Complicated relationships between *Taenia saginata*, *Taenia asiatica*, and their hybrids

Taenia saginata、*Taenia asiatica* と、その交雑体における複雑な系統関係

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Abstract

The genus *Taenia* consists of nearly 50 species, including three currently-known "human-*Taenia*" spp., *Taenia solium*, *Taenia saginata* and *Taenia asiatica*. *Taenia solium* is one of the most important cestodes concerning human health, but *T. saginata* and *T. asiatica* are also important for the zootechnical and veterinary sciences due to economic loss caused by them; during larval stages, *T. saginata* parasitizes the muscle of cattle, while *T. asiatica* parasitizes the viscera of pig.

It was a long-standing puzzle that adult taeniid tapeworms expelled from people in Asian countries seemed to be *T. saginata*, although these people ate pork rather than beef. Taiwan, Indonesian and Korean researchers studied the *T. saginata*-like tapeworm energetically, including experimental infections, and concluded that this parasite was an independent new species. However, several others working on molecular difference between "Asian *Taenia*" and *T. saginata* rejected this idea. In 1993 Eom and Rim described this Asian *Tania* as a new species, *T. asiatica*, based on morphological observations. However, due to the morphological similarity and a very small difference in the mitochondrial DNA sequences between *T. saginata* and *T. asiatica*, it has been debated whether these two taxa belong to the same species or are indeed two distinct species. Ernst Mayr defined a species as follows: "species are groups of interbreeding natural populations that are reproductively isolated from other such groups." In other words, if reproductive isolation is incomplete, hybridization between species that were considered to be distinct species should occur. And if hybridization occurred once,

nuclear-mitochondrial discordance should be detected in their descendants.

In chapter 1, five *Taenia* tapeworms collected from humans in Tibetan Plateau, Sichuan, China, where three species of human *Taenia* are sympatrically endemic, were examined for the mitochondrial *cox1* gene and two nuclear genes, *ef1* and *elp*. Phylogenetic analyses of these genes revealed that two adult worms showed nuclear-mitochondrial discordance, suggesting that they originated from hybridization between *Taenia saginata* and *Taenia asiatica*. One of two worms had *T.asiatica*-type mtDNA, whereas another worm had *T.saginata*-type mtDNA, indicating that reciprocal hybridization between *T. saginata* and *T. asiatica* could occur. The worm having *T. asiatica*-type mtDNA was heterozygous at both nuclear loci with *T. saginata*-type alleles and *T. asiatica*-type alleles. In another worm, the *ef1* locus was heterozygous with the *T. saginata*-type allele and the *T. asiatica*-type allele, while the *elp* locus was homozygous with *T. saginata*-type alleles. Self-fertilization is the main reproductive method of the genus *Taenia*. Since self-fertilization represents a type of inbreeding, each locus in the offspring would become homozygous over generations with genetic drift. The fact that some nuclear loci are still heterozygous means that hybridization might have occurred recently. Hybridization between *T. asiatica* and *T. saginata* is probably an ongoing event in many areas in which they are sympatrically endemic.

In chapter 2, partial sequences of the DNA polymerase delta (*pold*)

gene from *T. saginata*-like adult worms including samples used in chapter 1 were sequenced. Phylogenetic analysis revealed that *pold* gene sequences were clearly divided into two clades, differing from each other in five to seven nucleotides. There is little doubt that *T. saginata* and *T. asiatica* were once separated into two distinct taxa as has been concluded in previous studies. On the other hand, most of the adult worms, which were identified as *T. asiatica* using mitochondrial DNA, were homozygous for allele that originated from the allele of *T. saginata* via single nucleotide substitution. These results indicate that most of the adult worms, which had been called *T. asiatica*, are not actually "pure *T. asiatica*" but instead originated from the hybridization of "pure *T. saginata*" and "pure *T. asiatica*".

General introduction

The genus *Taenia* consists of nearly 50 species [1-6], including three currently-known "human-*Taenia*" spp. [3], *Taenia solium*, *Taenia saginata* and *Taenia asiatica*. Needless to say, *Taenia solium* is one of the most important cestodes concerning human health because it causes neurocysticercosis, a serious public health problem worldwide. But *T. saginata* and *T. asiatica* are also important for the zootechnical and veterinary sciences due to economic loss caused by them. In this study, I deal with this two speices, *T. saginata* and *T. asiatica*.

T. saginata is distributed worldwide, especially Africa, Eastern Europe, Asia, and Central and South America. Humans are definitive host, and during larval stages, *T. saginata* parasitizes the muscle of cattle. Because of eating uncooked or undercooked beef, humans harbor adult worms.

On the other hand, *T. asiatica* was first recognaized in Taiwan aboriginies and subsequently from Asian countries: Korea, Indonesia, Vietnam, and China, and called as Taiwan *Taenia* or Asian *Taenia* [7]. In 1993, Eom and Rim [8] described Asian *Taenia* as a new species, *Taenia asiatica*, based on the morphological observation. *Taenia asiatica* adult worm inhabits the small intestine of humans, and during larval stages, inhabits the visceral organs such as liver, lung and omentum of pigs.

T. saginata and *T. asiatica* are morphologically very similar, as are their mitochondrial DNA (mtDNA) sequences [9]. On the other hand, the two taxa clearly differ in biological features including host specificity and organotropism [7]. Because of the issures mentioned above, it has been

debated whether these two taxa belong to the same species or are indeed two distinct species [10-14].

There are many species concepts, and the definition of a species is also varied. Of these, the biological species concept is the most widely accepted. It defines species in terms of their ability to interbreed. For instance, Ernst Mayer [15] defined a species as follows: "species are groups of interbreeding natural populations that are reproductively isolated from other such groups." This definition is obviously imperfect when it is applied to the entire organism; however, it is widely accepted for organism with sexual reproduction. That is, if *T. asiatica* is a same species of *T. saginata*, hybridization between *T. asiatica* and *T. saginata* should occur in sympatric areas. Evidence for hybridization is often found by study of nuclear loci and comparison of these with mitochondrial data. Introgression (the infiltration of genes from the gene pool of one species into that of another) can be inferred from the finding of mitochondrial sequences typical of one species in an organism with the nuclear alleles of another. Alternatively, hybridization can be demonstrated by the presence of nuclear alleles in a single individual that are typical of more than one species.

Okamoto et al. [16] reported that two adult taeniid worms from Kanchanabri Province, Thailand showed nuclear-mitochondrial discordance, i.e., in spite of their possession of sequences typical of the *T. saginata* mitochondrial gene, some nuclear loci were homozygous for the alleles typically found in *T. asiatica*. Although their observations seem to provide the evidence of historical hybridization between *T. saginata* and *T. asiatica*,

the possibility of retention of ancestral polymorphism cannot be completely dismissed by their data alone.

So in chapter 1, additional individuals were surveyed in Tibetan Plateau, Sichuan, China, which had been originated from hybridization between *T. saginata* and *T. asiatica*. Those worms were heterozygous at some nuclear loci. I relate in detail about that on chapter 1.

In chapter 2, more samples in addition to samples which was used in study of Okamoto et al. [16] and chapter 1 were practiced about DNA sequencing and data analysis. Furthermore, additional nuclear loci were examined in this study.

These results suggest complicated relationships between *T. saginata*, *T. asiatica* and their hybrids.

Chapter 1

Recent hybridization between *Taenia asiatica* and *Taenia saginata*

Introduction

With regard to the definition of species, many researchers, such as Mayer [15], emphasized the possibility of hybridization. That is, a species is defined as a group of individuals that are actually or potentially interbreeding, and that are reproductively isolated from other such groups. This definition is obviously imperfect when it is applied to all of organisms; however, it is widely accepted for organism with sexual reproduction. Evidence of hybridization is often found through study of nuclear loci and comparison of these with mitochondrial data. Introgression can be inferred from the finding of mitochondrial sequences typical of one species in an organism with the nuclear alleles of another species. Alternatively, hybridization can be demonstrated by the presence of nuclear alleles in a single individual that are typical of more than one species. Okamoto et al. [16] reported that two individual adult worms of human *Taenia* from Kanchanabri Province, Thailand showed nuclear-mitochondrial discordance, i.e., in spite of their possession of sequences typical of the *T. saginata* mitochondrial gene, some nuclear loci were homozygous for the alleles typically found in *T. asiatica*. Although their observations seem to provide evidence for historical hybridization between *T. saginata* and *T. asiatica*, the possibility of retention of an ancestral polymorphism cannot be completely dismissed by their data alone.

In this study, I found additional individual worms in the Tibetan Plateau, Sichuan, China, which appear to have originated through

hybridization between *T. saginata* and *T. asiatica*. Three species of human *Taenia* have been reported to be sympatrically endemic in the Tibetan Plateau [17]. Worms examined here were heterozygous at some nuclear loci, which might indicate that hybridization has recently occurred.

Materials and methods

Parasite samples

We distributed questionnaire surveys were in Danba, Tibetan Plateau, Sichuan, China, in October 2008, which revealed several suspected carriers of taeniasis. Following treatment with traditional Chinese medicine [18], five adult tapeworms, which were morphologically similar to *T. saginata* or *T. asiatica*, were collected from 5 patients. All samples were stored in 70% ethanol until they were required for DNA extraction.

DNA preparation and multiplex PCR for *Taenia* species identification

Genomic DNA was individually extracted from immature proglottids using a DNeasy tissue kit (QIAGEN, Germany) in accordance with the manufacturer's instructions, and then used as a template for polymerase chain reaction (PCR).

Multiplex PCR based on the mitochondrial cytochrome c oxidase subunit 1 (*cox1*) gene is an easy method for identification of human *Taenia* [19]. Samples were first screened by this method for the tentative identification of species. According to the results, the sample code "Tasi" (*T. asiatica*) or "Tsag" (*T. saginata*) was added to the sample ID number. It is important to note that this code refers to the identification from the mitochondrial genome.

DNA sequencing and data analysis

Multiplex PCR yields products of taxon-specific lengths that can be visualized in a gel. For finer genetic discrimination, the complete sequence of the *cox1* gene was obtained for each individual. Partial sequences of two nuclear genes, elongation factor-1-alpha (*ef1*) and ezrin-radixin-mosein (ERM)-like protein (*elp*), were also obtained. Methods for PCR amplification and sequencing of those genes were the same as described previously [16]. At least two independent PCR products were used for sequencing. Samples that could not be directly sequenced were subjected to cloning using a TOPO TA Cloning Kit (invitrogen, USA), and more than fifteen clones were sequenced per sample.

DNA sequences from present samples were combined with previous data (database accession numbers AB465211-AB465248 for the *cox1* gene, AB462851-AB462890 for the *ef1* gene and AB462811-AB462850 for the *elp* gene) [16], and aligned using the CLUSTAL W computer program [20]. A method for phylogenetic analysis was the same as the previous study [16]. Phylogenetic trees were constructed by the neighbor- joining (NJ) method [21] using the MEGA4.0 computer program [22]. Evolutionary distances were computed using the Maximum Composite Likelihood Method, which was a high-precision method for the NJ analysis [23]. Each of the phylogenetic trees was evaluated using a bootstrap test based on 1000 resamplings [24]. Sequences of *Taenia solium* from Kanchanaburi Province were used as outgroups (AB066487 for *cox1*, AB505027 for *ef1* and AB505025 for *elp*) to indicate the location of the root of the ingroup. For

presentation purposes, the long branch leading to the outgroup is not shown in any trees.

Results

The mtDNA-based multiplex PCR tentatively assigned our samples to *T. asiatica* (the individual clade number TasiT041) or *T. saginata* (TsagT038, TsagT040, TsagT042 and TsagT043). The complete mitochondrial *cox1* gene sequences (1620 bp in length) were obtained from all samples by direct sequencing of PCR products. The mitochondrial haplotype of TasiT041 was the same as that reported previously by Okamoto et al. [16]. Three haplotypes were found in "Tsag", and all of them were also reported previously [16].

Okamoto et al. [16] reported that there were three alleles (*ef1*A, *ef1*B and *ef1*C) at the *ef1* locus. Two of them (*ef1*A, *ef1*B) occurred in *T. asiatica,* and *ef1*C was found in *T. saginata* [16]. As shown in Table 1, TsagT040, TsagT042 and TsagT043 were homozygous for the *ef1*C allele at this locus. DNA sequences of the *ef1* alleles from TsagT038 and TasiT041 could not be determined by direct sequencing because there were double peaks at several sites in electropherograms. The PCR products were then cloned using a TA cloning kit and the resultant clones were sequenced. Seventeen clones from TsagT038 were classified into 8 clones with *ef1*B and 9 clones with *ef1*C, and 18 clones from TasiT041 were classified into 8 clones with *ef1*B and 10 clones with *ef1*C. These indicated that TsagT038 and TasiT041 should be heterozygous at the *ef1* locus with *ef1*B and *ef1*C.

In the case of the *elp* locus, four alleles (*elp*A, *elp*B, *elp*C and *elp*D) have been reported, with two of them (*elp*A, *elp*B) originating from *T.*

asiatica and the remaining two (*elp*C, *elp*D) originating from *T. saginata* [16]. As summarized in Table 1, TsagT038, TsagT040, TsagT042 and TsagT043 were homozygous with the *elp*C allele. Only TasiT041 could not be determined by direct sequencing because there were double peaks at several sites in electropherograms. The PCR product was again cloned in a TA cloning kit and the cloned PCR products were sequenced. Eighteen clones from TasiT041 were classified into 8 clones with *elp*A and 10 clones with *elp*C. This indicated that TasiT041 should be heterozygous with *elp*A and *elp*C.

Fig. 1 shows neighbor-joining trees inferred from each of three genes. Each figure was simplified by omitting some of the sample names, which have been reported in previous study [16]. The topologies of these trees are almost the same as those reported previously [16].

Table 1.

Samples used, their geographical origins and genotypes[a].

Samples	mtDNA type	Genotype at *ef1* locus	Genotype at *elp* locus
TasiT041Sichuan_CN	*T. asiatica* type	***ef1B/ef1C***	***elpA/elpC***
TsagT038Sichuan_CN	*T. saginata* type	***ef1B/ef1C***	*elpC/elpC*
TsagT040Sichuan_CN		*ef1C/ef1C*	*elpC/elpC*
TsagT042Sichuan_CN		*ef1C/ef1C*	*elpC/elpC*
TsagT043Sichuan_CN		*ef1A/ef1A*	*elpC/elpC*

[a] See the text for abbreviations of mitochondrial haplotypes and alleles. The number after the species code identifies the sample ID used in the Asahikawa Medical University or Tottori University. Each sample code is followed by a locality name and country name (CN, China).

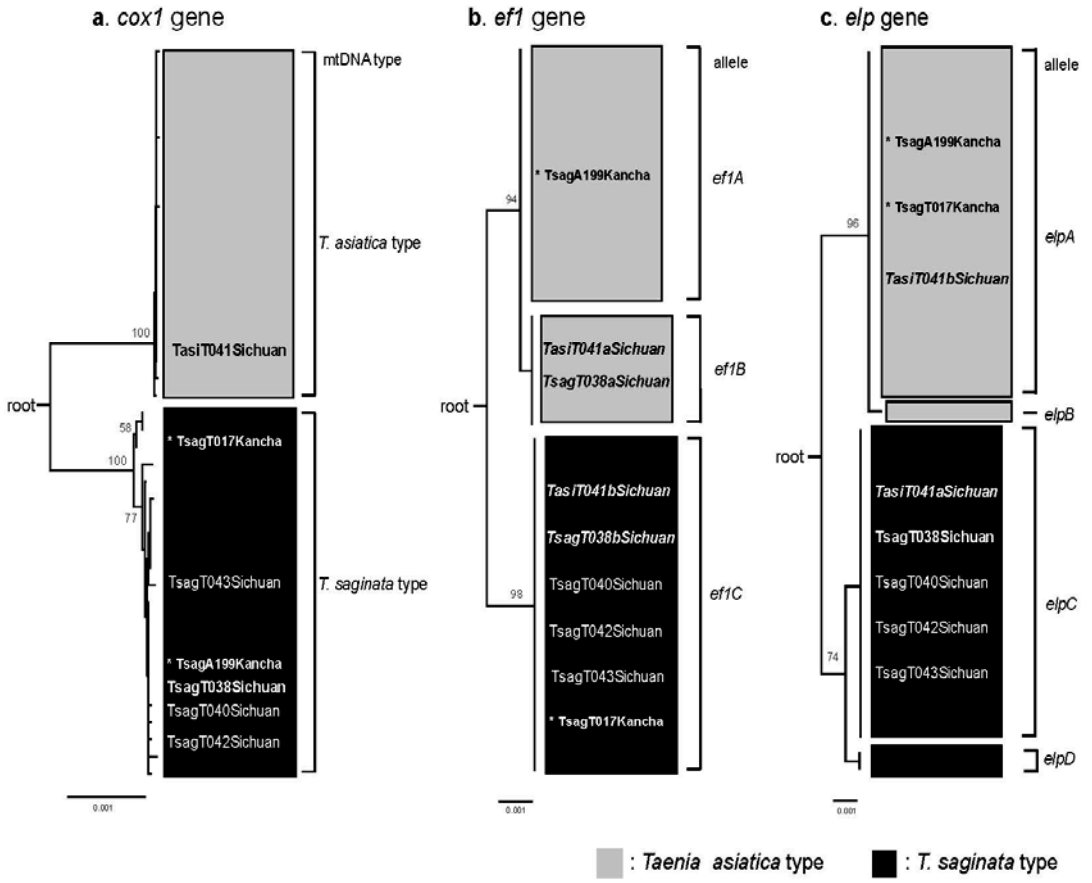


Fig.1. Neighbor-joining phylogenetic trees of the mitochondrial cytochrome *c* oxidase subunit 1 gene (a: *cox1)*, nuclear genes for elongation factor-1-alpha (b: *ef1*), and ezrin/radixin/moesin-like protein (c: *elp*).

Samples in italic type represent heterozygotes that displayed two alleles. Samples in bold type showed contradictions in the phylogeny between the mitochondrial gene and one or both of the nuclear genes. The asterisk (*) indicates the data from the previous study [16]. Numbers on the nodes represent bootstrap values. Each scale bar represents the evolutionary distances. The number after the species code identifies the sample ID used in the Asahikawa Medical University or Tottori University. Each sample

code is followed by a locality name. Country names (CN: China, TH: Thailand) are omitted.

Discussion

Nuclear-mitochondrial discordance is commonly used to identify putative hybrids [25]. In the previous study, both of the two adult worms, which had been collected from Kanchanaburi Province, Thailand, had *T. saginata* type mtDNA, but one worm was homozygous at two nuclear loci with alleles typical of *T. asiatica* (*ef1*A, *elp*A) and the other displayed such allele at one locus (*elp*A) [16]. Although occasional crossing between *T. asiatica* and *T. saginata* was proposed as an explanation for these observations, the possibility of retention of ancestral polymorphism could not be completely dismissed by their data [16]. The facts that additional worms with nuclear-mitochondrial discordance are found at another site in which these worms are sympatrically endemic will support the occurrence of hybridization between these two taxa. In this study, I found two worms (TasiT041 and TsagT038) showing nuclear-mitochondrial discordance, at Tibetan Plateau, Sichuan, China. Such individual worms have not been found in any other location except in these sympatric endemic areas. This could be supporting evidence for the hybridization of these two taxa. We have never found evidence of hybrid-derived offspring, which had *T. asiatica* type mtDNA at Kanchanaburi, but one of two worms from Tibetan Plateau had *T. asiatica*-type mtDNA. This indicates that hybridization between *T. saginata* and *T. asiatica* could occur reciprocally.

In many cases, only a single taeniid cestode is found alone in the

definitive host, but fertilized eggs are also found in the feces of the same host. In all likelihood, self-fertilization is the main reproductive method of the genus *Taenia*. However, outcrossing within or between species has also been reported for taeniid cestodes [16,26]. Fig. 2 shows the simplest process in theory whereby relevant four genotypes from Kanchanaburi and Tibetan Plateau would occur after hybridization between *T. saginata* and *T. asiatica*. Although this figure shows the processes until F1 or F2, these genotypes would occur in subsequent generations or by backcrossing. In this regard, however, incidence of these genotypes would decrease over time. As we all know, the F1 hybrid between species should be heterozygous at all nuclear loci. The two hybrid-derived worms from Kanchanaburi could not be F1 hybrids, because they were homozygous at the *ef1* and *elp* loci [16]. Those genotypes could occur in the F2 generation or through backcrossing between F1 and *T. asiatica*. To the contrary, TasiT041 from Tibetan Plateau was heterozygous at both *ef1* and *elp* loci with *T. saginata*-type alleles and *T. asiatica*-type alleles (*ef1*B/*ef1*C, *elp*A/*elp*C). Although the genotype of TasiT041 could appear at the F2 or later generation by self-fertilization, it cannot be ruled out that TasiT041 was a F1 hybrid. Additional investigations of other loci are needed for clarification.

In the case of TsagT038, although the *ef1* locus was heterozygous with the *T. saginata*-type allele and *T. asiatica*-type allele (*ef1*B/*ef1*C), the *elp* locus was homozygous (*elp*C/*elp*C). This genotype could not occur from backcross between F1 and parental species, but could occur in the

F2 generation theoretically (Fig. 2). Self-fertilization represents a type of inbreeding and each locus in the offspring becomes homozygous over generations with genetic drift. The fact that some nuclear loci are still heterozygous means that hybridization might have occurred recently. Hybridization between *T. asiatica* and *T. saginata* is probably an ongoing event in any areas in which these species are sympatrically endemic.

The data presented in this report clearly show that reproductive isolation between *T. saginata* and *T. asiatica* is incomplete.

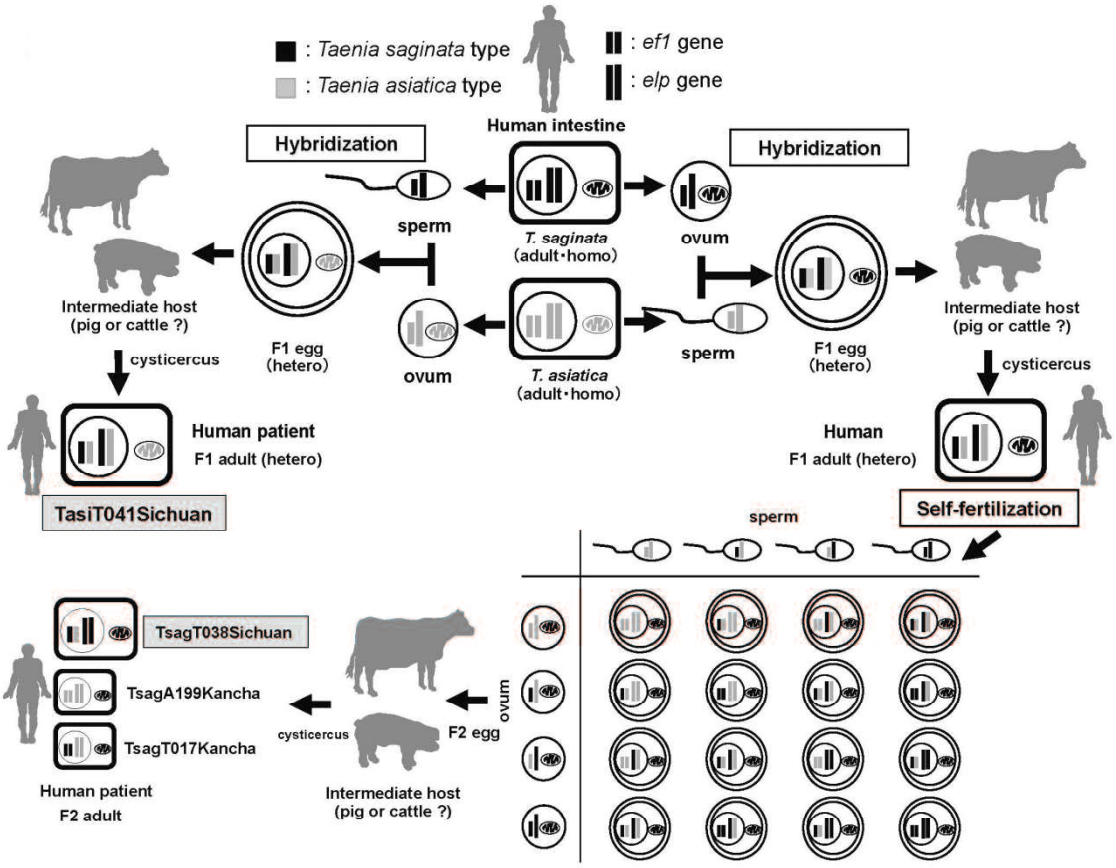


Fig. 2. The simplest processes of emergence of relevant genotypes after hybridization between *T. saginata* and *T. asiatica*.

First, some patient was simultaneously infected with *T. saginata* and *T. asiatica*. Since TasiT041 had *T. asiatica* type mtDNA and was possible to be F1, *T. asiatica* should have provided an ovum, and *T. saginata* should have supplied sperm for the F1, because mtDNA is maternally inherited. On the contrary, TsagT038, TsagA199 and TsagT017 were not F1. In these cases, *T. saginata* should have provided an ovum, and *T. asiatica* should have supplied sperm for the F1. The fertilized egg of each genotype was ingested by some intermediate host (cattle or pig), and developed into the mature cysticercus. Then, the patient of TasiT041 ate this cysticercus and adult worm developed in the intestine. Each egg with other genotypes was also

ingested by humans and developed into an adult worm. When F1 generated fertile eggs by self-fertilization, the probability that each genotype of TsagT038, TsagA199 and TsagT017 has produced is one-sixteenth judging from Mendel's law. Then, those eggs infected some intermediate host, and developed mature cysticerci, and each patient, who had isolate TsagT038, TsagA199 or TsagT017, ate one of those cysticerci.

Chapter 2

Genotypic relationships between *Taenia saginata*, *Taenia asiatica* and their hybrids

Introduction

It has been a long-standing puzzle that adult taeniid tapeworms expelled from people in Asian countries seemed to be *T. saginata*, although these people ate pork rather than beef [2,27,28]. Taiwan, Indonesian and Korean researchers energetically studied the *T. saginata*-like tapeworm, including experimental infections, and concluded that this parasite was an independent new species [29,30,31,32]. Several others working on molecular differences between 'Asian *Taenia*' and *T. saginata* rejected this idea [9,28,33].

In previous reports, four adult worms showing nuclear-mitochondrial discordance which suggest that hybridization between *T. saginata* and *T. asiatica* occurred were found in areas in which these taxa are sympatrically endemic [16,34]. The data presented in those reports clearly showed that reproductive isolation between *T. saginata* and *T. asiatica* was incomplete. Based on Mayr's biological species concept [15], it can thus be considered that *T. asiatica* is the same species as *T. saginata*. Concrete evidence is still lacking, however, because only 4 worms of hybrid origin have yet been identified. In addition, only two nuclear loci were examined in these previous studies. Examination of other nuclear loci should lead to the further discovery of the evidence of nuclear-mitochondrial discordance. To this end, I developed further polymerase chain reaction (PCR) and sequencing methods for the DNA polymerase delta (*pold)* gene and examined the *pold* loci from both taxa in this study. My results suggest

complicated relationships between *T. saginata*, *T. asiatica* and their hybrids which are discussed here.

Materials and methods

Parasite samples

In this study, I examined a total of 67 adult tapeworms which were morphologically similar to *T. saginata* collected from humans in 11 countries (Brazil, Ecuador, Ethiopia, Japan, South Korea, Philippines, China, Taiwan, Cambodia, Thailand and Indonesia). Those worms did not necessarily have a scolex and often we were only able to obtain just a few proglottids, then species were not identified exactly. Approximately two-thirds of the samples came from individuals who provided samples in our previous studies [16, 34]. Samples were stored in 70% ethanol until they were required for DNA extraction.

DNA preparation

Genomic DNA was individually extracted from mature or immature proglottids using a QIAamp DNA Mini Kit or a DNeasy tissue kit (QIAGEN, Germany) in accordance with the manufacturer's instructions, and then used as a template for PCR.

Multiplex PCR for *Taenia* species identification

Multiplex PCR based on the mitochondrial cytochrome c oxidase subunit 1 (*cox1*) gene is an easy method for identification of human taeniid cestodes [19,35]. Samples were screened by this method for the tentative identification of species.

DNA sequencing

Partial sequences of the DNA polymerase delta (*pold*) gene were amplified from the total DNA by PCR using the primer pair: *pold*/F_169: ATCCTGCACCTCCATAATGC and *pold*/R_1417: GCTTGATGGGGTTCACA-AAT. PCR was carried out in 15 μl reaction mixtures containing 1 μl template, 200 μM of each dNTP, 0.2 μM of each primer, 0.3U of Ex Taq polymerase (TaKaRa, Japan) and manufacturer-supplied reaction buffer. Thermal cycling was performed for 35cycles of denaturation (94 °C for 30 sec), annealing (60 °C for 30 sec), and extension (72 °C for 90 sec). The PCR products were purified using MinElute PCR Purification Kits (QIAGEN) or were enzymatically cleaned with calf intestine alkaline phosphatase (TOYOBO) and Exonuclease I (TaKaRa). Direct sequencing was performed with a Dye Terminator Cycle Sequencing Kit and an ABI 3130xl Generic Analyzer (Applied Biosystems, USA). At least two independent PCR products were used for sequencing.

In cases of double peaks in the sequencing reaction, thermal cycling was performed using PrimeSTAR GXL DNA polymerase according to the manufacturer's instructions. PCR products were subjected to cloning using TArgetClone-Plus- (TOYOBO, Japan), and more than ten clones were sequenced per sample.

Data analysis

DNA sequences obtained were aligned using the CLUSTALW computer program [20]. Phylogenetic trees were constructed by the

neighbour joining (NJ) method [21] using the MEGA5.1 computer program [36]. Evolutionary distances were computed using the Maximum Composite Likelihood Method [23]. Phylogenetic tree was evaluated using a bootstrap test based on 1000 resamplings [24]. Sequences of *Taenia ovis* (Acc. No.: FN568374), *T. multiceps* (Acc. No.: FN568373) and *T. serialis* (Acc. No.: FN568372) were used as out-groups to indicate the location of the root of the in-group. For presentation purposes, the long branch leading to the out-group is not shown in the tree.

The parsimonious network of *pold* haplotypes was drawn by using TCS 1.2 software [37] using statistical parsimony [38]. The network estimation was run at 95% connection limit.

Results

The mtDNA-based multiplex PCR assigned our samples to *T. saginata* (n = 28) or *T. asiatica* (n = 39). Since it is certain that there are some worms which originated from hybridization between *T. saginata* and *T. asiatica* [16,34], we could not identify the species of those samples using only mitochondrial genotypes. According to the results, the codes 'Tasi' (*T. asiatica*) or 'Tsag' (*T. saginata*) were added to the sample ID. It is important to note that these codes refer to the identification determined by the mitochondrial genome.

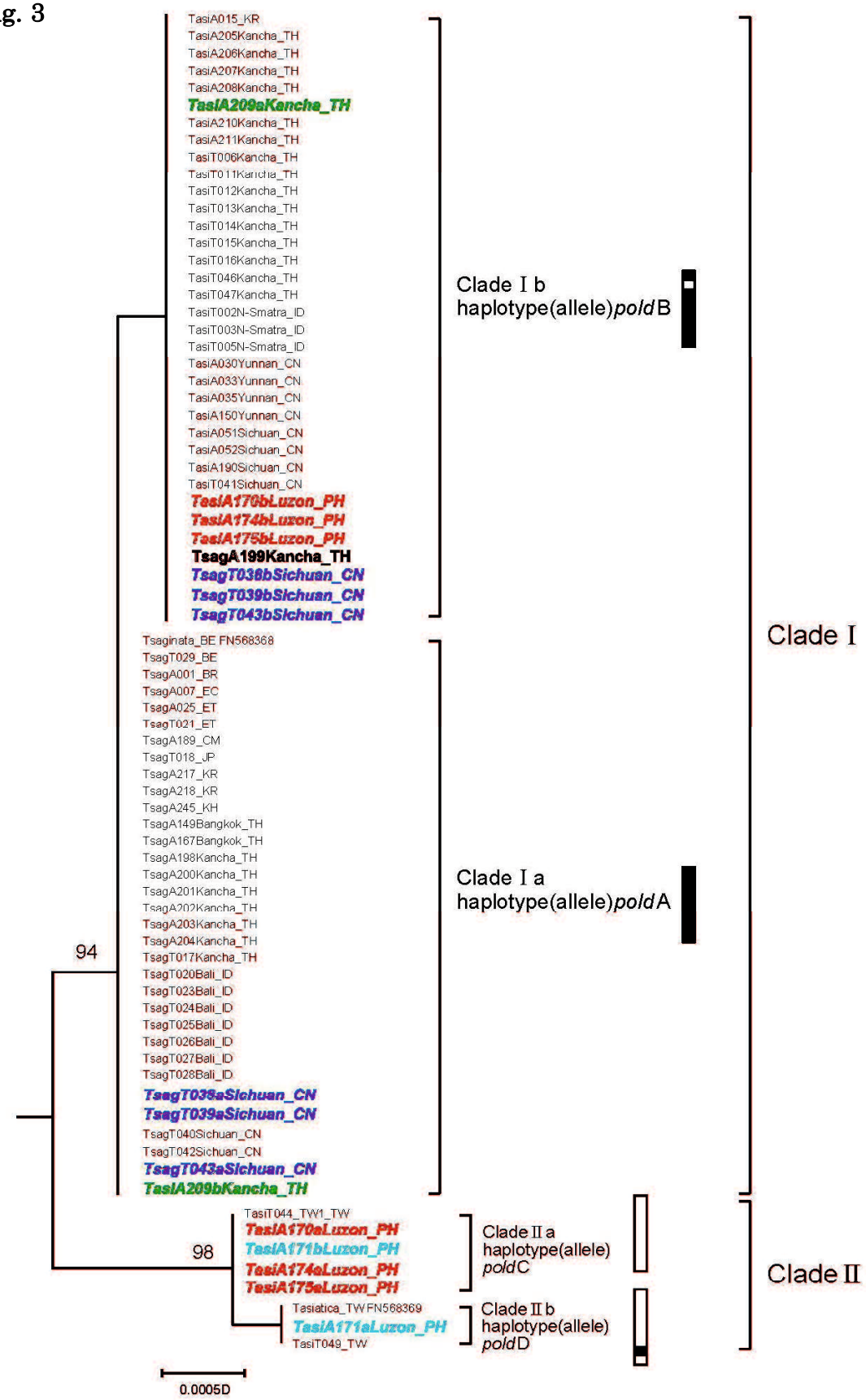
Partial sequences of *pold* gene (1200 bp in length) were obtained from all except 8 samples by direct sequencing of PCR products. There was no indel among all samples. Unfortunately, consistent sequences could not be obtained from the remaining 8 samples (TasiA209Kancha_TH, TasiA170Luzon_PH, TasiA171 Luzon_PH, TasiA174 Luzon_PH, TasiA175 Luzon_PH, TsagT038Sichuan_CN, TsagT039 Sichuan_CN, TsagT043 Sichuan_CN), because there were double peaks at several nucleotide positions in electropherograms. After cloning and sequencing, two independent sequences (haplotypes) were obtained from each of these 8 samples. Each haplotype was distinguished by adding 'a' or 'b'.

Fig. 3 shows the neighbour-joining phylogenetic tree inferred from *pold* gene sequences. Four haplotypes, which corresponded to four alleles, (*pold*A, *pold*B, *pold*C, *pold*D) were detected at the *pold* locus. These haplotypeswere clearly divided into two clades (Clade I, Clade II), which

differed by five to seven nucleotides from each other. 'Most of the Tsag' were included in the Clade Ia (*pold*A), while 'Most of the Tasi' were included in the clade Ib (*pold*B). On the other hand, Clade IIa (*pold*C) and Clade IIb (*pold*D) included only Tasi samples.

Fig. 4 shows the parsimonious network of *pold* haplotypes of human *Taenia* examined. It indicates that the *pold*B haplotype was derived from the *pold*A haplotype by the occurrence of single nucleotide substitution. Similarly, the *pold*D haplotype was derived from the *pold*C haplotype.

Fig. 3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TasiA205Kancha_TH
TasiA206Kancha_TH
TasiA207Kancha_TH
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TasiA210Kancha_TH
TasiA211Kancha_TH
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TsagA001_BR
TsagA007_EC
TsagA025_ET
TsagT021_ET
TsagA189_CM
TsagT018_JP
TsagA217_KR
TsagA218_KR
TsagA245_KH
TsagA149Bangkok_TH
TsagA167Bangkok_TH
TsagA198Kancha_TH
TsagA200Kancha_TH
TsagA201Kancha_TH
TsagA202Kancha_TH
TsagA203Kancha_TH
TsagA204Kancha_TH
TsagT017Kancha_TH
TsagT020Bali_ID
TsagT023Bali_ID
TsagT024Bali_ID
TsagT025Bali_ID
TsagT026Bali_ID
TsagT027Bali_ID
TsagT028Bali_ID
TsagT038aSichuan_CN
TsagT039aSichuan_CN
TsagT040Sichuan_CN
TsagT042Sichuan_CN
TsagT043aSichuan_CN
TasiA209bKancha_TH
Clade Ⅰa
haplotype(allele)poldA
94
Clade Ⅰ
TasiT044_TW1_TW
TasiA170aLuzon_PH
98
TasiA171bLuzon_PH
TasiA174aLuzon_PH
TasiA175aLuzon_PH
Clade Ⅱa
haplotype(allele)
poldC
Tasiatica_TW FN568369
TasiA171aLuzon_PH
TasiT049_TW
Clade Ⅱb
haplotype(allele)
poldD
Clade Ⅱ
0.0005D

Fig. 3. Neighbor-joining phylogenetic trees of the partial sequence of the nuclear the DNA polymerase delta (*pold*) gene. Samples in bold type show nuclear-mitochondrial discordance. Samples in italic type represent heterozygotes that displayed two alleles (red: Tasi represents heterozygous with *pold*B and *plod*C; blue: Tsag represents heterozygous with *pold*A and *plod*B; green: Tasi represents heterozygous with *pold*A and *plod*B; aqua: Tasi represents heterozygous with *pold*C and *plod*D). Numbers on the nodes represent bootstrap values. Scale bar represents the evolutionary distances. The number after the species code (e.g. A030) identifies the sample ID used in the Asahikawa Medical University or Tottori University. Each sample code is followed by a locality name (absent from some) and country name (abbreviated). Abbreviations of country names are as follow: BR, Brazil; CN, China; EC, Ecuador; ET, Ethiopia; ID, Indonesia; JP, Japan; KH, Cambodia; KR, South Korea; PH, Philippines; TH, Thailand; TW, Taiwan. See the text for abbreviations of mitochondrial types and alleles.

Fig. 4

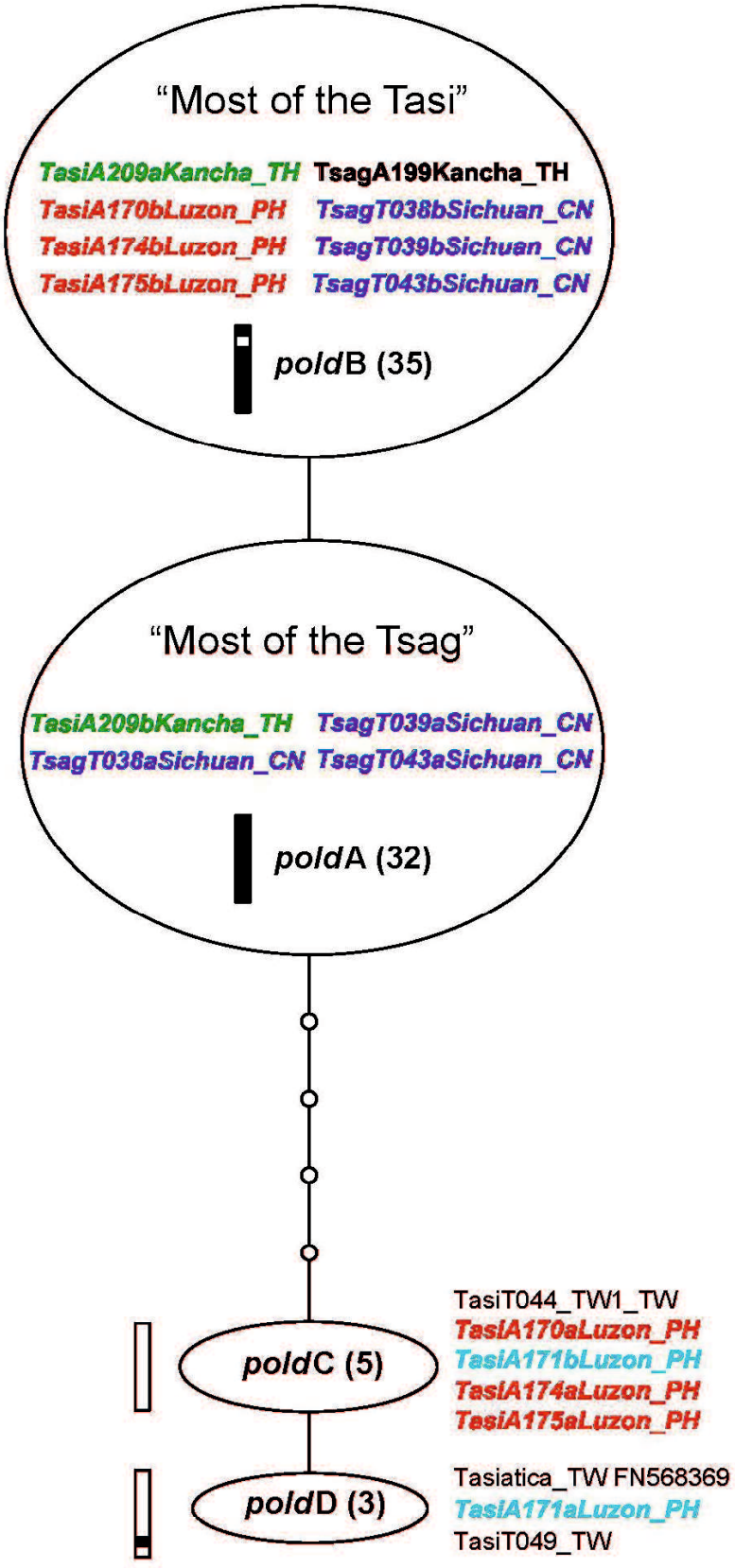
"Most of the Tasi"
TasiA209aKancha_TH
TsagA199Kancha_TH
TasiA170bLuzon_PH
TsagT038bSichuan_CN
TasiA174bLuzon_PH
TsagT039bSichuan_CN
TasiA175bLuzon_PH
TsagT043bSichuan_CN
poldB (35)
"Most of the Tsag"
TasiA209bKancha_TH
TsagT039aSichuan_CN
TsagT038aSichuan_CN
TsagT043aSichuan_CN
poldA (32)
poldC (5)
TasiT044_TW1_TW
TasiA170aLuzon_PH
TasiA171bLuzon_PH
TasiA174aLuzon_PH
TasiA175aLuzon_PH
poldD (3)
Tasiatica_TW FN568369
TasiA171aLuzon_PH
TasiT049_TW

Fig. 4. The parsimonious networks of *pold* gene haplotypes of *T. saginata*-like human *Taenia*. Samples in bold type show nuclear-mitochondrial discordance. Samples in italic type represent heterozygotes that displayed two alleles (red: Tasi represents heterozygous with *pold*B and *plod*C; blue: Tsag represents heterozygous with *pold*A and *plod*B; green: Tasi represents heterozygous with *pold*A and *plod*B; aqua: Tasi represents heterozygous with *pold*C and *plod*D). The size of the circles indicates the frequency of the haplotypes, and the actual numbers of haplotypes (>1) are shown in parentheses.

Discussion

We previously found four adult *T. saginata*-like worms that showed nuclear-mitochondrial discordance [16,34]. Namely, some individuals had *T. saginata* type mitochondrial DNA but had alleles originated from *T. asiatica* in some nuclear loci and vice versa. In light of these results, we came to four conclusions. First, phylogenetic analyses of both mitochondrial and two nuclear genes yielded trees consisting of two rather uniform clades corresponding to either *T. asiatica* or *T. saginata* and considerable differences between the mitochondrial lineages indicated a long period of separation between these two taxa. Second, although taeniid cestodes are primarily self-fertilizers, the presence of a few heterozygous individuals suggests that outcrossing also occurs. Third, since these four worms showed nuclear-mitochondrial discordance, reproductive isolation between *T. saginata* and *T. asiatica* remains incomplete, and hybrid breakdown has not yet occurred. Finally, since some nuclear loci remain heterozygous, hybridization might have occurred recently, and probably continues in areas where *T. saginata* and *T. asiatica* are sympatrically endemic.

In the present study, *pold* gene sequences were also clearly divided into two clades (Clade I and Clade II), differing from each other in five to seven nucleotides (Fig. 3). Since Clade II included only 'Tasi' samples, I might consider that Clade II corresponds to the allele from *T. asiatica* and that the other (Clade I) corresponds to that from *T. saginata*. Since the presence of several nucleotide substitutions in nuclear genes means

prolonged separation after speciation, there is little doubt that *T. saginata* and *T. asiatica* were once separated into two distinct taxa as has been concluded in previous studies. On the other hand, I demonstrate here one significant difference from the results of these previous reports; i.e. that 'Most of the Tasi' are homozygous for *pold*B allele. As indicated in the haplotype network tree (Fig. 4), there is no doubt that *pold*B derived from *pold*A with a single nucleotide substitution. Since all 'Tsag' except TsagA199Kancha_TH showed the *pold*A allele, *pold*A should be the original allele from 'pure *T. saginata*'. In contrast, it appears that *pold*C and *pold*D originated from 'pure *T. asitacia*', because each is found only in 'Tasi' collected from Taiwan and Philippines.

These results indicate that most of the adult worms which had been called *T. asiatica* are not actually 'pure *T. asiatica*' but instead originated from the hybridization of 'pure *T. saginata*' and 'pure *T. asiatica*', even if previously identified as *T. asiatica* using mitochondrial DNA. In other words, worms distributed everywhere other than the Philippines and Taiwan are all descendants of this hybridization. The genotypes of worms were examined and some of their possible relationships were inferred from the results of the present and previous studies [16,34] are shown in Fig. 5. A likely scenario for this event is as follows. At some point in the past, hybridization between 'pure *T. saginata*' and 'pure *T. asiatica*' occurred, producing a worm with *T. asiatica*-type mitochondrial DNA and heterozygous at the *pold* locus with the *pold*A and *pold*C alleles. When alternation of generations was repeated by self-fertilization, the *pold* locus

was fixed at the *pold*A allele in some worms due to genetic drift. At the same time, the *pold*A allele mutated to *pold*B via single nucleotide substitution. The descendants of such worms, which had *T. asiatica* type mitochondrial DNA and the *pold*B alleles, have since spread throughout southeast Asia. Of course, our results do not allow dismissal of the possible retention of ancestral polymorphism within 'pure *T. saginata*', but this is unlikely because, with the exception of TsagA199, no 'Tsag' were homozygous for *pold*B allele at all. Finally, 'pure *T. asiatica*', which would only have the alleles *pold*C and *pold*D, probably remains only in the Philippines and/or Taiwan, even if it still exists.

I found several adult worms whose *pold* locus was heterozygous. Of these, four worms, TasiA209Kancha_TH, TsgT038Sichuan_CN, TsagT039Sichuan_CN, TsagT043Sichuan_CN, were heterozygous with the *pold*A and *pold*B alleles. As mentioned above, the *pold*A allele is considered to be a major allele of 'pure *T. saginata*'. Although the *pold*B allele is a major allele of 'Tasi', it is not an allele from 'pure *T. asiatica*' but originated instead from the descendant of hybridization between 'pure *T. saginata*' and 'pure *T. asiatica*'. Therefore, it is highly possible that heterozygosity at the *pold* locus in these four worms cannot have been caused by hybridization between 'pure forms' but instead by the backcrossing between 'pure *T. saginata*' and 'Tasi' with the *pold*B allele ('Most of Tasi'). In general, when hybridization happens once and alternation of generations is repeated, their descendants show various genotypes. In the cases of *T. asiatica* and *T. saginata*, since a variety of descendants must have been produced after

hybridization, we cannot deny the possibility that relevant genotypes have occurred in such descendants. In previous studies, both the *ef1* and *elp* loci of all 'Tasi' examined, except one individual (TasiT041), were homozygous for *T. asiatica*-type alleles. This fact indicates that 'Tasi' spread in southeast Asia have limited variation as a result of a population bottleneck. It is thought that the above-mentioned scenario with relevant genotypes coming from back-crossing is not irrelevant.

In previous reports, both the *ef*1 and *elp* loci in TasiA209Kancha_TH and TsgT038Sichuan_CN were homozygous, so at least in these cases the two adults did not result from the backcross 1 (BC1) generation. Three other adults examined from Luzon, Philippines (TasiA170Luzon_PH, TasiA174 Luzon_PH and TasiA175 Luzon_PH) also originated from the backcrossing between "pure *T. asiatica*" and "Tasi" with the *pold*B allele.

TasiA171 was also heterozygous but had the *pold*C and *pold*D alleles, which were alleles originated from "pure *T. asiatica*". Although I cannot make sure to decide which allele was original, the parsimonious network indicates that *pold*C was likely to be original (Fig. 4). If alternation of generations was repeated by self-fertilization after single nucleotide substitution, the *pold* locus should be fixed at two *plod*D alleles in some worms (e.g. TasiT049). And it might be still heterozygous for *pold*C and *plod*D alleles in others. TsagA171 is possible to be such individual or the descendant of outcrossing. In any case, I cannot say that TasiA171 was an individual derived from hybridization between two taxa.

Fig. 5

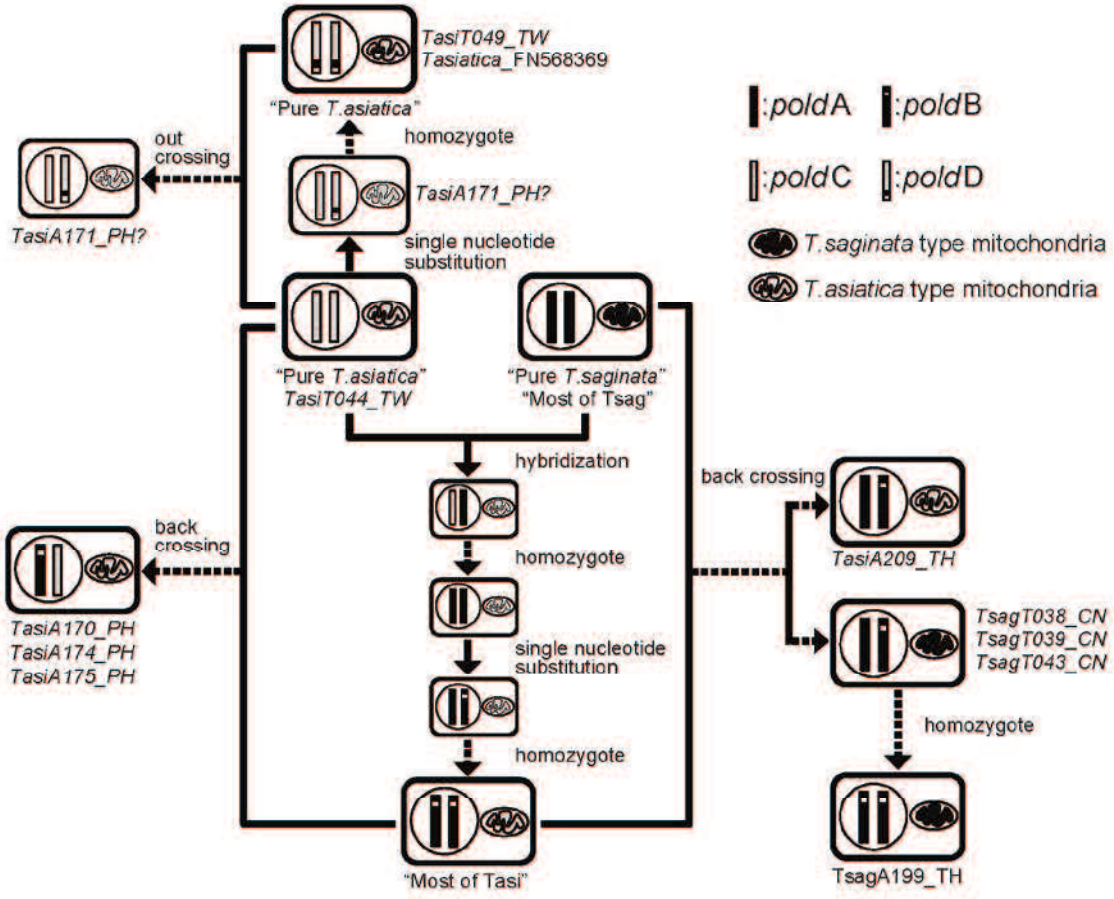

Fig. 5. Genotypes of worms examined and their possible relationships. Relevant genotypes appeared after hybridization between 'pure *T. saginata*' and 'pure *T. asiatica*'. Samples in italics represent heterozygotes that displayed two alleles. For details see text.

General conclusion

I examined the nucleotide sequences of one mitochondrial gene (*cox1*) and of alleles at three nuclear loci (*ef1, elp* and *pold*) from human taeniid worms. Phylogenetic analyses of those genes yielded trees consisting of two rather uniform clusters in each case. Therefore I concluded that *T. saginata* and *T. asiatica* were once separated into two taxa, "pure *T. saginata*" and "pure *T. asiatica*". There is little doubt about that the considerable difference between the mitochondrial lineages indicates a long period of separation between *T. asiatica* and *T. saginata*. Regardless of the species identified, most individuals are homozygous at the nuclear loci. This is to be expected because taeniids are primarily self-fertilizers [39,40], a mating system that will lead to increase homozygosity. The presence of a few heterozygous individuals in our study suggests that outcrossing also occurs in *Taenia* species.

In chapter 1, I concluded that four worms (TsagA199Kancha_TH, TsagT017Kancha_TH, TsagT038Sichuan_CN and TasiT041Sichuan_CN) from areas where *T. saginata* and *T. asiatica* are sympatrically endemic originated from hybrids between those two taxa [16,34], because nuclear-mitochondrial discordance was observed in those four worms. In chapter 2, however, since all four of those worms had neither the *pold* C nor the *pold* D (Fig. 3), there is a high possibility that they were not direct descendants of the "pure *T. saginata*" and "pure *T. asiatica*" hybrid. TsagA199Kancha_TH, Tsag038Sichuan_CN and TasiT041Sichuan_CN might be the products of backcrossing between descendants of the hybrid and "pure *T. saginata*". And TsagT017Kancha_TH appeared as a result of on

and after F2 generation. At present, I have not found a F1 hybrid of the "pure *T. saginata*" and "pure *T. asiatica*" , which should have the *pold*A and *pold*C or *pold*D alleles. Although it is certain that hybridization between pure forms once occurred, it is unclear whether or not such hybridization occurs even now. Therefore further investigations are necessary to clarify the relationship between *T. saginata*, *T. asiatica* and their hybrids, especially in Philippines and Taiwan.

In chapter 1 and chapter 2, I reached the conclusion that "pure *T. asiatica*" was able to hybridize with "pure *T. saginata*". According to the Mayer's Biological Species Concept [15], it is considered that *T. asiatica* is a same species as *T. saginata*. However I should not equate *T. asiatica* with *T. saginata*, even if they are same species. Because each taxon has its own biological identity. In particular, host specificity for intermediate hosts is quite different. The difference is very important for us because the intermediate host is the source of infection in humans. In addition, it is very important to know what kinds of animals could be the intermediate hosts of hybrid-derived worms. It is clear that *T. saginata* is parasitic in beef and that *T. asiatica* is parasitic in the viscera of pig, but not in pork [41,42]. However, it was reported that larval stage of *T. asiatica* could partly develop in the viscera of cattle [41] and that cycticerci of *T. saginata* have been found in the liver of cattle or zebu [43,44]. Although I cannot deny the possibility that those worms were hybrid-derived offspring, and host specificity for intermediate hosts or organotrophism of both taxa may not be strict. If the host specificity is not regulated by a single gene, but instead determined by

multiple genes, the problem of the intermediate host of hybrid-derived worms may be complicated.

Since the definitive host of *T. saginata* and *T. asiatica* is limited to humans, sexual reproduction of both parasites can occur only in the human intestinal tract. Since their reproductive isolation is incomplete even in the present, it is unlikely that sympatric reproductive isolation due to mutation has occurred in the past. Therefore, there is a strong possibility that ancient isolation between the two taxa was the result of geographical or ecological isolation. Because of the above mentioned, there may be considerable validity in the proposal by McManus that *T. asiatica* should be regarded as a subspecies of *T. saginata*, and be termed *T. saginata asiatica* [9,13]. However, it is actually impossible to demonstrate its type locality. Moreover, the present coexisting situation of both taxa makes it difficult to delineate subspecies. On the other hand, the ecological isolation includes human behaviors for food habit of flesh-eating and for domestication of cattle and pigs. There is a possibility that sympatric speciation could occur in human *Taenia* under the ecological isolation.

If humans had not interfered in the relationship between these taxa, each would have evolved into a distinct species. However, they have come into contact with each other prior to complete speciation and the consequent hybrid-derived populations have been appeared in Kanchanaburi Province, Thailand, in Tibetan Plateau, Sichuan, China and so on. Furthermore in Philippines and Taiwan where there is some possibility of existence of "pure *T. asiatica*", it is necessary to conduct more investigation. It may be

interesting to observe whether such populations evolve into a third, new species, or instead become extinct in the future.

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