■ BRIEF COMMUNICATION

Practical Algorisms for PCR-RFLP-Based Genotyping of *Echinococcus granulosus* Sensu Lato

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Abstract: *Echinococcus granulosus* sensu lato (s.l.) is a causative agent of cystic echinococcosis or cystic hydatid disease in humans and domestic and wild animals. The disease is a serious health problem in countries associated with poverty and poor hygiene practices, particularly in livestock raising. We introduced a practical algorism for genotyping the parasite, which may be useful to many developing countries. To evaluate the efficiency of the algorism, we genotyped 3 unknown strains isolated from human patients. We found that unknowns 1 and 3 were included in G1, G2, and G3 genotypes group and unknown 2 was included in G4 genotype (*Echinococcus equinus*) according to the algorisms. We confirmed these results by sequencing the 3 unknown isolates *cox1* and *nad1* PCR products. In conclusion, these new algorisms are very fast genotype identification tools that are suitable for evaluating *E. granulosus* s.l. isolated from livestock or livestock holders, particularly in developing countries.

Key words: *Echinococcus granulosus*, practical algorism, genotyping, *cox1*, *nad1*

Echinococcus granulosus sensu lato (s.l.) is a causative agent of cystic echinococcosis or cystic hydatid disease in humans and domestic and wild animals [1]. This disease is a serious health problem in countries associated with poverty and poor hygiene practices, particularly livestock raising [2]. Until recently, the taxonomy of *E. granulosus* s.l. spp. has been controversial because of the limited availability of morphological diagnostic characters in both adult and larval stages [3]. The incomplete development of metacestodes in aberrant hosts has further limited the identification of causative organisms. In the 1990s, an Australian research group [4-6] conducted molecular taxonomic analyses of *E. granulosus* s.l. using short mitochondrial DNA sequences of genes for cytochrome *c* oxidase subunit 1 (*cox1*) and NADH dehydrogenase subunit 1 (*nad1*). The analyses showed that *Echinococcus multilocularis*, *Echinococcus vogeli*,

and *Echinococcus oligarthra* are distinct from each other and that *E. granulosus* s.l. can be divided into genotypes G1-G8 [7]. These genotypes correspond to the following strains: *E. granulosus* sensu stricto (s.s.) G1 (sheep strain), G2 (Tasmanian sheep strain), G3 (buffalo strain); *Echinococcus equinus* G4 (horse strain); *Echinococcus ortleppi* G5 (cattle strain); *Echinococcus canadensis* G6 (camel strain), G7 (pig strain), and G8 (cervid strain) [3,8]. Currently, these genotypes are used as standards for molecular classification when considering the epidemiology of cystic echinococcosis [9]. Another *E. canadensis* G9 (unidentified strain) and G10 (Fennoscandian cervid strain) were subsequently characterized [10,11], while the status of G9 remains unclear [3,8].

Although entire or partial gene sequencing is effective for determining genotypes, specialized equipment is required to conduct these costly and time-consuming procedures. In many developing countries, the supply of such equipment is insufficient, and thus PCR-RFLP is frequently used [12,13]. In fact, most countries in which this parasite is prevalent are economically underdeveloped, and thus a simple PCR-RFLP method is necessary. However, PCR-RFLP results must be compared with

previously reported PCR-RFLP results. Using this method, some under-digested DNA fragments may be confused with fully digested fragments, limiting the interpretation of PCR-RFLP results. To overcome this limitation, we developed a PCR-RFLP algorism involving only 2 or 3 restriction enzymes that produce only 1 or 2 fragments in PCR-RFLP, which is highly helpful for improving the quality of the results.

The *cox1* and *nad1* sequences from 9 *E. granulosus* s.l. geno-

types (G1-G10, except G9) were downloaded from GenBank (https://www.ncbi.nlm.nih.gov/nucgss). After multiple alignment (MEGA software version 6), we searched for new PCR target lesions that could produce 1 or 2 fragments after restriction endonuclease digestion using NEBcutter version 2.0 (http://nc2.neb.com/NEBcutter2/). The PCR primer sets for *cox1* and *nad1* are constructed like as cox-f [CC(C/T) GG(A/G) TTT GGT(A/G) T(A/T) ATT], cox-r [ATC(A/G) TG(C/T) AA
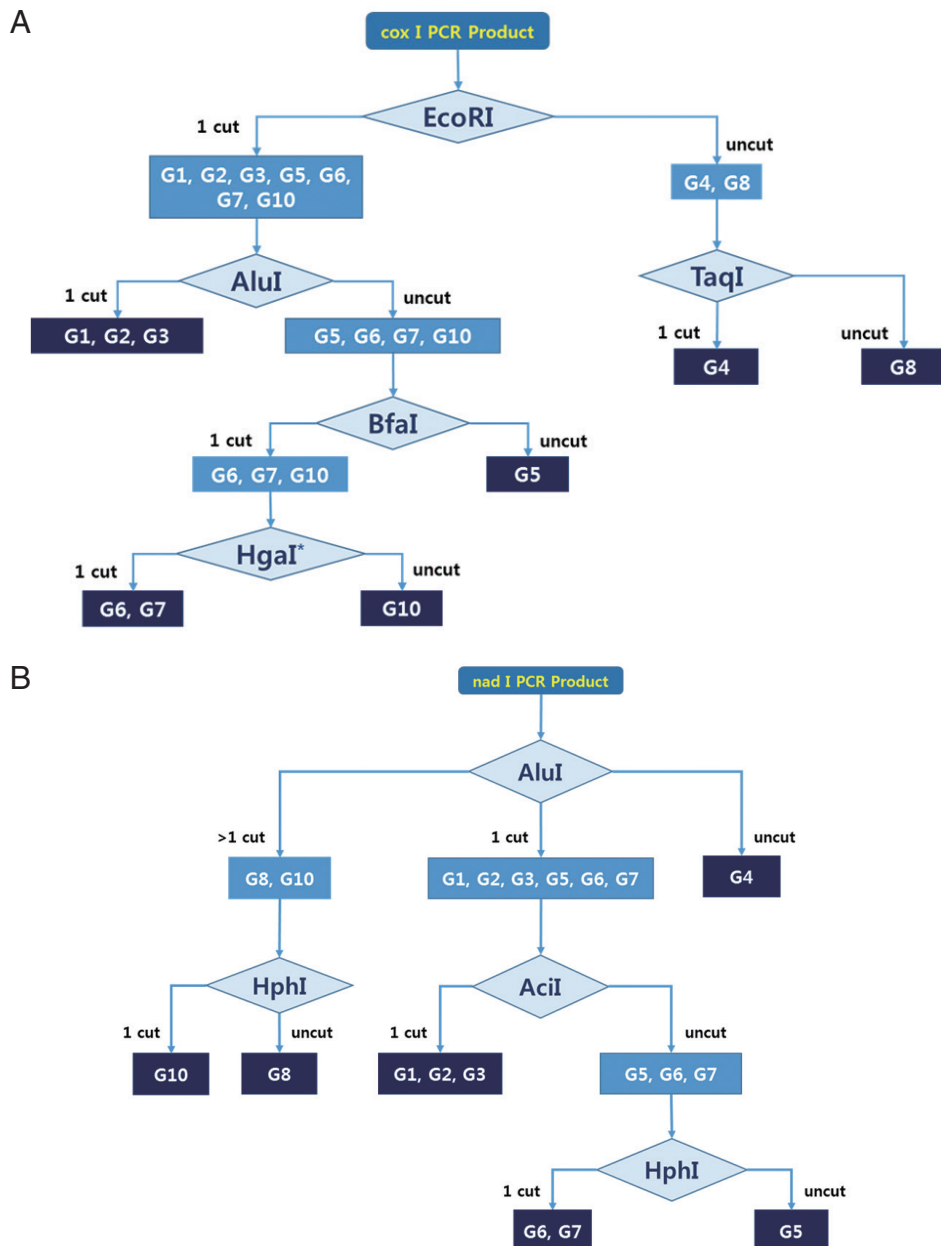

Fig. 1. A practical algorism for *E. granulosus* s.l. genotyping using PCR-RFLP of *cox1* and *nad1*. Schematic representation of hypothetical RFLP by several restriction enzymes. (A) Algorism for *cox1* PCR-RFLP genotyping. *Hga*I* could be replaced with *Tsp*45I or *Bsa*HI. (B) Algorism for *nad1* PCR-RFLP genotyping.

AA(C/T) ATT ATC], nad-f [A(G/A)(A/T) TTC GTA AGG G(G/C) C CTA ATA], and nad-r [(A/T)CC(A/T) CT AAC(T/C) AA TTC ACT TTC]. We predicted hypothetical PCR-RFLP fragments based on various endonuclease recognition sites, and selected appropriate restriction enzyme sets for each *cox1* and *nad1* PCR RFLP algorism. We used more than 10 restriction enzymes for hypothetical RFLP, as it was not possible to identify all genotypes using only 1 enzyme. Our goal was to develop an algorism that could identify all genotypes using the fewest enzymes. Most genotypes could be distinguished by using a specific restriction enzyme (Supplementary Fig. 1). However, we could not distinguish between the G1-G2, G1-G3, G2-G3, and G6-G7 genotypes using *cox1* and *nad1* PCR-RFLP in this study. Finally, we determined the best algorism for 2 mitochondrial genes (*cox1*, *nad1*) by comparing band patterns (Fig. 1). To evaluate the algorism, unidentified genomic DNA was isolated from 3 *Echinococcus* protoscoleces. Three hydatid cyst fluids were collected from patients who underwent medical treatment at Samarqand Hospital in Uzbekistan. The protoscoleces were obtained after centrifugation for 5 min at 2,000

×g. Total genomic DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer instructions. The genomic DNA was eluted in 200 µl of water and stored at -20℃ until PCR amplification. The extracted genomic DNA was amplified using noble primer sets for 2 mitochondrial DNA genes. PCR was carried out in a final volume of 50 µl containing 5 µl genomic DNA, 10X EF-Taq Reaction Buffer (25 mM MgCl$_2$ mixed), 2 µl of 10 mM dNTPs, 5 µl of 10 pmol of each primer, and 1 µl (2.5 U) of Di-aStar™ EF-Taq DNA Polymerase in a thermocycler (Takara, Shiga, Japan).

The following temperature profile was used for *cox1* amplification: initial denaturation at 94℃ for 30 sec; followed by 35 cycles of denaturation at 94℃ for 30 sec, annealing at 42℃ for 45 sec, and elongation at 72℃ for 35 sec. This was followed by final extension at 72℃ for 10 min. The following temperature profile was used for *nad1* amplification: initial denaturation at 94℃ for 30 sec; followed by 35 cycles of denaturation at 94℃ for 30 sec, annealing at 45℃ for 45 sec, and elongation at 72℃ for 35 sec. Final extension was performed at 72℃ for 10 min.
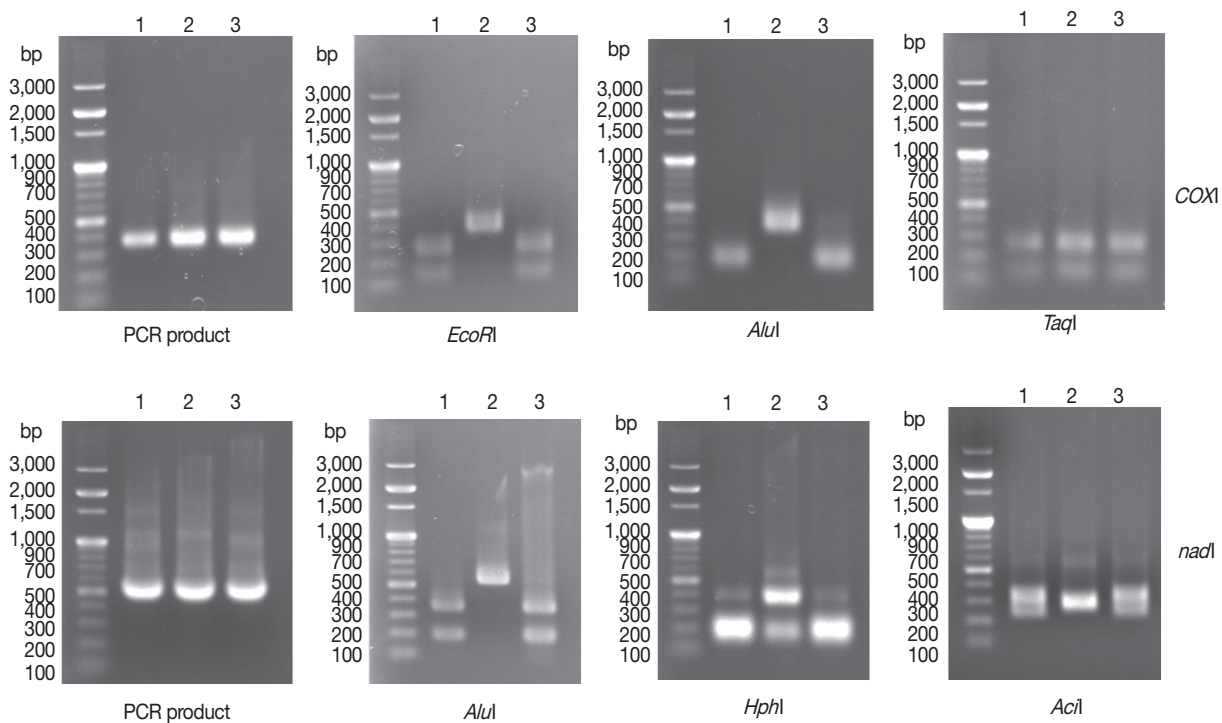

Fig. 2. Agarose gel electrophoretogram of PCR products and PCR-RFLP of 3 unidentified *Echinococcus* sp. isolated from echinococcosis patients. After PCR, each 5 µl PCR products of *cox1* and *nad1* were loaded onto a 1.0% agarose gel with 100-bp DNA ladder. The *cox1* gene PCR products were digested with *Eco*RI, *Alu*I, and *Taq*I according to noble genotyping algorism. For *nad1* PCR products, *Alu*I, *Hph*I, and *Aci*I were used for PCR-RFLP algorism. No. 1 and No. 3 isolates were identified as *E. granulosus* s.s. (G1-G3), but No 2. isolate was identified as *E. equinus* (G4). The 100-bp DNA Ladder Marker (Enzynomics) was used as a size marker.

PCR products were confirmed on 1.0% agarose gels under ultraviolet (UV) light. The PCR products were digested for 2-3 hr at 37°C by 4 restriction enzymes (*Alu*I, *Eco*RI, *Aci*I, and *Hph*I) and at 65°C by the restriction enzyme *Taq*I. Restriction fragments were separated on 1.5% agarose gels and photographed under UV light. After 1% agarose gel electrophoresis of *cox1* and *nad1* PCR products, we obtained 350-base pair (bp) (expected size 366 bp) *cox1* PCR products and 500-bp (expected size 530 bp) *nad1* PCR products (Fig. 2) from 3 human *Echinococcus* sample. After digestion of *cox1* gene PCR products with *Eco*RI, 2 PCR-RFLP patterns were observed. The genotypes of the 2 samples (unknowns 1 and 3) were likely G1, G2, G3, G5, G6, G7, or G10, while that of unknown 2 was likely G4 or G8. After *Alu*I digestion, we found that unknowns 1 and 3 were included in *E. granulosus* s.s (G1, G2, and G3 genotypes). Finally, we found that unknown 2 was included in *E. equinus* (G4 genotype) based on *Taq*I digestion. For the *nad1* PCR-RFLP genotyping algorism, the same results were observed for 3 different restriction enzymes (*Alu*I, *Hph*I, and *Aci*I) (Fig. 2).

To confirm the PCR-RFLP algorism, *nad1* and *cox1* PCR products of unidentified genotypes were sequenced after purifying the PCR products with Expin™ PCR SV (GeneAll Biotech, Seoul, Korea). Sequences and the reference *E. granulosus* s.l. genotype sequences were subjected to multiple alignment and phylogenetic distances were calculated using BLAST (http://www.ncbi.nlm.nih.gov/) and MEGA software version 6. Phylogenetic trees were constructed using the neighbor-joining method in MEGA software version 6.0. DNA sequencing yielded consensus sequences of the *cox1* and *nad1* genes. Key reference sequences for genotyping were used according to previous studies [14,15]. Sequences analysis of *cox1* PCR products using alignment and phylogenetic analysis with reference sequences indicated that unknowns 1 and 3 were very similar to the sequences of G1 (99% identity), G2 (99% identity) and G3 (99% identity). The entire sequence of *cox1* PCR product of unknown 2 was identical to that of the G4 genotype (Fig. 3). For *nad1* gene sequence analysis, although there were some insignificant differences, we obtained similar results as those obtained for *cox1* (Fig. 3).

Based on morphological characteristics, 4 species are currently recognized in the genus *Echinococcus: E. multilocularis, E. vogeli, E. oligarthra*, and *E. granulosus* s.l. Other species such as *E.*
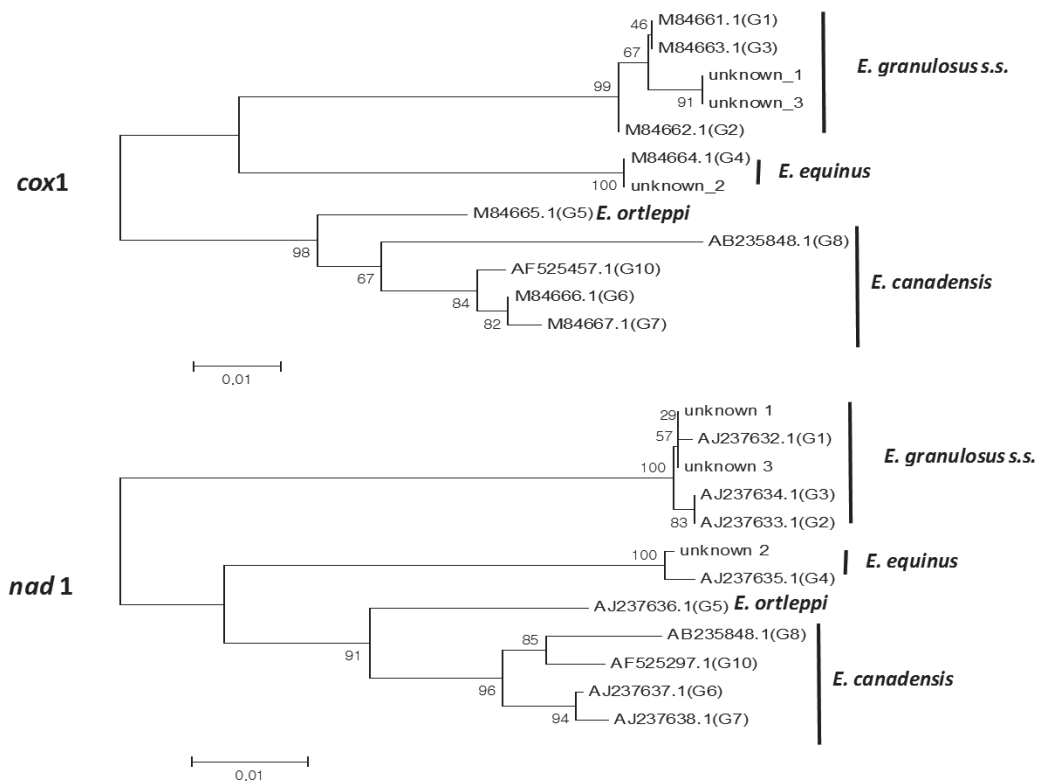

Fig. 3. Phylogenetic relationship of isolates and other genotypes previously identified. The *cox1* and *nad1* PCR products of *E. granulosus* isolates were sequenced and aligned with reference sequences.

shiquicus, E. felidis, E. ortleppi, E. equinus, E. canadensis and *E. intermedius* are currently classified as *E. granulosus* s.l. [3,8,16,17]. We constructed practical algorism for rapid genotyping of *E. granulosus* s.l. isolated from human and domestic animals using only 1-3 restriction enzymes. This method is less cost and more rapid than sequencing analysis. Although G1, G2, and G3 were not distinguished by this algorism, these genotypes have been recognized as the same species, *E. granulosus* s.s. [15,18,19]. The algorism distinguished G8 and G10 from the G6/G7 complex, but *E. canadensis* G6 and G7 could not be distinguished. Previous studies were also unable to separate the G6/G7 complex using mitochondria DNA, ITS1, and 18S ribosomal DNA sequencing [8,20].

There were some limitations to this study. The algorisms were developed for only 9 genotypes (G1-G10, except G9) of *E. granulosus* s.l. and not for *E. felidis, E. multilocularis, E. shiquicus, E. oligarthra,* and *E. vogeli,* which use wild animals as their intermediated hosts [8]. However, among these species, only *E. multilocularis* is known to commonly infect humans and is not found in domestic animals. Additionally, the morphological characteristics of their hydatid cysts are quite different from those of *E. granulosus* s.l. [20]. In these cases, genetic classification may be unnecessary for specific identification. Interestingly, we identified the first human infection case of *E. equinus* (Fig. 3). No human infections by the G4 genotype have been detected previously [21].

ACKNOWLEDGMENT

CONFLICT OF INTEREST

We have no conflict of interest related to this study.

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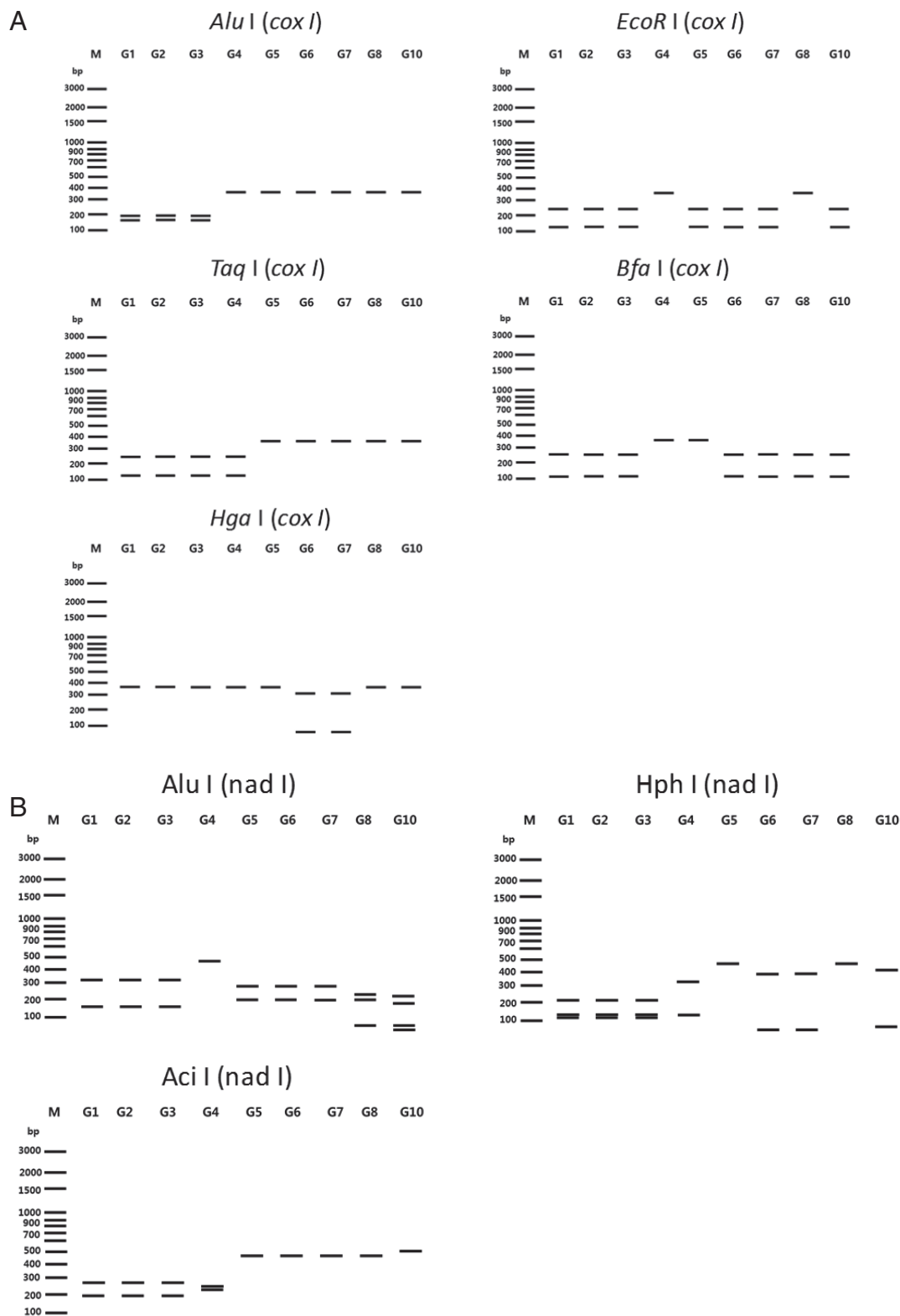
Supplementary Fig. 1. Schematic representation of hypothetical RFLP of *E. granulosus* s.l. species using four restriction enzymes. (A) The hypothetical pattern of bands using PCR product of *cox*I by *Alu*I, *EcoR*I, *Taq*I, *Bfa*I, and *Hga*I. (B) The hypothetical pattern of bands using PCR products of and 1 gene by *Alu*I, *Hph*I, and *Aci*I. The 100 bp DNA Ladder Marker (Enzynomics, Daejeon, Korea) was used as a size marker.