkeys (*Saimiri* spp.) in order to elucidate the transmission dynamics of *G. pulchrum* in domestic and wild ruminants and other wild animals in Japan.

1. 3 Materials and methods

1. 3. 1 Collection and examination of cattle and wild animals

Full-length oesophagi of 638 cattle from farms located in 27 prefectures of Japan were collected from a bovine viscera-processing company (Nanko-zouki Co. Ltd, Nanko-minami, Siminoe-ku, Osaka) during February and March 2009. Most of the cattle were Japanese black breed (2.3-2.8 years old) or F1 between Japanese black and Holstein-Friesian breed (1.6-2.5 years old). Full-length oesophagi of two infected cattle of Holstein-Friesian breed from farms in Hokkaido were supplied by Professeur H. Furuoka, Obihiro University of Agriculture and Veterinary Medicine. These two cattle were sacrificed on 19

and 26 November 2008. Twenty-seven Honshu sika deer (*Cervus nippon centralis*) were shot at three localities in Hyogo Prefecture during March 2008 and 12 sika deer (either Kyushu sika deer (*C. nippon nippon*) or Honshu sika deer) were shot in Kochi Prefecture on Shikoku Island during the hunting season of 2008-2009. In addition, 132 and 24 Honshu sika deer were collected between July 2009 and May 2010 in Hyogo Prefecture and between March 2009 and April 2011 in Wakayama Prefecture, respectively. Furthermore, oesophagi of four Hokkaido sika deer (*C. nippon yesoensis*) shot in Nakasatsunai Village, Hokkaido in May 2009 and a single dead body of a young Yaku sika deer (*C. nippon yakushimae*) found on Yakushima Island, Kagoshima Prefecture on 7 August 2010 were examined for the parasite. During the hunting seasons of 2009-2011, 132 wild boars were shot in Hyogo Prefecture. Ten Japanese macaques were collected in Nara Prefecture during November and December 2010 with the prefectural permission. Suspensions of lingual scraping in physiological saline from Bolivian squirrel monkeys (*Saimiri boliviensis*) and common squirrel monkeys (*Saimiri sciureus*) kept in two different zoological gardens on Kyushu Island were individually collected according to the method described by Sato et al. (2005) during the winter of 2009 to 2010. A feral Reeves's muntjac collected on Izu-oshima Island, Tokyo in June 2009 was provided by Dr. S. Ozawa, Oshima Island Park, Tokyo Prefectural Office.

The whole oesophagi of cattle, usually ten oesophagi samples per plastic bag, were transported at 4°C to the Laboratory of Parasitology, Yamaguchi University. The oesophagi or carcasse of sika deer, wild boars, Japanese macaques and the feral Reeves's muntjac were sent as frozen materials. In the laboratory, each oesophagus was opened longitudinally and the mucosal layers were peeled from the underlying tissues. The mucosal surface was then carefully checked with the naked eye and, particularly for sika deer samples, worm collections were conducted under a dissection microscope. Individual worms were carefully removed from the oesophageal epithelium using fine forceps and

fixed in 70% alcohol or 10% neutral-buffered formalin, except for worms for DNA extraction. Microtubes containing lingual scrapings from the squirrel monkeys were centrifuged and the supernatant was discarded except for 40 µl of physiological saline and precipitate (Sato et al., 2005). The precipitate in the microtube was resuspended and 10-20 µl of the suspension was examined under a light microscope.

1. 3. 2 Morphological examination of the gullet worm

Specimens fixed in 10% neutral-buffered formalin solution were cleared by the addition of several drops of glycerol. Six females and six males adults from each host category were observed under a light microscope and figures were drawn with the aid of a camera lucida. Measurements were performed on these drawn figures using a digital curvimeter type S (Uchida Yoko, Tokyo, Japan) when necessary. Collected specimens were deposited in the National Museum of Nature and Science, Tokyo, Japan under specimen numbers NSMT-As3501-As3527, As3608-As3639 and As3647-As3671.

1. 3. 3 DNA extraction, polymerase chain reaction and sequencing

Parasite DNAs were extracted by using single worms each taken from 18 cattle, eight Honshu sika deer, two Kyushu/Honshu sika deer collected in Kochi Prefecture, two Hokkaido sika deer, one Yaku sika deer, four wild boars, two Japanese macaques and one feral Reeves's muntjac, and by using *G. pulchrum* eggs collected from two colonies of captives squirrels monkeys. In addition, DNA was extracted from two or three worms taken from four Honshu sika deer. DNA extraction was performed usin an Illustra^{TM} tissue & cells genomicPrep Mini Spin Kit (GE Haealthcare UK, Buckinghamshire, UK) according to the manufacturer's instructions.

Polymerase chain reaction (PCR) amplification of overlapping rDNA fragments was performed as previously described (Sato et al., 2006; Halajian et

al., 2010) using different primer combinations (Table 1). PCR products for sequencing were purified using a High Pure PCR Cleanup Micro Kit (Roche Diagnostics GmbH, Mannheim, Germany). After direct sequencing of PCR amplicons, sequences were assembled manually with the aid of the CLUSTAL W multiple alignment program (Thompson et al., 1994). For rDNA segments containing internal transcribed spacers (ITS) 1 and 2, the amplicon was cloned into a plasmid vector, pTA2 (Target Clone^{TM} Toyobo, Osaka, Japan), and transformed into *Escherichia coli* JM109 (Toyobo) according to the manufacturer's instructions. Following propagation, plasmid DNA was extracted using a NucleoSpin® Plasmid kit (Macherey-Nagel GmbH, Düren, Germany) and inserts from multiple independent clones were sequenced using universal M13 forward and reverse primers. Clones were sequenced as follows: six clones for a single *G. pulchrum* from sika deer, wild boars and Japanese macaques, nine clones for a single *G. pulchrum* from a steer in Okayama/Tottori Prefecture, 11 clones for a single *G. pulchrum* from a steer in Kagoshima Prefecture and five clones each for *G. pulchrum* eggs from captive Bolivian and common squirrels monkeys.

The COI region of *G. pulchrum* mtDNA was amplified by a primer pair of BpCoxI-F1 (5'-TTTGGTCCTGAGGTTTATATT-3') and BpCoxI-R1 (5'-ATGAAAATGTCTAACTACATAATAAGTATC-3'). PCRs were conducted in a thermal cycler using the following cycling protocol: 3min at 94°C, followed by 40 cycles at 94°C for 45s, 52°C for 1min and 72°C for 1min, then a final extension at 72°C for 7min. The number of worms examined for COI included 18 from cattle in Japan, two from cattle in Iran (Halajian et al., 2010), 14 from Honshu sika deer, two from Kyushu/Honshu sika deer collected in Kochi Prefecture, two from Hokkaido sika deer, five from wild boars, four from Japanese macaques and two from different squirrel monkey colonies. Relationships of different haplotypes based on 369-bp long COI nucleotide sequences were visualized using an automated haplotype network layout and

visualization software, HapStar, download from http://fo.am/hapstar (Teacher & Griffiths, 2011).

Nucleotide sequences reported in the present study are available from the DDBJ/EMBL/GenBank database under the accession numbers AB495389-AB495402, AB513707-AB513730, AB645978-AB646106 and AB646110-AB646129.

1. 4 Results

1. 4. 1 Infection levels of *G. pulchrum* in different hosts

Prevalences of adult *G. pulchrum* in different hosts are summarized in the table 2. Of 638 oesophagi from cattle, 34 had *G. pulchrum* embedded in the mucosa. These infected cattle occurred in 12 of 27 prefectures examined. A single worm infection was detected in 17 oesophagi and two worms were recovered from five oesophagi. From the remaining 12 oesophagi, more than three worms were found and the maximum recovery was 109 (58 females and 51 males) from a steer of Japanese black breed. Up to 33 of 159 Honshu sika deer shot in Hyogo Prefecture were infected with *G. pulchrum*. The prevalence of *G. pulchrum* varied with locality, with all 20 deer shot on Awaji Island being infected, whereas in other localities in Hyogo Prefecture, infections were rare or absent. Five individual of the 33 infected deer were parasitized with 134-420 gullet worms and the mucosal surface of the oesophagi had grossly visible roughness through the complete length of the organ. The remaining deer were infected with 1-38 gullet worms and the oesophageal mucosa had no gross lesions except for the intramucosal localization of the parasites. Of 24 Honshu sika deer collected in Wakayama Prefecture, one harboured three gullet worms in its oesophagus. From two of 12 Kyushu/Honshu sika deer shot in Kochi Prefecture on Shikoku Island, one and two gullet worms were recovered. From two of four Hokkaido sika deer, one and three gullet worms were recovered. One gullet worm was collected from the Yaku sika deer. Of 132 wild boars shot

in Hyogo Prefecture, five individuals harboured 1-5 gullets worms. Of 10 Japanese macaques collected in Nara Prefecture, three animals were infected with one, two or five gullet worms. Microscopic examination of lingual scrapings revealed *Gongylonema* eggs in 14.2% (16/113) or 12.5% (12/96) of captive Bolivian squirrel monkey and in 45.7% (21/46) or 46.0% (23/50) of captive common squirrel monkeys in 2009 and 2010, respectively. From the oesophagus of the feral Reeves's muntjac, only a single juvenile worm was found.

1. 4. 2 Measurements of *G. pulchrum* from sika deer and cattle

Specimens collected from the oesophageal mucosa were characterized by verruciform protrusions on the cuticle near the anterior end. Asymmetrical caudal alae with 4-6 pairs each of pre- and post-anal papillae and distinct lengths of left and right spicules were present in the posterior end of male worms. Consistently, smaller sized worms were collected from sika deer compared to those from cattle. For example, worm length of male worms from sika deer, 21.2-26.9 mm (average 24.0 mm, n=6) versus those from cattle, 30.7-44.9 mm (average 36.7 mm, n=6), and worm length of female worms from sika deer, 35.7-51.6 mm (average 43.6 mm, n= 6) versus those from cattle, 67.9-85.1 mm (average 78.5 mm, n = 6). Several other measurements of the worms from sika deer and cattle were clearly different (Table 3, Fig.1) and were in accordance with previous reports for parasites collected from each host species (Kudo et al. 1992; Yokohata & Suzuki, 1993).

1. 4. 3 rDNA of *G. pulchrum* from different hosts

Approximately 6,100-bp-long sequences from 18S to 28S rDNA of *G. pulchrum* from different hosts and localities were compared to identify any unique nucleotide changes related to their origin. The rDNA sequences of *G. pulchrum* were generally well conserved, with few nucleotide changes detected

in 18S, 5.8S and 28S regardless of their origin. My analysis revealed that there were some unique nucleotide changes in the rDNA of *G. pulchrum* from cattle and from other origins, which were mainly observed in ITS1 and ITS2 (Table 4). The ITS1 and ITS2 regions of *G. pulchrum* had several repeats units, such as 'GA', 'A', 'CA' and 'GCT', in certain regions of their sequences. Sequence analyses of several clones of an amplicon of the ITS region for an individual worm showed that such repeat units were often repeated erratically. However, I did identify a few critical differences in the rDNA nucleotide sequences of gullet worms collected from cattle and sika deer. Specifically, cattle-derived worm genotype (termed 'cattle-type') variants were found in the worms from cattle, zoo-kept squirrel monkeys, the feral Reeves's muntjac and Hokkaido sika deer, whereas deer-derived worm genotype (termed 'deer-type') variants were found in the worms from the majority of sika deer, wild boars and Japanese macaques (Table 4).

1. 4. 4 COI of *G. pulchrum* from different hosts

A partial COI region, 369 bp length, of *G. pulchrum* mtDNA was successfully sequenced and compared (Table 5). The COI nucleotide sequences of *G. pulchrum* from different origins were divided into ten haplotypes (Table 5 and Fig. 3). Seven COI haplotype (#1-#6 and #8) were found in *G. pulchrum* from a majority of sika deer and wild boars examined in my study. An identical COI haplotype (#5) was shared by a single sika deer from Wakayama Prefecture and Japanese macaques from Nara Prefecture.

Three COI haplotypes (#7, #9 and #10) were differentiated in *G. pulchrum* collected from cattle. Haplotypes #9 and #10, differing at five base positions along their 369-bp length, were found in *G. pulchrum* collected from cattle reared in nine and six prefectures in Japan, respectively. Both COI haplotypes were distributed randomly in Japan, regardless of cattle breed or region. Except for one nucleotide, the 369-bp long COI sequence of *G. pulchrum* from cattle in

Iran was identical to that of haplotype #9 *G. pulchrum* collected from cattle in Japan. This haplotype (#7) was also found in *G. pulchrum* collected from Hokkaido sika deer and *G. pulchrum* eggs from two open-spaced colonies of Bolivian squirrel monkeys and common squirrel monkeys kept in zoological gardens. The amino acid sequences of *G. pulchrum* COI obtained in my study were identical except in two worms, one from a Kyushu/Honshu sika deer shot in Kochi Prefecture (at the 26th amino acid position, alanine was substituted by threonine) and the other from a wild boar shot in Hyogo Prefecture (the 57th amino position, arginine was substituted by glycine). The COI sequences of the gullet worm from the feral Reeves's muntjac and from the Yaku sika deer were not analysed due to unsuccessful PCR amplification of the gene or exhaustion of the samples.

1. 5 Discussion

Suzuki et al. (1992) reported different monthly prevalences of *G. pulchrum* infection in cattle in Hokkaido, Japan, ranging from 4.9 to 13.2%, and Kudo et al. (1992) reported different local prevalences of the infection in cattle from the northern part of Japan, ranging from 0 to 24.0%. The age of the examined cattle appears to affect the prevalence of *G. pulchrum* infection, with Kudo et al. (1992) reporting a prevalence of 3.3% (8/242) in cattle aged 2 years or younger, 17.0% (40/235) in 3-year-old cattle and 60.6% (57/94) in cattle aged 4 years or older. Accordingly, the distribution of *G. pulchrum* in cattle in Japan might not be limited to 12 of the 27 prefectures examined in my study, since only cattle younger than 3 years old were examined and the number of cattle examined from each prefecture was relatively low. However, my study demonstrates that *G. pulchrum* occurs widely in cattle in Japan, in addition to the northern part of Japan, such as Aomori and Hokkaido (Kudo et al., 1992, Suzuki et al., 1992). Prevalences of *G. pulchrum* in sika deer were variable in different localities, as suggested previously (Yokohata & Suzuki, 1993). Similarly, *G. pulchrum* was

found in only five of a total of 132 wild boars, with all five positive wild boars coming from one locality. High prevalences of *G. pulchrum* infection in pigs and wild boars have been reported in the USA (Zinter & Migaki, 1970; Smith et al., 1982) and Iran (Eslami & Farsad-Hamdl, 1992). Coombs & Springer (1974) reported a higher detection rate of the gullet worm in the tongue (6/9) than in the oesophagus (1/10). As I examined only the oesophagi of wild boars, then it is highly possible that the results presented here are an underestimation of the prevalence of *G. pulchrum* in wild boar populations in Japan. In contrast, squirrel monkeys in captivity were infected at a high prevalence, as reported previously (Sato et al., 2005), even though the lingual scraping technique detects the minimal number of infected monkeys when considering that parasitism of *G. pulchrum* in oesophagi could not be detected by this technique.

As mentioned previously, *G. pulchrum* is found in a variety of mammals, such as cattle, goats, camels, pigs, equines, cervids, rodents, bears and primates, that serves as the definitive hosts (Lichtenfels, 1971; Kirkpatrick et al., 1986; Anderson, 1992). Furthermore, successful experimental infections of *G. pulchrum* L3 of ruminant origin have been reported in rabbits, guinea-pigs and rat (Lucker, 1932; Alicata, 1935; Kudo et al., 2003, 2005). Based on the apparent low host specificity of *G. pulchrum*, it is reasonable to speculate that the transmission cycle of the gullet worm may be shared by domestic and wild mammals through accidental ingestion of infected dung beetles serving as an intermediate host. However, my present analyses demonstrate that the rDNA genotypes as well as the COI mtDNA haplotype of *G. pulchrum* prevalent in wild animals (including sika deer, wild boar and Japanese macaques) and domestic/captive mammals in Japan are clearly different (Tables 4 and 5; Fig. 2). My finding indicates that at present *G. pulchrum* has multiple origins in Japan and its transmission cycle is different between wild and domestic animals. Within the wildlife in Japan, sika deer and Japanese macaques are two known major hosts for the gullet worm (Uni et al., 1992; Yokohata & Suzuki, 1993). As

I examined *G. pulchrum* from a limited number of Japanese macaques at a single locality, the significance of a special COI haplotype (#5, see Fig. 3) of the gullet worms from macaques is unclear, i.e. it is not known whether this haplotype is prevalent in Japanese macaques or multiple haplotypes are shared by macaques and sika deer. Since the rDNA genotype and COI haplotypes of *G. pulchrum* from wild boar were essentially similar to those found in sika deer and the infection in wild boars was relatively low, these two wild mammals may share the same group of parasites. The rDNA genotype and COI haplotype of *G. pulchrum* in captive squirrel monkeys appear to be identical to not only those from cattle in Iran, but also those from Hokkaido sika deer. These alien monkeys and/or other captive animals in zoos might introduce the original infection when imported, maintaining its cycle within the zoo facility. Alternatively, squirrel monkeys and/or other animals may newly acquire the infection in the zoo facility.

The sika deer in Japan have been divided into two distinct lineages, the northern and southern groups, by nucleotide sequencing of the D-loop region of mtDNA (Tamate et al., 1998; Nagata et al., 1999; Nabata et al., 2004). The northern Japan group is distributed from Hokkaido Island to Hyogo Prefecture on Honshu Island, whereas the southern Japan group is in the westernmost part of Honshu Island, Kyushu Island and southward. On the fourth mainland of Japan, Shikoku Island, the distribution of the two sika deer groups is intermingled (Yamada et al., 2006). As far as my study's analyses are concerned, I could not find any *G. pulchrum* genotype or haplotype unique to each sika deer group. Interestingly, however, Hokkaido sika deer were found to be parasitized by *G. pulchrum* having an rDNA cattle-genotype and #7 COI haplotype. My finding appears to be contradictory to the view mentioned above. Hokkaido sika deer have history of drastic population decline/near extinction occurring more than 100 years ago as a result of the overexploitation and overharvesting of deer, beginning in 1869, and heavy snowfalls during the winters of 1879 and 1903

(Inukai, 1952; Nabata et al., 2004). This is reflected in a reduced genetic variation of current Hokkaido sika deer population (Nagata et al., 1999; Nabata et al., 2004). It could be speculated that this severe population decline history of host sika deer induced the extension of their original *G. pulchrum*, which was followed by recent re-acquisition of the parasite common in the Holstein-Friesian breed in dairy farms popular in Hokkaido. Indeed, Hokkaido sika deer frequently graze in fields for dairy cattle of the Holstein-Friesian breed. This hypothesis would also account for the scarcity of *G. pulchrum* parasitism in Hokkaido sika deer reported by Kitamura et al. (1997).

When accidental *G. pulchrum* infection is seen in humans, only a single worm or a fragment of the parasite is available for identification of the causative species (Crusz & Sivalingam, 1950; Eberhard & Bustillo, 1999; Wilson et al., 2001; Haruki et al., 2005). Additionally, a stunted development of the parasite in non-bovine host (Schwartz & Lucker, 1931; Kirkpatrick et al., 1986; Yokohata & Suzuki, 1993; Kudo et al., 2005; Sato et al., 2005) and its wide variation of morphological characters dependent on host (Lucker, 1932; Lichtenfels, 1971) makes its identification more difficult. Therefore, a molecular technique-based diagnosis of the species would be advantageous and reliable. This rDNA-based diagnosis of the species may facilitate the identification of *G. pulchrum* isolated from variety of animal sources, even under the limitation of sampling for morphological species diagnosis. Furthermore, as shown in my study, such molecular technique-based diagnosis could speculate the possible source of the gullet worm, maintained in either a domestic or sylvatic transmission cycle, at least in Japan.

Table 1. Primers used to amplify and sequence eight overlapping segments of rDNA of *Gongylonema pulchrum*.

Segment no.	Primer name[a] For amplifying	Primer name[a] For sequencing	Sequence	Position of 5'-end[b]
1	F: F-47		5'-CCCGATTGATTCTGTCGGC-3'	1 (18S)
	R: 18S-1192R/20		5'-CAGGTGAGTTTTCCCGTGTT-3'	1211 (18S)
		R: SSU9R	5'-AGCTGGAATTACCGCGGCTG-3'	588 (18S)
2	F: SSU22F		5'-TCCAAGGAAGGCAGCAGGC-3'	414 (18S)
	R: NSR1787/18		5'-CGACGGGCGGTGTGTACA-3'	1643 (18S)
		F: SSU24F	5'-AGAGGTGAAATTCGTGGACC-3'	899 (18S)
		R: 18S-1192R/20	(see above)	1211 (18S)
3	F: SSU23F		5'-ATTCCGATAACGAGCGAGACT-3'	1312 (18S)
	R:NC13(ITS1)/R		5'-GCTGCGTTCTTCATCGAT-3'	43 (5.8S)
4	F:NC5(ITS1)/F		5'-GTAGGTGAACCTGCGGAAGGATCATT-3'	1778 (18S)
	R: 28S-408R/20		5'-TTCACGCCCTCTTGAACTCT-3'	413 (28S)
5	F: 28S/F		5'-AGCGGAGGAAAAGAAACTAA-3'	50 (28S)
	R: 28S-1270R/22		5'-CAGCTATCCTGAGGGAAACTTC-3'	1240 (28S)
6	F:28S-839F/20		5'-TATCCGACCCGTCTTGAAAC-3'	855 (28S)
	R: 28S-1670R/20		5'-TACCACCAAGATCTGCACCA-3'	1809 (28S)
7	F: 28S-1449F/27		5'-GGAAGTCGGAATCCGCTAAGGAGTGTG-3'	1522 (28S)
	R: NLR2781/19		5'-CCGCCCCAGYCAAACTCCC-3'	3059 (28S)
		F: NLF1999/19	5'-CCGCAKCAGGTCTCCAAG-3'	1719 (28S)
		R: 28S-2132R/20	5'-AGAGGGCTGTTCACCTTGGAG-3'	2271 (28S)
		R: NLR2362/20	5'-ACATTCAGAGCACTGGGCAG-3'	2633 (28S)
8	F: NLF2551/21		5'-GGGAAAGAAGACCCTGTTGAG-3'	2816 (28S)
	R: NLR3284/21		5'-TTCTGACTTAGAGGCGTTCAG-3'	3565 (28S)

[a]Universal primers used for amplifying and sequencing a certain segment are shown as 'for amplifying' and those only for sequencing as 'for sequencing'. F: forward and R: reverse.

[b]Relative position of the 5'-end of each primer in *G. pulchrum* rDNA sequence (DDBJ/EMBL/GenBank accession no. AB495389). The 5' -end of F-47 primer is considered as the beginning of 18S rDNA here.

Table 2. Recovery of *Gongylonema pulchrum* from the oesophagus of a variety of hosts in Japan.

	No. of hosts examined	Prevalence (%)	Worm range	Deposited specimens[a]
Cattle				
27 Prefectures	638	5.3	1-109	As3608-As3636
Honshu sika deer				
Hyogo Prefecture	159	20.8	1- 420	As3501-As3527, As3647-As3658
Wakayama Prefecture	24	4.2	3	As3659
Kyushu/Honshu sika deer				
Shikoku Island	12	16.7	1 - 2	As3662-As3638, As3661
Hokkaido sika deer				
Hokkaido Island	4	50	1 - 3	As3662-As3663
Yaku sika deer				
Kagoshima Prefecture	1	100	1	As3660
Wild boar				
Hyogo Prefecture	132	3.8	1 - 5	As3664-As3668
Japanese macaque				
Nara Prefecture	10	30	1 - 5	As3669-As3671
Feral Reeve's muntjac				
Kanagawa Prefecture	1	100	1	-

[a]Specimen ID numbers at the National Museum of Nature and Science, Tokyo.

Table 3. Comparison of measurements (mm) of *Gongylonema pulchrum* collected from Cattle and Deer[a]

Host	Cattle	Deer
Locality	Japan	Japan
Reference	Sato (2009)	Sato (2009)
Male worm		
Number of worms examined	(n=6)	(n=6)
Body length	30.7-44.9 (36.8±5.8)	21.2-26.9 (24.0±2.8)
Max. body width	0.26-0.30 (0.28±0.02)	0.146-0.192 (0.174±0.016)
Pharynx	0.048-0.056 (0.052±0.004)	0.050-0.055 (0.052±0.003)
Oesophagus	4.50-6.64 (5.58±0.43)	5.01-6.37 (5.57±0.47)
Muscular portion	0.29-0.51 (0.43±0.08)	0.492-0.542 (0.518±0.018)
Glandular portion	4.02-6.21 (5.05±0.89)	4.47-5.85 (5.05±0.48)
Cervical lateral papillae[b]	0.112-0.176 (0.143±0.024)	0.122-0.149 (0.133±0.015)
Nerve ring[b]	0.220-0.288 (0.259±0.031)	0.282-0.382 (0.318±0.038
Excretory pore[b]	0.352-0.496 (0.432±0.065)	0.409-0.492 (0.458±0.039)
Left spicule	14.19-20.36 (17.60±2.20)	6.28-7.72 (6.78±0.57)
Relative length of left spicule/body length	39.5-64.1 (48.9)%	24.6-32.3 (28.4)%
Right spicule	0.096-0.160 (0.132±0.024)	0.097-0.163 (0.115±0.57)
Gubernaculum	0.072-0.125 (0.099±0.015)	0.077-0.089 (0.083±0.005)
Number of precloacal papillae	4 - 5	4 - 5
Number of postcloacal papillae	5 - 6	5
Tail length	0.240-0.300 (0.270±0.028)	0.282-0.321 (0.300±0.018)
Female worm		
Number of worms examined	(n=6)	(n=6)
Body length	67.90-85.07 (78.47±7.20)	35.7-51.6 (43.6±5.9)
Max. body width	0.30-0.34 (0.33±0.02)	0.216-0.280 (0.240±0.023)
Pharynx	0.040-0.072 (0.057±0.012)	0.044-0.061 (0.056±0.006)
Oesophagus	6.75-7.41 (7.12±0.24)	6.86-7.72 (7.28±0.38)
Muscular portion	0.50-0.86 (0.68±0.14)	0.554-0.765 (0.672±0.097)
Glandular portion	6.16-6.64 (6.43±0.19)	6.30-6.96 (6.60±0.29)
Cervical lateral papillae	0.124-0.160 (0.145±0.015)	0.122-0.166 (0.141±0.015)
Nerve ring	0.480-0.640 (0.568±0.067)	0.327-0.421 (0.369±0.038
Excretory pore	0.480-0.640 (0.568±0.067)	0.598-0.837 (0.734±0.100)
Vulva[c]	2.72-4.08 (3.15±0.50)	1.88-2.99 (2.38±0.36)
Tail length	0.260-0.340 (0.307±0.033)	0.266-0.316 (0.281±0.022)
Egg	0.056-0.060 (0.059±0.002) x0.032-0.036 (0.035±0.002)	0.056-0.062 (0.059±0.002) x0.032-0.038 (0.035±0.001)

[a]Measurements are expressed as range (mean±SD)
[b]Distance from the anterior end.
[c]Distance from the posterior end.

Table 4. Nucleotide changes observed in rDNA of *Gongylonema pulchrum* of different origins.

Host	Locality	DDBJ/EMBL/GenBank accession no.	No. of worms examined	Nucleotide position where any changes in rDNA are observed[a] 18S 13	18S 829	ITS1 31	ITS1 59/60	ITS1 122-132	ITS1 285	ITS1 302-	ITS2 66-67	ITS2 68-	ITS2 85	28S 542	28S 897	Genotype
Deer																
	Awaji Island, Hyogo Prefecture, Japan	AB495394-AB495396	1	A	A	C	-	(GA) x 5	C	(A) x 12-19 (CA) x 5 (TA) x 1 (CA) x 4	TT	(GCT) x 8	-	C	T	Deer-type
	Shiso, Hyogo Prefecture, Japan	AB495397-AB495400	1	A	A	C	-	(GA) x 5	C	(A) x 15-18 (CA) x 4-5 (TA) x 1-2 (CA) x 4	TT	(GCT) x 8	-	C	T	Deer-type
		AB646036-AB646044	2			C	-	(GA) x 5	C	(A) x 15-18 (CA) x 2-6 (TA) x 1 (CA) x 4-5	TT	(GCT) x 6-8	-			Deer-type
	Tamba, Hyogo Prefecture, Japan	AB645978-AB645982 AB645994-AB646006	4			C	-	(GA) x 5	C	(A) x 13-20 (CA) x 2-6 (TA) x 1 (CA) x 4	TT	(GCT) x 6-17	-	C	T	Deer-type
	Asago, Hyogo Prefecture, Japan	AB646007-AB646035	4			C	-	(GA) x 5	C	(A) x 13-20 (CA) x 2-6 (TA) x 1 (CA) x 4	TT	(GCT) x 6-11	-	C	T	Deer-type
	Tanabe, Wakayama Prefecture, Japan	AB645983-AB645993	3			C	-	(GA) x 5	C	(A) x 17-19 (CA) x 2-6 (TA) x 1 (CA) x 4-5	TT	(GCT) x 6-8	-			Deer-type
	Kami, Kochi Prefecture, Japan	AB646045-AB646049	1			C	CAA(4/5)	(GA) x 5	C	(A) x 16-22 (CA) x 5-6 (TA) x 1 (CA) x 4	TT	(GCT) x 9-17	-			Deer-type
	Yaku Island, Kagoshima Prefecture, Japan	AB646050-AB646054	1	G	A	C	-	(GA) x 5	C	(A) x 13-18 (CA) x 5-6 (TA) x 1 (CA) x 4	TT	(GCT) x 5	T-	(GCT) x 2-11	A	C	Deer-type
	Yaku Island, Kagoshima Prefecture, Japan	AB646055-AB646068	1	G	A	C	-	(GA) x 5	C	(A) x 14-20 (CA) x 4-7 (TA) x 1 (CA) x 4	TT	(GCT) x 5-8	-	-	A	C	Deer-type
	Naka-satsunai, Hokkaido Prefecture, Japan	AB646059-AB646063	2	G	A	C	-	(GA) x 5	C	(A) x 12-13 (CA) x 3 (TA) x 0-1 (CA) x 5-8	CT	(GCT) x 2	GCG	(GCT) x 2	A	C	Cattle-type
Wild boar																
	Tanba, Hyogo Prefecture, Japan	AB646064-AB646094	7	G	A	C	-	(GA) x 5	C	(A) x 10-20 (CA) x 2-6 (TA) x 1 (CA) x 4	TT	(GCT) x 6-16	-	-	C	C	Deer-type
Japanese macaque																
	Kamikitayama, Nara Prefecture, Japan	AB646095-AB646105	2			C	-	(GA) x 5	C	(A) x 15-19 (CA) x 4-5 (TA) x 1 (CA) x 5	TT	(GCT) x 8	-	-			Deer-type
										(A) x 16-19 (CA) x 4-6 (TA) x 1 (CA) x 4-5							
Feral Reeves's muntjac																
	Izu-ohshima, Kanagawa Prefecture, Japan	AB646106	1	G	A	C	-	(GA) x 4	C	(A) x 14 (CA) x 13 - -	CT	(GCT) x 2	GCG	(GCT) x 2			Cattle-type
Cattle																
	Okayama or Tottori Prefecture, Japan	AB513707-AB513710	1	G	A	C	-	(GA) x 4	C	(A) x 14-15 (CA) x 3 (TA) x 1 (CA) x 7-9	CT	(GCT) x 2	GCG	(GCT) x 2	A	T	Cattle-type
	Oita Prefecture, Japan	AB513711-AB513718	1	G	A	C	-	(GA) x 4	C	(A) x 13-17 (CA) x 3-4 (TA) x 1 (CA) x 4-10	CT	(GCT) x 2	GCG	(GCT) x 2	A	T	Cattle-type
	Kagoshima Prefecture, Japan	AB513719-AB513723	1	A	A	C	-	(GA) x 4	C	(A) x 12-14 (CA) x 3-4 (TA) x 1 (CA) x 6-8	CT	(GCT) x 2	GCG	(GCT) x 2	A	T	Cattle-type
	Iran	AB495389-AB495393	1	A	A	C	-	(GA) x 4	C	(A) x 12-14 (CA) x 3 (TA) x 1 (CA) x 3-8	CT	(GCT) x 2	GCG	(GCT) x 2	A	T	Cattle-type
Squirrel monkey																
	Captive in Japan	AB495401-AB495402	2	G	W	C	-	(GA) x 4	C	(A) x 12-13 (CA) x 3 (TA) x 1 (CA) x 7-8	CT	(GCT) x 2	GCG	(GCT) x 2	A	T	Cattle-type

[a]Nucleotide positions is expressed relative to the rDNA sequence of *G. pulchrum* collected from a deeer caught on Awaji Island (DDBJ/EMBL/GenBank accession no. AB495394). Gaps are indicated by '-' and blanks indicate no data.

Table 5. Nucleotide substitutions observed in COI mtDNA of *Gongylonema pulchrum* of different origins.

Host	Locality	No. of worms	DDBJ/EMBL/GenBank accession no.	Nucleotide position where any changes in COI mtDNA are observed[a]																	Haplotype
				33	46	69	76	84	114	117	132	165	169	207	216	234	327	337	351	354	
Deer																					
	Hyogo Prefecture, Japan	7	AB513724, AB646110, AB646111	A	T	T	G	G	A	T	T	T	T	C	G	G	T	C	G	A	**1**
	Hyogo Prefecture, Japan	1	AB646112	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	**2**
	Hyogo Prefecture, Japan	2	AB646113 -AB646114	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	**3**
	Hyogo Prefecture, Japan	1	AB646115	.	.	.	.	.	G	.	.	.	.	T	.	.	.	.	.	.	**4**
	Wakayama Prefecture, Japan	3	AB646116 -AB646118	G	.	C	.	.		.	.	.	.	.	.	.	.	T	A	.	**5**
	Kochi Prefecture, Japan	1	AB513725	.	.	.	A	.	.	.	.	.	.	.	.	.	.	T	.	.	**6**
	Kochi Prefecture, Japan	1	AB646119	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	**3**
	Hokkaido Prefecture, Japan	2	AB646120	.	C	.	.	.	.	C	C	.	.	T	A	A	.	T	.	.	**7**
Wild boar																					
	Hyogo Prefecture, Japan	2	AB646121 -AB646122	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	**3**
	Hyogo Prefecture, Japan	1	AB646123	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	**1**
	Hyogo Prefecture, Japan	1	AB646124	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	**4**
	Hyogo Prefecture, Japan	1	AB646125	.	.	C	.	.	.	.	.	.	.	.	.	.	.	T	A	.	**8**
Japanese macaque																					
	Nara Prefecture, Japan	4	AB646126 -AB646129	G	.	C	.	.	G	.	.	.	.	.	.	.	.	T	A	.	**5**
Cattle																					
	Japan	11	AB513727	.	C	.	.	.	.	.	C	.	.	T	A	A	.	T	.	.	**9**
	Japan	7	AB513728	.	C	.	.	.	.	C	C	C	.	T	.	A	C	T	.	G	**10**
	Iran	2	AB513726	.	C	.	.	.	.	C	C	.	.	T	A	A	.	T	.	.	**7**
Squirrel monkey																					
	S. boliviensis captive in Japan	1	AB513729	.	C	.	.	.	.	C	C	.	.	T	A	A	.	T	.	.	**7**
	S. boliviensis captive in Japan	1	AB513730	.	C	.	.	.	.	C	C	.	.	T	A	A	.	T	.	.	**7**

[a]Nucleotide position is expressed relative to the 5' -end of COI sequence of *G. pulchrum* collected from a deer caught on Awaji Island, Hyogo Prefecture, Japan (DDBJ/EMBL/GenBank accession no. AB513724). Dots denote an identical base to that of *G. pulchrum* collected from the deer in Hyogo.

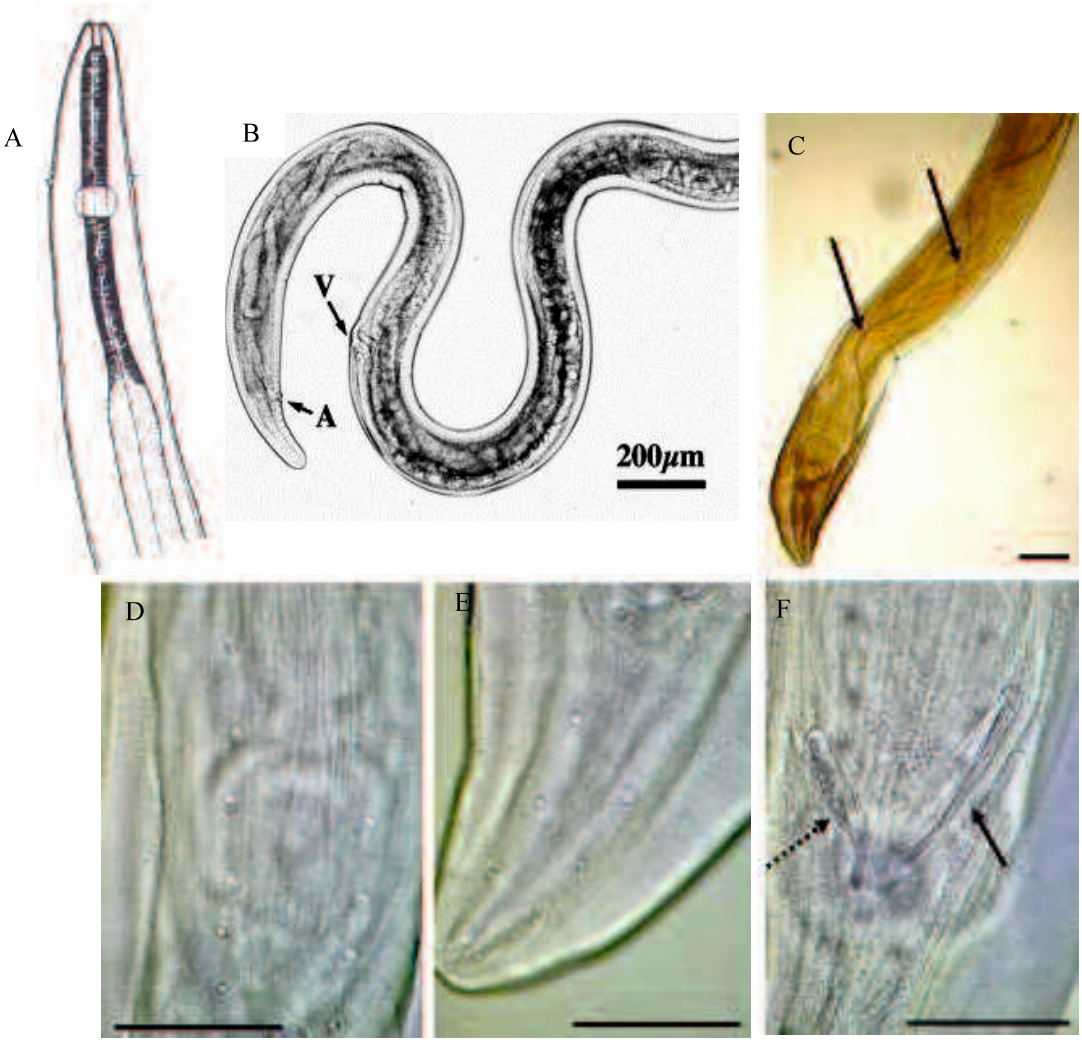

Figure 2. ***Gongylonema pulchrum***.

A, anterior end of male showing cervical papillae, nerve ring and oesophagus. **B**, posterior end of female showing vulva (V) and anus (A). **C**, posterior end and left spicule (arrows). **D**, preclocal papillae. **E**, post cloacal papillae. **F**, posterior end: right spicule (arrow) and gubernaculum (dotted arrow) (**A**: Ashour & Lewis, 1986; **B**: Sato et al., 2005; **C-F**: Pesson et al., 2013)

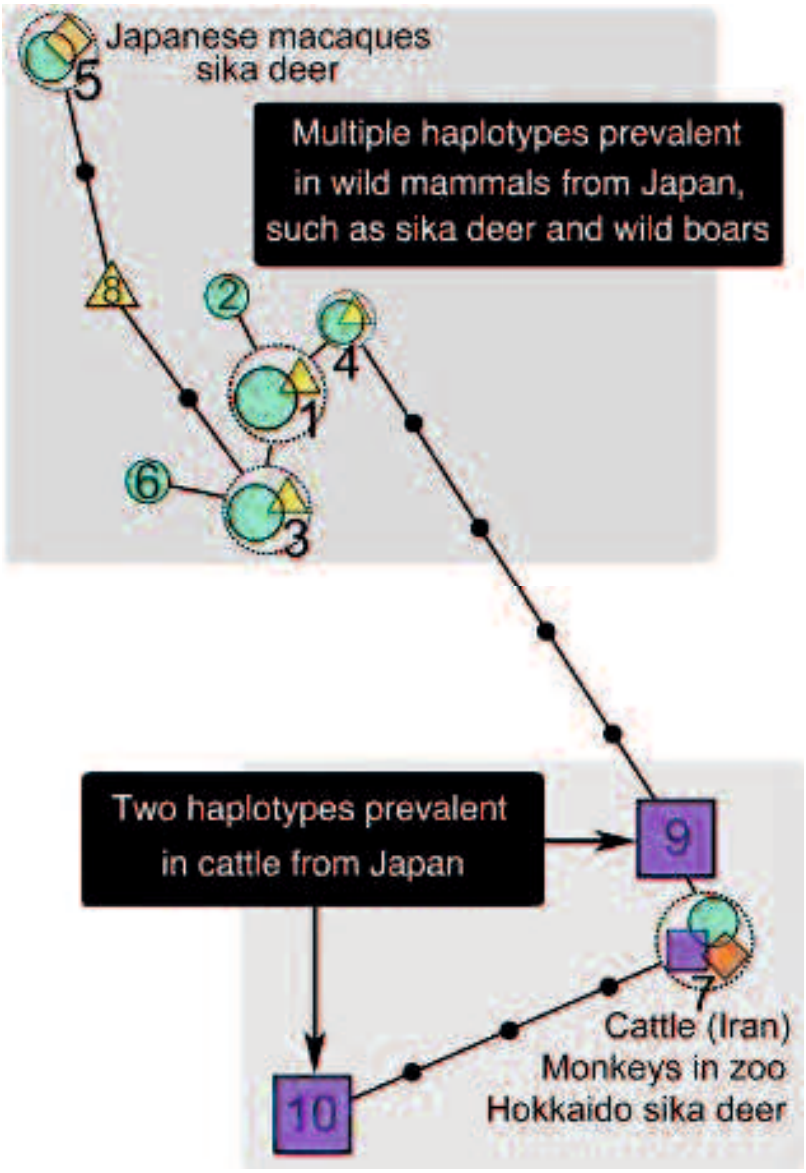

Figure 3. Relationship of COI haplotypes (#1-#10, see table 5) of *Gongylonema pulchrum*

The parasites were found in sika deer (open circle), wild boars (open triangle), Japanese macaques (open diamond), cattle (open square) and captive squirrel monkeys (open diamond). Sizes of symbols are arbitrary, just showing higher frequency in larger symbols. Symbols within a dotted circle denote the same haplotype.

CHAPTER II

A distinct genetic population of *Gongylonema pulchrum* from water buffaloes in Nepal

The work described in the chapter has been published as follows;

Makouloutou, P., Rana, H. B., Adhikari, B., Devkota, B., Dhakal, I. P. and Sato, H. (2013) : A distinct genetic population of *Gongylonema pulchrum* from water buffaloes in Nepal. *J. Parasitol.* 99: 669-676.

2. 1 Abstract

Whole-length esophagi of 111 Murrah cross water buffaloes (*Bubalus bubalis*) were collected in the Kathmandu and Chitwan districts of Nepal from December 2009 to February 2010. Gullet worms showing a typical epithelium-dwelling character were detected in 13 of 53 (24.5%) buffaloes in Kathmandu and in 5 of 58 (8.6%) buffaloes in Chitwan. The worms' morphology and measurements were identical to those of *Gongylonema pulchrum* Molin, 1857, except for the length of the left spicules relative to the body length. Scanning electron microscopy did not detect any further morphological differences regarding the collected specimen from Nepal compared with *G. pulchrum*. The ribosomal RNA gene (rDNA), including internal transcribed spacer (ITS) 1 and 2, and a partial region of the cytochrome *c* oxidase subunit I (COI) of mitochondrial DNA of the worms were characterized and compared with those of *G. pulchrum* collected from cattle, deer, wild boars, and monkeys in Japan and from cattle in Iran. The 18S, 5.8S, and 28S rDNA nucleotide sequences of the buffalo-collected worms had 99.8% (1,779/1,782), 100% (158/158), and 98.3–98.8% (3,494–3,507/3,551) identities, respectively, with those of *G. pulchrum* from the other host mammals. The ITS regions exhibited higher variations between the buffalo-collected worms and *G. pulchrum* from the other host mammals (85–88% identity for ITS1 and 56–80% identity for ITS2). The COI also showed lower identities (89.2–90.2%), although only a single amino acid substitution was noted compared with the majority of *G. pulchrum* samples collected in Japan. Based on these molecular genetic characters in the rDNA and COI mitochondrial DNA, together with a shorter left spicule length relative to body length, the gullet worms isolated from buffaloes in Nepal might belong to a distinct local or buffalo-preferring population of *G. pulchrum*, although its geographical distribution on the continent and host specificity remain to be clarified.

2. 2 Introduction

The gullet worm, *Gongylonema pulchrum* Molin, 1857, is a thread-like spirurid nematode localized in the oesophageal epithelium of mammals, particularly ruminants, worldwide. Various mammals, such as cattle, sheep, goats, camels, buffaloes, cervids, equines, pigs, wild boars, rodents, bears, and primates, have been recorded as the definitive host, and beetles serve as the intermediate host (Alicata, 1935; Zinter and Migaki, 1970; Lichtenfels, 1971; Kirkpatrick et al., 1986; Anderson, 1992). In the previous chapter, I demonstrated that *G. pulchrum* have several lineages transmitted independently in domestic mammals (cattle and zoo-kept monkeys) and wild mammals (deer, wild boar, and macaques). Despite independent transmission cycles in nature of several rDNA genotypes and COI haplotypes of *G. pulchrum* in Japan, I did not identify a distinct population within the examined gullet worms based on morphological and genetic perspectives, and their phenotypic and genetic characters were essentially well conserved.

In this chapter, I examined the morphology and genetic background of gullet worms collected from water buffaloes (*Bubalus bubalis*) in Nepal as part of a phylogeographical study of the worldwide distribution of *G. pulchrum*.

2. 3 Material and methods

2. 3. 1 Animals examined and parasite collection

Whole-length esophagi of 111 Murrah cross water buffaloes were collected at the site of slaughter or butcher shops in Kathmandu valley and Chitwan district of Nepal from December 2009 to February 2010. Materials were transported to the laboratory without freezing. In the laboratory of Institute of Agriculture and Animal Science, Tribhuvan University, in Chitwan, Nepal, or in the institute of Animal Care in Kathmandu, Nepal, each esophagus was cut open longitudinally and the mucosal surface was rigorously checked with the naked

eye within 6 hr after collection. Individual worms were carefully removed from the esophageal epithelium using fine forceps, killed in hot water at 70° C, and fixed in 70% alcohol. For morphological and genetic analyses, the fixed specimens were sent to the Laboratory of Parasitology, Yamaguchi University, Yamaguchi, Japan.

2. 3. 2 Morphological examination of the gullet worm

The specimens fixed in 70% alcohol were cleared by the addition of a few drops of glycerol. Eight representative adult nematodes of each sex were observed under a light microscope and drawn with the aid of a camera lucida. Measurements were performed on these drawn figures using a digital curvimeter type S (Uchida Yoko, Tokyo, Japan) when necessary. Regression analyses between two morphometric variables were conducted using StatView®-J 5.0 (SAS Institute Japan, Tokyo, Japan). Collected specimens were deposited in the National Museum of Nature and Science, Tokyo, under specimen numbers NSMT-As3672-As3691.

Morphological observation using scanning electron microscopy (SEM) was conducted to visualize the en face view of the parasite and other cuticular surface characters. In brief, the preserved sample was washed three times in 0.2 M Na_2HPO_4-NaH_2PO_4–buffered solution, pH 7.8 (PB) and immersed in 2.5% glutaraldehyde in PB overnight. After three washes in PB, the sample was postfixed in 1% (w/v) osmium tetroxide in PB for 1 hr. After washing three times in PB, the sample was dehydrated through a graded alcohol series, immersed in warmed *t*-butyl-alcohol, and then cooled at 4° C. The sample was subsequently freeze-dried with a freeze-drying apparatus (model JFD-300; JEOL, Tokyo, Japan) and then mounted onto a stub and sputter-coated with gold-palladium at 200 Å (model JFC- 1500 ion sputtering device, JEOL). Samples were examined using a scanning electron microscope (model JSM-

6100; JEOL) at an accelerating voltage of 15 kV. *G. pulchrum* specimens collected from cattle in Japan and preserved in 10% neutral-buffered formalin were observed in a similar manner (Sato, 2009).

2. 3. 3 DNA extraction, polymerase chain reaction (PCR) and sequencing

Parasite DNA was extracted separately from three worms using an Illustra^{TM} tissue and cells genomicPrep Mini Spin Kit (GE Healthcare UK, Little Chalfont, Buckinghamshire, U.K.) according to the manufacturer's instructions. PCR amplification of overlapping rDNA fragments, purification of PCR products and sequencing were performed as describe previously in chapter I. For rDNA segments containing ITS 1 and 2, the amplicon was cloned into the plasmid vector pTA2 (Target Clone^{TM}; TOYOBO, Osaka, Japan) as described in chapter I. Similarly, the COI region of mitochondrial DNA was amplified as described previously in chapter I. The nucleotide sequences reported in the present study are available from the DDBJ/EMBL/GenBank databases under the accessions AB646107-AB646109 (rDNA) and AB646130-AB646131 (COI).

2. 4 Results

2. 4. 1 Occurrence of the gullet worm in buffaloes

The gullet worm was detected in 13 of 53 (24.5%) buffaloes (13/48 females and 0/5 males) in Kathmandu and in 5 of 58 (8.6%) buffaloes (2/38 females and 3/20 males) in Chitwan. The worms were fully embedded in the epithelium of the esophageal mucosa, causing no additional pathological changes to their surroundings (Fig. 4). The infection intensity ranged from 1 to 4 worms per host.

2. 4. 2 Measurements of the gullet worm from buffaloes

Collected nematodes from the esophageal mucosa were characterized by verruciform protrusions of the cuticle in the anterior portion, cervical alea on

both sides, and asymmetric caudal alae with 5 or 6 pairs each of pre- and post-anal papillae and distinct lengths of left and right spicules in the posterior portion of males. Body size and other morphometrics, excluding the length of the left spicule, appeared to be consistent with those of worms collected from cattle with different geographical origins, whereas the body size of worms collected from sika deeer in Japan was markedly smaller (Table 6; Yokohata & Suzuki, 1993). Irrespective of the different body sizes of worms from different host origins, the length of the right spicule was fairly consistent. However, the length of the left spicule was distinct in the hosts, i.e., markedly shorter in worms collected from buffaloes and deer, worms collected from these two hosts had comparable left spicule lengths (Table 6). Figure 5 shows the relationship between body length and left spicule length of each worm from the different host origins. With the exception of worms collected from buffaloes, the parasites collected from cattle and deer showed a statistically significant correlation between body length and left spicule length, and the following regression line was applicable: $Y = -43.602 + 16.581 \times \ln(X)$ ($P < 0.05$).

The *en face* views obtained by SEM of gullet worms from different host origins were identical, together with other body surface structures (Fig. 6).

2. 4. 3 rDNA of the gullet worms from different hosts

The partial rDNA sequence of the gullet worms newly isolated from water buffaloes in Nepal contained 1,782-bp 18S rDNA (excluding a primer flanking sequence of 19-bp length at the 5' terminus), 406/408-bp ITS1, 158-bp 5.8S rDNA, 236-bp ITS2, and 3,523/3,536-bp 28S rDNA sequences. Compared with the 18S, 5.8S, and 28S rDNA of *G. pulchrum* obtained from sika deer, wild boars, and captive squirrel monkeys in Japan as well as cattle in Japan and Iran, the gullet worm from buffaloes in Nepal had 3 (99.83% identity), 0 (100% identity), and at least 15 base substitutions along with base deletions and

insertions (98.34-98.76% identity) in the respective rDNA regions (Table 7).

Gullet worms collected from a variety of mammals in Japan had 378- to 397-bp ITS1, whereas those from buffaloes had 406/408-bp ITS1. As described previously in chapter I, the ITS1 region of *G. pulchrum* rDNA has several repeats of a few nucleotide units such as A, CA, and TA, with the numbers of individual repeats resulting in different sequence lengths and variations. This variation was even observed within the same worm and the identity of ITS1 sequence of Japanese isolates ranged from 98 to 100%. The worm collected from buffaloes in Nepal exhibited the basic sequence design of the ITS1 region of Japanese isolates of *G. pulchrum*, but with different numbers of individual repeats, many nucleotide substitutions, and several indels. Major nucleotide variations found in the sequence of ITS1 and ITS2 regions are shown in Table 8. The identity of the ITS1 rDNA region between the gullet worms collected in Nepal and Japan ranged from 85 to 88%. The ITS2 rDNA region of Japanese gullet worms had only a single repeat nucleotide unit of GCT and the length ranged from 225 to 268 bp, with few substitutions in other parts. Intra-individual variation of the GCT repeat was observed in Japanese gullet worms. The gullet worm isolated in Nepal had no such nucleotide repeat, and base changes including substitutions, insertions, and deletions occurred frequently compared with Japanese isolates (Table 8). Consequently, the identity of the ITS2 rDNA region between the gullet worms collected in Nepal and Japan ranged from 56 to 80%.

2. 4. 4 COI of the gullet worms from different hosts

A partial COI region, 369 bp, of the gullet worm mitochondrial DNA was successfully sequenced and compared with those of *G. pulchrum* collected from cattle, deer, wild boars, and monkey in Japan (see chapter I). A maximum of 12 base substitutions was observed between *G. pulchrum* isolates collected in Japan

(e.g. AB646126 vs AB513728), whereas 36-40 base substitutions in the COI region were observed between the gullet worms collected in Nepal and Japan. Despite such a high frequency of nucleotide substitutions, only a single amino acid substitution was detected between the gullet worm collected from buffaloes and the majority of the gullet worms collected in Japan; isoleucine was substituted by valine at the 112th amino acid position. Namely, the majority of nucleotide variations occurred at the third nucleotide position of the codon, and different isolates had an almost identical acid sequence. Among 49 worms collected from a variety of mammals, only two worms collected from a deer (AB513725) and wild boar (AB646122) showed a single amino acid substitution at a different position compared with the majority of *G. pulchrum* isolates.

2. 5 Discussion

G. pulchrum is found in a variety of mammals, including humans (Alicata, 1935; Zinter & Migaki, 1970; Lichtenfels, 1971; Kirkpatrick et al., 1986; Anderson, 1992; Haruki et al., 2005; Sato et al., 2005). Half a century ago, Yamaguti (1961) listed 34 recorded *Gongylonema* spp. parasitizing mammals. Multiple species, at least 10 in number, on the list subsequently became junior synonyms of *G. pulchrum*. Sato (2009) listed 23 *Gongylonema* spp. from mammalian host and 9 *Gongylonema* spp. from avian hosts as nominal species of the genus, mainly according to Skrjabin et al. (1967). The latest taxonomy recognizes three *Gongylonema* spp. dwelling in the epithelium of the upper digestive tract of ruminants: *G. pulchrum* Molin, 1857 in the tongue, mouth mucosa, and esophagus of various mammals worldwide; *G. verrucosum* Giles, 1892 in the rumen, reticulum, and omasum of various ruminants from India, Africa, and North America; and *G. monnigi* Baylis, 1926 in the rumen of sheep from Africa. The two latter species are morphologically differentiated from *G. pulchrum* based on the following points: (1) unilateral (left side) versus bilateral

cervical alae, (2) unilateral versus bilateral distribution of cuticular verruciform protrusions in the anterior portion, and (3) different range of the right spicule length according to the species (0.26-0.32 mm for *G. verrucosum*, 0.213-0.250 mm for *G. monnigi*, and 0.084-0.180 mm for *G. pulchrum*) and different gubernaculum size ranges. With reference to a long history of taxonomic arguments and current taxonomic criteria based on morphology, the gullet worm isolated from buffaloes in the present study might be classified tentatively as *G. pulchrum*.

A wide spectrum of morphometric values of *G. pulchrum* growing in different host species is well recognized. Lichtenfels (1971) concluded that the most useful morphometric characters, i.e., those showing relatively little variation, for distinguishing *G. pulchrum* from other species of the genus are the left spicule length and body length. As evident from Table 6 and Figure 5, the sizes of body and other structures, excluding the left spicule, were comparable between worms collected from buffaloes and cattle, whereas the left spicule length of worms from buffaloes was comparable to the distinctly smaller worms collected from deer. There are only a few reports on the prevalences of *G. pulchrum* in buffaloes (Tüsdil, 1939; Islam et al., 1992); however, no morphometrics of the worm were provided by these studies. Therefore, my observation that *G. pulchrum* in buffaloes from Nepal has a markedly shorter left spicule relative to its body size requires confirmation in the same host species from different areas or in different host species from the same area. Nepal is primarily Hindu, and a survey on gullet worms in cattle could prove difficult to conduct. Nevertheless, despite intensive SEM studies of the en face view of the worms and body surface structure, I was unable to identify further morphological characters unique to the gullet worms from buffaloes.

In the chapter one, I characterized the rDNA genotypes and COI mitochondrial DNA haplotypes of *G. pulchrum* prevalent in cattle and wild

animals such sika deer, wild boar, and Japanese macaques. The rDNA and COI nucleotide sequences of the gullet worms isolated from buffaloes in Nepal were close to, but distinct from, *G. pulchrum* analyzed previously. As evident from nucleotide sequences of the ITS region of rDNA, the observed variations were remarkably high. Coupled with the rather shorter spicule, the gullet worms found in buffaloes from Nepal might be considered as a distinct population of *G. pulchrum*, or a cryptic *Gongylonema* species. Because host specificity and geographical distribution of the special *G. pulchrum* population remain to be clarified in future studies, I'm currently not in a position to term this population local or host-specific, or a possibility of a cryptic species. Nevertheless, I should be aware that so-called "*G. pulchrum*" that exhibits a cosmopolitan and inhabits a wide range of mammalian hosts is not a homologous species, but rather is composed of multiple populations or cryptic species showing minor but distinct morphological variation(s) as well as genetic divergences.

Table 6. Comparison of measurements (mm) of *Gongylonema pulchrum* collected from ruminants[a]

Host	Buffalo	Cattle	Cattle	Cattle	Deer
Locality	Nepal	Japan	Iran	Japan	Japan
Reference	The present study	Sato (2009)	Halaijan et al. (2010)	Kudo et al. (1992)	Sato (2009)
Male worm					
Number of worms examined	(n= 8)	(n=6)	(n=6)	(n=60)	(n=6)
Body length	37.0-46.6 (41.1±3.5)	30.7-44.9 (36.8±5.8)	36.7-48.6 (41.9±4.8)	24.1-52.4	21.2-26.9 (24.0±2.8)
Max. body width	0.20-0.28 (0.23±0.03)	0.26-0.30 (0.28±0.02)	0.22-0.26 (0.24±0.02)	0.208-0.296	0.146-0.192 (0.174±0.016)
Pharynx	0.039-0.050 (0.044±0.004)	0.048-0.056 (0.052±0.004)	0.045-0.056 (0.048±0.005)	0.042-0.066	0.050-0.055 (0.052±0.003)
Oesophagus	4.73-7.80 (6.22±1.08)	4.50-6.64 (5.58±0.43)	4.96-6.08 (5.58±0.43)	4.89-7.44	5.01-6.37 (5.57±0.47)
Muscular portion	0.45-0.63 (0.56±0.06)	0.29-0.51 (0.43±0.08)	0.55-0.65 (0.61±0.04)	0.478-0.697	0.492-0.542 (0.518±0.018)
Glandular portion	4.28-6.62 (5.43±0.87)	4.02-6.21 (5.05±0.89)	4.40-5.47 (4.97±0.43)	4.35-6.78	4.47-5.85 (5.05±0.48)
Cervical lateral papillae[b]	0.122-0.169 (0.144±0.019)	0.112-0.176 (0.143±0.024)	0.134-0.171 (0.150±0.013)	0.116-0.186	0.122-0.149 (0.133±0.015)
Nerve ring[b]	0.256-0.372 (0.296±0.039)	0.220-0.288 (0.259±0.031)	0.266-0.322(0.298±0.020)	0.264-0.352	0.282-0.382 (0.318±0.038
Excretory pore[b]	0.461-0.583 (0.512±0.046)	0.352-0.496 (0.432±0.065)	0.495-0.599(0.537±0.039)	0.408-0.616	0.409-0.492 (0.458±0.039)
Left spicule	5.89-7.94 (7.02±0.81)	14.19-20.36 (17.60±2.20)	10.60-27.86 (18.87±6.46)	11.1-22.7	6.28-7.72 (6.78±0.57)
Relative length of left spicule / body length	15.6-21.1 (18.4)%	39.5-64.1 (48.9)%	21.8-65.6 (46.3)%	-	24.6-32.3 (28.4)%
Right spicule	0.111-0.153 (0.133±0.014)	0.096-0.160 (0.132±0.024)	0.137-0.168 (0.157±0.014)	0.118-0.160	0.097-0.163 (0.115±0.57)
Gubernaculum	0.072-0.125 (0.099±0.015)	0.072-0.125 (0.099±0.015)	0.072-0.104 (0.083±0.011)	0.106-0.142	0.077-0.089 (0.083±0.005)
Number of precloacal papillae	5 - 6	4 - 5	5 - 6	5 - 7	4 - 5
Number of postcloacal papillae	5	5 - 6	5 - 6	5 - 6	5
Tail length	0.267-0.417 (0.325±0.048)	0.240-0.300 (0.270±0.028)	0.172-0.336 (0.275±0.062)	0.240-0.376	0.282-0.321 (0.300±0.018)
Female worm					
Number of worms examined	(n=8)	(n=6)	(n=6)	(n=60)	(n=6)
Body length	60.0-91.6 (72.7±10.0)	67.90-85.07 (78.47±7.20)	68.5-107.3 (82.4±14.4)	46.0-111.5	35.7-51.6 (43.6±5.9)
Max. body width	0.24-0.41 (0.33±0.05)	0.30-0.34 (0.33±0.02)	0.29-0.38 (0.32±0.04)	0.272-0.424	0.216-0.280 (0.240±0.023)
Pharynx	0.050-0.069 (0.060±0.008)	0.040-0.072 (0.057±0.012)	0.045-0.064 (0.055±0.007)	0.044-0.073	0.044-0.061 (0.056±0.006)
Oesophagus	7.58-9.89 (8.91±0.84)	6.75-7.41 (7.12±0.24)	6.46-8.39 (7.58±0.72)	5.80-9.46	6.86-7.72 (7.28±0.38)
Muscular portion	0.47-0.81 (0.62±0.10)	0.50-0.86 (0.68±0.14)	0.63-0.88 (0.80±0.10)	0.576-0.936	0.554-0.765 (0.672±0.097)
Glandular portion	6.93-9.58 (8.29±0.82)	6.16-6.64 (6.43±0.19)	5.83-7.52 (6.77±0.63)	5.20-8.60	6.30-6.96 (6.60±0.29)
Cervical lateral papillae	0.150-0.192 (0.167±0.017)	0.124-0.160 (0.145±0.015)	0.168-0.224 (0.189±0.020)		0.122-0.166 (0.141±0.015)
Nerve ring	0.272-0.417 (0.343±0.069)	0.480-0.640 (0.568±0.067)	0.324-0.453 (0.382±0.049)		0.327-0.421 (0.369±0.038
Excretory pore	0.539-0.722 (0.608±0.069)	0.480-0.640 (0.568±0.067)	0.632-0.862 (0.743±0.087)		0.598-0.837 (0.734±0.100)
Vulva[c]	3.42-4.58 (3.9±0.40)	2.72-4.08 (3.15±0.50)	2.69-5.18 (3.63±0.96)	1.97-6.06	1.88-2.99 (2.38±0.36)
Tail length	0.167-0.272 (0.216±0.031)	0.260-0.340 (0.307±0.033)	0.246-0.414 (0.307±0.058)	0.202-0.380	0.266-0.316 (0.281±0.022)
Egg	0.055-0.058 (0.057±0.001)	0.056-0.060 (0.059±0.002)	0.058-0.060 (0.059±0.001)	0.050-0.063	0.056-0.062 (0.059±0.002)
	x0.030-0.032 (0.031±0.001)	x0.032-0.036 (0.035±0.002)	x0.032-0.034 (0.033±0.001)	x0.032-0.038	x0.032-0.038 (0.035±0.001)

[a]Measurements are expressed as range (mean±SD)

[b]Distance from the anterior end.

[c]Distance from the posterior end.

Table 7. Nucleotide changes observed in the 18S and 28S rDNA of *Gongylonema pulchrum* with different origins[a]

Host	Locality	DDBJ/EMBL/GenBank accession no.	Nucleotide position where any changes in rDNA sequences are observed*																																
			18S				28S																														
			13	211	695	1,676	182	485	542	588	598	601	604-608	668	713	735	750	785	797	810-816	897	961	987	988	990/991	1,01	1,5	1,658-1,660	1,679/1,680	1,696-1,698	2,473	2,479	2,491	2,963
Deer																																			
	Hyogo, Japan	AB495394	A	C	G	T	T	C	C	G	G	A	TTTTT	T	G	C	G	A	C	GATTCAA	T	T	G	T	—	C	C	AAA	—	TGT	T	T	G	T
	Hyogo, Japan	AB495397	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
	Kagoshima, Japan	AB646050	G	•	•	•	•	•	A	•	•	•	•	•	•	•	•	•	•	•	C	•	•	•	•	•	•	•	•	•	•	•	•	•	•
	Hokkaido, Japan	AB646059	G	•	•	•	•	•	A	•	•	•	•	•	•	•	•	•	•	•	C	•	•	•	•	•	•	•	•	•	•	•	•	•	•
Wild boar																																			
	Hyogo, Japan	AB646064	G	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	C	•	•	•	•	•	•	•	•	•	•	•	•	•	•
Squirrel monkey																																			
	Captive in Japan	AB495401	G	•	•	•	•	•	A	•	•	•	•	•	•	•	•	•	•	•	C	•	•	•	•	•	•	•	•	•	•	•	•	•	•
Cattle																																			
	Okayama or Tottori, Japan	AB513707	G	•	•	•	•	•	A	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
	Oita, Japan	AB513711	G	•	•	•	•	•	A	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
	Kagoshima, Japan	AB513719	•	•	•	•	•	•	A	•	•	•	•	•	•	•	•	•	•	•	C	•	•	•	•	•	•	•	•	•	•	•	•	•	•
	Iran	AB495389	•	•	•	•	•	•	A	•	•	•	•	•	•	•	•	•	•	•	C	•	•	•	•	•	•	•	•	•	•	•	•	•	•
Buffalo																																			
	Nepal	AB646107	G	T	A	A	C	T	A	A	A	G	—	A	•	T	A	T	T	—	C	A	A	A	C	•	T	—	CACGCACA	—	C	C	A	C
		AB646109	G	T	A	A	C	T	A	A	A	G	—	A	A	T	A	T	T	—	C	A	A	A	C	G	T	A	CACGCACA	—	•	C	•	C

[a] Nucleotide position is expressed relative to rDNA of *G. pulchrum* collected from a deer (DDBJ/EMBL/GenBank accession no. AB495394). An identical nucleotide to the uppermost sequence is indicated by '•', and gaps are indicated by '—'.

Table 8. Major nucleotide changes observed in the ITS region of DNA of *Gongylonema pulchrum* with different origins.

Host	Locality	DDBJ/EMBL/GenBank accession no.	Nucleotide position where major changes in rDNA are observed[a]																					
			ITS1										ITS2											
			57/58	64/65	119/120	243-250	304-				358/349	386/387	9/10	19/20	35/36	54-55	64-67	68-			160/161	226	231-237	
Deer																								
	Hyogo, Japan	AB495394-AB495396	—	—	—	AAATTTTA	(A) x 12-19	(CA) x 5	(TA) x 1	(CA) x 4	—	—	—	—	—	GT	TATT	(GCT) x 8	—	—	—	C	TATTAAT	
	Hyogo, Japan	AB495397-AB495400	•	•	•	•	(A) x 15-18	(CA) x 4-5	(TA) x 1-2	(CA) x 4	•	•	•	•	•	•	•	(GCT) x 8	—	—	•	•	•	
	Kagoshima, Japan	AB646050-AB646054	•	•	•	•	(A) x 13-18	(CA) x 5-6	(TA) x 1	(CA) x 4	•	•	•	•	•	•	•	(GCT) x 5	T--	(GCT) x 2-11	•	•	•	
	Hokkaido, Japan	AB646059-AB646063	•	•	•	•	(A) x 12-13	(CA) x 3	(TA) x 0-1	(CA) x 5-8	•	•	•	•	•	•	TA-T	(GCT) x 2	GCG	(GCT) x 2	•	•	•	
Wild boar																								
	Hyogo, Japan	AB646064-AB646094	•	•	•	•	(A) x 10-20	(CA) x 2-6	(TA) x 1	(CA) x 4	•	•	•	•	•	•	•	(GCT) x 6-16	—	—	•	•	•	
Squirrel monkey																								
	Captive in Japan	AB495401-AB495402	•	•	•	•	(A) x 12-13	(CA) x 3	(TA) x 1	(CA) x 7-8	•	•	•	•	•	•	TACT	(GCT) x 2	GCG	(GCT) x 2	•	•	•	
Cattle																								
	Okayama or Tottori, Japan	AB513707-AB513710	•	•	•	•	(A) x 14-15	(CA) x 3	(TA) x 1	(CA) x 7-9	•	•	•	•	•	•	TACT	(GCT) x 2	GCG	(GCT) x 2	•	•	•	
	Oita, Japan	AB513711-AB513718	•	•	•	•	(A) x 13-17	(CA) x 3-4	(TA) x 1	(CA) x 4-10	•	•	•	•	•	•	TACT		GCG	(GCT) x 2	•	•	•	
	Kagoshima, Japan	AB513719-AB513723	•	•	•	•	(A) x 12-14	(CA) x 3-4	(TA) x 1	(CA) x 6-8	•	•	•	•	•	•	TACT	(GCT) x 2	GCG	(GCT) x 2	•	•	•	
	Iran	AB495389-AB495393	•	•	•	•	(A) x 12-14	(CA) x 3	(TA) x 1	(CA) x 3-8	•	•	•	•	•	•	TACT	(GCT) x 2	GCG	(GCT) x 2	•	•	•	
Buffalo																								
	Nepal	AB646107-AB646108	AAGCA	ATGTTGCTGC	GGTGCATA	T-------	(A) x 12	(CA) x 5-6	(TA) x 1	(CA) x 1	AAATAAT or AAAAAAATAAT	AGAA	GAAAAG	ATC	CAA	—	--CT	(GCT) x 1	—	—	ATC	GTGGT	CAAAACTGAAAA	

[a] Nucleotide position is expressed relative to the rDNA sequence of *G. pulchrum* collected from a deer (DDBJ/EMBL/GenBank accession no. AB495394). An identical nucleotide to the uppermost sequence is indicated by '•', and gaps are indicated by '—'.
Repeats of a few nucleotides units surrounded by squares are unique to the worms collected mainly from cattle (see Chapter I). Substitutions and indels involving single nucleotide are omitted from this table.

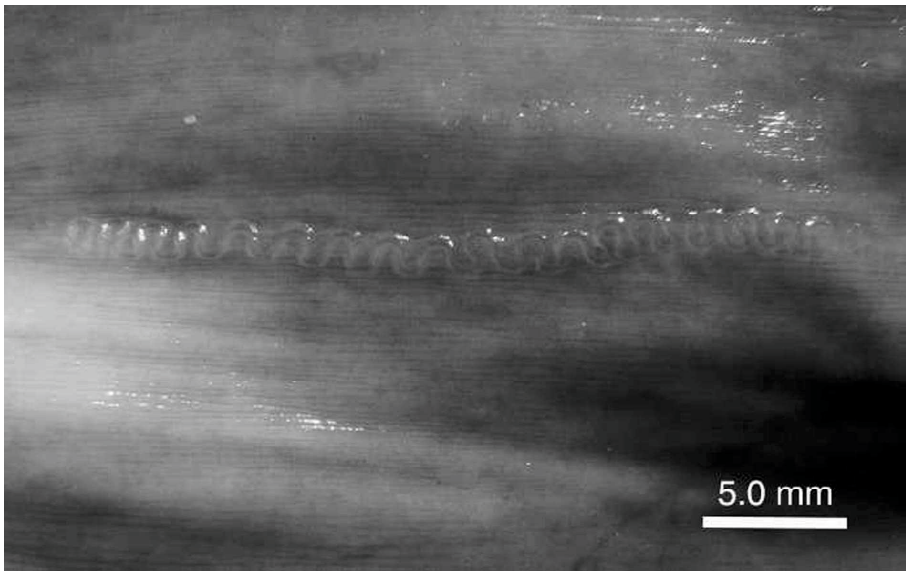

Figure 4. Adult gullet worm embedded in the epithelium of the esophageal mucosa of a buffalo in a zig-zag pattern. No pathological changes except for the presence of the worm are evident.

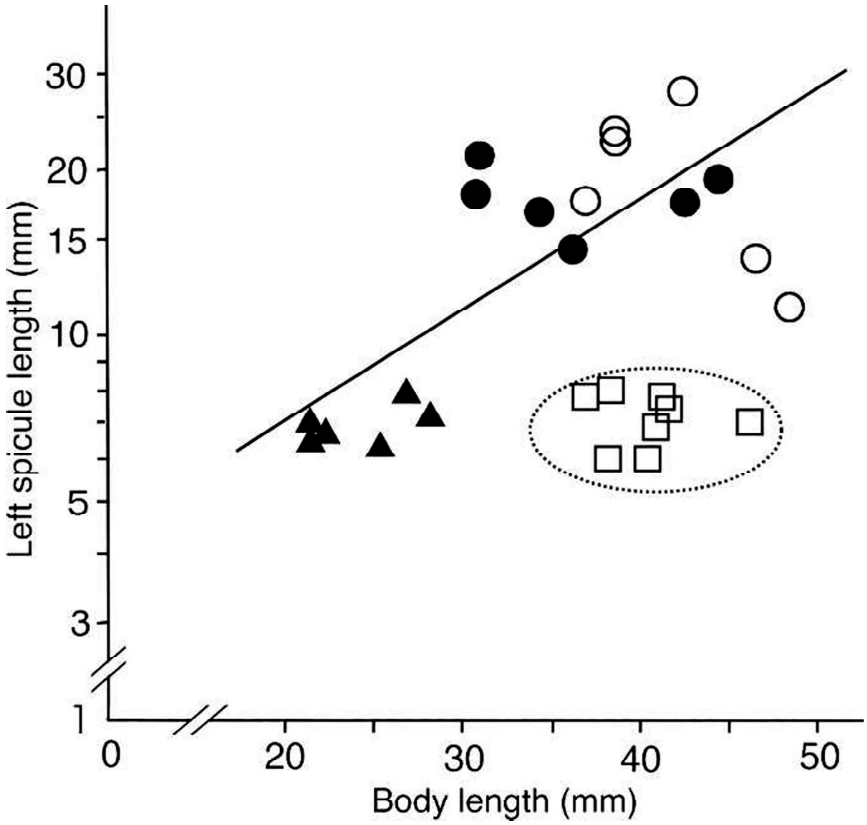

Figure 5. Relationship between body length and left spicule of male gullet worms collected from cattle in Japan (closed circle), cattle in Iran (open circle), deer in Japan (closed triangle), and buffaloes in Nepal (open square). With the exception of buffalo-collected worms (circled by a broken line), the following regression line is applicable to this relationship: $Y = -43.602 + 16.581 \times \ln(X)$ ($P < 0.05$).

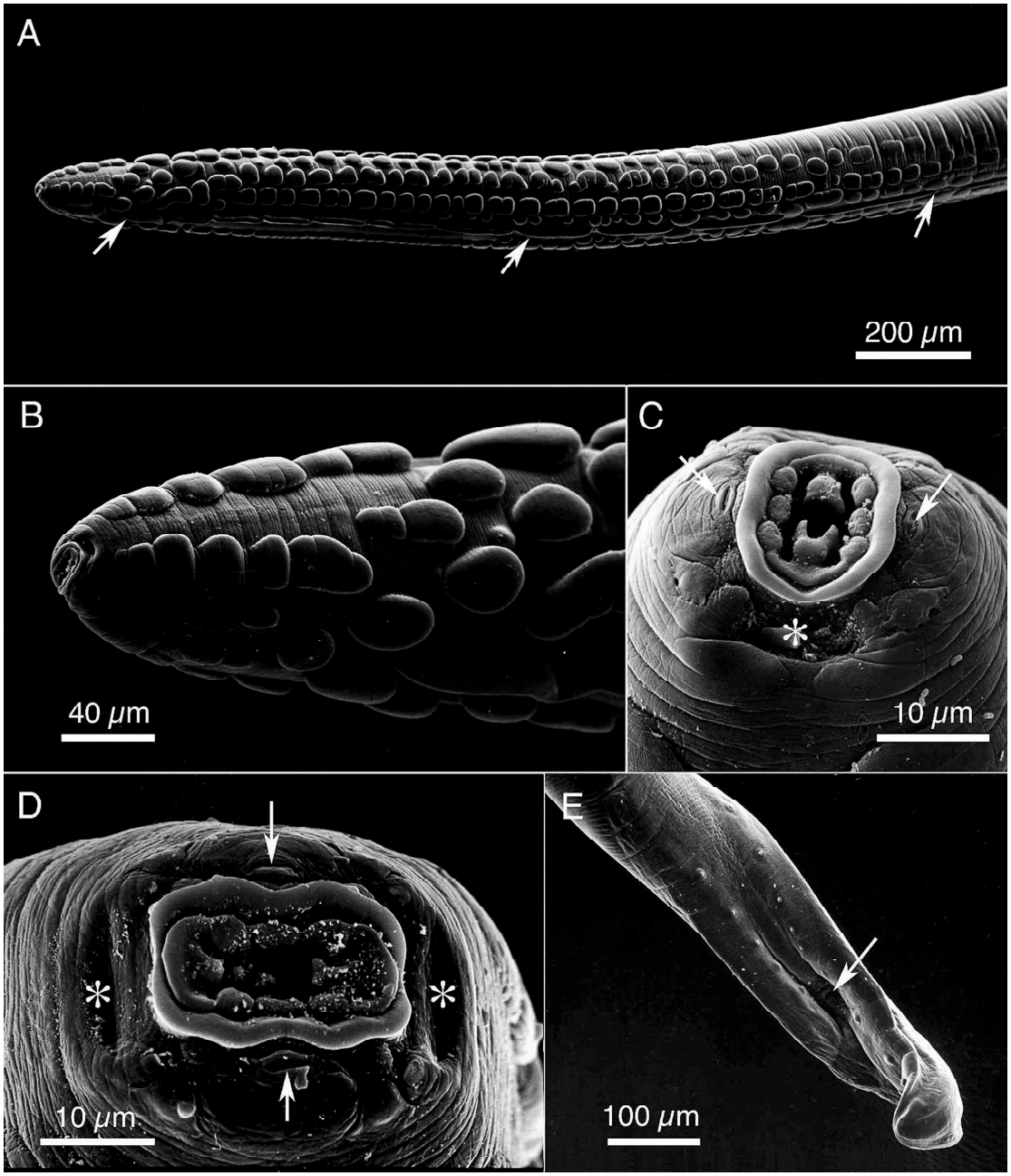

Figure 6. SEM view of adult male gullet worms collected from buffaloes in Nepal. (**A**) Anterior portion of the cuticle with verruciform protrusions and cervical alae on both sides (small arrows). (**B**) Higher magnification of panel A. (**C-D**) Anterior extremity with amphidial pores (arrows) and semilunar depression (asterisks). (**E**) Posterior end with asymmetric caudal alae with 5 or 6 pairs each of pre- and post-anal papillae. A cloacal opening is indicated (arrow).

CHAPTER III

Involvement of two genetic lineages of *Sarcoptes scabiei* mites in a local mange epizootic of wild mammals in Japan.

The work described in the chapter has been published as follows;

Makouloutou, P., Suzuki, K., Tran, B. T., Yokoyama, M., Takeuchi, M. and Sato, H. (2014): Involvement of two genetic lineages of *Sarcoptes scabiei* mites in a local mange epizootic of wild mammals in Japan. J. Wildl. Dis. (in acceptance at 23 May 2014).

3. 1 Abstract

Similar to wild mammals on the continents, mange caused by *Sarcoptes scabiei* (Acari: Sarcoptidae) is spreading in wild mammals such as foxes, raccoon dogs, wild boars, and Japanese serows through most parts of Japan. Pieces of crusted and/or alopetic skin tissues were collected from 120 raccoon dogs, three raccoons, six Japanese badgers, one Japanese marten, one stray dog, four wild boars, and one Japanese serow mainly in an area where mangy wild animals have been increasingly noted in the last four years. The second internal transcribed spacer (ITS2) region of the ribosomal RNA gene and the partial 16S and cytochrome *c* oxidase subunit I (*cox-1*) genes of mitochondrial DNA (mtDNA) were characterized in these skin samples. ITS2 sequencing (404-bp length) revealed the causative mite for mangy skin lesions of 128 animals to be *S. scabiei* regardless of host origin, while *Notoedres cati* was identified as the causative mite for one raccoon dog and one raccoon. The majority of mites had almost identical ITS2 nucleotide sequences to those recorded in a variety of mammals worldwide. Partial 16S and *cox-1* fragments of mtDNA amplified and sequenced successfully (331 bp and 410 bp in length, respectively) showed an identical nucleotide sequence except for one site ('C' vs. 'T') for the former and four sites ('G', 'C', 'C', 'C' vs. 'A', 'T', 'T', 'T', respectively) for the latter fragment. Furthermore, these substitutions were always synchronized, with the two mtDNA haplotypes, i.e. 'C/GCCC' and 'T/ATTT', appearing to separately colonize in small geographical units. Moreover, the 'T/ATTT' haplotype was cladded into a branch where animal-derived mites worldwide dominated, whereas the 'C/GCCC' haplotype formed a geographical branch unique to Japanese isolates. These results suggest that heterologous populations of monospecific *S. scabiei* are expanding their populations and distributions regardless of host species in an apparently local mange epizootic of wild mammals in Japan.

3. 2 Introduction

Epizootic outbreaks of sarcoptic mange are known to occur in a wide variety of domestic and wild mammals worldwide, similar to endemic scabies in humans (Arlian, 1989; Burgess, 1994; Bornstein et al., 2001; Pence & Ueckermann, 2002; Walton et al., 2004b; Walton & Currie, 2007; Currier et al., 2012). Host-specific varieties of morphologically indistinguishable *Sarcoptes scabiei* (Acari: Sarcoptidae) occur in different animal species, exhibiting a low degree of cross infectivity in general (Fain, 1968, 1978; Samuel, 1981). The disease, often manifested as hyperkeratotic or crusted skin, severely debilitates and reduces populations of affected wild mammals (León-Vizcaíno et al., 1999; Bornstein et al., 2001; Pence & Ueckermann, 2002; Ryser-Degiorgis et al., 2002; González-Candela et al., 2004; Rossi et al., 2007; Soulsbury et al., 2007; Oleaga et al., 2008; Jimenez et al., 2010; Sarasa et al., 2011; Millán et al., 2012).

Over the last decade, several molecular perspectives aimed at clarifying the genetic relationship of different host-associated mites causing sarcoptic mange or scabies have been pursued. Studies focusing on the second internal transcribed spacer (ITS2) of the ribosomal RNA gene (rDNA) concluded that morphologically defined *S. scabiei* mite isolates from a variety of mammals on different continents are a single but heterogeneous species (Zahler et al., 1999; Gu & Yang, 2008; Alasaad et al., 2009). Other studies concentrating on mitochondrial DNA (mtDNA) genes (e.g. 12S and 16S ribosomal RNA and cytochrome *c* oxidase subunit I [*cox-1*]) detected three major groups of *S. scabiei* consisting of human-derived mites from Panama, human-derived mites from Aboriginal communities in northern Australia, and a mixed group of animal- and human-derived mites (Berrilli et al., 2002; Skerratt et al., 2002; Walton et al., 2004a). Likewise, a similar genetic separation of human-derived mites, carnivore-derived mites, herbivore-derived mites, and omnivore-derived mites was determined by multilocus microsatellite analysis (Walton et al., 1999;

Soglia et al., 2007; Rasero et al., 2010; Alasaad et al., 2011a). Using the same molecular technique, however, Gakuya et al. (2011) showed that lions and cheetahs in Masai Mara, Kenya, did not share an identical *S. scabiei* population, but did share identical mite populations with their favorite herbivorous prey, Thomson's gazelles and/or wildebeests.

During the mid-1980s, sarcoptic mange started to spread in wild mammals in Japan, affecting, for example, raccoon dogs (*Nyctereutes procyonoides*), foxes (*Vulpes vulpes japonica* and *V. vulpes schrencki*), wild boars (*Sus scrofa leucomystax* and *Sus scrofa riukiuanus*), and Japanese serows (*Capricornis crispus*) (Yamamoto et al., 1998; Takahashi et al., 2001a, b; Shibata et al., 2003; Nakagawa et al., 2009; Kido et al., 2013). In the central part of Wakayama Prefecture, Japan, where the majority of mangy animals examined in the present study were collected, a raccoon dog with severe sarcoptic mange was first noticed in July 2009. Subsequently, affected animals were increasingly observed throughout the year, particularly in winter to early spring. In addition to raccoon dogs, new cases of mange affecting Japanese badgers (*Meles anakuma*), wild boars, and Japanese serows were noted in the region. Consequently, in the present study we have analyzed ITS2 rDNA and mtDNA markers in order to understand more clearly the epidemiology of the vigorously expanding mite population(s) in wild mammals in the central part of Wakayama Prefecture, Japan.

3. 3 Materials and methods

3. 3. 1 Collection of mangy animals

In the central part of Wakayama Prefecture, Japan, mangy wild mammals, particularly raccoon dogs, were first noticed in July 2009. Subsequently, the number of affected animals has increased and their distribution has expanded (Fig. 7). Preceding this (April 2008), the same problem was already well

recognized in wild mammals in the northern part of the same prefecture. Mangy animals examined in this study included 120 raccoon dogs, three feral raccoons (*Procyon lotor*), six Japanese badgers, one Japanese marten (*Martes melampus*), one stray dog (*Canis lupus familiaris*), four wild boars, and one Japanese serow (Table 9; Fig. 8). Of these animals, three mangy raccoon dogs and three wild boars were collected in other prefectures (Saga, Yamaguchi, Shimane and Hyogo Prefectures) in the western part of Japan. As negative controls, skin samples of four raccoon dogs killed by road traffic accidents in the central part of Wakayama Prefecture were also examined. Mangy or normal skin samples were stored at –20˚C prior to microscopic examination and genetic analyses.

3. 3. 2 Preparation of mite DNA

Following 10% KOH digestion of several mangy skin samples, microscopic examination revealed abundant numbers of *S. scabiei* at various life stages. Consequently, 1–2-mm^3 pieces of the crusted or alopetic skins were individually trimmed off using new blades. The skin pieces were placed in individual Eppendorf tubes and gently freeze-dried. Parasite DNA was extracted from each skin piece using an Illustra™ tissue & cells genomicPrep Mini Spin Kit (GE Healthcare UK, Buckinghamshire, UK) according to the manufacturer's instructions.

3. 3. 3 Polymerase chain reaction (PCR) amplification of the ITS2 region of rDNA

The entire length of the ITS2 region of rDNA was amplified by PCR using the primer pair of RIB-18 (5'-GGGCTGCAGTATCCGATGGCTTCGT-3') and RIB3 (5'-CGGGATCCTTCRCTCGCCGYTACT-3') flanking the 3'-terminus of 5.8S rDNA and the 5'-terminus of 28S rDNA, respectively (Zahler et al. 1999). The following PCR cycling protocol was used: 2 min at 94˚C, then 40 cycles at

94˚C for 30 sec, 60˚C for 30 sec, and 72˚C for 90 sec, followed by a final extension at 72˚C for 7 min. PCR products of 453-bp length were visualized in a 1.3% agarose gel containing a few drops of ethidium bromide.

3. 3. 4 PCR amplification of the partial 16S and *cox-1* genes of mtDNA

The partial 16S gene fragments of mtDNA were amplified by PCR using the primer pair of 16SD1 (5'-CTAGGGTCTTTTTGTTCTTGG-3') and 16SD2 (5'-GTAAGTATACGTTGTTATAAC-3') according to Walton et al. (2004a). The following PCR cycling protocol was used: 2 min at 94˚C, then 40 cycles at 94˚C for 30 sec, 52˚C for 45 sec, and 72˚C for 60 sec, followed by a final extension at 72˚C for 7 min. The partial *cox-1* gene fragments of mtDNA were amplified by PCR using the primer pair of 772 (5'-TGATTTTTTGGTCACCCAGAAG-3') and 773 (5'-TACAGCTCCTATAGATAAAAC-3') according to Navajas et al. (1994). The following PCR cycling protocol was used: 2 min at 94˚C, then 40 cycles at 94˚C for 30 sec, 51˚C for 45 sec, and 72˚C for 60 sec, followed by a final extension at 72˚C for 7 min. PCR products of 16S and *cox-1* gene fragments of 373-bp length and 453-bp length, respectively, were visualized in a 1.3% agarose gel containing a few drops of ethidium bromide.

3. 3. 5 PCR product purification, sequencing, and cloning

PCR products were purified using a FastGene Gel/PCR Extraction Kit (NIPPON Genetics Co., Tokyo, Japan) and sequencing of the majority of the samples was conducted bi-directionally using primers used for the PCR amplification. When direct sequencing was unsuccessful, purified amplicons were cloned into a plasmid vector, pTA2 (Target Clone™; TOYOBO, Osaka, Japan), and transformed into *Escherichia coli* JM109 (TOYOBO) according to the manufacturer's instructions. Following propagation, plasmid DNA was extracted using a FastGene Plasmid Mini Kit (NIPPON Genetics Co.) and

inserts from multiple independent clones were sequenced using universal M13 forward and reverse primers. At least three clones per product were sequenced. The nucleotide sequences reported in the present study are available in the DDBJ/EMBL/GenBank databases under the accession numbers AB820960–AB820994 (ITS2), AB820995–AB821003 (16S), and AB821004–AB821012 (*cox-1*).

3. 3. 6 Alignment, construction of putative secondary structure of ITS2, and sequence relationship analysis of ITS2

The newly obtained sequences and related ones of *S. scabiei* retrieved from the DDBJ/EMBL/GenBank databases were aligned using the CLUSTAL W multiple alignment program (Thompson et al., 1994). For predicting the secondary structure of ITS2 molecules using the energy minimization approach for each sequence, the mfold web server (http://mfold.rna.albany.edu/) (Zuker 2003) and the University of Vienna's RNAfold web server (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi) (Hofacker, 2003; Gruber et al., 2008) were employed. The newly obtained 404-bp long sequences, referred to as 'ITS2' in the present study, consisted of 16-bp long 5.8S rDNA, 306-bp long ITS2, and 82-bp long 28S rDNA fragments. Relationships of different ITS2 sequences were visualized using an automated haplotype network layout and visualization software, HapStar, downloaded at http://fo.am/hapstar (Teacher and Griffiths, 2011).

3. 3. 7 Phylogenetic analysis

For phylogenetic analysis, the newly obtained 16S and *cox-1* mtDNA sequences and those of *S. scabiei* mites from various mammals, including

humans, around the world retrieved from the DDBJ/EMBL/GenBank databases were aligned using the CLUSTAL W multiple alignment program mentioned above, with subsequent manual adjustment; 304 characters, of which 30 were variable, remained for the subsequent analysis of 16S mtDNA. Similarly, 410 characters, of which 53 were variable, remained for the subsequent analysis of *cox-1* mtDNA. Maximum likelihood (ML) analysis was performed with the program PhyML (Guindon & Gascuel, 2003; Dereeper et al., 2008) provided on the Phylogeny.fr website (http://www.phylogeny.fr/). The probability of inferred branch was assessed by the approximate likelihood ratio test, an alternative to the non-parametric bootstrap estimation of branch support (Anisimova & Gascuel, 2006).

3. 4 Results

3. 4. 1 PCR amplification of *S. scabiei* DNA fragments

The majority of animals, belonging to seven genera but mostly raccoon dogs (Table 9), showed typical severe mange with thickly crusted skin lesions. From one raccoon dog and one feral raccoon, cat mites (*Notoedres cati*) were detected microscopically and genetically. In this study, we used skin pieces of mangy animals to amplify the ITS2 region of rDNA as well as 16S and *cox-1* gene fragments of mtDNA. Successful DNA amplifications were achieved for the majority of samples, excluding PCR for the 16S gene fragment. However, no amplification was attained for skin pieces from the four healthy raccoon dogs killed by road traffic accidents.

3. 4. 2 Diversity of the ITS2 sequence of *S. scabiei*

The newly obtained 128 ITS2 sequences of *S. scabiei*, 404 bp in length, showed nucleotide variations at 20 sites. Regardless of the geographical and host

origins of the sample, 30 ITS2 genotypes were observed; the first genotype included 56 cases, the second genotype 25 cases, the third genotype eight cases, the fourth genotype six cases, and the remaining 26 genotypes included 35 cases. A total of 156 retrieved ITS2 sequences of *S. scabiei* collected from wild and domestic animals around the world (mainly Europe), 402–404 bp in length, showed nucleotide variations at 83 sites. Essentially, all mites around the world regardless of host mammals were found to share almost identical ITS2 sequences (Figs. 9 & 10).

3. 4. 3 Diversity of the 16S and *cox-1* genes of mtDNA of *S. scabiei*

PCR amplification of 16S mtDNA fragments was partially successful, with new sequences of 331-bp length being obtained for 41 wild mammals (38 raccoon dogs, one Japanese marten, one stray dog, and one Japanese serow). All 16S mtDNA sequences obtained in the present study were identical except for a single variable nucleotide (either C or T) at base position 242 of the representative sequence (DDBJ/EMBL/GenBank accession no. AB820995). Sequences with 'T' at base position 242 (15 mite samples) were identical to a retrieved 16S sequence of *S. scabiei* var. *canis* in Australia (accession no. AY493410), whereas sequences with 'C' at base position 242 (26 mite samples) formed a clade exclusively of Japanese isolates, although two such haplotypes of the 16S sequence belonged to a major clade of *S. scabiei* isolated from various domestic and wild animals in the Australian, European, and American continents (Fig. 11A).

PCR amplification of the partial *cox-1* gene of mtDNA was successful, with new sequences of 410-bp length being obtained for 122 wild mammals (112 raccoon dogs, two raccoons, three Japanese badgers, one stray dog, one Japanese marten, two wild boars, and one Japanese serow). Sequences were

divided into two types by nucleotide variations at base positions 104 (A/G/R), 215 (T/C/Y), 332 (T/C/Y), and 350 (T/C/Y) of the representative sequence (DDBJ/EMBL/GenBank accession no. AB821004); combinations of nucleotides at these four base positions were either 'ATTT' (39 mite samples), 'GCCC' (75 mite samples), or 'RYYY' (eight mite samples). Furthermore, all *S. scabiei* samples with 'T' at base position 242 of the 16S mtDNA fragment mentioned above (T type 16S) had 'ATTT' type of the *cox-1* mtDNA sequence, whereas all *S. scabiei* samples showing 'C' type 16S mtDNA fragment had 'GCCC' type *cox-1* sequence. The 'ATTT' type *cox-1* sequences were identical to retrieved *cox-1* sequences of *S. scabiei* from a wombat, wallaby, and dog in Australia or USA (accession nos. AY493397, AY493398, and AY493393), whereas the 'GCCC' type *cox-1* sequences formed a special clade exclusively of Japanese isolates, although two such haplotypes of the *cox-1* sequence belonged to a major clade of *S. scabiei* isolated from various domestic and wild animals in the Australian, European, and American continents (Fig. 11B).

3. 4. 4 Geographical distribution of *S. scabiei* with two different mtDNA haplotypes

As mentioned above, two different haplotypes of mtDNA genes (16S and *cox-1*) were detected in *S. scabiei* mites collected from wild animals. Their distributions are plotted on a map of the area covered by the present study (Fig. 12). The distributions of the two different haplotypes were found to have close associations with certain localities. For example, the majority of wild mammals in the regions Ryujin (RJN), Nakahechi (NHC), Kamitonda (KTD), and Shirahama (SRH) were infested with *S. scabiei* of the 'C/GCCC' haplotype, whereas the majority of wild mammals in the regions Hongu (HNG) and Ohto (OHT) were infected with mites of the 'T/ATTT' haplotype regardless of host

origin.

3. 5 Discussion

As shown in Fig. 7, wasted mangy raccoon dogs have frequently been observed in every winter season since 2009 in the central part of Wakayama Prefecture, but major hot spots of the epizootic mange have changed each year; NHC and HNG for the first year, RJN for the second year, RJN and Tanabe (TNB) for the third year, and TNB and OHT for the fourth year. Rapid geographical expansion of the disease is likely to be associated with a special genetic lineage of *S. scabiei* with a high virulence. It is also unclear whether such a *S. scabiei* variety is linked to the recent occurrence of mange in mammals other than raccoon dogs. To address these issues, we conducted molecular genetic analyses of *S. scabiei* prevalent in wild mammals in the region.

In the present study, parasite DNA was extracted from a mass of mites localized in small pieces of mangy or alopetic skin. This method of processing resulted in minimal problem(s) concerning overlapped heterologous sequences of rDNA or mtDNA. For instance, only 6.6% (8/122) of skin pieces contained two heterologous sequences of the *cox-1* gene. Alasaad et al. (2008) could not demonstrate a skin site preference (e.g. head, back, or abdomen) of *S. scabiei* subpopulations by multilocus microsatellite analysis. Thus, two different 16S/*cox-1* haplotypes could represent a dominant mite population colonizing an individual host rather than reflecting sampled skin sites. On the other hand, the presence of two heterologous mtDNA gene sequences of mites even in a small skin piece could indicate two or more colonizations of mites in a single host. The real frequency of such repeated or overlapped infestations is not known for our materials because only a single skin piece was taken from each individual host.

As concluded by previous studies on different *S. scabiei* isolates from a variety of mammals worldwide (Zahler et al., 1999; Gu & Yang, 2008; Alasaad et al., 2009), the mite is monospecific having almost identical ITS2 rDNA nucleotide sequences. Minor but variable ITS2 nucleotide sequences, 26 in number, were found in 27% (35/129) of skin samples, and a proportion of them may represent minor local lineages of mites in Japan.

The 16S and *cox-1* nucleotide sequences had a limited number of nucleotide variations, one base site across 331-bp length and four base sites across 410-bp length, respectively. All these base substitutions were absolutely synchronized, i.e. all mites examined in the present study had either 'T/ATTT' haplotype or 'C/GCCC' haplotype of mtDNA genes. In addition to our study area of the central part of Wakayama Prefecture, these two haplotypes may be dominant in other regions of Japan since they were detected in mites collected from two raccoon dogs in the northern part of Wakayama Prefecture and from three raccoon dogs and one wild boar in localities distant from the prefecture (Shimane, Yamaguchi, and Saga Prefectures). As shown in Fig. 11, the 'T/ATTT' haplotype mites from various wild mammals in Japan formed a clade with *S. scabiei* var. *canis* from Australia and USA as well as *S. scabiei* var. *wallaby* and var. *wombati* from Australia, whereas the 'C/GCCC' haplotype mites from raccoon dogs and a stray dog in Japan formed a unique branch, although both Japanese mite haplotypes were localized in a mixed group of human- and animal-derived *S. scabiei* isolates worldwide in the sense of Walton et al. (1999, 2004a, b). Berrilli et al. (2002) reported three 16S ribosomal RNA haplotypes of *S. scabiei* collected from the Alpine chamois (*Rupicapra rupicapra*) in northeastern Italy, Pyrenean chamois (*Rupicapra pyrenaica*) in northwestern Spain, and red fox (*Vulpes vulpes*) in northwestern Italy and northeastern Spain. They provided 407-bp long 16S sequences which contained two variable base sites at positions 58 (G vs. A) and 224 (T vs. C) of their

sequences (accession nos. AF387675–AF387702). Unfortunately, the 16S fragments sequenced by Berrilli et al. (2002) and our group partially overlap for only 139 bp from the 5'-terminus of sequences provided by Berrilli et al. and the 3'-terminus of our sequences. All 16S fragments of *S. scabiei* from chamois and foxes in Europe consistently show 'T' at base position 242 of our sequences. However, there is no information on the two variable nucleotide sites reported by Berrilli et al. (2002) concerning the 16S fragments of mites from wild mammals in Japan.

Another intriguing finding is that the two mtDNA haplotypes of Japanese *S. scabiei* preferentially colonized wild mammals in certain geographical units (Fig. 12). In other words, one mtDNA haplotype often dominated in one small geographical unit. Berrilli et al. (2002) considered that their three 16S haplotypes of *S. scabiei* might reflect local populations due to a limited gene exchange across geographical barriers rather than a differential adaptation to host species. Skerratt et al. (2002) put forward a similar proposal to explain their results of 12S mtDNA gene variations of mites found in wombats, dogs, and humans in Australia.

A high resolution of multilocus microsatellite markers visualizes the transmission dynamics of *S. scabiei* in different mammalian hosts (Walton et al., 1999; Soglia et al., 2007; Rasero et al., 2010; Alasaad et al., 2011a; Gakuya et al., 2011). When considering the 'host taxon' law for the transmission of the mite (Walton et al., 1999; Soglia et al., 2007; Rasero et al., 2010; Alasaad et al., 2011a) along with the 'prey-to-predator-wise' transmission (Gakuya et al., 2011), limited gene flow between some host-associated populations of mites is ascribed to multiple factors including geographical and host behavioral limits as well as mite adaptation degree to host physiological differences, as clearly summarized by Holt and Fischer (2013). Involvement of possible cosmopolitan

and local populations of *S. scabiei* in wild mammals in a limited region, depicted in the present study as 'T/ATTT' and 'C/GCCC' mtDNA haplotypes of Japanese mites, respectively, stimulates our interest in their phylogeographical nature. Analyses of nucleotide sequences covering a long length of a single gene or multiple genes of mtDNA are required to understand the phylogeography of *S. scabiei* distributed worldwide (Criscione et al., 2005; Alasaad et al., 2011b; Currier et al., 2012).

Table 9. Animals used for the genetic analyses.

Host				No. of	Evident	ITS2	mtDNA	mtDNA haplotype		
Species	Locality	Date of collection	Sex	animals	mange	PCR	PCR	C/GCCC	T/ATTT	Mixed
Raccoon dog (*Nyctereutes procyonoides*)										
	Central Wakayama	July 2009-April 2012	Male	68	65	63*	62	70	34	3
			Female	51	50	46	45			
	Northern Wakayama	March 2008	Male	1	1	1	1	1	0	1
		April 2008	Female	1	1	1	1			
	Oki Is., Shimane	—**	—	2	2	2	2	2	0	0
	Imari//Takeo, Saga	October—November 2009	—	1	1	1	1	0	1	0
Raccoon (*Procyon lotor*)										
	Central Wakayama	June 2009—November 2009	Male	2	2	2*	1	0	0	2
			Female	1	1	1	1			
Japanese badger (*Meles anakuma*)										
	Central Wakayama	May 2010—April 2012	Male	4	4	4	2	1	0	2
			Female	2	2	2	1			
Japanese marten (*Martes melampus*)										
	Central Wakayama	February 2010	Male	1	1	1	1	0	1	0
Stray dog (*Canis lupus familiaris*)										
	Central Wakayama	October 2010	—	1	1	1	1	1	0	0
Wild boar (*Sus scrofa leucomystax*)										
	Central Wakayama	January 2011	—	1	1	1	1	0	1	0
	Asago, Hyogo	January 2008	—	2	2	2	0	0	0	0
	Hagi, Yamaguchi	January 2011	—	1	1	1	1	0	1	0
Japanese serow (*Capricornis crispus*)										
		September 2010	Male	1	1	1	1	0	1	0

* Including one case each of cat mange caused by *Notoedres cati*.

** Bar '—' means no information.

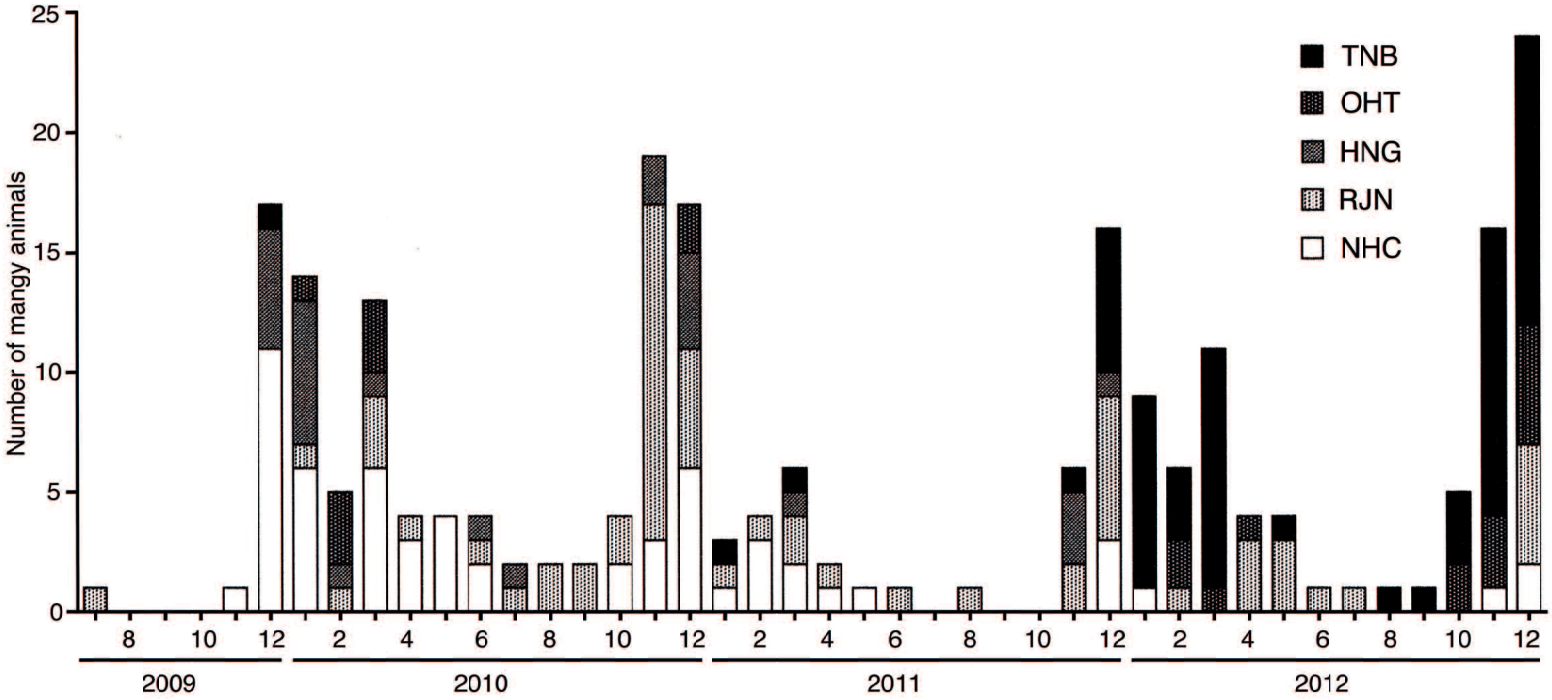

Fig. 7. Temporal changes of occurrence of mangy raccoon dogs in the central part of Wakayama Prefecture, Japan. Numbers of animals are summed by regions: TNB, Tanabe; OHT, Ohto; HNG, Hongu; RJN, Ryujin; and NHC, Nakahechi.

Fig. 8. Photographs of a mangy raccoon dog with crusted skin (upper panel) and a mangy skin sheet from a Japanese serow (lower panel).

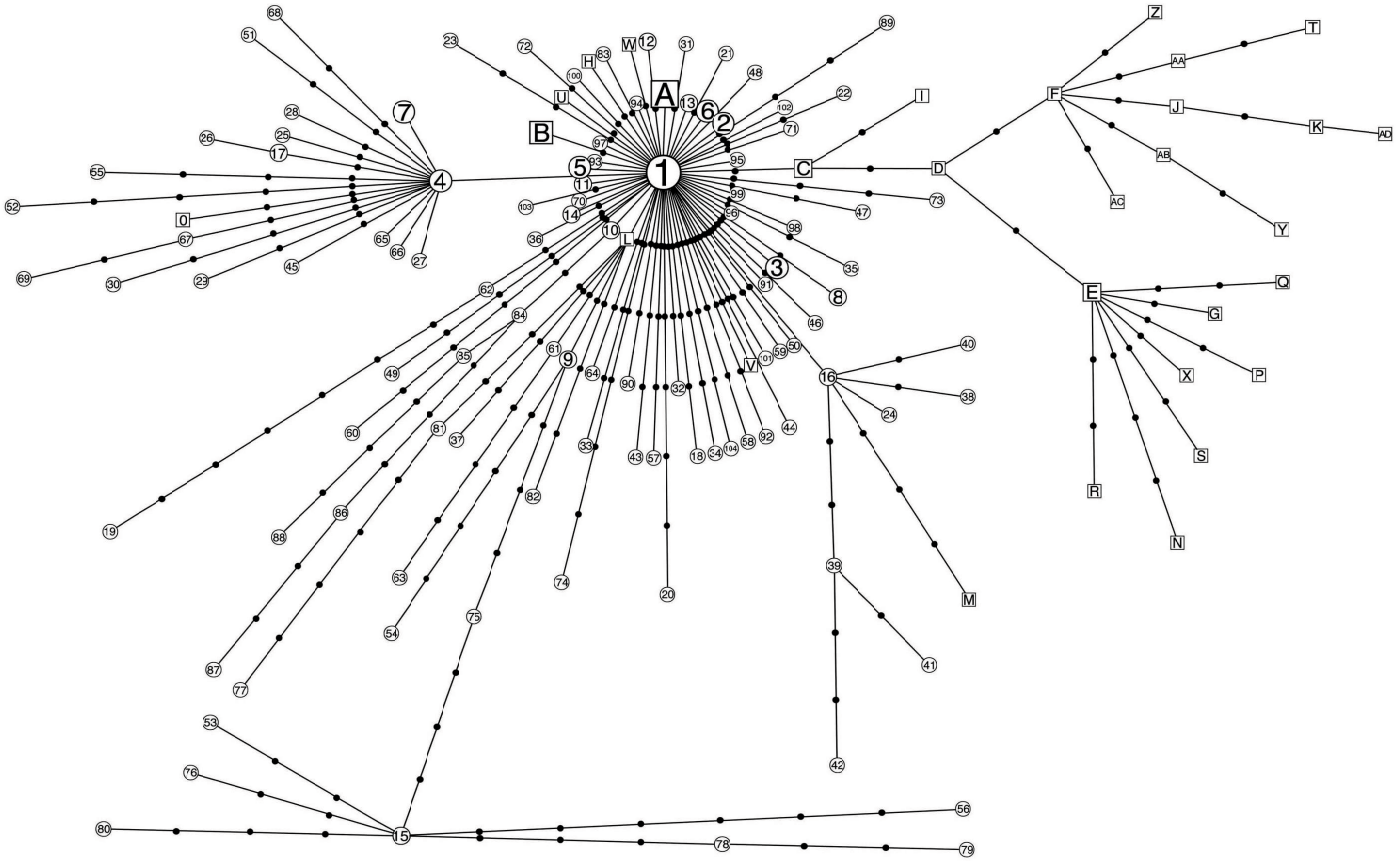

Fig. 9. Relationships of 5.8S–ITS2–28S rDNA genotypes of *Sarcoptes scabiei* from a variety of mammals in Japan (squares labeled from A to AD [n=30]) and other locations around the world (circles labeled from 1 to 104 [n=104]). Sizes of symbols are arbitrary, only denoting higher frequency in larger symbols.

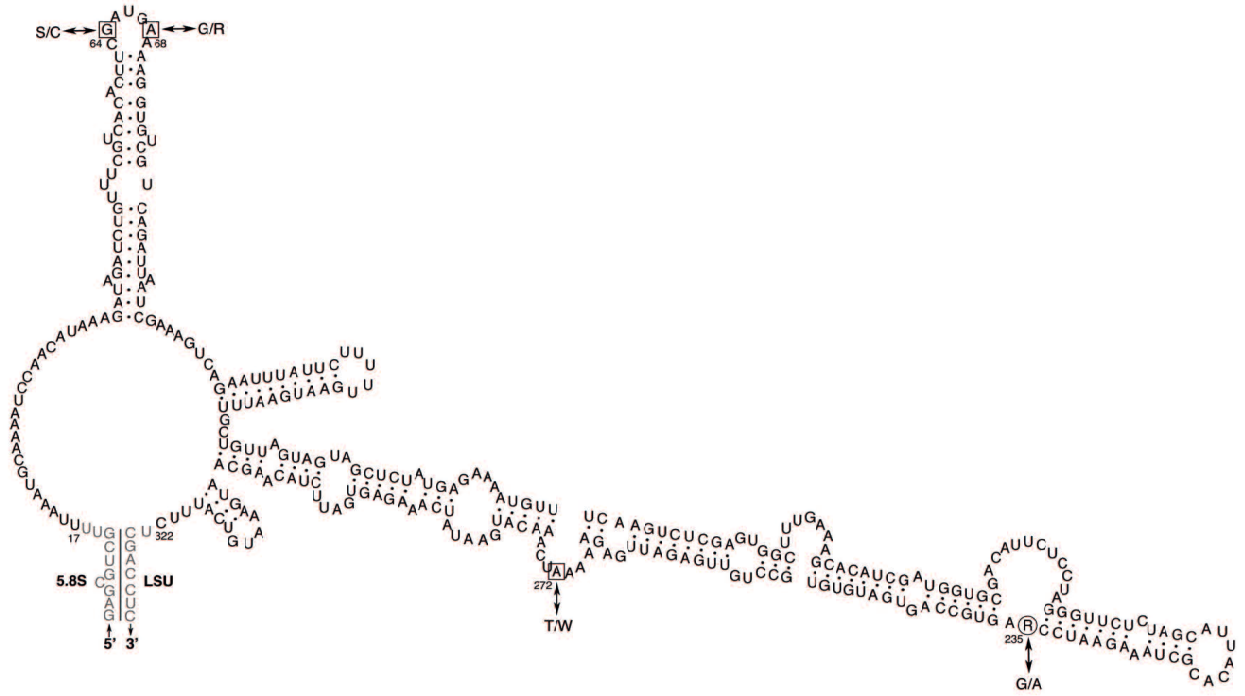

Fig. 10. Illustration of the putative secondary structure of the ITS2 region of *Sarcoptes scabiei* rDNA with plots of highly variable nucleotide sites.

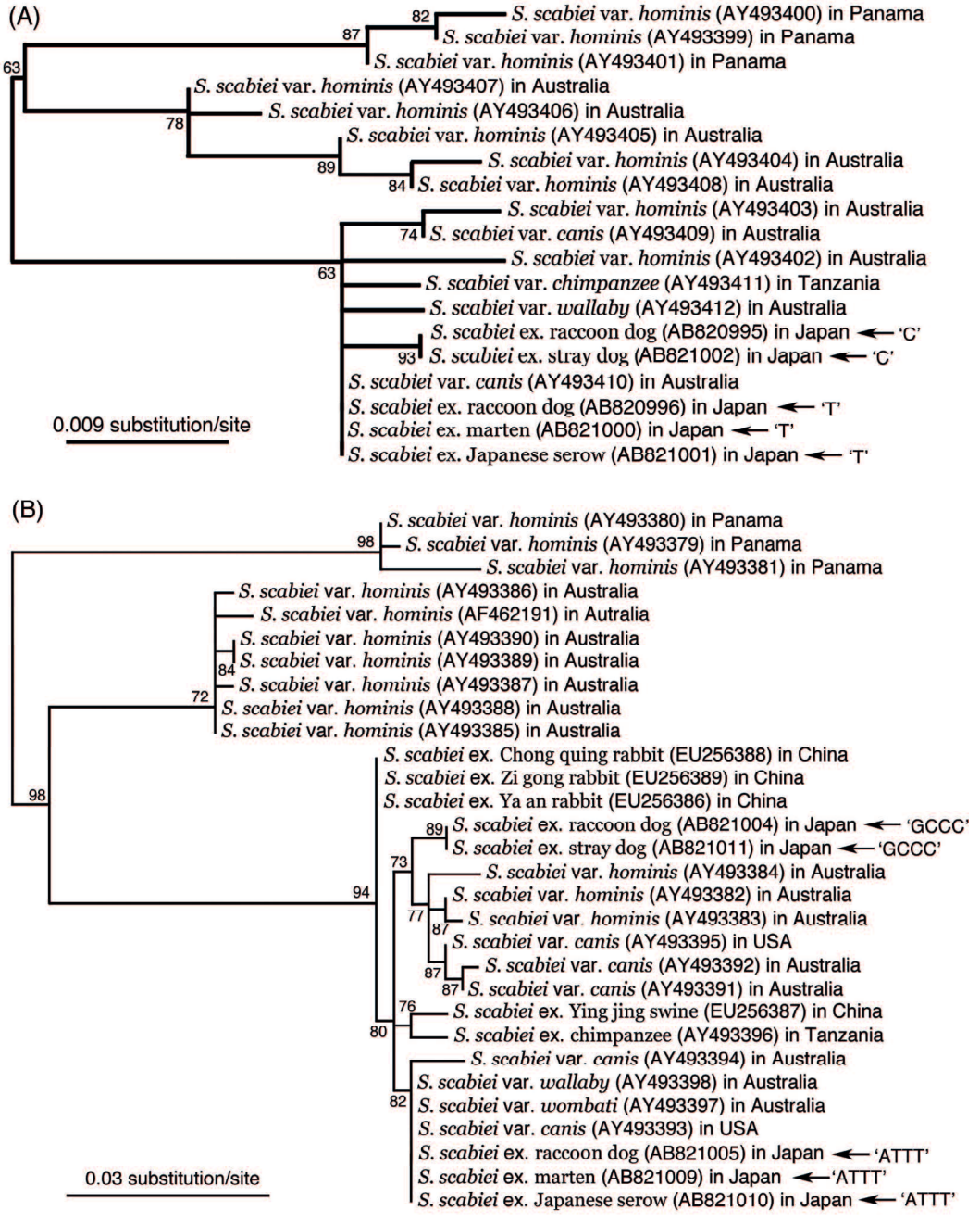

Fig. 11. PhyML phylogenetic trees based on the 16S (**A**) and *cox-1* (**B**) gene sequences of mtDNA. Newly obtained sequences in the present study are indicated by arrows. Labeling with 'C' in (A) or 'GCCC' in (B) denotes the 'C/GCCC' haplotype, and 'T' in (A) or 'ATTT' in (B) denotes the 'T/ATTT' haplotype.

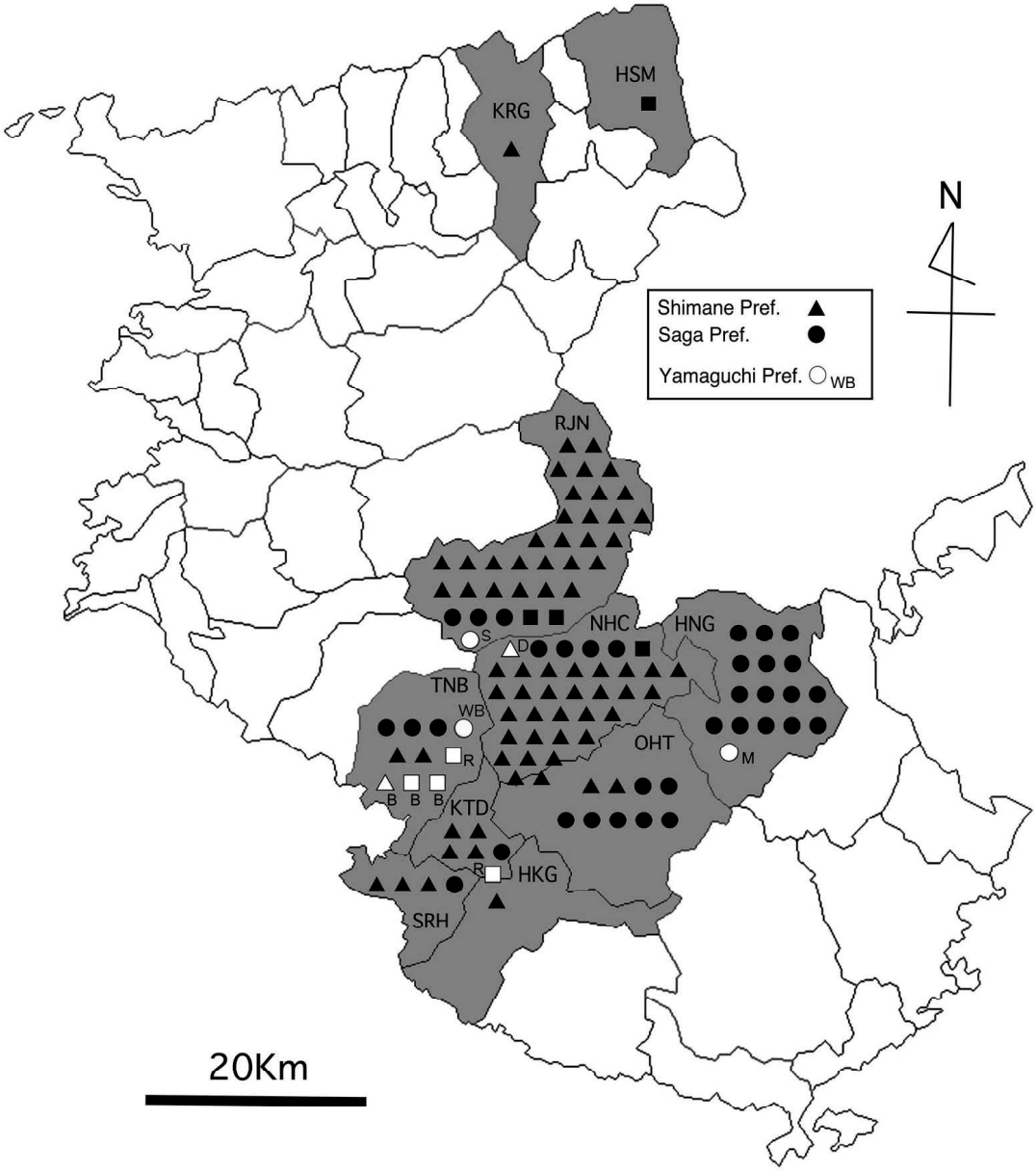

Fig. 12. Geographical distribution of two major mtDNA haplotypes in wild mammals in the central part of Wakayama Prefecture. The 'C/GCCC' haplotype in raccoon dogs (closed triangle) and other mammals (open triangle); the 'T/ATTT' haplotype in raccoon dogs (closed circle) and other mammals (open circle); and the mixture of both haplotypes in raccoon dogs (closed square) and other mammals (open square). Letter beside the open symbol denotes animal species: B, badger; D, stray dog; M, marten; R, raccoon; S, serow; and WB, wild boar. Region abbreviation: HKG, Hikigawa; HNG, Hongu; HSM, Hashimoto; KRG, Katsuragi; KTD, Kamitonda; NHC, Nakahechi; OHT, Ohto; RJN, Ryujin; SRH, Shirahama; and TNB, Tanabe.

CHAPTER IV

Prevalence and genetic diversity of *Oesophagostomum stephanostomum* in wild lowland gorillas at Moukalaba-Doudou National Park, Gabon

The work described in the chapter has been published as follows;
Makouloutou, P., Nguema, P. P. M, Fujita, S., Takenoshita, Y., Hasegawa, H., Yanagida, T. and Sato, H. (2014) : Prevalence and genetic diversity of *Oesophagostomum stephanostomum* in wild lowland gorillas at Moukalaba-Doudou National Park, Gabon. Helminthologia 51: 83-93.

4. 1 Abstract

Using a sedimentation method, the prevalence of the nodular worm *Oesophagostomum stephanostomum* (Nematoda: Strongylida) in western lowland gorillas at Moukalaba-Doudou National Park (MDNP), Gabon, was determined in fecal samples collected between January 2007 and October 2011, along with their coprocultures. Concurrently, possible zoonotic *Oesophagostomum* infections in villagers living near MDNP were assessed from their fecal samples collected during October and November of 2011. In the gorillas, strongylid (*Oesophagostomum* and/or hookworm) eggs were found in 47 of 235 fecal samples (20.0%) and *Oesophagostomum* larvae were detected in 101 of 229 coprocultures (44.1%). In the villagers, strongylid eggs were found in 9 of 71 fecal samples (12.7%), but no *Oesophagostomum* larvae were detected in coprocultures. The internal transcribed spacer (ITS) region of ribosomal RNA gene (rDNA) and cytochrome *c* oxidase subunit-1 (*cox-1*) region of mitochondrial DNA (mtDNA) of coprocultured *Oesophagostomum* larvae were amplified using parasite DNA extracted from 7–25 larvae/sample, cloned into *Escherichia coli*, and sequenced. Sequenced rDNA contained 353/354-bp long ITS1, 151-bp long 5.8S rDNA, and 227-bp long ITS2. Parts of clones showed variations at 1–3 bases in the ITS1 region at a frequency of 24/68 (35.3%) and at 1–2 bases in the ITS2 region at a frequency of 7/68 (10.3%), whereas the 5.8S rDNA was essentially identical. Sequenced *cox-1* gene of the parasites, 849 bp in length, showed a higher number of nucleotide variations, mainly at the third nucleotide position of the codon. The majority of clones (27/41 (65.9%)) had an identical amino acid sequence. These results suggest that at MDNP, Gabon, only a single population of *O. stephanostomum* with a degree of genetic diversity is prevalent in western lowland gorillas, without zoonotic complication in local inhabitants. The possible genetic variations in the ITS region of rDNA and *cox-1*gene of mtDNA presented here may be valuable when

only a limited amount of material is available for the molecular species diagnosis of *O. stephanostomum*.

4. 2 Introduction

Nematodes of the genus *Oesophagostomum*, termed nodular worms, cause oesophagostomiasis characterized by granuloma formation, caseous lesions or abscesses around encapsulated larvae in the intestinal wall of suids, ruminants, primates, and African rodents (Chabaud & Durette-Desset, 1974; Stewart & Gasbarre, 1989; Anderson, 1992; Polderman & Blotkamp, 1995; Krief et al.., 2008; Lichtenfels, 2009). Clinical oesophagostomiasis is evident worldwide in domestic animals and focally or sporadically in humans (Stewart & Gasbarre, 1989; Polderman & Blotkamp, 1995). Of the eight *Oesophagostomum* spp. recorded to date from non-human primates, *O. bifurcum*, *O. stephanostomum*, and *O. aculeatum* have also been reported in humans (Chabaud & Lariviere, 1958; Polderman & Blotkamp, 1995). Zoonosis caused by *O. bifurcum* is endemic in the northernmost part of Ghana and Togo situated in western Africa, and high prevalences of the disease in human residents have been recorded (Blotkamp et al., 1993; Polderman & Blotkamp, 1995; Pit et al.., 1999; Yelifari et al.., 2005; Gasser et al., 2006). Recently, however, multifaceted genetic analyses have demonstrated that different genotypes of *O. bifurcum* are prevalent in human patients and local non-human primates (Mona monkeys, Patas monkeys, Green monkeys, and Olive baboons) in Ghana and Togo (de Gruijter et al., 2004, 2005; reviewed by Gasser et al., 2006, 2009), supporting parallel assumptions based on epidemiological and morphological analyses (van Lieshout et al., 2005; de Gruijter et al., 2006).

Genetic characterization of *Oesophagostomum* spp. facilitates not only reliable diagnosis of the species without morphological observation of the adult

parasite, but also transmission dynamics of the parasite in different animal hosts distributed in the same area, as seen in preceding intensive studies on *O. bifurcum* (de Gruijter et al., 2004, 2005; Gasser et al., 2006, 2009). In the present study, we collected fecal samples from western lowland gorillas (*Gorilla gorilla gorilla*) and human residents at the Moukalaba-Doudou National Park (MDNP), Gabon, to evaluate the current status of oesophagostomiasis in local primates, i.e. gorillas and humans. Furthermore, *Oesophagostomum* isolates were genetically characterized based on the internal transcribed spacer (ITS) region of ribosomal RNA gene (rDNA) as well as the partial sequence of cytochrome *c* oxidase subunit-1 (*cox-1*) gene of mitochondrial DNA (mtDNA) in order to define the genetic diversity of locally prevalent *Oesophagostomum* parasites. Elucidation of the possible genetic variation of the most frequently used markers, namely the ITS region of rDNA and *cox-1* gene of mtDNA, should facilitate the specific diagnosis of parasites, particularly when limited numbers of specimens are available for this purpose.

4. 3 Materials and methods

4. 3. 1 Study area and sample collection

Sample collection was carried out in a national park in southern Gabon, Moukalaba-Doudou National Park (2˚26'S, 10˚25'E), covering an area of 5,028 km^2 with humid rain forest and savannah grasslands (Fig. 13). It is home to western lowland gorillas and chimpanzees (*Pan troglodytes*), and a program for the habituation of a group of gorillas (Group Gentil, GG) localized mainly on the southwestern side of the park (approximately 30 km^2) has been successfully conducted since 2003 by researchers from Kyoto University, Japan, and their Gabonese colleagues of L'Institut de Recherches en Ecologie Tropicale (IRET) (Ando et al., 2008). During the habituation and all-day follows of GG gorillas, fresh feces of the members, ca. 20 in total, were collected between January 2007

and October 2011 for this study, along with samples of gorillas belonging to unknown groups or solitary individuals and a few chimpanzees. Fecal samples of villagers living near the park were collected during October and November of 2011 with the permission of Le Centre National de la Recherche Scientifique et Technologique (CENAREST) and the chiefs of two villages. We explained the purpose of our study to all participants and obtained signed documents from every participant which allowed us to use their feces for research purposes. The Moukalaba River is a natural barrier between the villages and the home range of animals in the park, particularly great apes.

Individual fresh fecal samples of gorillas and villagers were divided into three parts: 1) one part was fixed in 10% neutral-buffered formalin; 2) one part was fixed in 80% ethanol; and 3) one part was used for coproculture as described below. Each sample tube was carefully labeled with collection date, host species, and location. Although individual identification of GG gorillas had been established (Ando et al., 2008), individual ID numbers were not available for samples collected for this study.

A modified Harada-Mori fecal culture technique using a filter paper strip and a disposable tea bag according to Hasegawa (2009a) was conducted at an open research cabin in the park. After keeping the coprocultures for 1–2 weeks, grown larvae that emerged into the water were fixed in 80% ethanol.

4. 3. 2 Parasitological examination

Fixed fecal samples and coproculture products were transported to the Laboratory of Parasitology, Yamaguchi University, Japan, and microscopic examinations were performed as follows. An approximate 1–2 g amount of formalin-fixed feces was examined microscopically following processing with a standard egg sedimentation method. Referring to Hasegawa (2009b), helminth

eggs were identified under a light microscope. Apart from trophozoites of the ciliate *Troglodytella abrassarti*, protozoan infections were not assessed in this study. Coprocultured nematode larvae were separated under a dissection microscope and detailed morphology was observed under a light microscope with reference to van Wyk et al. (2004).

4. 3. 3 DNA extraction, polymerase chain reaction (PCR), and sequencing

From individual fecal cultures, 7–25 *Oesophagostomum* larvae were arbitrarily selected and washed three times in distilled water. Parasite DNA was extracted from such a pool of larvae using an Illustra™ tissue & cells genomicPrep Mini Spin Kit (GE Healthcare UK, Buckinghamshire, UK) according to the manufacturer's instructions.

PCR amplification of the entire ITS1–5.8S–ITS2 region of rDNA was performed using a combination of a forward primer (NC5/F2; 5'-GTAGGTG-AACCTGCGGAAGGATCAT-3') and a reverse primer (NC2; 5'-TTAGTTTC-TTTTCCTCCGCT-3') flanking the 3'-terminus of 18S rDNA and the 5'-terminus of 28S rDNA, respectively (Newton et al., 1998). The following PCR cycling protocol was used: 2 min at 94˚C, then 40 cycles at 94˚C for 30 sec, 60˚C for 30 sec, and 72˚C for 90 sec, followed by a final extension at 72˚C for 7 min, as described above in Chapter I. PCR products were purified using a FastGene Gel/PCR Extraction Kit (NIPPON Genetics Co., Tokyo, Japan), cloned into a plasmid vector, pTA2 (Target Clone™; TOYOBO, Osaka, Japan), and transformed into *Escherichia coli* JM109 (TOYOBO) according to the manufacturer's instructions. Following propagation, plasmid DNA was extracted using a FastGene Plasmid Mini Kit (NIPPON Genetics Co.) and inserts from multiple independent clones were sequenced using universal M13 forward and reverse primers. For the majority of larval samples, at least three clones per culture were sequenced. The partial *cox-1* gene of parasite mtDNA

was amplified by a primer pair of StrCoxAfrF (5'-GTGGTTTTGGT-AATTGAATGGTT-3') and MH28R (5'-CTAACTACATAATAAGTATCA-TG-3') described by Hasegawa et al. (2010), but a different cycling protocol was used as follows: 2 min at 94˚C, then 40 cycles at 94˚C for 30 sec, 51˚C for 45 sec, and 72˚C for 1 min, followed by a final extension at 72˚C for 7 min. Amplicons were sequenced after genomic cloning as detailed above.

In order to identify the species of filariform larvae in coprocultures, two overlapping fragments of 18S rDNA were amplified by primer pairs of S.r.18S-F1/S.r.18S-R1 and SSU22F/SSU23R and sequenced as described previously (Sato et al., 2006). Parasite DNA was extracted from a pool of filariform larvae (8–50 filariform larvae, average 16.3) collected from individual coprocultures.

The newly obtained sequences of *Oesophagostomum* larvae and those of related strongylid species retrieved from the DDBJ/EMBL/GenBank databases were aligned using the CLUSTAL W multiple alignment program (Thompson et al., 1994). Likewise, the newly obtained sequences of the filariform larvae and those of *Strongyloides* spp. retrieved from the aforementioned databases were similarly aligned. The nucleotide sequences reported in the present study are available in the DDBJ/EMBL/GenBank databases under accession numbers AB821013–AB821046.

4. 3. 4 Putative secondary structure of ITS2

Based on the structural association between 5.8S and 28S rDNA (Hwang & Kim, 2000; Gottschling & Plötner, 2004), a putative secondary structure of the 5.8S rDNA of *Oesophagostomum* spp. was drawn. For predicting the secondary structure of ITS2 molecules using the energy minimization approach for each sequence, the mfold web server (http://mfold.rna.albany.edu/) (Zuker, 2003) and the University of Vienna's RNAfold web server (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi) (Hofacker, 2003 ; Gruber et al., 2008) were employed.

4. 3 . 5 COI haplotype analysis

Relationships of different haplotypes based on *cox-1* nucleotide sequences were visualized using an automated haplotype network layout and visualization software, HapStar, downloaded at http://fo.am/hapstar (Teacher & Griffiths, 2011).

4. 4 Results

4. 4. 1 Prevalence of gastrointestinal parasites in gorillas and humans residents at MDNP

From microscopic examination of 205 fecal samples from GG gorillas and 30 fecal samples from gorillas belonging to other groups or solitary individuals, strongylid eggs were found at highest frequency (average 20.0%) among helminth (Table 10). Since fecal samples examined in my study could not be traced back to individual animals, and GG members were approximately 20 in total number, these prevalence data represent repeated examinations – on average 10 times- at different opportunities. In 71 villagers living near MDNP, whipworm infection was the most common helminthiasis, being prevalent in all four age and sex categories of people (23.1-47.4%; Table 10). Strongylid eggs were also detected in the fecal samples of villagers, but mainly in men over 40 years of age (29.2%)

In order to identify the strongylid species, coproculture of 229 fecal samples from gorillas and 19 fecal samples from villagers was performed (Table 11). Representing the highest frequency (44.1%), *Oesophagostomum* larvae were detected in 101 coprocultures from gorillas, whereas hookworm larvae were found in 52 coproculture (22.7%). Filariform *Strongyloides* larvae were found in 35 coprocultures from gorillas (15.3%). Partial 18S rDNA fragments, 1,142 bp in length, were successfully sequenced for 14 coprocultures of the feces obtained from gorillas, in addition to two coprocultures of the feces of

chimpanzees. All these 16 sequences were absolutely identical to each other, and to a deposited 18S rDNA sequence of *Strongyloides fuelleborni* from a Japanese mammalogist visiting Tanzania (DDBJ/EMBL/GenBank accession no. AB453320; Hasegawa et al., 2010). Identities of the newly obtained sequence with those previously recorded from a gorilla and a chimpanzee at MDNP (accession nos. AB453322 and AB453321, respectively) were 99.64% and 99.38%, respectively. From coprocultures of 19 villagers, only hookworm larvae and *Strongyloides* larvae were detected at rates of 57.9% (11/19) and 21.1% (4/19), respectively. Species identification of these nematodes was not performed in the present study.

Sheathed *Oesophagostomum* larvae obtained from coproculture of gorillas' feces had characteristic morphological features: triangular intestinal cells, a very long and finely tapered sheath tail, and prominent transverse striations on the sheath throughout most of its length. These characters were used to differentiate them from hookworm larvae. Larvae excluding the sheath were 597-927 (average 689 [n=7]) μm long by 20-24 (22) μm wide. Larvae including the sheath were 791-977 (879) μm long by 28-32 (30) μm wide. The buccal cavity was short, 8.5 μm in depth, the oesophagus was strongyliform, 136-152 (143) μm long, the nerve ring was located 88-113 (103) μm from the anterior end; the excretory pore was found 80-102 (91) μm from the anterior end; triangular intestinal cells were 30 to 32 in number; tails were almost conical, 61-72 (66) μm long; and the tail part of sheaths was 202-347 (242) μm long.

4. 4. 2 ITS1-5.8S-ITS2 rDNA sequences of *Oesophagostomum* larvae

An 827/828-bp long fragment was successfully amplified in 29 of 56 larval samples containing 4-34 (average 13.4) larvae/coproculture. Using these amplicons, 68 clones for 21 larval were established and sequenced; 3-6 clones for 12 samples, two clones for six samples, and one clone for three samples.

Concurrently, three clones from the coproculture of a chimpanzee's fecal sample (6 larvae) were obtained.

Amplicons contained 353/354-bp long ITS1, 151-bp long 5.8S rDNA, and 227-bp long ITS2 sequence, based on putative secondary structure of each region of rDNA (Fig. 14). The majority of clones showed an identical nucleotide sequence regardless of their origins (Table 12): 42/68 (61.8%) for ITS1, 61/67 (91.0%) for ITS2, and almost all (95.6%) for 5.8S rDNA. In comparison to retrieved ITS2 sequences of nine *Oesophagostomum* spp., the sequences I obtained in the present study were almost identical to that of *O. stephanostomum* recovered from a chimpanzee in Tanzania (DDBJ/EMBL/GenBank accession no. AF136576), except for three polymorphic nucleotide positions 122, 182 and 203 in the deposited sequence (Table 12). Closely related ITS sequences were found in primate nodular worms, such as *O. bifurcum* (AF136575, Y11733) and *O.* cf. *aculeatum* (AB586134), and a nodular worm of sheep and goats, *O. columbianum* (AJ006150). However, the ITS2 sequences of two other *Oesophagostomum* spp. of sheep and goats (*O. asperum* and *O. venulosum*) were rather different, as were species of pigs (*O. dentatum* and *O. quadrispinilatum*) and cattle and water buffaloes (*O. radiatum*). Positions of recorded nucleotide substitutions and indels of *O. stephanostomum* and *O. bifurcum* are plotted on the putative secondary structure of ITS2 (Fig. 14B), demonstrating the conservation of its main stem structure in such primate nodular worms. As far as examined at present, the ITS1 sequence of *Oesophagostomum* spp. showed lower interspecific variations than the ITS2 region.

4. 4. 3 COI mtDNA sequence of *Oesophagostomum* larvae

An 895-bp fragment containing 46-bp long primer regions was successfully amplified in 16 of 56 larval samples containing 6-25 (average 12.5)

larvae/coproculture. Using these amplicons, 36 clones for 12 larval samples were established and sequenced; 3-5 clones for 6 samples, 2 clones for 4 samples, and one clone for 2 samples. In addition, 5 clones from the coproculture of a chimpanzee's fecal sample were sequenced.

Forty-one newly obtained COI sequences of 849-bp length, coding 283 amino acids (AA), showed 25 different sequences due to nucleotide variations at 68 positions; 6 positions for the first nucleotide of the codon, 5 positions for the second, and 57 positions for the third. Using the first and second nucleotides for each codon of the *cox-1* gene, 12 haplotypes of 597-bp length were determined and their relationships were analyzed along with 2 haplotypes of *O. dentatum* and a single haplotype of *O. quadrispinulatum* (Table 13; Fig. 15A). A part of coprocultured larvae from a chimpanzee showed an identical haplotype with coprocultured larvae from three fecal samples from gorillas. In order to include more sequences from the two latter species, another shorter COI fragment of 392-bp length from the three *Oesophagostomum* spp. was retrieved from the DDBJ/EMBL/GenBank databases and trimmed by removing the third nucleotide for each codon; 4 haplotypes of *O. dentatum* (20 sequences) and 3 haplotypes of *O. quadrispinulatum* (6 sequences). Coprocultured *Oesophagostomum* larvae examined in my study showed 9 haplotypes (41 sequences) (Fig. 15B). Haplotypes of each *Oesophagostomum* sp. were closely related, although *O. dentatum* showed two groups of haplotypes with at least three nucleotide differences through trimmed sequences of 262-bp length. Irrespective of such a higher number of nucleotide variations, 27 clones had the same AA sequence and the remaining 14 clones had eight different AA sequences (Table 13).

4. 5 Discussion

In the present study, coprology and coproculture were applied to survey helminth infection of western lowland gorillas at MDNP, Gabon, and villagers

living near MDNP. Although strongylid infection was the most prevalent in both gorillas and villagers, gorillas were infected with *O. stephanostomum* and hookworms, whereas villagers were infected solely with hookworms. *Oesophagostomum* spp. are the most common nematode of Old World monkeys and apes, including lowland gorillas. Freeman et al. (2004) reported a high prevalence (100%) of strongyles / trichostrongyles for their samples from western lowland gorillas at Bai Hokou, Central African Republic. From non-invasive parasitological surveys of chimpanzees and seven other non-human primate species at Kibale National Park, Uganda, high prevalences of *Oesophagostomum* eggs were observed (8.3–85%) (Krief et al., 2005; Bezjian et al., 2008; Ghai et al., 2014). From similar surveys of chimpanzees in Gombe Stream and Mahale National Parks, Tanzania, fairly high prevalences of *Oesophagotomus* eggs were recorded (38–91%) (File et al., 1976; Huffman *et* al., 1996, 1997). For *Oesophagostomum* samples collected from Mahale chimpanzees, the species identification as *O. stephanostomum* had been made based on morphology of expelled adult worms in the feces after natural medical plant use of the primate, as well as coprocultured larvae (Huffman et al., 1996, Huffman & Caton, 2001).

Recent molecular genetic studies have demonstrated that the ITS2 nucleotide sequence of rDNA allows an unequivocal identification of a range of strongylid nematode species, irrespective of the developmental stage of parasites. Based on this molecular strategy of species identification, we identified the prevalent helminth parasite of western lowland gorillas at MDNP as *O. stephanostomum*, in addition to the *Oesophagostomum* morphology of coprocultured L3 larvae. Prior to the present study, a single ITS2 nucleotide sequence of *O. stephanostomum* was deposited in the DDBJ/EMBL/GenBank databases (accession no. AF136576). This sequence was based on three worms collected from a chimpanzee in Tanzania (Gasser et al., 1999b), and it contained

polymorphic bases at just three positions throughout its entire length. Furthermore, our preliminary direct sequencing of the ITS1–5.8S–ITS2 rDNA of seven coproculture samples generated two relatively clear forward sequences and five problematic forward sequences due to overlapped nucleotides by reading slips for all lengths, whereas all reverse sequences were relatively clear (unpublished data). Based on these findings, we assumed the presence of genetic variations in the ITS region and attempted to clarify the range and rate of nucleotide sequence diversity of *O. stephanostomum* found in gorillas at MDNP using the technique of DNA cloning. As shown in Table 12, a nucleotide insertion at a position between 6 and 7 of ITS1, which was found at a frequency of 13/68 (19.1%), could cause unsuccessful direct forward sequencing of ITS1–5.8S–ITS2 of the parasite. It is not clear whether the observed variations were intraindividual or interindividual ones, because we extracted parasite DNA from 4–34 worms for PCR amplification of the ITS1–5.8S–ITS2 region. Nevertheless, the ITS region of *O. stephanostomum* at MDNP was variable to some extent; 1–3 nucleotide variations occurred in the ITS1 region at a rate of 35.3% and 1–2 nucleotide variations occurred in the ITS2 region at a rate of 10.3%. In contrast to our study, the quite recent work by Ghai et al. (2014) found, however, no nucleotide variations or polymorphic nucleotide sites in the ITS2 region of *O. stephanostomum* collected from 8 chimpanzees, 5 blue monkeys, 2 black and white colobus, 2 gray-cheeked mangabey, 3 red colobus and 12 red-tailed quenon at Kibale National Park, Uganda, and all sequences deposited by them to the DDBJ/EMBL/GenBank databases are similar to the most prominent *O. stephanostomum* ITS2 sequence in western lowland gorillas in our study (see Table12).

Nucleotide sequences of the partial *cox-1* gene, 849 bp in length, showed a high number of variations, particularly at the third nucleotide of codons. Indeed, substitutions of the first and second nucleotides of codons were quite limited

(16.2% of all substitutions). Consequently, despite a high number of genetic variations, AA sequences of the parasites were well conserved (Table 13). Regarding the 113th AA (Leu), two alternative codon usages of *O. stephanostomum* in gorillas at MDNP, i.e. UUR and CUA, were found. However, as with other different substitutions of a minority of clones, its significance with respect to population genetics is currently unknown. In the case of *O. dentatum*, we found that four haplotypes appeared to be divided into two groups (Fig. 15B). Although this segregation has no geographical distinction (Lin *et al.,* 2012b**)**, it is worth pursuing the background of this genetic divergence through the collection of more parasite samples from across the world. In contrast to these nucleotide divergences within species or between species, we found that AA sequences were often shared by different species or were different even within species (Table 13). There was no relationship between *O. bifurcum cox-1* haplotype groupings and the specific primate host infected (de Gruijter et al., 2002), in contrast to the genetic segregation of this species to each host species, as demonstrated by amplified fragment length polymorphism and random amplified polymorphic DNA analyses (de Gruijter et al., 2004, 2005; reviewed by Gasser et al., 2006, 2009). Species identification or phylogenetic study of *Oesophagostomum* spp. using *cox-1* sequences should therefore be conducted with care.

Based on our genetic analyses of the ITS region of rDNA and *cox-1* gene of mtDNA, it is concluded that at MDNP, Gabon, only a single population of *O. stephanostomum* with notable molecular variations is prevalent in western lowland gorillas and probably chimpanzees, without zoonotic complication in local inhabitants. The possible genetic variations of the ITS region of rDNA and *cox-1*gene of mtDNA presented here may be valuable when only a limited amount of material is available for genetic analyses for *O. stephanostomum* species diagnosis. Furthermore, different spectra of minor genetic variations of

such gene sequences might be seen at different localities, since polymorphic nucleotide sites are different between *O. stephanostomum* collected in Tanzania, Uganda and Gabon (Gasser et al., 1999b; Ghai et al., 2014; the present study). Or, as having been demonstrated by Ghai et al. (2014), genetic approaches can definitely find cryptic *Oesophagostomum* sp. For the morphological characterization of such new genotype(s) of the species, we might use living adult nematodes expelled by apes in the process of self-medication, i.e. leaf-swallowing, as was done previously (Huffman *et* al., 1996, 1997; Huffman & Caton, 2001).

Table 10. Microscopic examination of fecal samples from gorillas and human residents at MDNP, Gabon*

Host	Western lowland gorillas (n=235)		Villagers living near MDNP (n=71)			
	Habituated Group Gentil (GG)	Other groups or solitary	Men, >40-yr-old	Women, > 40-yr-old	Youths, 16 to 30-yr-old	Children, <15-yr-old
No. of samples examined	205	30	24	19	15	13
Strongylid eggs	37 (18.0%)	10 (33.3%)	7 (29.2%)	1 (5.3%)	0	1 (7.7%)
Strongyloides eggs	13 (6.3%)	0	0	0	0	0
Ascaris eggs	1 (0.5%)	0	1 (4.2%)	3 (15.8%)	2 (13.3%)	1 (7.7%)
Trichuris eggs	1 (0.5%)	1 (3.3%)	10 (41.7%)	9 (47.4%)	6 (40.0%)	3 (23.1%)
Fasciola-like trematode eggs	12 (5.9%)	0	1 (4.2%)	0	0	0
Streptopharagus eggs	1 (0.5%)	0	0	0	0	0
Schistosoma haematobium eggs	0	0	0	1 (5.3%)	0	0
Troglodytella abrassarti	86 (42.0%)	9 (30.0%)	0	0	0	0

*Number of positive samples (%).

Table 11. Coproculture of fecal samples from gorillas and human residents at MDNP, Gabon*

Host	Western lowland gorillas (n=229)		Villagers living near MDNP (n=19)			
	Habituated Group Gentil (GG)	Other groups or solitary	Men, >40-yr-old	Women, > 40-yr-old	Youngs, 16 to 30-yr-old	Children, <15-yr-old
(No. of samples examined	198	31	11	1	5	2
Oesphagostomum larvae	88 (44.4%)	13 (41.9%)	0	0	0	0
Hookworm larvae	46 (23.2%)	6 (19.4%)	7 (63.6%)	0	2 (40.0%)	2 (100%)
Strongyloides larvae	33 (16.7%)	2 (6.5%)	3 (27.3%)	0	1 (20.0%)	0

*Number of positive samples (%).

Table 2. Nucleotide variation in the ITS1 and 5.8S (A) and ITS2 (B) regions of rDNA of *Oesophagostomum stephanostomum* at MDNP, Gabon, and *Oesophagostomum bifurcum*

(A) ITS1 and 5.8S rDNA

Species	Locality	Host (No. of fecal samples)	No. of clones	DDBJ/EMBL/GenBank accession no.	Reference	ITS1*							5.8S*		
						6/7	7	14	137	138	202-204	233	76	83	91
O. stephanostomum	MDNP, Gabon	Gorillas (18) and chimpanzee (1)	42	AB821013-AB821017	The present study	–	A	C	C	C	GCC	T	A	A	A
O. stephanostomum	MDNP, Gabon	Gorilla (1)	1	AB821018	The present study	.	.	.	.	.	...	.	.	.	G
O. stephanostomum	MDNP, Gabon	Gorilla (1)	1	AB821019	The present study	.	.	.	.	.	...	.	G	.	.
O. stephanostomum	MDNP, Gabon	Gorillas (6)	8	AB821020, AB821021	The present study	C	C	.	.	.	...	.	.	.	.
O. stephanostomum	MDNP, Gabon	Gorilla (1)	1	AB821022	The present study	C	C	.	.	.	...	.	.	G	.
O. stephanostomum	MDNP, Gabon	Gorillas (3)	4	AB821023, AB821024	The present study	.	.	G	.	.	...	.	.	.	.
O. stephanostomum	MDNP, Gabon	Gorilla (1) and chimpanzee (1)	3	AB821025, AB821026	The present study	T	.	.	.	.	...	.	.	.	.
O. stephanostomum	MDNP, Gabon	Gorilla (1)	3	AB821027	The present study	.	.	G	.	.	...	A	.	.	.
O. stephanostomum	MDNP, Gabon	Gorilla (1)	3	AB821028	The present study	.	.	.	.	.	---	.	.	.	.
O. stephanostomum	MDNP, Gabon	Gorilla (1)	1	AB821029	The present study	.	.	.	T	T	...	.	.	.	.
O. stephanostomum	MDNP, Gabon	Gorilla (1)	1	AB821030	The present study	T	.	.	T	T	...	.	.	.	.

(B) ITS2

Species	Locality	Host (No. of fecal samples)	No. of clones	DDBJ/EMBL/GenBank accession no.	Reference	ITS2*																
						34	54	62	82	100	105	111	118	122	123	137	162	165	168	179	182	203
O. stephanostomum	MDNP, Gabon	Gorillas (20) and chimpanzee (1)	61	AB821013, AB821017-AB821021, AB821023, AB821024, AB821026-AB821030	The present study	G	T	G	G	T	C	G	A	A	G	A	C	C	C	T	G	C
O. stephanostomum	MDNP, Gabon	Gorilla (1)	3	AB821015	The present study	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.
O. stephanostomum	MDNP, Gabon	Gorilla (1)	2	AB821014, AB821025	The present study	A	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.
O. stephanostomum	MDNP, Gabon	Gorilla (1)	1	AB821016	The present study	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.
O. stephanostomum	MDNP, Gabon	Chimpanzee (1)	1	AB821022	The present study	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.
O. stephanostomum	Kibalek, Uganda	Chimpanzee (8), blue monkeys (5), black and white colobus (2), gray-cheeked mangabey (2), red colobus (3), and red-tailed guenon (12)	32	KF250585-KF250588, KF250592, KF250594, KF250637-KF250644, KF250647, KF250648**	Ghai *et al.*, 2014	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
O. stephanostomum	Tanzania	Chimpanzee (1)	3	AF136575***	Gasser *et al.*, 1995b	.	.	.	.	.	.	.	.	W	.	.	.	.	.	.	R	M
O. bifurcum	Togo	Human (1)	1	Y11733***	Ob10; Romstad *et al.*, 1997	A	.	R	A	G	Y	R	R	T	R	.	T	.	Y	A	.	.
O. bifurcum	Togo	Human (2)	2	Y11733***	Ob11, Ob14; Romstad *et al.*, 1997	A	.	M	A	G	T	R	.	T	.	.	T	.	.	A	.	.
O. bifurcum	Togo	Human (1)	1	Y11733***	Ob12; Romstad *et al.*, 1997	A	.	A	A	G	Y	R	R	T	R	.	T	.	.	A	.	.
O. bifurcum	Ghana and Togo	Human and Mona monkey	9	AF136575***	T1; Gasser *et al.*, 1999a	A	.	A	A	G	T	A	.	T	.	.	T	.	.	A	.	.
O. bifurcum	Ghana	Human	3	AF136575***	T3; Gasser *et al.*, 1999a	A	.	R	A	G	T	A	.	T	R	.	T	.	.	A	.	.
O. bifurcum	Ghana and Togo	Human	1	AF136575***	T4; Gasser *et al.*, 1999a	A	.	C	A	G	T	A	.	T	.	.	T	.	.	A	.	.
O. bifurcum	Ghana	Mona monkey	1	AF136575***	T2; Gasser *et al.*, 1999a	A	.	.	A	G	.	.	.	T	R	.	T	.	.	A	.	.
O. bifurcum	Togo	Human	2	AF136575***	T5; Gasser *et al.*, 1999a	A	.	.	A	G	.	.	G	T	A	.	T	.	.	A	.	.
O. bifurcum	Togo	Human	1	AF136575***	T6; Gasser *et al.*, 1999a	A	Y	.	A	G	Y	.	R	T	.	.	T	.	.	A	.	.
O. bifurcum	Togo	Human	9	AF136575***	T7; Gasser *et al.*, 1999a	A	.	.	A	G	Y	R	R	T	R	.	T	.	.	A	.	.
O. bifurcum	Togo	Human	1	AF136575***	T8; Gasser *et al.*, 1999a	A	Y	M	A	G	T	A	.	T	.	.	T	.	Y	A	.	.
O. bifurcum	Togo	Human	1	AF136575***	T9; Gasser *et al.*, 1999a	A	.	.	A	G	Y	.	R	T	R	.	T	.	.	A	.	.

* Nucleotide position is expressed relative to the 5'-terminus of each region of *O. stephanostomum* (DDBJ/EMBL/GenBank accession no. AB821013). Dots denote an identical base to that of the uppermost sequences, and gaps are indicated by '—'.

** There are additional sequences such as KF250595, KF250598, KF250599, KF250605, KF250606, KF250612-KF250619, KF250631, KF250634, KF250635 and KF250653.

*** Representative sequence deposited by the original authors.

Table 13. Amino acid (the first and second nucleotides of a codon) variation in a partial *cox-1* region of mtDNA of *Oesophagostomum stephanostomum* at MDNP, Gabon, and two other *Oesophagostomum* spp.

Species	Haplotype	DDBJ/EMBL/GenBank accession no.	Host (No. of fecal samples)	Reference	No. of clones	Amino acid (AA) position*																				
						3	35	37	43	44	54	66	90	113	125	187	188	189	194	204	205	230	242	244	275	279
O. stephanostomum	S1	AB821032	Gorillas (6)	The present study	15	Asp (GAU)	Val (GUU)	Met (AUA)	Trp (UGA)	Thr (ACU)	His (CAY)	Leu (CUU)	Ser (AGA)	Leu (UUR)	Leu (UUR)	Leu (CUR)	Gly (GGU)	Met (AUR)	Leu (UUR)	Trp (UGC)	Ala (GCY)	Val (GUS)	Thr (ACW)	Phe (UUU)	Ser (UCU)	Leu (UUR)
O. stephanostomum	S2	AB821033	Gorilla (1)	The present study	2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	Leu (CUG)
O. stephanostomum	S3	AB821034; AB821035	Gorillas (3) and chimpanzee (1)	The present study	9	.	.	.	.	.	.	.	.	Leu (CUA)	.	.	.	.	.	.	.	.	.	.	.	.
O. stephanostomum	S4	AB821036	Gorilla (1)	The present study	1	.	.	.	.	.	.	.	.	Leu (CUA)	.	Leu (UUA)	.	.	.	.	.	.	.	.	.	.
O. stephanostomum	S5	AB821037	Gorilla (1)	The present study	1	.	.	.	.	.	.	.	.	Leu (CUA)	.	.	.	.	.	.	.	.	.	Tyr (UAU)	.	.
O. stephanostomum	S6	AB821038	Gorilla (1)	The present study	1	.	.	.	.	.	.	.	.	Leu (CUA)	.	.	.	.	.	.	.	.	.	.	Pro (CCU)	.
O. stephanostomum	S7	AB821039	Gorilla (1)	The present study	1	.	.	.	.	Ala (GCU)	.	.	.	Leu (CUA)	.	.	.	.	.	.	.	.	.	.	.	.
O. stephanostomum	S8	AB821040	Gorilla (1)	The present study	1	.	.	.	Arg (CGA)	.	.	.	Thr (ACA)	.	.	.	.	.	.	.	.	.	.	.	.	.
O. stephanostomum	S9	AB821041	Gorilla (1)	The present study	2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	Met (AUG)	.	.	.	.	.
O. stephanostomum	S10	AB821042	Gorilla (1)	The present study	5	.	.	Thr (ACA)	.	.	.	.	.	Leu (CUA)	.	.	.	.	.	.	Asp (GAU)	.	.	.	.	.
O. stephanostomum	S11	AB821043	Gorilla (1)	The present study	1	.	.	Thr (ACA)	.	.	.	.	.	.	.	.	.	.	.	Ter (UAG)	.	.	.	.	.	.
O. stephanostomum	S12	AB821044	Chimpanzee (1)	The present study	2	.	.	.	.	.	Arg (CGC)	.	.	Leu (CUA)	.	.	.	Thr (ACG)	.	.	.	.	.	.	.	.
O. dentatum	D1	GQ888716	An experimentally infected pig in Denmark	Jex et al., 2010	–	.	.	.	.	.	.	Leu (UUA)	.	.	Leu (CUG)	Leu (UUG)	.	.	Leu (CUA)	.	.	.	.	.	.	.
O. dentatum	D4	FM161882	A slaughtered pig in China	Lin et al., 2012a	–	.	.	.	.	.	.	Leu (UUA)	.	.	.	Leu (UUG)	Asp (GAU)	.	.	.	.	.	.	.	.	.
O. quadrispinulatum	Q2	FM161883	A slaughtered pig in China	Lin et al., 2012a	–	Asn (AAU)	Ala (GCU)	.	.	.	.	Leu (UUA)	.	.	Leu (CUU)	Leu (UUA)	.	.	.	.	.	.	Ala (GCU)	.	.	.

* Nucleotide position is expressed relative to the 5'-terminal of each region of *O. stephanostomum* (DDBJ/EMBL/GenBank accession no. AB821032). Dots denote an identical base to that of the uppermost sequence. Distinct AA from the uppermost sequence is written in squares.

Figure 13. Localization of Moukalaba-Doudou National Park (2°26'S, 10°25'E), in southern Gabon (Ando et al., 2008)

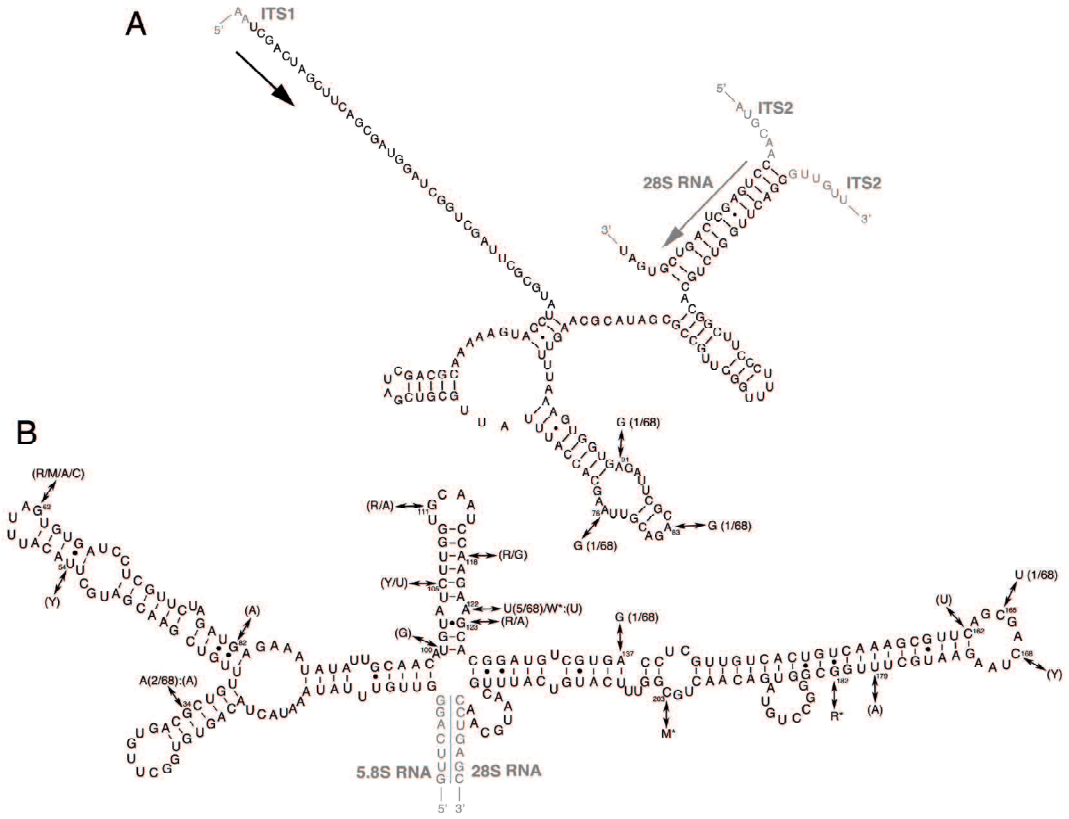

Figure 14. Putative secondary structure of the 5.8S rDNA (**A**) and ITS (**B**) of *O. stephanostomum* (DDBJ/EMBL/GenBank accession no. AB821013), with indications of possible nucleotide substitutions in *O. stephanostomum* (nucleotide followed by a frequency in parentheses): *O. bifurcum* (nucleotide in parenthese). Substitutable nucleotide with an asterisk are those reported by Gasser et al. [1999b] (refer to Table 12 for details). M=A or C; R=A or G; W= A or U; and Y= C or U.

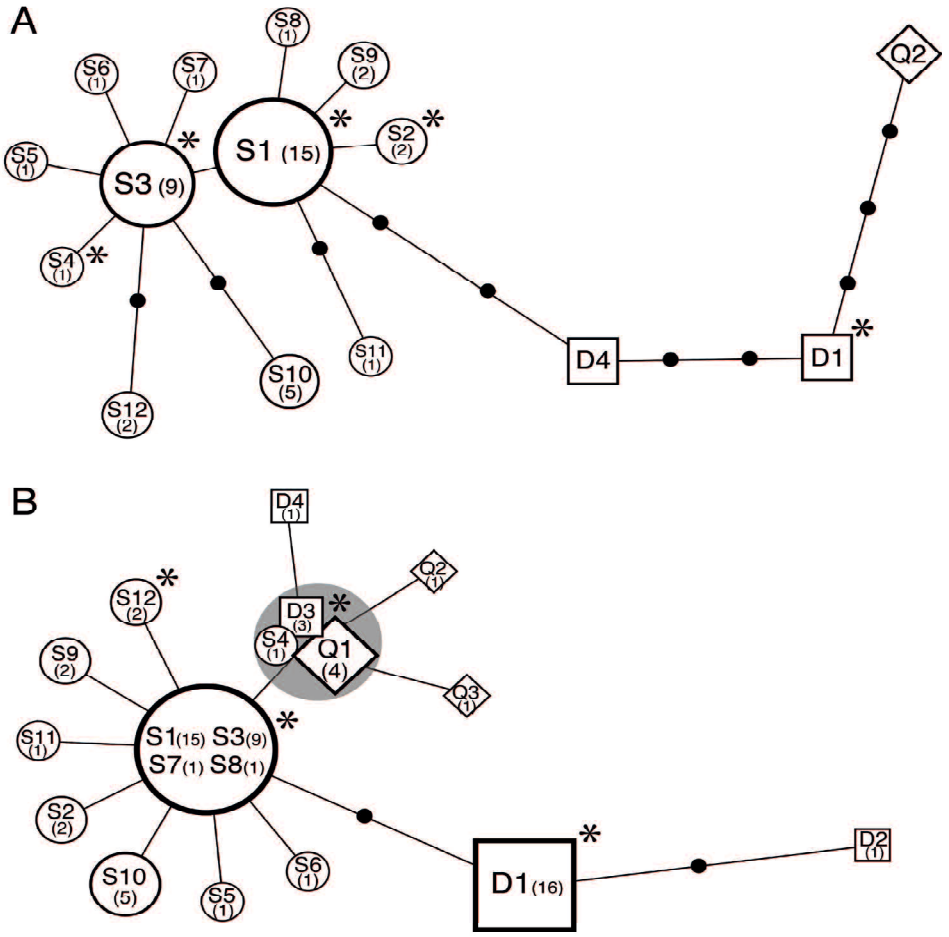

Figure 15. Relationship of COI haplotypes of three *Oesophagostomum* spp. based on trimmed 597-bp long szquences (**A**) and trimmed 262-bp long sequences (**B**). *O. stephanostomum*, ○ (S1-S12 haplotypes); *O. dentatum*, □ (D1-D4 haplotypes); and *O. quadrispinulatum*, ◇ (Q1-Q3 haplotypes). Numbers in parentheses after the haplotypes indicate the number of clones. Asterisks denote an identical AA sequence shared by different haplotypes.

General discussion and conclusion

The gullet worm, *Gongylonema pulchrum* Molin, 1875, is a thread-like spirurid nematode found worldwide. A variety of mammals, such as cattle, sheep, goats, camels, pigs, equines, cervids, rodents, bears and primates, serves as the definitive host and dung beetles plays a role as the intermediate host (Lichtenfels, 1971; Kirkpatrick et al., 1986; Anderson, 1992). More than one hundred cases of accidental human infection with the gullet worm have been also recorded (reviewed by Xu et al., 2000; Haruki et al., 2005). Furthermore, successful experimental infections of *G. pulchrum* L3 of ruminant origin have been reported in rabbits, guinea-pigs and rat (Lucker, 1932; Alicata, 1935; Kudo et al., 2003, 2005). Based on the apparent low host specificity of *G. pulchrum*, it is reasonable to speculate that the transmission cycle of the gullet worm may be shared by domestic and wild mammals living sympatrically through accidental ingestion of infected dung beetles serving as an intermediate host.

In the study described in Chapter I, I challenged the aforementioned speculation based on multiple circumstantial evidences by analyzing directly the rDNA and COI of mtDNA of the gullet worm specimens from cattle, sika deer, wild boars, Japanese macaques, a feral Reeves' muntjac and captive squirrel monkeys in order to elucidate the transmission dynamics of *G. pulchrum* in domestic and wild ruminants and other wild animals in Japan. Finally, I could demonstrate for the first time that the rDNA genotypes as well as the COI mtDNA haplotype of *G. pulchrum* prevalent in wild animals and domestic/captive mammals in Japan are clearly different. This finding also indicates that *G. pulchrum* distributed in Japan has multiple origins.

Despite independent transmission cycles in nature of several rDNA genotypes and COI haplotypes of *G. pulchrum* in Japan, I did not identify a distinct population within the examined gullet worms based on morphological

and genetic perspectives, and their phenotypic and genetic characters were essentially well conserved. To pursue the phylogeography of the worldwide distribution of different *G. pulchrum* COI haplotypes or rDNA genotypes, I examined the morphology and genetic background of gullet worms collected from water buffaloes (*Bubalus bubalis*) in Nepal as described in Chapter II.

The latest taxonomy recognizes three *Gongylonema* spp. dwelling in the epithelium of the upper digestive tract of ruminants: *G. pulchrum* Molin, 1857 in the tongue, mouth mucosa, and esophagus of various mammals worldwide; *G. verrucosum* Giles, 1892 in the rumen, reticulum, and omasum of various ruminants from India, Africa, and North America; and *G. monnigi* Baylis, 1926 in the rumen of sheep from Africa. The two latter species are morphologically differentiated from *G. pulchrum*, even if considering a wide spectrum of morphometric values of *G. pulchrum* growing in different host species (Lichtenfels, 1971). As evident from Table 6 and Figure 5, the sizes of body and other structures, excluding the left spicule, were comparable between worms collected from buffaloes and cattle, whereas the left spicule length of worms from buffaloes was comparable to the distinctly smaller worms collected from deer. Since there are only a few reports on the prevalences of *G. pulchrum* in buffaloes and no morphometrics of the worm from them, my observation that *G. pulchrum* in buffaloes from Nepal has a markedly shorter left spicule relative to its body size requires confirmation in the same host species from different areas or in different host species from the same area. The rDNA and COI nucleotide sequences of the gullet worms isolated from buffaloes in Nepal were close to, but distinct from, *G. pulchrum* analyzed in cattle and sika deer in Japan. Coupled with the rather shorter spicule, the gullet worms found in buffaloes from Nepal might be considered as a distinct population of *G. pulchrum*, or a cryptic *Gongylonema* species. Here, I would like to emphasize that so-called "*G. pulchrum*" that exhibits a cosmopolitan and inhabits a wide range of mammalian

hosts is not a homologous species, but rather is composed of multiple populations or cryptic species showing minor but distinct morphological variation(s) as well as genetic divergences.

In Chapter III, I focused on the epizootic outbreak of sarcoptic mange involving a variety of wildlife and domestic mammals, like endemic scabies in humans (Arlian, 1989; Burgess, 1994; Bornstein et al., 2001; Pence & Ueckermann, 2002; Walton et al., 2004b; Walton & Currie, 2007; Currier et al., 2012). Host-specific varieties of morphologically indistinguishable *Sarcoptes scabiei* (Acari: Sarcoptidae) occur in different animal species, exhibiting a low degree of cross infectivity in general (Fain, 1968, 1978; Samuel, 1981). The disease severely debilitates and reduces populations of affected wild mammals (León-Vizcaíno et al., 1999; Bornstein et al., 2001; Pence & Ueckermann, 2002; Ryser-Degiorgis et al., 2002; González-Candela et al., 2004; Rossi et al., 2007; Soulsbury et al., 2007; Oleaga et al., 2008; Jimenez et al., 2010; Sarasa et al., 2011; Millán et al., 2012). Molecular studies focusing on mtDNA genes such as 12S and 16S ribosomal RNA and COI detected three major groups of *S. scabiei* consisting of human-derived mites from Panama, human-derived mites from Aboriginal communities in northern Australia, and a mixed group of animal- and human-derived mites (Berrilli et al., 2002; Skerratt et al., 2002; Walton et al., 2004a).

In the study conducted, pieces of crusted and/or alopetic skin tissues were collected from 120 raccoon dogs, three raccoons, six Japanese badgers, one Japanese marten, one stray dog, four wild boars, and one Japanese serow mainly in an area where mangy wild animals have been increasingly noted in the last four years. The majority of mites had almost identical ITS2 nucleotide sequences to those recorded in a variety of mammals worldwide. Partial 16S and *cox-1* fragments of mtDNA amplified and sequenced successfully, demonstrating that the two mtDNA haplotypes of Japanese *S. scabiei*

preferentially colonized wild mammals in certain geographical units. These results suggest that heterologous populations of monospecific *S. scabiei* are expanding their populations and distributions regardless of host species in an apparently local mange epizootic of wild mammals in Japan.

In the study described in Chapter IV, I conducted a field survey of helminth infection of western lowland gorillas at MDNP, Gabon, and villagers living near MDNP. Although strongylid infection was the most prevalent in both gorillas and villagers, gorillas were infected with *O. stephanostomum* and hookworms, whereas villagers were infected solely with hookworms. Based on our genetic analyses of the ITS region of rDNA and COI gene of mtDNA, it is concluded that at MDNP, Gabon, only a single population of *O. stephanostomum* with notable molecular variations is prevalent in western lowland gorillas and probably chimpanzees, without zoonotic complication in local inhabitants.

Various studies have reported the value of ribosomal DNA sequencing to allow the detection of cryptic species (Chilton et al., 1995; Hung et al., 1996). For instance, Chilton et al. (1995) determined the sequence of ITS2 for three members of the nematode *Hypodontus macropi* complex from three species of Australian macropodid marsupial (*Petrogale persephone*, *Macropus robustus robustus* and *Thylogale billardierii*). Sequences difference between nematode from the different host species ranged from 25.0 to 28.3%, suggesting that *H. macropi* from *P. persephone* represents a different species to those of in *M. robustus robustus* and *Thylogale billardierii*. The unique feature of this study is that it represents a comparison of the rDNA sequences of nematode species which are morphologically indistinguishable but genetically distinct. In this study, a distinct genetic population *G. pulchrum* was isolated from buffaloes in Napel. This population exhibited minor but distinct morphological variation(s) as well as genetic divergences to *G. pulchrum* from cattle and deer. Although, the morphological variations require confirmation in the same host species from

different areas or in different host species from the same area, the so-called "*G. pulchrum"* may represent a cryptic species. This result demonstrates clearly that morphological features alone are often inadequate for species identification.

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