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SHORT COMMUNICATION

Taenia solium cysticercosis in Bali, Indonesia: serology and mtDNA analysis

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KEYWORDS
Cysticercosis;
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Summary An active *Taenia solium* cysticercosis case in Bali, Indonesia, was followed-up by serology and computed tomography. Serology using semi-purified glycoprotein and recombinant antigens showed a drastic drop in titers after calcification of the cysts. Three paraffin-embedded cysts, prepared for histopathological examination, from three other patients were used for mtDNA analysis. The sequences of *cox1* gene from *T. solium* cysticerci from Bali differed from those in Papua and other Asian countries.

1. Introduction

Taenia solium cysticercosis is rather uncommon in Indonesia. It is thought that this is associated with the lack of consumption of pork by the Muslim majority population. However, cysticercosis occurs in some areas or islands, where most people are either Christians or Hindu (Ito et al., 2006; Wandra et al., 2003, 2006). It has been shown that cysticercosis is a very serious public health issue in Papua (formerly Irian Jaya, Indonesia) (Wandra et al., 2003). It has been suggested that *T. solium* was introduced into Papua from Bali in 1969 or subsequently, when Papua was governed by Indonesia (Wandra et al., 2003). However, there is no molecular evidence to test this hypothesis.

In Bali, taeniasis due to either *T. saginata* or *T. solium* is found in all nine districts. Historically, cysticercosis was rather common, but has been found only sporadically for at least the past three decades. By contrast, taeniasis due to *T. saginata* is now rather common in Bali (Wandra et al., 2006). Unfortunately, there are no ethanol-fixed *T. solium* cysticercus specimens available from Bali; however, histopathological paraffin-embedded specimens can be used to analyze mtDNA (Ito et al., 2006; Yamasaki et al., 2005). Recently, we obtained three such cysticercus samples from three patients in Bali.

In this study, the *cox1* gene sequences of mtDNA were analyzed using the specimens from Bali and compared with those from Papua and several other Asian countries. A follow-up study of a patient who showed good serological

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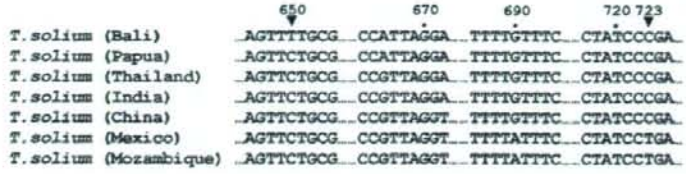

Figure 1 Comparison of nucleotide sequences of *cox1* of the *Taenia solium* isolate from a Balinese cysticercosis patient and those of *cox1* from known human *Taenia* cestodes. Nucleotides at positions 690 and 723 serve as differential markers for two genotypes of *T. solium*, *T. saginata* and *T. asiatica* (Yamasaki et al., 2005). A thymine base at position 650 is unique to *T. solium* isolated from Bali, Indonesia. The nucleotide sequences of *T. solium* from Bali (Indonesia), Papua (Indonesia), Thailand, India, China, Mexico and Mozambique are from AB271234, AB066488, AB066487, AB066489, AB066485, AB066490 and AB066493, respectively.

dynamics for detection of active cysticercosis is also briefly discussed, since serology to detect active cysticercosis is still under debate (Ito and Craig, 2003).

2. Serological follow-up of active cysticercosis

A 36-year-old Balinese man, from a rural village, Gianyar District, southern Bali, Indonesia, was diagnosed with disseminated cysticercosis in June 2003. A computed tomography scan of his brain showed multiple active lesions in the left frontal lobe and parieto-occipital region. Histopathological examination of a nodule resected from his tongue in January 2004 revealed a typical taeniid cysticercus. After treatment with albendazole, only calcified lesions were detected in February 2006. Serology by ELISA and immunoblots, using highly specific semi-purified glycoprotein antigens (pH 8.1) (Sako et al., 2000), for detection of active cysticercosis was strongly positive in June 2003 and January 2004 but weakly positive in February 2006. Optical density values for ELISA using the same antigens increased from 0.221 (June 2003) to 0.254 (January 2004) and dropped to 0.093 (February 2006) (cut-off = 0.051). Such dynamic antibody responses during treatment for 3 years were also confirmed by ELISA using recombinant chimeric antigen (Sako et al., 2000). Optical density values were 0.411 (June 2003), 0.528 (January 2004) and 0.165 (February 2006) (cut-off = 0.093).

3. mtDNA

Three paraffin-embedded nodules from 36, 36 and 26 year-old men in Bali were available for mtDNA analysis. DNA was extracted from thin sections (four sections of 5-μm thickness) using a DEXPAT kit (TaKaRa Shuzo, Shiga, Japan) (Yamasaki et al., 2005). In brief, 10 drops of lysis solution from the kit were added to paraffin sections in a microtube, and lyzed at 100°C for 2 h. After centrifugation, the resultant supernatants (~100 μl) were used as template DNA samples for PCR. For the amplification of a mitochondrial cytochrome *c* oxidase subunit 1 (*cox1*), PCR was performed using forward (5′-ATGACTAATATATTTTCTCGTAC-3′, positions 520–542) and reverse (5′-ATTAACACATAAACCTCGGGA-3′, positions 740–720) primers according to the method described previously (Yamasaki et al., 2005). The PCR-amplified *cox1* was run on a 10% polyacrylamide gel and DNA sequencing was performed on an ABI PRISM 310 Genetic Analyzer.

The *cox1* fragment of 224-bp was successfully amplified from the three samples analyzed. DNA sequencing of the PCR product revealed the cysticercus *T. solium* Asian genotype based on the differential nucleotides at positions 690 and 723 (Figure 1). In addition, the nucleotide at position 650 was thymine (T) instead of cytosine (C). As far as is known, this is unique to *T. solium* isolates from Bali, Indonesia.

4. Discussion

This serological follow-up study revealed a good correlation between neuroimaging and serology during curative chemotherapy. The results strongly suggested that the serology used for this case was very useful for detection of active cysticercosis and monitoring the patient's progress (Ito et al., 2006; Sako et al., 2000).

Due to the possibility that *T. solium* was introduced in to Papua from Bali when Papua was governed by Indonesia (Wandra et al., 2003, 2006), we are interested in comparing *T. solium* from Bali and Papua. The mtDNA analysis using three paraffin-embedded specimens revealed that *T. solium* isolated from Bali differed from isolates from Papua, China, India and Thailand at position 650 of the *cox1* fragment of 224 bp. African/American genotypes differed from Asian ones at positions 690 and 723 (Figure 1). So far, we have been able to check only three metacestodes of *T. solium* resected from three patients in Bali. Therefore, further similar studies based on the full sequencing of mtDNA genes and using more *T. solium* specimens from Bali and Papua, and from other areas of Indonesia, such as East Nusa Tenggara, located between Bali and Papua, where cysticercosis is recognized as not rare (T. Wandra et al., unpublished data), are necessary for a full phylogeographic study. Further subtyping of *T. solium* in Asia and/or America/Africa genotypes may be interesting for tracking back where cysticercosis patients from non-endemic countries were exposed to eggs of *T. solium* in endemic countries.

Authors' contributions: TW and AI designed the study protocol and carried out field surveys in Indonesia once or twice every year from 1996; TW and AI discussed the cysticercosis cases in the hospital at Udayana University with AARS from 2002 and carried out serological analysis; AARS treated the patients; AA carried out the histopathological examinations; AN and TW carried out the molecular work; AI prepared the manuscript with help from TW and AARS. All authors read and approved the final manuscript. AI is guarantor of the paper.

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Conflicts of interest: None declared.

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Genetic characterization and phylogenetic position of *Echinococcus felidis* Ortlepp, 1937 (Cestoda: Taeniidae) from the African lion ☆

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Abstract

Echinococcus felidis had been described in 1937 from African lions, but was later included in *Echinococcus granulosus* as a subspecies or a strain. In the absence of any genetic characterization, most previous records of this taxon from a variety of large African mammals remained unconfirmed due to the lack of diagnostic criteria and the possible confusion with the sympatric *E. granulosus* sensu stricto, *Echinococcus ortleppi* and *Echinococcus canadensis*. In this study, we obtained taeniid eggs from lion feces in Uganda and amplified DNA from individual eggs. Mitochondrial and nuclear DNA sequences showed similarities with those of other *Echinococcus* spp., but high values of percentage divergence of mitochondrial genes indicated the presence of a distinct species. In a second step, we compared this material with the preserved specimens of adult *E. granulosus felidis*, which had been identified morphologically approximately 40 years ago in South Africa. All DNA fragments (<200 bp) that could be amplified from the adults showed 100% similarity with the Ugandan material. In the phylogenetic tree of *Echinococcus* which was constructed from the mitochondrial genes, *E. felidis* is positioned as a sister taxon of *E. granulosus* sensu stricto. The data obtained will facilitate the development of diagnostic tools necessary to study the epidemiology of this enigmatic parasite.

Keywords: *Echinococcus felidis*; *Panthera leo*; Phylogeny

1. Introduction

Cestodes of the genus *Echinococcus* are causative agents of various forms of zoonotic echinococcosis. The adult tapeworms are only 2–7 mm in length and inhabit the small intestine of carnivorous definitive hosts belonging to the families Canidae, Felidae and Hyaenidae (Nelson et al., 1965; Rausch and D'Alessandro, 2002; Eckert and Deplazes, 2004). The gravid segments are released into the environment with the feces of these carnivores. Herbivorous intermediate hosts including humans become infected with the larvae of *Echinococcus* spp. following ingestion of the eggs. The development of massive hydatid cysts, mainly in the liver and lungs, cause severe pathological effects in the intermediate hosts. In spite of the medical and veterinary importance of these cestodes (Budke et al., 2006), their taxonomic classification is still controversial, particularly concerning the cryptic species complex of

☆ Nucleotide sequence data reported in this paper are available in DDBJ/EMBL/GenBank databases under Accession Nos. EF558355–EF558360.

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Echinococcus granulosus sensu lato (Bowles et al., 1995). Recently, a robust molecular phylogeny of *Echinococcus* spp. was reconstructed from their complete mitochondrial genomes (Ito et al., 2007; Nakao et al., 2007), and *E. granulosus* sensu lato was split into *E. granulosus* sensu stricto (genotypes G1–G3), *Echinococcus equinus* (genotype G4), *Echinococcus ortleppi* (genotype G5) and *Echinococcus canadensis* (genotypes G6–G10). Only the enigmatic 'lion strain' from Africa (Thompson and McManus, 2002) has eluded any study of its taxonomic position and DNA profile due to the unavailability of suitable isolates.

The lion strain was first described as *Echinococcus felidis* in 1937 by Ortlepp (1937) from the lion, *Panthera leo*, in South Africa. The basis for its description as a new species was the marked rugosity of the rostellar hooks and its occurrence in a felid as definitive host, which is unique for a member of the *E. granulosus* complex. In 1963, Rausch and Nelson considered this taxon to be conspecific with *E. granulosus*. However, Verster (1965) re-examined the morphology of *Echinococcus* spp., and noted the distribution of testes anterior to genital pore in the mature segment of the lion parasite. She proposed a subspecific status, as *E. granulosus felidis*, but this was considered invalid due to the sympatric occurrence of various such 'subspecies' of *E. granulosus* in southern Africa (Rausch, 1967). To the present, the 'lion strain' has been viewed as a form of *E. granulosus* of uncertain taxonomic status, and which is transmitted between lions and large wild herbivores in Africa (Macpherson and Wachira, 1997).

Hydatid cysts allocated to the lion strain have been recorded from a large number of wild mammals such as wildebeest, warthog, bushpig, zebra and various antelopes (reviewed by Macpherson and Wachira, 1997). Most of these records are of doubtful validity due to the lack of specific diagnoses. Likewise, a number of transmission experiments was performed using hydatid cysts from wild mammals in Africa; some were successful in infecting dogs, while others were not (Macpherson et al., 1983; Macpherson and Wachira, 1997). These ambiguous results are possibly due to confusion with the other taxa of *E. granulosus* sensu lato, which may also be present in African wildlife. The issue can only be clarified by molecular characterization of adult *Echinococcus* worms from lions which correspond morphologically with *E. felidis*. However, obtaining intestinal worms from lions in the wild has proven difficult. Therefore, we collected taeniid eggs from fecal samples of lions in Uganda, and tested the conspecificity of these isolates with *E. felidis* by comparison with short DNA fragments amplified from the original adult worm material from South Africa, which had been preserved for approximately 40 years.

2. Materials and methods

2.1. Parasite specimens

Freshly deposited fecal samples from lions were collected in the Queen Elizabeth National Park in Uganda in 2005. Since lions are strictly protected there, the samples were collected from the ground without any manipulation of the animals. In total, 52 fecal samples were collected, originating from an unknown number of animals. The identification of the feces was made by experienced field workers, based on shape, colour, odour, ingested hair (from grooming) and the presence of field signs such as footprints (Breuer, 2005). The feces were kept refrigerated for 3 weeks, were placed at −80 °C for at least 5 days for safety reasons and subsequently stored at −20 °C until use.

The preserved adult specimens of *E. felidis* are in the Department of Veterinary Tropical Diseases, University of Pretoria in South Africa. The material consisted of a piece of small intestine taken from a lion in South Africa about 40 years ago. Many adult worms were still attached to the mucosal surface. Worms from this collection had been morphologically identified by Anna Verster, who had described the morphology of this taxon. When we received the material, the tissue was stored in alcohol. However, the difficulty of DNA purification by a commercial kit using proteinase K suggested that the tissue had originally been fixed in formalin.

2.2. DNA amplification and sequencing

Taeniid eggs were recovered from lion feces by zinc chloride flotation (Mathis et al., 1996), and then suspended in 10 mM Tris–HCl, pH 7.5 containing 1 mM EDTA (TE buffer). To reduce the risk of mixed parasitic infections (Müller-Graf, 1995), individual eggs were isolated by using a capillary pipette. As reported previously (Nakao et al., 2003a), a single egg was lysed in 10 μl of 0.02 N NaOH at 95 °C for 10 min, and used directly as a template for PCR.

The intestinal tissue including *E. felidis* adults was washed several times with distilled water and immersed in excess TE buffer overnight to remove preservative agents. The adults were recovered from the intestinal mucosa with a fine needle. Whole lysates with alkaline solution were made without further DNA purification. Each of the adults was placed in a microtube containing 25 μl of 0.02 N NaOH, and crushed with a pestle. After heating at 95 °C for 10 min, the adult homogenate was used as a template for PCR.

Template preparations and PCR for the eggs and the adults were performed using different rooms, divided reagents and individual pipettes to avoid DNA contamination. DNA amplifications were mostly performed using nested PCR because of the minute amount of DNA in single taeniid eggs (Rishi and McManus, 1987) and the severe damage caused to DNA in long-preserved samples by formalin or other acid preservatives. For the first PCR, a 50 μl reaction mixture containing 0.2 μM of each external primer, 200 μM of each dNTP and 1 U of Ex-Taq polymerase (Takara, Japan) was prepared as recommended by the manufacturer. Whole parasite lysates were individually added to the reaction mixture. The added volumes were 1 μl in the egg lysate and 3 μl in the adult lysate. After amplification, 1 μl of the first PCR was transferred to a

second PCR consisting of the same reaction mixture, but instead of external primers, each internal primer was added at 0.2 μM. All thermal reactions were performed for 35 cycles of denaturation (94 °C for 30 s), annealing (55 °C for 30 s) and extension (72 °C for 30–90 s).

Mitochondrial genes for cytochrome *c* oxidase subunit 1 (*cox1*), NADH dehydrogenase subunit 1 (*nad1*), cytochrome *b* (*cob*) and rRNA (*rrn*), and nuclear genes for elongation factor 1 alpha (*ef1a*) and ezrin-radixin-moesin (ERM)-like protein (*elp*) were amplified from taeniid eggs by using primers listed in Table 1. The *rrn* includes the large (*rrnL*) and small (*rrnS*) subunit rRNA genes. The external and internal primers for these mitochondrial genes were designed from the conserved regions of *E. granulosus* mtDNA (database Accession No. AF297617) (Le et al., 2002). In the case of the preserved *E. felidis* adults, short mtDNA fragments (<200 bp) of *cox1*, *nad1* and *cob* were amplified by using additional primers (Table 1).

Amplification products were purified by using the QIAquick PCR purification kit (QIAGEN, Germany) and used as a template for direct sequencing. The sequencing reaction was carried out using BigDye terminator ver. 1.1 (Applied Biosystem, USA) and the resultant ladders were read by ABI PRISM 377 genetic analyser (Applied Biosystem, USA). Long DNA templates were sequenced by primer walking.

2.3. Data processing

Complete mitochondrial genomes, which were available from 10 taxa of *Echinococcus* spp. (Le et al., 2002; Nakao et al., 2002, 2007) and from *Taenia solium* (Nakao et al., 2003b), served as comparative sequences (Accession Nos. AB018440, AB086256, AB208063-4, AB208545-6, AB235846-8, AF297617 and AF346403). The nuclear DNA sequences of *elp* (Xiao et al., 2005; Hüttner et al., unpublished data) and *ef1a* (Nakao et al., unpublished data) in *Echinococcus* spp. were also used for comparisons (Accession Nos. AB159141-3, EF660053-4 and AB306933-40). The mtDNA sequences of *cox1*, *nad1* and *cob* were translated to amino acid sequences by the echinoderm mitochondrial genetic code (Nakao et al., 2000; Telford et al., 2000).

Multiple alignments of nucleotide and amino acid sequences were made gene by gene, using the Clustal W program via the internet (http://clustalw.ddbj.nig.ac.jp). The alignment of *rrn* was corrected by deleting ambiguous positions such as loops and indels. The conversion of the aligment format to PHYLIP or NEXUS was done by the program Seqret of EMBOSS (Rice et al., 2000). The percentage divergence values of nucleotide sequences were computed by PAUP4.0b10 (Swofford, D.L., 2002. PAUP: phylogenetic analysis using parsimony (and other methods) 4.0 beta. Sinauer Associates, Sunderland, Massachusetts, USA.) using Kimura's two parameter model (Kimura, 1980) with a γ-shape parameter ($a = 1$).

The nucleotide and amino acid sequences derived from mitochondrial genes (*cox1*, *nad1*, *cob* and *rrn*) were used for phylogenetic analyses. The alignments of nucleotide sequences were concatenated into a DNA data set (total 5170 sites). Likewise, the alignments of amino acid sequences were concatenated into a protein data set (total

Table 1
Primer pairs used for PCR

Target genes	Primers for the first PCR (5′–3′)[a]	Primers for the second PCR (5′–3′)
cox1 (mtDNA)	F: TTACTGCTAATAATTTTGTGTCAT	–
	R: GCATGATGCAAAAGGCAAATAAAC	–
nad1 (mtDNA)	F: TGGAACTCAGTTTGAGCTTTACTA	F: TATTAAAAATATTGAGTTTGCGTC
	R: ATATCAAAGTAACCTGCTATGCAG	R: TCTTGAAGTTAACAGCATCACGAT
cob (mtDNA)	F: GTGGTATTGACATTTTGTAGATTA	F: GTTACTAATAGGTTAGTTTAAACT
	R: AATTTCCAGAGTAGAAATCACCAT	R: CGCCCAACAGTTAAAAAATAACTA
rrn (mtDNA)	F: TCCTGTAGCTTGTCATAATGATTA	F: ACTTATGGTGTGTATTATATGCGT
	R: CTGATTCTCACTACCAAATTTAAC	R: ATAAATACACAAACCGCCACAATA
ef1α (nuclear DNA)	F: TGGGCAAGTCCTCTTTCAAGTACG	F: CTTGATAAGCTCAAGGCTGAGCGT
	R: GCGACAGTCGATCTCATGTCACGA	R: ATTGGTTTGCTGGGCACCATTCTA
elp (nuclear DNA)	F: ATGCGCGTGAGAGTCTTCAGAAGA[b]	F: GAGATGAACAGAAAGCTGAAGGAG
	R: ATTCTGCGAAGCTCAGCTTCA[b]	R: CTTGGCCATGGCGACCTTCTGAGC
cox1 (mtDNA)[c]	F: ACTGTTGGGTTGGATGTTAAGACG	F: TAGTTCTGTTACTATGATTATAGG
	R: CATAACATAATGAAAATGAGCCA	R: GTATCATGTAAAACATTATCCAAC
nad1 (mtDNA)[c]	F: TGTTTTTGAGATCAGTTCGGTGTG	F: CAGTTCGGTGTGCTTTTGGGTCTG
	R: CATAATCAAACGGAGTACGATTAG	R: GAGTACGATTAGTCTCACACAGCA
cob (mtDNA)[c]	F: TTATGCTATACTTCGGTGTATTA	F: TCGGTGTATTAATTCGAAGATTG
	R: ATAAGGATACTCCGGATGACAAC	R: GATGACAACCACCCAAATAAGTC

[a] F, forward primer; R, reverse primer.
[b] Primers previously designed by Xiao et al. (2005).
[c] Primers used for historical collections of *Echinococcus felidis* adults.

1188 sites). PAUP4.0b10 was employed for maximum likelihood (ML) analysis using the DNA data set. The substitution model GTR + G + I and its parameters were determined by Akaike Information Criterion (AIC) implemented in MODELTEST 3.7 (Posada and Crandall, 1998). A full heuristic search algorithm was run to estimate the ML tree. The ML analysis using the protein data set was implemented using Tree-Puzzle 5.2 (Schmidt et al., 2002) with the substitution model JTT + G + I. In both analyses, the robustness of inferred trees was tested by bootstrapping with 1000 replicates.

Partitioned Bayesian analysis was carried out by MrBayes 3.1 (Ronquist and Huelsenbeck, 2003). The DNA data sets were divided into four partitions. The nucleotide substitution models GTR + G + I for *cox1*, *nad1* and *rrn* partitions and HKY + G + I for *cob* partition were selected by AIC implemented in MrModeltest 2.2 (modified from MODELTEST by Johan Nylander, Uppsala University, Sweden). The protein data sets were divided into three partitions, and their substitution models were determined by AIC in PROTTEST 1.26 (Abascal et al., 2005) as follows: Mtmam + G + I for *cox1* protein, JTT + G for *nad1* protein and Mtmam + G for *cob* protein. Using each of the data sets, a Metropolis-coupled Markov chain Monte Carlo analysis was run for 1 million generations to estimate the posterior probabilities of trees. The tree sampling was undertaken every 1000 generations and all tree samples before the chain reached a stationarity were discarded as burn-in.

In all the phylogenetic analyses, the trees were rooted with *T. solium* as the outgroup taxon.

3. Results

3.1. DNA profile of taeniid eggs from lion feces

Parasite eggs of Diphyllobothriidae, Taeniidae, Ancylostomatidae and Trichuridae were detected in the lion feces, in accordance with a previous survey in the region (Müller-Graf, 1995). Out of 52 fecal samples, 36 contained taeniid eggs. Based on preliminary result of *cox1*-sequencing, one fecal sample was selected and the eggs purified from this sample were used for the present genetic study.

Using *Echinococcus*-derived primers (Table 1), the DNA fragments of four complete mitochondrial genes (*cox1*, *nad1*, *cob* and *rrn*) and two partial nuclear genes (*ef1a* and *elp*) were amplified from each of three taeniid eggs. The length of nucleotide sequences determined were 1608 bp in *cox1*, 891 bp in *nad1*, 1068 bp in *cob*, 1603 bp in *rrn*, 973 bp in *ef1a* and 907 bp in *elp*. The sequences of each gene were completely identical among the three eggs. As shown in Table 2, all the sequences of the taeniid eggs were compared with the known sequences of *Echinococcus* spp. and *T. solium*. The mitochondrial gene sequences obtained from the eggs were similar to those of *Echinococcus* spp., but the pairwise divergence values showed that the taeniid egg species is distinct from all known species of the genus. In the nuclear genes, the pairwise divergence values were very low when compared with *Echinococcus* spp., but the sequences of the eggs were clearly distinguishable from those of *E. granulosus* sensu stricto, *E. ortleppi* and *E. canadensis* (genotype G6), which are known to be present in eastern Africa. In conclusion, the eggs were found to belong to a taxon of *Echinococcus*, which had not yet been genetically characterized.

3.2. Species identification

To test the hypothesis that the eggs belong to *E. felidis*, we examined the historical specimen of *E. felidis*. Standard PCR protocols did not succeed in amplifying mitochondrial genes from the long-preserved adults of *E. felidis*. However, the use of alkaline lysate as a template for nested PCR and the size reduction of target DNA to <200 bp succeeded in amplifying short regions of *cox1*, *nad1* and *cob*. The nucleotide sequences of *cox1* from position 913 to

Table 2
Pairwise divergence values (%) of mitochondrial and nuclear DNA sequences between lion-derived taeniid egg and confirmed species

Lion-derived taeniid egg compared with	Mitochondrial genes				Nuclear genes	
	cox1	*nad1*	*cob*	*rrn*	*ef1α*	*elp*
Echinococcus granulosus sensu stricto	8.4	17.1	11.6	6.6	1.4	1.7
Echinococcus canadensis G6	10.6	17.9	14.8	9.2	1.0	1.6
Echinococcus canadensis G7	10.5	17.9	15.0	9.2	1.0	–
Echinococcus canadensis G8	11.1	19.3	15.0	8.6	0.9	–
Echinococcus equinus	8.1	16.7	12.4	7.6	–	–
Echinococcus ortleppi	10.6	18.4	14.8	8.9	0.9	1.5
Echinococcus vogeli	10.0	17.8	14.6	8.4	1.8	–
Echinococcus oligarthrus	11.1	20.0	18.1	10.1	2.4	–
Echinococcus multilocularis	10.4	19.3	15.2	10.4	2.9	5.1
Echinococcus shiquicus	10.4	22.9	15.7	12.1	2.2	4.2
Taenia solium	21.6	37.4	30.2	28.3	–	–
	(11.2)[a]	(20.8)	(15.1)	(11.0)	(3.3)	(5.4)

The complete sequences of mitochondrial genes and the partial sequences of nuclear genes were used for comparison.
[a] Percentage divergences between *E. multilocularis* and *E. granulosus* sensu stricto are shown in parentheses.

1110, *cob* from position 818 to 988 and *nad1* from position 405 to 589 were used for comparison. The positions were numbered from each start codon, based on the complete mtDNA sequences (Accession No. AF297617) of *E. granulosus* sensu stricto. These short mtDNA sequences of *E. felidis* adults were clearly distinguishable from those of other *Echinococcus* spp., and showed a 100% identity with those of the taeniid eggs from Uganda (Supplementary Fig. S1). Therefore, we conclude that both the taeniid eggs from Ugandan lion feces and the preserved worms from South Africa belong to the same taxon, *E. felidis*.

Unfortunately, we were unable to reconfirm the morphological characteristics of the adult *E. felidis*, because the long-preserved worms were too hard, brittle and incomplete for observation of the diagnostic morphological features.

3.3. Phylogenetic analyses

Using the long mtDNA sequences (*cox1*, *nad1*, *cob* and *rrn*) from the taeniid eggs, the taxonomic status of *E. felidis* and its phylogenetic relationship with other *Echinococcus* spp. were determined by ML and partitioned Bayesian analyses. The bootstrapped phylogenetic trees were identical in both analyses (Supplementary Fig. S2A). As reported previously (Nakao et al., 2007), the neotropical endemic species *Echinococcus oligarthrus* and *Echinococcus vogeli* occupied basal positions. Sister species relationships between *E. canadensis* and *E. ortleppi* and between *E. multilocularis* and *E. shiquicus* were also reconfirmed in these analyses. In addition, a new pair of sister species was recognized, *E. granulosus* sensu stricto and *E. felidis*. The high values of bootstrap proportions and Bayesian posterior probabilities support these relationships. The species pair *E. felidis* and *E. granulosus* sensu stricto forms the most basal lineage among the examined taxa except for *E. oligarthrus* and *E. vogeli*. A phylogram obtained by ML analysis shows the degree of genetic relatedness (Fig. 1).

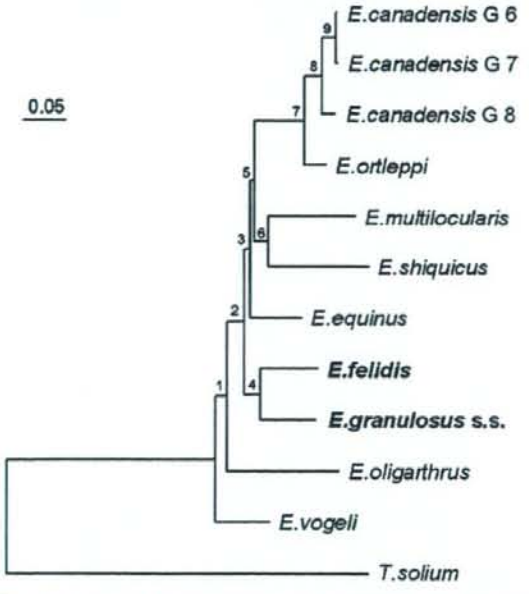

Fig. 1. Phylogram of *Echinococcus* species obtained using the maximum likelihood method. The scale bar represents the estimated number of nucleotide substitutions per nucleotide site. The sister species relationship between *Echinococcus felidis* and *Echinococcus granulosus* sensu stricto is shown in boldface. The bootstrap proportions (%) of maximum likelihood (ML) and the posterior probabilities (%) of Bayesian analyses (PB) for nodes Nos. 1–9 are: (1) 44/50, (2) 85/100, (3) 82/100, (4) 97/100, (5) 49/82, (6) 94/100, (7) 100/100, (8) 100/100, (9) 100/100, (ML/PB).

The phylogeny of *Echinococcus* spp. was also reconstructed from mitochondrial proteins deduced from *cox1*, *nad1* and *cob*. Maximum likelihood and partitioned Bayesian analyses using the protein data set resulted in a cladogram with some differences relating to the position of *E. equinus* and the positions of *E. vogeli* and *E. oligarthrus* relative to each other. However, the sister species relationship between *E. felidis* and *E. granulosus* sensu stricto was still highly supported by both the analyses (Supplementary Fig. S2B).

4. Discussion

In recent years, the phylogenetic tree of *Echinococcus*, mainly based on mtDNA, has been gradually completed by inclusion of most of the described taxa (Thompson et al., 2006; Nakao et al., 2007; Ito et al., 2007). The present study is a further step towards an understanding of the phylogeny of the genus by including one of its most elusive members, *E. felidis*. Our analysis shows that *E. felidis* is an independent taxon, as its genetic distance (pairwise divergence values of mt genes) to its closest relative, *E. granulosus* sensu stricto, is in the range of the distance between *E. multilocularis* and *E. shiquicus*, and far exceeds the distance between *E. ortleppi* and genotypes of *E. canadensis* (Nakao et al., 2007). Divergence values are also far higher than between the well accepted species pair *Taenia saginata* and *Taenia asiatica* (Jeon and Eom, 2006; Nakao et al., 2007). The recent proposition to split *E. granulosus* sensu lato, of which *E. felidis* is doubtlessly a member, into various species is presently under debate. It is acknowledged that there are a number of details which still need further clarification, e.g. the status of genotypes in the cluster *E. ortleppi–E. canadensis* and the extent of intergradation between various species in sympatric situations. In the case of *E. felidis* we have no doubt about the species status due to the maintenance of genetic identity over a wide geographical range (isolates from South Africa collected in the 1960s versus isolates from Uganda collected in 2007), the distinct morphology (Verster, 1965), and the unique use of a felid as definitive host.

Recently, the phylogenetic utility of mitochondrial protein-coding genes was assessed by using six taxa of cestodes and five taxa of trematodes (Hardman and Hardman,

2006). They demonstrated that reliable nodes were recovered when 4.0 kb had been sampled and recommended *nad2* or *cox1* as a region to include in a sampling program. In this study, three protein-coding genes including *cox1* (total 3567 nucleotide sites) and ribosomal RNA genes (1603 nucleotide site) were sampled to reconstruct the phylogeny of *Echinococcus* spp. Maximum likelihood analysis using the mtDNA data set resulted in two unreliable nodes with low bootstrap values (Fig. 1 and Supplementary Fig. S2A), suggesting that our sampling was insufficient. However, three nodes involving sister species pairs were robust in the resultant tree. In particular, the sister species relationship between *E. felidis* and *E. granulosus* sensu stricto was supported further by partitioned Bayesian analyses using nucleotide and amino acid sequences.

In cladistic taxonomy, the discovery of sister species relationships is important in studying the evolutionary processes of closely related organisms. As reported in the genus *Taenia* (Hoberg, 2006), sister species and their geographical distribution play a crucial role in understanding the evolutionary origin of human parasites. In this study, molecular phylogenetic analyses demonstrated a sister species relationship between *E. felidis* and *E. granulosus* sensu stricto. Human cystic echinococcosis caused by *E. granulosus* sensu stricto has a global impact (Budke et al., 2006), and its evolutionary origin remains to be determined. A recent molecular phylogenetic study revealed that modern Felidae arose in Asia (Johnson et al., 2006). According to their estimation, Asian-derived *Panthera* species spread into America (jaguar and lion) and into Africa (lion and leopard) in late Pliocene (3–2 million years ago). Because of the lack of a molecular clock in tapeworms, we cannot estimate the molecular date of bifurcation between *E. felidis* and *E. granulosus* sensu stricto exactly. However, the high values of pairwise divergence in their mitochondrial genes (Table 2) indicate that the bifurcation had occurred already in Asia, when the general estimation of base substitution in mtDNA (2% divergence per million years) was applied (Avise et al., 1992). In this scenario, *E. felidis* invaded Africa together with lions in the late Pliocene. The dog tapeworm *E. granulosus* sensu stricto utilizes sheep as the main intermediate host, and the populations of moufflon (*Ovis gmelini*) in Asia and Asia Minor are widely accepted as the ancestors of domestic sheep (Pedrosa et al., 2005). This information also supports the hypothesis that *E. granulosus* sensu stricto originated in Asia. Further studies on host range and geographical distribution are needed to test these hypotheses. In contrast to *E. granulosus* sensu stricto which was globally transferred with livestock, *E. felidis* as been less subject to anthropogenic re-distribution, and any record of its presence should reflect the natural range.

In Africa, the dog tapeworms *E. granulosus* sensu stricto, *E. ortleppi* and *E. canadensis* (genotype G6) occur in sheep, cattle and camels, respectively (McManus and Thompson, 2003). The distribution of these domesticated mammals overlaps with the natural habitats of wildlife hosts for *E. felidis*. Since the metacestodes of these parasites develop into similar unilocular cysts, the morphological identification of cystic larvae is impossible in domestic and wildlife intermediate hosts. Likewise, taeniid eggs in the feces of African lions are microscopically indistinguishable, and at least six species of taeniid tapeworms have been recorded from *Panthera* (Dinnik and Sachs, 1972; Loos-Frank, 2000). Due to this diagnostic uncertainty, there exist very few confirmed data on host range and geography of the parasite. We do not know if lions are exclusive definitive hosts, or if other felids, canids or hyaenids are also involved in the lifecycle. Likewise, we know very little about the intermediate host range. There is, of course, no doubt that transmission occurs via the important prey species of lions. However, lions have a very catholic diet that can differ substantially between regions. Hayward and Kerley (2005) compiled data from 32 studies on the diet of lions from different locations and found that the diet included 42 prey species, mainly ungulates of the orders Artiodactyla and Perissodactyla. Almost 20 species of wild ungulates are on record as harbouring hydatid cysts (Macpherson and Wachira, 1997). Not all of these cysts necessarily belong to *E. felidis*, as was recently demonstrated by the recovery of *E. granulosus* sensu stricto (G1) from a wild warthog in East Africa (Hüttner et al., unpublished data). Zebra (*Equus quagga*) can be considered as an intermediate host, as experimental infection of lions with cysts from zebra has resulted in successful infections (Young, 1975).

At present, there are no data available on the pathogenicity of *E. felidis* to humans. Its close relationship with *E. granulosus* sensu stricto suggests a zoonotic potential, as the latter species is apparently responsible for the majority of human cases worldwide (Jenkins et al., 2005). However, the public health impact of this species is expected to be minimal, as lions are largely restricted to national parks and game reserves with limited human activity. It may have some impact on pastoralists in East Africa who still coexist with wildlife such as the Maasai in Kenya and Tanzania. Ultrasound scanning surveys demonstrated the frequent occurrence of cystic echinococcosis among the Maasai of northern Tanzania (prevalence 1.1%) (Macpherson et al., 1989). These pastoralists settle on the eastern border of the Serengeti where wildlife is common even outside the protected area. An exposure to lion-derived *Echinococcus* eggs is therefore feasible, but further epidemiological studies are required to evaluate the pathogenicity of *E. felidis* to humans.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijpara.2007.10.013.

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Short Communication

Molecular identification of *Echinococcus* isolates from Peru

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ABSTRACT

Genetic variations in tapeworms causing cystic echinococcosis in Peru were investigated. Seventy one larval isolates collected from different intermediate hosts and geographic regions were identified by the DNA sequencing of genes for mitochondrial cytochrome *c* oxidase subunit 1 (*cox1*) and nuclear elongation factor 1 alpha (*ef1a*). The G7 genotype (*E. canadensis* pig strain) was found for the first time in pigs reared in the city of Lima. *Echinococcus granulosus* sensu stricto (sheep strain or G1) was the most prevalent in human patients, sheep, and cattle and the G6 genotype (*Echinococcus canadensis* camel strain) was found in goats and in one human patient. These findings may inform prevention strategies and control programs against echinococcosis in Peru.

Key words:
Echinococcus
genetic variation
cox1
ef1a
haplotypes
Peru

Cystic echinococcosis (CE) is caused by the larval stage of tapeworms belonging to the cryptic species complex of *Echinococcus granulosus* sensu lato. CE occurs in sheep rearing regions of Peru where it is considered a major public health and economic problem [1]. Surveys in endemic villages have shown human infection prevalences raging from 5.5-9.3% [1,2]. Molecular studies using mitochondrial DNA sequences have identified 10 distinct genotypes (G1-10) within *E. granulosus* sensu lato [3,4]. These include two sheep strains (G1 and G2), two bovid strains (G3 and G5), a horse strain (G4), a camel strain (G6), two pig strain (G7 and G9) and cervid strains (G8 and G10). Because the taxon *E. granulosus* sensu lato is paraphyletic, the following taxonomic revision has been made: *E. granulosus* sensu stricto (G1-3), *E. equinus* (G4), *E. ortleppi* (G5) and *E. canadensis* (G6-G10) [5]. However, some controversy continues to exist regarding the taxonomical status of *E. canadensis*. Lavikainen et al. [4] and Moks et al. [6] have emphasized the close relationship between *E. ortleppi* (G5) and *E.canadensis* (G6-G10) whereas Thompson [8] suggests G6 and G7 could represent a species of its own, *E.intermedius*, distinct from *E. canadensis* (G8 and G10). *E. granulosus* sensu stricto is most commonly associated with human infections worldwide. Knowledge of the frequency, distribution in different host species and geographic distribution of these genotypes is important for development of control strategies because some of them have different growth and development characteristics. This study describes a survey of *Echinococcus* isolates from different hosts and regions in Peru.

Hydatid cysts were obtained from different animal hosts slaughtered at the Yerbateros abattoir in the city of Lima (table 1). In addition, human hydatid cysts excised from human patients who had surgery for CE at the Department of Thoracic Surgery at Hospital Nacional Hipolito Unanue in Lima were also examined. Ethical approval was obtained from Ethical Committees of Asociación Benéfica PRISMA and Hospital Nacional Hipólito Unanue in Lima, Perú. Animal and human samples were collected during October 2006-January 2007. An *Echinococcus* isolate was defined as protoscoleces obtained from a single hydatid cyst. The genomic DNA of each isolate was prepared from 70% ethanol-preserved protoscoleces by using DNeasy blood and tissue kit (Qiagen, Germany), and used as a template for polymerase chain reaction (PCR). Regions targeted for PCR were a mitochondrial gene for cytochrome *c* oxidase subunit 1 (*cox1*) and a nuclear gene for elongation factor 1 alpha (*ef1a*). The PCR was carried out by using a published primer set for *cox1* [8] or an original primer set for *ef1a* (5'-AATGGATGCAG

TTGATTACAGTGA-3' and 5'-GCAGGGTTAGTCTCCTTGA CCTGA-3'). Thermal reactions were performed for 35 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 60 sec. Each PCR product was directly sequenced as reported previously [5]. The nucleotide lengths determined were 789 bases in *cox1* and 656 bases in *ef1a*. The phylogenetic tree of *cox1* genotypes was constructed by neighbour-joining method [9].

A total of 80 larval *Echinococcus* isolates were obtained from 32 sheep, 16 cattle, 21 pigs, 6 goats, and 5 humans. The PCR amplification of *cox1* was successful in 71 isolates. The negative results of 9 isolates from pigs were probably due to the low concentration or the degeneration of DNA. Table 1 shows the geographic origins and hosts of 71 *Echinococcus* isolates identified by *cox1* genotyping. Fifty seven (80%) isolates were identified as *E. granulosus* sensu stricto, which included 5 haplotypes (designated as *Egra*-A to *Egra*-E). Six (9%) isolates corresponded to the G6 genotype of *E. canadensis* with 2 haplotypes (*Ecan*-A and *Ecan*-B), and 8 (11%) isolates to the G7 genotype of *E. canadensis* with 1 haplotype (*Ecan*-C). The phylogenetic relationships of these haplotypes were illustrated in Figure 1. The mitochondrial haplotypes found in this study were deposited into DDBJ/EMBL/GenBank databases under the accession numbers **AB458672-5** and **AB470527** (*Egra*-A to *Egra*-E) and **AB458676-8** (*Ecan*-A to *Ecan*-C).

Two species of *Echinococcus* were found from humans. As shown in Table 1, 4 of 5 patients were infected with *E. granulosus* sensu stricto, and the remaining one with *E. canadensis* (G6 genotype). All patients were males with an age range 10 to 28 years (mean 20 years). Two patients had a non-complicated lung cyst and 3 had a complicated lung cyst. No distinguishing clinical features were observed in the patient infected with the G6 genotype. All 32 sheep and 16 cattle were infected with *E. granulosus* sensu stricto. The dominant parasite in 6 goats was *E. canadensis* (G6 genotype), although one isolate was identified as *E. granulosus* sensu stricto (G1 genotype). In pigs, 8 of 12 isolates were identified as *E. canadensis* (G7 genotype), while 4 isolates as *E. granulosus* sensu stricto. Seven of the 12 isolates were originated from liver, 1 from lung, and 4 from kidney. The liver-specific tropism was found in pigs infected with the G7 genotype. In the PCR amplification of nuclear *ef1a*, positive results were obtained from 67 isolates. The PCR positive rate of nuclear *ef1a* was smaller than that of mitochondrial *cox1*, probably due to the low copy number of the nuclear gene. The DNA sequencing of *ef1a* proved 55 isolates to be *E. granulosus* sensu stricto and 12 isolates to be *E. canadensis*. The G6 and G7

genotypes of *E. canadensis* showed the identical sequences of *ef1a*. Mitochondrial introgression was not observed in all isolates examined.

We identified the G7 genotype for the first time in pig isolates from Peru. A previous study in Peru reported the presence of the G1 and G6 genotypes in human isolates [10], however in our study we examined a larger number of hosts from very diverse geographic regions of Peru and this allowed us to identify the G7 genotype. In our study, all pig isolates originated from districts surrounding the city of Lima, and this finding alone is cause of concern as it demonstrates *Echinococcus* transmission in the city of Lima considered to be a low endemic area for this cestode. In most areas of Peru, pigs are raised along with other livestock and are allowed to roam freely in the fields where they can have easy access to feces of dogs infected with *Echinococcus* spp. In some highly endemic areas, the prevalence of swine echinococcosis can be as high as 83% [11]. It is important to determine if the G7 genotype is as prevalent in other *Echinococcus* endemic areas of Peru as it is in Lima pigs or if Lima pigs present a unique situation that favours the occurrence of this genotype alone given the absence of other intermediate hosts in Lima. The G7 genotype has been previously described in pig isolates from Argentina [12-14] and Mexico [15] but thus far it has not been isolated in humans in South America. An interesting finding was the predominance of liver hydatid cysts among pigs infected with the G7 genotype which is in agreement with previous findings that report a predominant liver localization for cysts of the G7 genotype [16]. Although it would appear that the pig strain is poorly infective to humans, further studies should help define the distribution of the G7 genotype in Peru and its presence in the human population.

We found one Peruvian patient to be infected with the G6 genotype, and we did note that most of the G6 isolates occurred in goats, suggesting this species can be one of the reservoirs for this genotype. The other Latin America country where the G6 genotype has been described is Argentina [12]. Future studies with human and lower animal isolates from different geographic regions of Peru should further elucidate the frequency of the G6 genotype in human patients and other intermediate hosts. The finding of the G6 and G7 genotypes in Peru has important implications for public health. In dogs, for example, both genotypes develop into adults more quickly than the more common *E. granulosus* sensu stricto [16,17]. Intervention programs based on periodic anthelminthic treatment of canine hosts infected with the G6 and G7 genotypes may require shorter treatment intervals than those directed at other, more slowly maturing strains of

Echinococcus. This may mean greater expenditures for purchasing anthelmintics which could add financial constraint to control programs in developing countries.

In summary, the G7 genotype of *E. canadensis* (pig strain) has been found for the first time in Peruvian pigs from the city of Lima, demonstrating the involvement of *E. canadensis* in the urban transmission cycle of *Echinococcus*. *E. granulosus* sensu stricto (sheep strain) is predominant in animal and human hosts in Peru, but the G6 genotype of *E. canadensis* (camel strain) is also present in goats and can cause human infection.

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