Morphological and molecular study on gastrointestinal parasites of Asian elephants in Myanmar

(ミャンマーのアジアゾウにおける消化管内寄生虫に関する形態学的および分子学的研究)

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ABBREVIATIONS

IUCN	International Union for Conservation of Nature
MTE	Myanma Timber Enterprise
HYG	Hmaw Yaw Gyi
TK	Taung Kya
DNA	deoxyribonucleic acid
PCR	polymerase chain reaction
ITS	internal transcribed spacer
COI	mitochondrial cytochrome *c* oxidase subunit I
DDBJ	DNA Data Bank of Japan
EMBL	European Molecular Biology Laboratory
MEGA	Molecular Evolutionary Genetics Analysis
ML	maximum likelihood
CDS	coding sequence
EMBOSS	European Molecular Biology Open Software Suite
Mya	million years ago
SE	standard error
mm	millimeter
µm	micrometer
No.	number
sp.	species

GENERAL INTRODUCTION

Elephants which were gradually spread to all northern continents and South America, originated in Africa in the late Eocene Epoch. Despite of numerous proboscideans development, many species became extinct. Nowadays, African savannah elephant, *Loxodonta africana*, African forest elephant, *Loxodonta cyclotis*, and Asian elephant, *Elephas maximus* are the only species surviving into the current epoch (Fowler, 2006; Roca et al., 2015). Two elephant genera, *Elephas* (Asia) and *Loxodonta* (Africa), were considered to diverge 5 million years ago, and African savannah elephant (*L. africana*) and forest elephant (*L. cyclotis*) diverged approximately 2.63 (± 0.94) million years ago from fossil evidence (Roca et al., 2015).

E. maximus is recognized as an endangered species and listed in the International Union for Conservation of Nature (IUCN) Red list. They are extant in Bangladesh, Bhutan, Cambodia, China, India, Indonesia, Lao, Malaysia, Myanmar, Nepal, Sri Lanka, Thailand, and Vietnam (https://www.iucnredlist.org). In Myanmar, elephants have been domesticated and are used for timber logging. There are approximately 3,000 captive Asian elephants and half of those are owned by the governmental institution, Myanma Timber Enterprise (MTE) (www.mte.com.mm). As Asian elephants are an endangered species, both wild and captive elephants must be well cared for ensuring their health (Leimgruber at el., 2011). Advanced biological and medical studies, including investigations of pathogens, are valuable for elephant conservation (Fowler and Mikota at el., 2006).

Asian and African elephants harbor the same genera of parasites, but species found are usually different (Fowler, 2006). Gastrointestinal parasites are extremely

common and cause gastritis and enteritis, especially in younger elephants (Leimgruber et al, 2011; Oo, 2012). The parasites in the gastrointestinal tract including many species of nematodes, trematodes and bot fly larvae often produce a protein-losing gastroenteropathy, causing hyperalbuminemia, anaemia, enteritis and even death in younger elephants (Fowler, 2006).The nematode species and bot fly larvae reported in *E. maximus* and *L. africana* are listed in Table 1 (Fowler, 2006). Among them, the most common parasites found in gastrointestinal tracts are cyathostimine nematodes and stomach bot fly larvae according to the report of MTE when the veterinarians diagnose the cause of death in MTE elephants by post mortem examinations. They note as only cyathostomine nematode and stomach bot fly infection in their records and they have not identified the species of the parasites.

From captive and zoo Asian elephant populations in India and China, the following species of nematodes belonging to subfamily Cyathostominae, were reported: six *Murshidia* species, *M. elephasi*, *M. falcifera* (*M. falcifer*), *M. indica*, *M. murshida*, *M. lanei*, and *M. neveulemairei* (*M. neveu-lemairei*), five *Quilonia* species, *Q. edentata*, *Q. guptai*, *Q. renniei* (*Q. quilona*), *Q. simhai*, and *Q. travancra*, and one species of *Khalilia*, *K. pileate* (Lane, 1914; Ware, 1924; Witenberg, 1925; Wu, 1934; Gupta and Jaiswal, 1984; Gupta and Trivedi, 1984; Zhang and Xie, 1992). No other species of cyathostomine nematodes so far have been reported in Asian elephants. On the other hand, 17 species of *Murshidia*, eight species of *Quilonia* and one species of *Khalilia* have been listed in African elephants (Fowler, 2006). All of the cyathostomine nematodes found in African elephants are different from those found in Asian elephants.

In the family Gastrophilidae, *Cobboldia elephantis* which has been reported in different parts in India (Javare Gowda at el., 2017; Panda at el., 2005; Venu at el.,

2015), Thailand (Sanyathitiseree at el., 2009) and Indonesia (Matsuo and Suprahman, 1997), is the only one stomach bot fly found in Asian elephants. *Rhodhainomya roverei* and *Platycobboldia loxodontis* (*Cobboldia loxodotis*) parasitize in the stomach of African elephants. Adult flies of *Cobboldia* species lay their eggs near the mouth or base of the tusks and the larvae hatch in the mouth cavity and later move to the stomach causing gastric myiasis (Cobbold, 1882; Fowler and Mikota at el., 2006).

Diagnostic keys for species identification of such parasites are provided with old illustrations in the form of line drawings. Accordingly, the identification of parasite species requires expert skills in morphology. It can be considered that photomicrographs of key structures for parasite identification can facilitate diagnosis. Unfortunately, the photomicrographs have been reported only on several species such as *M. falcifera* and *M. neveulemairei* found in Thailand (Carreno et al., 2001) and *Q. travancra* from Indonesia (Prahardani et al., 2019). If the photomicrographs of many species of parasites are available, it can assist diagnosis with lesser requirement of expert skills in morphology.

Alternatively, genetic tools have a potential for diagnostic identification of parasite species (McLean et al., 2012) in which expert skill in morphology is no longer required. The nucleotide sequence of some cyathostomine nematode species have been reported in African savannah elephants. The nucleic ribosomal DNA (ITS1, 5.8S and ITS2) and mitochondrial cytochrome *c* oxidase subunit I gene (*COI*) were determined for three species of *Murshidia (M. africana*, *M. linstowi*, and *M. longicaudata*), one species of *Quilonia (Q. africana)*, and one species of *Khalilia (K. sameera*) (McLean et al., 2012). Yan et al. (2019) also reported the mitochondrial genetic data of *C. loxodontis* in the evolutionary study of stomach bot flies. However,

there is no genetic information of cyathostomine nematodes and stomach bot flies of Asian elephants.

Considering those situations, this study was aimed to investigate what species of cyathostomine parasites and bot flies are prevalent in Asian elephants in Myanmar; to provide the photomicrographs of morphological key structures that can facilitate the diagnosis of parasite species and to obtain the genetic information of those parasites of which nucleotide sequences are deposited for molecular diagnosis and the phylogenetic relationship is analyzed with the sequences of the parasites found in African elephants.

For these reasons, in chapter I, the nematode worms were collected from faeces of Asian elephants in two elephant camps in Myanmar, and identified in morphology with providing photomicrographs of key structures. Then, the mitochondrial gene sequences of the parasite species were analyzed and compared to those of related species from African elephants.

In chapter II, a third-instar fly larva excreted from a captive Asian elephant imported to Maruyama zoo from Myanmar was collected. The larva was identified for the species by morphological characteristics and the photomicrographs were taken. Then, the phylogenetic relationship of the bot fly larva and related species of African elephants and other animals was evaluated based on the *COI* gene sequences.

Table 1. Nematode species in order Strongylida and arthropod species in order Diptera reported in *Elephas maximus* and *Loxondata africana.*

Parasites	*Elephas maximus*	*Loxondata africana*
Nematodes in order Strongylida	**Family Strongylidae** **Subfamily Strongylinae** *Chonianguin* *C. epistomum* *C. magnostomum* *Equinubria* *E. spunculiformis* *Decrusia* *D. additictia* *D. decrusi* **Subfamily Cyanthostominae** *Murshidia* *M. falcifera* *M. neveu-lemairei* *M. murshida* *M. elaphasi* *M. indica* *M. lanei* *Quilonia* *Q. renniei* *Q. travencra* *Khalilia* *K. pileata* **Family Ancylostomidae** *Bunostomum* *B. foliatum* Bathmostumum *B. saneri* *Grammocephalus* *G. varedatus* *G. hybridatus* **Family Syngamidae** *Mammomonogamus* *M. indicus* **Family Atractidae** *Leiperenia* *L. galebi*	**Family Strongylidae** **Subfamily Strongylinae** *Chonianguin* *C. algericum* *Equinubria* *Decrusia* **Subfamily Cyanthostominae** *Murshidia* *M. linstowi* *M. hadia* *M. longicaudata* *M. brachyscelis* *M. africana* *M. anisa* *M. dawoodi* *M. omoensis* *M. brevicapulatus* *M. memphisia* *M. aziza* *M. loxodontae* *M. soundanensis* *M. brevicaudata* *M. vuylstekae (cyclotis)* *M. witenbergi (cyclotis)* *Quilonia* *Q. apiensis* *Q. africana* *Q. uganda* *Q. brevicauda* *Q. ethiopica* *Q. khalili* *Q. loxodontae* *Q. magna* *Khalilia* *K. sameera* **Family Ancylostomidae** *Bunostomum* *B. brevispiculum* *B. hamatum* *Gammocephalus* *G. clathrates* *G. intermedius* **Family Syngamidae** *Mammomonogamus*

		M. loxodontus **Family Atractidae** *Leiperenia* *L. leiperi* *L. morelli*
Arthropods in order Diptera, flies	**Family Oestroidae** **Subfamily Gasterophilinae** *Cobboldia elephantis*	**Family Oestroidae** **Subfamily Gasterophilinae** *Platycobboldia loxodontis* *Rhodhainomya roverei* *Ruttenia loxodontis* *Neocuterebra squamosa*

CHAPTER I

Morphological and molecular identification of cyathostomine gastrointestinal nematodes of *Murshidia* and *Quilonia* species from Asian elephants in Myanmar

1. Introduction

Gastrointestinal nematode parasites have long been recognized in Asian elephants. The most common parasites belong to the subfamily Cyathostominae of the family Strongylidae, which are small to medium-sized with a cylindrical buccal capsule surrounded by coronal leaflets. In subfamily Cyathostominae, six species of *Murshidia*, five species of *Quilonia* and one *Khalilia* species have been found in Asian elephants, *Elephas maximus* (Lane, 1914; Ware, 1924; Witenberg, 1925; Wu, 1934; Gupta and Jaiswal, 1984; Gupta and Trivedi, 1984; Zhang and Xie, 1992) and 17 species of *Murshidia*, eight species of *Quilonia* and one species of *Khalilia* have been listed in African elephants, *Loxodonta africana* (Fowler, 2006). However, no same species are shared by these two elephant species. In Asian elephants, diagnostic keys of those parasites are provided from old illustrations in the form of line drawings. There are very few photomicrographs of key structures for morphological identification of *Mushidia* and *Quilonia* species found in Thailand and Indonesia (Carreno et al., 2001; Prahardani et al., 2019). However, the prevalence of those parasites has not been studied in Myanmar. Concerning with the molecular information, five cyathostomine nematode species were genetically identified in African savannah elephants (McLean et al., 2012). In Asian elephants, the genetic information of cyathostomine nematodes is not still available.

Thus, the study in chapter I aimed to investigate what species of cyathostomine nematodes are prevalent in Asian elephants in Myanmar, to provide the morphological key structures with photomicrographs and finally to deposit the gene sequences for molecular diagnosis and analyze the phylogenetic relationship of those parasites between Asian and African elephants.

2. Materials and methods

2.1 Sample collection

The fresh elephant faecal boluses were collected as described by Lyndale et al. (2015). Faeces were collected in December 2018 from two elephants in the Taung Kya (TK) elephant camp (N19°55', E96°30') and from eight elephants in the Hmaw Yaw Gyi (HYG) elephant camp (N18°22', E96°24') one day after oral albendazole administration at a dose of 5-7 mg/kg of body weight. Faecal boluses were kept in a cooler box and transported to a laboratory at the University of Veterinary Science, Nay Pyi Taw. Each faecal bolus in a container was diluted with a large amount of tapped water. Nematode worms were searched using naked eyes in diluted faecal materials and placed parasites in Petri dishes using forceps. Most worms were already dead or immobile, but some were still alive and motile. The worms were separated into males and females, and a total of 47 adult worms (22 males and 25 females) that seemed to be alive and not denatured, were randomly selected. The worms were fixed with absolute alcohol for subsequent morphometric, morphological, and genetic examinations. After measuring worm body length and width, the worms were dissected into three parts, anterior, middle, and posterior portions. Anterior and posterior parts were cleared in glycerol and examined under a light microscope. The middle parts were used for DNA isolation.

2.2 Morphological and morphometric identification

The morphometric data and optical micrographs of parasites were taken using an Olympus SZX16 stereo microscope, CKX41 inverted microscope, and BX50 upright microscope (Olympus Corp., Tokyo, Japan), equipped with a DP26 digital camera (Olympus) and cellSens. The morphological identification of the parasite

species were performed by comparing to previous descriptions (Lane, 1914; Witenberg, 1925; Van der Westhuysen, 1938; Chabaud, 957; Popova, 1965; Prahardani et al., 2019).

2.3 DNA extraction, PCR, and sequencing

The total genomic DNA was extracted from the middle parts of all 47 specimens using a DNA extraction kit (DNeasy Blood & Tissue Kit, Qiagen, Hilden, Germany) and DNA concentration was measured using a NanoDrop 2000 (ThermoFisher Scientific, MA, USA). To amplify the *COI* gene, two new primers were designed as COIQF (5' CCACCAGTTCTAGGATCAAA 3') and COIQR (5' CTATTATTACGGCTCATGC 3') based on the alignment sequences of mitochondrial *COI* genes of cyathostomine nematodes from African elephants (McLean et al., 2012) because the preliminary experiments showed that some specimens were not amplified using PCR with the universal barcoding primers, LCO-1490 and HCO-2198 (Folmer et al, 1994). The PCR mixture contained 0.3 μM of each primer, 10 ng/μL of template DNA, 0.025 U/μL of Tks GFlex^{TM} DNA polymerase (Takara Bio Inc., Tokyo, Japan), and 1× GFlex buffer in a volume of 10 μL. After initial denaturation at 94°C for 1 min, the reaction was carried out with 40 cycles of denaturation at 98°C for 10 s, annealing at 52°C for 15 s, and extension at 68°C for 45 s, followed by a final extension at 68°C for 5 min, with the SimpliAmp thermal cycler (Applied Biosystems Japan, Tokyo, Japan). PCR products were run on 1.2% agarose gels, stained with GelRed Nucleic Acid Staining Solution (Biotium, Hayward, CA, USA), and photographed under LED light. PCR products were purified with NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel AG, Düren, Germany) and subjected to direct sequencing with COIQF and COIQR primers using

an Applied Biosystems 3130 Genetic Analyzer with a Big Dye v3.1 Terminator cycle sequencing kit (Applied Biosystems, Inc., Foster City, CA, USA).

2.4 Phylogenetic analysis

The phylogenetic analysis was conducted for the *COI* gene nucleotide sequences of all 47 nematodes. The ingroup comprised of *K. sameera*, *M. africana*, *M. linstowi*, *M. longicaudata*, and *Q. africana* from African elephants. *Strongyloides stercoralis* was included as an outgroup. The *COI* sequences were aligned using the MUSCLE (codon) option within MEGA7 (Kumar et al., 2016), and constructed a maximum likelihood (ML) tree. The Tamura-Nei+G model was adapted as it showed the best fit to all datasets. Bootstrap values were determined by 500 replicates.

The genetic distance and diversity of *COI* genes were analyzed using MEGA7 with the Tamura-Nei model (Tamura and Nei, 1993). Pairwise sequence comparisons were made to determine the average number of base-pair differences per site between all groups. Standard error was calculated with 1,000 bootstrap replications. The analysis between groups involved 47 nucleotide sequences in 411 positions in the final dataset. The average number of base-pair differences per site within each group in 471 positions in the final dataset was also analyzed. The nucleotide sequences of *COI* genes determined in this study are available in DDBJ/EMBL/GenBank databases under accession numbers LC513767-86, 88, 89, 91-99, LC513800-4, 6-16.

3. Results

3.1 Morphometric description and morphological identification

A total of 115 male and 158 female worms were obtained from faeces of 10 elephants, and approximately, the half of the parasites was collected from one elephant (TK-1) in TK camp (Table 1.1). Among them, morphometric data of 47 parasites that seemed alive and not denatured are shown in Table 1.2. Five cyathostomine nematode species, *M. falcifera* (Figure 1.1), *M. indica* (Figure 1.2), *M. neveulemairei* (Figure 1.3), *Q. renniei* (Figure1.4), and *Q. travancra* (Figure 1.5) were identified. The number of parasites identified was three for *M. falcifera*, one for *M. indica*, 10 for *M. neveulemairei*, 29 for *Q. renniei*, and four for *Q. travancra* (Table 1.1).

3.2 Phylogenetic analysis

The PCR amplifications produced ~469 bp nucleotide sequences for the *COI* gene of *Murshidia* and *Quilonia* species with a primer set of COIQF and COIQR. A ML phylogenetic tree constructed from CDS (~453 bp) of the *COI* genes of 47 specimens from Myanmar elephants and five ingroup sequences from African elephants in GenBank clearly showed that the genera *Murshidia* and *Quilonia* formed in separate clades (Figure1.6). A close genetic relationship was found between *Q. renniei* from Asian elephants and *Q. africana* from African elephants, and likewise between *M. indica* and *M. africana*. However, *M. falcifera* and *M. neveulemaire*i were in different clades from *M. linstowi* and *M. longicaudata* of African elephants. All 29 *Q. renniei* specimens formed one clade that was separated from *Q. africana*. Four *Q. travancra* specimens formed a clade separated from *Q. renniei*.

3.3 Nucleotide diversity

Mean distances of nucleotide sequences of *COI* genes between parasite species in this study were in the range of 0.104–0.166 (Table 1.3). The highest level of interspecific nucleotide diversity (16.6%) was detected between *M. neveulemairei* and *Q. travancra*. The mean diversity (± SE) within species were 0.0071 ± 0.0030, 0.0101 ± 0.0033, 0.0099 ± 0.0019, and 0.0149 ± 0.0040 for three *M. falcifera*, ten *M. neveulemairei*, 29 *Q. renniei*, and four *Q. travancra* specimens, respectively. Namely, the range was 0.71%–1.49% across species, and *Q. travancra* had the highest level of nucleotide diversity.

4. Discussion

In this study, three species of *Murshidia* (*M. falcifera*, *M. indica*, and *M. neveulemairei*) and two species of *Quilonia* (*Q. renniei*, and *Q. travancra*) were detected by morphological and genetic identification from Asian elephants in Myanmar for the first time. The photomicrographs of these adult parasites were shown almost 100 years after their original drawings, but photomicrographs and scanning electron photomicrographs were reported for *Q. travancra* from Sumatran elephants in Indonesia (Prahardani et al., 2019). The photomicrographs taken by optical microscopy here clearly showed species-specific morphological characteristics, including mouth collar, buccal capsule, plumose sculpturing on the oesophagus, and dorsal bursal rays of the parasites.

Phylogenetic analysis of *COI* genes from *Murshidia* species in Asian and African elephants suggested that *M. indica* and *M. africana* were closely related species. The ancestor of these species may have evolved to *M. indica* in *E. maximus* and to *M. africana* in *L. africana*. The early divergence of *M. falcifera* was suggested

taking together with a proposed evolutionary lineage of *M. africana, M. linstowi*, and *M. longicaudata* in African elephants (McLean et al., 2012). This was followed by an ancestor of *M. linstowi* and *M. longicaudata* before *M. neveulemairei*. However, a detailed *Murshidia* lineage will be required for further genetic studies because the lack of *M. elephasi* and *M. murshida COI* gene sequences from Asian elephants and other *Murshidia* species from African elephants. Currently, only three of 17 African *Murshidia* species have accessed *COI* gene sequences (McLean et al., 2012).

Phylogenetic tree of *COI* genes from *Quilonia* species suggested that *Q. renniei* from Asian elephants was closely related to *Q. africana* from African elephant rather than *Q. travancra* from Asian elephants. Likewise, better understanding of the evolutionary lineage between *Quilonia* species in elephants awaits further genetic studies because of the lack of sequence data from *Q. edentata*, *Q. simhai*, and *Q. guptai* from Asian elephants and the other seven *Quilonia* species from African elephants.

The number of coronal leaflets is one of the major taxonomic keys for species identification, but the numbers were very close to each other 20, 24, 20-22, and 18, for *Q. edentata*, *Q. guptai*, *Q. simhai*, and *Q. renniei*, respectively. The morphology of *Q. edentata* was like *Q. simhai* in general features, but it can be distinguished by shapes like cervical papillae, gubernaculum, and buccal capsules in both sexes (Zhang and Xie, 1992). Furthermore, the variation in the number of coronal leaflets in *Q. simhai* was accepted by the authors (Gupta and Trivedi, 1984). Thus, the genetic analysis of *Q. edentata*, *Q. guptai,* and *Q. simhai* will be required to elucidate whether these species are real species or variations in *Quilonia* species. Here, 29 *Q. renniei* specimens showed a lower level of nucleotide diversity in the *COI* gene (0.99 ± 0.19% at 471 positions of CDS sequences) and no obvious separation in

the phylogenetic tree, indicating intraspecific variation in the gene. Meanwhile, for six *Q. africana* specimens from African elephants, intraspecific nucleotide diversity in the *COI* gene was 2.20 ± 0.39% at 655 positions (McLean et al., 2012) and 2.22 ± 0.38% at 654 positions of CDS sequences by the present study. Determination of the precise number of coronal leaflets of these *Q. renniei* specimens using scanning microscopy is needed to clarify whether leaflets variation exists within *Q. renniei* populations in Myanmar. The consistency of ten coronal leaflets in *Q. travancra* was shown using scanning electron microscopy (Prahardani et al., 2019). Thus, further scanning electron microscopic studies will clarify an association between the number of coronal leaflets and genetic separation of *Quilonia* species from Asian elephants.

Two elephant genera, *Elephas* (Asia) and *Loxodonta* (Africa), diverged 5 million years ago, and African savannah elephants (*L. africana*) and forest elephant (*L. cyclotis*) diverged approximately 2.63 (± 0.94) million years ago from fossil evidence (Roca et al., 2015). Here, the molecular evidence of the presence of the same genus but different species of *Murshidia* and *Quilonia* nematode parasites was shown in Asian (*E. maximus*) and African (*L. africana*) elephants.

A much higher number of *Quilonia* species was detected compared to *Murshidia* species in Myanmar elephants. Both *Quilonia* and *Murshidia* species were detected in the HYG elephant camp whereas only *Quilonia* species were found in the TK camp. Particularly, one elephant excreted various *Quilonia* and *Murshidia* species in the HYG camp. The reasons for parasite species geographic separation are unknown. These two elephant camps are located at greater than 150 km distance, and elephants in camps are cared for under semi-captive conditions, in which elephants can eat plants freely in the surrounding forest. More than 30 MTE elephant camps are currently scattered throughout Myanmar and artificial movement of elephants

between camps is common for natural mating. In the preliminary experiments, the *COI* gene sequences from the single third stage larva after the cultivation of elephant faeces, indicate that *Q. renniei* parasites were infested in both TK and HYG camps (unpublished). Thus, development of genetic markers using nematode eggs and subsequent cultured larvae will facilitate identification of parasite species. Nationwide distribution and abundance of cyathostomine parasite species await further epidemiological surveys in different elephant camps of Myanmar.

In conclusion, this study demonstrated the morphological and genetic identification of *Murshidia* and *Quilonia* species from Asian elephants in elephant camps in Myanmar. Phylogenetic analysis of *COI* gene sequences was performed for 47 adult nematode specimens. The results showed that *Murshidia* and *Quilonia* species in Asian elephants were closely related to but different from those in African elephants. Further studies, including genetic analysis of more samples in other elephant camps in Myanmar as well as Asian elephants in other countries, are needed to elucidate control strategies, evolution and parasitism of gastrointestinal parasites in elephants.

5. Summary

Gastrointestinal nematode parasites have long been recognized in Asian elephants. The most common parasites belong to the subfamily Cyathostominae of the family Strongylidae, which are small to medium-sized with a cylindrical buccal capsule surrounded by coronal leaflets. Diagnostic keys of such parasites are provided from old illustrations in the form of line drawings. However, there are very few photomicrographs and no genetic information of these parasites is available. In the present study, 47 adult worm specimens were obtained from faeces of Asian elephants after anthelmintic treatment in two elephant camps in Myanmar. This study provided photomicrographs for five cyathostomine parasites, *Murshidia falcifera*, *Murshidia indica*, *Murshidia neveulemairei*, *Quilonia renniei*, and *Quilonia travancra* almost 100 years after their original drawings. In addition, the mitochondrial *COI* gene sequences of these species were analyzed for genetic information. Phylogenetic analysis of the *COI* genes of *Murshidia* and *Quilonia* species from Asian and African elephants revealed parasite speciation in each elephant host. The results also indicated that several *Murshidia* and *Quilonia* species were harbored in Asian elephants in Myanmar. Further studies are required to provide new insight into control strategies and more species identification are necessary for better understanding of evolution of cyathostomine gastrointestinal parasites in elephants.

Table 1.1. The number of *Murshidia* and *Quilonia* adult worms identified in Asian elephants in Myanmar.

Elephant			No. of worms collected from faeces		No. of worms identified									
ID	Sex	Age in years			*M. falcifera*		*M. indica*		*M. neveulemairei*		*Q. renniei*		*Q. travancra*	
			M[1)]	F[2)]	M	F	M	F	M	F	M	F	M	F
HYG-1	F	4	5	2					2	1				
HYG-2	F	14	12	15	1	2	1		4	2	1	5	2	1
HYG-3	F	58	3	2								1		
HYG-4	M	7	3	5						1		1	1	
HYG-5	M	8	12	23										
HYG-6	F	67	1	1										
HYG-7	F	8	16	21							3	7		
HYG-8	M	1	7	6							1	2		
TK-1	F	30	47	83							2	2		
TK-2	M	6	9								4			
Total			115	158	1	2	1	0	6	4	11	18	3	1

[1)] M, male; [2)] F, female.

HYG, Hmaw Yaw Gyi elephant camp; TK, Taung Kya elephant camp

Table 1.2. Morphometrical data of *Murshidia* and *Quilonia* species from Asian elephants in Myanmar.

Species	*M. falcifera*		*M. indica*	*M. neveulemairei*		*Q. renniei*		*Q. travancra*	
	M (1) [1)]	F (2)[2)]	M (1)	M (6)	F (4)	M (11)	F (18)	M (3)	F (1)
Total length (mm)	24	29.6-32	15.7	17-23	25-28.8	15-17.2	19.7-26.4	16.6-19	25.8
Mean total length (mm)		30.8		21.1	27.2	16	22.7	18	
Maximum diameter (mm)	0.7	1.0-1.3	0.54	0.6-0.8	0.9-1.1	0.6-0.7	0.8-1.1	0.7	0.9
Diameter of head (µm)	245	243	135	134-144	136-149	199-232	242-322	231-261	322
No. of coronal leaflets	~80	~80	~40	~40	~40	12~18	16~18	8~10	8~10
Length of buccal capsule (µm)	114	120-122	64	67-101	84-113	44-79	50-104	53-85	136
Diameter of buccal capsule (µm)	131	111-136	65	53-64	55-65	123-149	158-211	94-121	198
Length of oesophagus (µm)	792	956-1058	517	698-776	724-880	611-667	668-812	746-790	956
Maximum diameter of oesophagus (µm)	238	290-328	146	182-231	192-270	158-234	206-284	175-229	221
Cervical papillae from anterior end (µm)				1154		670			
Excretory pore from anterior end (µm)				1147-1169	1383	509-564	713	909-1228	
Nerve ring from anterior end (µm)	411	497-526	213	353-384	391-413	267-375	317-470	405-413	504
Length of spicules (right) (µm)	1750		939	1213-1338		559-1055		932-958	
Length of spicules (left) (µm)			954	1209-1321		613-1024		937	
Length of gubernaculum (µm)				117-215		87-266		217-248	
Length of female tail (µm)		1763-1880			1326-1584		1527-2177		1600
Vulva from tail end (µm)		2575-3150			1767-2079		4873-6859		2663

[1)] M, male (no. of specimens examined); [2)] F, female (no. of specimens examined).

Table 1.3. Mean distance (± SE) of nucleotides of the *COI* genes between *Murshidia* and *Quilonia* species from Asian elephants in Myanmar.

Species (no. of specimens)	1	2	3	4
1. *M. falcifera* (3)				
2. *M. indica*(1)	0.108 ± 0.018			
3. *M. neveulemairei* (10)	0.104 ± 0.016	0.129 ± 0.020		
4. *Q. renniei* (29)	0.119 ± 0.019	0.129 ± 0.019	0.134 ± 0.020	
5. *Q. travancra* (4)	0.127 ± 0.020	0.124 ± 0.019	0.166 ± 0.023	0.110 ± 0.016

Pairwise sequence comparisons were made to determine the average number of base-pair differences per site between all groups using MEGA7 with the Tamura-Nei model. Standard error was calculated with 1,000 bootstrap replications. The analysis involved 47 nucleotide sequences in 411 positions in the final dataset.

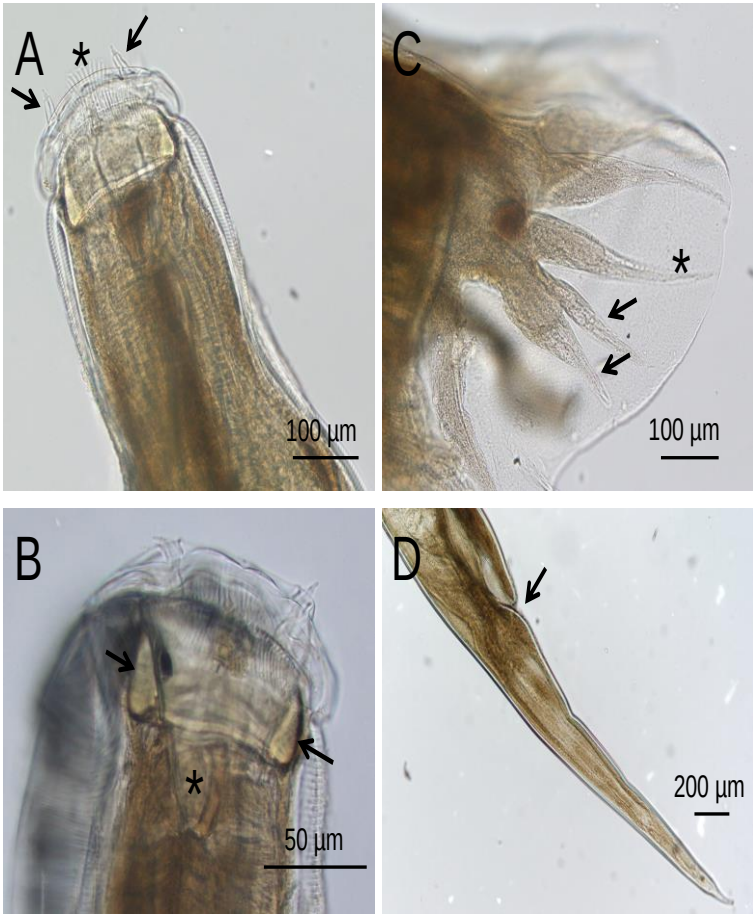

Figure1.1. Photomicrographs of *Murshidia falcifera.*

A, anterior end of a female, showing the appearance of two lateral lips of mouth collar with prominent head papillae (arrows) and coronal leaflets (*); B, head of a male, showing cuticular lining of buccal capsule (arrows) and funnel-shaped throat (*); C, dorsal ray of bursa of a male, showing three branches, in which anterior branch is composed of two sub-branches (arrows) and the posterior one is longer (*); D, posterior end of a female, showing anus (arrow).

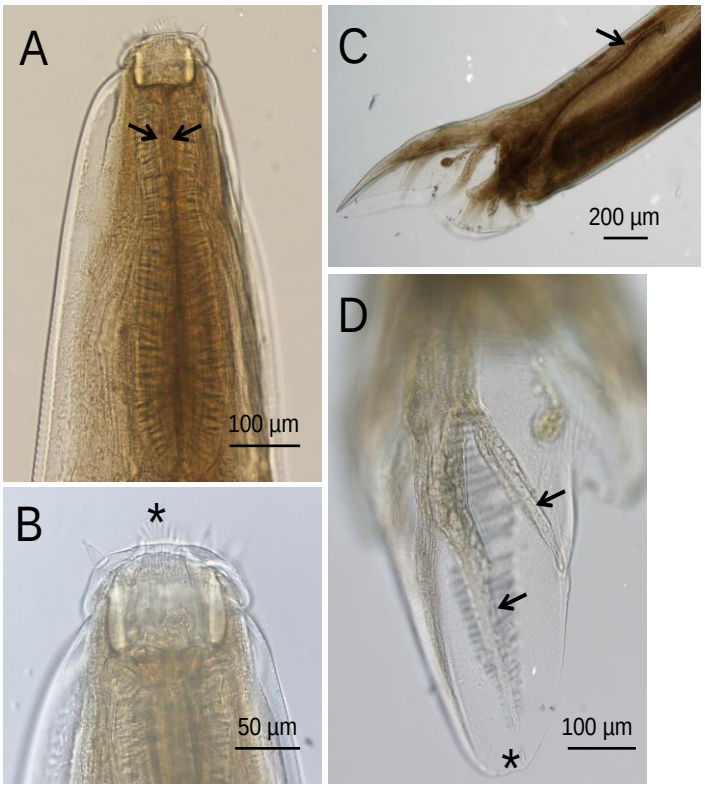

Figure1.2. Photomicrographs of *Murshidia indica*.

A, anterior end of a male, showing the appearance of plumose sculpturing on anterior portion of oesophagus (arrows); B, head of a male, showing coronal leaflets (*); C, copulatory bursa and spicules (arrow) of a male; D, dorsal ray of bursa of a male, showing two branches (arrows), in which the posterior branch has a pointed extremity (*).

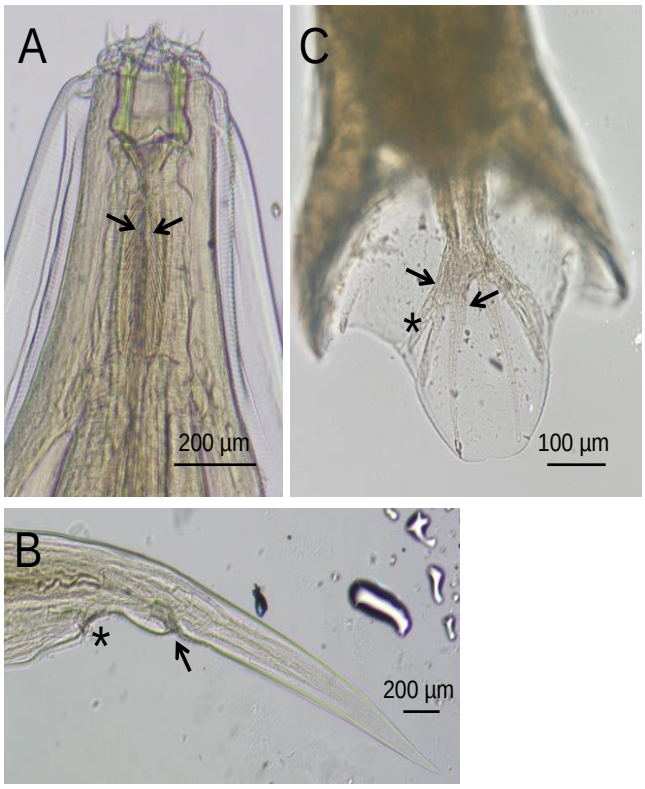

Figure 1.3. Photomicrographs of *Murshidia neveulemairei.*

A, anterior end of a female, showing the appearance of plumose sculpturing on anterior portion of oesophagus (arrows); B, posterior extremity of a female, showing anus (arrow) and vulva (*); C, dorsal ray of bursa of a male, showing two branches (arrows), in which the anterior branch is bifurcated in the distal half (*).

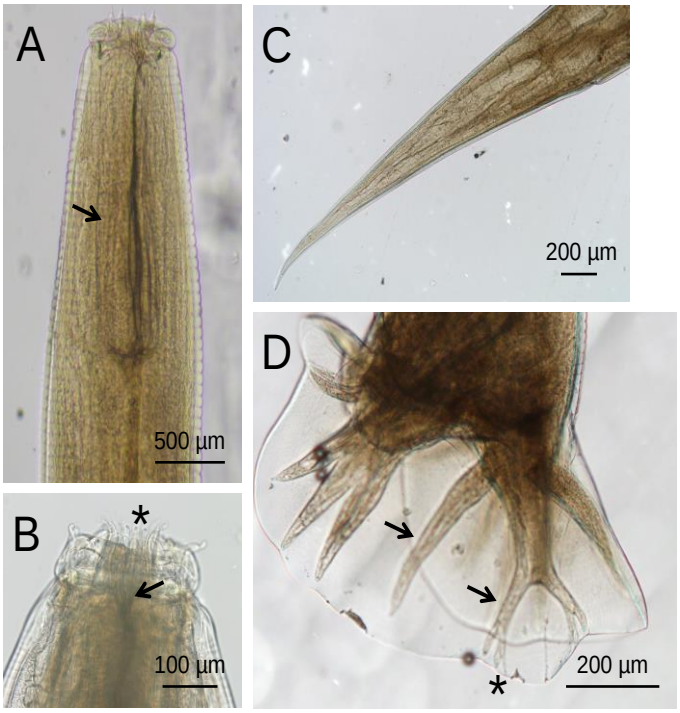

Figure 1.4. Photomicrographs of *Quilonia renniei*.

A, anterior end of a female, showing cylindrical shape of oesophagus (arrow); B, head of a female, showing a small buccal capsule (arrow) and curved coronal leaflets project above head (*); C, posterior end of a female; D, dorsal ray of bursa of a male, showing two branches (arrows), in which the posterior branch divided two sub-branches and the inner sub-branch is slightly bifid at the extremity (*).

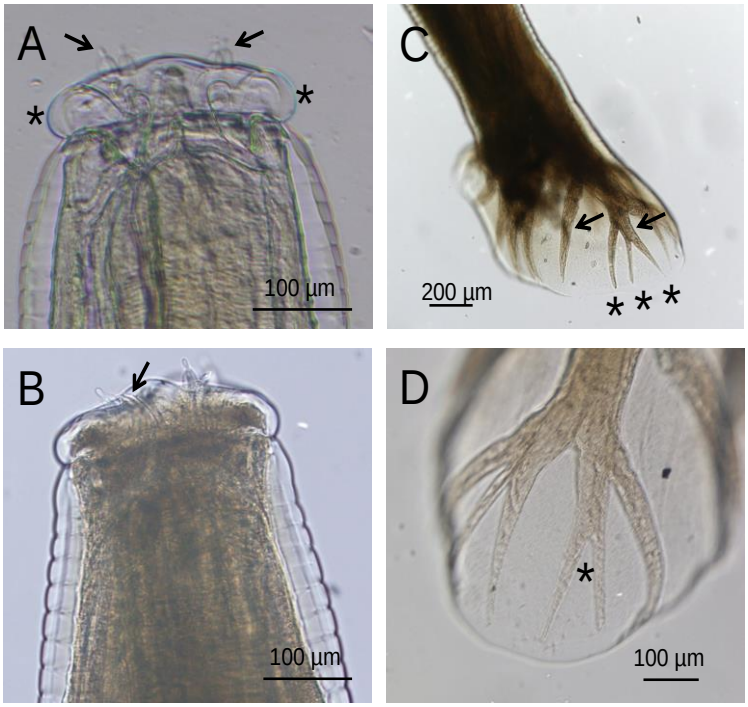

Figure 1.5. Photomicrographs of *Quilonia travancra.*

A, anterior end of a male, showing head papillae (arrows) and mouth collars (*); B, head of a male, showing crown leaflets (arrow); C, bursa of a male, lateral view, showing two branches (arrows) and three sub-branches of the posterior branch at approximately the same length (*); D, posterior branch of dorsal ray of bursa of a male, showing an appearance of trifurcation although the median and internal sub-branches are fused (*).

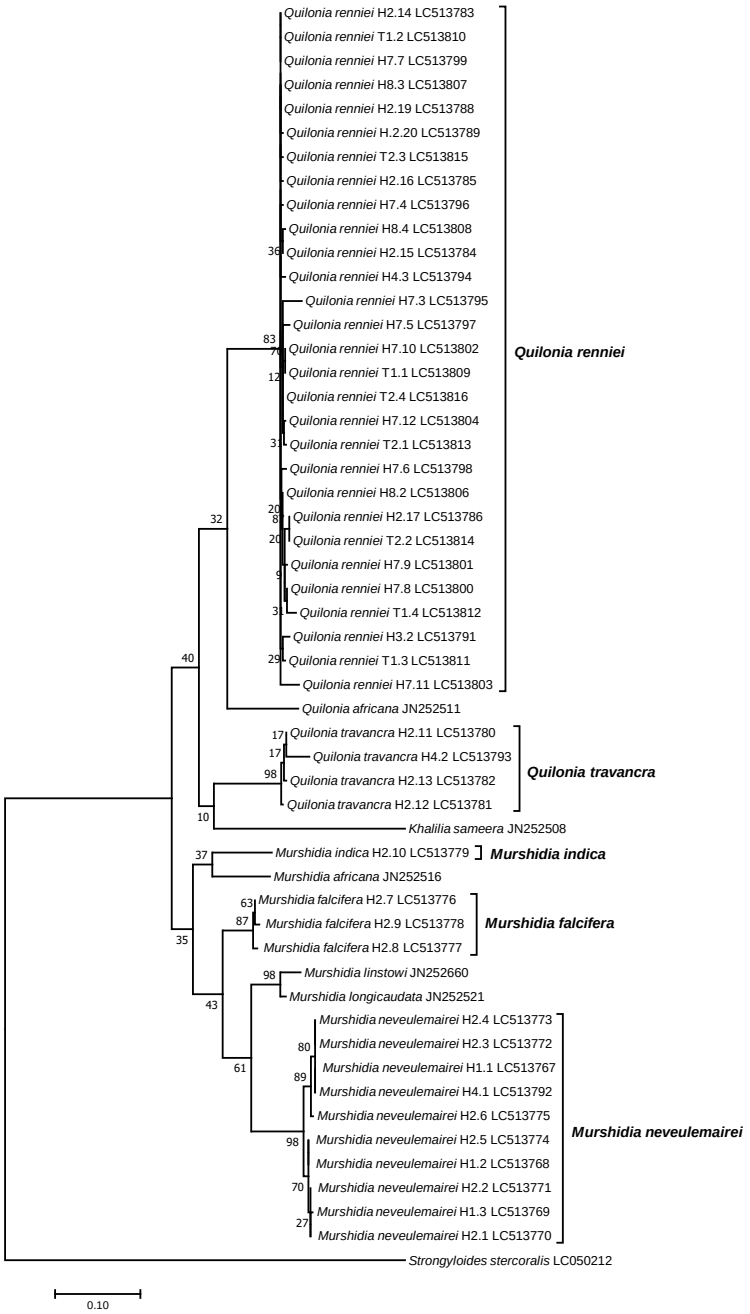

Figure 1.6. Molecular phylogenetic analysis of *COI* gene sequences of cyathostomine species using the maximum likelihood method in MEGA7.

The percentage of trees in which associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. GenBank accession numbers are indicated alongside taxa name.

CHAPTER II

First record and analysis of the *COI* gene of *Cobboldia elephantis* obtained from a captive Asian elephant from Myanmar

1. Introduction

Myiasis is defined as the parasitic infestation of humans and animals with dipterous fly larvae, and has been recognized since ancient times. It causes severe impacts in animal husbandry with significant economic losses in many countries (Francesconi and Lupi, 2012; Zumpt, 1965). The Brachycera contains all species that cause specific myiasis, notably those within the families Muscoidae, Oestroidae, Calliphoridae, and Sarcophagidae (Francesconi and Lupi, 2012). Anatomical classifications of myiasis include sanguinivorous, dermal/subdermal, nasopharyngeal, intestinal, and urogenital infestations (Zumpt, 1965). Among intestinal bot flies, the stomach bot flies belong to three genera, *Gasterophilus, Gyrostigma* and *Cobboldia* (family Oestroidae: subfamily Gasterophilinae) (Zumpt, 1965; Yan et al., 2019). Zumpt (1965) described nine *Gasterophilus* species, which larvae parasitize the alimentary tract of equids, including horses, donkeys, and zebras, and cause often serious pathogenicity for horses. In the three known species of *Gyrostigma*, the third instar larvae develop in the stomach of both white and black rhinoceroses (Zumpt, 1965). In the genus *Cobboldia, Cobboldia elephantis* in Asian elephants, *Cobboldia loxodontis* (*Platycobboldia loxodontis*) in African savannah elephants and *Cobboldia russanovi* in extinct woolly mammoth were reported (Grunin, 1973; Cobbold, 1882). All three stages of larvae (L1, L2, and L3) may be observed on the stomach wall causing gastric myiasis (Fowler and Mikota at el., 2006).

C. elephantis infestations in Asian elephants have been reported in India (Javare Gowda at el., 2017; Panda at el., 2005; Venu at el., 2015), Thailand (Sanyathitiseree at el., 2009) and Indonesia (Matsuo and Suprahman, 1997). However, there is still no report of the bot fly species prevalent in Myanmar and the

genetic information is also lacking in Asian elephants. The purposes of this chapter were to identify the stomach bot fly species collected from a captive Asian elephant with providing the photomicrographs, and to obtain the gene sequences and apply to phylogenetic analysis of bot fly species.

2. Materials and methods

2.1 Sample collection and morphological characterization

Four Asian elephants were imported into Sapporo Maruyama Zoo, Sapporo, Japan on November 30, 2018 from Hmaw Yaw Gyi elephant camp in the Bago region of Myanmar. Because gastrointestinal helminth infections are very common in Myanmar, elephants at each elephant camp are regularly treated with anthelmintic 2–3 times a year. Thus, the four imported elephants had received albendazole and ivermectin approximately two months prior to transportation to Japan, but not after transportation. On April 4, 2019, a fly larva was found on the floor of the elephant house at the zoo. It was thought to have been excreted with the feces of a five-year-old female elephant calf, which had soft stools that time. The dry specimen was morphologically examined and measured under Shimadzu STZ-171 (Shimadzu Corp., Kyoto, Japan) and Olympus SZX16 (Olympus Corp., Tokyo, Japan) stereoscopic microscopes with a DP26 digital camera and CellSens Imaging software (Olympus). The posterior spiracles were observed after fixation of the specimen with 70% alcohol.

2.2 DNA extraction, PCR, and sequencing

Total DNA was extracted from segments 7 and 8 of the larva using a DNA extraction kit (DNeasy Blood & Tissue Kit, Qiagen, Hilden, Germany). Two primer

pairs were initially used to amplify the *COI* gene: the universal barcoding primers LCO-1490 and HCO-2198 (Folmer at el., 1994), and LepF1 and LepR1 designed for insects and amphibians (Hebert, 2004). Subsequently, two new primers were designed, CeF1 and CeR1, by identifying conserved nucleotides in the LepF1 and LepR1 regions of sequences from various myiasis-producing fly species obtained from data bases; *Cephenemyia auribarbis* (KX146909), *Cochliomyia aldrichi* (KX529529), *Cochliomyia hominivorax* (KX529557), *Cochliomyia macellaria* (EU418551), *Cochliomyia minima* (KX529547), *Cuterebra fontinella* (JF439549), *Dermatobia hominis* (JQ246701), *Gasterophilus intestinalis* (AF257117), *Hypoderma bovis* (AF257115), *Hypoderma lineatum* (NC013932), and *Oestrus ovis* (AF257118) (Figure2.1). In addition, an internal primer pair, CeInF1 and CeInR1, were also designed (Figure2.1) as alternatives to MF1 and MH-MR1 described by Haijabaei et al. (2006). Finally, a second internal forward primer CeInF2 (5'-GCTTTCCCACGAATAAATAATT-3') was used to confirm the *COI* gene sequences.

The first round of PCRs was performed using the primer sets LCO-1490 and HCO-2198, or LepF1 and LepR1. The PCR mixture contained 0.3 μM of each primer, 10 ng/μl of template DNA, 0.025 U/μl of Tks GFlex^{TM} DNA polymerase (Takara Bio Inc., Tokyo, Japan) and 1× GFlex buffer in a volume of 10 μl. After an initial denaturation at 94°C for 1 min, the reaction was carried out with 40 cycles of denaturation at 98°C for 10 s, annealing at 52°C for 15 s, and extension at 68°C for 45 s, followed by a final extension at 68°C for 5 min, with the SimpliAmp thermal cycler (Applied Biosystems Japan, Tokyo, Japan). PCR products were run on 1.2% agarose gels, stained with Red Safe Nucleic Acid Staining Solution (iNtRON Biotechnology Inc., Sungnum, Korea) and photographed under LED light.

Subsequent PCRs with primers CeF1 and CeR1 used annealing temperatures of 50°C and 55°C, and were otherwise performed as described above. PCR products were purified with the NucleoSpin® Gel and PCR Clean-up Kit (Macherey-Nagel AG, Düren, Germany) and subjected to direct sequencing with CeF1, CeR1, CeInF1, CeInF2, and CeInR1 primers using an Applied Biosystems 3130 Genetic Analyzer with a Big Dye v3.1 Terminator cycle sequencing kit (Applied Biosystems, Inc., Foster City, CA, USA).

2.3 Phylogenetic analysis

Phylogenetic analysis of *COI* gene sequences from stomach bot flies was conducted referring to Yan et al. (2019). Sequences from *Dermatobia hominis* (human bot fly) and *Hypoderma lineatum* (common cattle grub) were included as outgroups, and the ingroup comprised six *Gasterophilus* bot fly species (*Gasterophilus haemorrhoidalis*, nose bot fly; *G. inermis*; *G. intestinalis*, horse bot fly; *G. nasalis*, nose bot fly; *G. nigricomis*; and *G. pecorum*), one *Gyrostigma* species (*G. rhinocerontis*, Rhinoceros stomach botfly), and two *Cobboldia* species (*C. loxodontis*, African elephant stomach bot fly; and the *C. elephantis*, Asian elephant stomach bot fly sequenced in this study). The *COI* barcoding sequences were aligned using the MUSCLE (codon) option within MEGA7 (Kumar at el., 2016), and maximum likelihood (ML) trees were constructed. The GTR+G+I and mtREV+G models were adapted for nucleotide and amino acid sequences, respectively, as they showed the best fit to all datasets. Bootstrap values were determined by 1,000 replicates. Distance analysis was also performed using MEGA7. The nucleotide sequence of the *COI* gene of *C. elephantis* determined in this study is available in the DDBJ/EMBL/GenBank databases under accession number LC492460.

3. Results

3.1 Morphological characterizations

The larva was approximately 1.5 cm in length and 0.5 cm in width (Figure 2.1A). The body consisted of 12 segments with small spines. A belt-like row of triangular shaped spines was prominent between segments, particularly in abdominal segments 5 to 8 (Figure 2.1A and 2.1B). The anterior end had two powerful curved oral hooks (Figure 2.1C). A cephalo-pharyngeal skeleton was not observed in the raw sample. Six papillae were observed at the posterior end (Figure 2.1D). Posterior spiracles of the larva were not observed in the raw sample, but they were found after removal of the tissue, showing three longitudinal parallel slits (Figure 2.1E). These morphological characteristics were almost identical to published descriptions of *Cobboldia elephantis* (Zumpt, 1965; Cobbold, 1882; Javare Gowda at el., 2017; Panda at el., 2005; Venu at el., 2015).

3.2 PCR and sequencing

The initial PCR amplifications of *C. elephantis* with primer sets LCO-1490 and HCO-2198 or LepF1 and LepR1 were unsuccessful; however, PCR products of the expected size (701 bp) were amplified using the CeF1 and CeR1 primer set annealing temperatures of both 50°C and 55°C.A total of 658 bp of nucleotide sequences (657 bp for coding sequences) were obtained using the CeF1, CeInF2, CeR1, and CeInR1 primers. An NCBI BLAST search revealed that the *C. elephantis COI* gene nucleotide sequences determined in this study showed the highest nucleotide identity (83.1%) to *Aplomya confinis* (KX843839), a northern European tachinid fly species. Pairwise sequence alignment using EMBOSS Needle (https://www.ebi.ac.uk/Tools/psa/ emboss_needle/) revealed that the identity between

the *COI* coding sequences of *C. elephantis* and *C. loxodontis (*Yan et al., 2019) was 76.6% and 83.6% at the nucleotide and amino acid levels, respectively, although the *C. loxodontis COI* gene sequence contained 10 consecutive unknown nucleotides. Mean amino acid p-distances between the bot fly species was shown in Table 2.1. The distances between *C. elephantis* and *Gyrostigma* plus *Gasterophilus* species were in the range of 0.0822 to 0.0959, whereas the distances between *C. loxodontis* and *Gyrostigma* plus *Gasterophilus* species showed much higher values, ranging from 0.1674 to 0.1860. The distances between *Gyrostigma* and *Gasterophilus* species were relatively low, ranging from 0.0183 to 0.0274, although the distance between the *C. elephantis* and *C. loxodontis COI* proteins was 0.1488 (Table 2.1).

3.3 Phylogenetic analysis

A ML phylogenetic tree constructed from protein sequences of the *COI* barcoding region showed that a clade containing the two *Cobboldia* species diverged earlier than *Gyrostigma* and *Gasterophilus* in the phylogenetic lineage of stomach bot flies (Figure2.3).

4. Discussion

The stomach is the intestinal attachment site for third instar fly larvae of *C. elephantis* in Asian elephants, *C. loxodontis* in African elephants, *G. rhinocerontis* in rhinoceros, and *G. intestinalis* in equids. The similarity of the intestinal parasitic lifestyles of these species provides significant insights into the evolution of parasitism. Recently, Yan et al. (2019) described the evolutionary history of stomach bot flies by analyzing the mitogenomes of *C. loxodontis*, *G. rhinocerontis*, and six *Gasterophilus* species. They proposed that attachment of third instar fly larvae occurred initially in the stomach and then moved to duodenal and large intestinal

positions. In the present study, the *COI* gene was sequenced from *C. elephantis*, the Asian elephant stomach bot fly species. Together with the data from Yan et al. (2019), the results of this study revealed that despite relatively low sequence homology between *C. elephantis* and *C. loxodontis*, these two *Cobboldia* species formed a clade distinct from the *Gyrostigma* and *Gasterophilus* species. Phylogenetic analysis inferred a phylogenetic lineage of stomach bot flies from *Cobboldia* to *Gyrostigma* and then *Gasterophilus*. This is consistent with the hypothesis that host shifts of stomach bot flies occurred from elephants to rhinoceroses and then from rhinoceroses to equids and that the flies spread with their hosts from the Afrotropical region into the Palearctic and Oriental regions (Yan et al., 2019).

Mean genetic distance analysis revealed that sequences of the *C. loxodontis COI* gene/protein showed the highest divergence from those of related intestinal bot fly species. However, these results are based on genetic analysis of only one *C. elephantis* larva from Myanmar in the present study and one adult fly of *C. loxodontis* from Namibia (Yan et al., 2019). For better understanding of the evolution of *Cobboldia* species, analysis of a more comprehensive set of samples from different areas worldwide is required.

Considering the evolution of elephants, the divergence date between forest elephant (*Loxodonta cyclotis*) and savanna elephant (*Loxodonta africana*) mitochondrial genomes is estimated as 5.51 (4.26–7.24) Mya, which is comparable to the divergence between Asian elephant and mammoth mitochondrial genomes estimated as 6.01 (4.71–7.17) Mya. Three subspecies of Asian elephant are generally accepted, namely *Elephas maximus maximus*, *E. m. indicus*, and *E. m. sumatranus*, although more than a dozen have been proposed (Roca at el., 2015). Asian elephants are distributed across a large geographical area, including northern and southern

India, Nepal, Bhutan, Myanmar, Laos, Thailand, Cambodia, Vietnam, Peninsular Malaysia, Sumatra, Borneo, and southern China. Asian elephant mitochondrial DNA haplotypes form two major clades, A and B, estimated to have diverged approximately 1.35 Mya (Vidya at el., 2009). It is important to note that there is incomplete geographic partitioning of the two clades, suggesting allopatric divergence and secondary admixture. The allopatric divergence may have occurred in different glacial refugia, with one clade in the Myanmar region and the other clade possibly in southern India–Sri Lanka, with a later isolation in the Sunda Island region in Brunei, east Timor, Indonesia, and Malaysia (Vidya at el., 2009; Zhang at el., 2015). Given the wide geographical distribution of Asian elephants encompassing a variety of climate conditions, genetic variation and haplotype diversity can be expected in *C. elephantis*, as was previously found in *Gasterophilus* populations (Zhang at el., 2018). Furthermore, it is possible that new species or subspecies of *Cobboldia* will be discovered.

In the present study, it was obvious that Asian elephants carried stomach bot fly larvae from Myanmar to Japan despite having been administered ivermectin. Thus, officials should be aware of the risk of importing flies when transporting livestock and wild animals. Veterinary care should be provided before and after animal transport to prevent the spread of parasites.

In conclusion, *C. elephantis*, the stomach bot fly, was reported from a captive Asian elephant bred in Myanmar. Further, the *COI* gene was amplified by PCR using newly designed primers and compared with those sequences from other related fly species. The results showed limited sequence homology between stomach bot fly species from Asian elephants and African elephants, but the two *Cobboldia* species formed a clade distinct from the stomach bot fly species found in rhinoceros and

equids. Further studies, including molecular analysis of stomach bot flies from Asian elephants distributed in other countries, are necessary to elucidate the evolution and parasitism of stomach bot flies in elephants.

5. Summary

The stomach bot fly species in Asian elephants has long been known as *Cobboldia elephantis*. However, there is no genetic information available for this species to date. In this study, a third-instar fly larva was excreted from a captive Asian elephant four months after export from an elephant camp in Myanmar to a zoological garden in Japan. Morphological characteristics of the larva were coincident with published descriptions of *C. elephantis*. The mitochondrial cytochrome *c* oxidase subunit I (*COI)* gene was amplified from the larva by PCR using primers modified from those designed for DNA barcoding of insects and amphibians. The *COI* gene of *C. elephantis* showed 76.6% and 83.6% identity at the nucleotide and amino acid levels, respectively, to that of *C. loxodontis,* the stomach bot fly species in African elephants. Phylogenetic analysis of the *COI* genes of several stomach bot fly species revealed that the two *Cobboldia* species formed a clade separate from the stomach bot fly species found in rhinoceros and equids.

Table 2.1 Mean genetic distances between myiasis-producing fly species on mitochondrial cytochrome *c* oxidase subunit I protein.

Species	DI	HL	GP	GH	GIM	GIN	GNS	GNC	GR	CL
DI (*Dermatobia hominis*)	-									
HL (*Hypoderma lineatum*)	0.0365									
GP (*Gasterophilus pecorum*)	0.0457	0.0502								
GH (*Gasterophilus haemorrhoidalis*)	0.0502	0.0639	0.0137							
GIM (*Gasterophilus inermis*)	0.0502	0.0639	0.0137	0.0000						
GIN (*Gasterophilus intestinalis*)	0.0457	0.0594	0.0091	0.0046	0.0046					
GNS (*Gasterophilus nasalis*)	0.0685	0.0594	0.0274	0.0320	0.0320	0.0274				
GNC (*Gasterophilus nigricornis*)	0.0548	0.0548	0.0228	0.0274	0.0274	0.0228	0.0228			
GR (*Gyrostigma rhinocerontis*)	0.0411	0.0502	0.0274	0.0228	0.0228	0.0183	0.0274	0.0228		
CL (*Cobboldia loxodontis*)	0.1767	0.1767	0.1767	0.1860	0.1860	0.1814	0.1674	0.1767	0.1721	
CE (*Cobboldia elephantis*)	0.0868	0.0913	0.0868	0.0913	0.0913	0.0868	0.0959	0.0822	0.0868	0.1488

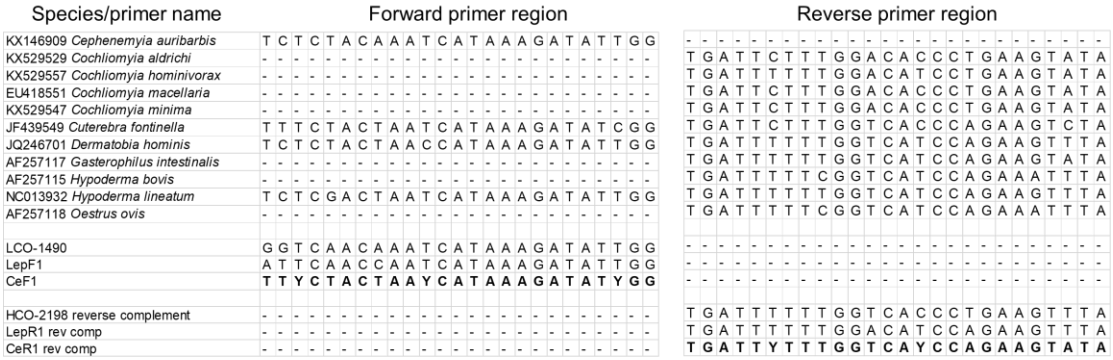

Figure 2.1. Alignment of PCR primer regions for barcoding of the mitochondrial cytochrome oxidase c subunit I (*COI*) genes from some myiasis-producing fly species.

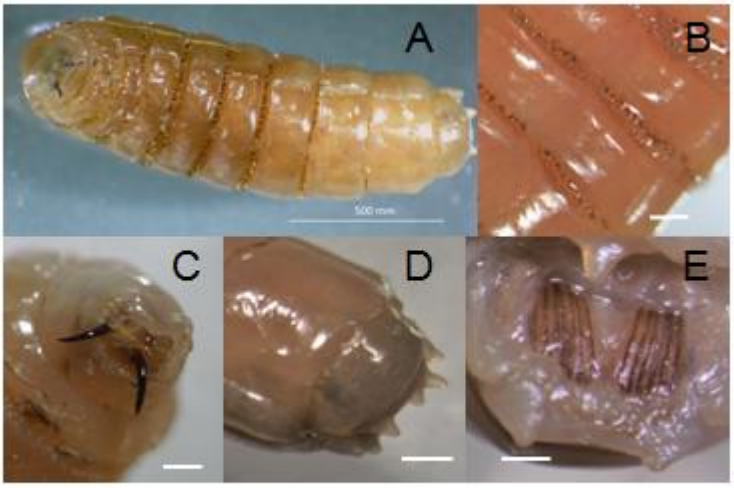

Figure 2.2. Morphological features of the third stage instar larva of *C. elephantis* excreted from a captive elephant from Myanmar.

A: ventral view of whole body, B: spine pattern on the segments, C: oral hooks at the anterior end, D: six papillae at the posterior end, E: posterior spiracles, showing three longitudinal parallel slits. Scale bars in B–E: 1.0 mm.

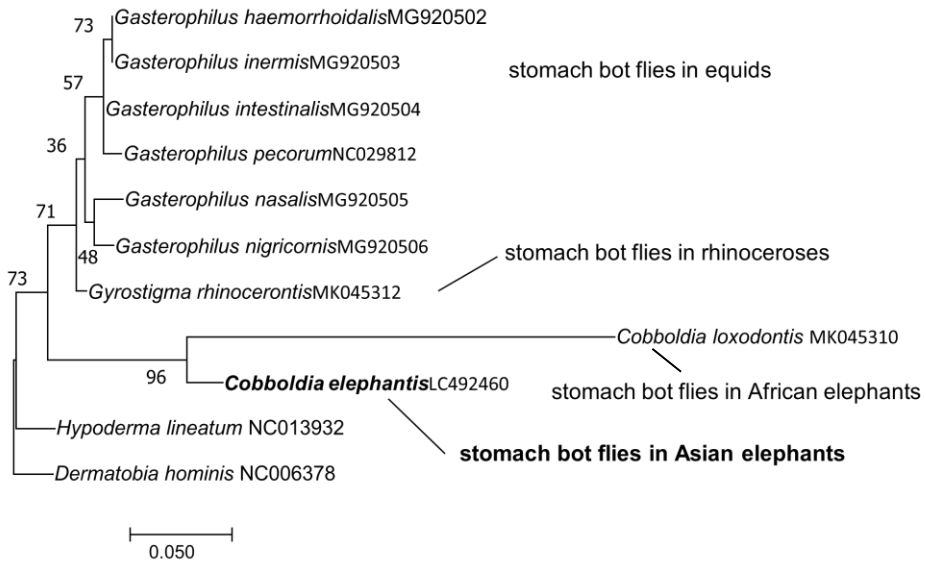

Figure 2.3. Molecular phylogenetic analysis of the COI protein sequences of stomach bot flies using the maximum likelihood method in MEGA7.

The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. GenBank accession numbers are indicated alongside taxa names.

GENERAL CONCLUSION

In Myanmar, there are approximately 3,000 captive Asian elephants and half of these are owned by MTE. Since Asian elephants are endangered species, both wild and captive elephants must be conserved in the current situation. According to MTE records, gastrointestinal parasites including nematodes and stomach bot flies are extremely common and cause gastroenteritis and even death especially in younger elephants. Despite the importance as the causative agents, study on prevalence of those parasites in Myanmar has not been conducted. There are few studies on morphological identifications of some nematodes and bot fly species providing with photomicrographs in some Asian countries. As molecular aspects, although genetic data of some nematode parasites and stomach bot flies have been reported from African elephants, there is no genetic information from Asian elephants. Thus, this study was conducted to investigate the prevalence of gastrointestinal parasites in Asian elephants in Myanmar, to provide the photomicrographs describing the key structures of morphology, and finally, to deposit the genetic sequences of those parasites for the analysis of the phylogeny with those in African elephants.

In chapter I, 47 adult nematodes were collected from two elephant camps in Myanmar. Then the species were identified morphologically and microphotographs of key structures such as the shape of buccal capsule, oesophagus and bursal rays were taken to provide the materials for morphological diagnosis of the parasites. As the result, three *Murshidia* species and two *Quilonia* species were identified. Finally, the *COI* genes of those parasites were sequenced for phylogenetic analysis. Phylogenetic analysis revealed that *Murshidia indica* and *Quilonia renniei* have a close relationship

to *Murshidia africana* and *Quilonia africana* reported from African elephants, respectively.

In chapter II, a stomach bot fly larva was collected from a captive Asian elephant and was morphologically identified as *Cobboldia elephantis*. Then, the *COI* gene was amplified and sequenced for phylogenetic analysis in comparison with other stomach bot flies reported from African elephants, equids and rhinoceroses. *C. elephantis* from this study and *C. loxodontis* from African elephants are in the same clade in the phylogenetic tree.

In conclusion, this study was the first report of the prevalence of five cyathostomine nematode species and one species of stomach bot fly in Asian elephants in Myanmar. This study could provide the photomicrographs of key structures for morphological identification of three *Murshidia*, two *Quilonia* and one *Cobboldia* species 100 years after the original drawings. The *COI* gene sequences of *M. falcifera, M. indica, M. neveulemairei, Q. renniei, Q. travancra,* and *C. elephantis* were deposited as the first time for genetic information and the phylogenetic analysis was conducted with the parasite species found in African elephants. The findings are useful for future molecular survey of parasites in Asian elephants and African elephants. Further studies such as the interaction between host, parasites, and drugs are required to provide the new insight into control strategies and more species identifications are necessary in other elephant camps in Myanmar as well as in other countries for better understanding of the evolution of gastrointestinal parasites in elephants.

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ABSTRACT

In Myanmar, there are approximately 3000 captive Asian elephants, *Elephas maximus*, in which cyathostomine gastrointestinal nematodes and stomach bot fly cause enteritis and death especially in younger animals. Despite the importance as the causative agents, there is no study on the prevalence of those parasites in Myanmar and there are few studies with the photomicrographs or molecular aspects so far. In this study, 47 nematodes and a bot fly larva were obtained from the faeces of Asian elephants in Myanmar after anthelmintic treatment and subjected to morphological identification and molecular analysis. In the results, five cyathostomine nematode species, *Murshidia falcifera* (n=3), *Murshidia indica* (1), *Murshidia neveulemairei* (10), *Quilonia renniei* (29) and *Quilonia travancra* (4), and one bot fly species, *Cobboldia elephantis* (1), were identified by morphology with providing the photomicrographs of key structures for morphological diagnosis. For molecular study, the partial sequences of the *COI* gene were determined for each species. Phylogenetic analysis revealed that *Murshidia indica, Quilonia renniei* and *Cobboldia elephantis* have close relationship to *Murshidia africana, Quilonia africana* and *Cobboldia loxodontis* reported from African elephants, respectively. It was also suggested that *Murshidia falcifera* and *Murshidia neveulemairei* constructs a clade with *Murshidia linstowi* and *Murshidia longicaudata* reported from African elephants. This clade is divided into three sub-clades, one by *Murshidia falcifera,* one by *Murshidia neveulemairei* and the last by *Murshidia linstowi* and *Murshidia longicaudata*. *Quilonia travancra* makes one separate clade. This study was the first report of the prevalence of five cyathostomine nematode species and one species of stomach bot fly in Asian elephants in Myanmar. This study could provide the photomicrographs of key structures for morphological identification of three *Murshidia*, two *Quilonia* and

one *Cobboldia* species 100 years after the original drawings. The *COI* gene sequences of *M. falcifera, M. indica*, *M. neveulemairei, Q. renniei, Q. travancra,* and *C. elephantis* were deposited as the first time for genetic information in Asian elephants and the phylogenetic analysis was conducted with the parasite species found in African elephants. The findings are useful for future molecular survey of parasites in Asian elephants and African elephants. Further studies such as the interaction between host, parasites, and drugs are required to provide the new insight into control strategies and more species identifications are necessary in other elephant camps in Myanmar as well as in other countries for better understanding of the evolution of gastrointestinal parasites in elephants.