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Detection of genetic polymorphism among and within *Echinococcus granulosus* strains by heteroduplex analysis of a microsatellite from the U1 snRNA genes

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ABSTRACT. Polymerase chain reaction of a pentanucleotide microsatellite in the U1 snRNA gene complex generated a multiple band pattern due to the priming of paralogous sequences. Denaturation and slow renaturation of polymerase chain reaction products allow the formation of heteroduplex DNA that can be detected by its differential mobility in polyacrylamide gel electrophoresis. Heteroduplex analysis was used to determine if the U1 snRNA microsatellite could be a useful genetic marker in *Echinococcus granulosus*. A U1 snRNA microsatellite fragment from *E. granulosus* was isolated and characterized by Southern blot and sequencing. Four *E. granulosus* strains were analyzed: sheep, Tas-

manian sheep, cattle, and camel strains. The former two showed polymorphism and shared three of the six patterns found for sheep strain. The cattle strain displayed two patterns, and the camel strain was monomorphic. The electrophoretic profiles were used for statistical analysis in order to determine genetic distance and the relationship among strains. Heteroduplex analysis can be helpful in genotyping *E. granulosus* strains and is useful in detecting polymorphism within strains.

Key words: *Echinococcus granulosus*, Microsatellite markers, Heteroduplex DNA, U1 snRNA gene

INTRODUCTION

Echinococcus granulosus is an endoparasitic flatworm that is the causative agent of cystic hydatid disease in intermediate hosts (wild and domestic herbivores), which is one of the most important and widespread zoonoses (Thompson and Lymbery, 1995; McManus et al., 2003).

To date, molecular studies using mainly mtDNA sequences [cytochrome oxidase subunit 1 (CO1) and NADH dehydrogenase 1 (NADH1) genes] have identified ten distinct genotypes (G1-G10) within *E. granulosus* (Bowles et al., 1992; Scott et al., 1997; Lavikainen et al., 2003). This categorization follows very closely the patterns of strain variation emerging from biological and epidemiological traits (Thompson and McManus, 2002). According to Thompson and Lymbery (1988), a strain is a variant that differs statistically from other groups of the same species in gene frequencies and in one or more traits of actual or potential significance to the epidemiology and control of echinococcosis.

Several techniques using molecular markers such as restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA-polymerase chain reaction (PCR) and single-strand conformation polymorphism (SSCP-PCR) have been used in order to show the high degree of differentiation in the genus *Echinococcus* (Eckert and Thompson, 1997; Thompson and McManus, 2001). However, it has been shown that genetic variability within strains is present mainly in the *E. granulosus* G1 genotype or sheep strain (Haag et al., 1999; Kamenetzky et al., 2002).

The U1 snRNA is involved in RNA splicing. A study in *E. multilocularis* demonstrated that the U1 snRNA gene is more than 50 times tandemly repeated in the tapeworm genome, and that all copies are localized in one cluster. The gene repeat unit is 1,300 bp long and consists of a transcribed region of 156 bp and spacers in which microsatellites of three, four and five nucleotides are found (Bretagne et al., 1991).

Bretagne et al. (1996) analyzed polymorphism of the pentanucleotide microsatellite repeat number by examining patterns of amplification peaks of that sequence for *E. multilocularis*, which showed agreement with the geographical distribution of the samples. Besides, a further evaluation for *E. granulosus* of this microsatellite characterized it as a polymorphic molecular marker to genotype strains of *E. granulosus* in agreement with CO1 sequence analysis (Bart et al., 2004).

Amplification of the pentanucleotide microsatellite in the U1 snRNA gene complex could allow heteroduplex formation among the paralogous sequences, which differ in number and sequences of repetitive units. Heteroduplex DNA has different mobility in polyacrylamide gels due to the single-stranded loops within the heteroduplexes and can be visualized as additional bands in addition to the homoduplex fragments. The greater the heterogeneity among U1 snRNA microsatellite sequences, the greater the number of expected heteroduplex bands. It could work as an alternative to make good use of U1 snRNA microsatellites as potential molecular markers. To our knowledge, no attempts to analyze repeated loci by heteroduplex formation have been made to date.

With the aim of verifying the applicability of heteroduplex pattern analysis to a multiple repeated sequence (the pentanucleotide microsatellite in the internal spacer of the *E. granulosus* U1 snRNA gene complex), we isolated and characterized this sequence and studied the heteroduplex band patterns of PCR amplification products for four human infective *E. granulosus* strains: the sheep (G1), Tasmanian sheep (G2), cattle (G5), and camel (G6) strains, in order to detect polymorphism among these strains and within isolates of the same strain.

MATERIAL AND METHODS

Echinococcus granulosus total DNA extraction

Total DNA was extracted from protoscoleces of single hydatid cysts as described earlier (McManus et al., 1985), and the strain determination was performed by sequencing of mitochondrial COI gene (Kamenetzky et al., 2002) or by SSCP-PCR of six different DNA segments (Haag et al., 1999).

Isolation of a U1 snRNA gene segment containing a pentameric microsatellite

A forward primer designed by Bretagne et al. (1996) for *E. multilocularis* U1 snRNA gene flanking the pentanucleotidic repeat and a reverse primer designed in this study were used to amplify the pentameric microsatellite from *E. granulosus* (sheep strain).

PCR was carried out in a 50-µL reaction volume containing 20 ng *E. granulosus* total DNA, 2.5 units *Taq* DNA polymerase (Cenbiot), 100 µM of each dNTP, 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, and 20 pmol of each primer (U1 snRNA F - 5'ATTGTCGTTGCCAT CTCTCC3' and U1 snRNA R - 5'GCTCTCCATCACCACACATC3').

The samples were subjected to 20 cycles consisting of 1 min denaturation at 94°C, 1 min annealing at 50°C and 1 min extension at 72°C with a touchdown of 1°C at every cycle, followed by 20 more cycles at an annealing temperature of 40°C and a final 5 min extension at 72°C.

The PCR product was used as a probe in the Southern experiment and also cloned for sequencing.

Southern hybridization

Total DNA obtained from protoscoleces of a single hydatid cyst was completely digested with *Hin*dIII, *Eco*RI, *Rsa*I, *Taq*I, or *Alu*I, electrophoresed on 0.8% agarose gels, trans-

ferred to nylon membranes (Hybond N^+, Amershan-Pharmacia) and hybridized overnight at 60°C using 100 ng of a ^{32}P-labelled U1 snRNA probe according to standard protocols (Sambrook and Russel, 2001). The filter was washed for 20 min with 5X, 2X, 1X, and 0.2X SSC/0.1% SDS at 50°C.

Cloning of PCR products and sequencing

Amplified products were cloned into the pGEM-T Easy Vector (Promega), according to the manufacturer's instructions. Clones were sequenced using "Thermo Sequenase Radiolabeled Terminator Cycle Sequencing Kit" (USB) and the universal primers T7 and SP6.

U1 snRNA microsatellite analysis from different isolates

DNA samples from 45 *E. granulosus* isolates were used: four of camel strain, six of cattle strain, five of Tasmanian sheep strain, and 30 of sheep strain.

PCR conditions were the same as those described above, except for the cycling program. The samples were subjected to 30 cycles of 1 min at 94°C, 1 min at 55°C, 1 min at 72°C, and then 7 min at 72°C for final extension.

The cloned U1 snRNA sequence was used as the PCR positive control. Negative control was carried out without DNA.

Prior to electrophoresis, amplified DNA samples were denatured at 95°C for 5 min and slowly cooled at room temperature for 1 h to allow heteroduplex formation. Products were analyzed by electrophoresis on 6% polyacrylamide gels stained with $AgNO_3$.

Statistical analysis

For the cluster analyses, the heteroduplex DNA bands were assigned 0 or 1, depending on the absence or presence, respectively, of each band in the isolates of the analyzed strains. The numerical analysis of heteroduplex results was performed using the NTSYSpc program (Rohlf, 1998). The Jaccard coefficient was used to obtain a similarity matrix, and the SAHN (sequential, agglomerative, hierarchical, and nested clustering) method (Sneath and Sokal, 1973) was applied to obtain the corresponding dendrogram. A cophenetic value matrix was produced by COPH and used by the MXCOMP program to measure the goodness of fit of a cluster analysis to the similarity matrix produced by SAHN.

RESULTS

Detection and isolation of U1 snRNA microsatellite in the *E. granulosus* genome

Primers from *E. multilocularis* U1 snRNA sequence used to isolate the microsatellite of *E. granulosus* genomic DNA amplified a nearly 260-bp segment. The hybridization of the U1 snRNA probe to digested DNA generated a pattern of single (*Hin*dIII and *Eco*RI) or multiple (*Rsa*I, *Taq*I and *Alu*I) bands (Figure 1).

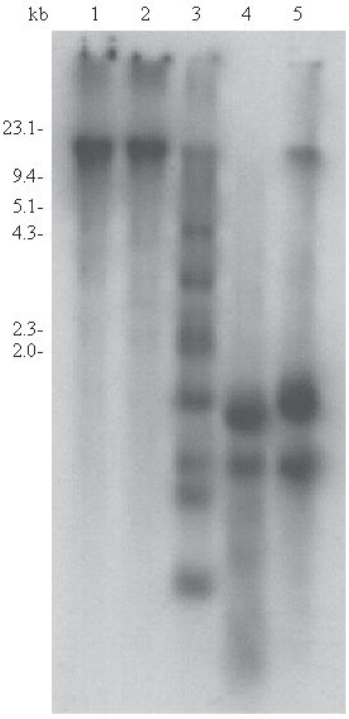

Figure 1. U1 snRNA presence in *Echinococcus granulosus* genome. Total DNA obtained from protoscoleces of a single hydatid cyst was digested with *Hin*dIII (lane 1), *Eco*RI (lane 2), *Rsa*I (lane 3), *Taq*I (lane 4), or *Alu*I (lane 5), electrophoresed on 0.8% agarose gels, transferred to a nylon membrane and hybridized to 100 ng of the U1 snRNA probe.

Cloning of PCR products

Six different clones containing the microsatellite sequence and its flanking regions were obtained, but each one different from the others and showing identity (91% for clone U1 snRNA-1 and 92% for the remainders) to the *E. multilocularis* U1 snRNA previously described sequence (Bretagne et al., 1996). Cloned products varied in repeat unit copy number as well as in the sequence of the tandemly repeated unit within the array. Two clones showed imperfect microsatellites while one showed a complex repeat with the main array (GACGA). Two sequences showed compound microsatellites (GACGA)(GCGAG) and one sequence showed another compound microsatellite (GACGA)(GGCGA) (see Table 1).

Heteroduplex analysis of U1 snRNA microsatellite amplification products in different *E. granulosus* isolates

Analysis of the PCR products by polyacrylamide gel electrophoresis after renaturation showed several bands of different mobilities between 200 and 400 bp, corresponding to heteroduplex DNA. The 260-bp fragment, which was more intensely stained for some samples, probably corresponds to homoduplex DNA (Figure 2).

Table 1. Pentanucleotide repeated sequences of the U1 snRNA microsatellite from the six clones obtained and their respective GenBank accession numbers.

Clone	Microsatellite sequence	Classification	Accession number
U1 snRNA-1	$(GACGA)_6(GGCGA)$	Imperfect	AY619589
U1 snRNA-2	$(GACGA)_4(GGCAG)(GCAGG)_2$ $(GCGAG)_3(ACGAG)(GCGAG)_2$	Complex	AY619590
U1 snRNA-3	$(GACGA)_8(GGCGA)$	Imperfect	AY619591
U1 snRNA-4	$(GACGA)_4(GGCGA)_3$	Compound	AY619592
U1 snRNA-5	$(GACGA)_5(GCGAG)_5$	Compound	AY619593
U1 snRNA-6	$(GACGA)_4(GCGAG)_4$	Compound	AY619594

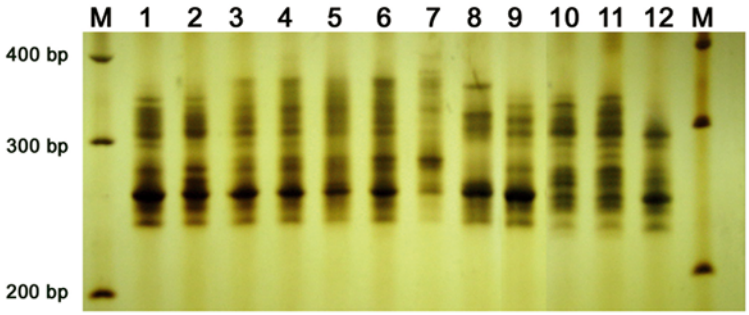

Figure 2. Polyacrylamide gel showing U1 snRNA microsatellite amplified products and different heteroduplex DNA band patterns formed in *Echinococcus granulosus* strains. M = molecular weight marker (100-bp ladder); lane 1 = pattern o1; lane 2 = o2; lane 3 = o3; lane 4 = o4; lane 5 = o5; lane 6 = o6; lane 7 = o7; lane 8 = o8, and lane 9 = o9, all of sheep and Tasmanian sheep strains; lane 10 = b1 and lane 11 = b2 of cattle strain, and lane 12 = c of camel strain. The numbers on the right are the lengths of the marker bands.

The four analyzed isolates of camel strain showed the same band migration pattern, called c (Figure 2 and Table 2).

The cattle strain had two very similar heteroduplex DNA migration pattern (Figure 3) among the six isolates analyzed. The cattle strain pattern is easily distinguishable from the others.

Sheep strain samples showed nine different heteroduplex DNA band patterns, three of which (o1, o3 and o6) were shared with Tasmanian sheep isolates (Table 2 and Figure 3).

DNA fragments amplified in independent experiments by PCR, using the same DNA sample and conditions, showed exactly the same heteroduplex patterns, indicating that the patterns are reproducible for each isolate (data not shown).

The statistical analysis of the dendrogram and the similarity-dissimilarity matrix showed the early separation of camel, cattle and sheep strains. The camel pattern was more closely related to cattle than sheep strain patterns. The genetic distance of pattern o8 to the other sheep strain patterns was as great as that between camel and cattle patterns. The other sheep and Tasmanian sheep patterns displayed a close relationship (Figure 3).

Table 2. *Echinococcus granulosus* strains, geographic origin, intermediate host, and heteroduplex DNA band patterns of a pentanucleotide microsatellite in U1 snRNA gene amplification products of the isolates analyzed.

Isolate	Strain	Origin	Intermediate host	Pattern
1	Camel	Argentina, Neuquém	Human	c
2	Camel	Argentina, Neuquém	Human	c
3	Camel	Argentina, Neuquém	Human	c
4	Camel	Argentina, Neuquém	Human	c
5	Cattle	Brazil, Cacequi	Bovine	b1
6	Cattle	Brazil, Tupanciretã	Bovine	b2
7	Cattle	Brazil, Santiago	Bovine	b2
8	Cattle	Brazil, São Pedro	Bovine	b2
9	Cattle	Brazil, Lavras do Sul	Bovine	b2
10	Cattle	Brazil, Lavras do Sul	Bovine	b2
11	Sheep	Brazil, Cacequi	Bovine	o2
12	Sheep	Brazil, Uruguaiana	Bovine	o1
13	Sheep	Brazil, Itaqui	Bovine	o9
14	Sheep	Brazil, Cacequi	Bovine	o2
15	Sheep	Brazil, Uruguaiana	Bovine	o2
16	Sheep	Brazil, Itaqui	Bovine	o8
17	Sheep	Brazil, Jaguarão	Bovine	o1
18	Sheep	Brazil, Bagé	Ovine	o7
19	Sheep	Brazil, Bagé	Ovine	o7
20	Sheep	Brazil, Bagé	Ovine	o2
21	Sheep	Brazil, Bagé	Ovine	o8
22	Sheep	Brazil, Bagé	Ovine	o7
23	Sheep	Brazil, Bagé	Ovine	o3
24	Sheep	Brazil, Bagé	Ovine	o5
25	Sheep	Brazil, Bagé	Ovine	o1
26	Sheep	Brazil, Bagé	Ovine	o3
27	Sheep	Argentina, Santa Cruz	Human	o7
28	Sheep	Argentina, Santa Cruz	Human	o7
29	Sheep	Argentina, Tucuman	Human	o2
30	Sheep	Argentina, Tucuman	Human	o9
31	Sheep	Argentina, Rio Negro	Human	o9
32	Sheep	Argentina, Rio Negro	Human	o3
33	Sheep	Argentina, Rio Negro	Human	o4
34	Sheep	Argentina, Rio Negro	Human	o3
35[1]	Sheep	Argentina, Rio Negro	Human	-
36	Sheep	Argentina, Neuquém	Human	o6
37	Sheep	Argentina, Neuquém	Human	o8
38	Sheep	Argentina, Neuquém	Human	o8
39	Sheep	Argentina, Santa Fé	Ovine	o2
40	Sheep	Argentina, Chubut	Ovine	o3
41	Tasmanian sheep	Argentina, Tucuman	Human	o6
42	Tasmanian sheep	Argentina, Tucuman	Human	o1
43	Tasmanian sheep	Argentina, Tucuman	Human	o6
44	Tasmanian sheep	Argentina, Tucuman	Human	o3
45	Tasmanian sheep	Argentina, Tucuman	Human	o1

[1]No amplification was obtained for isolate No. 35.

	o1	o2	o3	o4	o5	o6	o7	o8	o9	b1	b2	c
o1	1.0000000											
o2	0.8333333	1.0000000										
o3	0.5714286	0.4666667	1.0000000									
o4	0.5333333	0.4375000	0.9166667	1.0000000								
o5	0.3750000	0.3750000	0.5714286	0.6428571	1.0000000							
o6	0.4666667	0.4666667	0.8333333	0.9166667	0.6923077	1.0000000						
o7	0.4375000	0.4375000	0.6428571	0.7142857	0.6428571	0.7692308	1.0000000					
o8	0.2857143	0.2857143	0.2857143	0.2666667	0.3846154	0.2857143	0.3571429	1.0000000				
o9	0.5833333	0.4615385	0.5833333	0.5384615	0.3571429	0.4615385	0.4285714	0.2500000	1.0000000			
b1	0.1578947	0.1578947	0.1578947	0.1500000	0.1578947	0.1578947	0.2105263	0.1250000	0.1176471	1.0000000		
b2	0.0952381	0.0952381	0.0952381	0.0909091	0.0952381	0.0952381	0.1428571	0.1176471	0.0526316	0.7692308	1.0000000	
c	0.0666667	0.0666667	0.0666667	0.0625000	0.0666667	0.0666667	0.0625000	0.0909091	0.0833333	0.1428571	0.1333333	1.0000000

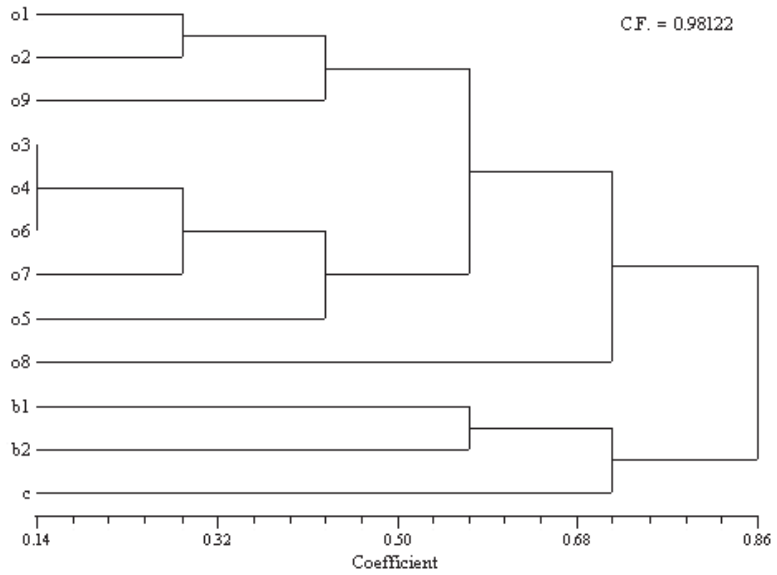

Figure 3. Genetic distance matrix and dendrogram obtained from heteroduplex DNA patterns of the U1 snRNA gene amplification, using NTSYSpc program. C.F. = cophenetic correlation coefficient.

DISCUSSION

The similarity of the U1 snRNA gene between *E. multilocularis* and *E. granulosus* was demonstrated by sequence analysis showing 91-92% identity within a segment of a 1300-bp sequence previously described in *E. multilocularis* (Bretagne et al., 1991). Besides, Southern blot analysis showed the same band pattern of the *E. multilocularis* U1 snRNA gene sequence, except for *Rsa*I which showed seven hybridized fragments instead of four as shown for *E. multilocularis.*

In the study of U1 snRNA pentanucleotide microsatellite of *E. multilocularis* isolates from Europe, Japan and North America, Bretagne et al. (1996) found three electrophoretic profiles, which were in agreement with geographic region. The authors also showed through PCR cloning of each profile that differences among peaks were due to the variation in the number of repeated units as well as the sequences of the arrays.

In the present study, a great variability in U1 snRNA pentanucleotide microsatellites was also evident for *E. granulosus.* The most common array $(GACGA)_n(GGCGA)_n$ was the

same as that sequenced by Bart et al. (2004) for all their samples. However, our clones were shown to be more polymorphic due to the presence of other motifs (Table 1).

As previously demonstrated (Bartholomei-Santos et al., 2003), there is no polymorphism in a microsatellite locus among protoscoleces that reproduce asexually within a single hydatid cyst, so that protoscoleces from one cyst can be pooled and analyzed as one isolate. Thus, the different U1 snRNA cloned sequences and the amplification of DNA segments with different sizes cannot be attributed to the variation among protoscoleces from the same cyst.

Analysis of the heteroduplex migration patterns in several isolates demonstrated genetic polymorphism among *E. granulosus* strains. Camel strain monomorphism had already been described in a previous study using a microsatellite (Bartholomei-Santos et al., 2003) with the same samples as in the present study. We have found the same U1 snRNA pattern among four isolates. Besides, the camel strain pattern showed less heteroduplex bands than did the other strains analyzed (Figure 2), which could be interpreted as a greater homogeneity in the several paralogous sequences.

The cattle strain showed two similar patterns. Several studies have found the cattle strain to be monomorphic (Haag et al., 1998; van Herwerden et al., 2000; Kamenetzky et al., 2002; Bartholomei-Santos et al., 2003); thus, the presence of two patterns in only six isolates analyzed, even with slight differences, is an interesting finding.

The greatest diversity of heteroduplex DNA patterns formed by U1 snRNA microsatellite amplification was found in the sheep strain which showed nine different patterns, highlighting sheep intrastrain variability. This finding could be due to the greater number of sheep strain isolates analyzed (30) compared to the four camel and the six cattle strain isolates which were shown to be less polymorphic.

However, these results are in agreement with previous studies, in which the sheep strain was the most polymorphic among the strains analyzed by SSCP-PCR of nuclear and mitochondrial genes (Haag et al., 1999; Kamenetzky et al., 2002) and by a dinucleotide microsatellite locus (Egmsca1) analysis (Bartholomei-Santos et al., 2003). Moreover, microsatellite alleles of an Egmsca1 locus were shared between sheep and Tasmanian sheep strains. The proximity of these two strains was already described in parsimonious trees built from mitochondrial data (Bowles et al., 1995).

Differences regarding morphology, prepatency period and allozyme frequencies (Kumaratilake et al., 1983; Lymbery and Thompson, 1988; Thompson and Lymbery, 1988) support the establishment of Tasmanian sheep as a distinct strain from the common sheep strain. A molecular approach based on comparison of mitochondrial DNA sequences demonstrated that only 3 of the 366 nucleotide sites examined in the Tasmanian sheep strain sample (G2) differ from the standard sheep strain sequence (G1) (Bowles et al., 1992). According to our results, it is not possible to differentiate Tasmanian sheep from sheep strain samples through heteroduplex pattern comparison, just as it was not possible by conventional RFLP (Hope et al., 1991), PCR/RFLP technique (Bowles and MacManus, 1993) and the microsatellite locus Egmsca1 analysis (Bartholomei-Santos et al., 2003), which were able to identify other strains. Coincidentally, in a recent study (Obwaller et al., 2004) it was suggested that differentiation between sheep and Tasmanian sheep strains using CO1 and NADH1 mitochondrial genes is questionable or unreliable.

Heteroduplex pattern analysis of U1 snRNA microsatellite provided good information about genetic polymorphism among and within *E. granulosus* strains. Moreover, this approach can be used in strain genotyping, at least for the strains studied in the present work. In addition,

heteroduplex analysis of other microsatellite loci as EMms1 and EMms2 (Nakao et al., 2003) may be evaluated as a method for conducting population genetic studies in *E. granulosus*.

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