

Molecular discrimination of *Echinococcus granulosus* and *Echinococcus multilocularis* by sequencing and a new PCR-RFLP method with the potential use for other *Echinococcus* species

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Background/aim: To develop a novel polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) protocol using a new genomic marker sequence and a novel set of restriction enzymes in order to detect and discriminate 2 *Echinococcus* species, *E. granulosus* and *E. multilocularis*, found in formalin-fixed paraffin-embedded (FFPE) human tissues.

Materials and methods: DNA was isolated from 11 FFPE human tissue samples positive for cystic echinococcosis or alveolar echinococcosis. A mitochondrial genomic marker region was amplified and sequenced using a novel primer pair and a new PCR-RFLP protocol was developed for the detection and discrimination of *E. granulosus* and *E. multilocularis* using a set of restriction enzymes including *AccI*, *MboI*, *MboII*, and *TsoI*.

Results: The selected marker region was amplified using DNA isolated from FFPE human tissue samples positive for cystic echinococcosis or alveolar echinococcosis and the discrimination of *E. granulosus* and *E. multilocularis* was accomplished by use of the novel PCR-RFLP method.

Conclusion: In this PCR-RFLP protocol, use of any single restriction enzyme is enough for the discrimination of *E. granulosus* and *E. multilocularis*. The PCR-RFLP protocol can be potentially used for the discrimination of 5 other *Echinococcus* species: *E. oligarthus*, *E. shiquicus*, *E. ortleppi*, *E. canadensis*, and *E. vogeli*.

Key words: FFPE tissues, *Echinococcus granulosus*, *Echinococcus multilocularis*, sequencing, PCR-RFLP

1. Introduction

Echinococcosis is a cosmopolitan zoonosis caused by adult or larval stages of cestodes belonging to the genus *Echinococcus*. The 2 major species of medical and public health importance are *Echinococcus granulosus* and *Echinococcus multilocularis*, which cause cystic echinococcosis (CE) and alveolar echinococcosis (AE), respectively (1–4).

The diagnostic procedure for CE as well as for AE includes clinical, radiological, and particularly serological examinations (2). Ultrasonography, computer tomography, and magnetic resonance are used in radiological diagnosis (5,6). Indirect hemagglutination, indirect fluorescent antibody test, and enzyme linked immunosorbent assay are used in serological

diagnosis (7,8). However, the definitive diagnosis is usually based on pathological-histological analysis of particularly periodic acid–Schiff-stained specimens of surgically resected tissues (9). On the other hand, histological examinations are hampered by some difficulties. First, sometimes *Echinococcus* cysts are sterile, making the diagnosis of cysts difficult. It is also possible in some cases to have difficulties in the discrimination of *E. granulosus* and *E. multilocularis*. In addition, subgenotypes of *E. granulosus* (G1–G10) and subspecies of *E. multilocularis* can be discriminated using molecular methods, although discrimination is not possible using histological methods (10).

Molecular methods, unlike classical histopathological methods, can both detect and discriminate *Echinococcus*

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species. Cyst liquid, cyst membrane, and protoscolex were used in many previous studies on *Echinococcus* phylogenetics (11). However, fresh materials from humans are only available in regions where the disease is endemic (12). Therefore, instead of fresh material, formalin-fixed and paraffin-embedded tissues (FFPETs) are commonly used in molecular studies. FFPETs represent the most important specimens for diagnostic surgical pathology and, moreover, have proven to be an invaluable source for retrospective studies. Moreover, paraffin blocks are easy to store for long periods and to transport to the laboratory (11,12).

In contrast to classical histopathological examinations, molecular methods provide the opportunity to conclusively determine species as well as strains within the genus *Echinococcus*, which causes serious diseases worldwide. Molecular examinations mostly focus on mitochondrial and nuclear genomic sequences and sequences such as *cox1*, cytochrome c oxidase subunit 1; *nad1*, NADH dehydrogenase subunit 1; *atp6*, ATPase subunit 6; *cob*, cytochrome b; and *rrnL*, large-subunit rRNA, and these sequences have been used as markers. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) is one of the common methods to discriminate *Echinococcus* species and subspecies (13–15). In PCR-RFLP, a marker region is amplified by PCR and the amplified DNA fragment is digested by specific restriction enzymes. Different sizes of digestion products will discriminate between different species. Lately, in depth analyses of DNA by sequencing and multiplex PCR have been extensively performed for the identification and phylogenetic classification of *Echinococcus* species. These studies utilized the complete mitochondrial genome or nuclear and mitochondrial markers (16–18).

In this study, we designed a novel primer pair for sequencing analysis. We developed a new PCR-RFLP protocol using a new genomic marker sequence and a novel set of restriction enzymes in order to detect and discriminate 2 *Echinococcus* species, *E. granulosus* and *E. multilocularis*, found in human FFPETs.

2. Materials and methods

2.1. Collection of samples

In this study FFPET samples were used. Ten FFPET samples histomorphologically positive for CE and 1 FFPET sample positive for AE were obtained. Genomic DNA for *E. multilocularis* was a kind gift from Dr Bruno Gottstein. All tissue samples were confirmed to be *Echinococcus*-positive by histomorphological analysis. Samples were obtained from the Department of Pathology of the Medical Faculty of Firat University and from the Medical Faculty of Erciyes University. This study was approved by the Elazığ Clinical Investigations Institutional Ethics Committee.

2.2. DNA isolation

Deparaffinization and dehydration of FFPET samples were performed. Seven embedded tissue slices of 10 µm in size were collected. These pieces were deparaffinized in 1 mL of xylene at 65 °C for 10 min. The samples were centrifuged at 3500 rpm for 5 min and the supernatant was discarded (this step was repeated 5 times). Dehydration was performed using 70%, 80%, 90%, and 100% ethanol, respectively.

DNA isolation from FFPET samples was performed using the Wizard Genomic DNA Purification Kit (Promega, USA) by modifying the proteinase K step. The modified step was as follows: each sample was incubated in 600 µL of EDTA/Nuclei Lysis Solution and 15 µL of proteinase K (Sigma, Germany) at 55 °C in a shaker water bath for 48 h. At 12-h intervals, 5 µL of fresh proteinase K (50 mg/mL) was added. In the last step, DNA was resuspended in 100 µL of DNA rehydration solution and stored at –20 °C.

2.3. Primer design and PCR

The mitochondrial genomic region of 871 bp was PCR-amplified using the genomic DNA extracted from FFPET samples. The amplified genomic region spans the last part of a large ribosomal RNA gene, the cysteine tRNA gene, and the beginning of a small ribosomal RNA gene. We designed novel primers as follows: PEmg F (5'-GTTAAGTTACCCTAGGGATAAC-3') and PEmg R (5'-TACTATTAAGTCCTAAA-3') (Figures 1A and 1B).

In PCR reactions, 12.5 µL of 2X master mix (Vivantis, Malaysia), 2 µL of 20 pmol PEmg FR primers, 1 µL of genomic DNA, and 9.5 µL of distilled water were used for 25 µL of reaction mix. PCR conditions were as follows: 1) hot start at 95 °C for 5 min; 2) 35 cycles of denaturation at 94 °C for 1 min, annealing at 42 °C for 1 min, and extension at 72 °C for 1 min; and 3) final extension at 72 °C for 10 min. Amplicons obtained by PCR amplification were run in 1.5% agarose gel at 120 V for 30 min and visualized in Gel Logic 212 PRO (Carestream, USA). Amplicons were purified using the GF-1 AmbiClean Kit (Vivantis) before moving forward to the restriction digestion analysis.

2.4. DNA sequencing

PCR products were sequenced and confirmed. The purified products were subjected to cycle sequencing by the dideoxynucleotide chain termination method using the DYEnamic ET Terminator Cycle Sequencing Kit (Amersham, USA), with PEmg F and PEmg R primers as sequencing primers. The products were separated in an automated DNA genetic analyzer (MegaBACE 500 Genetic Analyzer). The GenBank BLAST program was used for comparisons.

2.5. Restriction fragment length polymorphism

Purified PCR products were cut by the following restriction enzymes: *TsoI* (Fermentas, USA), *MboI* (BIORON,

Germany), *MboII* (Fermentas), and *AccI* (Fermentas). Restriction reactions were performed in 20 µL using approximately 0.5–1 µg DNA with the following conditions for each enzyme: *TsoI*, 3 units at 55 °C for 16 h; *MboI*, 10 units at 37 °C for 1.5 h; *MboII*, 2.5 units at 37 °C for 1.5 h; and *AccI*: 10 units at 37 °C for 16 h. Digestion products were separated in 2% (w/v) agarose gels, visualized in Gel Logic 212 PRO, and photographed.

3. Results

3.1. Amplification of the mitochondrial genomic region of *E. granulosus* and *E. multilocularis*

Genomic DNA from FFPET samples was isolated and used for the following experiments. A pair of novel primers was designed to amplify a selected mitochondrial genomic region of both *E. granulosus* (9171–10,042 bp) and *E. multilocularis* (11,585–12,461 bp). The genomic region of approximately 875 bp spans the last part of a large ribosomal RNA gene, cysteine tRNA gene, and the beginning of a small ribosomal RNA gene. Restriction enzymes that differentially digest the amplified mitochondrial genomic region of *E. granulosus* and *E. multilocularis* were selected. The DNA sequence of the amplified genomic region of *E. granulosus* and *E. multilocularis* and restriction enzyme sites used on this sequence are depicted in Figure 1. PCR fragments amplified using the designed primers were sequenced for confirmation. *E. granulosus* and *E. multilocularis* were clearly discriminated based on sequencing results (Figure 1). Confirmed PCR products were purified and used for the RFLP analysis.

3.2. Restriction fragment length polymorphism

Four DNA restriction enzymes were selected to digest the amplified genomic region. PCR fragments amplified using *E. multilocularis* and *E. granulosus* genomic DNA were digested with each of selected restriction enzymes. Restriction digestion products and uncut controls that were not digested with restriction enzymes were visualized. The PCR fragment amplified using *E. multilocularis* genomic DNA as the template was digested by *MboI* and the digestion resulted in DNA fragments with sizes of 788 bp and 89 bp. The PCR fragment amplified using *E. granulosus* genomic DNA as the template was digested by *MboI*; the digestion did not result in any digested DNA fragments and only the uncut full-length PCR fragment was visualized (Figure 2). The PCR fragment amplified using *E. multilocularis* genomic DNA as the template was digested by *MboII* and the digestion resulted in DNA fragments with sizes of 690 bp and 187 bp. The PCR fragment amplified using *E. granulosus* genomic DNA as the template was digested by *MboII*; the digestion did not result in any digested DNA fragments and only the uncut full-length PCR fragment was visualized (Figure 3). The PCR fragment amplified using *E. multilocularis* genomic DNA as the template was digested by *AccI* and the digestion resulted in DNA fragments with sizes of 660 bp and 217 bp. The PCR fragment amplified using *E. granulosus* genomic DNA as the template was digested by *AccI*; the digestion did not result in any digested DNA fragments and only the uncut full-length PCR fragment was visualized (Figure 4). In contrast, the PCR fragment amplified using *E. multilocularis* genomic DNA as the template was digested

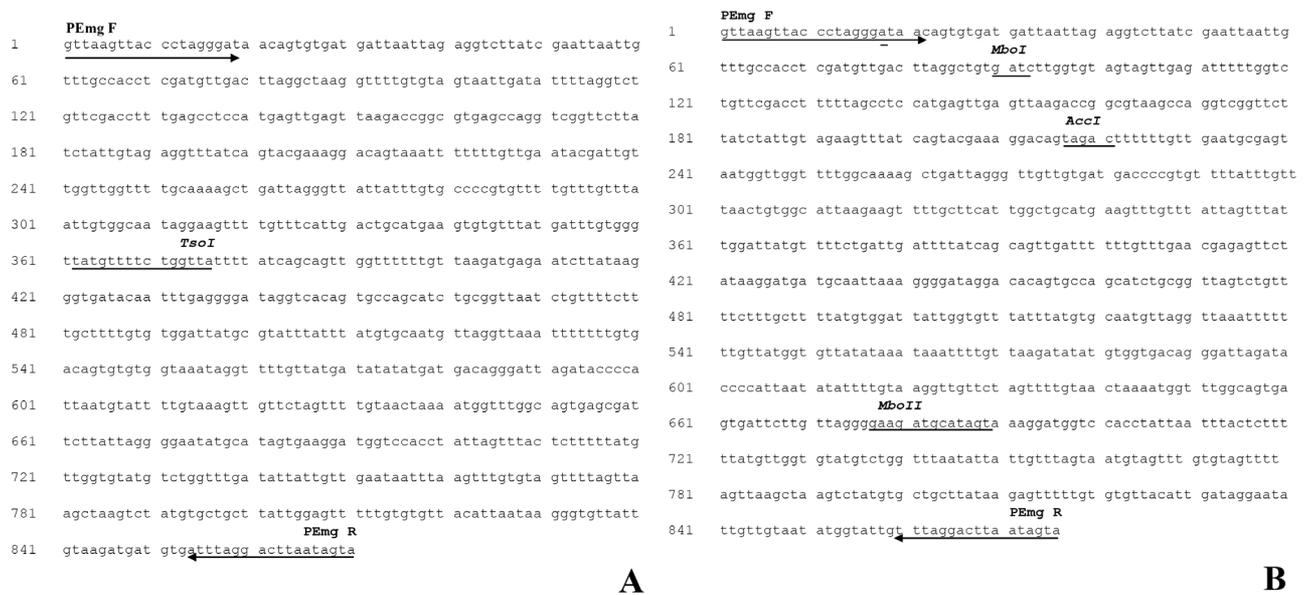


Figure 1. Map of restriction enzyme sites in the amplified mitochondrial genome region of A) *E. granulosus* and B) *E. multilocularis*.

MboI

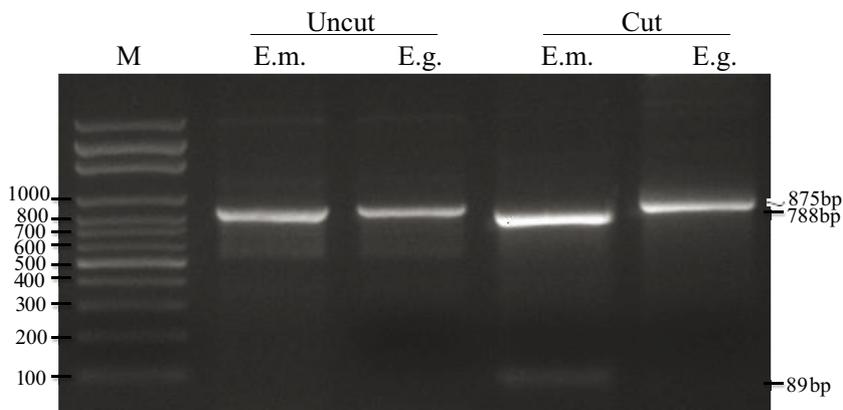


Figure 2. A mitochondrial genomic region was amplified using *E. multilocularis* (E.m.) and *E. granulosus* (E.g.) genomic DNA, digested with *MboI*, and visualized. From left to right, lane M is the DNA ladder, the second and third lanes are uncut controls, and the fourth and fifth lanes are PCR fragments digested with *MboI*. The digestion of the PCR fragment produced using E.m. genomic DNA as the template resulted in 2 digestion products of 788 bp and 89 bp as visualized in the fourth lane. The digestion of the PCR fragment produced using E.g. genomic DNA as the template did not result in any restriction fragment as visualized in the fifth lane.

MboII

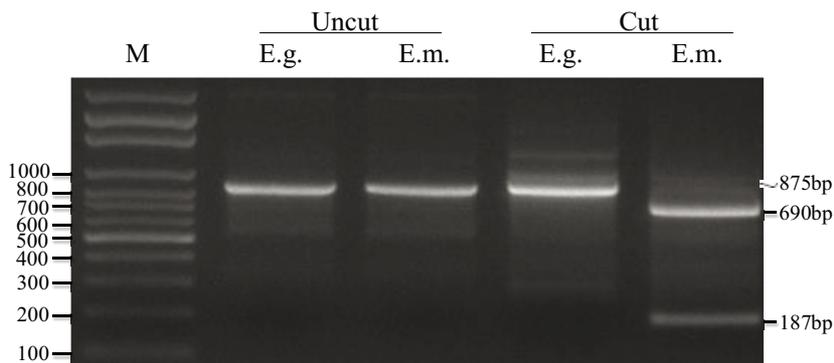


Figure 3. A mitochondrial genomic region was amplified using *E. multilocularis* (E.m.) and *E. granulosus* (E.g.) genomic DNA, digested with *MboII*, and visualized. From left to right, lane M is the DNA ladder, the second and third lanes are uncut controls, and the fourth and fifth lanes are PCR fragments digested with *MboII*. The digestion of the PCR fragment produced using E.m. genomic DNA as the template resulted in 2 digestion products of 690 bp and 187 bp as visualized in the fifth lane. The digestion of the PCR fragment produced using E.g. genomic DNA as the template did not result in any restriction fragment as visualized in the fourth lane.

by *TsoI* and the digestion did not result in any digested DNA fragments. The PCR fragment amplified using *E. granulosus* genomic DNA as the template was digested by *TsoI* and the digestion resulted in DNA fragments with sizes of 362 bp and 510 bp (Figure 5).

4. Discussion

Genomic DNA was obtained from FFPET samples. FFPET samples are among the most commonly used and available samples. Use of FFPET samples in molecular studies has increased significantly. It is crucial to be able to use

AccI

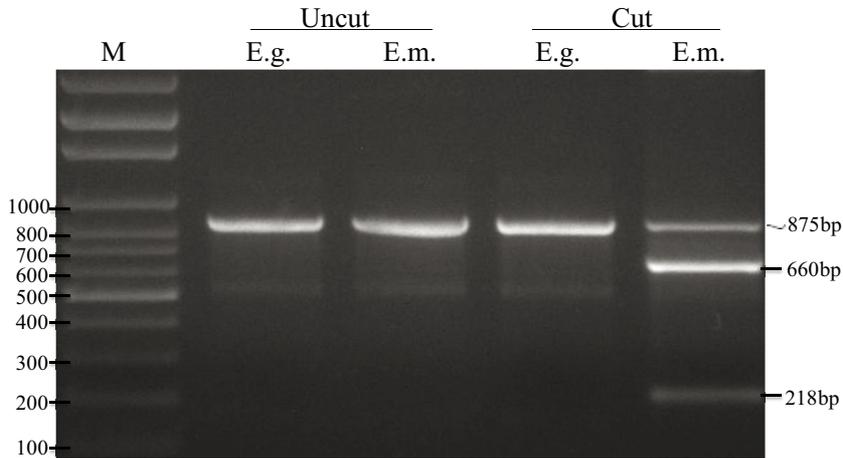


Figure 4. A mitochondrial genomic region was amplified using *E. multilocularis* (E.m.) and *E. granulosus* (E.g.) genomic DNA, digested with *AccI*, and visualized. From left to right, lane M is the DNA ladder, the second and third lanes are uncut controls, and the fourth and fifth lanes are PCR fragments digested with *AccI*. The digestion of the PCR fragment produced using E.m. genomic DNA as the template resulted in 2 digestion products of 660 bp and 217 bp as visualized in the fifth lane. The digestion of the PCR fragment produced using E.g. genomic DNA as the template did not result in any restriction fragment as visualized in the fourth lane.

TsoI

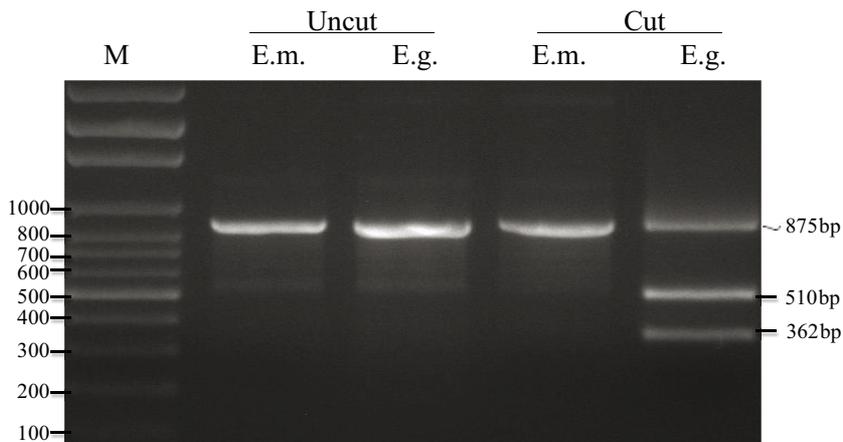


Figure 5. A mitochondrial genomic region was amplified using *E. multilocularis* (E.m.) and *E. granulosus* (E.g.) genomic DNA, digested with *TsoI*, and visualized. From left to right, lane M is the DNA ladder, the second and third lanes are uncut controls, and the fourth and fifth lanes are PCR fragments digested with *TsoI*. The digestion of the PCR fragment produced using E.g. genomic DNA as the template resulted in 2 digestion products of 510 bp and 362 bp as visualized in the fifth lane. The digestion of the PCR fragment produced using E.m. genomic DNA as the template did not result in any restriction fragment as visualized in the fourth lane.

DNA isolated from FFPET samples to do further PCR amplifications and RFLP analysis (10). In this study, it was

possible to amplify the target genomic region using the DNA from FFPET samples.

Molecular methods were developed in order to detect and discriminate *Echinococcus* species by making use of mitochondrial and nuclear genomic sequences. Characterization of *E. granulosus* was attempted by PCR-RFLP using an ITS-1 DNA sequence in Iran, an endemic region (19). The discrimination of *E. granulosus* and *E. multilocularis* and specific discrimination of *E. granulosus* strains have been performed by PCR-RFLP (20). Mitochondrial and nuclear markers (cox1, cytochrome c oxidase subunit 1; nad1, NADH dehydrogenase subunit 1; atp6, ATPase subunit 6; cob, cytochrome b; rrnL, large-subunit rRNA) were used in the genetic characterization of *Echinococcus shiquicus* (21). The discrimination of taeniid cestode species including *E. granulosus* and *E. multilocularis* has been done by PCR-RFLP of ITS-2 ribosomal DNA (22). Another PCR-RFLP method for the identification of the 3 species of *E. granulosus*, *E. multilocularis*, and *E. shiquicus* utilizes the species-specific *SspI* digestion of a large subunit of ribosomal RNA gene *rrnL* (23).

In this study, we designed a novel primer pair that can amplify a genomic marker region containing the last part of a large ribosomal RNA gene and the beginning of a small ribosomal RNA gene in both *E. multilocularis* and *E. granulosus*. Additionally, it is possible to amplify the same genomic region with the same primer pair using the genomic DNA of several other species such as *E. oligarthus*, *E. shiquicus*, *E. orteppi*, *E. canadensis*, and *E. vogeli*. We sequenced the amplified PCR product using the DNA isolated from *Echinococcus*-positive human FFPET samples and did the molecular discrimination of *E. multilocularis* and *E. granulosus* based on sequencing results (Figure 1). It is also possible to discriminate other *Echinococcus* species by PCR and sequencing using this novel set of primer pairs.

We also developed a new PCR-RFLP using the same genomic region amplified by our primer set to discriminate *E. multilocularis* and *E. granulosus*. This PCR-RFLP method can be used to discriminate other *Echinococcus* species including *E. oligarthus*, *E. shiquicus*, *E. orteppi*, *E. canadensis*, and *E. vogeli* (Table). We used 4 different restriction enzymes to discriminate *E. multilocularis* and *E. granulosus*. Three of these enzymes (*MboI*, *MboII*, and *AccI*) were only able to digest the PCR fragment produced using the genomic DNA of *E. multilocularis* and the other enzyme (*TsoI*) was only able to digest the PCR fragment produced using the genomic DNA of *E. granulosus*. We showed that it is possible to decide whether a sample contains *E. multilocularis* or *E. granulosus* in 4 different ways using our new PCR-RFLP protocol. It is also possible to perform a sequential double digest using *TsoI* and 1 of the other 3 restriction enzymes (*MboI*, *MboII*, or *AccI*). By doing so, one can decide rapidly whether it is *E. multilocularis* or *E. granulosus* by visualizing DNA fragments in a single reaction. Moreover, combinations of these enzymes can also be used to decide whether the sample contains *E. shiquicus* (digestion is possible only with *MboII* and *TsoI*), *E. oligarthus* (digestion is possible only with *MboI*), or the other 3 species (for *E. orteppi*, *E. canadensis*, and *E. vogeli*, digestion is possible only with *MboII*) (Table).

Although there are numerous studies on the molecular discrimination of *Echinococcus* species utilizing sequencing, PCR-RFLP, and multiplex PCR, our study provides: 1) an alternative procedure using a single PCR reaction for amplification of the marker genomic region in 7 *Echinococcus* species, and 2) a PCR-RFLP method that can potentially identify 7 *Echinococcus* species using 4 restriction enzymes. In this study, the method was successfully applied for the identification of

Table. Positions of restriction enzyme sites in amplified genomic region for different species of *Echinococcus*.

Restriction enzymes	<i>AccI</i>	<i>MboI</i>	<i>MboII</i>	<i>TsoI</i>
<i>E. multilocularis</i> (AB018440.2)	217	89	690	–
<i>E. granulosus</i> (AF297617.1)	–	–	–	362
<i>E. oligarthus</i> (AB208545.1)	–	40	–	–
<i>E. shiquicus</i> (AB208064.1)	–	–	691	20
<i>E. orteppi</i> (AB235846.1)	–	–	689	–
<i>E. canadensis</i> (AB208063.1)	–	–	695	–
<i>E. vogeli</i> (AB208546.1)	–	–	686	–

E. multilocularis and *E. granulosus*, and further studies for the application of this method to identify the other 5 *Echinococcus* species are warranted.

In conclusion, we developed a new PCR-RFLP protocol for the detection and discrimination of *E. granulosus* and *E. multilocularis* using the genomic region containing the last part of a large ribosomal RNA gene, the cysteine tRNA gene, and the beginning of a small ribosomal RNA gene as the marker and making use of a novel set of restriction enzymes.

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