Table 1
Fecal egg examination for intestinal helminths in Kanchanaburi Province, Thailand.

Village	No. examined	No. positive (%)	Parasites species (%)[a]				
			Hookworm	*Ascaris*	*Trichuris*	*Taenia*	Others
Village A	265	76 (28.7)	70 (26.4)	2 (0.8)	6 (2.3)	2 (0.8)	1 (0.4)[b]
Village B	402	113 (28.1)	88 (21.9)	2 (0.5)	29 (7.2)	2 (0.5)	6 (1.5)[c]
Total	667	189 (28.3)	158 (23.7)	4 (0.6)	35 (5.2)	4 (0.6)	7 (1.1)

[a] Some cases have multiple infections.
[b] *Enterobius vermicularis*.
[c] Intestinal trematodes suspected to be belonging to Heterophyidae.

The area was composed largely of highlands and a water reservoir south of Vajiralongkorn Dam. The study sites were 2 rural villages A and B close to the Thai–Myanmar border. Sympatric occurrence of 3 human *Taenia* species was previously confirmed in the same district including village A [7], whereas there was no information in village B. As people in village A purchased piglets from village B, we chose village B for the second year survey. The villages were surrounded in the south by a water basin, and in the others by heavy forest behind the partial plain area. Communication to and from nearby villages and district centers is limited to travel by boat. Each village has about 800–1000 residents. The majority of the population is Karen in origin with Mon, Myanmar, Lao, and Thai minorities. A number of residents are legal or illegal immigrants mainly from Myanmar. Therefore, actual village names are not specified. The main economical activities are agriculture and fishery. The study was approved by the Ethics Committee of the Faculty of Tropical Medicine, Mahidol University, Thailand. Informed consents were obtained at the time of stool and blood collections.

2.2. Fecal egg examination for intestinal helminths including *Taenia* spp.

The survey was conducted in December 2006 (village A) and November 2007 (village B). Stool samples were collected from residents aged ≥5 years, of both sexes, who were willing to participate in the study. Community members with histories/memories on *Taenia* proglottids in their stools were encouraged to receive examination. Kato–Katz stool examination was applied for microscopic detection of helminth eggs [10]. Three fecal *Taenia* eggs-positive persons and 3 other persons who had a recent (<1 year) history of discharging worm segments in their stools were treated with 2 g niclosamide early in the morning pre-meal followed by saturated magnesium sulfate (60 ml) 2 h post treatment. Four to five whole bowel movements were collected to detect the worms. Evacuated *Taenia* segments from each carrier were fixed in 80% ethanol for molecular study. All scolices expelled were fixed in 10% formalin for staining and morphological study. A part of individual fecal samples were kept frozen in Eppendorf tubes for DNA analysis.

2.3. Molecular study

Frozen stool samples and *Taenia* segments expelled from the participants kept in 80% ethanol were analyzed by multiplex PCR at Asahikawa Medical College (AMC), Japan. DNA was extracted from worms and stool by using DNeasy tissue kit and QIAamp DNA Stool Mini kit (Qiagen, Hilden, Germany), respectively. Cytochrome c oxidase subunit 1 (*cox1*) gene in mitochondrial DNA of the worms and eggs in stool were PCR-amplified and analyzed [6].

2.4. Serological tests for cysticercosis

Blood samples were obtained from 159 (114 of 8–66 years old from village A and 45 of 8–80 years old from village B) out of 667 residents who provided fecal samples. The serum samples were tested at AMC, Japan, for cysticercosis by ELISA using partially purified glycoprotein antigens (GPsAg) by preparative isoelectric focusing of *T. solium* cysts [11]. The cut-off ELISA value was 3 times of the optical density of pooled healthy control sera. Then, ELISA-positive samples were examined by immunoblot using recombinant chimeric antigens (RecAg) prepared from *T. solium* metacestodes [12].

3. Results

3.1. Intestinal helminth infections and taeniasis

A total of 667 (265 and 402 from villages A and B) stool samples were examined. The results for intestinal helminth infections were similar in the 2 villages (Table 1). *Taenia* eggs were found in 4 persons (2 each from villages A and B). Three other persons from village A had a recent history of discharging proglottids in their stools. Among these 7 persons being suspected of having taeniasis, 6 (nos. 1–6 in Table 2) of them were treated with 2 g niclosamide followed by purgatives. One patient was not treated because of severe ascites. Three participants (nos. 3–5) expelled strobilae with a single scolex each and 2 others (nos. 2, and 6) expelled those without scolices. One patient (no. 6) expelled big biomass, which was estimated as 6 worms

Table 2
Summary of the taeniasis cases from Kanchanaburi Province, Thailand, detected by various methods.

Village	Carriers (age in years, gender)	Helminth eggs-positive	*Taenia* egg-positive	History of expelled proglottids	No. of worms expelled		Multiplex PCR	
					Scolex	Proglottids	Segments	Feces
A	1 (32, male)	Hookworm[a]	+	–	0	0	ND	*T. saginata*
	2 (40, female)	–	+	–	0	1	*T. asiatica*	*T. asiatica*
	3 (44, male)	–	–	+	With hooks	1	*T. solium*	*T. solium*
	4 (35, male)	–	–	+	No hook	1	*T. saginata*	*T. saginata*
	5 (33, female)	ND	ND	+	No hook	1	*T. asiatica*	No band[b]
B	6 (30, female)	–	+	+	0	6[c]	*T. asiatica*	*T. asiatica*
	7 (76, female)[d]	Hookworm[a]	+	+	ND	ND	ND	*T. asiatica*

ND – not done.
[a] Numbers of eggs/gram of feces were 3450 (carrier 1) and 60 (carrier 7).
[b] The stool for coproDNA was collected only after given magnesium salt.
[c] Estimated due to the sizes of the strobilae.
[d] No treatment due to her severe ascites.

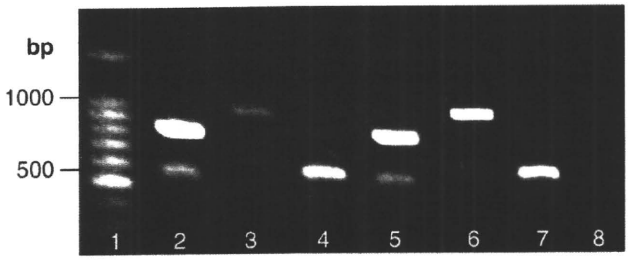

Fig. 1. Multiplex PCR of *Taenia* worms from 3 carriers from village A. Lane 1: DNA markers, Lane 2: *T. saginata* control, Lane 3: *T solium* control, Lane 4: *T. asiatica* control. Lane 5: carrier no. 4, Lane 6: carrier no. 3, Lane 7: carrier no. 5, Lane 8: negative control.

based on the sizes of the strobilae. One of the 3 scolices had hooklets on the rostellum. One *Taenia* egg-positive person (no. 1) did not expel any worms within 5 bowel movements of a day post-treatment.

3.2. Molecular identification of Taenia species

The identification of taeniid worms was carried out by multiplex PCR (Fig. 1, Table 2). The segment from a worm with hooklets on the scolex was identified as *T. solium* (carrier no. 3). Segments from worms without hooklets on the scolex were *T. saginata* and *T. asiatica* (carrier nos. 4 and 5). All other worms including 6 estimated worms from one person were *T. asiatica*.

The 22 available stool samples including those of all 5 and 2 taeniasis patients from villages A and B, respectively (Table 2) were analyzed also by multiplex PCR. Six fecal samples from *Taenia* carriers (nos. 1–4, 6–7) exclusively showed coproDNA-positive results, whereas no. 5 was negative. CoproDNA results from 4 carriers (nos. 2–4, 6) showed the same results from multiplex PCR using the worms.

3.3. Serological tests for cysticercosis and clinical follow-up

Five of 114 people of 8–66 years old from village A and 4 of 45 people of 8–80 years old from village B were sero-positive by ELISA using GPsAg. Five ELISA positives and 1 additional border line case from village A were checked by confirmative immunoblot using RecAg. Three of the five ELISA positives and one border line case showed the specific diagnostic band (Fig. 2). The sample on the border line showed the weakest but visible band. Two of the 4 ELISA positive samples from village B were also positive with RecAg immunoblotting (data not shown). All seven *Taenia* worm/egg-positive cases listed in Table 2 were sero-negative by ELISA, suggesting no person being suffered from taeniasis and cysticercosis simultaneously.

The clinical histories of these 4 serologically confirmed cysticercosis cases from village A were reviewed, and 3 persons underwent brain CT examination. Due to political problem, one patient could not go out from the village. The 42 year-old woman, (lane 3, Fig. 2), had

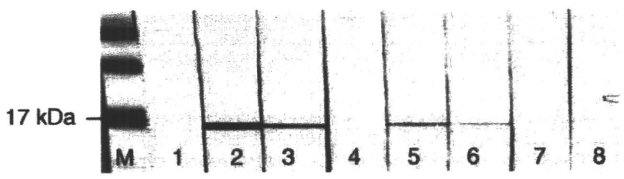

Fig. 2. Immunoblot using RecAg for 6 persons of village A including 5 ELISA-GPsAg positive (lanes 3–7) and one ELISA-GPsAg border line sample (lane 8). Lane M: prestained protein markers to confirm the quality of transblotting and molecular sizes, lane 1: negative control, lane 2: positive control, lanes 3–8: sera from 6 persons suspected by ELISA using GPsAg. Lanes 3, 5, 6 and 8 were positive. Lane 3 was symptomatic NCC case.

clinical symptoms related to neurocysticercosis (NCC), *i.e.* headache, stiff neck, seizure, and vomiting. Brain CT images showed small ill-marginated hypodense areas in the left frontal lobe, at the cortical medullary junction close to the brain surface at left frontal lobe, with minute nodular contrast enhancement (Fig. 3). This person had a past (>1 years ago) history of expelling proglottids. Two other persons were clinically normal and showed no obvious CT-image abnormality.

4. Discussion

Through this study in the two villages in Kanchanaburi Province, central Thailand, we have found a total of 7 *Taenia* carriers (Table 2) detected by fecal egg examination and deworming with molecular speciation by multiplex PCR for worms and/or stool samples. Among those 7 cases, 5 (nos. 2–6, Table 2) were confirmed by treatment with niclosamide. The *Taenia* species were *T. solium* ($n=1$), *T. saginata* ($n=1$) and *T. asiatica* ($n=8$ from 3 carriers). Two persons (nos. 1 and 7) were *Taenia* egg-positive by microscopic examination and coproDNA, the latter method have proven that one (no. 1) was positive for *T. saginata* and the other (no. 7) for *T. asiatica*. However, the no. 1 did not expel any worms after niclosamide treatment and the no. 7 was not treated due to her serious ascites. The present results reconfirmed our previous finding of the sympatric occurrence of three *Taenia* species in village A [7]. In the present study, two worm carriers in village B had *T. asiatica* only.

Serological screening for cysticercosis by ELISA revealed that 9 persons (5 in village A and 4 in village B) were sero-positive against GPsAg. Among them, 5 persons were clearly positive by immunoblot using RecAg (Fig. 2). At the moment, ELISA using GPsAg is recommended for screening of cysticercosis, and the immunoblot using RecAg is strongly recommended for the confirmative serodiagnosis [12,13], since immunoblot using RecAg is 100% specific to *T. solium* cysticercosis [12,14]. Among the immunoblot positive cases, one woman was confirmed to be symptomatic NCC, whereas the others showed no critical clinical abnormalities. Therefore, we concluded that these other cases of antibody-positive to RecAg by immunoblot were asymptomatic cysticercosis cases. As subcutaneous cysticercosis (SCC) is also common clinical manifestation of cysticercosis in Asia, we have to be keen to check SCC as well as NCC. Since identification of cysticercosis cases is not always easy without histopathological specimens [13,15], combination of both radio-imaging figures and serology using highly reliable antigens are the essential tools for screening and diagnosis [8,16].

In this study, all persons including the confirmed and suspected cysticercosis were not taeniasis carriers. However, two of them including confirmed NCC case had a history of expelling proglottids in feces which ceased more than one year ago, suggesting that these two persons might have been infected with *T. solium* in the past. As the carrier of *T. solium* is the highest risk person for cysticercosis, we should pay attention not only for the carriers themselves but also for their family members and neighbors to detect hidden cases of cysticercosis [17–19].

In contrast to the diagnosis for cysticercosis, detection of taeniasis cases is rather easy, since we can detect eggs or proglottids in feces. However, detection of eggs under microscope is not sensitive enough [20]. History of expulsion of proglottids is, in general, not always clear or reliable, especially for *T. solium*, and is highly variable in the communities [21]. In the present study, however, 4 of 6 *Taenia* carriers had a history of expulsion of proglottids (Table 2) and they expelled either *T. solium* (no. 3), *T. saginata* (no. 4) or *T. asiatica* (nos. 5, and 6) after niclosamide treatment. Therefore, the carriers' memories were highly reliable in these villages. Speciation of *Taenia* worms can be easily done by molecular tools. Even the carriers who did not expel worms were diagnosed at species level by coproDNA test (no. 1). Therefore, coproDNA test is expected to be more sensitive than microscopic detection of eggs so far [6]. Nonetheless, multiplex PCR is

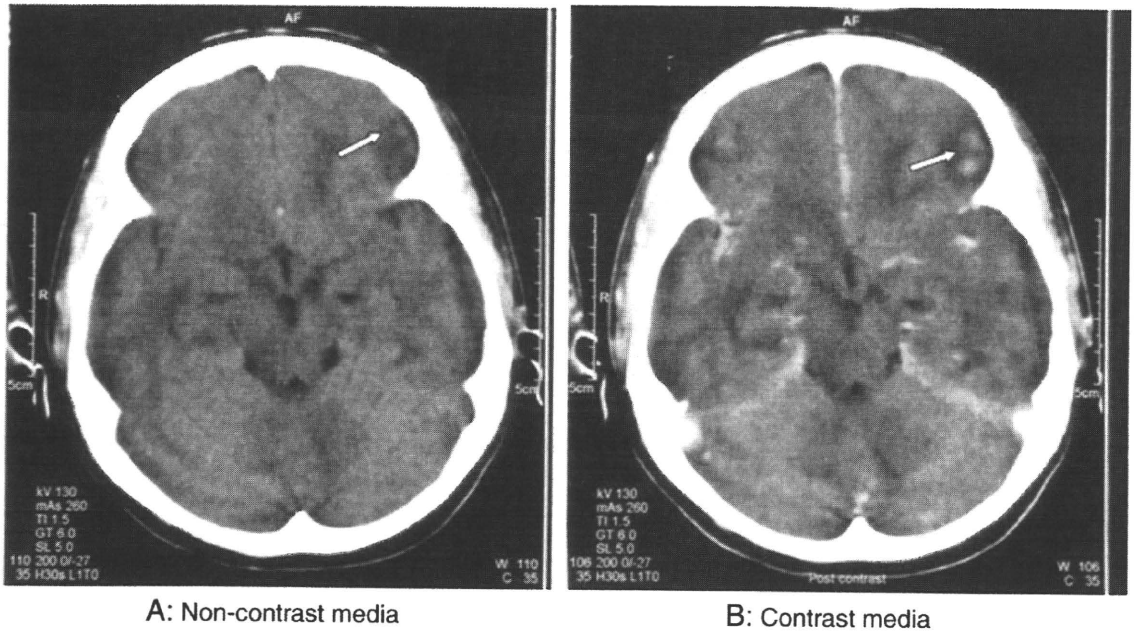

Fig. 3. CT image of 42 year-old woman with clinical symptoms related to neurocysticercosis. A: a poorly defined low attenuation area is noted (arrow) in left frontal gray–white matter junction in non contrast scans. B: Small ring enhancement is noted (arrows) with perifocal edema in contrast enhanced scans. Cysticercosis is one of the differential diagnosis (other possibility include: brain abscess, brain metastasis and granulomatous lesion).

not always applicable in the rural communities where we cannot expect stable electric power supply. Moreover, fecal samples may contain some inhibitors against multiplex PCR [22]. In order to clear these problems, introduction of a new molecular tool, loop-mediated isothermal amplification (LAMP) method [23] should be considered in near future.

In the present study, we found 2 carriers of *T. asiatica* in village B, and 5 carriers of 3 *Taenia* species in village A where the hybrid worms of *T. saginata* and *T. asiatica* have been found [24]. Further studies are necessary to clarify the biological background as to where and how such hybrid *Taenia* is produced. Also, more extensive survey, treatment and prevention are necessary in such areas where sympatric distribution of two or more *Taenia* species is observed.

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PLOS NEGLECTED TROPICAL DISEASES

Serological Studies of Neurologic Helminthic Infections in Rural Areas of Southwest Cameroon: Toxocariasis, Cysticercosis and Paragonimiasis

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Abstract

Background: Both epilepsy and paragonimiasis had been known to be endemic in Southwest Cameroon. A total of 188 people (168 and 20 with and without symptoms confirmed by clinicians, respectively, 84.6% under 20 years old) were selected on a voluntary basis. Among 14 people (8.3%) with history of epilepsy, only one suffered from paragonimiasis. Therefore, we challenged to check antibody responses to highly specific diagnostic recombinant antigens for two other helminthic diseases, cysticercosis and toxocariasis, expected to be involved in neurological diseases. Soil-transmitted helminthic infections were also examined.

Methodology/Principal Findings: Fecal samples were collected exclusively from the 168 people. Eggs of *Ascaris lumbricoides*, *Trichuris trichiura* and hookworms were found from 56 (33.3%), 72 (42.8%), and 19 (11.3%) persons, respectively. Serology revealed that 61 (36.3%), 25 (14.9%) and 2 (1.2%) of 168 persons showed specific antibody responses to toxocariasis, paragonimiasis and cysticercosis, respectively. By contrast, 20 people without any symptoms as well as additional 20 people from Japan showed no antibody responses. Among the 14 persons with epilepsy, 5 persons were seropositive to the antigen specific to *Toxocara*, and one of them was simultaneously positive to the antigens of *Paragonimus*. The fact that 2 children with no history of epilepsy were serologically confirmed to have cysticercosis strongly suggests that serological survey for cysticercosis in children is expected to be useful for early detection of asymptomatic cysticercosis in endemic areas.

Conclusions/Significance: Among persons surveyed, toxocariasis was more common than paragonimiasis, but cysticercosis was very rare. However, the fact that 2 children were serologically confirmed to have cysticercosis was very important, since it strongly suggests that serology for cysticercosis is useful and feasible for detection of asymptomatic cysticercotic children in endemic areas for the early treatment.

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Introduction

Parasitic infections are serious public health problems in many developing countries [1,2]. These diseases can affect various tissues and organs including the brain leading to neurological dysfunction. Cysticercosis caused by *Taenia solium* metacestodes has been assumed to be the most common parasitic infection of the brain worldwide including Cameroon [3–5]. As cysticercosis is one of the major causative agents of the late-onset of epilepsy, the major work on cysticercosis has been carried out for adults but not for children in endemic areas, and other causative agents of epilepsy still remain unclear. Therefore, we were lead to obtain more information on the causative agents of epilepsy in developing countries, since many helminthic diseases including toxocariasis, paragonimiasis, onchocerciasis etc., and also protozoan diseases including malaria, toxoplasmosis and others may cause epilepsy [4–6]. Among these neglected helminthic diseases, toxocariasis is expected to have cosmopolitan distribution, since dogs and cats

Author Summary

A total of 188 people (168 and 20 with and without symptoms confirmed by clinicians, respectively, 84.6% under 20 years old) were selected on a voluntary basis in Cameroon. Soil transmitted helminthic infections were prevalent among persons surveyed as is common in developing countries, since eggs of *Ascaris lumbricoides*, *Trichuris trichiura* and hookworms were found from 56 (33.3%), 72 (42.8%) and 19 (11.3%) persons, respectively. Serological analyses revealed that 61 (36.3%), 25 (14.9%) and 2 (1.2%) persons were positive to the diagnostic antigens specific for toxocariasis, paragonimiasis and cysticercosis, respectively. Among 14 people with epilepsy, 5 persons were seropositive to the antigen of *Toxocara* and one of them was simultaneously positive to the antigens of *Paragonimus*. Serological confirmation of cysticercosis in two children is very important, and we suggest that further serologic surveys of cysticercosis be carried out in both children and adults in this area for the promotion of a better quality of life including control and early treatment.

are companion animals with close contact with people in the world [7,8]. Although there are no data on the prevalence of human toxocariasis in Cameroon, its prevalence in dogs in Cameroon is high [9]. Simultaneously, there is poor information on cysticercosis in children in Cameroon, although it seems to be rather common in the adult population [4,5].

Tombel health district in South West Province in Cameroon (Figure 1) is known as an endemic focus of epilepsy and is also highly endemic for paragonimiasis [10,11]. Our previous report in this area showed that 8.3% of enrolled people (14/168) suffered from epilepsy but only one of the epileptic patients simultaneously suffered from paragonimiasis [11]. Therefore, we concluded that paragonimiasis was not the major cause of epilepsy in children in this area.

In this study, we used the same 188 samples examined for paragonimiasis [11] and additional 20 samples from Japan, where cysticercosis and paragonimiasis have long been eradicated and toxocariasis is very rare [12], as healthy controls. We performed serosurveys using highly specific recombinant antigens for toxocariasis and cysticercosis, and simultaneously analyzed the unpublished data on microscopic observation of soil-transmitted helminthic (STH) infections. Serological data on paragonimiasis for this study were modified from published data [11]. Although onchocerciasis was known to be endemic in Cameroon and might be involved in neurological disorder, we could not examine simply because the lack of serological tools [13,14].

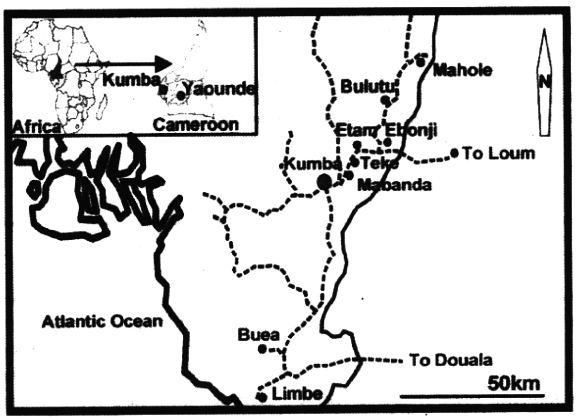

Figure 1. Locations of Bulutu, Ebonji, Etam and Teke in Tombel sub-Division, Southwest Province, Cameroon.
doi:10.1371/journal.pntd.0000732.g001

Materials and Methods

Study sites

Four villages in rural areas, Bulutu, Ebonji, Etam and Teke, were selected for this study. They are located in the Tombel Health District (50,000–100,000 inhabitants) in the rain forest zone about 40 km northwest of Kumba, Manengouba Department, South West Province of Cameroon (4°3′N, 9°3′W). The annual average temperature is 24°C and the relative humidity varies from 52% to 74%. Agriculture is the principal economic activity; hunting and fishing are also practiced (Figure 1).

Ethical statement

The survey, approved by the National Ethics Committee of Cameroon, was conducted in the general population in January 2004 and February 2006 in villages mentioned above.

Human samples

The chief of each village was informed about the study and participants or parents/guardians were asked to give informed consent for participation. A total of 188 people ranged in age from 4 to 78 years (14.9±7.8 years in males and 13.1±6.1 years in females) were examined by clinicians and were asked whether they had experienced symptoms such as cough, haemoptysis, headache, epilepsy, chest pain, and eye disorder and whether they consumed raw and/or undercooked fresh water crabs or pork. Our study population with symptoms ranged from 0–10 years (80 persons), 11–20 years (63 persons), and >21 years (25 persons). Following the questionnaire, serum, sputum and fecal samples were collected from 168 people who accepted to participate to the study voluntarily (28, 52, 55 and 33 from Bulutu, Ebonji, Etam, and Teke villages, respectively). By contrast, 20 healthy persons [5 persons from each village including 11 females and 9 males ranged from 6 to 34 years (13.0±3.7 in males and 15.1±7.5 in females)] confirmed by clinicians donated serum samples exclusively; these serum samples were used as expected healthy controls. An additional 20 serum samples from students at Asahikawa Medical College (AMC), Japan, were used as confirmed healthy controls. Sputum was examined for eggs of *P. africanus* [11]. Fecal samples were examined by flotation techniques for the presence of eggs to provide a diagnosis of helminthic infections.

Serology

A total of 208 serum samples were examined by ELISA. A recombinant antigen of *T. canis* second-stage larvae (0.5 μg/ml) was used for toxocaraisis [15]. Glycoproteins (GPs) (1.0 μg/ml) from *T. solium* cyst fluid purified by preparative isoelectric focusing (pH 9.2–9.6) were used for screening of cysticercosis by ELISA [16]. Immunoblot using a recombinant chimeric antigen, 100% specific to cysticercosis (0.5 μg/mini gel) was applied for serological confirmation of cysticercosis [16–19]. Somatic antigens of *P. africanus* adult worms (5μg/ml), which showed few cross reactivity with other parasitic infections were used for paragonimiasis [11]. Briefly, 96-well microtiter plates (Maxisorp; Nunc, Roskilde, Denmark) were coated with each of the antigens described above in PBS and incubated at 4°C overnight. The

plates were probed with diluted serum samples. Serum dilutions were in 1:200 with bicarbonate buffer for toxocariasis, and 1:100 and 1:200 with blocking buffer for cysticercosis and paragonimiasis, respectively, according to the original papers for these diseases described above. Peroxidase-conjugated rec-Protein G (Zymed, San Francisco, USA) diluted in 1:1000 with blocking buffer was added into each well. Peroxidase activity was revealed by adding 0.4 mmol/l 2,2-azino-bis 3 ethybenz-thiazoline-6-sulphonic acid in 0.1 mol/l sodium citrate buffer, pH 4.7 containing 0.003% H_2O_2 at room temperature. The optical density (OD) was monitored at 405 nm on a microplate reader (ImmunoMini, model NJ-2300; Nalgene Nunc International, Tokyo, Japan). The cut-off value was calculated for each antigen based on the means+3SD of 40 healthy donors from the local areas in Cameroon (n = 20) and from Japan (n = 20).

Statistical analyses

To obtain adjusted odds ratios (ORs) of paragonimiasis and toxocariasis seropositivities for each symptom, we performed multivariate logistic regression analysis adjusted for age (−10, 11–20, 21year) and sex. Because the number of cysticercosis seropositivity was rather small (n = 3), we did not analyze the ORs of cysticercosis seropositivity. For all statistical analyses, a 5% level of significance was applied. All statistical analyses were conducted using SPSS for Windows version 18.0 (SPSS, Inc., Chicago, U.S.A.).

Results and Discussion

In this study, the samples used for paragonimiasis [11] were also tested for toxocariasis and cysticercosis and also the data of STHs were analyzed. The enrolled persons (168: 78 males and 90 females) were diagnosed suffering from cough (n = 135, 80.3%), haemoptysis (n = 18, 11.3%), chest pain (n = 80, 47.6%), epilepsy (n = 14, 8.3%), visual impairment (n = 30, 17.8%) and headache (n = 106, 63.0%) and had histories of eating raw or undercooked crabs (n = 137, 81.5%) or pork (n = 135, 80.3%). Microscopic examination revealed *Paragonimus* eggs in sputum from 16 (9.5%) persons but no eggs from feces [11], whereas *A. lumbricoides*, *T. trichiura*, and hookworms were found in feces from 56 [33.3%; 30 (53.5%) males and 26 (46.4%) females], 72 [42.8%; 38 (52.7%) males and 34 (47.2%) in females] and 19 [11.3%; 14 (73.6%) males and 5 (26.3%) females] persons, respectively. Among these helminthic infections, hookworm infection exclusively showed statistically significant difference between the genders ($p<0.05$). The difference in prevalence between males and females for hookworm infection may be due to the barefoot roaming behavior of males but further investigation of this topic is needed. The highest multiple infections were found in 3 kids infected with 3 STHs and were simultaneously seropositive for paragonimiasis and toxocariasis as well.

ELISA for diagnosis of toxocariasis, paragonimiasis and cysticercosis indicated that 61 [36.3%; 31 (50.8%) males and 30 (49.1%) females], 25 [14.9%; 10 (40%) males and 15 (60%) females] [11], and 3 [1.8%; 2 boys of 13 and 11 year-old, and one girl, 4 year-old] persons were positive (Figure 2). Persons with cough and haemoptysis were more likely to have paragonimiasis (Table 1, OR = 7.19 and 2.28 respectively, $p<0.001$), whereas there was a relative risk with other symptoms. As none of the symptoms were specific for toxocariasis, the probability to have the infection was equally likely in exposed and control group as OR values were close to 1 (Table 1). The likelihood for cysticercosis to occur was not included due to the low number of seropositive persons. Nonetheless, there were crucial differences in antibody

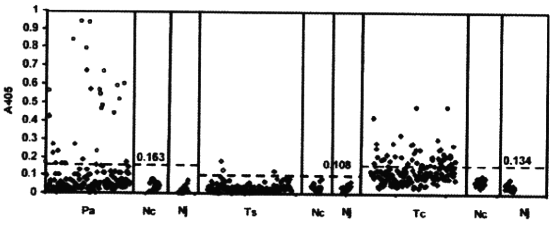

Figure 2. ELISA results for paragonimiasis (Pa), cysticercosis (Ts) and toxocariasis (Tc) from 208 persons. The surveyed persons (n = 208) include 168 and 40 (20 persons from Cameroon (Nc) and 20 from Japan (Nj)) with and without symptoms, respectively. ○, samples with *Paragonimus* eggs in the sputum. Serology could detect more cases than microscopical examination of sputum and was expected to be more sensitive for detection of paragonimiasis including immature adult stage [11]. ◇, samples positive against the recombinant antigen 100% specific to cysticercosis by immunoblot [17]. The broken line denotes the respective cut-off value for each disease and each cut-off value is shown.
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responses between the two groups. Furthermore, there was no difference in OD values between healthy controls from endemic Cameroon and from non-endemic, Japan where we expected no positive samples from students at AMC. Therefore, we concluded that the serological findings indicated specific responses to these three helminthic infections. The ELISA system applied for paragonimiasis in this study was much more sensitive for diagnosis than detection of eggs as already shown (Figure 2) [11]. As it has already been shown that the ELISA for toxocariasis in this study showed no cross-reactions with ascariasis patients in Asia and Latin America [20,21], we consider that it is highly specific to toxocariasis. As children are the most risky population for toxocariasis and the prevalence of *T. canis* in dogs in Cameroon was very high [9], we expected that 61 persons (36.3%) were really exposed to eggs of *Toxocara* [22,23]. Among these seropositive persons, 11 persons were concluded to have dual infection of both *Toxocara* and *Paragonimus*.

Table 1. Odds ratio of positive serological test for each symptom among 188 subjects.

Type of symptom	Serological test	Odds ratio*	95%CI	P value
Headache	Paragonimiasis	1.22	0.52–2.87	0.999
	Toxocariasis	1.12	0.60–2.08	0.729
Haemoptysis	Paragonimiasis	7.19	2.68–19.30	<0.001
	Toxocariasis	0.99	0.40–2.46	0.977
Cough	Paragonimiasis	2.28	0.50–10.29	0.284
	Toxocariasis	1.97	0.75–5.16	0.167
Chest pain	Paragonimiasis	1.44	0.61–3.43	0.406
	Toxocariasis	1.33	0.71–2.48	0.371
Eye disorder	Paragonimiasis	0.91	0.23–3.64	0.910
	Toxocariasis	1.38	0.06–4.31	0.521
Epilepsy	Paragonimiasis	0.51	0.52–3.70	0.539
	Toxocariasis	1.30	0.39–4.28	0.667

*Adjusted for age (−10y, 11–20y, >21y) and sex.
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Three children (1.7%) showing weak responses to the GPs of *T. solium* by ELISA (Figure 2) were further analyzed using the recombinant antigen for serological confirmation of cysticercosis, since there are no false positive antibody responses to the recombinant antigen by immunoblot [17–19]. Two of them showing higher OD values by ELISA (Figure 2) exhibited positive response with the recombinant antigen by immunoblot (Figure 3) [17,18]. Therefore, these two cases are considered as asymptomatic cysticercosis and are important targets for cysticercosis studies in the future. We believe that further epidemiological surveys for neurocysticercosis in the adult population should be carried out in the same areas, since 1) the late-onset epilepsy due to cysticercosis is expected to be detectable more common from senior people [3–6,24,25], 2) cysticercosis prevalence in Cameroon ranges from 2.5% to 13% [4,5] and 3) more than the half of epileptic adult patients show antibodies against cysticercosis in West and North West regions in Cameroon using the same serology [5].

In Papua, Indonesia, one of the most serious endemic areas of cysticercosis in the world, more than 80% and 70% of people over 18 years old, who had history of epileptic seizures with or without subcutaneous nodules, were confirmed as having cysticerci, respectively [26,27]. Approximately 30% of asymptomatic healthy people were serologically identified as positive for cysticercosis and follow up investigations revealed that many of them had detectable subcutaneous nodules. Furthermore, the most recent retrospective study using molecular tools has revealed that a cysticercus of *T. solium* survived at least for 10 years in a patient's brain [28].

According to these data mentioned above, the most important implication on cysticercosis from this serological study is that asymptomatic cysticercosis can be detected from children in endemic areas. Therefore, introduction of serological screening of children becomes highly informative for detection of asymptomatic cases and for getting better and early treatment for them [29]. Follow-up studies on these 2 boys using neuroimaging tools are necessary for further evaluation. We recommend highly reliable serological screening for cysticercosis for all pupils in the primary school, if possible, or all teenagers at least in highly endemic areas. As risk factors associated with human cysticercosis include the occurrence of cysticercosis in pigs, detection of adult worm carriers should be investigated. For the future survey of taeniasis carriers, both copro-ELISA [30] and copro-DNA tests [31] are expected to be introduced in this area, Cameroon, and in any other areas where cysticercosis is highly endemic.

Figure 3. Immunoblot using the recombinant antigen of the 3 samples showing weak positive response by ELISA. Sera were in 1:20 dilutions. Lane 1: negative control, lane 2: positive control, lanes 3–5: samples exhibited weak positive response by ELISA. Lanes 3 and 4 corresponding to the samples showing higher OD value by ELISA were positive to the recombinant antigen by immunoblot.
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Participants in the study were selected on a voluntary basis and may not be representative for the population as the whole but the numbers of children younger than 20 years were approximately 84.6% of surveyed persons. Therefore, the results are highly informative as a preliminary study identifying areas for further investigation of all these helminthic infections in this area.

In conclusion, toxocariasis, paragonimiasis and cysticercosis have been serologically confirmed among surveyed persons. Five of 14 epilepsy cases were sero-positive for toxocariasis. Correlation between epilepsy and these helminthic infections should be further evaluated, since screening of children for these parasitic diseases may become more important and feasible for the early treatment and prevention of these infections and promotion of better quality of life in the future.

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Author Contributions

Conceived and designed the experiments: RMS AI. Performed the experiments: AN YS SI. Analyzed the data: AN YS YS JK AI. Contributed reagents/materials/analysis tools: YS SI AKM CNN HY MN KN RMS AI. Wrote the paper: AN YS AI.

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Evaluation of a Loop-Mediated Isothermal Amplification Method Using Fecal Specimens for Differential Detection of *Taenia* Species from Humans▽‡

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We compared the performance of loop-mediated isothermal amplification (LAMP) with that of a multiplex PCR method for differential detection of human *Taenia* parasites in fecal specimens from taeniasis patients. The LAMP method, with no false positives, showed a higher sensitivity (88.4%) than the multiplex PCR (37.2%). Thus, it is expected that the LAMP method has a high value for molecular diagnosis of taeniasis.

Differential detection of *Taenia* species (*Taenia saginata*, *T. asiatica*, and *T. solium*) is a key point for control and prevention of taeniasis/cysticercosis in areas where *Taenia* disease is endemic. The identification of the carriers of *T. solium* tapeworms is most important for the prevention of cysticercosis, the most severe *Taenia* disease. Diagnosis of taeniasis by stool examination to detect taeniid eggs, the most common method, lacks both sensitivity and specificity because the eggs of *Taenia* species are morphologically indistinguishable. Moreover, *Taenia* species identification relying on comparative morphology of proglottids also lacks specificity.

The coproantigen detection method has proved to be a useful application in epidemiological surveys (1, 5), but this method is genus specific, not species specific. Recently, a *T. solium*-specific coproantigen enzyme-linked immunosorbent assay (ELISA) has been developed and shown to be potentially useful for mass screening (4). However, this test fails to identify *T. saginata* and *T. asiatica* taeniasis patients. Therefore, molecular tools are more reliable for differential identifications of taeniid parasites. Several PCR technique-based detection methods for *Taenia* species in fecal samples, including the multiplex PCR method with mitochondrial DNA (18), the PCR-restriction fragment length polymorphism method with mitochondrial DNA (15, 16), and the nested-PCR method with the Tso31 gene encoding the *T. solium* oncosphere-specific protein (10), have been reported. We have recently developed a loop-mediated isothermal amplification (LAMP) method targeting cytochrome *c* oxidase subunit 1 (cox1) and cathepsin L-like cysteine peptidase (clp) genes for differential detection of *Taenia* species (13). This method utilizes a *Bst* DNA polymerase with strand replacement activity and four primers that recognize six sequences on the target DNA under isothermal conditions. This method has proved to be simple and highly sensitive and specific for differential detection of *Taenia* species by using DNA prepared from proglottids, cysticerci, and fecal samples of taeniasis patients (12) without using sophisticated and expensive equipment. In the present study, we evaluated its sensitivity and specificity with fecal specimens from taeniasis patients by comparison of the results obtained by the LAMP method with those obtained by the multiplex PCR method.

Thirty-six fecal samples were collected in China from 26 *T. saginata* taeniasis patients, 5 *T. asiatica* taeniasis patients, and 5 *T. solium* taeniasis patients, and 7 fecal samples from Indonesia were obtained from *T. saginata* taeniasis patients after ethical approvals were received from the local health bureaus in both countries. The fecal samples were collected from patients prior to treatment with antiparasitic drugs to expel the worm, and both fecal samples and recovered parasites were stored in 70% ethanol for further analysis. The expelled tapeworm from each patient was identified by multiplex PCR (18). In addition, taeniid egg-negative fecal samples ($n = 11$) from persons without a history of tapeworm expulsion were used as negative controls. Copro-DNAs were extracted by using the QIAamp DNA stool Mini kit (Qiagen, Hilden, Germany) as described previously (13), and the extracted DNA was stored at −20°C until use. Moreover, to confirm the specificity of the LAMP method, DNAs prepared from parasite tissues of *Ascaris lumbricoides*, *Enterobius vermicularis*, *Hymenolepis nana*, and hookworms by using the DNeasy tissue kit (Qiagen) were used. Multiplex PCR and LAMP reactions were carried out as described previously (13, 18). In the multiplex PCR, the diagnostic DNA fragments with sizes of 827, 269, 720, and 984 bp were produced in *T. saginata*, *T. asiatica*, American/African

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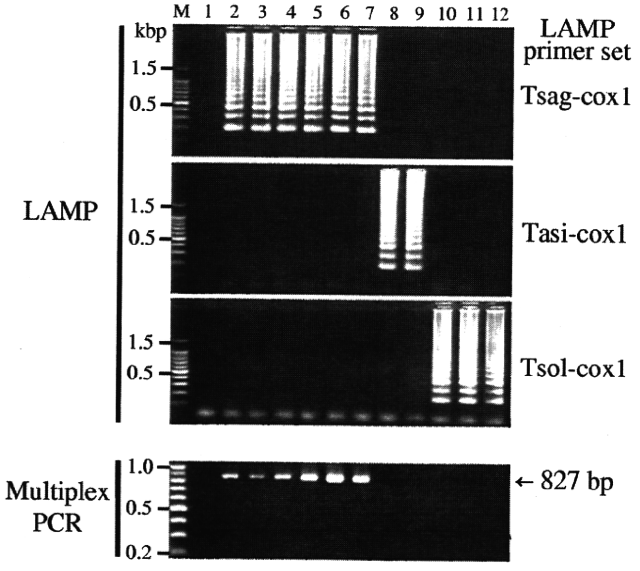

FIG. 1. Differential detection of human *Taenia* species by LAMP with cox1 primer sets and multiplex PCR. The figure shows a representative subset of the data. Lane M, 100-bp DNA ladder marker (Promega); lane 1, negative control without DNA; lanes 2 to 12, copro-DNA from each taeniasis patient. Tsag, *T. saginata*; Tasi, *T. asiatica*; Tsol, *T. solium*.

genotype *T. solium*, and Asian genotype *T. solium*, respectively. To verify the specificity of LAMP amplification, the sequences of LAMP amplicons were determined. Briefly, the LAMP products were digested with HinfI (37°C) or ApoI (50°C) enzymes (New England Biolabs, Beverly, MA) and purified by using the NucleoSpin ExTract kit (Macherey-Nagel, Düren, Germany). The digested products were cloned into the pGEM-T vector (Promega, Madison, WI), after filling in sticky ends to provide blunt-ended DNA and the addition of adenine to the ends by *Taq* DNA polymerase. The ligation mixtures were used to transform *Escherichia coli* DH5α, and each colony was analyzed by PCR using vector primers. The PCR products were sequenced as described previously (13). McNemar's test was applied to compare the sensitivities of LAMP and multiplex PCR.

Figure 1 shows the results of LAMP with cox1 primer sets and multiplex PCR, and Table 1 shows results for all methods with all samples. The LAMP products appeared as a ladder-like pattern on the agarose gel due to their characteristic stem-loop structure. The LAMP with cox1 primer sets could differentially detect target DNA in 37 out of 43 (86.0%) samples, which had *T. saginata* (n = 30), *T. asiatica* (n = 4), and *T. solium* (n = 3). The LAMP with clp primer sets differentially detected target DNA in 13 (30.2%) samples, which had *T. saginata* (n = 11), *T. asiatica* (n = 1), and *T. solium* (n = 1). No amplification from negative control fecal samples was observed by LAMP with cox1 and clp primer sets or by multiplex PCR (data not shown). All samples positive by LAMP with clp primer sets were positive by LAMP with cox1 primer sets except one sample. The differences between detection rates for the cox1 gene and the clp gene may be responsible for the number of copies of each target gene within samples, since a large amount of mitochondrial DNA exists in a cell, and the amount of mitochondrial DNA is one criterion for selection as a target DNA for detection. Although the cox1 gene was targeted, multiplex PCR could differentially identify *Taenia* parasites in only 16 samples, 1 sample after the first PCR and 15 samples after the second PCR. The samples negative by the multiplex PCR method were also negative by PCR using one individual primer set specific to each parasite cox1 gene (data not shown). The LAMP products from the multiplex PCR-negative samples were confirmed to be specific for *Taenia* parasites by sequencing (data no shown), which indicated the high sensitivity of the LAMP methods. *Taq* DNA polymerase used in PCR is often inactivated by inhibitors present in biological samples, which sometimes cause problems related to sensitivity and reproducibility. Due to the tolerance of the *Bst* DNA polymerase to inhibitors, in contrast to the *Taq* DNA polymerase, LAMP appears as a gold standard method to detect pathogens in fecal samples (2, 3, 7–9, 17, 19). The fact that LAMP is less affected by inhibitory substances in biological materials (6) is of great advantage when using fecal specimens that are known to contain a large amount of inhibitors.

Distribution of other intestinal helminthic parasites in areas where taeniasis is endemic raises the possibility of a mixed infection with *Taenia* and other helminthic parasites. Thus, the specificity of the LAMP method was assessed with DNAs extracted from parasites such as *Hymenolepis nana*, *Enterobius vermicularis*, *Ascaris lumbricoides*, and hookworms. All DNAs from these parasites were negative by LAMP with cox1 and clp primer sets (data not shown). LAMP has been shown to be a highly specific method because the amplification occurs when the four primers specifically recognize the six regions within the target sequence, a unique feature of LAMP (11, 14).

Other interesting advantages of LAMP are as follows: (i) it is possible to distinguish a positive LAMP reaction from a negative LAMP reaction by visual endpoint judgment of the turbidity caused by magnesium pyrophosphate as a by-product of the reaction, and (ii) the LAMP method, performed within 90 min, is faster than the multiplex PCR, which takes at least 4 h for completion of the two-round PCR, because LAMP is carried out under isothermal conditions by using a simple incubator, such as a water bath or a block heater.

In this study, we demonstrated that the LAMP method for identification of taeniid DNA in fecal specimens is more sen-

TABLE 1. Results of LAMP and multiplex PCR

Taenia sp.	No. of samples examined	No. of samples positive by method with indicated primer type(s)			
		LAMP			Multiplex PCR (cox1)
		cox1	clp	cox1 and/or clp	
T. saginata[a]	33	30	11	30	16
T. asiatica	5	4	1	4	0
T. solium	5	3	1[b]	4	0
Total	43	37	13	38	16

[a] For *T. saginata* samples, significant differences were observed between LAMP with cox1 and LAMP with clp and between LAMP with cox1 and multiplex PCR ($P < 0.001$).

[b] This sample was negative by LAMP with cox1 primer sets.

sitive, easier, and faster than the multiplex PCR method. In addition to these findings, the simplicity and cost-effectiveness of LAMP may make it easily applicable for a clinical practice as well as an epidemiological survey in areas of disease endemicity, especially developing countries. Therefore, the LAMP method as a molecular diagnosis tool will provide the successful control and prevention of taeniasis/cysticercosis in areas of endemicity.

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Review

State-of-the-art *Echinococcus* and *Taenia*: Phylogenetic taxonomy of human-pathogenic tapeworms and its application to molecular diagnosis

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ABSTRACT

The taxonomy of tapeworms belonging to the family Taeniidae has been controversial because of the paucity of adult phenotypic characters and the great plasticity of larvae in intermediate hosts. The family consists of the medically important two genera *Echinococcus* and *Taenia*, which are closely related to each other. Cladistic approaches using the molecular data of DNA and the numerical data of morphologic characters are clarifying phylogenetic relationships among the members of these genera. The nucleotide data of worldwide taeniid parasites accumulated in public DNA databases may provide a basis for the development of molecular diagnostic tools, and make it possible to identify the parasites, at least the human *Taenia* spp. by non-morphologists. Furthermore, the detection of intraspecific genetic variations prompts evolutionary and ecological studies to address fundamental questions of parasite distributional patterns. Here, we introduce the recent advances of taeniid phylogeny and its application to molecular diagnosis.

Contents

1. Introduction

The combination of morphological taxonomy, molecular genetics and evolutionary ecology is required to better understand the biodiversity of parasitic organisms. Fundamental information on their phenotypic and genotypic characteristics is especially important to control human and animal parasitic diseases. Moreover, regional and global phylogeographic surveys establish a basis of understanding for the evolutionary history of parasites.

The species identification of pathogenic organisms is essential for the diagnosis and treatment of infectious diseases. Multicellular parasites have traditionally been classified by morphological taxonomists, who provide a prerequisite fund of knowledge to differentiate etiologic agents isolated from humans and animals. However, the delineation of sibling or cryptic species is a difficult issue in morphological taxonomy. In addition to identifying species, exploring intraspecific variations has become a scientific imperative to characterize the local populations of parasites. The knowledge of parasite diversity at intraspecific level is necessary to understand the difference of clinical manifestations, and become a basis for the development of vaccines and immunodiagnostic antigens.

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The classification of tapeworms belonging to the family Taeniidae has been controversial because of the paucity of adult phenotypic characters and the great plasticity of larvae developed in various intermediate hosts. Recent advances on biochemical tools for DNA amplification and sequencing have provided a basis for the development of molecular taxonomy. Database catalogs known as "DNA barcoding" (Hebert et al., 2003) make it possible to identify parasites by non-morphologists. A DNA-based approach to the identification of parasites has also prompted the fields of molecular epidemiology and ecology. In this review, we deal especially with the phylogenetic taxonomy of human taeniid tapeworms and summarize the molecular diagnosis of the parasites.

2. Basic knowledge of taeniid parasites

Table 1 shows a brief list of human-pathogenic taeniid species and their closest relatives. The family Taeniidae is a medically important group of tapeworms constituted of the two genera *Echinococcus* Rudolphi 1801 and *Taenia* Linnaeus 1758. Two mammalian hosts showing predator–prey relationships are necessary to maintain the life cycle of the parasites. Carnivores are mostly definitive hosts for hermaphroditic adults, and herbivorous mammals serve as intermediate hosts for bladder larvae. When the intermediate hosts are eaten by the definitive hosts, ingested scolices attach to the intestinal mucosa and develop into adult tapeworms consisting of a chain of proglottids with genital organs. The gravid proglottids containing embryonated eggs are released into external environment. The intermediate hosts orally ingest the eggs, and hatched oncospheres invade various tissues to develop into fluid-filled bladder larvae. The larvae of both hydatid (*Echinococcus* spp.) and coenurus (*Taenia* spp.) enlarge their sizes in connection with the asexual reproduction of scolices in the bladders, whereas cysticercus (*Taenia* spp.) containing an invaginated scolex is mostly regarded as a non-multiplying larva.

All *Echinococcus* spp. are tiny tapeworms within several millimeters in length, but numerous tapeworms are parasitic on a canine definitive host because of the predation of infected animals containing many protoscolices. Medically important pathogens are the dog tapeworm *Echinococcus granulosus* sensu stricto (s. s.) and the fox tapeworm *Echinococcus multilocularis* (Eckert and Deplazes, 2004). Humans are vulnerable to the larval infestation through oral ingestion of eggs derived from feces of canine definitive hosts. Cystic echinococcosis caused by *E. granulosus* s. s. occurs worldwide, particularly in African, Asian and South American countries, whereas alveolar echinococcosis by *E. multilocularis* is restricted in the endemic areas of the Northern Hemisphere. Because sheep is a main intermediate host for *E. granulosus* s. s., a pastoral environment in which working dogs ingest the viscera of sheep is essential for maintaining the synanthropic cycle of the parasites. In human cystic echinococcosis, the liver and lungs are main target organs, and it takes many years to enlarge the spherical hydatid larvae. Alveolar echinococcosis caused by *E. multilocularis* exhibits a contrast to cystic echinococcosis. The parasite principally utilizes foxes as definitive hosts and voles as intermediate hosts, but humans are involved as an aberrant host. In humans, alveolar hydatid usually occurs in the liver, and minute vesicles proliferate slowly in the manner of exogenous budding. The invasive larval development is lethal to humans and animals. The disease is hyperendemic in the Tibetan communities of China (Schantz et al., 2003; Craig et al., 1992, 2008; Craig, 2006), but sporadically occurs even in industrialized European countries (Romig et al., 2006) and in Japan (Ito et al., 2003a).

Among the members of the genus *Taenia*, only *Taenia solium*, *Taenia saginata* and *Taenia asiatica* use humans as a definitive host. The latter species was formerly treated as a geographic race of *T.*

Table 1
Human-pathogenic species of the family Taeniidae and their closest relatives.

Species (genotype)[a]	Human infections[b]	Main hosts for		Distribution[c]
		Adult	Larva	
The genus *Echinococcus*				
E. granulosus (G1)	Common*	Dog	Sheep	Cosmopolitan
E. equinus (G4)	Unknown	Dog	Horse	PA
E. ortleppi (G5)	Rare*	Dog	Cattle	PA, ET and NT
E. canadensis (G6, G7)	Rare*	Dog	Camel, pig	PA, ET and NT
E. canadensis (G8, G10)	Rare*	Wolf	Cervid	PA and NA
E. felidis	Unknown	Lion	Warthog?	ET
E. multilocularis	Common*	Fox	Rodents	PA and NA
E. shiquicus	Unknown	Fox	Pika	PA (Tibet)
E. oligarthrus	Very rare*	Wild felids	Rodents	NT
E. vogeli	Not rare*	Bush dog	Rodents	NT
The genus *Taenia*				
T. saginata	Common#	Human	Cattle	Cosmopolitan
T. asiatica	Common#	Human	Pig	OR and PA (Asia)
T. solium	Common#,*	Human	Pig	Cosmopolitan
T. multiceps	Rare*	Canids	Ungulates	Cosmopolitan
T. serialis serialis	Rare*	Canids	Lagomorphs	Cosmopolitan
T. serialis brauni	Rare*	Canids	Lagomorphs	ET
T. crassiceps	Very rare*	Canids	Rodents	PA and NA
T. taeniaeformis	Very rare*	Felids	Rodents	Cosmopolitan
T. hyaenae	Unknown	Hyena	Ungulates	ET
T. crocutae	Unknown	Hyena	Ungulates	ET
T. simbae	Unknown	Felids	Ungulates	ET

[a] Data of each species are mainly taken from Verster (1969), Gasser et al. (1999), Loos-Frank (2000), Hoberg (2006), McManus and Thompson (2003), Xiao et al. (2005), Hüttner et al. (2008, 2009), Lavikainen et al. (2008), Moks et al. (2008), Saarma et al. (2009) and D'Alessandro and Rausch (2008). The genotypes G6 and G7 of *E. canadensis* were considered as a distinct species, namely *E. intermedius* (Thompson, 2008). The final taxonomic revision awaits the completion of ongoing studies.

[b] Asterisks indicate the accidental invasion of larvae into internal tissues, and hash marks denote the parasitism of adults in the small intestine. The categories of "very rare", "rare", "not rare" mean "few", "around 10 or more", and "around 100 or more" records have been reported, respectively.

[c] The areas are shown as zoogeographic regions; ET, Ethiopian; NA, Nearctic; NT, Neotropical; OR, Oriental; PA, Palearctic.

saginata, but now is considered as a distinct species (Eom and Rim, 1993). The human *Taenia* spp. attain a length of several meters. Swine serve as an intermediate host for *T. solium* and *T. asiatica*, while cattle do the intermediate host for *T. saginata*. Both *T. solium* and *T. saginata* are distributed worldwide through the trade of livestock and the migration of humans. The most severe illness is caused by *T. solium* because its larvae also parasitize human tissues such as subcutis, muscle and brain, resulting in cysticercosis (Ito et al., 2006; Sinha and Sharma, 2009). Eggs released from the tapeworm carriers cause cysticercosis to themselves (autoinfection) and other persons.

3. Phylogeny of *Echinococcus* spp.

Since *E. granulosus* was historically considered as the cause of both unilocular and alveolar echinococcosis, the species validity of *E. multilocularis* had been uncertain until 1950s. Following the discovery of alveolar cysts from microtine voles on St. Lawrence island, Alaska, Rausch and Schiller (1954) described the new species *Echinococcus sibiricensis* and considered it to be a causative agent for alveolar echinococcosis. However, Vogel (1957) directly demonstrated *E. multilocularis* to be a valid taxon, based on the morphological observation of adult tapeworms originated from the alveolar lesions of human cases in Germany. The Alaskan *E. sibiricensis*, therefore, was regarded as a synonym of *E. multilocularis*. The etiologic agent of alveolar echinococcosis in Russia was formerly described as *Alveococcus multilocularis* (Lukashenko, 1968), but this revision was not accepted widely. After the evaluation of *E. multilocularis*, the taxonomic status of *E. granulosus* still remained controversial because morphologically similar taxa were inadequately proposed based mainly on the host specificity (Williams and Sweatman, 1963; Verster, 1965). Rausch (1967) regarded most of them as synonyms of *E. granulosus*, and a subsequent classification permitted only four morphospecies, namely *E. granulosus*, *E. multilocularis*, *Echinococcus oligarthrus* and *Echinococcus vogeli* (Rausch and Bernstein, 1972). From ecological and epidemiological standpoints, *E. granulosus* was divided into "strains", depending on the usage of particular ungulates as intermediate hosts (Thompson et al., 1995).

The amplification of DNA fragments by polymerase chain reaction (PCR) has greatly accelerated taxonomic studies on parasites. In the early phase of the studies, universal PCR primers were designed from the conserved regions of DNA. Mitochondrial DNA (mtDNA) is always a major target to differentiate closely related taxa because of its rapid evolution (Brown et al., 1979). Molecular analyses using the mtDNA sequences of genes for cytochrome *c* oxidase subunit 1 (cox1) and NADH dehydrogenase subunit 1 (nad1) showed that *E. multilocularis*, *E. vogeli* and *E. oligarthrus* maintain their genetic identities and that *E. granulosus* can be divided into mainly 10 genotypes (G1–G10), corresponding to the strain definition (Bowles et al., 1992; Bowles and McManus, 1993; Scott et al., 1997; Lavikainen et al., 2003). Recent taxonomic revisions indicated that *E. granulosus* was an oversimplified species in which four or five cryptic species were intermixed (Thompson and McManus, 2002; Nakao et al., 2007). Reviving the names of synonyms and subspecies, the species has been split into *E. granulosus* s. s. (genotypes G1–G3), *Echinococcus equinus* (G4), *Echinococcus ortleppi* (G5) and *Echinococcus canadensis* (G6–G10). However, the species status of *E. canadensis* is still controversial. Thompson (2008) stated his opinion that the genotypes G6 and G7 (pig and camel strains) should be separated into *Echinococcus intermedius*. However, this nomenclature seems to be unsuitable because the original description of *E. intermedius* did not make a reference to the specificity of its intermediate hosts (López-Neyra and Soler Planas, 1943). No one has seen any study which could morphologically, genetically or ecologically connect with G6/G7 (the pig and camel strains) to *E. intermedius* (described form dogs). Therefore, comparative genetic studies on the genotypes G6–G10 are prerequisite for the taxonomic reconsideration of *E. canadensis* in order to clear the discrepancies among the papers (Le et al., 2002; Nakao et al., 2007; Lavikainen et al., 2008; Moks et al., 2008; Thompson, 2008; Saarma et al., 2009). As G9 reported by Scott et al. (1997) has not been confirmed yet (Kedra et al., 1999) or we have no chance to examine it, we do not deal with it.

Other valid species recently confirmed are *Echinococcus shiquicus* from the Tibetan fox (Xiao et al., 2005) and *Echinococcus felidis* from the African lion (Hüttner et al., 2008). Although their pathogenicity to humans is still unknown, both species are very important in reconstructing the phylogeny of *Echinococcus*. Recently, Tang et al. (2007) described a new species of *Echinococcus russicensis* from the corsac fox in Inner Mongolia. However, a comparative genetic study regarded it as a synonym of *E. multilocularis* (Nakao et al., 2009; Ito et al., 2010).

Bowles et al. (1995) at first analyzed the phylogenetic relationships among species and strains of *Echinococcus* using the sequence data of mtDNA (cox1 and nad1) and nuclear DNA (ribosomal ITS1). The results showed that *E. granulosus* was a species complex containing at least three evolutionarily diverse groups and that the Neotropical species *E. vogeli* and *E. oligarthrus* appeared distinct from the other species and strains. However, the data was less informative for inferring the phylogenetic position of *E. multilocularis* due to the short mtDNA sequences examined and the heterogeneous sequences of ITS1 observed in a single isolate.

The sequencing of complete mitochondrial genomes was greatly valuable in phylogenetic studies on *Echinococcus*. The comparison of the genomes of *E. granulosus* (sheep and horse strains) (Le et al., 2002) and *E. multilocularis* (Nakao et al., 2002b) triggered off a taxonomic revision (Thompson and McManus, 2002). Subsequently, a robust phylogenetic tree was reconstructed from the mitochondrial genomes of representative taxa (Nakao et al., 2007). The tree provides the following topics: (1) the Neotropical species *E. oligarthrus* and *E. vogeli* are basal lineages; (2) *E. multilocularis* and *E. shiquicus* are sister species; (3) *E. granulosus* s. s. is distantly related to *E. equinus*; (4) *E. ortleppi* and the genotypes G6, G7 and G8 are a monophyletic group. In particular, the close relationship of the G6, G7 and G8 suggested that these genotypes (camel, pig and cervid strains, respectively) should be unified into *E. canadensis*. Furthermore, Hüttner et al. (2008) showed that *E. granulosus* s. s. and *E. felidis* are sister species. These phylogenetic interrelationships (Nakao et al., 2007; Hüttner et al., 2008) are summarized in Fig. 1a. However, it is a little bit different from Lavikainen et al. (2008).

On the contrary, Saarma et al. (2009) presented a novel phylogenetic tree for *Echinococcus*, based on the DNA sequences of nuclear genes from published data (Xiao et al., 2005; Hüttner et al., 2008). The tree was inferred from the concatenated DNA data set of genes for elongation factor 1 alpha (ef1a), ezrin-radixin-moesin-like protein (elp), transforming growth factor beta receptor kinase (tgf), thioredoxin peroxidase (th) and calreticulin (cal). Although there was no sequence data of tgf, th and cal in the taxa of *E. ortleppi*, *E. shiquicus*, *E. felidis*, *E. vogeli* and *E. oligarthrus*, the full nuclear phylogeny was based at least on two genes, ef1a and elp, which provided large proportion of the phylogenetic signals. When compared with the result of mitochondrial genes, the nuclear tree showed the translocation of *E. multilocularis* and *E. shiquicus* into basal nodes (Fig. 1b). Further studies on these genotypes especially G5–G10 in Europe are important to get better resolution in the future.

Most recently, a new standpoint of phylogeny for *Echinococcus* was presented by using the EG95 antigen gene (eg95), which may influence host specificity by positively selected amino acids (Haag et al., 2009). The antigen is highly expressed in oncospheres

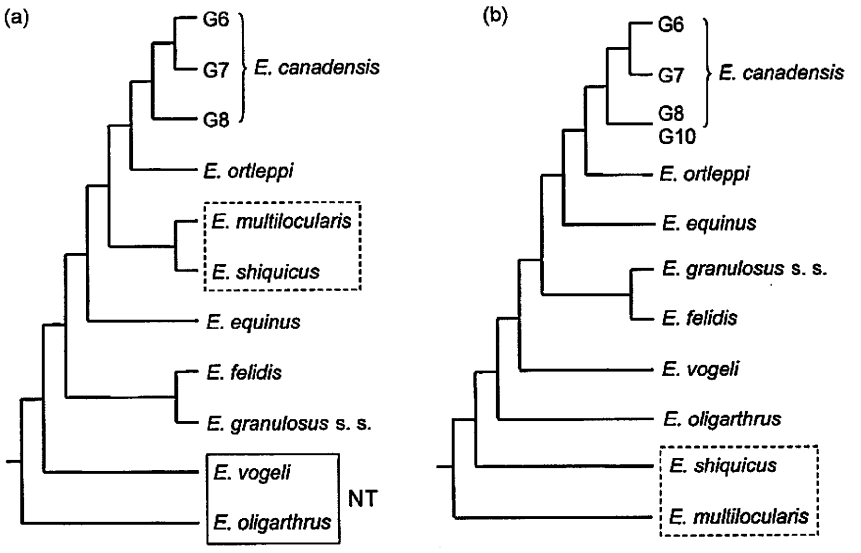

Fig. 1. Phylogenetic relationships of *Echinococcus* tapeworms summarized from recent publications. (a) The cladogram inferred by the nucleotide data of mitochondrial genes (Nakao et al., 2007; Hüttner et al., 2008). Neotropical (NT) species are surrounded by solid line. (b) The cladogram inferred by the nucleotide data of nuclear genes (Saarma et al., 2009). The translocation of taxa is shown by dotted line.

invading into the intermediate hosts, and mainly used as a vaccine for sheep. The evolution of EG95 isoforms was convergent with regard to the number of beta-sheets and alpha-helices, and a phylogenetic association existed between the degree of intermediate host specificity and the number of eg95 variants identified for each species.

4. Phylogeny of human *Taenia* and the closest relatives

Verster (1969) surveyed 70 *Taenia* spp., and showed 32 species to be valid. She divided the valid species into two groups, based on the arrangement of the genital ducts. The ducts of the group I pass between lateral excretory canals, but those of the group II pass the canals ventrally. The group I included human *Taenia* and other species infecting modern canines and felines with the exception of *Taenia taeniaeformis*. The group II consisted of *T. taeniaeformis* and other species infecting the primitive carnivores belonging to the families Viverridae and Mustelidae (e.g., *Taenia martis*, *Taenia mustelae* and *Taenia parva*). Formerly, several genera had been described within the family Taeniidae. The genera Multiceps Goeze 1782, Hydatigera Lamarck 1861 and Tetratirotaenia Abuladse 1964 were characterized with their larval morphology, whereas the genera Taeniarhynchus Weinland 1858, Monordotaenia Little 1967 and Fossor Honess 1937 were differentiated from *Taenia* only by the variations of adult rostellar hooks. All of these genera were treated as *Taenia* in the Verster's monograph. Currently, 42 species have been recognized within this genus (Eom and Rim, 1993; Loos-Frank, 2000; Rausch, 2003a; Hoberg, 2006).

Monophyly of *Taenia* spp. was demonstrated by a numerical taxonomic analysis using 26 morphological characters and 1 organotropic character in larval stage (Hoberg et al., 2000). The comprehensive phylogenetic tree including nearly all species of *Taenia* demonstrated that the above-mentioned old genera should be unified into *Taenia* as synonyms. As concerns human *Taenia* spp., the tree clarified that *T. solium* is distantly related to the sister species pair *T. saginata* and *T. asiatica*. It was also demonstrated that the zoonotic species *Taenia multiceps*, *Taenia serialis*, *Taenia crassiceps* and *T. taeniaeformis* (see Table 1) have no direct relation to the human *Taenia* spp. The phylogenetic structures of *Taenia* spp. supported the "out of Africa" view of human evolution and suggested a dietary change from herbivory to carnivory, which occurred among early hominids (Hoberg et al., 2001; Hoberg, 2006). As summarized in Fig. 2a, the obligate human parasite *T. solium* is connected directly with *Taenia hyaenae* and *Taenia crocutae*, the African tapeworms in hyenas. Moreover, the other human parasites *T. saginata* and *T. asiatica* are close relatives of *Taenia simbae*, the African tapeworm in lion. These phylogenetic relationships and divergence data analyses showed that taeniid tapeworms colonized hominids twice independently in Africa, and that the occurrence of human *Taenia* spp. predated the domestication events of pigs and cattle (Clutton-Brock, 1999; Larson et al., 2005). Thus, the phylogeny of *Taenia* spp. is also attractive to paleoanthropologists for its implication to ancient hominid lifeforms. However, the hypothesis of Hoberg et al. (2001) is totally dependent on the morphological data of *Taenia*, particularly the African species *T. hyaenae*, *T. crocutae* and *T. simbae*. The DNA profiles of those species are completely unknown because of the difficulties involved in sampling from African wildlife. Molecular phylogenetic studies are required to verify the "out of Africa" hypothesis for human *Taenia*.

Using the short mtDNA sequences of cox1 gene, Okamoto et al. (1995) initiated the molecular phylogenetic analysis of *Taenia* tapeworms. However, the phylogenetic position of human *Taenia* was not clarified because of the small number of taxa examined. Partial mtDNA sequences of cox1 and nad1 genes allowed to make phylogenetic trees in which three species of human *Taenia* were included (Gasser et al., 1999; Zhang et al., 2007). The trees clearly show that *T. saginata* and *T. asiatica* are closely related to each other, and that *T. solium* is distant from them. Furthermore, von Nickisch-Rosenegk et al. (1999) reconstructed a taeniid phylogenetic tree using the partial mtDNA sequences of small subunit rRNA gene (rrnS). The tree shows that *Taenia* spp. are divided into two groups, corresponding to the morphological division of Verster (1969). The basal nodes of the tree were occupied by the group of *T. taeniaeformis*, *T. martis*, *T. mustelae* and *T. parva*, which mainly utilizes primitive carnivores as definitive hosts, suggesting that *Taenia* had coevolved with carnivores. The arrangement of genital ducts in proglottid indicated by Verster (1969) seems to be a synapomorphic character in the evolution of *Taenia* spp. Using relatively many taxa of the genera *Taenia* and *Echinococcus*, a more reliable phylogenetic tree has recently been reconstructed from the concatenated mtDNA data set of nad1 and cox1 genes

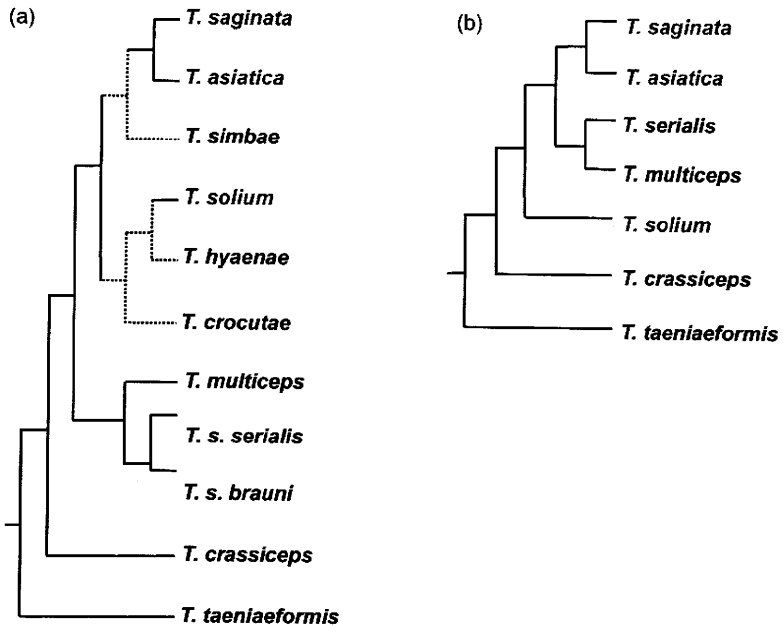

Fig. 2. Phylogenetic relationships of human-pathogenic *Taenia* spp. and their closest relatives. Closed circles indicate human intestinal parasites, while zoonotic species are marked with open circles. (a) The cladogram summarized from the data of numerical morphological analysis (Hoberg et al., 2001; Hoberg, 2006). Dotted branches indicate African lineage. (b) The cladogram summarized from the data of molecular analysis using the nucleotide sequences of mitochondrial genes (von Nickisch-Rosenegk et al., 1999; Gasser et al., 1999; Zhang et al., 2007; Lavikainen et al., 2008). Species in red are human *Taenia*. Species in blue are African species. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

(Lavikainen et al., 2008). Although it is a new challenge to reconstruct molecular phylogenetic tree on the two genera (Lavikainen et al., 2008), it is still preliminary and more data using more extensive mitochondrial and nuclear genes. Nevertheless, the tree shows that *Echinococcus* is a monophyletic genus but *Taenia* is paraphyletic because of the remarkable genetic similarity of *T. mustelae* to *Echinococcus*. Also in the numerical taxonomic analysis by Hoberg (2006), this strange species was the most divergent among *Taenia* taxa. These data may imply that a new genus will be described for *T. mustelae* and its relatives. Based on the published data, a molecular phylogenetic relationship among medically important *Taenia* spp. is summarized in Fig. 2b. Unfortunately, this tree is less informative than the morphology-based tree (Fig. 2a) because of the lack of taxa originated from African wildlife. However, the molecular tree showed that the enzootic species *T. serialis* and *T. multiceps* are genetically related to the human-pathogenic species *T. saginata* and *T. asiatica*. The phylotaxonomy of *Taenia* other than human *Taenia* spp. is still in its infancy and needs serious further investigation.

The complete DNA sequences of mitochondrial genomes have been determined in all species of human *Taenia* (Nakao et al., 2003a; Jeon et al., 2005, 2007). Gene arrangements in the genomes are totally identical with one another. However, the pairwise comparison of sequence divergence clearly shows that *T. solium* is distantly related to *T. saginata* and *T. asiatica*. The overall sequence difference between *T. saginata* and *T. asiatica* was 4.6%, while that between *T. saginata* and *T. solium* was 11% (Jeon et al., 2007). The sequence divergence of mitochondrial genes is frequently used as a yardstick for closely related species. For instance, most of congeneric species showed greater than 2% divergence (Hebert et al., 2003). This criterion supports the species validity of *T. asiatica*.

5. Molecular clinical diagnosis

The clinical diagnosis of echinococcosis in humans has been carried out based on the morphological characteristics of macro and microstructures, which are classified into cystic, alveolar and polycystic forms (Eckert and Deplazes, 2004). The pathological identification of the causative species is difficult in the cases of aberrant forms. Such lesions should be subjected to molecular diagnosis for species identification. However, simple differential tests have not been developed for the etiologic agents of *Echinococcus* because there are too many species and genotypes to easily confirm their genetic identity. At present time, clinical samples taken at biopsy are subjected to PCR, and the amplified fragments of mitochondrial and nuclear DNA are subsequently sequenced (Scott et al., 1997; Kedra et al., 1999; McManus et al., 2002; Kamenetzky et al., 2002; McManus and Thompson, 2003; Yang et al., 2005). Sequence homology search in public DNA databases via internet is the easiest way to identify the species and genotypes. PCR-based single-strand conformation polymorphism (SSCP) and restriction fragment length polymorphism (RFLP) analyses were tentatively performed at local scale, where the number of target species was limited (Zhang et al., 1999; Dinkel et al., 2004; Li et al., 2008).

In human *Taenia* infections, patients excrete proglottids and eggs together with feces. The parasite debris of tegumental turnover is also excreted into feces. The genetic identification of these parasite samples is a main purpose for molecular diagnosis. In the view of public health, detecting the carriers of *T. solium* tapeworms is the most important for the prevention of cysticercosis. The carriers discharge characteristic proglottids, but the morphological identification of fecal eggs is practically impossible. Molecular diagnosis, therefore, is needed to clarify causative species. Before the widespread use of PCR technique, Southern blot hybridization using specific DNA probes was utilized to distinguish between *T. solium* and *T. saginata* (Rishi and McManus, 1987; Flisser et al., 1988; Chapman et al., 1995). The assay was highly sensitive and specific, but the isotope-labeled probes were impractical in actual use. The method of PCR-RFLP was subsequently developed to diagnose both human taeniases, based on the sequence variations of nuclear DNA (Mayta et al., 2000; González

et al., 2002) and mtDNA (Rodriguez-Hidalgo et al., 2002). Among the forms of cestode larvae in human cerebral tissue, a proliferating acephalous form is referred to as "racemose" which could be either a larva of *T. multiceps*, *T. serialis*, *T. solium* or unknown *Taenia*. The sequence comparison of parasite DNA clearly reveals that the racemose is an aberrant larva of *T. solium* (Chung et al., 2005; Hinojosa-Juarez et al., 2008).

The widespread distribution of *T. asiatica* in Asian countries prompted us to genetically differentiate it from *T. saginata* (Ito et al., 2003b). Because of the close relationship between *T. asiatica* and *T. saginata*, variable mtDNA was selected as a target for PCR amplification. Based on the richness of thymine-base in cestode mtDNA, a base excision sequence scanning thymine-base method clearly differentiated *T. asiatica* from *T. saginata* and *T. solium* (Yamasaki et al., 2002). The differentiation method of the three human pathogens was further improved to a multiplex PCR method (Yamasaki et al., 2004a), which is applicable to the detection of *Taenia*-specific DNA in feces (copro-DNA). The molecular tools for identification or differentiation of human *Taenia* spp. and genotypes of *T. solium* (Nakao et al., 2002a,b) were overviewed by Ito et al. (2009). The multiplex PCR method is very simple, but requires a thermocycler in modern laboratories. For field use, a loop-mediated isothermal amplification (LAMP) method without using the PCR machine was developed to classify the human taeniid tapeworms (Nkouawa et al., 2009). Positive reactions of the LAMP can be confirmed by naked eyes without electrophoresis (Ito et al., in press).

6. Molecular epidemiology and ecology

Since echinococcosis and taeniasis are zoonotic diseases, prevalence data in both human and animal populations are essential for epidemiological surveillance in various endemic areas. In particular, the accurate diagnosis of definitive hosts (canines for *Echinococcus* and humans for *Taenia*) provides a key information to control the diseases. Immunological methods to detect copro-antigens or serum antibodies are applied for mass screening (Ito and Craig, 2003; Ito et al., 2007; Torgerson and Deplazes, 2009), but the immunodiagnosis is generally unable to differentiate pathogens at species level. In contrast, molecular diagnostic tools can provide differential data even in sympatric areas where several congeneric species coexist (Li et al., 2008). Furthermore, intraspecific variations detected by the molecular tools prompt evolutionary and ecological studies to address fundamental questions of parasite distributional patterns.

Stool examinations to detect taeniid eggs lack accuracy for epidemiological applications because eggs of all species belonging to the family Taeniidae are morphologically indistinguishable. Since canines serve as definitive hosts for many species of taeniid tapeworms, a PCR-based differentiation assay is required for canine surveys to confirm *Echinococcus* infections at species level. The detection of copro-antigens by enzyme-linked immunosorbent assay (ELISA) is now the best method for mass screening, and copro-PCR should be conducted for confirmatory purposes of antigen-positive samples (Torgerson and Deplazes, 2009). The sensitivity and specificity of PCR to detect *Echinococcus* DNA from canine feces have been evaluated under various conditions (Dinkel et al., 1998; Stefanić et al., 2004; Trachsel et al., 2007).

In ecological studies on *Echinococcus* spp., genetic markers are still needed to trace their spatial spread in synanthropic and non-synanthropic habitats. A commonly applied marker for the assessment of population genetic structures is highly polymorphic microsatellite, consisting of short tandemly repeated DNA. Single locus microsatellite markers are especially important in analyzing the kinship of parasites. Although the single locus markers have been isolated from *E. multilocularis* (Nakao et al., 2003b) and *E. granulosus* (Bartholomei-Santos et al., 2003), but their total number is insufficient for ecological use. Bart et al. (2006) found a microsatellite within anonymous multicopy DNA from a genomic library of *E. multilocularis*. This multi-loci microsatellite marker has been widely used to classify *E. multilocularis* individuals into genotypes (Knapp et al., 2009). However, a panel of single locus microsatellite markers should be prepared to depict the high-resolution genetic structures of *Echinococcus* populations. A haploid maternally inherited mtDNA marker has been alternatively used for population genetic studies. A mtDNA-based phylogeographic study demonstrated that *E. multilocularis* populations are generally divided into European, Asian and North American clades (Nakao et al., 2009). It seems likely that those clades were caused by the vicariance of foxes in the Pleistocene ice ages. Moreover, the statistical parsimony network of mtDNA haplotypes illustrated that recent founder effects arose in the Chinese populations of *E. granulosus* s. s. and *E. multilocularis*, whereas *E. shiquicus* which requires the Tibetan fox and plateau pika as the definitive and intermediate host, respectively (Xiao et al., 2005) were completely different from the former two species (Nakao et al., 2010).

A minimal genetic variability within *T. solium* populations was at first found in the short nucleotide sequences of a mitochondrial gene for cox1 and a nuclear gene for the diagnostic antigen Ts14 (Hancock et al., 2001). However, another survey using the complete nucleotide sequences of mitochondrial genes for cox1 and cob revealed that *T. solium* is divided into two main geographic clades (Nakao et al., 2002a). The one is widely distributed in Latin America and Africa, but the other is restricted in Asia. Such a geographic pattern suggests a possibility that *T. solium* was introduced recently into Latin America and Africa from Europe during the colonial age, which started 500 years ago, and that the tapeworm of another origin independently spread in Asia. Recently, this hypothesis was further supported by a new analysis of published data (Martinez-Hernandez et al., 2009). Population genetic studies using random amplified polymorphic DNA (RAPD) show that the local population of *T. solium* maintains a clonal structure, and suggest that the occurrence of local lineages is due to genetic recombination under limited gene flow (Vega et al., 2003; Maravilla et al., 2003). Microsatellite markers have not been isolated from human *Taenia* in spite of their usefulness (Campbell et al., 2006).

Parasite specimens and pathological tissues have been kept for long time in museums, hospitals and institutional laboratories. Molecular retrospective analyses using formalin-fixed or paraffin-embedded samples are important for studying the natural history of past epidemics. The success of PCR is uncertain in the long-term preserved samples because their genomic DNA is highly fragmented by formalin or other preservatives. Short DNA less than 300 base pairs can be generally amplified by using the fragmented DNA as a template for PCR (Schneider et al., 2008). A molecular retrospective study demonstrated the sympatric distribution of three human *Taenia* spp. in Thailand (Anantaphruti et al., 2007) and in Korea (Jeon et al., 2008). A similar PCR diagnosis was also performed in the clinical cases of cysticercosis (Yamasaki et al., 2004b; Yanagida et al., in press) and echinococcosis (Yamasaki et al., 2008).

7. Perspectives of taeniid phylogeny

In metazoan animals, Ernst Mayr's biological species concept (BSC) defines species as "groups of actually or potentially interbreeding natural populations, which are reproductively isolated from other such groups" (de Queiroz, 2005). However, modern molecular systematics develops the phylogenetic species concept (PSC) defining species as the smallest set whose members are descended from a common ancestor (Nixon and Wheeler, 1990). The monophyly-based PSC is less restrictive than the classic

BSC, in possible hybridization between closely related species. The strict application of the classic BSC to taeniid tapeworms may preclude the reliable detection of species.

The members of taeniid tapeworms have unique reproduction systems. The adult tapeworms of human *Taenia* spp. are hermaphroditic, and most infected persons harbor a single adult, suggesting that selfing is a main breeding manner. The reproduction system of *Echinococcus* spp. is more complex. Selfing also occurs in the adults, and asexual reproduction further occurs in the larval stage. These breeding manners are responsible for the genetic uniformity of local populations (Lymbery, 1992; Haag et al., 1999). Recently, Badaraco et al. (2008) indicated a possibility that gene introgression occurs infrequently among *E. granulosus* s. s., *E. ortleppi* and *E. canadensis*. Our research group also found the possible cases of gene introgression between *T. saginata* and *T. asiatica* (Nkouawa et al., 2009; Okamoto et al., 2010). These observations strongly suggest that reproductive isolation is incomplete among closely related species.

In the case of the sympatric species *T. saginata* and *T. asiatica*, "subspecies" is unsuitable for them because this category is for intraspecific populations which evolved allopatrically. The concept of "semispecies" (incipient species showing incomplete reproductive isolation) (Mayr, 1978), therefore, seems the most suitable for them. However, the semispecies is not a rank of the Linnaean taxonomic system. The populations of semispecies must be classified as the same species or different species. Biomedical researchers need pragmatic approaches to species concepts (Tibayrenc, 2006). Since each of *T. saginata* and *T. asiatica* maintains an identity on host specificity, both parasites should not be equated even though they can crossbreed.

As mentioned above, the classification of *E. canadensis* including the genotypes G6–G10 is a debatable issue. Although mitochondrial DNA markers can differentiate among the genotypes (Bowles et al., 1995; Lavikainen et al., 2003), their population genetic structures must be evaluated by using several nuclear DNA markers. The monophyly of genetic variants and the cessation of gene flow are critical points in considering the validity of species. In case the genotypes G6 and G7 (camel and pig strains) keep particular haplotypes at nuclear loci, they should be ranked as a distinct species as mentioned by Nakao et al. (2007) and Thompson (2008). If nuclear haplotypes are relatively common in all members of the genotypes, a rank of subspecies can be used to recognize their minor biological differences because the genotypes G8 and G10 (cervid strains) are ecologically and geographically segregated from the other genotypes by colonizing sylvan wildlife (Rausch, 2003b).

Concerning the phylogeny of the genus *Taenia*, the genetic makeups of the African species *T. hyaenae*, *T. crocutae* and *T. simbae* are absolutely necessary to clarify the evolutionary history of human *Taenia* spp. Chronological analyses based on the molecular phylogeny of the tapeworms may lead to a new paradigm in considering human evolution.

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