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A phylogenetic hypothesis for the distribution of two genotypes of the pig tapeworm *Taenia solium* worldwide

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SUMMARY

Genetic polymorphism was determined among 13 isolates of *Taenia solium* from various regions using PCR-amplified sequences of 2 mitochondrial genes: *cytochrome c oxidase subunit 1* and *cytochrome b*. The 2 phylogenies obtained were similar to each other regardless of the genes examined. The isolates from Asia (China, Thailand, Irian Jaya and India) formed a single cluster, whereas the isolates from Latin America (Mexico, Peru, Ecuador, Bolivia and Brazil) combined with those from Africa (Tanzania, Mozambique and Cameroon) to form an additional cluster. These results and historical data of swine domestication, distribution of pigs and colonization suggest that *T. solium* was introduced recently into Latin America and Africa from different regions of Europe during the colonial age, which started 500 years ago, and that the tapeworm of another origin independently spread in Asian countries.

Key words: *Taenia solium*, distribution, phylogeny, DNA polymorphism.

INTRODUCTION

The tapeworm *Taenia solium* is one of the most important human parasites because of its serious pathogenicity. *T. solium* completes its 2-host life-cycle, including humans as the definitive host and the domestic pig *Sus scrofa* as the intermediate host. Three pathological types of *T. solium* infection exist in humans: (a) an intestinal taeniasis due to ingestion of undercooked pork contaminated with cysticerci, (b) neurocysticercosis (NCC) in the brain with or without cysticercosis in subcutaneous, muscle, eye and other tissues or organs due to ingestion of viable eggs released from the worm carriers (taeniasis patients). Another type, (c) is a combination of (a) and (b) in the same patients. Type (c) has been thought to be due to autoinfection and the possibility of independent infections of (a) following (b) is often ignored but the latter might be more common in endemic regions. It is speculated based on (1) the immune responses to the tissue and intestinal phases of *Hymenolepis nana* in mice. Immune responses to the tissue stage in the intermediate host (mice) do not affect the intestinal stage in the definitive host (same mice) (Ito, 1997) and (2) antibody responses in taeniasis differ from those in cysticercosis in *T. solium* infection in humans (Wilkins *et al*. 1999). Although pigs are the most common and well-known intermediate hosts, humans may become intermediate hosts with NCC. However, humans do not become the functional intermediate host like pigs for transmission of its life-cycle, except through cannibalism. Most taeniid species develop into adult worms in carnivorous mammals but *T. solium*, *T. saginata* and *T. asiatica* require humans as the definitive host (Loos-Frank, 2000).

It is believed that humans acquired *Taenia* tapeworms coincidental with the domestication of cattle and swine which began about 10000 years ago (Epstein & Bichard, 1984; Bradley *et al*. 1996). However, recent phylogenetic studies on the origin and evolution of the human-parasitic *Taenia* species show another scenario. A molecular study by De Queiroz & Alkire (1998) has indicated that the 3 species of *Taenia* in humans do not form a clade (*T. saginata* is related to *T. asiatica* and is unrelated to *T. solium*) and that taeniid tapeworms have switched from carnivorous definitive hosts to primate definitive hosts twice independently in their history. One is an ancestor of *T. solium* and the other is an ancestor of *T. saginata* and *T. asiatica*. Hoberg *et al*. (2001) have suggested that the occurrence of human-parasitic *Taenia* species pre-dates the development of agriculture and animal domestication. Based on their hypothesis, African hominids in the Pliocene/Pleistocene (approximately 1–5 million years ago), which scavenged or preyed upon antelopes and other bovids in the Savannah, became definitive hosts for taeniid tapeworms instead of carnivores including

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hyaenids and felids. Accordingly, it seems likely that ancestors of human-parasitic taeniids spread from the African Continent to Eurasia together with *Homo erectus* or early *Homo sapiens*.

In the present day, *T. solium* is prevalent in the regions where pigs are domesticated and man consumes raw or insufficiently cooked pork. Its life-cycle was eradicated in Europe but is still maintained in developing countries in Asia, Latin America and Africa and is spreading wider (Craig *et al*. 1996; Simanjuntak *et al*. 1997; Schantz, Wilkins & Tsang, 1998; Singh *et al*. 2002). It is, therefore, now expected to be re-introduced into EU countries (European Commission, 2000) and any other developed countries by the increase in number of immigrants, refugees and tourists as demonstrated in the USA (Schantz *et al*. 1992, 1998). The expansion of human agriculture and pig breeding may have played a significant role in the global distribution of *T. solium*; however, no data are available for explanation of the recent distribution of the tapeworm after its ancestor came out of Africa. In the study described here, we examined the genetic polymorphism of *T. solium* in various localities by comparing sequences of 2 mitochondrial genes: *cytochrome c oxidase subunit 1* (*Cox1*) and *cytochrome b* (*Cytb*). The main purpose of this study is to infer how the tapeworm has expanded its distribution throughout the world.

MATERIALS AND METHODS

Parasite and DNA extraction

A total of 13 isolates of *T. solium* cysticerci from China, Thailand, Irian Jaya, India, Mexico, Peru, Ecuador, Bolivia, Brazil, Tanzania, Mozambique and Cameroon, collected from 1996 through 2001, were examined for this study (Table 1). All cysticerci samples were obtained from muscles of domesticated pigs by ourselves and collaborators (see acknowledgements) and were preserved in ethanol until DNA extraction. Genomic DNA was extracted from a single cysticercus by using a spin column kit (DNeasy tissue kit; QIAGEN) as recommended by the manufacturer. DNAs from *Taenia asiatica* cysticercus from Taiwan and *Taenia saginata* adult segment from China were also used for this study.

DNA amplification and sequencing

The entire *Cox1* and *Cytb* genes were amplified by the polymerase chain reaction (PCR). Two sets of PCR primers were designed from the complete sequence of *T. solium* mitochondrial genome (unpublished data). Primers Cox1/F (5′-GTTATGTTAGACTAGATGTTTTCA-3′) and Cox1/R (5′-TCCACTAAGCATAATGCAAAAGGC-3′) allowed us to amplify *Cox1* genes of *T. solium*, *T. saginata*, and *T. asiatica*. *Cytb* genes of the 3 species could be amplified by using primers Cytb/F (5′-ATAAACTGATAGATTGTGGTTC-3′) and Cytb/R (5′-CATATGACTGTCTAATGAAGAAAA-3′). PCR was carried out in a 50 μl reaction mixture containing 2 μl of template DNA, each dNTP at 200 μM, each primer at 0·5 μM, 1 U of DNA polymerase (Ex *Taq*; TaKaRa Biomedicals, Kyoto, Japan) and Ex *Taq* reaction buffer. For PCR amplification, we employed 30 thermal cycles (94 °C for 30 s, 56 °C for 30 s and 72 °C for 90 s) for *Cox1* gene and 30 cycles (94 °C for 30 s, 58 °C for 30 s and 72 °C for 60 s) for *Cytb* gene. Prior to DNA sequencing, each amplified product was purified by using the QIAquick PCR purification kit (QIAGEN). A dye terminator cycle sequencing kit (Thermo Sequenase II; Amersham Pharmacia Biotech) and a fluorescent automated sequencer (ABI PRISM 377; PE Applied Biosystems) were used as recommended by the manufacturers. Both strands of DNA were directly sequenced by primer walking. Sequencing reaction mixtures were primed with a series of custom-synthesized primers.

Data processing

Nucleotide sequences determined in this study have been deposited in the GenBank/EMBL/DDBJ international databases (Table 1). Sequences of *Cox1* and *Cytb* genes of the tapeworm *Echinococcus multilocularis* (database accession no AB018440) were also used for this study. Amino acid sequences of mitochondrial genes were deduced using the flatworm codon table modified for cestodes (Nakao *et al*. 2000). Multiple alignment of sequences was achieved using the CLUSTAL W program (Thompson, Higgins & Gibson, 1994) available over the World Wide Web (http://www.ddbj.nig.ac.jp/E-mail/homology.html). Phylogenetic trees were inferred with the neighbor-joining method (Saitou & Nei, 1987) using *E. multilocularis* as an outgroup. Percentage divergences were corrected by Kimura's 2 parameter model (Kimura, 1980). Confidence values for each branch of the resultant trees were determined by 1000 bootstrap replications. Sequences of *Cox1* and *Cytb* genes of the mouse *Mus musculus* (Bibb *et al*. 1981) and the rat *Rattus norvegicus* (Gadaleta *et al*. 1989) were utilized to estimate dates of divergence for taeniid tapeworms.

RESULTS

The full lengths of *T. solium* mitochondrial genes sequenced in this study were 1620 base pairs (bp) in *Cox1* gene and 1068 bp in *Cytb* gene. No deletions of nucleotides were observed in both genes among 13

Table 1. Thirteen geographical samples of *Taenia solium* and two other taeniid tapeworms examined for mitochondrial nucleotide sequences

Samples or species	Locations	Database accession numbers	
		Cox1 gene	*Cytb* gene
CHI1	China	AB066485	AB066570
CHI2	China	AB066486	AB066571
THA	Thailand	AB066487	AB066572
IRI	Irian Jaya	AB066488	AB066573
IND	India	AB066489	AB066574
MEX	Mexico	AB066490	AB066575
PER	Peru	Same as MEX	Same as MEX
ECU	Ecuador	AB066491	AB066576
BOL	Bolivia	Same as ECU	Same as ECU
BRA	Brazil	AB066492	AB066577
TAN	Tanzania	AB066493	AB066578
MOZ	Mozambique	Same as TAN	Same as TAN
CAM	Cameroon	Same as MEX	AB066579
T. asiatica	Taiwan	AB066494	AB066580
T. saginata	China	AB066495	AB066581

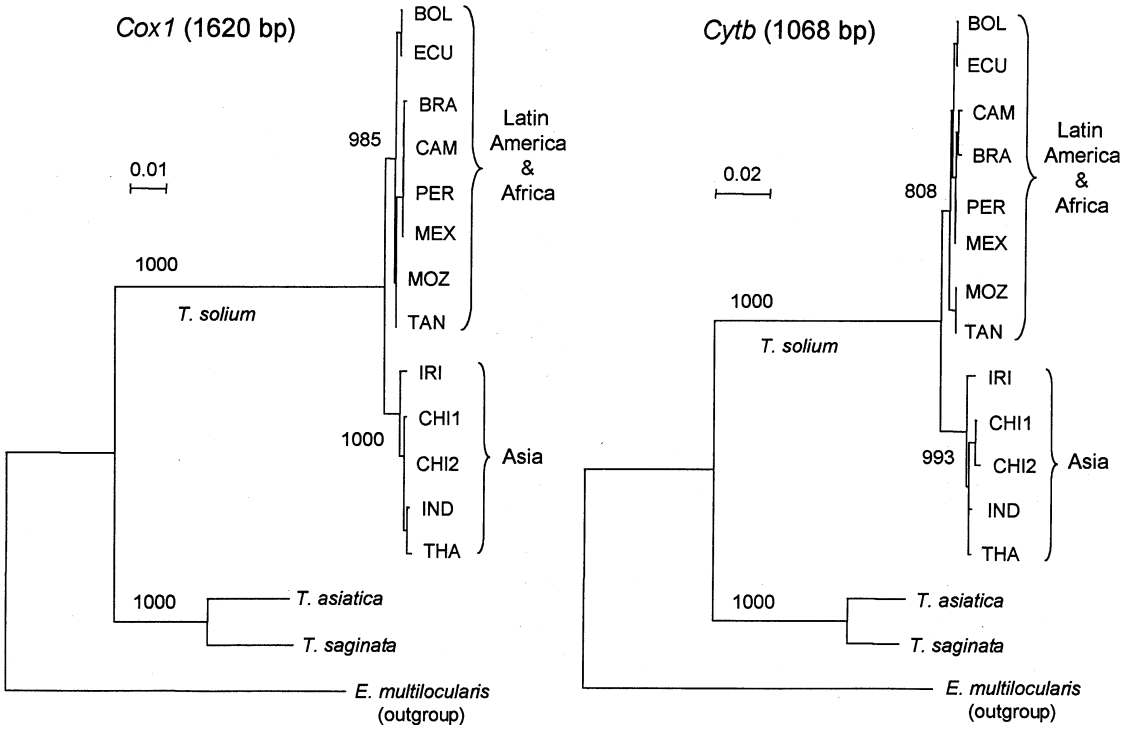

Fig. 1. The neighbor-joining phylogenetic trees of 2 genotypes of *Taenia solium* from 13 isolates from various regions inferred from nucleotide sequences of *Cox1* and *Cytb* genes. The scale bar represents the estimated number of nucleotide substitutions per nucleotide site. Numbers at individual nodes are the bootstrap confidence values obtained after 1000 replicates. The sample names of *T. solium* are shown in Table 1.

isolates of *T. solium*. The other human-specific taeniid tapeworms *T. saginata* and *T. asiatica* showed the same gene lengths as *T. solium*. However, *Cox1* and *Cytb* genes of *E. multilocularis* have been determined to be 1608 bp and 1068 bp in length, respectively. The intraspecific variation of mitochondrial genes was observed among 13 isolates of *T. solium*. In *Cox1* gene, 28 variant nucleotide positions (1·7% of total length) were detected among the 13 isolates. Similarly, 31 nucleotide positions were found in *Cytb* gene (2·9% of total length). The difference in the rates of variant positions denoted that *Cox1* gene is more conservative than *Cytb* gene. Transitional substitutions repeatedly occurred in these variant positions and their frequencies were 92·9% in *Cox1* gene (26/28) and 83·9% in *Cytb* gene (26/31).

The neighbor-joining phylogenetic trees were constructed for human taeniid tapeworms from *Cox1* and *Cytb* gene sequences (Fig. 1). The resultant 2

trees were similar to each other regardless of the genes examined. In both trees, 13 isolates of *T. solium* from various geographical regions were divided into 2 genotypes. Five Asian isolates formed a single cluster; however, 5 Latin American isolates combined with 3 African isolates to form an additional cluster. Pairwise divergences between the 2 genotypes ranged from 0·9 to 1·3 % for *Cox1* gene and from 1·6 to 2·1 % for *Cytb* gene. The separation into 2 genotypes was reliable because each node of the trees showed high bootstrap values. Phylogenies were reconstructed by using deduced amino acid sequences; however, the intraspecific variation of *T. solium* is ambiguous because of synonymous nucleotide substitutions (data not shown). With regard to other human-specific taeniid tapeworms, our results were in better agreement with previous views that *T. saginata* is related to *T. asiatica* (Bowles & McManus, 1994; De Queiroz & Alkire, 1998; Hoberg *et al*. 2000). Pairwise divergences between these closely related species reached 4·7 % for *Cox1* gene and 4·0 % for *Cytb* gene. We assumed that these are limited values to differentiate species in taeniid tapeworms. It seemed to be reasonable that the divergence values of *T. solium* samples observed in this study are intraspecific levels when compared with those of *T. saginata* and *T. asiatica*.

DISCUSSION

Our results demonstrate that isolates of *T. solium* are mainly divided into 2 genotypes of 2 different geographical distributions. One is restricted to Asia and the other is widespread in both Africa and Latin America. Date of divergence for these 2 genotypes was roughly estimated by the method of Depres *et al*. (1992). As there are no fossil records of tapeworms, a time calibration was carried out by using a pair model of mouse and rat which diverged 9–12 million years ago (MYA) (Jaeger, Tong & Denys, 1986). Pairwise divergences between these rodents were 19·2 % in *Cox1* gene and 18·8 % in *Cytb* gene. We have to assume that the rates of evolution of *Cox1* and *Cytb* genes are almost the same in rodents and tapeworms, and estimated that dates of divergence in the 2 genotypes of *T. solium* are 0·4–0·8 MYA in *Cox1* gene and 0·8–1·3 MYA in *Cytb* gene. If the mtDNA of the tapeworm evolves even 10 times faster than that of the rodents, the shortest divergence date is 40000 years ago in *Cox1* gene and far beyond 10000 years when domestication of pigs started (Epstein & Bichard, 1984; Bradley *et al*. 1996). The divergence-date analysis, therefore, suggests that the intraspecific variation of *T. solium* occurred before the domestication of pigs which began about 10000 years ago. Based on these results and reference to other information on the history of domestication of pigs and distribution of pigs (Epstein & Bichard, 1984; Bradley *et al*. 1996) and the origin of *T. solium* (De Queiroz & Alkire, 1998; Hoberg *et al*. 2001), we propose the following scenario. *Homo erectus* or early *Homo sapiens* came out of Africa into Eurasia together with the prototype of *T. solium* (Hoberg *et al*. 2001). The tapeworm was maintained and diverged in early human colonies through a 'human–human' cycle (cannibalism) and a 'human–wild suids' cycle (hunting) (Epstein & Bichard, 1984; Bradley *et al*. 1996). The wild boar *Sus scrofa* is wildly distributed in the Palearctic region. During boar domestication in Asia, Asia Minor, and Europe (Epstein & Bichard, 1984; Bradley *et al*. 1996), the tapeworm secondarily acquired it as an obligatory intermediate host and a modern synanthropic 'human–pig' cycle was established. Natural selection in these processes would have increased the genetic variations among isolates of *T. solium*. Since the dawn of history, the Moslem and Jewish religions, in which pork eating is prohibited, would be a barrier for the spreading of the tapeworm. In the colonial age, which started 500 years ago, the European isolates of *T. solium* were extensively introduced into Latin American and African countries together with pigs and humans (Epstein & Bichard, 1984). Genetic divergences of Latin American and African samples (0–0·4 % in *Cox1* and 0–0·6 % in *Cytb*) suggest that introduced populations were variable and originated from different regions of Europe. Indeed, pigs came into Latin American countries from Europe with Spanish and Portuguese colonists, and all pigs owned by Africans in southern African countries are descended from stock imported by Europeans (Epstein & Bichard, 1984). On the other hand, tapeworms of a different origin independently spread in Asian countries. Genetic divergences of Asian isolates (0·1–0·5 % in *Cox1* and 0·2–0·8 % in *Cytb*) suggest that minor changes occurred in different regions of Asia. A more complete sampling in Africa is necessary for concluding that the tapeworm was reintroduced into Africa. According to the numerical taxonomic data of Hoberg *et al*. (2000), *T. solium* is closely related to *T. hyaenae*, which is a tapeworm of hyenas. Molecular phylogenetic analyses on this species should be conducted to complement the 'out of Africa' hypothesis of *T. solium*.

Clinical manifestations in human cysticercosis appear to be well correlated with the genotypes of *T. solium*. Subcutaneous involvement by cysticerci in patients with neurocysticercosis is frequently found in Asia (Feng *et al*. 1979; Simanjuntak *et al*. 1997) but is rare in Latin America (Cruz *et al*. 1994). In Africa, cysticercosis is uncommon among the Moslem populations; however, some African countries have a higher prevalence and neurological disorders are the main symptoms in patients (Boa *et al*. 1995; Vilhena, Santos & Torgal, 1999). A possible reason for the clinical difference might correlate with the variation in genotypes of *T. solium*, although other

factors such as nutritional status and ethnic difference of patients cannot be ignored. The variation in genotypes of *T. solium* should be considered also in serological diagnosis. We recommend that if specimens of *T. solium* from NCC patients or taeniasis solium patients from any regions or any hospitals are received, it would be appropriate to analyse the DNA and refer to the results described in this paper for further analysis on where the patients lived or travelled, since the parasite moves with the human, the worm carriers.

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