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Research Article

Phylogenetic characterization of the pork tapeworm *Taenia solium* in Japan: implications for the enigmatic evolutionary history

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Note: Nucleotide sequence data reported in this paper are available in GenBank/DDBJ/EMBL databases under accession numbers LC844894–LC844917 and LC847175–LC847180.

Abstract

Taenia solium is a tapeworm of the family Taeniidae that causes neurocysticercosis, a serious zoonotic disease in humans. Its life cycle involves pigs and wild boars as intermediate hosts and humans as the sole definitive hosts. Since poor sanitation and free-roaming pigs contribute to maintaining its life cycle, cysticercosis is endemic in developing countries across Asia, Africa, and Latin America, while local transmission is generally absent in developed countries. However, we unexpectedly identified three cases of cysticercosis in wild boars in Japan between 2014 and 2023. Genetic analyses were performed on six cysticerci collected from two wild boars independently captured in 2023. Phylogenetic analysis using three nuclear DNA markers confirmed that the cysticerci were indeed *T. solium*. Mitogenome sequencing from these cysticerci yielded six complete mitogenomes, each 13,712 bp in length and identical to each other. Haplotype network analysis using mitochondrial *cox1* and *cob* sequences revealed that the cysticerci in Japan possess a haplotype distinct from haplogroups in other endemic regions, i.e., haplogroups in Asia, Africa/America, and Bhutan, indicating that the *T. solium* population is divided into at least four haplogroups. Subsequent phylogenetic inference from the mitochondrial 12 protein-coding sequences demonstrated that the Japanese haplotype

diverged from both the Asian and African/American haplogroups before the divergence of these two major haplogroups in the early to middle Pleistocene. Our findings indicate that the *T. solium* life cycle can be maintained in regions generally considered non-endemic, highlighting an overlooked risk of local transmission in developed countries. Furthermore, the genetic diversity and phylogenetic relationships revealed in this study may provide evidence for revisiting the "Out of Africa" hypothesis for *T. solium*. Comprehensive mitogenomic analyses based on additional specimens would hold the key to unraveling the evolutionary history of this tapeworm, which currently uses humans as its sole definitive host.

Keywords

Taenia solium; Zoonosis; Cysticercosis; Mitogenome; Phylogenetic analysis; Evolution

1. Introduction

Taenia solium is a zoonotic tapeworm of the family Taeniidae (Cestoda: Cyclophyllidea). The parasite's life cycle involves pigs and wild boars (*Sus scrofa*) as intermediate hosts and humans as the only definitive hosts (Dixon et al., 2021). The intermediate hosts become infected by oral ingestion of the embryonated eggs excreted in human feces, leading to the development of cysticerci primarily in the muscles. Human infection occurs through the consumption of undercooked or raw pork meat containing cysticerci, which mature into adult worms within the intestinal tract and release numerous eggs in the feces (Pawlowski, 2002). Importantly, humans can also serve as the intermediate host by ingesting parasite eggs from the environment, such as contaminated food or water, or by auto-infection (Rasamoelina-Andriamanivo et al., 2013). In such cases, cysticerci develop in the muscles and other organs, resulting in human cysticercosis. Cysticerci in the central nervous system are responsible for neurocysticercosis (NCC), a neglected tropical disease that leads to severe neurological disorders such as epilepsy in endemic regions (Garcia et al., 1993; Ndimubanzi et al., 2010).

Human cysticercosis is highly endemic in developing countries across Asia, Africa, and Latin America (Donadeu et al., 2022). Poor sanitation, free-roaming pigs, and open defecation still persist in these areas, allowing the full life cycle of *T. solium* to be maintained. Previous studies using mitochondrial DNA sequences have demonstrated that *T. solium* is divided into

two major haplogroups corresponding to their geographical distributions: the Asian haplogroup and the African/American haplogroup, which likely diverged from each other in the early to middle Pleistocene (Nakao et al., 2002; Michelet et al., 2010). The Asian haplogroup is widely distributed in Asian countries such as China, Nepal, Thailand, India, and Indonesia, while the African/American haplogroup is widespread in sub-Saharan Africa and Meso and South America, which include Zambia, Tanzania, Mexico, Peru, and Brazil (Nakao et al., 2002; Martinez-Hernandez et al., 2009; Michelet et al., 2010; Yanagida et al., 2014, 2021; Jiménez-Avalos et al., 2023). Phuentshok et al. (2021) recently reported a case of neurocysticercosis in a wild Bengal tiger (*Panthera tigris tigris*) in Bhutan and found that the cysticerci had unique haplotypes, which were distinct from both the Asian and American/African haplogroups (Phuentshok et al., 2021). The discovery of these novel haplotypes suggests that the genetic diversity of *T. solium* may be more extensive than previously considered.

Japan is currently regarded as a non-endemic country for cysticercosis (Coral-Almeida et al., 2015; Donadeu et al., 2022). Okinawa, the southernmost prefecture of Japan, was once a highly endemic area for tapeworm transmission due to the use of pig toilets, especially up until World War II. However, improved hygiene across Japan, including Okinawa, has likely prevented the tapeworm transmission cycle (Ito et al., 2003a, 2019). A literature review reported that 41 cysticercosis cases were documented during 1997–2011 and suggested that most of the cases were imported from abroad (Yamasaki, 2013). Mitochondrial DNA analysis

supports this perspective, showing that the cysticerci from patients with overseas travel histories had genotypes found in other endemic regions, such as India and Nepal (Yanagida et al., 2010; Katurahara et al., 2013). However, several patients identified in Japan after 1994 had no history of international travel, suggesting domestic transmission of *T. solium* (Yanagida et al., 2012). It is hypothesized that such patients got infected from food imported from other endemic countries or environments contaminated by *T. solium* carriers (Araki, 1994; Yanagida et al., 2012).

Here, we present three independent cases of cysticercosis which were identified in wild boars captured in 2014 and 2023 in Nagano Prefecture, Japan. We collected six cysticerci from two wild boars captured in 2023 and performed genetic analysis based on nuclear DNA to confirm the species. Furthermore, we aimed to gain insight into their phylogenetic relationships with previously reported genogroups by analyzing their mitochondrial genomes.

2. Materials and methods

2.1. Cases

Three wild boars hunted in Nagano Prefecture, Japan, were found to have cysticerci in their skeletal and cardiac muscles (Cases 1－3). The first case was found in the Daihoku region, in November 2014 (Case 1), and the other two cases were detected in the Kiso region in January 2023 and the Daihoku region in June 2023, respectively (Cases 2 and 3) (Fig. 1 and Supplementary Fig. S1). Parasite specimens were collected from the two cases in 2023 for genetic analysis.

2.2. Parasite DNA samples

Six cysticerci in the muscle or the heart wall were collected from two wild boars (three each) hunted in 2023. After washing three times with phosphate-buffered saline (PBS), DNA extraction from each cysticercus was performed using DNeasy® Blood & Tissue kit (Qiagen, Hilden, Germany). The six DNA samples eluted in 100 µl of elution buffer each were used as template DNA in subsequent analysis.

2.3. Multiplex PCR for taeniid cestodes

We performed a multiplex PCR assay for taeniid tapeworms that can distinguish *Echinococcus multilocularis*, *E. granulosus*, and *Taenia* spp. based on the amplicon size (Trachsel et al., 2007). The multiplex PCR was performed with five primers, Cest1-5, targeting the mitochondrial *nad1* of *E. multilocularis* and the mitochondrial small subunit of ribosomal

RNA of *E. granulosus* and *Taenia* spp. (Table S1). The reaction mixture (25.0 µl) consisted of 12.5 µl of 2 ×QIAGEN Multiplex PCR Master Mix (Qiagen), 9.0 µl of water, 2.5 µl of primer mix (2 µM of primers Cest1, Cest2, Cest3, Cest4 and 16 µM of Cest5), and 1.0 µl of extracted DNA. The PCR conditions used in this study are listed in Supplementary Table S1. PCR products were analyzed by electrophoresis on an agarose gel.

2.4. PCR and Sanger sequencing for nuclear genes of cysticerci

We performed PCRs targeting the genes encoding RNA polymerase II second largest subunit (*rpb2*), phosphoenolpyruvate carboxykinase (*pepck*), DNA polymerase delta (*pold*), and a low molecular weight backbone protein of the cystic glycoproteins (*Ag2*) (Knapp et al., 2011; Sato et al., 2011). The primer sequences and cycling conditions are summarized in Table S1. The reaction mixture contained 12.5 µl of 2×Gflex PCR buffer (TaKaRa Bio Inc., Shiga, Japan), 0.5 µl of each 10 µM primer, 0.5 µl of Tks Gflex DNA Polymerase (TaKaRa Bio Inc.), 10.0 µl of water, and 1.0 µl of template DNA. PCR products were analyzed as described above. Following purification with the ExoSAP-IT PCR Product Cleanup Reagent (Thermo Fisher Scientific, Waltham, MA, USA), the products were sequenced by primer walking using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA).

2.5. Phylogenetic analysis using nuclear genes

Phylogenetic analysis based on nuclear genes of *rpb2*, *pepck*, and *pold* was performed

following the methodology by Knapp et al. (2011). After multiple alignments by MAFFT v7.450 software (Katoh and Standley, 2013), putative exon regions were extracted from each gene sequence according to the exon-intron map of *E. multilocularis* previously determined (Knapp et al., 2011). We used the exon data set consisting of 9 *Echinococcus*, 12 *Taenia*, 1 *Versteria*, 2 *Hydatigera* species, and the 6 cysticerci detected in this study for Bayesian phylogenetic analysis.

The best-fit models of three nuclear genes were estimated by a nucleotide substitution model selection script, Kakusan4 (Tanabe, 2011). The Bayesian inference was performed using MrBayes v3.2.6 (Ronquist et al., 2012) in MrBayes5D (Tanabe, 2008) via the Markov Chain Monte Carlo (MCMC) algorithm with 1,000,000 generations with a sample frequency of 500 and a burn-in of 10%. The stationary and effective sample sizes were assessed by Tracer v1.6 (Drummond and Rambaut, 2007). The generated trees were used for the 50% majority rule consensus tree, which was visualized by FigTree v1.4.3.

2.6. Next-generation sequencing for mitogenome construction

The complete mitogenome sequences were constructed using a method similar to that previously developed for *E. multilocularis* (Hayashi et al., 2023) (Supplementary Fig. S2). Two sets of primers (Ts_MtG_F1 and TsMtG_R1, TsMtG_F2 and TsMtG_R2) were designed by aligning the mitogenome sequences of *T. saginata* (AF445798), *T. solium* (AB086256), *T. asiatica* (AF445798), *T. ovis* (AB731675), and *T. serialis* (AB731674). The reaction mixture

consisted of 5.0 µL of 5× PrimeSTAR GXL Buffer (TaKaRa Bio Inc.), 2.0 µl of dNTP mixture (2.5 mM), 0.5 µl of each primer (10 µM), 0.5 µl of PrimeSTAR GXL DNA Polymerase (TaKaRa Bio Inc.), and 1.0 µl of template DNA and PCR was performed with the cycling conditions indicated in Table S1. After purification of the products using NucleoSpin® Gel and PCR Clean-up Kit (Macherey-Nagel, Düren, Germany), the Illumina sequencing library was prepared with the Nextera XT DNA Library Preparation Kit (Illumina, Hayward, CA, USA). The library was sequenced on the Miseq platform, and the sequencing reads were mapped to the reference mitogenome of *T. solium* from China (AB086256) using CLC genomics workbench v23.0.2 (Qiagen).

2.7. Haplotype network analysis

The cytochrome *c* oxidase subunit I (*cox1*) and cytochrome *b* (*cob*) gene sequences were extracted from each constructed mitogenome. We collected the sequences of *cox1* and *cob* genes of *T. solium* from Asian, African, and Latin American countries deposited in the NCBI database. The number of samples per haplotype was retrieved from the previous studies (Table 1) (Nakao et al., 2002, 2003; Michelet and Dauga, 2012; Yanagida et al., 2014, 2021; Phuentshok et al., 2021; Sadlowski et al., 2021; Jiménez-Avalos et al., 2023). If the number of samples was not specified in their reports, it was assumed to be one in our analysis. Following sequence alignment, a median-joining network was constructed and visualized using PopART v1.7 (Leigh and Bryant, 2015).

2.8. Phylogenetic analysis based on mitochondrial genes

The phylogenetic analysis based on all mitochondrial coding sequences was performed as previously described (Nakao et al., 2013). We extracted the 12 protein-coding sequences from 18 mitochondrial genomes derived from 11 species within the genus *Taenia*. The nucleotide sequences of each gene were translated into amino acid sequences using the echinoderm and flatworm mitochondrial code (translation table 9). Each amino acid sequence was aligned using ClustalW in MEGA7 (Kumar et al., 2016), followed by the removal of amino acid deletions and stop codons. For the phylogenetic analysis based on nucleotide sequences, the amino acid alignments were subsequently converted back to nucleotide sequences. A total of 12 nucleotide and 12 amino acid sequences were concatenated into two datasets, resulting in 9,903 nucleotide sites and 3,301 amino acid sites from 18 mitochondrial genomes.

All of the third codon positions in the nucleotide alignments were removed using PAUP 4.0 software to avoid substitution saturation, which frequently occurs at these positions (Nakao et al., 2013; Terefe et al., 2014; Yilmaz et al., 2016). The best-fit model for phylogenetic inference was estimated by Kakusan4 for nucleotide sequences and by Aminosan for amino acid sequences, respectively (Tanabe, 2011). The GTR+Γ model was selected for the nucleotide sequences and the amino acid model mtMam+F+Γ was applied to the mitochondrial protein data set. Bayesian phylogenetic inference was performed using

MrBayes v3.1.2. with 1,000,000 generations with a sample frequency of 500 and a burn-in of 25%.

We also reconstructed the molecular phylogeny of *T. solium* based on two mitochondrial gene sets, *rrnS*, and large subunit ribosomal RNA (*rrnL*), *cox1* and *cob*. Briefly, the gene sequences of *T. solium* from Asian, African, and Latin American countries were collected as described above. Following the model selection by Kakusan4, a phylogenetic inference by MrBayes v3.1.2 was performed under the GTR+ Γ for the ribosomal RNA genes and the HKY model for *cox1* and *cob*, respectively, with 1,000,000 generations with a sample frequency of 500 and a burn-in of 25%. The generated consensus trees were visualized by FigTree v1.4.

2.9. Data accessibility

The dataset used in this study, including sequence alignments and software settings, have been deposited to Mendeley Data: https://data.mendeley.com/preview/spsfvcspnk?a=1b2e39a7-fd16-46e8-bf6c-785d0504b02a. Any additional information is available from the corresponding authors upon request.

3. Results

3.1. Morphological features of the cysticerci are consistent with those of *T. solium*

In Cases 1－3, the wild boars had a number of cysticerci in the muscles (Fig. 1). The cysticerci, approximately 5–10 mm in length, exhibited an ovoid to pointed shape with a white, opaque, and spherical structure in the midsection. Microscopic examination of flattened specimens collected from the boars in Cases 2 and 3 revealed that the parasites had four suckers and hooks on each scolex (Fig. 1F, I). The rostellum was armed with 14 large hooks (184–195 μm long) and 14 small hooks (120–135 μm long) (Fig. 2). These morphological features are consistent with those of *T. solium* cysticercus found in pigs (Verster, 1969; Šlais, 1970).

3.2. The cysticerci belong to the genus *Taenia*

A multiplex PCR assay previously developed for taeniid cestodes was performed to confirm the genus of the cysticerci. The multiplex PCR, targeting the mitochondrial small subunit ribosomal RNA (*rrnS*) gene, yielded single clear bands at the same position for *Hydatigera taeniaeformis* (formerly *Taenia taeniaformis*), indicating that the cysticerci obtained from two wild boars belong to the genus *Taenia* (Supplementary Fig. 3). The sequences of the PCR products from each cysticercus (217 bp) were completely identical to each other, showing a high similarity of 99.1–100.0% to that of *T. solium* deposited in the NCBI database. In contrast, the identity of the sequences to other *Taenia* spp. was lower than 92.7%.

3.3. Nuclear gene analysis identifies the cysticerci as *T. solium*

In line with the result of mitochondrial sequence, the sequences of two nuclear genes, *rpb2* and *pepck* were completely identical to those of *T. solium* from Ecuador (accession nos. FN566856 and FN567996). For the sequences of *pold*, one cysticercus collected in June 2023 had an identical sequence to that of *T. solium* from Ecuador (FN568367), while the remaining five cysticerci exhibited two nucleotide substitutions. We performed a phylogenetic analysis using these genes, which can be used for species identification of *Taenia* spp. (Knapp et al., 2011; Terefe et al., 2014). Bayesian phylogenetic inference revealed that our samples formed a distinct clade with *T. solium* from Ecuador (Fig. 3), indicating that the cysticerci obtained from wild boars in Japan are *T. solium*. The nucleotide sequences were deposited in the DNA Data Bank of Japan under accession numbers LC844894–LC844899 for *rpb2*, LC844900–LC844905 for *pepck*, and LC844906–LC844911 for *pold*.

3.4. Mitogenome sequences of the cysticerci have multiple nucleotide substitutions compared to the previously reported sequences

We successfully obtained six mitogenomes from the cysticerci using the amplicon-based strategy (Fig S2). The six complete mitogenomes were deposited in the DNA Data Bank of Japan under accession numbers LC847175–LC847180. They were 13,712 bp in length and completely identical to each other. The mitogenomes consisted of 12 protein-coding genes, 22 tRNA genes, and 2 ribosomal RNA genes and showed no rearrangements of the gene order compared to the reference sequence (AB086256). In contrast to the results of nuclear gene

analysis, the mitogenome sequences had multiple mismatch sites compared to those previously reported from China and Peru (Fig. 4).

3.5. The cysticerci have a unique mitochondrial haplotype

Among the 12 protein-coding genes, we used *cox1* and *cob* genes for the haplotype network analysis as they were the most frequently sequenced genes in previous studies. The median-joining haplotype network using the concatenated sequences (2,688 bp in length) revealed that *T. solium* obtained from wild boars in Japan were genetically distant from both Asian and African-American haplogroups (Fig. 5). We designated the haplotype in Japan as Ts_JPN. The pairwise divergences between Ts_JPN and Asian haplogroup and African-American haplogroup were 1.86–2.01% and 1.79–2.05%, respectively, while those between the two haplogroups were 1.19–1.53% (Fig. 6 and Table S2). *Taenia solium* collected from a Bengal tiger in Bhutan, potentially representing a novel haplogroup (Phuentshok et al., 2021), exhibited 1.97–2.05%, 1.26–1.53%, and 1.45–1.71% of the pairwise divergences to Ts_JPN, Asian haplogroup, and African-American haplogroup, respectively (Fig. 6 and Table S2).

3.6. The obtained haplotype is distinct from the Asian and African/American haplogroups

The genetic relationship among *T. solium* haplotypes was further evaluated through phylogenetic analysis using mitochondrial gene sequences. Bayesian inference based on nucleotide and amino acid sequences of 12 protein-coding sequences (CDSs) revealed that *T. solium* in Japan belonged to a clade distinct from both Asian and African/American

haplogroups (Fig. 7), which is consistent with the result of haplotype network analysis. The clade of Japanese haplotypes diverged from the Asian and African/American haplogroups prior to the divergence between the two major haplogroups.

Bayesian inference using *rrnS*, *rrnL*, *cox1* and *cob* genes also showed that *T. solium* in Japan were positioned in a separate branch to the Asian and African/American haplogroups (Fig. S4). However, the tree topologies differed from those based on 12 CDSs. The Japanese haplotype diverged from the Asian haplogroup in the ribosomal RNA gene phylogenetic tree (Supplementary Fig. S4A). In contrast, the Japanese haplotypes shared a common ancestor with African/American haplogroups in the phylogenetic tree from *cox1* and *cob* genes, though the posterior probability of the node was not high (< 0.85) (Supplementary Fig. S4B).

3.7. *Ag2* gene sequence of the cysticerci is identical to *T. solum* in the Asian genogroup

We sequenced a DNA marker, Ag2, which was previously associated with the segregation between Asian and African/American genogroups (Sato et al., 2011), to further characterize the genetic background of *T. solium* in Japan. The 336-bp sequences of the six cysticerci collected in Japan were completely identical (accession numbers LC844912–LC844917). The multiple sequence alignment showed that the cysticerci had sequences identical to the Asian genogroup, which was detected from Indonesia, India, and China, having three nucleotide substitutions compared to the African genogroup (Fig. 8).

4. Discussion

Taenia solium has not been considered prevalent in Japan for a long time due to the high standards of sanitation and hygiene (Singh et al., 2002; Ito et al., 2003a; Donadeu et al., 2022). However, the present study revealed that the cysticerci collected from wild boars in 2014 and 2023 in Nagano, Japan, were *T. solium*. Given that the life span of adult *T. solium* in definitive hosts is considered to be under 5 years (Garcia et al., 2014), the detections of cysticerci in Nagano Prefecture, with a 9-year interval, suggest the maintenance of the life cycle of *T. solium* in Japan.

Only humans are known to harbor the adult *T. solium* in nature (Allan et al., 2002). Experimental infections have demonstrated that gravid proglottids containing infective eggs were not recovered when cysticerci were inoculated into several mammalian species, including rhesus monkeys, pigs, dogs, cats, white rabbits, white rats, golden hamsters and guinea pigs (Flisser et al., 2010). Thus, one can speculate that humans serve as the definitive hosts in Japan, supporting the full life cycle of *T. solium*. In certain areas of Nagano Prefecture, between 5 to 15 percent of the population have inadequate sewage disposal systems, such as sewers and septic tanks (Nagano Prefecture, 2023). The access of wild boars to human feces in such areas might contribute to the maintenance of the life cycle. Although the consumption of undercooked or raw boar meat is not common in Japan, the emergence of domestic infections with *T. asiatica* in Tokyo, Japan (Yamasaki et al., 2021), may support the possible maintenance of the *T. solium*

life cycle by humans in Japan. On the other hand, previous studies revealed that chinchillas (*Chinchilla laniger*) can harbor adult *T. solium* worms with mature eggs under severe immunosuppressed conditions (Maravilla et al., 1998, 2011). The establishment of infection in this experimental animal might indicate that it is premature to conclude that humans are the sole definitive host in nature. Wild animals, particularly carnivores—as is the case with other Taeniidae cestodes— could serve as the definitive hosts in Japan. The largest carnivore in Japan, *Ursus thibetanus* (the Asian black bear), has been reported to eat wild boars (Oi et al., 2012; Mori et al., 2018; Anezaki, 2021). Unfortunately, the endoparasite fauna of wildlife, particularly in large carnivores, remains far from being fully understood (Hwang et al., 2021; Moriyoshi et al., 2025). Accumulating data on endoparasites in wildlife would be crucial for elucidating the potential and unrecognized transmission of *T. solium*, a parasite that was not expected to exist in Japan today.

The most striking finding of this study is that *T. solium* detected in Nagano Prefecture has a unique genetic background, distinct from previously reported haplogroups, as shown in the haplotype network analysis (Fig. 5). The pairwise divergences between the haplotype from Japan (Ts_JPN) and other haplogroups were higher than those observed among other haplogroups (Fig. 6), which highlights its genetic uniqueness. Bayesian inferences further indicated that the Japanese haplotype forms a separate branch and likely diverged first from the other haplogroups (Fig. 7). These findings demonstrate that the Japanese haplotype constitutes

a novel haplogroup of *T. solium*. Furthermore, the haplotypes from Bhutan also formed another distinct clade with high pairwise divergences (Figs. 6 and 7), consistent with the previous study (Phuentshok et al., 2021). Collectively, our data show that the *T. solium* population is divided into at least four genetically differentiated groups, highlighting that the genetic diversity of this parasite is still far from being fully understood.

The life cycle of *Taenia* is maintained by predator-prey relationships between definitive hosts (Carnivora) and intermediate hosts. This fact suggests that the ancestral tapeworms of *T. solium*, *T. saginata*, and *T. asiatica*, which currently use humans as the sole definitive host (known as human-*Taenia*), originally had other definitive hosts and later underwent host-switching to humans along with a shift in human diet, i.e., from herbivory to omnivory/carnivory (Baer, 1940; Hoberg et al., 2001). However, the evolutionary history of *T. solium*, including its origin and the timing of host-switch, remains largely unknown. Morphology-based analysis has shown that *T. solium* is closely related to *Taenia* species in Africa, suggesting that the host-switch to hominids occurred in sub-Saharan Africa (Hoberg et al., 2001). Nakao et al., who first identified the Asian and African/American haplogroups of *T. solium*, indicated that the diversification into these two haplogroups occurred well before the domestication of boars around 10,000 years ago (Nakao et al., 2002). The authors hypothesized that the prototype of *T. solium* came out of Africa along with *Homo erectus* or early *H. sapiens* migration, and that subsequent natural selection during boar domestication (establishment of

the ‘human–pig’ cycle) in Asia, Asia Minor, and Europe would have contributed to the genetic diversity of *T. solium* (Nakao et al., 2002). On the other hand, phylogenetic analysis using mitochondrial genome sequences by Terefe et al. showed that *T. solium* is distantly related to *Taenia* species in Africa, suggesting that *T. solium* originated in the Palearctic region rather than in Africa (Terefe et al., 2014). The novel haplogroups identified in the Asian region, particularly the Japanese haplotype which diverged possibly in the early Pleistocene, may support the case for revisiting the “Out of Africa” hypothesis for *T. solium*. The host-switching from previous definitive hosts (unknown carnivores or early humans) to modern humans might have occurred in various parts of the Palearctic.

Previous studies have estimated the divergence date between the two major groups, Asian and African/American haplogroups, using evolutionary rates derived from other *Taenia* species and mammals (Nakao et al., 2002; Michelet et al., 2010; Michelet and Dauga, 2012). In this study, the phylogenetic analysis using mitochondrial 12 CDSs showed that the haplotype in Japan was located at the basal position of the clade consisting of the *T. solium* haplogroups (Fig. 7). In contrast, the Japanese haplotype clustered within the clade containing the African/American haplogroup based on the phylogenetic analysis using *cox1* and *cob* sequences (Supplementary Fig. S4). This incongruence made it challenging to accurately estimate the divergence time of *T. solium* in Japan. Such instability in the tree topologies could be partly attributed to the differences in sequence lengths used for analysis. Given the higher pairwise

divergence between the haplotype in Japan and other haplogroups (Fig. 6), it is plausible that the analysis based on 12 CDSs more accurately represents the phylogenetic relationships. Genetic analyses using longer mitogenomic sequences are known to provide higher phylogenetic resolution compared to shorter mitochondrial DNA datasets and potentially more suitable for evaluating genetic relationships (Kinkar et al., 2017, 2018; Lallemand et al., 2024). Nevertheless, previous studies have largely relied on one or two CDSs to assess the phylogenetic relationships of *T. solium* haplotypes (Nakao et al., 2002; Michelet et al., 2010; Yanagida et al., 2021), leaving a significant gap in mitogenomic data unaddressed. Further efforts are required to collect comprehensive mitogenomic data for *T. solium* to refine its phylogenetic relationships and to trace its divergence history. The two sets of specific primers designed in this study would facilitate the expansion of the genetic database.

Our data cannot provide definitive evidence to determine when and how the parasites were introduced to the mainland of Japan. However, we can propose several plausible scenarios based on previous surveys and historical records. The first hypothesis is that *T. solium* was introduced relatively recently by human activities. It is well known that *T. solium* can be transmitted by the movements of infected humans. For example, migrant workers carrying adult tapeworms are suspected to have caused *T. solium* cysticercosis in domestic pigs in Colorado, USA (Theis et al., 1996). In Japan, migrant workers are considered to have been infected with *T. asiatica*, which has a similar life cycle involving pigs and humans, thereby serving as sources

of infection for domestic pigs (Yamasaki et al., 2021). It is conceivable that travelers or immigrants from other endemic countries in recent years may have brought *T. solium* into Japan and caused cysticercosis in wild boars. The second scenario is that the introduction of *T. solium* occurred earlier, in the 20th century along with human migration. British soldiers repatriated from India likely brought *T. solium* and caused a cysticercus emergence in England in the 1920s to 1940s, although the disease was almost non-existent in the country (Dixon and Smithers, 1934; Dixon and Hargreaves, 1944; Del Brutto and García, 2015). Similarly, soldiers or infected pigs from neighboring regions apparently introduced *T. solium* into West New Guinea in the 1960s to 1970s, where cysticercosis was previously absent until 1972, leading to a severe endemic situation (Wandra et al., 2000; Del Brutto and García, 2015). In Japan, many individuals repatriated from other endemic countries, including India, Myanmar, Indonesia, and the Philippines during and after World War II. Masuda et al. mentioned that some people were infected with adult tapeworms abroad before the end of World War II and returned to Japan (Masuda and Huse, 1965). Incidentally, Okinawa, the southernmost prefecture of Japan, was a highly endemic area of cysticercosis until the 1940s to 1950s due to the use of pig toilets (Ito et al., 2019). Migrations from endemic areas in the mid-20th century could have triggered the introduction of *T. solium*. Unfortunately, none of these hypotheses is strongly supported by evidence. Genetic investigations, particularly in areas with scarce genetic data, may provide insights into the hypotheses described above. Such attempts to trace the origins and

transmission routes of parasites using genetic data have been successful for other taeniid tapeworms, *Echinococcus granulosus* and *E. multilocularis* (Kinkar et al., 2018; Hayashi et al., 2023).

Cases of cysticercosis in the patients without overseas travel history in Japan might be related to the tapeworms reported in this study. Unfortunately, there is a significant lack of genetic information on human cysticercosis in Japan due to insufficient efforts to genotype parasites in clinical cases (Matsunaga et al., 2002; Masuda et al., 2009; Kwasi et al., 2018), making it challenging to analyze genetic relationships. Further investigations are needed to collect genetic information on cysticerci, particularly those from patients who have never visited endemic countries.

The identification of novel genetic groups of *T. solium* poses potential concerns regarding cysticercosis control. It has been suggested that different genetic variants of *T. solium* exhibit different pathogenicities and clinical manifestations (Nakao et al., 2002; Vega et al., 2003; Ito and Budke, 2021). Variations in clinical manifestations have also been known from a number of case reports of human cysticercosis prior to the advent of molecular studies: neurocysticercosis identified in patients from Asian countries were often accompanied with subcutaneous cysticercosis (Dixon and Smithers, 1934; Dixon and Hargreaves, 1944; Feng et al., 1979), whereas cases without subcutaneous cysticercosis were common in the Americas and certain regions of Africa (Nieto, 1956; Cruz et al., 1994; Ito and Budke, 2021). Furthermore,

the antigenicity of *T. solium* metacestode proteins which might influence the results of serodiagnosis differed between Asian and African/American genogroups (Ito et al., 2003b; Sato et al., 2006). Analyzing the phenotypes and antigenic profiles of the newly identified genotypes, such as those from Japan and Bhutan, would be essential for the control of cysticercosis.

In conclusion, this study provides new insights into the transmission dynamics and phylogenetics of *T. solium*. Three independent cases of cysticercosis in wild boars in Japan, with a 9-year interval (one in 2014 and two in 2023), suggest that the *T. solium* lifecycle can be maintained in Japan. This finding highlights an overlooked risk of local infection of *T. solium* even in developed countries. Furthermore, the genetic data revealed that *T. solium* in Japan had a unique genetic background and diverged first from other known haplogroups, likely in the early Pleistocene. These results support the case for revisiting the “Out of Africa” hypothesis for *T. solium*, which posits a sub-Saharan African origin of the tapeworm with subsequent dispersal along with human migration. Further comprehensive genomic analyses will be required to unravel the enigmatic evolutionary history of *T. solium*.

CRediT authorship contribution statement

Naoki Hayashi: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Software, Visualization, Writing – original draft, Writing – review & editing. **Ryo Kuwamoto**: Investigation, Resources, Writing – review & editing. **Mitsuhiro Okada**: Investigation, Resources, Writing – review & editing. **Kenta Suzuki**: Investigation, Resources, Writing – review & editing. **Takaya Hoketsu**: Methodology, Writing – review & editing. **Samuel Kelava**: Methodology, Writing – review & editing. **Yuma Ohari**: Methodology, Writing – review & editing. **Munehiro Okamoto**: Conceptualization, Funding acquisition, Writing – review & editing. **Kinpei Yagi**: Conceptualization, Writing – review & editing. **Nariaki Nonaka**: Conceptualization, Funding acquisition, Project administration, Writing – review & editing. **Ryo Nakao**: Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Visualization, Writing – review & editing.

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Figure legends

Fig. 1. Macroscopic and microscopic images of cysticerci found in wild boars in Nagano Prefecture, Japan. (A－C) cysticerci in the skeletal muscles found in November 2014. (D, E) Cysticerci detected in a wild boar muscle in January 2023 and (F) a microscopic image of the parasite. (G, H) Cysticerci detected in a heart in June 2023 and (I) a microscopic image of the parasite.

Fig. 2. Representative images of rostellar hooks. (A) Rostellum armed with 14 large hooks and 14 small hooks. Scale bar = 100 μm. (B) Large hook. Scale bar = 50 μm. (C) Small hook. Scale bar = 50 μm.

Fig. 3. A phylogenetic tree reconstructed by Bayesian inference using the exon sequences of RNA polymerase II second largest subunit (*rpb2*), phosphoenolpyruvate carboxykinase (*pepck*), and DNA polymerase delta (*pold*) of taeniid cestodes.

Nodes with black dots have posterior probabilities > 0.85.

Fig. 4. Comparison of the mitogenome sequences between *Taenia solium* from Japan, China, and Peru.

The outermost circle represents the mitogenome of *T. solium* from Japan, with 12 protein-coding genes (red), 2 ribosomal RNA genes (blue), and 22 transfer RNA genes (green). Purple, light blue, and yellow lines on the circles respectively depict mutations in non-coding

regions, synonymous mutations, and non-synonymous mutations between the sequences from Japan and Peru (African-American genotype, inner blue backbone) or between the sequences from Japan and China (Asian genotype, outer red backbone). The segments shaded in light orange represent *cox1* and *cob* genes, which were used for the haplotype network analysis.

Fig. 5. Mitochondrial haplotype network of *Taenia solium* using *cox1* and *cob* gene sequences.

The color and size of each circle represent the location where the haplotype was detected and the number of haplotypes, respectively. The haplotype detected in this study (Ts_JPN) is shown in red. The analysis included previously reported haplotypes from 15 different countries in Asia, Africa, and South America.

Fig. 6. Distribution of pairwise divergence between *Taenia solium* haplogroups. Each dot represents the pairwise divergence between two haplotypes from different *T. solium* haplogroups (see Table S2). The analysis used the concatenated sequences of mitochondrial *cox1* (1,620 bp) and *cob* (1,068 bp) genes.

Fig. 7. Mitochondrial genome-based phylogeny of *Taenia* spp. depicted by Bayesian inference. The nucleotide sequences (A) and amino acid sequences (B) of 12 protein-coding sequences from *Taenia* spp. were used for analysis. *Taenia regis* and *T. hydatigena* was used

as an outgroup to root the trees. Dots on the nodes represent posterior probabilities > 0.85.

Fig. 8. The exon-intron structure of nuclear *Ag2* gene of *Taenia solium* and the nucleotide alignment of the intron.

The number above the exon-intron structure indicates the nucleotide position of the PCR products of partial sequence of *Ag2*. Nucleotides highlighted in black indicate the substitution positions.

Supplementary Fig. S1. A map showing regions where *Taenia solium* were detected from wild boars. Nagano prefecture is highlighted in gray. The upper left inset shows regions where wild boars infected with cysticerci were found. The lower left region, filled in red, shows Okinawa Prefecture, where cysticercosis was highly endemic until the 1940s and 1950s.

Supplementary Fig. S2. PCR strategy to amplify the mitogenome sequence of *Taenia solium*. Arrowheads indicate the positions of primer annealing sites. Two overlapping fragments (orange and blue) were obtained.

Supplementary Fig. S3. Multiplex PCR using DNA from taeniid cestodes. The PCR products were analyzed using a 2.0% agarose gel. Lane M, DNA ladder; lane NC, negative control (water); lane 1, *Echinococcus multilocularis* Nemuro strain; lane 2, *Hydatigera*

taeniaeformis; lanes 3–5, cysticerci obtained in January 2023; lanes 6–8, cysticerci obtained in June 2023.

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Supplementary Fig. S4. Bayesian phylogenetic trees from mitochondrial gene sequences of *Taenia* spp.

Phylogenetic trees were reconstructed from (A) the *rrnS* and *rrnL* genes and (B) the *cox1* and *cob* genes. The trees were rooted with (A) *Taenia regis and T. hydatigena* and (B) *T. arctos* as

750 outgroups. Dots on the nodes represent posterior probabilities > 0.8

Table 1. Mitochondrial haplotypes of *Taenia solium* used for the genetic analysis

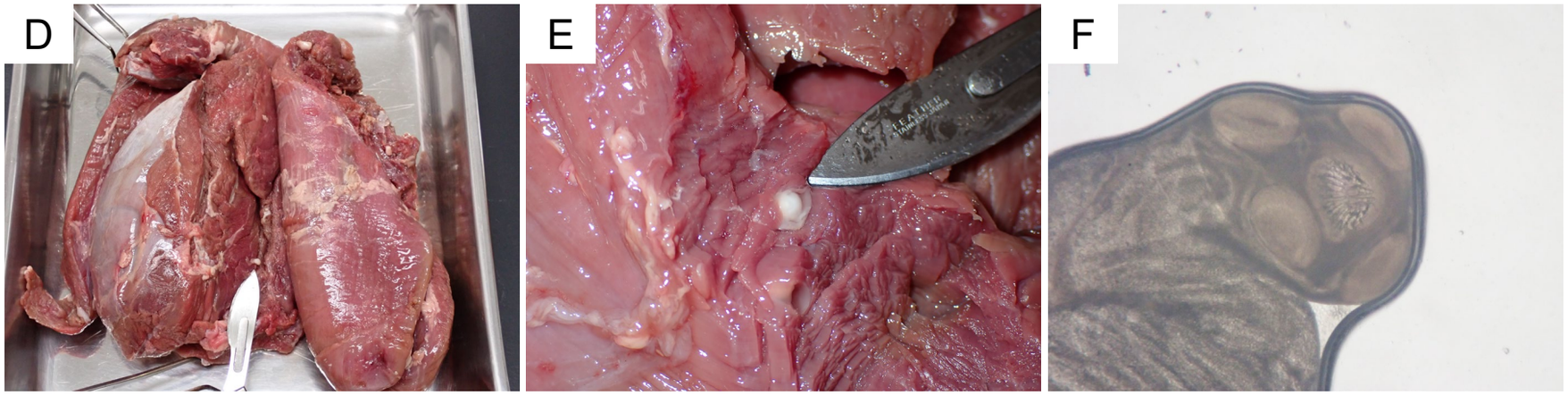
Haplotype ID	Country (No. of detected samples)	*cox1*	*cob*	Reference
Ts_JPN	Nagano, Japan (6)	LC847175–LC847180	LC847175–LC847180	This study
BT1[a]	Bhutan (1)	MT366763[a]	MT371084[a]	Phuentshok et al., 2021
BT2[a]	Bhutan (1)	MT366764[a]	MT371085[a]	Phuentshok et al., 2021
CHN1	China (1)	AB066485	AB066570	Nakao et al., 2002
CHN2	China (1)	AB066486	AB066571	Nakao et al., 2002
CHN_complete	China (1)	AB086256	AB086256	Nakao et al., 2003
IND	India (1)	AB066489	AB066574	Nakao et al., 2002
NPL1	Nepal (1)	AB491985	AB781746	Yanagida et al., 2010
NPL2 (MDG1)	Nepal (2), Madagascar (68)	AB491986	AB781362	Yanagida et al., 2010
IRI	Indonesia (1)	AB066488	AB066573	Nakao et al., 2002
ID_BA1	Indonesia (5)	LC378448	LC378444	Yanagida et al., 2021
ID_BA2	Indonesia (5)	LC378450	LC378444	Yanagida et al., 2021
ID_BA3	Indonesia (6)	LC378451	LC378444	Yanagida et al., 2021
ID_BA4	Indonesia (2)	LC378453	LC378444	Yanagida et al., 2021
ID_PA	Indonesia (10)	LC378454	LC378445	Yanagida et al., 2021
THA	Thailand (1)	AB066487	AB066572	Nakao et al., 2002
MDG2	Madagascar (1)	AB781356	AB781362	Yanagida et al., 2014
MDG3	Madagascar (3)	AB781355	AB781363	Yanagida et al., 2014
MDG4	Madagascar (1)	AB781357	AB781362	Yanagida et al., 2014
MDG5	Madagascar (9)	AB781358	AB781362	Yanagida et al., 2014
MDG6	Madagascar (2)	AB781359	AB781362	Yanagida et al., 2014
MDG7	Madagascar (25)	AB781360	AB781364	Yanagida et al., 2014
MDG8	Madagascar (2)	AB781361	AB781364	Yanagida et al., 2014
TZA	Tanzania (1)	AB066493	AB066578	Nakao et al., 2002
MOZ	Mozambique (1)	AB066493	AB066578	Nakao et al., 2002
ZAM	Zambia (1)	MW718881	MW718881	Sadlowski et al., 2021
CMR	Cameroon (1)	AB066490	AB066579	Nakao et al., 2002
CMR_North	Cameroon (9)	FN995665	FN995667	Michelet et al., 2012
CMR_West	Cameroon (4)	FN995666	FN995668	Michelet et al., 2012
MEX1	Mexico (25)	FN995657	FN995661	Michelet et al., 2012
MEX2	Mexico (8)	FN995658	FN995662	Michelet et al., 2012
MEX3	Mexico (2)	FN995659	FN995663	Michelet et al., 2012
Yucatan	Mexico (3)	FN995660	FN995664	Michelet et al., 2012
MEX	Mexico (1)	AB066490	AB066575	Nakao et al., 2002
ECU	Ecuador (1)	AB066491	AB066576	Nakao et al., 2002
BOL	Bolivia (1)	AB066491	AB066576	Nakao et al., 2002
BRA	Brazil (1)	AB066492	AB066577	Nakao et al., 2002
PER	Peru (1)	OM967033	OM967033	Jiménez et al., 2023

[a] In the study by Phuentshok et al. (2021), two cysticerci from a Bengal tiger were pooled before the TA cloning. The sequences of *cox1* and *cob* are tentatively concatenated for the phylogenetic analysis.

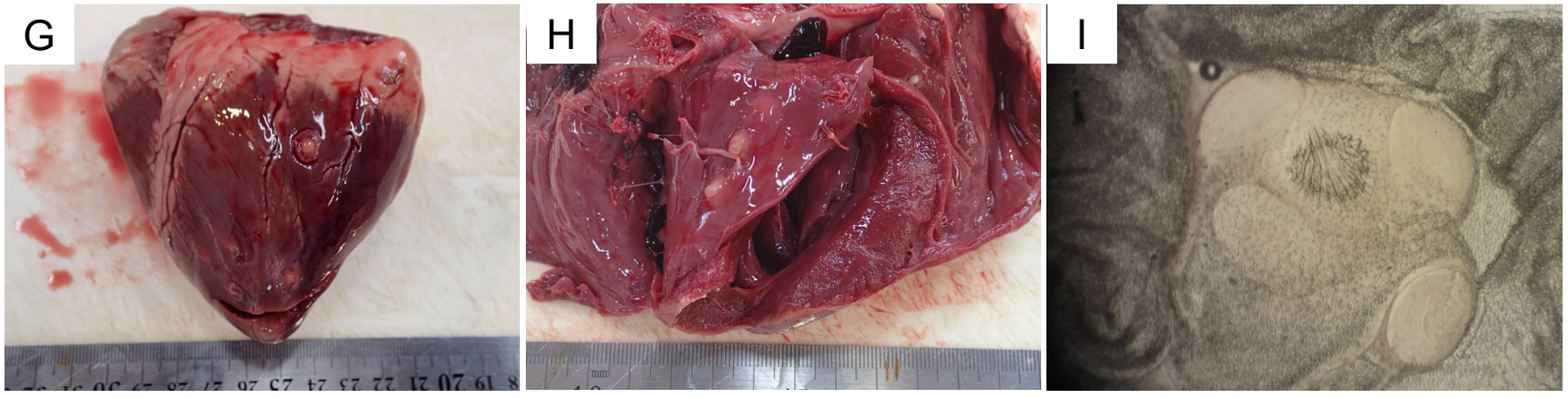
Case 1 (November, 2014)

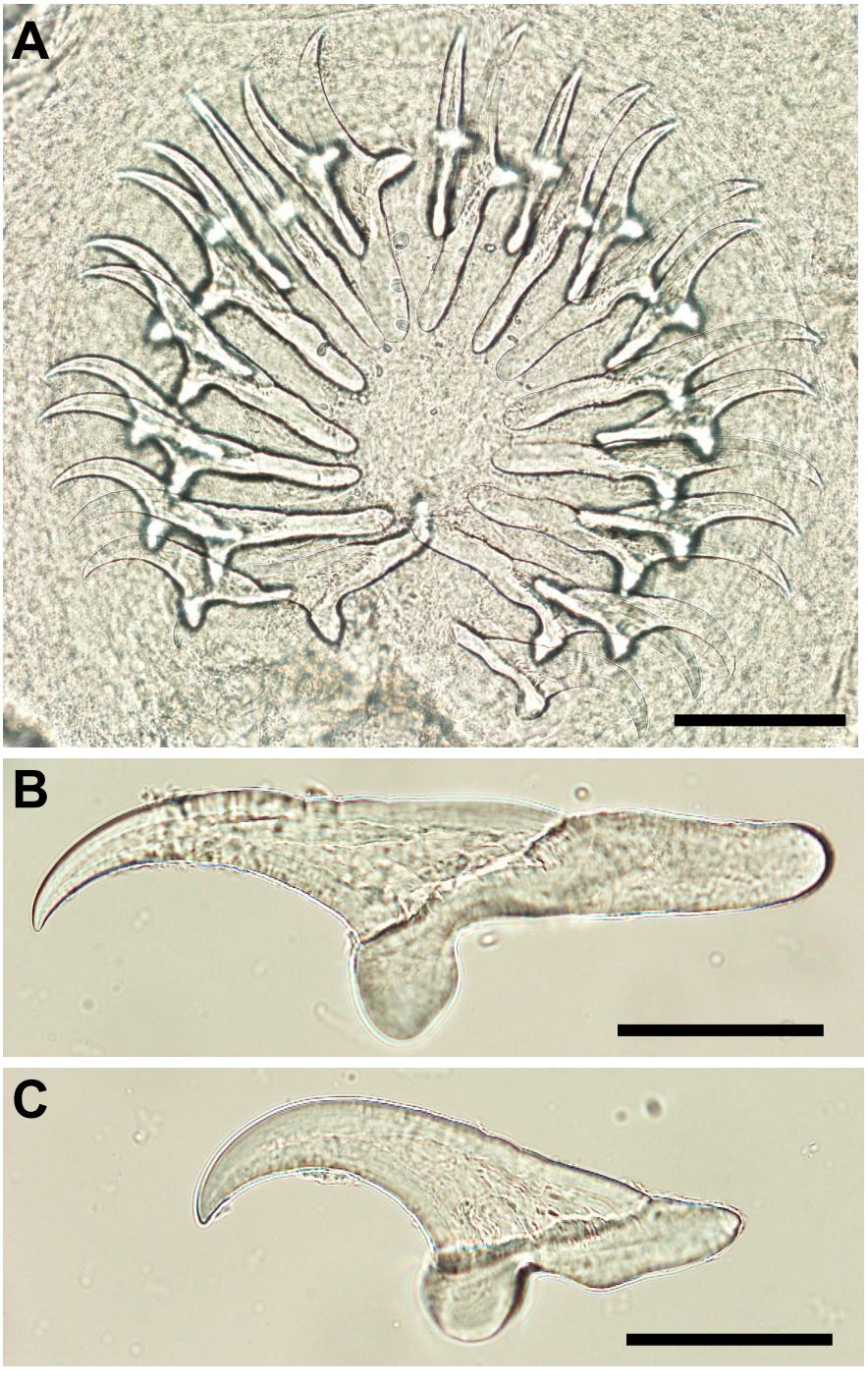


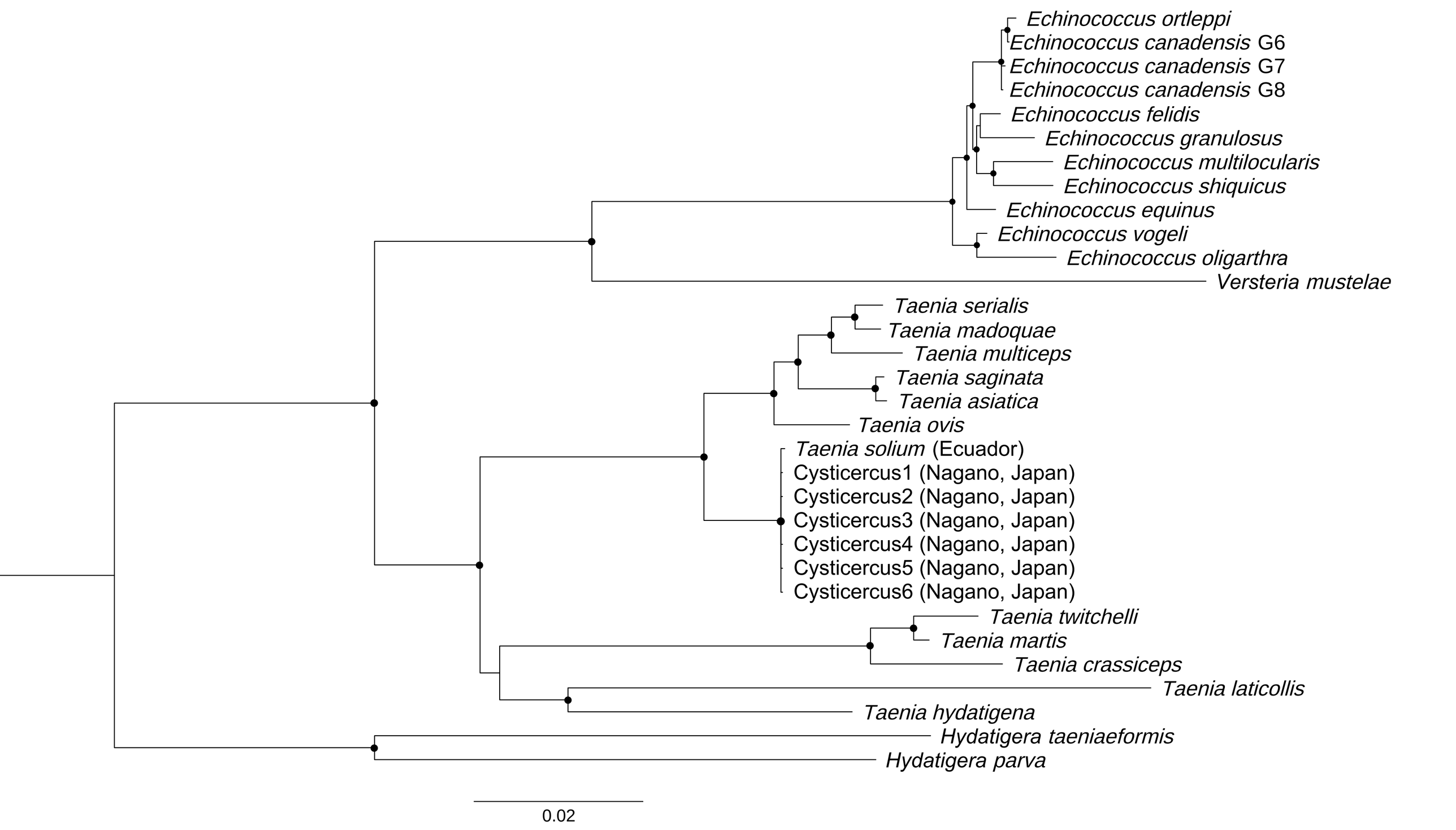
Case 2 (January, 2023)

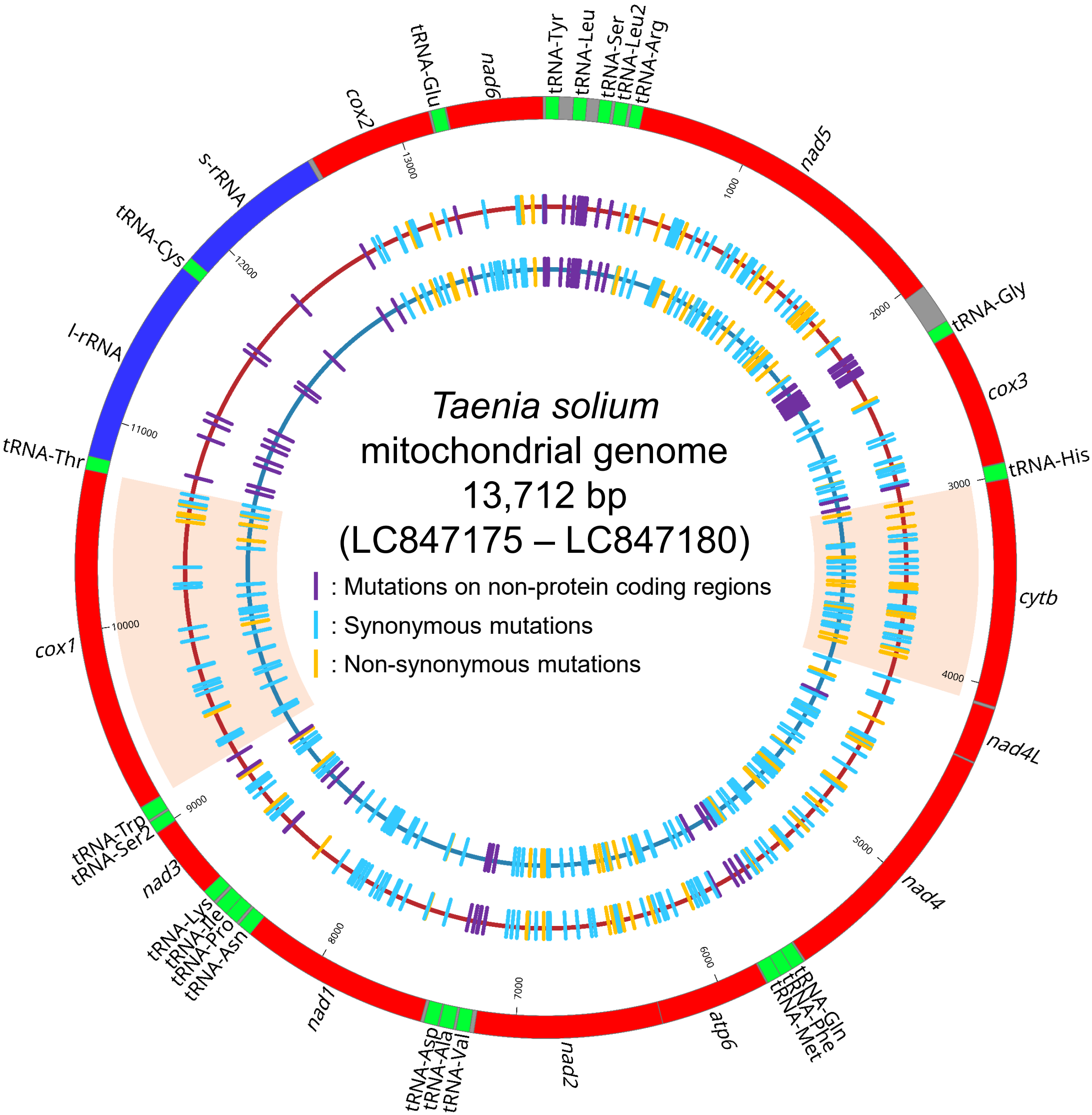


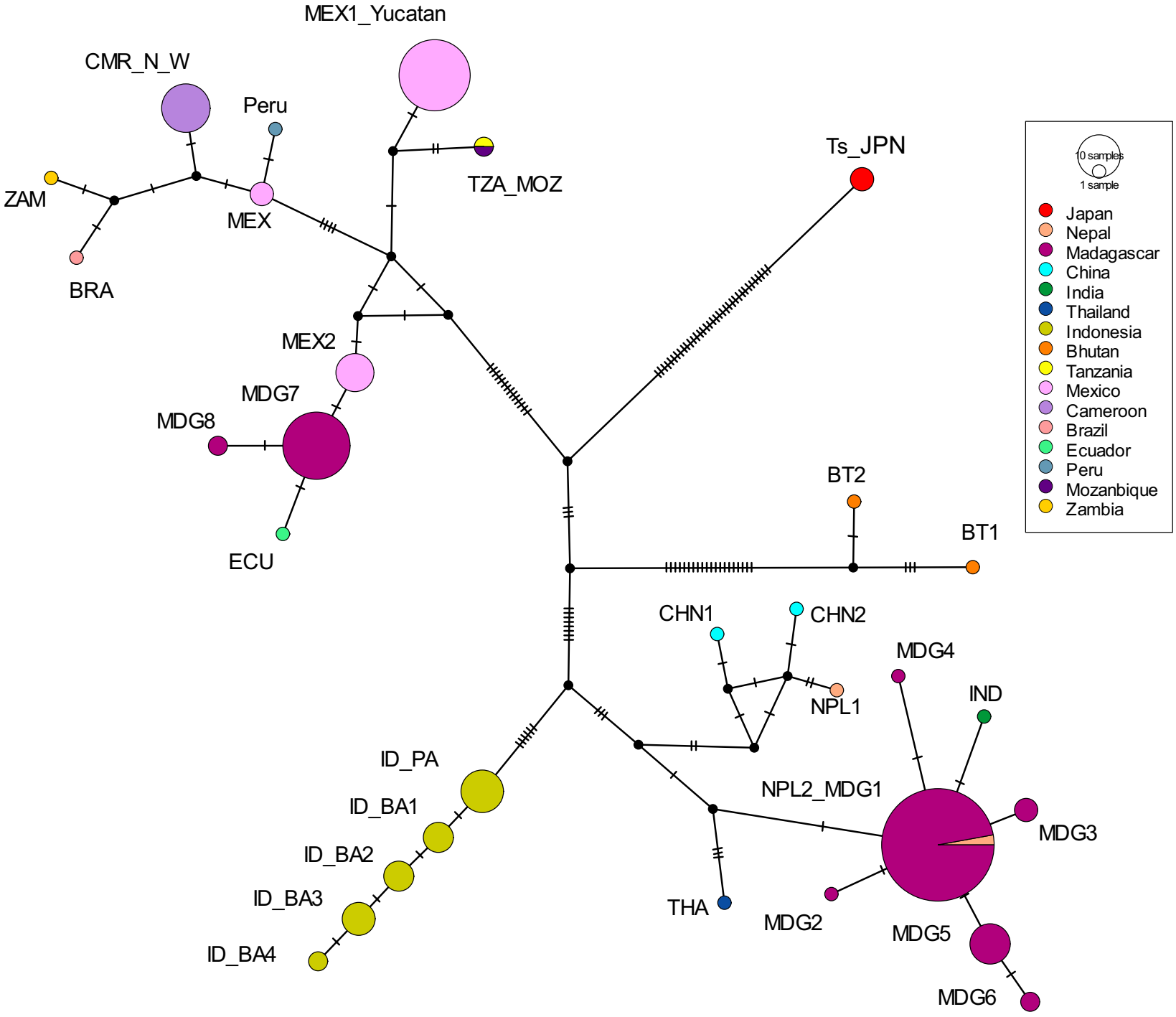
Case 3 (June, 2023)



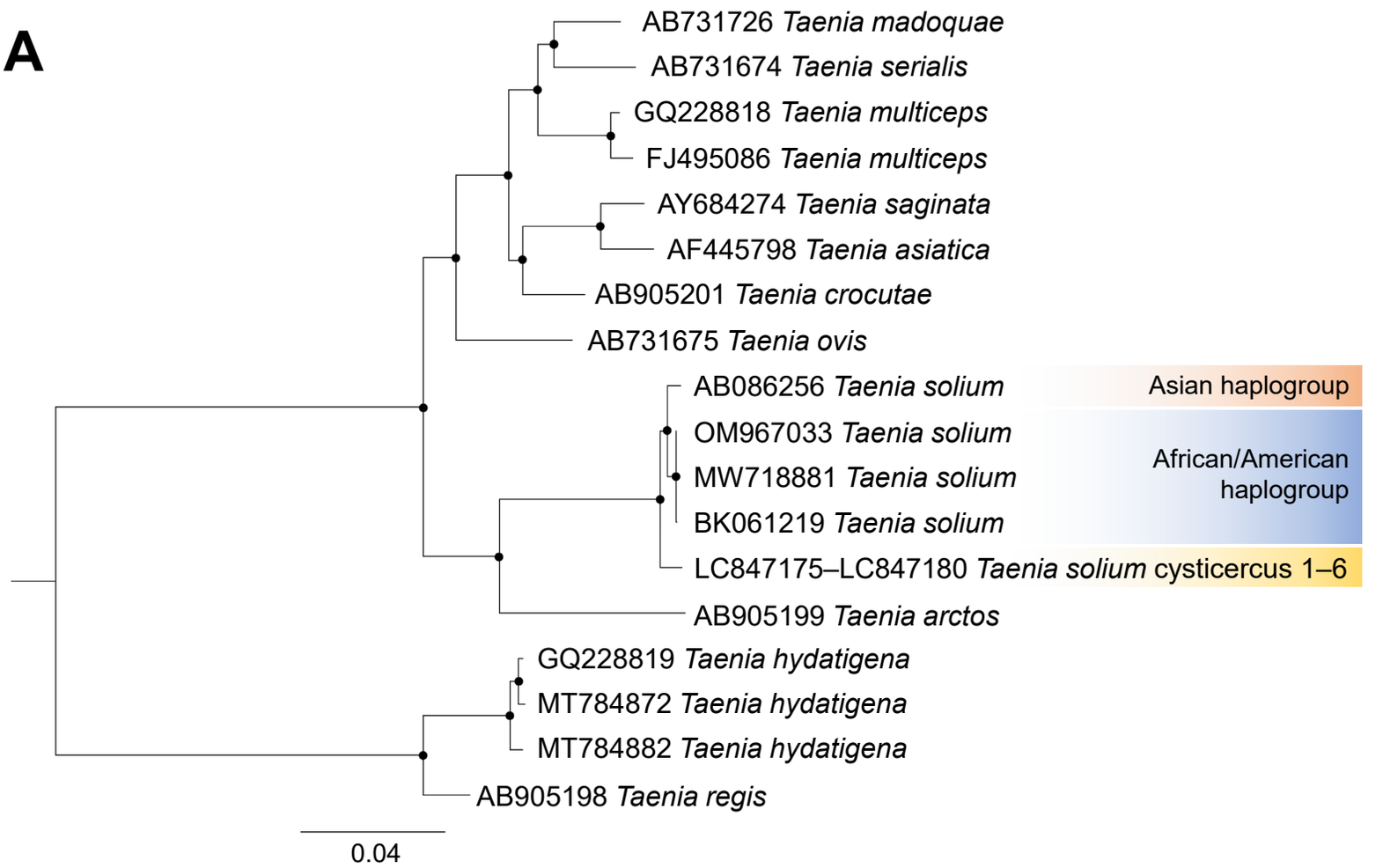

A
B
C

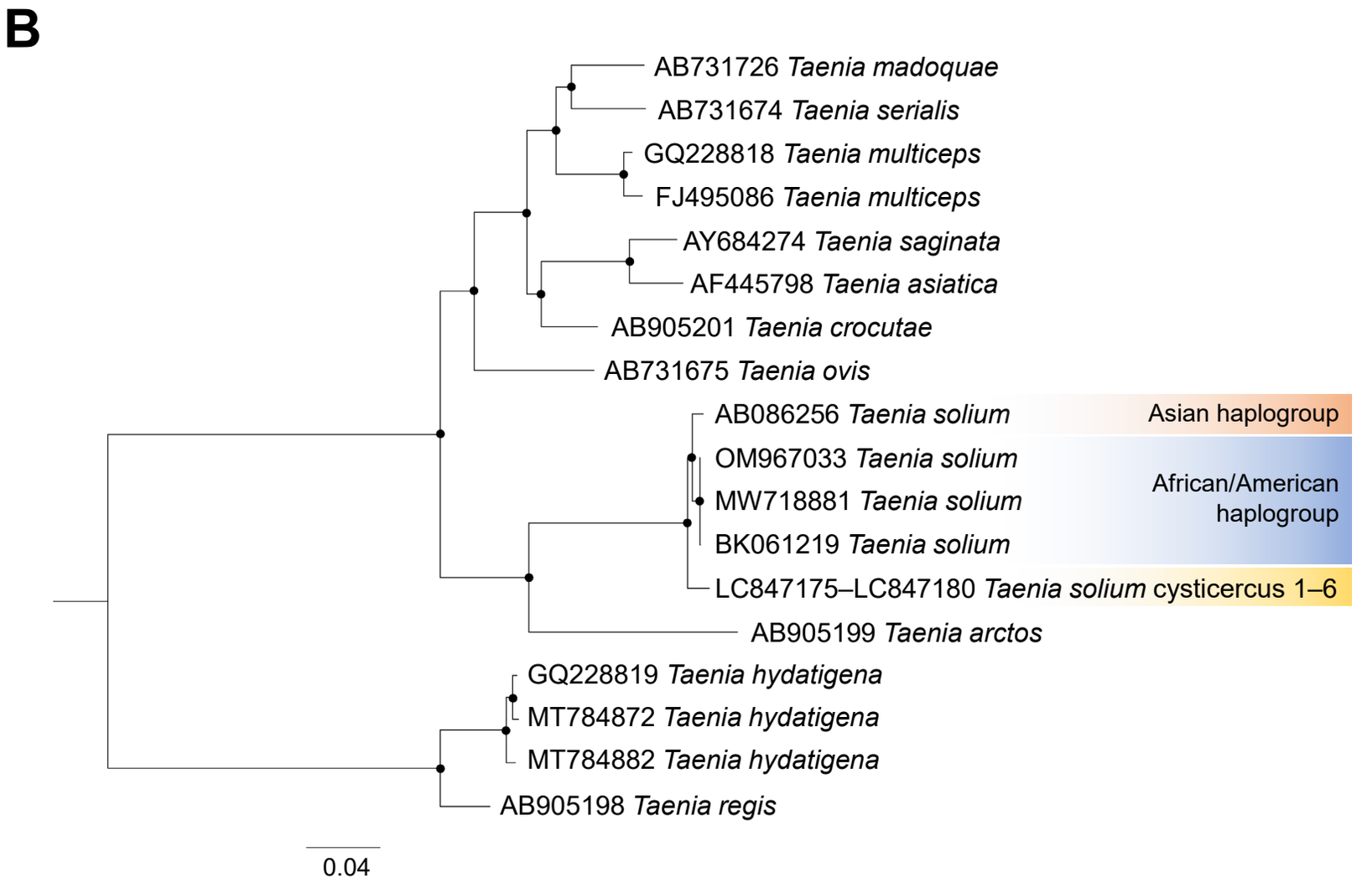

Echinococcus ortleppi
Echinococcus canadensis G6
Echinococcus canadensis G7
Echinococcus canadensis G8
Echinococcus felidis
Echinococcus granulosus
Echinococcus multilocularis
Echinococcus shiquicus
Echinococcus equinus
Echinococcus vogeli
Echinococcus oligarthra
Versteria mustelae
Taenia serialis
Taenia madoquae
Taenia multiceps
Taenia saginata
Taenia asiatica
Taenia ovis
Taenia solium (Ecuador)
Cysticercus1 (Nagano, Japan)
Cysticercus2 (Nagano, Japan)
Cysticercus3 (Nagano, Japan)
Cysticercus4 (Nagano, Japan)
Cysticercus5 (Nagano, Japan)
Cysticercus6 (Nagano, Japan)
Taenia twitchelli
Taenia martis
Taenia crassiceps
Taenia laticollis
Taenia hydatigena
Hydatigera taeniaeformis
Hydatigera parva
0.02

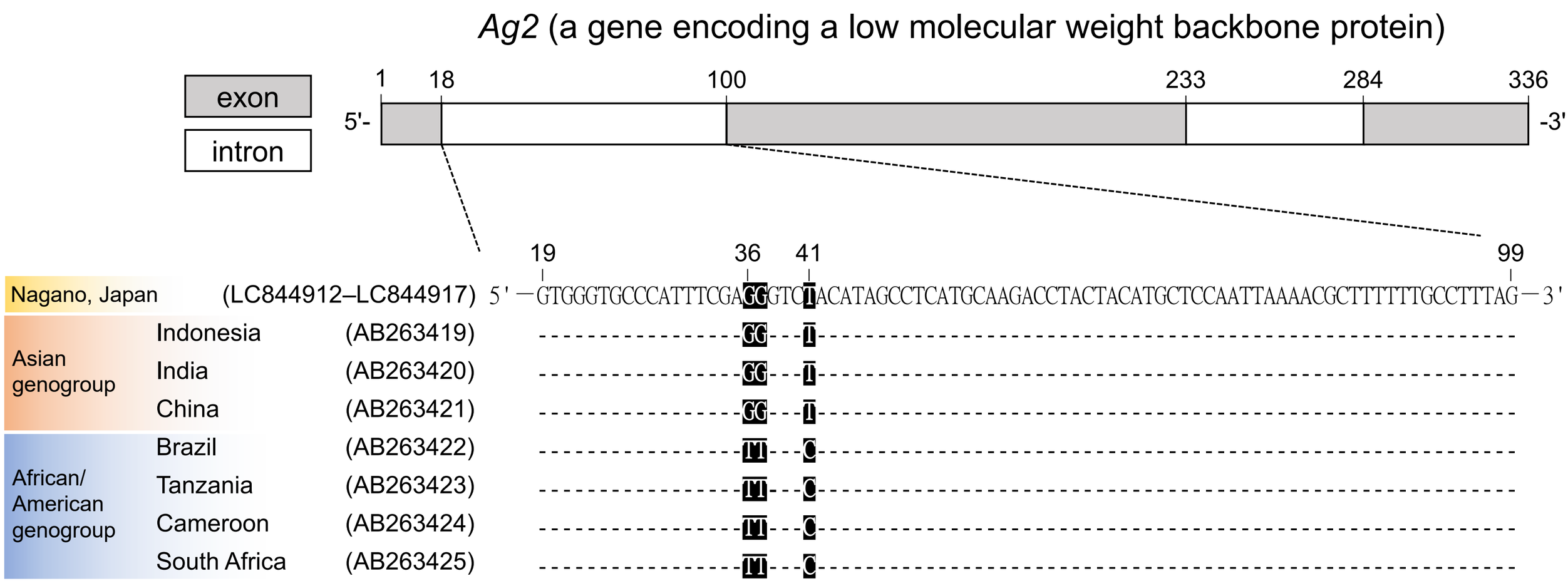

Taenia solium
mitochondrial genome
13,712 bp
(LC847175 – LC847180)
: Mutations on non-protein coding regions
: Synonymous mutations
: Non-synonymous mutations
cox2
tRNA-Glu
nad6
tRNA-Tyr
tRNA-Leu
tRNA-Ser
tRNA-Leu2
tRNA-Arg
nad5
tRNA-Gly
cox3
tRNA-His
cytb
nad4L
nad4
tRNA-Gln
tRNA-Phe
tRNA-Met
atp6
nad2
tRNA-Asp
tRNA-Ala
tRNA-Val
nad1
tRNA-Lys
tRNA-Ile
tRNA-Pro
tRNA-Asn
nad3
tRNA-Trp
tRNA-Ser2
cox1
tRNA-Thr
l-rRNA
tRNA-Cys
s-rRNA
1000
2000
3000
4000
5000
6000
7000
8000
9000
10000
11000
12000
13000


MEX1_Yucatan
CMR_N_W
Peru
ZAM
MEX
TZA_MOZ
Ts_JPN
BRA
MEX2
MDG7
MDG8
ECU
BT2
BT1
CHN1
CHN2
MDG4
IND
NPL1
ID_PA
ID_BA1
ID_BA2
ID_BA3
ID_BA4
NPL2_MDG1
MDG3
THA
MDG2
MDG5
MDG6
10 samples
1 sample
Japan
Nepal
Madagascar
China
India
Thailand
Indonesia
Bhutan
Tanzania
Mexico
Cameroon
Brazil
Ecuador
Peru
Mozanbique
Zambia


2.5
2.0
1.5
1.0
Pairwise divergence (%)
Ts_JPN – Asian haplogroup
Ts_JPN – African/American haplogroup
Asian haplogroup – African/American haplogroup
Haplotypes in Bhutan – Asian haplogroup
Haplotypes in Bhutan – African/American haplogroup


A
AB731726 Taenia madoquae
AB731674 Taenia serialis
GQ228818 Taenia multiceps
FJ495086 Taenia multiceps
AY684274 Taenia saginata
AF445798 Taenia asiatica
AB905201 Taenia crocutae
AB731675 Taenia ovis
AB086256 Taenia solium
OM967033 Taenia solium
MW718881 Taenia solium
BK061219 Taenia solium
LC847175–LC847180 Taenia solium cysticercus 1–6
AB905199 Taenia arctos
GQ228819 Taenia hydatigena
MT784872 Taenia hydatigena
MT784882 Taenia hydatigena
AB905198 Taenia regis
0.04
Asian haplogroup
African/American haplogroup


B
AB731726 Taenia madoquae
AB731674 Taenia serialis
GQ228818 Taenia multiceps
FJ495086 Taenia multiceps
AY684274 Taenia saginata
AF445798 Taenia asiatica
AB905201 Taenia crocutae
AB731675 Taenia ovis
AB086256 Taenia solium
OM967033 Taenia solium
MW718881 Taenia solium
BK061219 Taenia solium
LC847175–LC847180 Taenia solium cysticercus 1–6
AB905199 Taenia arctos
GQ228819 Taenia hydatigena
MT784872 Taenia hydatigena
MT784882 Taenia hydatigena
AB905198 Taenia regis
0.04
Asian haplogroup
African/American haplogroup


Ag2 (a gene encoding a low molecular weight backbone protein)
exon
intron
1
18
100
233
284
336
5'-
-3'
19
36
41
99
Nagano, Japan (LC844912–LC844917) 5'—GTGGGTGCCCATTTCGAGGGTCTACATAGCCTCATGCAAGACCTACTACATGCTCCAATTAAAACGCTTTTTTGCCTTTAG—3'
Asian genogroup
Indonesia (AB263419)
India (AB263420)
China (AB263421)
African/ American genogroup
Brazil (AB263422)
Tanzania (AB263423)
Cameroon (AB263424)
South Africa (AB263425)