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Coprological survey of alimentary tract parasites in dogs from Zambia and evaluation of a coproantigen assay for canine echinococcosis

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Faecal samples were collected from the rectum of 540 domestic dogs from four districts (Lusaka, Katete, Petauke and Luangwa) in Zambia between 2005 and 2006 and prevalences of canine alimentary tract parasites were determined by coprological examination. Thirteen different ova and parasites including strongyle (43.3%), *Spirocerca lupi* (18.7%), taeniid (13.1%), *Toxocara canis* (7.6%), *Sarcocystis* sp.* (7.5%), *Isospora* sp.* (5.7%), *Physaloptera* sp.* (4.6%), *Capillaria* sp.* (2.8%), *Dipylidium caninum* (2.2%), *Mesocestoides* sp.* (2.0%), *Ascaris* sp.* (1.7%), *Trichuris vulpis** (0.4%) and *Schistosoma mansoni** (0.4%) were detected, *Ascaris* and *Schistosoma* probably originating from coprophagy. The species with asterisks and later-described *Taenia multiceps* are for the first time reported from dogs in Zambia. A coproantigen enzyme-linked immunosorbent assay (CoproAg-ELISA) developed for *Echinococcus* spp. revealed 43 positive dogs and 37 of these harboured taeniid eggs. From 63 of the 71 taeniid egg-positive samples, eggs and DNA thereof were isolated and subjected to a multiplex polymerase chain reaction for differentiating *E. granulosus sensu lato*, *E. multilocularis* and *Taenia* spp. Amplicons indicative for *Taenia* spp. were obtained from 60 samples. Sequencing of amplicons spanning part of the mitochondrial cytochrome *c* oxidase subunit 1 gene, which was possible with 38 samples, revealed 35 infections with *T. hydatigena* and 3 with *T. multiceps*. Therefore, the CoproAg-ELISA showed some positives, but concrete evidence for the existence of canine *E. granulosus* infection could not be established. Comparison of the results of the CoproAg-ELISA and *Taenia* species identification indicated that the CoproAg-ELISA cross-reacts with patent infections of *T. hydatigena* (57%) and *T. multiceps* (33%).

INTRODUCTION

It is very common in most African countries to see free-ranging dogs under poor hygiene condition and in close contact with people, especially in rural settings. Although the potential role of dogs as source of zoonotic parasites to humans has been recognized as a significant public health problem, investigation on the prevalence of such parasite infections has hardly been conducted in most African countries. The conditions combined with poor veterinary services and a lack of awareness of zoonotic diseases exacerbate the risks of disease transmission from dogs to humans.

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Echinococcus spp. belonging to such canine zoonotic parasites can cause serious diseases in humans. Therefore, the precise diagnosis of definitive hosts that are shedding infective eggs is of primary importance for evaluating the endemicity and the risk posed to humans in a region, and for assessing control programs. For detecting *Echinococcus* adult infections, we developed a monoclonal antibody-based coproantigen detection assay (Morishima *et al.*, 1999), which in turn has been used for epidemiological studies in foxes, dogs and cats mainly in Hokkaido, Japan (Tsukada *et al.*, 2000; Nonaka *et al.*, 2009a). The assay showed high specificity against antigens of common canine parasites including *Toxocara canis*, *Dipylidium caninum*, *Spirometra erinaceieuropaei* and some *Taenia* species (Sakashita *et al.*, 1995); however, cross-reactivity of the assay was recognized against patent *T. hydatigena* infections (Malgor *et al.*, 1997). Nevertheless, there have been few practical problem in using this assay in Hokkaido because the prevalences of most *Taenia* species are very low there. Only *T. taeniaeformis* is commonly found in cats, but the assay showed no cross-reactivity with this parasite.

When considering using the assay in other regions of the world, its specificity should be further evaluated with samples from regions where common canine parasites, including *Echinococcus* and *Taenia* species, are prevalent. In Zambia, it has been estimated that *E. granulosus* G1 strain (sheep strain), which has a broad range of intermediate hosts including sheep, cattle, goats and pigs, is prevalent (Macpherson and Wachira, 1997); thus, the parasite might be present in most of the typical villages in Zambia where animal husbandry of cattle, goats, and pigs is common. Indeed, it has been reported that metacestodes of *E. granulosus* were routinely found in lungs and livers of cattle at abattoirs in Lusaka (Pandey, 1987; Pandey and Sharma, 1987). Unfortunately, no information is available on the prevalence of canine parasites from that region except for one report in which a parasitic helminth necropsy survey was conducted with 85 dogs in Lusaka, revealing the existence of various parasites such as *D. caninum* (25% in prevalence), *T. hydatigena* (18%), *Toxocara canis* (14%), *Ancylostoma caninum* (8%), *Toxascaris leonina* (7%), *Ancylostoma braziliensis* (2%), *Spirocerca lupi* (2%) and *E. granulosus* (1%) (Islam and Chizyuka, 1983).

In this survey, at first, in order to clarify the prevalence of alimentary tract parasites in dogs in Zambia, fresh faecal samples from dogs raised in rural villages and in Lusaka were examined for faecal helminth eggs and coccidian oocysts/sporocysts. Samples containing taeniid eggs were further characterized for identifying the species of taeniid cestodes by multiplex polymerase chain reaction (PCR) and nucleotide sequence analysis of part of the mitochondrial cytochrome *c* oxidase subunit 1 (CO1) gene. To evaluate its specificity, the developed coproantigen detection assay for canine echinococcosis was performed on all faecal samples.

SUBJECTS AND METHODS

Collection of Faecal Samples and Questionnaire

Since we obtained local information (unpublished) that *E. granulosus* cysts were found in cattle from the Tete Province in Mozambique, we selected for this study two provinces of Zambia that are located next to this province. Fresh faecal samples were collected per rectum from 540 dogs raised in rural villages in Katete and Petauke (eastern province) and Luangwa and Lusaka (Lusaka province) between 2005 and 2006 (Fig.). In order to facilitate the capture and holding of these free-ranging dogs, sample collections were announced to village residents 1 day before. The purpose of the survey was explained and a simple questionnaire was administered to dog owners at the day of sample collection. The questionnaire included the dog's age and sex.

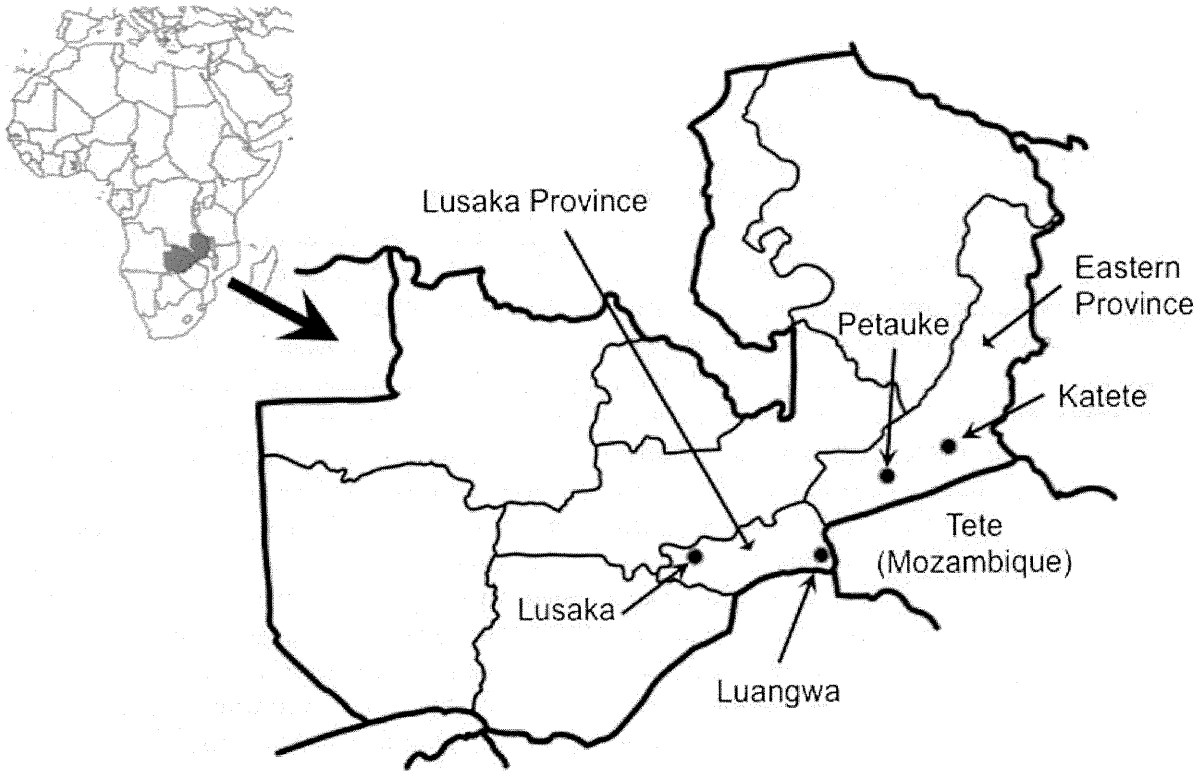

FIG. Study area in Zambia.

Examination for Faecal Helminth Eggs and Coccidian Oocysts/Sporocysts, Coproantigen ELISA

Coproscopic examination (ova-examination) and a coproantigen sandwich enzyme-linked immunosorbent assay (CoproAg-ELISA) were performed as described by Morishima *et al.* (1999). In order to kill *Echinococcus* eggs, all faecal samples were stored at −80°C for more than 7 days before examination. Briefly, 0.5 g of faecal samples were weighed and put into plastic tubes. Then 1% formalin containing 0.3% Tween 20 were added to make a total volume of 15 ml. After a centrifugation step (1000*g* for 10 minutes), the centrifugal sucrose (specific gravity =1.27) flotation method (Ito, 1980) was applied to the resultant sediments for ova-examination. The supernatants were used for CoproAg-ELISA developed for detecting *Echinococcus* coproantigen using the monoclonal antibody EmA9. Two cutoff values were used in CoproAg-ELISA to discriminate between negative and suspicious samples [mean (μ)+3 standard deviations (SD) of negative controls; OD=0.206] and between suspicious and positive samples (μ+5SD; OD=0.289). The negative controls used were faecal samples from 605 companion dogs raised and kept only on the main island of Japan that is free of *Echinococcus* infections.

Preparation of Egg DNA

From all samples in which taeniid eggs were detected, 1–10 eggs per sample were collected manually from the cover glass used for ova-examination under a stereomicroscope. Egg DNA was then extracted using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instruction.

Multiplex PCR for Discriminating *E. multilocularis*, *E. granulosus sensu lato* and *Taenia* species

The multiplex PCR for discriminating taeniid eggs was done as described by Trachsel *et al.* (2007) using egg DNA as template.

This method can distinguish *E. multilocularis, E. granulosus* s.l. and *Taenia* species. The amplified products [395 base pairs (bp) for *E. multilocularis*, 117 bp for *E. granulosus s.l.* and 267 bp for *Taenia* spp.] were examined by agarose gel electrophoresis. Positive controls of DNA extracted from metacestodes of *E. granulosus* genotype G1, *E. multilocularis* and *T. hydatigena* using QIAamp DNA Mini Kit (Qiagen) and a negative control without DNA were included in all the tests.

Nucleotide Sequencing

Egg DNA identified as *Taenia* sp. by the multiplex PCR was subjected to another PCR and sequence analysis. Part (491 bp) of the mitochondrial cytochrome CO1 gene was amplified using the primers PR-A (5′-TGG TTT TTT GTG CAT CCT GAG GTT TA-3′) and PR-B (5′-AGA AAG AAC GTA ATG AAA ATG AGC AAC-3′) according to Okamoto *et al.* (1995). Nucleotide sequences of the products were determined by a Beckman CEQ 8000 DNA analyser using a GenomeLab DTCS Quick Start kit (Beckman Coulter, Fullerton, CA, USA) following the manufacturer's instruction. The sequences obtained were subjected to BLAST sequence similarity search (National Center for Biotechnology Information, Bethesda, MD, USA) to identify the species of *Taenia*.

Statistical Analysis

Ninety-five per cent confidence intervals of prevalence were calculated on the basis of binomial distributions using the software program R (version 2.8.1, R Development Core Team, 2008). For the ova and parasites showing more than 5% in overall prevalence, logistic regression analysis was performed to evaluate the effect of site (district), sex and age of dogs on the prevalence using the software program JMP 8 (SAS Institute, Tokyo, Japan). Since sex was not recorded from the dogs from Lusaka, the data of these dogs were excluded in the above analysis. Dogs were grouped as younger or older than 6 months ($\leqslant$6 months old versus $>$6 months old) for comparing *T. canis* prevalences, and younger or older than one year ($\leqslant$1 year old versus $>$1 year old) for the other parasites. If the effects of interaction between sites and other factors were significant ($P<0.05$), the same analysis was further performed on the effect of sexes and ages at each site separately. For dogs from Lusaka, the difference in prevalence between age groups was analysed by Fisher's exact test using the software program R.

RESULTS

Faecal Examination for Helminth Eggs and Coccidian Oocysts/Sporocysts

From 540 canine faecal samples examined, 13 kinds of helminth eggs and coccidian oocysts/sporocysts were detected (Table 1). Among all the ova and parasites found, strongyle eggs were most abundant and were found in 43.3% of the dogs. The prevalence was, however, significantly lower in Luangwa district (17.0%) as compared to the other districts. Taeniid eggs were discovered in 71 (13.1%) dogs in total, and a significantly higher prevalence (33.9%) was observed in Luangwa district where goat husbandry is more prominent than in other districts. The number of taeniid eggs detected ranged from 1 to more than 1000 with a median of 20. Less than 10 eggs were found in 24 dogs. Eggs of the other potential zoonotic parasites such as *T. canis*, *D. caninum* and *Schistosoma mansoni* were also found in less than 10% of the dogs. The prevalence of *T. canis* was significantly higher in Lusaka (25.8%) than in the other districts. Interestingly, *Ascaris* sp. eggs were found in nine dogs (four in Katete and five in Lusaka), thus in 1.7% of the samples.

In the logistic regression analysis (n=339), significant differences in prevalences among districts were obtained for taeniids, *Spirocerca lupi* and strongyle eggs.

TABLE 1. *Prevalences based on detecting helminth eggs and coccidian oocysts/sporocysts in rectal faeces of free-ranging dogs from four districts in Zambia*

Parasite	Prevalence (%) (95% confidence interval)				
	Katete (n=224)	Petauke (n=89)	Luangwa (n=165)	Lusaka (n=62)	Total (n=540)
Strongyle*	60.7 (54.2–66.9)	48.3 (38.2–58.5)	17.0 (12.0–23.4)	43.5 (31.9–55.9)	43.3 (39.2–47.5)
Spirocerca lupi	7.1 (4.4–11.3)	41.6 (31.9–52.0)	26.1 (20.0–33.2)	8.1 (3.5–17.5)	18.7 (15.6–22.2)
Taeniid†	4.5 (2.2–8.1)	4.5 (1.2–11.1)	33.9 (27.2–41.5)	1.6 (0.0–8.7)	13.1 (10.6–16.3)
Toxocara canis	9.8 (6.6–14.4)	2.2 (0.6–7.8)	0.6 (0.0–3.3)	25.8 (16.6–37.9)	7.6 (5.6–10.1)
Sarcocystis sp.‡	ND‡	9.0 (4.6–16.7)	6.7 (3.8–11.5)	ND‡	7.5 (4.8–11.4)
Isospora sp.	6.3 (3.8–10.2)	10.1 (5.4–18.1)	4.2 (2.1–8.5)	1.6 (0.0–8.7)	5.7 (4.1–8.0)
Physaloptera sp.	4.0 (2.1–7.5)	4.5 (1.2–11.0)	7.3 (4.2–12.3)	0 (0.0–4.7)	4.6 (3.2–6.7)
Capillaria sp.	2.2 (1.0–5.1)	11.2 (6.2–19.5)	0 (0.0–1.8)	0 (0.0–4.7)	2.8 (1.7–4.5)
Dipylidium caninum	2.2 (1.0–5.1)	4.5 (1.2–11.0)	1.2 (0.1–4.3)	1.6 (0.0–8.7)	2.2 (1.3–3.8)
Mesocestoides sp.	2.7 (1.2–5.7)	4.5 (1.2–11.0)	0.6 (0.0–3.3)	0 (0.0–4.7)	2.0 (1.1–3.6)
Ascaris sp.	1.8 (0.5–4.5)	0 (0.0–3.3)	0 (0.0–1.8)	8.1 (3.5–17.5)	1.7 (0.9–3.1)
Trichuris vulpis	0 (0.0–1.3)	1.1 (0.0–6.1)	0 (0.0–1.8)	1.6 (0.0–8.7)	0.4 (0.0–1.3)
Schistosoma mansoni	0.4 (0.0–2.5)	0 (0.0–3.3)	0.6 (0.0–1.8)	0 (0.0–4.7)	0.4 (0.0–1.3)

*Strongyle possibly includes the genera *Ancylostoma*, *Uncinaria* and others whose eggs cannot be distinguished morphologically.
†Taeniid possibly includes the genera *Taenia* and *Echinococcus* whose eggs cannot be distinguished morphologically.
‡The samples of Katete and Lusaka were not carefully examined for the small sporocysts of *Sarcocystis* sp.

Differences in prevalences between age groups were significant for *S. lupi* in Luangwa, and *Sarcocystis* sp. in Petauke. However, in Lusaka (n=51), no parasites showed significant differences between age groups. Moreover, differences in prevalences between sex groups were not significant for any parasite.

Among the 540 samples, 160 (29.6%) samples showed neither parasite, a single parasite kind was diagnosed in 244 (45.2%) samples and multiple different ova and parasites were found in 136 (25.2%) samples (two kinds in 100, three kinds in 25, four kinds in 10 and five kinds in 1).

Multiplex PCR and Nucleotide Sequencing

From 63 of the 71 samples containing taeniid eggs, DNA was isolated from the eggs. PCR products were obtained from 60 samples in the multiplex PCR, and all were of the size specific for *Taenia* spp. There were 18 samples with less than 10 eggs detected, and three of them showed no products in the multiplex PCR.

PCR targeting part of the mitochondrial CO1 gene was successful with 38 samples out of the available 63 DNA samples. Sequencing and BLAST sequence similarity searches showed that 35 sequences had 98.4–100% identity with those of *T. hydatigena* registered in GenBank (accession no. DQ995656, AM503318 or EU544552). The sequences of three samples revealed *T. multiceps* with identities of 94.4–100% (GenBank accession no. EF393620). For the remaining 25 samples, no amplicons (n=11) were obtained in the PCR with primers PR-A and PR-B or no clear sequences (n=14) were obtained by direct sequencing. *T. hydatigena* was found in three districts (Katete, Petauke and Luangwa), whereas *T. multiceps* was identified only in the Luangwa district.

CoproAg-ELISA

The CoproAg-ELISA for *Echinococcus* spp. was positive in 43 dogs and suspicious in 6 dogs, while 37 and 5 of those had taeniid eggs, respectively, after coproscopic examination

(Table 2). The multiplex PCR performed on taeniid egg DNA of the 32 CoproAg-ELISA-positive and the 4 CoproAg-ELISA-suspicious samples, however, showed that the eggs of all samples were those of *Taenia* spp.

Among the 38 samples identified as harbouring taeniid species by nucleotide sequence analysis, 20 of 35 samples (57%) identified as *T. hydatigena* and 1 of 3 samples (33%) as *T. multiceps* showed positive reactions in the CoproAg-ELISA.

DISCUSSION

The coproscopic examination revealed that the dogs in the study area of Zambia were infected with a variety of zoonotic parasites. In the previous study conducted in Lusaka, Islam and Chizyuka (1983) found nine helminth species, of which all but two (*T. leonina* and *E. granulosus*) also were identified in the present study. In addition, *Sarcocystis* sp., *Isospora* sp., *Physaloptera* sp., *Capillaria* sp., *Mesocestoides* sp., *T. vulpis*, *S. mansoni*, *Ascaris* sp. and *T. multiceps* were observed, which are thus the first reports from dogs of Zambia.

The highest prevalence was observed for strongyles (43.3%). From the study of Islam and Chizyuka (1983), it can be speculated that *A. caninum* and *A. braziliense* are the dominant species among strongyles. Since *A. braziliense* is more frequently involved in cutaneous larva migrans than *A. caninum* (Bowman, 2009), species identification of *Ancylostoma* by molecular methods should be considered in future studies for public health risk assessment. In this study, we detected lower prevalence of strongyles in the Luangwa district than in other districts. Luangwa is a valley with a very hot and humid climate throughout the year. Therefore, taking into account that climatic conditions of Luangwa are suitable for parasite transmission, results obtained in this study need further clarification.

TABLE 2. *Comparison of the results of coproantigen ELISA and faecal examination for taeniid eggs*

Results of coproantigen examination	Results of faecal examination for taeniid eggs		
	Positive	Negative	Total
Positive	37	6	43
Suspicious	5	1	6
Negative	29	462	491
Total	71	469	540

For infections with *T. canis*, an age resistance limit of 6 months has been indicated (Webster, 1956) and therefore, prevalences for this parasite of age groups younger and older than 6 months were compared. However, no significant difference was observed in this study which is in agreement with recent findings (Fahrion *et al.*, 2008) demonstrating that a low infective dose of 100 embryonated eggs consistently induced patency in adult dogs with and without previous exposure to the parasite. Since an overall prevalence of 5.1% (19/371) was observed in dogs older than 6 months, infection with *T. canis* in older dogs may constantly be occurring in Zambia. It is noteworthy that a higher prevalence of *T. canis* was observed in Lusaka, but the reason for this observation was not further elucidated in this study. Nevertheless, *T. canis* is one of the most important zoonotic parasites, and thus, the high prevalence in Lusaka would be of significance in public health because this district is the most populated city in Zambia and holds many high-density residential areas.

Eggs of *S. mansoni* were found in two dogs, but without intact miracidium inside the eggs. In general, trematode eggs are not isolated by the flotation techniques. It is known that dogs are not an appropriate host for *S. mansoni*, and combined with the absence of an intact miracidium in our results, it is most probable that the observation could be a result of coprophagy of human faeces by the dogs. Coprophagy of either human or pig faeces by dogs is also suspected by the finding of *Ascaris* sp. eggs

in this study. A similar observation was reported from India (Traub *et al.*, 2005). Recently, infection of *A. lumbricoides* in dogs was reported in an endemic region for this human parasite (Shalaby *et al.*, 2010), suggesting that active infection with *A. lumbricoides* may occur in dogs in the study area. The finding of *S. mansoni* and *Ascaris* sp. eggs in dog faeces could also indicate their endemicity in the region.

It should be also noted that we used frozen samples for the faecal egg examination because of the biohazard concern of *Echinococcus* eggs. As it was observed that freezing of faeces reduced significantly the egg count of ovine gastro-intestinal strongyles in flotation techniques (van Wyk and van Wyk, 2002; Rinaldi *et al.*, 2011), the result obtained in this study may be an underestimation.

Taeniid eggs were found in 71 samples. Canine taeniid species include both zoonotic and non-zoonotic species. Since taeniid eggs cannot be distinguished by their morphology, we used immunological and molecular techniques that were recently developed for distinguishing the genus and species of taeniid cestodes, and the result was used for evaluating potential cross-reactivity of the CoproAg-ELISA.

Various coproantigen detection methods for adult *Echinococcus* spp. infection have been developed (Deplazes and Eckert, 1996; Benito and Carmena, 2005; Benito *et al.*, 2006; Huang *et al.*, 2007). The CoproAg-ELISA used was developed to detect adult infections of *E. multilocularis* (Sakashita *et al.*, 1995) and *E. granulosus* (Malgor *et al.*, 1997). The sensitivity and specificity of the test had been evaluated for *E. multilocularis* infection using fox samples in Hokkaido, measuring 92.2% in sensitivity and 96.6% in specificity (Morishima *et al.*, 1999; Yimam *et al.*, 2002). Cross-reactivity with patent *T. hydatigena* (Malgor *et al.*, 1997) and *T. pisiformis* infections (unpublished) has been observed, but not with *T. taeniaeformis* or *T. crassiceps* (Sakashita *et al.*, 1995). In this study, positive reactions in the CoproAg-ELISA were observed in 43 samples, of which taeniid eggs were detected in 37 samples. The multiplex PCR revealed that all of the egg DNAs extracted were those of *Taenia* spp. These results indicate that the prevalence of *E. granulosus* in the study area was low or negligible. On the other hand, among 35 samples containing *T. hydatigena* eggs, 20 (57%) showed positive reactions in the CoproAg-ELISA. In addition, one of three samples containing *T. multiceps* eggs was positive. These results suggest a cross-reactivity of the test in patent infections with *T. hydatigena* and *T. multiceps*. Higher specificities for the detection of *E. granulosus* coproantigen were reported for a test system using polyclonal antibodies (Deplazes *et al.*, 1992; Deplazes *et al.*, 1994).

The multiplex PCR on egg DNA yielded products in 60 out of 63 samples. It is known that PCR on copro-DNA is often unsuccessful because of the presence of a variety of PCR inhibitors in faeces (Monteiro *et al.*, 1997). In this study, individual eggs were picked up under a stereomicroscope, and this method for isolating taeniid eggs effectively excludes PCR inhibitors.

Sequence analysis of the partial CO1 gene revealed the occurrence of *T. hydatigena* and *T. multiceps*. Especially, in Luangwa where a significantly higher prevalence of taeniid eggs was observed than in other districts, both *Taenia* species were found with *T. hydatigena* being more prevalent. The observed higher prevalence of *Taenia* spp. in Luangwa could be related to the difference in the local animal husbandry system. In typical local villages in Zambia, it is common to raise cattle, goats and pigs. However, in Luangwa, goats, which can serve as an intermediate host of both *T. hydatigena* and *T. multiceps*, are the main animal in the local husbandry system, and self-consuming of goats is more popular. In contrast, pigs are the main animal in Katete and Petauke, whereas goat husbandry is less popular there. Although pigs can also serve

as an intermediate host of *T. hydatigena*, self-consuming of pigs, or even goats and cattle are limited in those districts. In Lusaka, the capital urban city, animal husbandry is not popular.

We tried to detect *E. granulosus* infection in dogs in the study area in Zambia using recently developed diagnostic techniques for *Echinococcus* spp.; however, concrete evidence of the infection could not be obtained. Nevertheless, the study revealed the potential cross-reactivity of the CoproAg-ELISA with patent *T. hydatigena* and *T. multiceps* infections. The previous experimental infection study showed that the cross-reactivity of the CoproAg-ELISA with *T. hydatigena* was only observed in its patency period (Malgor *et al.*, 1997). Therefore, the relationship of the cross-reactivity with *T. multiceps* and the patency should also be clarified. Moreover, the evaluation of the antigens playing a role in this cross-reactivity is also of future interest.

Since the CoproAg-ELISA showed cross-reactivity with patent infection with *Taenia* spp., similar surveys in future should be conducted in combination with molecular techniques that enable genus and species identification of taeniid eggs. A technique using PCR-based restriction fragment length polymorphism has been developed for this purpose (Trachsel *et al.*, 2007). With the PCR-based restriction fragment length polymorphism, mixed infection with multiple species of *Taenia* can be identified simultaneously.

For conducting a survey for gastrointestinal parasite infection, faeces would provide valuable information about the animal itself and the aetiological agents that it harbours (Nonaka *et al.*, 2009b). Moreover, a survey upon faeces is a non-invasive method, thus causing minimal disturbance to the animal condition, ecology and life. Classical approaches such as conducting faecal egg examination alone did not provide adequate information for parasite identification especially for the parasites producing morphologically similar eggs. However, as shown in this and in earlier studies (Bruzinskaite *et al.*, 2009; Davidson *et al.*, 2009; Ziadinov *et al.*, 2008), faecal egg examination in combination with the recently developed molecular techniques would provide results with more accuracy, and being a useful tool in surveys of zoonotic parasite infections in dogs.

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Development of PCR/dot blot assay for specific detection and differentiation of taeniid cestode eggs in canids

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ABSTRACT

We report the development of a colourimetric PCR/dot blot assay targeting the mitochondrial gene NADH dehydrogenase subunit 1 (*nad1*) for differential diagnosis of taeniid eggs. Partial sequences of the cestode *nad1* gene were aligned and new primers were designed based on conserved regions. Species-specific oligonucleotide probes (S-SONP) for canine taeniid cestodes were then designed manually based on the variable region between the conserved primers. Specifically, S-SONP were designed for the *Taenia crassiceps*, *T. hydatigena*, *T. multiceps*, *T. ovis*, *T. taeniaeformis*, *Echinococcus granulosus* (genotype 1), *E. multilocularis* and *E. vogeli*. Each probe showed high specificity as no cross-hybridisation with any amplified *nad1* fragment was observed. We evaluated the assay using 49 taeniid egg-positive samples collected from dogs in Zambia. DNA from 5 to 10 eggs was extracted in each sample. Using the PCR/dot blot assay, the probes successfully detected PCR products from *T. hydatigena* in 42 samples, *T. multiceps* in 3 samples, and both species (mixed infection) in the remaining 4 samples. The results indicate that the PCR/dot blot assay is a reliable alternative for differential diagnosis of taeniid eggs in faecal samples.

1. Introduction

Canids, such as dogs, dingoes, foxes, wolves and jackals, harbour the adult stage of important taeniid cestode species, including *Echinococcus granulosus*, *E. multilocularis*, *Taenia ovis*, *T. multiceps* and *T. hydatigena*. Whereas the dangers caused by larval stages of *Echinococcus* species to public health are well known [1], the potential risk associated with zoonotic infection by metacestodes of several *Taenia* spp. such as *T. multiuceps* (*Coenurus cerebralis*) [2] and *T. crassiceps* (*Cysticecus longicollis*) [3] is poorly clarified. In fact, several *Taenia* species have been reported to be highly prevalent in many countries including Uruguay [4], Ethiopia [5], and Italy [6] where tons of carcasses and offal are discarded every year due to infection of domestic livestock by taeniid larvae.

The lack of accurate diagnostic methods for *Taenia* species differentiation in live canids further hinders our understanding of the biology and host–parasite interaction of these parasites. Since canids can harbour several species of *Taenia* and *Echinococcus* simultaneously, developing a method for detecting and distinguishing between taeniid eggs present in faeces is considered to be essential. While the detection of coproantigens by ELISA (coproELISA) [7,8] and PCR (copro-DNA) [9] had previously been used to accurately diagnose infection by *Echinococcus* spp., analogous methods for distinguishing between species of *Taenia* in canids have not yet been developed. Although Gasser and Chilton [10] and Trachsel et al. [11] successfully discriminated *Taenia* spp. by PCR-RFLP, their methods had not been used in survey studies.

The PCR/dot blot assay is a widely used hybridisation technique that has been applied to the identification and genotyping of pathogens such as *Mycobacterium tuberculosis* [12], *Chlamydia psittaci* [13], and Echovirus [14]. The simplicity of this hybridisation assay enables simultaneous and rapid screening of several samples and is capable of species differentiation using species-specific oligonucleotide probes. Lavikainen et al. [15] reported that *Taenia* spp. can be differentiated based on the polymorphisms of the cytochrome *c* oxidase subunit 1 (*cox1*) gene and NADH dehydrogenase subunit 1 (*nad1*) gene sequences. Among these two genes, we chose *nad1* as a candidate for oligonucleotide probes design on the basis of the higher variability range [16]. In addition much more sequences of this gene are currently registered in the GenBank database compared to other genes studied to date.

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The aim of this study was therefore to develop a colourimetric PCR/dot blot assay using species-specific oligonucleotide probes targeting the *nad1* gene to detect and identify taeniid eggs present in canid faeces.

2. Materials and methods

2.1. Parasite sample collection

Cestode adults and larvae used in this study were collected from a variety of hosts in several countries (Table 1). After collection and identification using morphological characters under a light microscope, samples were stored in 70% ethanol until use. Taeniid eggs were isolated from 49 dog faeces samples collected in Lusaka and the Eastern province of Zambia from 2005 to 2007. Briefly, the faecal samples were collected from the rectums of each dog and stored at −80 °C for 10 days and then at −40 °C until egg isolation. Then, 0.5 g of faeces from each dog was placed into a 15 ml plastic tube (Asahi Glass Co. Ltd., Japan) and suspended in sucrose solution (specific gravity: 1.27). After 10 min of centrifugation at 2000 rpm the tubes were laid vertically and filled to the edge of the bottle opening with additional sucrose solution. A glass coverslip was then placed on the top of each tube and left for 1 h to allow the eggs to float to the surface and attach to it. Five to ten taeniid eggs were then manually recovered from the glass coverslips under a stereomicroscope, placed in double distilled water (DDW), and stored at −40 °C until use.

2.2. DNA extraction

Genomic DNA was extracted from adult cestodes, larvae and eggs using QIAamp DNA mini kit (Qiagen K.K., Japan) following the manufacturer's instructions. The concentration of extracted DNA was measured using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific Inc., USA).

2.3. PCR amplification of nad1 gene

Common primers for the taeniid cestode *nad1* gene, *nad1*T-Fw (5′-GGK TAT TCT CAR TTT CGT AAG GG-3′) and *nad1*T-Rv (5′-ATC AAA TGG AGT ACG ATT AGT YTC AC-3′), were designed based on the conserved regions of 11 *Echinococcus* taxa and 10 *Taenia* taxa registered in GenBank (Table 2). The expected size of the amplicons was 507 bp. PCR amplifications were conducted in a reaction mixture consisting of 5 μl of 10× Ex *Taq* buffer, 2.5 mM of each dNTP, 1 U of Ex Taq™ (Takara Bio Inc., Japan), 1 μM of each primer (Hokkaido System Science Co. Ltd., Japan), 5 μl template DNA and up to 50 μl DDW. Amplification was performed using a thermal cycler (GeneAmp PCR system 9700, Applied Biosystems, USA) programmed for 35 cycles of denaturation at 94 °C for 30 s, annealing at 52 °C for 30 s and extension at 72 °C for 1 min. Five microlitres of PCR products were fractionated by electrophoresis on a 1.5% agarose gel in TAE buffer (40 mM Tris, 40 mM acetic acid, and 1 mM EDTA). The gels were then stained in a 1 μg/ml ethidium bromide solution before being photographed under UV light. The remaining 45 μl of PCR product were purified using QIAquick PCR purification kit (Qiagen K.K., Japan) following the manufacturer's instructions and stored at −40 °C until use. The concentration of the PCR products was measured using a NanoDrop 1000 spectrophotometer.

Table 1
Host and country of origin of the cestode species (adult/larvae) used in this study.

Species	Abbreviation	Country	Host
Dipylidium caninum	Dc	Uruguay	Dog
Mesocestoides vogae	Mv	Switzerland	Vole
Echinococcus granulosus (genotype 1)	Eg#1	Mauritania	Camel
Echinococcus granulosus (genotype 1)	Eg#2	Uruguay	Dog
Echinococcus canadensis (genotype 6)	Ec	Zambia	Cattle
Echinococcus multilocularis	Em#1	Austria	Vole
Echinococcus multilocularis	Em#2	Japan	Vole
Echinococcus multilocularis	Em#3	France	Human
Echinococcus multilocularis	Em#4	Japan	Vole
Echinococcus vogeli	Ev	Colombia	*Agouti paca*
Taenia hydatigena	Th#1	Japan	Dog
Taenia hydatigena	Th#2	China	Dog
Taenia hydatigena	Th#3	Japan	Sheep
Taenia hydatigena	Th#4	Switzerland	Dog
Taenia hydatigena	Th#5	Uruguay	Dog
Taenia crassiceps	Tc#1	Japan	Fox
Taenia crassiceps	Tc#2	Japan	Vole
Taenia multiceps	Tm#1	China	Dog
Taenia multiceps	Tm#2	China	Sheep
Taenia ovis	To	Switzerland	Dog
Taenia taeniaeformis	Tt#1	France	Vole
Taenia taeniaeformis	Tt#2	France	Vole
Taenia taeniaeformis	Tt#3	Japan	Rat

Table 2
Accession numbers of taeniid cestode nad1 gene sequences used for designing primers and oligonucleotide probes.

Parasite	Accession number
Echinococcus granulosus (genotype 1)	AF297617
Echinococcus equinus (genotype 4)	AF346403
Echinococcus ortleppi (genotype 5)	AB235846
Echinococcus canadensis (genotype 6)	AB208063
Echinococcus canadensis (genotype 8)	AB235848
Echinococcus canadensis (genotype 9)	AB235847
Echinococcus canadensis (genotype 10)	AF525297
Echinococcus multilocularis	AB018440
Echinococcus oligarthrus	AB208545
Echinococcus shiquicus	AB208064
Echinococcus vogeli	AB208546
Taenia crassiceps	AF216699
Taenia hydatigena	DQ995654
Taenia multiceps	AY669089
Taenia ovis	AJ239103
Taenia pisiformis	AJ239109
Taenia polyacantha	DQ408420
Taenia saginata	AY684274
Taenia serialis	DQ401137
Taenia solium	AB086256
Taenia taeniaeformis	EF179171

2.4. DNA sequencing and homology search

The sequences of PCR products were determined by direct sequencing using an automated sequencer (CEQ 8000, Beckman Coulter Inc., USA) and a GenomeLab™ DTCS quick start kit for dye terminator cycle sequencing (Beckman Coulter Inc., USA). A homology search of the obtained sequences was performed by conducting an online NCBI Basic Local Alignment Search Tool for nucleotides (BLASTN) search of the GenBank database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.5. Oligonucleotide probes

The species-specific oligonucleotide probes were designed by eye based on *nad1* sequences retrieved from the GenBank database. The theoretical specificity of the probes was assessed by a sequence homology search with the BLASTN algorithm. The probes designed in this study were labeled with digoxigenin (DIG) using the DIG oligonucleotide 3′-end labeling kit, 2nd Generation (Roche Diagnostics, Germany) and stored at −40 °C until use.

Table 3
List of species-specific oligonucleotide probes designed in this study.

Parasite	Probe name	Sequence (5′-3′)
E. granulosus (genotype 1)	EgG1-*nad1*	CCGCCAGAACATCTAGGTATT
E. multilocularis	Em-*nad1*	TTTGTTCTTTGTGTTACTGTAGGTA
E. vogeli	Ev-*nad1*	TGTTATGATTCTTAGCTGCTGC
T. crassiceps	Tcra-*nad1*	GTACGTAGAAATTATAGTTTATTAGGAGC
T. hydatigena	Thyd-*nad1*	GTTTATGGGTCTTATCATAGTTGTAG
T. multiceps	Tmul-*nad1*	TGTATATTATTCTTTTGTATATGGTGGTT
T. ovis	Tov-*nad1*	TGGTGTGATATTACTTGTTAATTTAGTT
T. taeniaeformis	Ttae-*nad1*	TTTATGTGGTTATGCTGTGTTATGT

2.6. Dot blot assay

Dot blot assays were carried out using the DIG nucleotide acid detection kit (Roche Diagnostics). Briefly, the *nad1* PCR products were heated at 95 °C for 10 min before being cooled on ice. Two microlitres of each denatured PCR product was then manually blotted in duplicate on positively charged nylon membranes previously activated with 2× SSC (0.3 M NaCl, 0.03 M sodium citrate) for 5 min. The membranes were then soaked in 0.4 M NaOH for 5 min, rinsed with 2× SSC for 10 min, and dried before use. The blots were hybridised with 10 pM oligonucleotide probes at 60 °C for 1.5 h. After hybridisation, each blot was washed twice for 5 min in 2× SSC/0.1% SDS (sodium dodecyl sulphate) at room temperature (RT) with gentle agitation, followed by additional two washes with pre-heated 0.5× SSC/0.1% SDS for 10 min at 60 °C. Blots were then washed in DIG Wash and Block Buffer Set for 2 min at RT before the blocking reaction was performed by incubating in blocking solution for 30 min. After the membranes were incubated in antibody solution containing 150 mU/ml alkaline phosphatase conjugated anti-digoxigenin antibody (anti-digoxigenin-AP) for another 30 min, they were washed twice in washing solution for 15 min before being equilibrated in 0.1 M Tris–HCl and 0.1 M NaCl (pH 9.5) for 3 min. For colourimetric detection of hybridisation, substrate solution (NBT/BCIP) was used as per the manufacturer's instructions. Colour development was performed for 1 h and the reaction stopped by washing the membrane with TE buffer (10 mM Tris–HCl (pH 8.0), and 1 mM EDTA) for 5 min.

3. Results

3.1. nad1 PCR with newly designed primers

The specificity of the new primer pair, *nad1*T-Fw/*nad1*T-Rv, was evaluated by using adult/larval cestode genomic DNAs as templates (Table 1). Fragments of the *nad1* gene were amplified from all of the taeniid cestode DNAs tested, as well as two non-taeniid cestodes, *Dipylidium caninum* and *Mesocestoides vogae*. Sensitivity tests of the

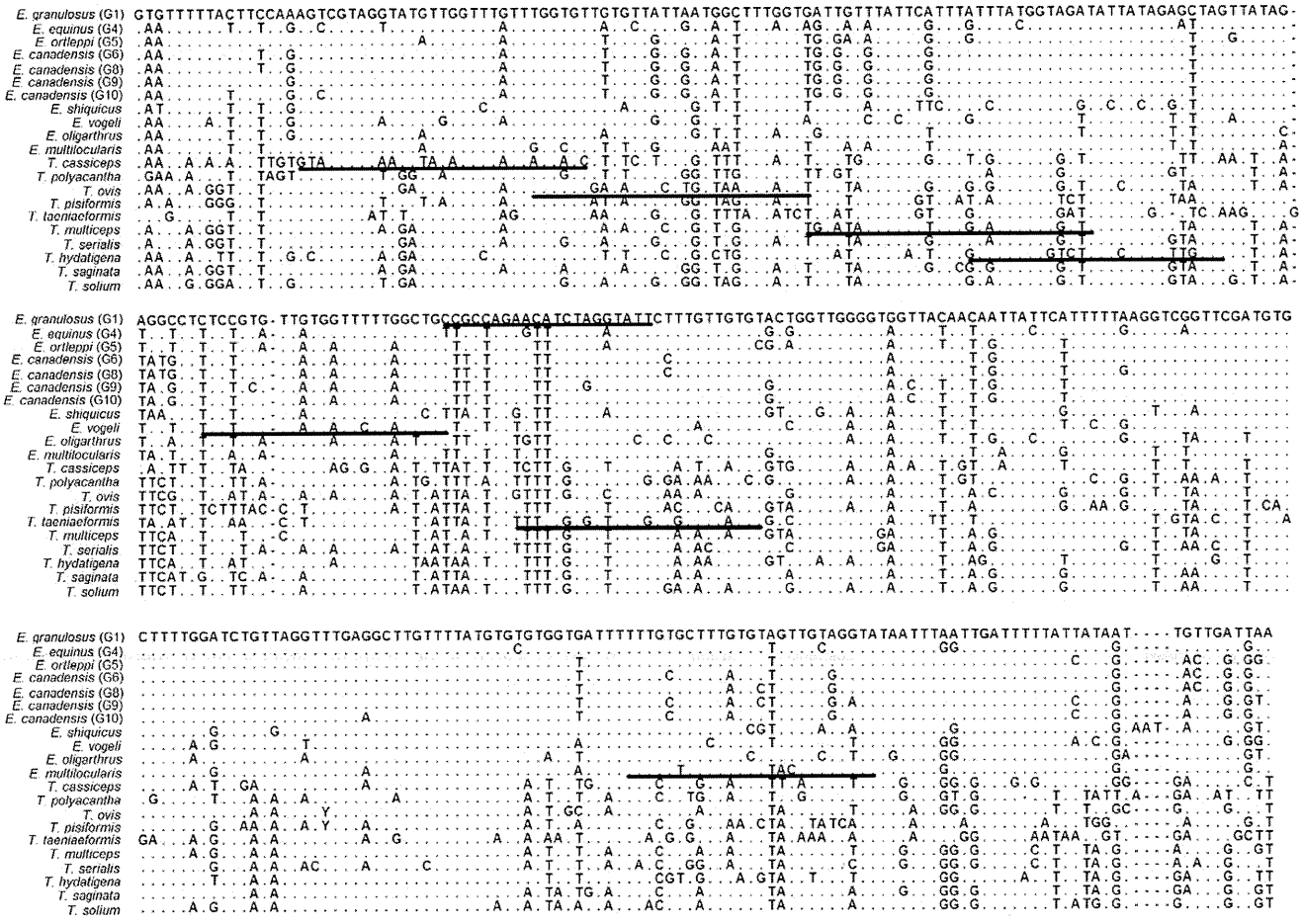

Fig. 1. Multiple alignment of the partial *nad1* sequences containing variable regions in different taeniid species. Sequences were obtained from GenBank. The position of species-specific oligonucleotide probes were underlined and dots represent bases identical to those in the *E. granulosus* genotype 1 sequence (first line).

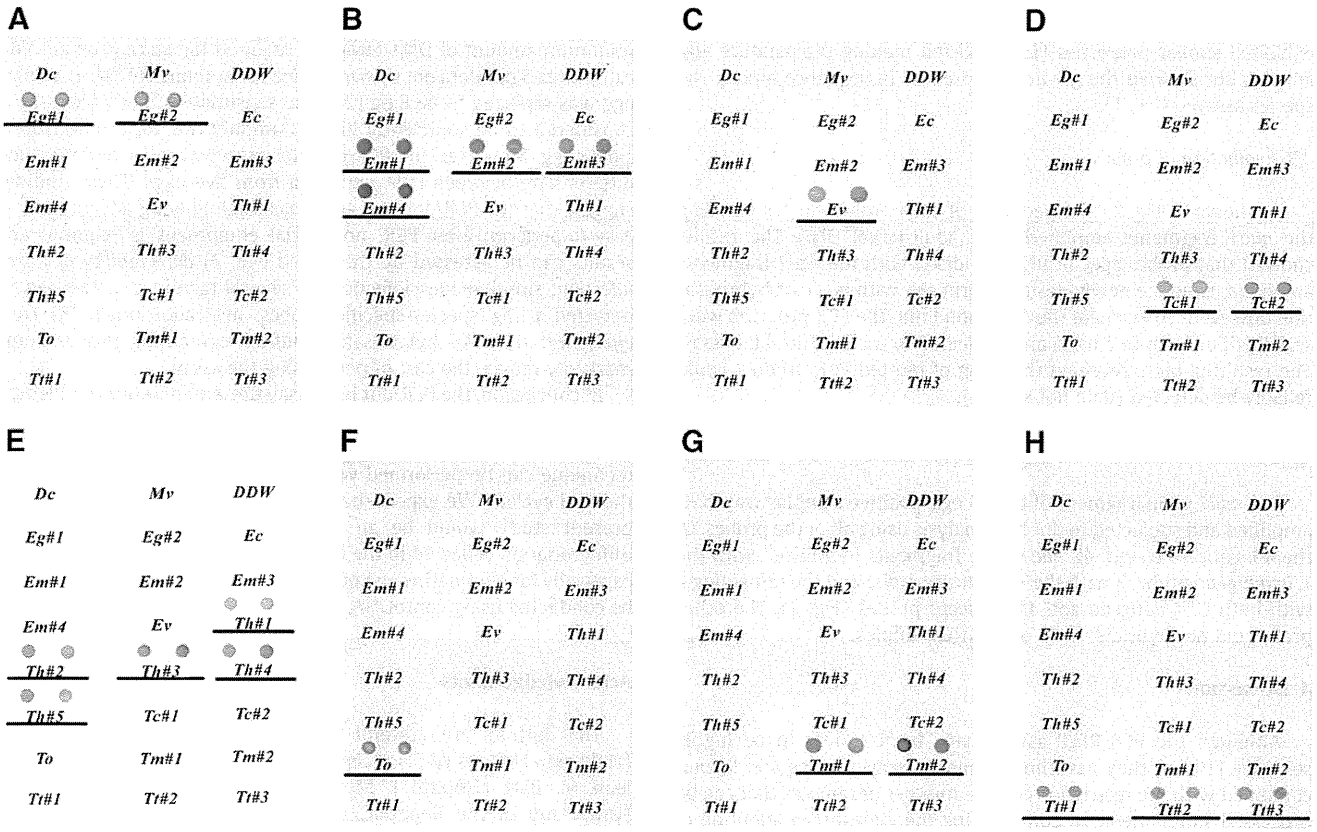

Fig. 2. Dot blot assay performed using adult/larval cestode *nad1* PCR products. Membranes A to H correspond to the *E. granulosus* (genotype 1), *E. multilocularis*, *E. vogeli*, *T. crassiceps*, *T. hydatigena*, *T. ovis*, *T. multiceps* and *T. taeniaeformis* probes, respectively. The PCR products blotted on these membranes are (from top to bottom) Dc, *D. caninum*; Mv, *M. vogae*; Eg, *E. granulosus* (genotype 1); Ec, *E. canadensis* (genotype 6); Em, *E. multilocularis*; Ev, *E. vogeli*; Tc, *T. crassiceps*; Th, *T. hydatigena*; To, *T. ovis*; Tm, *T. multiceps* and Tt, *T. taeniaeformis*. The species with more than one sample were numbered. Underline indicates the species-specific hybridisation. *D. caninum* and *M. vogae nad1* PCR products were included as negative control DNAs and DDW was applied in the blank space. No cross-reactions were observed between any of the probes.

primer pair was performed using serial dilutions of the cestode genomic DNA templates, revealing that 5 pg was sufficient for amplifying the *nad1* fragment in all of the species assayed (data not shown).

3.2. Oligonucleotide probes

DNA sequences of *nad1* genes registered in the GenBank database (Table 2) were aligned using BioEdit version 7.0.0. The oligonucleotide

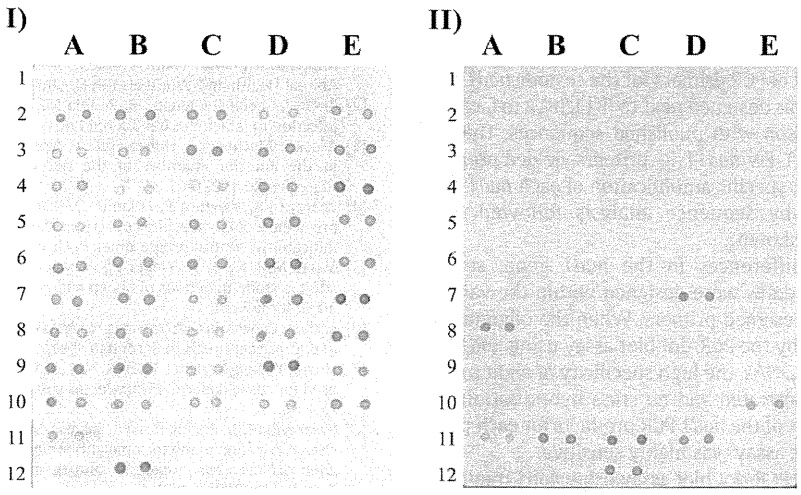

Fig. 3. Dot blot assay of 49 taeniid egg samples. Membrane I) was hybridised with *T. hydatigena* probe, and II) was hybridised with *T. multiceps* probe. As controls, PCR products of *E. granulosus* genotype 1, *E. canadensis* genotype 6, *E. multilocularis* and *E. vogeli* were applied to the first row (1A–1D), and PCR products from *T. crassiceps*, *T. hydatigena*, *T. multiceps*, *T. ovis* and *T. taeniaeformis* were applied to the bottom row (12A–12E). Samples were organised based on PCR/direct sequencing results with *T. hydatigena* samples on row 2A–10E and *T. multiceps* on row 11A–11D. DDW was applied on 1E and 11E. Of the 49 samples, 42 hybridised with *T. hydatigena* probe and 3 with *T. multiceps* probe, the other 4 samples (7D, 8A, 10E, and 11A) reacted with both *T. hydatigena* and *T. multiceps* probes.

probe candidates (Table 3) were selected based on whether they exhibited similar properties (i.e. predicted melting temperature and length), but showed the greatest differences in sequence among the species assayed (Fig. 1).

3.3. Evaluation of probes

As shown in Fig. 2, the specificity of each probe was tested using the *nad1* fragments amplified from adult/larval DNA. The results showed that probes specifically hybridised with the *nad1* fragments amplified using the newly designed primers, with no cross hybridisation observed. To evaluate the detection limit, the PCR products were serially diluted up to 1 ng/μl and subjected to the PCR/dot blot assay. The resulting blots revealed that 2 ng of blotted PCR product could reliably be detected (data not shown).

3.4. Egg differentiation

The *nad1* gene fragment of the 49 egg-positive samples was PCR-amplified and subjected to dot blot analysis using all of the probes. Of the 49 samples tested, 42 *nad1* gene fragments hybridised with the *T. hydatigena* probe, 3 with the *T. multiceps* probe and the remaining 4 with both *T. hydatigena* and *T. multiceps* probes (Fig. 3). The other probes did not hybridise with any of the samples.

4. Discussion

Although the PCR-RFLP assays have been shown to be highly sensitive [10,11], they have limitations in terms of time and labour, combined with the restrictions on the number of samples that can be processed simultaneously, complicating the widespread adoption of this technique using field samples.

Mitochondrial DNA sequencing has been used extensively to identify a variety of organisms, including parasites, and the *cox1* and *nad1* genes have frequently been used in phylogenetic studies [15,17,18]. In the case of taeniid species, the *cox1* gene had been shown to be more conserved than the *nad1* gene, with inter-taxon differences in both genes observed to range from 2.5 to 18% and 5.9 to 30.8%, respectively [16]. We therefore targeted the *nad1* gene for amplification and subsequent analysis using the dot blot detection assay with species-specific oligonucleotide probes to distinguish between taeniid species.

Most of taeniid *nad1* sequences registered in Genbank had been generated using the primer set JB11/JB12 [19]. However, we could not amplify several taeniid samples using this primer pair (data not shown). Therefore, a new set of PCR primers for the cestode *nad1* gene (*nad1*T-Fw and *nad1*T-Rv) was designed next to JB11/JB12 to facilitate the alignment and comparison with published sequences. The PCR amplification using the *nad1*T-Fw/*nad1*T-Rv primers yielded products of expected size. In addition, specific amplification of each *nad1* gene fragment was confirmed by sequence analysis followed by a homology search (data not shown).

Based on nucleotide differences in the *nad1* gene, several oligonucleotide probe candidates were designed within the variable region flank by the newly designed primers. When the oligonucleotide probes were evaluated by the PCR/dot blot assay using *nad1* PCR fragments from adult/larval DNAs, the high specificity of eight probes (Table 3) was clearly demonstrated and no cross hybridisation was observed. The detection limit of the *nad1* PCR products for each probe was 2 ng, indicating that the assay was highly sensitive.

Finally, we evaluated the PCR/dot blot assay using *nad1* fragments PCR-amplified from egg DNAs. In the PCR/dot blot system developed in this study, 42 samples hybridised only with *T. hydatigena* probe and 3 with *T. multiceps* probe, while the remaining 4 samples hybridised with both *T. hydatigena* and *T. multiceps* probes. We conclude that these 4 samples harboured eggs of both *T. hydatigena* and *T. multiceps*. Sensitivity tests of the PCR assay described herein revealed that the minimum amount of DNA template required for *nad1* gene amplification was 5 pg (data not shown). Since the amount of DNA in a single egg was reported to be 8 pg [20], the sensitivity of our PCR assay is considered to be sufficiently high to amplify the *nad1* gene from a single egg. Moreover, the PCR/dot blot assay was able to detect and differentiate between DNA extracted from five eggs. These findings suggest that the PCR/dot blot assay has the following advantages; 1) *easy-to-perform*; after PCR, no special equipment is required and results can be assessed by the naked eye, 2) *detectability of mixed infection*; simultaneous infection by several taeniid cestodes can be detected using species-specific probes, and additionally 3) *cost-effectivity*; reuse of hybridisation buffers containing probes could markedly reduce the cost of performing the assay.

In conclusion, the PCR/dot blot assay presented herein enables the detection and differentiation of eight of the most important taeniid cestodes in the areas of veterinary and public health. Moreover, this technique can be performed with relative ease in laboratories with thermal cyclers. We expect that PCR/dot blot assay described in the present study would be an alternative method for taeniid egg differentiation collected in the field. In order to improve the method, especially for eliminating egg purification step, further studies have to be conducted using coproDNA.

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Characterization of *Fasciola* spp. in Myanmar on the basis of spermatogenesis status and nuclear and mitochondrial DNA markers

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ABSTRACT

Fasciola spp. in Myanmar were characterized on the basis of spermatogenesis status and DNA markers of nuclear internal transcribed spacer 1 (ITS1) and mitochondrial NADH dehydrogenase subunit 1 (*nad1*). We collected 88 adult flukes from Yangon, Lashio, and Myitkyina. Spermatogenesis status was analyzed by the presence of sperm in the seminal vesicles, and 8 aspermic and 80 spermic flukes were detected. The flukes were identified on the basis of spermatogenesis status and ITS1 types which were analyzed by a PCR-RFLP method, and 80 spermic flukes were identified as *F. gigantica*. A very low detection rate of aspermic *Fasciola* sp. indicated that they are not established in Myanmar. In phylogenetic analyses, the 7 aspermic *Fasciola* sp. from Myitkyina displayed a haplotype in *nad1* sequence, which was identical to that of aspermic *Fasciola* sp. from other Asian countries including China. Therefore, they were probably introduced from China through an infected domestic ruminant. On the other hand, 17 *nad1* haplotypes detected in *F. gigantica* belonged to 2 clades unique to Myanmar, each with a distinct founder haplotype in a network analysis. This indicated a unique history of *F. gigantica* introduction into Myanmar involving ancient artificial movements of domestic ruminants.

1. Introduction

Fasciolosis results in significant economic losses in the livestock industry of endemic areas. It is caused by *Fasciola hepatica*, which occurs mainly in Europe, the Americas, and Oceania, and *F. gigantica*, which occurs mainly in Africa and Asia [1,2]. These 2 *Fasciola* species have normal spermatogenic ability and reproduce bisexually by fertilization, and a prominent feature is the presence of abundant mature sperm in the seminal vesicle, a male reproductive organ for temporary storage of self-produced sperm [3]. Although these species have been classified by morphological characteristics, such as body length and width, they can be differentiated by DNA sequences of the nuclear ribosomal internal transcribed spacer 1 (ITS1), ITS2, and 28S ribosomal RNA genes [4–7]. Furthermore, intraspecific phylogenetic relationships can be analyzed by mitochondrial DNA (mtDNA) markers, such as the NADH dehydrogenase subunit 1 (*nad1*) and cytochrome c oxidase 1 (*cox1*) genes [7].

On the other hand, *Fasciola* flukes that display an intermediate form between the 2 species, which cannot be classified by morphology, have been found in Asian countries, including Japan [8–10], India [11], Korea [12], the Philippines [13], and Iran [14]. Moreover, the intermediate flukes in Japan and Korea have a meiotic disorder affecting spermatogenesis [15], and there are few or no sperm in the seminal vesicle [7,16–18], which suggests that they are parthenogenetic. Therefore, the intermediate *Fasciola* flukes have been termed aspermic (parthenogenetic) *Fasciola* sp. [7,17–19]. These aspermic *Fasciola* sp. display 3 distinct DNA types in nuclear ITS1 sequences [7,17,19,20]. Two of the DNA types are identical to the sequences of *F. hepatica* and *F. gigantica* and are termed Fh type and Fg type, respectively. The third is termed Fh/Fg type, which has both sequences of *F. hepatica* and *F. gigantica*. Additionally, these aspermic *Fasciola* sp. show the 2 major *nad1* haplotypes of mtDNA. One of the *nad1* haplotypes of the *F. hepatica* clade, was detected in aspermic *Fasciola* sp. from Japan (Fsp1) [7], Korea (Kor1) [17], and China (Fh-C4) [19] and the other *nad1* haplotype of the *F. gigantica* clade, was detected in aspermic *Fasciola* sp. from Japan (Fsp2) [7,18], Korea (Kor2a) [17], China (Fg-C2) [19], and Vietnam (NDI-Fg5) [20]. These close phylogenetic relationships indicate that the 2 lineages of aspermic *Fasciola* sp. distributed in Asian countries are descendants of the 2 common maternal lineages [17]. This suggests that Asian *Fasciola* flukes, including aspermic *Fasciola* sp., should be characterized on the

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basis of not only spermatogenesis status and nuclear DNA markers but also mtDNA markers.

Fasciola gigantica is the primary cause of fasciolosis in Southeast Asia [1]. Recently, we reported that aspermic *Fasciola* sp. as well as *F. gigantica* occurred in Vietnam [20]. However, *Fasciola* flukes in other Southeast Asian countries, including Myanmar, have not been accurately characterized. Therefore, we identified species of *Fasciola* flukes in Myanmar based on spermatogenesis status and DNA sequences of the nuclear ITS1 region and conducted phylogenetic analyses based on mitochondrial *nad1* haplotypes.

2. Materials and methods

2.1. Fasciola flukes and microscopic observation

Eighty-eight adult *Fasciola* flukes were collected from the bile ducts of infected cattle and buffaloes at slaughterhouses in Yangon, Lashio, and Myitkyina from 2009 to 2010. The numbers of infected hosts and flukes obtained for each location are listed in Table 1. One or 2 adult flukes were analyzed per infected host, except for 1 host from Myitkyina in which all of the 7 adult flukes obtained were analyzed. The flukes were fixed in 70% ethanol between 2 glass slides under mild pressure. The anterior parts of the fixed flukes were removed and stained with hematoxylin-carmine solution and then examined with an optical microscope to check for sperm within the seminal vesicles.

2.2. DNA analysis

The posterior parts of the flukes without the uteri and ootypes, to exclude sperm from other flukes, were used for DNA extraction. Total DNA was extracted from individual flukes using High Pure PCR Template Preparation Kit (Roche, Mannheim, Germany), according to the manufacturer's instructions, and stored at −20 °C until use. DNA fragments of the nuclear ITS1 region, including partial 18S and 5.8S ribosomal RNA genes, and the partial mitochondrial *nad1* gene were amplified by polymerase chain reaction (PCR) using ITS1-F (5′-TTGCGCTGATTACGTCCCTG-3′) and ITS1-R (5′-TTGGCTGCGCTCTTCATCGAC-3′) for the ITS1 region, and Ita 10 (5′-AAGGATGTTGCTTTGTCGTGG-3′) and Ita 2 (5′-GGAGTACGGTTACATTCACA-3′) for the partial *nad1* gene [7]. PCR was performed in a 25 μL reaction volume containing 2 μL of DNA template, 0.2 mM of each dNTP, 0.1 μM of each primer, 1.25 U of GoTaq DNA polymerase (Promega, Madison, USA), and the manufacturer-supplied reaction buffer. The thermal program was 94 °C for 90 s, 30 cycles of 94 °C for 90 s, 55 °C for 90 s, and 72 °C for 2 min, and 72 °C for 10 min.

DNA types of the ITS1 amplicons were determined by a PCR-RFLP method [21] with slight modification. The reaction volume of 10 μL contained 5 μL of PCR amplicons, 1 U of the restriction enzyme, *Rsa*I, and 1 μL of manufacturer-supplied reaction buffer (Roche, Mannheim, Germany). After incubation at 37 °C for 3 h and heat inactivation of *Rsa*I at 65 °C for 15 min, DNA types were distinguished by fragment patterns detected on agarose gels.

PCR amplicons of the *nad1* gene were directly sequenced in both directions with BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, USA) using an additional forward primer, Ichi 1 (5′-AGGTGTTGGGTTATATGCA-3′), and Ita 2 as a reverse primer. The resultant sequencing ladders were read by the ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems, Foster City, USA).

2.3. Phylogenetic analysis

The *nad1* sequences were aligned by Clustal X version 2.0 software [22], and haplotypes were distinguished. Amino acid sequences were inferred using the platyhelminth mitochondrial code [23].

A neighbor-joining (NJ) phylogram was constructed by PAUP 4.0b10 [24]. In the tree construction, the sequence of *Paragonimus westermani* (accession no. AF219379) was used as an outgroup. Additionally, the sequences of *F. hepatica* from Uruguay, Australia, Ireland, Iran, Egypt, and China, *F. gigantica* from Zambia, Egypt, Thailand, Vietnam, and China, and aspermic *Fasciola* sp. from Japan, Korea, Vietnam, and China were used as references. Bootstrap analyses were conducted using 1000 replicates.

A median-joining (MJ) network of *nad1* haplotypes obtained in this study and reference haplotypes was illustrated by Network 4.5.1.6. software [25] and enhanced by Microsoft Office PowerPoint 2007. The reference haplotypes were from China [19], Vietnam [20], Japan [7,18], Korea [17], and Thailand [7], and they were classified into the Asian *F. gigantica* clade in the NJ phylogram. The frequencies of reference haplotypes were cited from our previous studies [7,17–20]. The frequency data of 137 Chinese *Fasciola* spp. remain unpublished. In the MJ network, a median vector is a hypothesized haplotype that is required to connect existing haplotypes within the network.

Table 1
Number of *Fasciola* flukes, sperm in their seminal vesicles, DNA types, and species identification.

Location	Number of infected hosts	Number of flukes [a)]	Sperm in seminal vesicle	DNA type		Species
				ITS1	*nad1* (accession number)	
Myitkyina	20	18	+	Fg	Fg-M1 (AB604007)	*F. gigantica*
		1	+	Fg	Fg-M11 (AB604017)	*F. gigantica*
		1	+	Fg	Fg-M12 (AB604018)	*F. gigantica*
		1	+	Fg	Fg-M13 (AB604019)	*F. gigantica*
		8	+	Fg	Fg-M15 (AB604021)	*F. gigantica*
		1	+	Fg	Fg-M16 (AB604022)	*F. gigantica*
		1	+	Fg	Fg-M17 (AB604023)	*F. gigantica*
		1	+	Fg	Fg-M18 (AB604024)	*F. gigantica*
		1	+	Fg	Fg-M19 (AB604025)	*F. gigantica*
		2	+	Fg	Fg-M20 (AB604026)	*F. gigantica*
		7 [b)]	-	Fh/Fg	Fg-M14 (AB604020)	*Fasciola sp.*
Subtotal		42				
Lashio	ND[c)]	1	+	Fg	Fg-M1 (AB604007)	*F. gigantica*
		1	+	Fg	Fg-M2 (AB604008)	*F. gigantica*
Subtotal		2				
Yangon	26	29	+	Fg	Fg-M1 (AB604007)	*F. gigantica*
		1	+	Fg	Fg-M3 (AB604009)	*F. gigantica*
		3	+	Fg	Fg-M4 (AB604010)	*F. gigantica*
		1	+	Fg	Fg-M5 (AB604011)	*F. gigantica*
		1	+	Fg	Fg-M6 (AB604012)	*F. gigantica*
		4	+	Fg	Fg-M7 (AB604013)	*F. gigantica*
		1	+	Fg	Fg-M8 (AB604014)	*F. gigantica*
		2	+	Fg	Fg-M9 (AB604015)	*F. gigantica*
		1	+	Fg	Fg-M10 (AB604016)	*F. gigantica*
		1	-	Fg	Fg-M3 (AB604009)	*Fasciola sp.*
Subtotal		44				
Total		88				

a) One or 2 specimens were collected per host.
b) The 7 specimens were collected from one host.
c) No data.

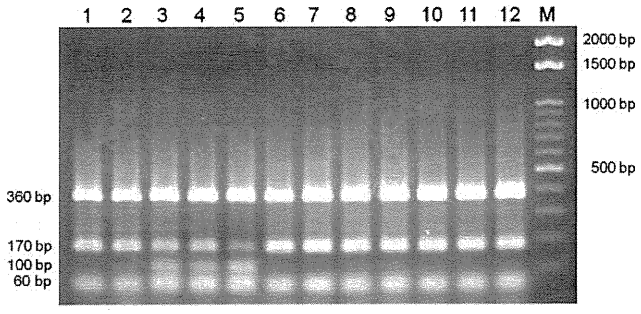

Fig. 1. Restriction fragment patterns of the nuclear ITS1 region of *Fasciola* flukes in Myanmar. Lanes 1, 2, and 6–12: Fg type. Lanes 3–5: Fh/Fg type. M: 100-bp DNA ladder.

The degree of gene flow between populations was estimated using the pairwise fixation index (Fst) calculated by Arlequin 3.5 [26]. The 3 *F. gigantica* populations from Myanmar, Vietnam, and China were used, and calculations were performed using the same data set as the MJ network. Fst values approaching 1 indicate extreme genetic differentiation between 2 populations.

3. Results

3.1. Species identification based on spermatogenesis and nuclear ITS1 types

The 7 flukes obtained from 1 host in Myitkyina and the 1 fluke from Yangon had no sperm in their seminal vesicles. These flukes were identified as aspermic *Fasciola* sp. because they were adult flukes with eggs in their uteri. On the other hand, the remaining 80 flukes had abundant sperm in their seminal vesicles and were identified as spermic species (Table 1). Fg type and Fh/Fg type were detected in this study using the PCR-RFLP method. Fg type displayed the same fragment pattern as *F. gigantica* (approximately 360, 170, and 60 bp), and Fh/Fg type displayed a mixed fragment pattern of *F. hepatica* and *F. gigantica* (approximately 360, 170, 100, and 60 bp) (Fig. 1). All of the 80 spermic flukes displayed Fg type, and were therefore identified as *F. gigantica*. The 7 aspermic *Fasciola* sp. from Myitkyina and the 1 aspermic *Fasciola* sp. from Yangon displayed Fh/Fg type and Fg type, respectively (Fig. 2).

3.2. Mitochondrial nad1 haplotypes

The partial nucleotide sequences (535 bp) of the *nad1* gene showed 27 substitution sites and yielded 20 haplotypes, represented by Fg-M1 to Fg-M20 (accession nos. AB604007–AB604026). The substitution sites included 9 transversions, 18 transitions, and 11 nonsynonymous substitutions. Fg-M1 was detected in *F. gigantica* from all 3 locations and showed the highest frequency (54.5%, 48 out of 88 flukes), followed by Fg-M15 (9.1%, 8 out of 88 flukes), which was detected in *F. gigantica* from Myitkyina. Fg-M14 was detected only in the 7 aspermic *Fasciola* sp. from Myitkyina, and Fg-M3 was detected in both *F. gigantica* and the 1 aspermic *Fasciola* sp. from Yangon (Table 1).

The NJ phylogram showed that the 20 *nad1* haplotypes from Myanmar belonged to the Asian clade of *F. gigantica*, which was clearly distinguished from the African clade with high bootstrap values (99.6%, Fig. 3).

The MJ network is illustrated in Fig. 4. The nucleotide sequence of Fg-M14 was identical to those of aspermic *Fasciola* sp. from Japan (Fsp2: AB207168), Korea (Kor2a: AB211240), Vietnam (NDI-Fg5: AB385619), and China (Fg-C2: AB477366). The sequence of Fg-M2 was identical to those of *F. gigantica* from Vietnam (NDI-Fg2: AB385616) and China

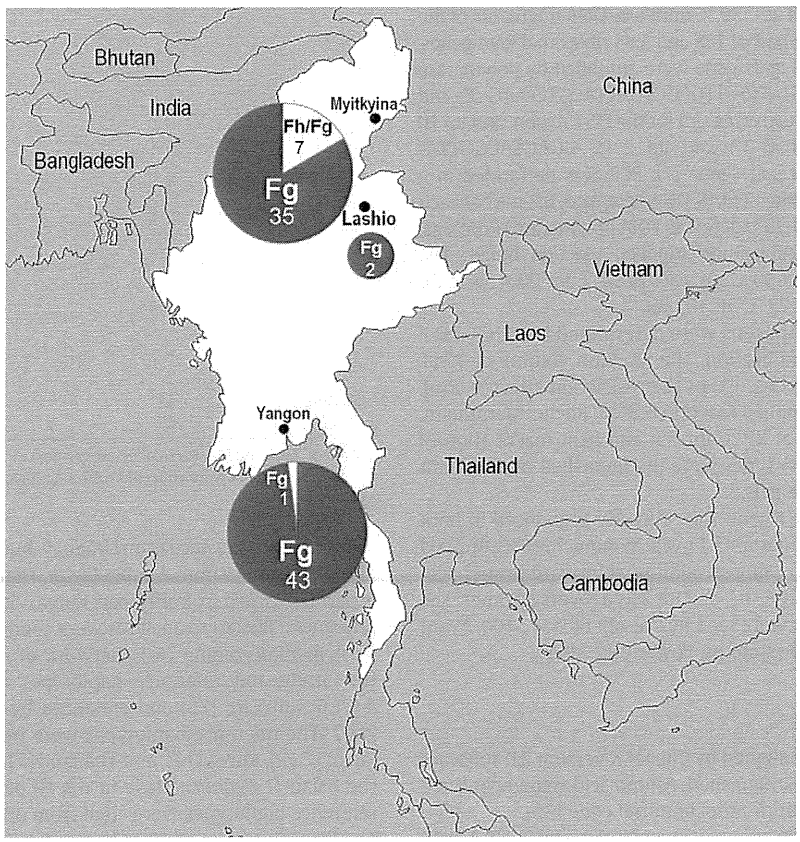

Fig. 2. Spermatogenesis status and nuclear ITS1 types of *Fasciola* flukes collected from 3 cities in Myanmar. White and black portions of circles indicate aspermic and spermic flukes, respectively. Sizes of circles correlate to the number of *Fasciola* flukes, and numeric characters indicate actual numbers.

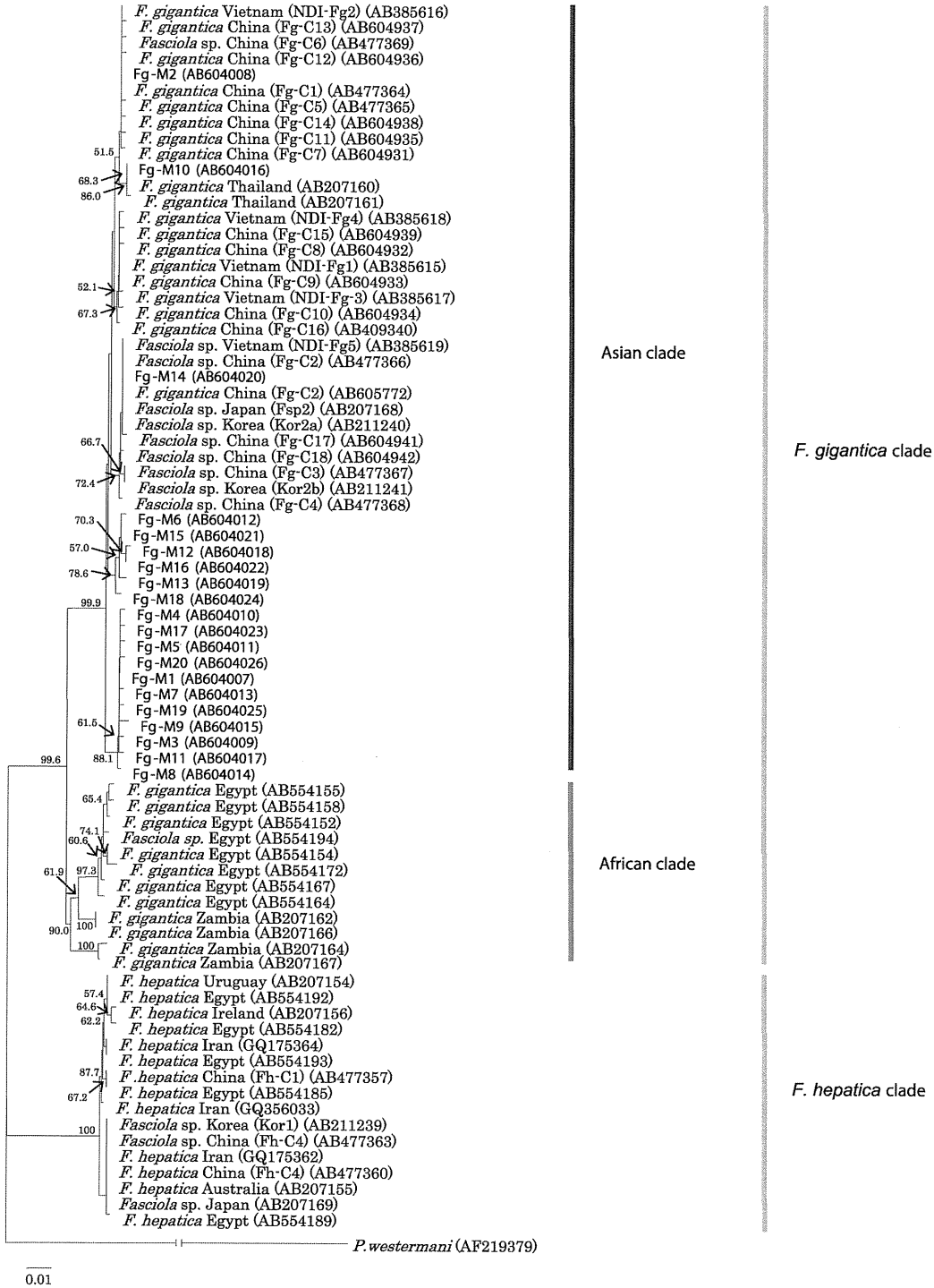

Fig. 3. A neighbor-joining phylogram of the mitochondrial *nad1* haplotypes of *Fasciola* spp. from Myanmar and other countries. Bootstrap values higher than 50% are shown on tree nodes. The haplotypes obtained in this study, Fg-M1 to Fg-M20, are represented by gothic font.

(Fg-C1: AB477364), and the sequence of Fg-M10 was identical to that of *F. gigantica* from Thailand (AB207160). The other 17 haplotypes belonged to 2 distinct clades unique to Myanmar, and a median vector connected the 2 Myanmar clades and the other clades. The maximum mutational steps within the 2 Myanmar clades were 11, which was more than that within the *F. gigantica* clade from China and Vietnam (4 and 3, respectively).

3.3. Genetic distance between *F. gigantica* populations

The Fst value was 0.581 between Myanmar and Vietnamese populations, and 0.534 between Myanmar and Chinese populations. In contrast, it was 0.105 between Vietnamese and Chinese populations (Table 2). This suggested that gene flows between Myanmar and the other populations had been maintained at a relatively low level.