Asian countries. From 1994 to 2010, there were 10 Japanese female patients whose travel histories could be ascertained, with 7 of these patients having traveled to endemic Asian countries such as Thailand, India, Nepal and Vietnam. Also, 4 NCC have been reported in foreign residents from India, Nepal, Cambodia and the Philippines. Based on mtDNA analysis of proglottids and on travel histories, it was concluded that infections in 2 of the Japanese cases were acquired in Nepal [9] and India (Case 3) during business trips. The former case was suspected to be NCC by neurologists but serology carried out in the hospital was negative. However, serology using specific diagnostic antigens carried out in Asahikawa was positive before surgery but negative one year after surgery [21].

In 2010, more than 20 taeniasis cases caused by *Taenia asiatica*, another human-infecting *Taenia* species which requires pigs as the intermediate host, were reported from Tokyo and surrounding areas [13]. *T. asiatica* is endemic in several Asian countries, such as China, Taiwan, Korea, Indonesia, Vietnam, the Philippines and Thailand where local people eat uncooked pig liver [1, 33, 39-41, 43]. In Japan, 2 taeniasis cases in 1968 and 1992 were retrospectively analyzed

and the expelled worms were confirmed as *T. asiatica*, [48, 49] but there was no crucial evidence of autochthonous infection. However, the taeniasis patients in the 2010 report [13] had no travel history to known endemic countries during the last decade, but instead ate raw indigenous pork or beef livers (sliced liver "liver sashimi") in Tokyo and surrounding areas. Therefore, these are considered to be cases of autochthonous taeniasis caused by contaminated domestic animals. A possible source of contamination is tapeworm carriers working on Japanese pig or cattle farms. Recently, foreign agricultural workers from Asian developing countries are common not only in Japan but also in Europe [50, 51] and New Zealand [52]. It is probable that at least some of these individuals are infected with tapeworms and are responsible for contaminating farming areas. This is especially likely since there are no regulations in place to check visitors to Japan for anything other than diarrhea or fever. Under the current conditions, it is likely that an outbreak of *T. solium* infection will also occur in the near future.

In our literature review for the years 1994-2010, there was a significant correlation between the types of treatment received and immunological diagnosis.

In most of the patients who received no immunological testing, mainly serology or showed negative responses on immunodiagnosis, cysts were misdiagnosed as brain tumors and surgically resected, whereas the majority of patients who were serologically confirmed to have cysticercosis received only chemotherapy. To avoid unnecessary operations, reliable serologic examinations should be applied to patients with suspicious neuroimaging results. However, NCC may not be suspected by clinicians working in non-endemic areas due to the lack of knowledge or experience about this disease. Another example of inadequate treatment was found in Case 2. This patient was treated with pyrantel pamoate, a drug used for nematodiasis, despite a diagnosis of cestodiasis. Therefore, education on parasitic diseases should be strengthened for clinicians and for the general public.

Conclusions

Although the incidence of cysticercosis in Japan has seemingly not increased over the past 35 years, diversification of the profile of patients was observed, such as

an increase in the percentage of female patients, wider age distributions and the risk of importing the disease from South and Southeast Asia. This change is likely the result of the globalization of business and increased tourism to remote areas in developing countries. Another important risk is the employment of foreign workers in agriculture. The agricultural population in Japan has been decreasing and therefore, immigrant labour from developing countries is becoming essential. To control this neglected parasitic disease in Japan, recognition of the possible risks is necessary. In addition, it would be desirable to include *T. solium* infection on Japan's list of notifiable diseases. Cooperation among clinicians, researchers and governmental officials is needed to address the prevention of the introduction of *T. solium* into Japan.

Competing interests

The authors have no conflicts of interest concerning the work reported in this paper.

Author's contributions

TY carried out the molecular diagnosis and analysis of the taeniasis/cysticercosis cases and drafted the manuscript and revised it. YS carried out immunological examination on the cases. MN carried out some of the molecular analysis. KN carried out histopathology of the cases. AI participated in designing the study and helped to draft the manuscript and revised it. All authors read and approved the final manuscript. Core MS was prepared based on TY's talk entitled "Taeniasis/cysticercosis of *Taenia solium* in developed countries introduced by people from developing countries: some Japanese cases" at the JSPS-AASP Symposium "Toward the prevention of taeniasis and cysticercosis in Asia: emergent problems from developing to developed countries" on 3 Dec 2010 at the Joint International Tropical Medicine Meeting 2010 (JITMM2010), Bangkok, Thailand. All authors read and approved the final manuscript.

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References

1. Ito A, Nakao M, Wandra T: **Human taeniasis and cysticercosis in Asia.** *Lancet* 2003, **362**:1918-1920.

2. Schantz PM, Wilkins PP, Tsang VCW: **Immigrants, imaging and immunoblots: the emergence of neurocysticercosis as a significant public health problem.** In: *Emerging Infections 2*. Edited by Scheld WW, Craig WA, Hughes JM. Washington DC: ASM Press; 1998:213-242.

3. Flisser A, Craig PS, Ito A: **Cysticercosis and taeniosis: *Taenia solium*, *Taenia saginata* and *Taenia asiatica*.** In: *Oxford Textbook of Zoonoses*. Edited by Palmer SR, Lord Soulsby, Torgerson PR, Brown DWG. Oxford: Oxford

University Press; 2011:625-642.

4. Dorny P, Praet N, Deckers N, Gabriel S: **Emerging food-borne parasites.** *Vet Parasitol* 2009, **163**:196-206.

5. Molyneux D, Hallaj Z, Keusch GT, McManus DP, Ngowi H, Cleaveland S, Ramos-Jimenez P, Gotuzzo E, Kar K, Sanchez A, Garba A, Carabin H, Bassili A, Chaignat CL, Meslin FX, Abushama HM, Willingham AL, Kioy D: **Zoonoses and marginalised infectious diseases of poverty: Where do we stand?** *Parasit Vectors* 2011, **4**:106.

6. Schantz PM, Moore AC, Muñoz JL, Hartman BJ, Schaefer JA, Aron AM, Perasaud D, Sarti E, Wilson M, Flisser A: **Neurocysticercosis in an Orthodox Jewish community in New York City.** *N Engl J Med* 1992, **327**:692-695.

7. Sorvillo F, Wilkins P, Shafir S, Eberhard M: **Public health implications of cysticercosis acquired in the United States.** *Emerg Infect Dis* 2011, **17**:1-6.

8. Hira PR, Francis I, Abdella NA, Gupta R, Al-Ali FM, Grover S, Khalid N, Abdeen S, Iqbal J, Wilson M, Tsang VCW: **Cysticercosis: imported and**

autochthonous infections in Kuwait. *Trans Roy Soc Trop Med Hyg* 2004, **98**:233-239.

9. Yanagida T, Yuzawa I, Joshi DD, Sako Y, Nakao M, Nakaya K, Kawano N, Oka H, Fujii K, Ito A: **Neurocysticercosis: assessing where the infection was acquired from.** *J Trav Med* 2010, **17**:206-208.

10. Jongwutiwes U, Yanagida T, Ito A, Kline SE: **Isolated intradural-extramedullary spinal cysticercosis: a case report.** *J Trav Med* 2011, **18**:284-287.

11. Ito A, Takayanagui OM, Sako Y, Sato MO, Odashima NS, Yamasaki H, Nakaya K, Nakao M: **Neurocysticercosis: clinical manifestation, neuroimaging, serology and molecular confirmation of histopathologic specimens.** *Southeast Asian J Trop Med Public Health* 2006, **27** (Suppl 3): 74-81.

12. Yamasaki H, Nakao M, Sako Y, Nakaya K, Ito A: **Molecular identification of *Taenia solium* cysticercus genotype in the histopathological specimens.** *Southeast Asian J Trop Med Public Health* 2005, **36** (Suppl): 131-134

13. Yamasaki H, Muto M, Morishima Y, Sugiyama H, Kawanaka M, Nakamura-Uchiyama F, Ohgame M, Kobayashi K, Ohnishi K, Kawai S, Okuyama T, Saito K, Miyahira Y, Yanai H, Matsuoka H, Haruki K, Miyoshi Y, Akao N, Akiyama J, Araki J: ***Taenia asiatica*** **infection occurring in Kanto District, 2010 [abstract in Japanese].** *The 80th Annual Meeting of the Japanese Society of Parasitology* 2011, p. 76.

14. Sako Y, Nakao M, Ikejima T, Piao XZ, Nakaya K, Ito A: **Molecular characterization and diagnostic value of *Taenia solium* low-molecular-weight antigen genes.** *J Clin Microbiol* 2000, **38**:4439-4444.

15. Yamasaki H, Allan JC, Sato MO, Nakao M, Sako Y, Nakaya K, Qiu DC, Mamuti W, Craig PS, Ito A: **DNA differential diagnosis of taeniasis and cysticercosis by multiplex PCR.** *J Clin Microbiol* 2004, **42**:548-553.

16. Nkouawa A, Sako Y, Nakao M, Nakaya K, Ito A: **Loop-mediated isothermal amplification method for differentiation and rapid detection of *Taenia* species.** *J Clin Microbiol* 2009, **47**:168-174.

17. **PubMed** [http://www.ncbi.nlm.nih.gov/pubmed/]

18. **Ichushi-Web, Japan Medical Abstract Society** [http://search.jamas.or.jp/]

19. Araki T: **President lecture: current trend of cerebral cysticercosis in Japan.**

Clin Parasitol 1994, **5**:12-24 (in Japanese).

20. Ohsaki N, Matsumoto A, Miyamoto K, Kondoh N, Araki K, Ito A, Kikuchi K: **Neurocysticercosis without detectable specific antibody.** *Internal Med* 1999, **38**:67-70.

21. Ito A, Nakao M, Ito Y, Yuzawa I, Morishima H, Kawano N, Fujii K: **Neurocysticercosis case with a single cyst in the brain showing dramatic drop in specific antibody titers within 1 year after curative surgical resection.** *Parasitol Int* 1999, **48**:95-99.

22. Ishikawa E, Komatsu Y, Kikuchi K, Yamasaki H, Kimura H, Osuka S, Tsurubuchi T, Ito A, Matsumura A: **Neurocysticercosis as solitary parenchymal lesion confirmed by mitochondrial deoxyribonucleic acid sequence analysis.** *Neuro Med Chir* (Tokyo) 2007, **47**:40-44.

23. Nakao M, Okamoto M, Sako Y, Yamasaki H, Nakaya K, Ito A: **A phylogenetic hypothesis for the distribution of two genotypes of the pig tapeworm *Taenia solium* worldwide.** *Parasitology* 2002, **124**:657-662.

24. Enander RT, Amaya AR, Enander RA, Gute DM: **Neurocysticercosis: risk and primary prevention strategies update.** *Int J Environ Health Res* 2010,

20:329-365.

25. del La Garza Y, Graviss EA, Daver NG, Gambarin KJ, Shandera WX, Schantz PM,White ACJr: **Epidemiology of neurocysticercosis in Houston, Texas.** *Am J Trop Med Hyg* 2005, **73**:766-770.

26. Burneo JG, Plener I, Garcia HH: **Neurocysticercosis in a patient in Canada.** *Canadian Med Assoc J* 2009, **180**:639-642.

27. Esquivel A, Diaz-Otero F, Gimenez-Roldan S: **Growing frequency of neurocysticercosis in Madrid (Spain).** *Neurologia* 2005, **20**:116-120.

28. Waloch M: **Cestode infections in Poland 2007.** *Przegl Epidemiology* 2009, **63**:267-9 (in Polish).

29. Croft AM, Flores AA, López HZ: **Cysticercosis in a female Nicaraguan traveler.** *J Travel Med* 2007, **14**:349-351.

30. Ito A, Plancarte A, Ma L, Kong Y, Flisser A, Cho SY, Liu YH, Kamhawi S, Lightowlers MW, Schantz PM: **Novel antigens for neurocysticercosis: simple method for preparation and evaluation for serodiagnosis.** *Am J Trop Med Hyg* 1998, **59**:291-294.

31. Ito A: **Serologic and molecular diagnosis of zoonotic larval cestode infections.** *Parasitol Int* 2002, **51**:221-235.

32. Ito A, Sako Y, Yamasaki H, Mamuti W, Nakaya K, Nakao M, Ishikawa Y:

Development of Em18-immunoblot and Em-18-ELISA for specific diagnosis of alveolar echinococcosis. *Acta Trop* 2003, **85**:173-182.

33. Ito A, Craig pS: **Immunodiagnostic and molecular approaches for the detection of taeniid cestode infections.** *Trends Parasitol* 2003, **19**:377-381.

34. Deckers N, Dorny P: **Immunodiagnosis of *Taenia solium* taeniasis/cysticercosis.** *Trends Parasitol* 2010, **26**:137-144.

35. Ito A, Okamoto M, Li T, Wandra T, Dharmawan NS, Swastika KI, Dekumyoy P, Kusolsuk T, Davvajav A, Davvasuren A, Dorjsuren T, Mekonnen SM, Hegasi ZH, Yanagida T, Sako Y, Nakao M, Nakaya K, Lavikainen AJ, Nkouawa A, Mohammadzadeh T: **The first workshop towards the control of cestode zoonoses in Asia and Africa.** *Parasit Vectors* 2011, **4**:114.

36. Ito A, Okamoto M, Wandra T, Wibisono H, Anantaphruti MT, Waikagul J, Li T, Qiu D: **The present situation of taeniasis and cysticercosis in Asia and the Pacific.** *Southeast Asian J Trop Med Public Health* 2007, **38** (Suppl 1): 119-124.

37. Ikejima T, Piao ZX, Sako Y, Sato MO, Bao S, Si R, Yu F, Zhang CL, Nakao M,

Yamasaki H, Nakaya K, Ito A: **Evaluation of clinical and serological data from *Taenia solium* cysticercosis patients in eastern Inner Mongolia Autonomous region, China.** *Trans R Soc Trop Med Hyg* 2005, **99**:625-630.

38. Tran DS, Odermatt P, Le Oanh T, Huc P, Phoumindr N, Ito A, Druet-Cabanac M, Perux PM, Strobel M: **Risk factors for epilepsy in rural Lao PDR: a case-control study.** *Southeast Asian J Trop Med Public Health* 2007, **38**:537-42.

39. Ito A, Craig PS, Schantz PM: **Taeniasis/cysticercosis and echinococcosis with focus on Asia and the Pacific.** *Parasitol Int* 2006, **55**:S1-S308.

40. Li T, Craig PS, Ito A, Chen X, Qiu D, Qiu J, Sato MO, Wandra T, Bradshaw H, Li L, Yang Y, Wang Q: **Taeniasis/cysticercosis in a Tibetan population in Sichuan province, China.** *Acta Trop* 2006, **100**:223-231.

41. Anantaphruti MT, Yamasaki H, Nakao N, Waikagul J, Wattanakulanich D, Nuamtanong S, Maipanich W, Pubampen S, Sanguankiat S, Muennoo C, Nakaya K, Sato MO, Sako Y, Okamoto M, Ito A: **Sympatric occurrence of *Taenia solium*, *T. saginata*, and *T. asiatica*, Thailand.** *Emerg Infect Dis* 2007,

13:1413-1416.

42. Swastika K, Dewiyani CI,Yanagida T, Sako Y, Sudamaja M, Sutisna P, Wandra T, Dharmawan NS, Nakaya K, Okamoto K, Ito A: **An ocular cysticercosis in Bali, Indonesia caused by *Taenia solium* Asian genotype.** *Parasitol Int* 2011 Nov 28.

43. Somers R, Dorny P, Geysen D, Nguyen LA, Thach DC, Vercruysse J, Ngouyen VK: **Human tapeworms in north Vietnam.** *Trans R Soc Trop Med Hyg* 2007, **101**:275-277.

44. Croker CC, Reporter R, Mascola L: **Use of statewide hospital discharge data to evaluate the economic burden of neurocysticercosis in Los Angeles County.** *Am J Trop Med Hyg* 2010, **83**:106-110.

45. Townes JM, Hoffman CJ, Kohn MA: **Neurocysticercosis in Oregon, 1995-2000.** *Emerg Infect Dis* 2004, **10**:508-510.

46. Wallin MT, Kurtzke JF: **Neurocysticercosis in the United States. Review of an important emerging infection.** *Neurology* 2004, **63**:1559-1564.

47. Barton Behravesh C, Mayberry LF, Bristol JR Cardenas VM, Mena KD, Martínez-Ocaña J, Flisser A, Snowden KF: **Population-based survey of**

taeniasis along the United States-Mexico border. *Ann Trop Med Parasitol* 2008, **102**:325-333.

48. Eom KS, Jeon HK, Rim HJ: **Geographic distribution of *Taenia asiatica* and related species.** *Korean J Parasitol* 2009, **47**: S115-S124.

49. Jeon HK, Kim KH, Eom KS: **Molecular identification of *Taenia* specimens after long-term preservation in formalin.** *Parasitol Int* 2011, **60**: 203-205.

50. Dorny P, Praet N: ***Taenia saginata* in Europe.** *Vet Parasitol* 2007, **149**:22-24.

51. Flutsch F, Heinzmann D, Mathis A, Herzberg H, Stephan R, Deplazes P: **Case-control study to identify risk factors for bovine cysicercosis on farms in Switzerland.** *Parasitology* 2008, **135**: 641-646.

52. McFadden AM, Heath DD, Morley CM, Dorny P: **Investigation of an outbreak of *Taenia saginata* cysts (*Cysticercus bovis*) in dairy cattle from two farms.** *Vet Parasitol* 2011, **176**:177-184.

Table 1. Summarized data on cysticercosis cases reported in Japan between 1976-1993 and 1994-2010.

*The total number and incidence (number of cases/year) of cases. **The mean and range (in parentheses) of patient ages. ***Percentage of East Asian patients. ND: no data.

	1976-1993	1994-2010
No. cases*	43 (2.4)	39 (2.3)
Sex (% Male)	71.8	43.6
Age**	ND (20-62)	39.9 (9-70)
Japanese (%)	60.5	56.4
E Asia (%)***	25.6	17.9

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Phylogenetic relationships within *Echinococcus* and *Taenia* tapeworms (Cestoda: Taeniidae): An inference from nuclear protein-coding genes

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ABSTRACT

The family Taeniidae of tapeworms is composed of two genera, *Echinococcus* and *Taenia*, which obligately parasitize mammals including humans. Inferring phylogeny via molecular markers is the only way to trace back their evolutionary histories. However, molecular dating approaches are lacking so far. Here we established new markers from nuclear protein-coding genes for RNA polymerase II second largest subunit (*rpb2*), phosphoenolpyruvate carboxykinase (*pepck*) and DNA polymerase delta (*pold*). Bayesian inference and maximum likelihood analyses of the concatenated gene sequences allowed us to reconstruct phylogenetic trees for taeniid parasites. The tree topologies clearly demonstrated that *Taenia* is paraphyletic and that the clade of *Echinococcus oligarthrus* and *Echinococcus vogeli* is sister to all other members of *Echinococcus*. Both species are endemic in Central and South America, and their definitive hosts originated from carnivores that immigrated from North America after the formation of the Panamanian land bridge about 3 million years ago (Ma). A time-calibrated phylogeny was estimated by a Bayesian relaxed-clock method based on the assumption that the most recent common ancestor of *E. oligarthrus* and *E. vogeli* existed during the late Pliocene (3.0 Ma). The results suggest that a clade of *Taenia* including human-pathogenic species diversified primarily in the late Miocene (11.2 Ma), whereas *Echinococcus* started to diversify later, in the end of the Miocene (5.8 Ma). Close genetic relationships among the members of *Echinococcus* imply that the genus is a young group in which speciation and global radiation occurred rapidly.

1. Introduction

Phylogeny reconstruction based on the hierarchy of relative recency of common ancestry is essential for the morphological and molecular recognition of species and eventually leads to the revision of the taxonomic units. Parasites and their host organisms may provide valuable phylogenetic case studies in the fields of paleontology, biogeography, conservation biology and disease ecology. Terrestrial and aquatic vertebrates specifically serve as definitive hosts for many species of tapeworms (Platyhelminthes: Cestoda), and the evolutionary background of each host provides key information to interpret the phylogeny of the parasites.

The largest taxonomic group within the Cestoda is the order Cyclophyllidea whose members are parasitic as adults in amphibians, reptiles, birds and mammals. The order includes at least 15 families with several thousand species (Jones et al., 1994). The cyclophyllidean family Taeniidae consists of the two genera *Taenia* Linnaeus 1758 and *Echinococcus* Rudolphi 1801 that are of medical and veterinary importance. The phylogeny of the Cyclophyllidea inferred from morphological characters suggests that the families Paruterinidae and Metadilepididae are the sister-group of the Taeniidae and that the enigmatic genus *Dasyurotaenia* Beddard 1912 occurring in Australian marsupials is the closest relative to the Taeniidae (Hoberg et al., 1999). On the contrary, the Paruterinidae is placed distant to the Taeniidae in a phylogeny inferred from 18S ribosomal DNA (rDNA) sequences (Foronda et al., 2004). Another study based on mitochondrial 12S rDNA indicates a sister-group relationship between the Taeniidae and Dipylidiidae (von Nickisch-Rosenegk et al., 1999a). These molecular phylogenies, however, were based on limited number of taxa used in the analyses. The ambiguity of close relatives outside the Taeniidae makes it difficult to determine an appropriate outgroup in taeniid phylogeny.

Taeniid parasites require two mammalian hosts to perpetuate their life cycles. Terrestrial predators act as definitive hosts for the adult worms, and their preys as intermediate hosts for the

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cystic larvae. The process of host switching in both adult and larval stages, namely the adaptive selection of susceptible variants, is a possible driving force in forming the new species of tapeworms (Hoberg, 2006; Emelianov, 2007). The splitting of tapeworm populations by the vicariance of their host mammals is important for speciation because it leads to reproductive isolation. However, mating between different individuals seems to be minor in tapeworms (Haag et al., 1999). Their major reproduction manner is autogamy (selfing in hermaphrodite), which causes the genetic uniformity of populations (Lymbery, 1992). A tendency toward a clonal population structure resulting from the selfing system might affect the speciation of tapeworms (Smyth and Smyth, 1964).

The genus *Echinococcus* is a small taxonomic group containing a cryptic species complex (Thompson and McManus, 2002). All the members of *Echinococcus* are quite similar to each other in having small number of proglottids in adult stage, and their larvae asexually multiply in vesicular cysts. These remarkable commonalities support an idea that *Echinococcus* is a monophyletic entity. A recent molecular phylogenetic analysis using complete mitochondrial genome data revealed that the genus consists of at least nine species (Nakao et al., 2007). However, there are significant discrepancies between phylogenies of mitochondrial DNA (mtDNA) and nuclear DNA. As summarized in Fig. 1, the mtDNA phylogeny suggests that a cryptic species complex consisting of *Echinococcus granulosus* sensu stricto (s.s.), *Echinococcus equinus*, *Echinococcus ortleppi*, *Echinococcus canadensis* (genotypes G6-G10) and *Echinococcus felidis* is paraphyletic and that *Echinococcus oligarthrus* is a sister taxon to all other *Echinococcus* species (Nakao et al., 2007; Hüttner et al., 2008; Moks et al., 2008). By contrast, the nuclear phylogeny inferred from protein-coding genes indicates that the cryptic species complex is a monophyletic entity and that *Echinococcus multilocularis* is sister to all other members (Saarma et al., 2009). Holarctic *E. multilocularis*, Tibetan *Echinococcus shiquicus*, African *E. felidis* and Neotropical *E. oligarthrus* and *Echinococcus vogeli* utilize indigenous wildlife as natural hosts. However, *E. granulosus* s.s., *E. equinus*, *E. ortleppi* and *E. canadensis* maintain synanthropic life cycles including domestic dogs and ungulate livestock in worldwide regions. The species status of *E. canadensis* remains controversial because it includes wild-type lineages (genotypes G8 and G10) using wolves and cervids as natural hosts (Rausch, 2003; Thompson, 2008).

The genus *Taenia* is a large group in which at least 42 species have been recognized as valid (Loos-Frank, 2000; Hoberg, 2006). The endemic species of *Taenia* are abundant in Africa but rare in the Australian and Neotropical regions (Loos-Frank, 2000). The common parasites of domestic dogs (e.g., *Taenia hydatigena* and *Taenia ovis*) and cats (*Taenia taeniaeformis*) are distributed worldwide. Among the members of *Taenia*, only *Taenia solium*, *Taenia saginata* and *Taenia asiatica* use humans as a definitive host, and are therefore termed "human *Taenia*". An "Out of Africa" hypothesis on the human *Taenia* explains that *Taenia* spp. of African carnivores colonized hominids before the emergence of modern humans (Hoberg et al., 2001). Historically, several genera had been created within the family Taeniidae, but all the genera except *Echinococcus* were eventually treated as *Taenia* by Verster (1969). In this monograph, *Taenia* was divided into two groups based on the arrangement of genital ducts in the adult stage. In the group I, the ducts pass between lateral excretory canals, whereas those of the group II pass the canals ventrally. The group I consists of human *Taenia* and other species using modern canines and felines as definitive hosts. The group II includes the cat tapeworm *T. taeniaeformis* and few species infecting the primitive carnivores of the families Mustelidae and Viverridae (e.g., *Taenia mustelae* and *Taenia parva*). This grouping is supported by a molecular phylogenetic analysis using mtDNA sequences (von Nickisch-Rosenegk et al., 1999b). A numerical taxonomic analysis using morphological characters indicates the monophyly of the genus *Taenia* (Hoberg et al., 2000). However, a recent study based on mtDNA sequences suggests a possibility that the genus is paraphyletic (Lavikainen et al., 2008).

The molecular phylogeny of taeniid tapeworms has been reconstructed mainly by analyzing the sequences of mtDNA (Bowles

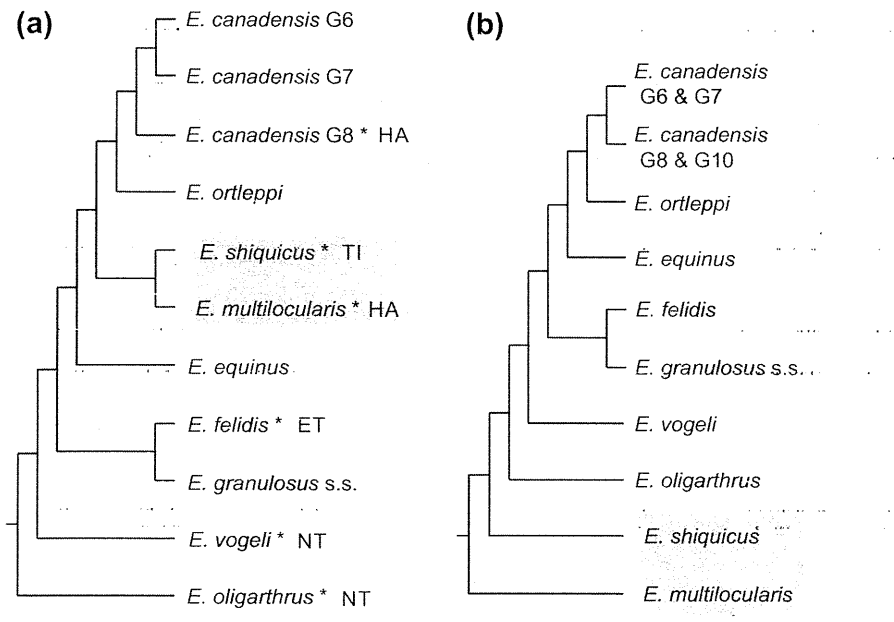

Fig. 1. The summary of recent publications for phylogenetic relationships among *Echinococcus* spp. (a) A cladogram based on the DNA sequences of mitochondrial genes (Nakao et al., 2007; Hüttner et al., 2008). (b) A cladogram based on the DNA sequences of nuclear protein-coding genes (Saarma et al., 2009). Shaded areas show the translocation of taxa. Asterisks indicate endemic species using wildlife mammals as their hosts. HA, Holarctic; TI, Tibet; ET, Ethiopian; NT, Neotropical. The other species using domestic mammals as their hosts are distributed worldwide.

et al., 1995; Okamoto et al., 1995; de Queiroz and Alkire, 1998; Gasser et al., 1999; Nakao et al., 2007; Zhang et al., 2007; Hüttner et al., 2008; Lavikainen et al., 2008; Jia et al., 2010). However, the construction of phylogenetic trees from mtDNA data alone involves a risk of misunderstanding evolutionary history since maternally inherited mtDNA does not necessarily match the true history of species (Ballard and Whitlock, 2004). Instead of employing mtDNA as an evolutionary marker, a broader use of multiple nuclear DNA markers is suggested. Nevertheless, phylogenetic studies using a set of nuclear markers are uncommon in tapeworms because of the lack of suitable markers. Furthermore, there are no fossil records of tapeworms to calibrate molecular clocks for estimating the divergence time of species within a lineage. One possible way to overcome this limitation would be to employ paleozoogeographic events, which caused the immigration and isolation of host mammals, as calibration points for time estimates.

In this study we established new nuclear DNA markers for the phylogenetic analyses of taeniid tapeworms. These markers are targeted to the genomic regions of protein-coding genes. Maximum likelihood phylogenetic inference and Bayesian molecular dating were carried out using the data sets of the nuclear DNA sequences. The main purposes of this study are (1) to reevaluate interspecific relationships among *Echinococcus* spp., (2) to infer intergeneric relatedness in the family Taeniidae and (3) to estimate divergence times of taeniid tapeworms.

2. Materials and methods

2.1. Taxon sampling and genomic DNA preparation

As shown in Table 1, 11 taxa of *Echinococcus* and 15 taxa of *Taenia* were used for this study. Most of them were obtained as ethanol-preserved specimens from collaborators. The *Echinococcus* taxa cover all known species in the genus. The *Taenia* taxa consist of representative species from the groups I and II of Verster (1969). The group II species *T. mustelae* was selected as an outgroup for the phylogeny of *Echinococcus* because a previous study showed this species to be the most related to *Echinococcus* (Lavikainen et al., 2008). *Moniezia expansa* (Anoplocephalidae) and *Dipylidium caninum* (Dipylidiidae) were used as outgroups for Taeniidae on a trial basis, although it is unknown which family of the Cyclophyllidea is the most suitable candidate.

By using a spin column kit (DNeasy Blood &Tissue Kit, Qiagen, Hilden, Germany), genomic DNA (gDNA) was extracted individually from the larval tissues of *Echinococcus* spp., *T. mustelae*, *Taenia martis* and *T. parva*, and from the adult proglottids of other *Taenia* spp., *M. expansa* and *D. caninum*. Only the gDNA of *E. felidis* was purified from eggs, which were isolated from lion feces (Hüttner et al., 2008). Each of the purified gDNAs was used as a template for polymerase chain reaction (PCR).

2.2. Nuclear gene targets and primer design

Genes for RNA polymerase II second largest subunit (*rpb2*), phosphoenolpyruvate carboxykinase (*pepck*) and DNA polymerase delta (*pold*) were selected as targets for nuclear markers in taeniid parasites because of their single-copy presence in many eukaryotic organisms (Friedlander et al., 1994; Loeb and Monnat, 2008), including *E. multilocularis* (*Echinococcus* genome project, Wellcome Trust Sanger Institute, http://www.sanger.ac.uk/). The *rpb2* codes for a protein subunit involved in the catalyses of the RNA transcription (Kornberg, 1999), the *pepck* plays an important role in glucose homeostasis (Hers and Hue, 1983) and the *pold* is responsible for both leading and lagging strand synthesis during DNA replication (Hubscher et al., 2002). The original sequences of these genes were obtained from the expressed sequence tags (ESTs) database of *E. multilocularis* (http://www.sanger.ac.uk/). Oligonucleotide primers for PCR were designed to highly conserved portions of the coding sequences (Table 2). In *Taenia* taxa, primers for *pepck* and *pold* were redesigned based on determined sequences.

2.3. PCR condition and DNA sequencing

PCR was performed in a final volume of 50 μL containing 100 ng of template DNA, 0.25 μM of each primer, 2.5 mM of each dNTP, 1 unit of Ex-*Taq* DNA polymerase (TaKaRa Bio Inc., Otsu, Japan) and the manufacturer-supplied reaction buffer. The DNA fragments of *rpb2*, *pepck* and *pold* were amplified under the following thermal conditions: an initial denaturing step at 94 °C for 30 s followed by 35 cycles of 94 °C for 30 s, 55 °C for 15 s and 72 °C for 90 s. The reaction terminated with a final extension step of 72 °C for 5 min. The annealing temperature was raised to 60 °C when non-specific bands appeared.

Both strands of the amplified DNA were directly sequenced by using BigDye terminator cycle sequencing kit and ABI PRISM 310 genetic analyzer (Applied Biosystems, Foster City, CA, USA). The PCR-amplified DNA fragments were completely sequenced by primer walking, using PCR primers and custom-synthesized primers. In cases of double peaks in the sequencing reaction, PCR products were ligated into pGEM-T plasmid vector (Promega Corp., Madison, WI, USA) and then introduced into *Escherichia coli* DH5α. At least 10 clonal colonies were picked from an agar plate and their insert DNAs were sequenced to confirm allelic polymorphism.

2.4. Rapid amplification of cDNA 3' end (3'-RACE)

The fresh protoscoleces of laboratory-maintained larval *E. multilocularis* were used as a source for mRNA. PCR for 3'-RACE was carried out in order to identify the exon–intron structures of genomic genes. After extracting total RNA by TRIzol (Invitrogen Corp., Carlsbad, CA, USA), mRNA was purified through oligo-dT latex particles (Oligotex-dT30, TaKaRa Bio). The first-strand complementary DNA (cDNA) was synthesized by a reverse transcription-PCR kit using PrimeScript RTase (TaKaRa Bio) and an oligo-dT adaptor primer (5′-CTGATCTAGAGGTACCGGATCCTTTTTTTTTTTTTTTTTT-3′). The cDNA of each target gene was amplified with a pair of the gene-specific forward primer (Table 2) and the adaptor primer (5′-CTGATCTAGAGGTACCGGATCC-3′). The cDNA amplicon was sequenced and then compared with the corresponding genomic sequence.

2.5. Phylogenetic and dating analyses

The multiple sequence alignment of PCR-amplified gDNA was made for each gene by the T-Coffee program (Notredame et al., 2000). Putative exon regions were extracted from each of the gDNA alignments under the guidance of the exon–intron maps of *E. multilocularis*. The sequence alignments of *rpb2*, *pepck* and *pold* genes were concatenated to make operational data sets. The gDNA data set (5008 nucleotide sites) and the exon data set (3726 sites) including 11 *Echinococcus* taxa and an outgroup (*T. mustelae*) were prepared for maximum likelihood (ML) analysis to examine relationships among *Echinococcus* spp. Only the exon data set (3726 sites) including 26 taeniid taxa (11 *Echinococcus* and 15 *Taenia* taxa) was used for Bayesian phylogenetic and dating analyses. The exon alignments of *rpb2*, *pepck* and *pold* including the 26 taxa were modified by adding the corresponding sequence of a cyclophyllidean species outside the Taeniidae. The additional taxa were *M. expansa* for *rpb2* and *D. caninum* for *pepck* and *pold*. These were treated as an outgroup for the construction of ML phylogenetic trees.

Table 1
Taxa of taeniid tapeworms (*Echinococcus* and *Taenia*) and their DNA sequences used for this study.

Species (genotypes)	Geographic origins	Database accession numbers of nuclear protein-coding genes		
		rpb2	*pepck*	*pold*
Echinococcus				
E. multilocularis	Japan	FN566845	FN567985	FN568356
E. shiquicus	China	FN566846	FN567986	FN568357
E. vogeli	Columbia	FN566847	FN567987	FN568358
E. oligarthrus	Panama	FN566848[b]	FN567988[b]	FN568359[b]
E. felidis	Uganda	FN566849	FN567989	FN568360
E. granulosus s.s.	China	FN566850	FN567990	FN568361
E. equinus	United Kingdom	FN566851	FN567991	FN568362
E. ortleppi	Argentina	FN566852	FN567992	FN568363
E. canadesis (G6)	Peru	FN566853	FN567993	FN568364
E. canadensis (G7)	Peru	FN566854	FN567994	FN568365
E. canadensis (G8)	North America	FN566855	FN567995	FN568366
Taenia				
T. solium	Ecuador	FN566856	FN567996	FN568367
T. saginata	Belgium	FN566857	FN567997	FN568368
T. asiatica	Taiwan	FN566858	FN567998	FN568369
T. crassiceps	Canada	FN566859	FN567999	FN568370
T. hydatigena	China	FN566860	FN568000	FN568371
T. serialis	Australia	FN566861	FN568001	FN568372
T. multiceps	undocumented	FN566862	FN568002	FN568373
T. ovis	New Zealand	FN566863	FN568003	FR869707[b]
T. madoquae	Kenya	FR869694	FR869699	FR869705
T. laticollis	Finland	FR869693	FR869697	FR869703
T. martis[a]	Croatia	FR852565	FR852569	FR869706
T. twitchelli[a]	Russia	FR852564	FR852568	FR869710
T. parva[a]	Spain	FR852566	FR869700	FR869708
T. taeniaeformis[a]	Japan	FR852567	FR869701	FR869709
T. mustelae[a]	Finland	FR869695	FR869698	FR869704
Outgroup taxa				
Dipylidium caninum	undocumented	–	FR869702	FR869711
Moniezia expansa	China	FR869696	–	–

[a] The group II species of Verster (1969).
[b] Cases of putative heterozygosity were detected. The accession numbers of the other allele are FN658827, FN658828, FN658829 and FN658833.

Table 2
PCR primer pairs designed to amplify the nuclear protein-coding genes of taeniid tapeworms.

Target genes	Primers (5′–3′)[a]
RNA polymerase II second largest subunit (*rpb2*)	General set for *Echinococcus* and *Taenia* F: AGGGATCACTATGGAAACAAACGA R: ACCATCATCGTCTAGTTTATCGTA
Phosphoenolpyruvate carboxykinase (*pepck*)	General set for *Echinococcus* and *Taenia* F: ATCTGCGACGGTAGCAAGGCCGAA R: AGCCCATTCAATCACACGGATATT Special set for *T. parva* and *T. taeniaeformis* F: CGACAAGATCACTGAAGAACT R: AGCCCATTCAATCACACGGATATT
DNA polymerase delta (*pold*)	General set for *Echinococcus* F: AACGCAGTTAGTTATCACTTCCTA R: CGTTTCAATACCTTTGCAATCCAT General set for *Taenia* F: CCTCTGCTCCTGTTGGATAAA R: CGTTTCAATACCTTTGCAATCCAT Special set for *T. taeniaeformis* F: CTGATGTCTGTTGCAAATAATGTC R: CTTGTCGTACTTGTCCGGTTT

[a] F, forward primer; R, reverse primer.

The degree of phylogenetic incongruence among three gene partitions (*rpb2*, *pepck* and *pold*) in both gDNA and exon data sets was assessed by the incongruence length difference (ILD) test (Farris et al., 1995). The partition-homogeneity test in PAUP 4.0b (Swofford, 2002) using a heuristic tree search with 1000 replicates was conducted to compute the probability of incongruence when the three gene partitions were used simultaneously. The significant probability values less than 0.05 was considered incongruent. In a phylogenetic context the data homogeneity can be defined as the sharing of a single history (Barker and Lutzoni, 2002), but the ILD test has only limited power to detect incongruence (Darlu and Lecointre, 2002).

The sequence characteristics of each gene marker were examined using the gDNA and exon data sets. Pairwise divergence values and their means within each genus were calculated by MEGA 4.0 (Tamura et al., 2007) under Kimura 2-parameter model (Kimura, 1980) with a gamma setting of 0.5. The pairwise divergence of deduced amino acid sequences was computed using Dayhoff's PAM matrix. The codon-based Z-test (Nei and Gojobori, 1986) of MEGA 4.0 was conducted to test the hypothesis of negative selection for each gene. The variance of difference between synonymous and nonsynonymous substitutions per site (d_S–d_N) was estimated using bootstrapping with 500 replicates. The resultant statistics of all sequence pairs were averaged within each genus.

The phylogenetic trees of *Echinococcus* were generated by the ML analysis of PAUP 4.0b using the gDNA and exon data sets including 11 *Echinococcus* taxa and an outgroup. Nucleotide substitution models were selected using Akaike information criterion (AIC) as implemented in Modeltest 3.7 (Posada and Crandall, 1998) running on PAUP 4.0b. The substitution models used for the *Echinococcus* phylogeny were TIM + G for the gDNA data set and TIMef + I for the exon data set. The ML trees of individual genes were also built from exon alignments including 26 taeniid taxa and an outgroup. The substitution models used for the taeniid phylogeny were TIM + I + G for *rpb2*, SYM + I + G for *pepck* and K81uf + G for *pold*. The likelihood settings of base frequencies, substitution rate matrix, gamma distribution shape and proportion of invariable sites were set according to the calculated parameters of the best-fit