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# Genotypic Characterization of Buffalo Isolates of *Echinococcus granulosus*

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**Cystic echinococcosis (CE) caused by the larval stage of the cestode *Echinococcus granulosus* is one of the most widespread zoonoses of veterinary and medical importance. Molecular techniques have allowed the identification of 10 different genotypes (G1-G10)of the parasite. Numerous studies have provided evidence that *Echinococcus granulosus* exists as a complex of different strains, that differ in wide variety of criteria that have impact on the epidemiology, pathology and control of cystic hydatid disease (CHD). On sequence analysis of mitochondrial COX1 and NADH genes showed the occurrence of the G1 genotype, the common sheep strain, G2 genotype, the Tasmanian sheep strain, the G3 genotype, the buffalo strain. The present study is an update of *E.granulosus* strains by genotyping a large samples of isolates and checking out genetic differentiation within and among the G1,G2,G3 genotype using additional gene marker, the RRNS gene. Sequencing of RRNS gene revealed fixed nucleotide substitution among G1,G2 and G3 genotypes. This study provides further evidence of the occurrence of the *E.granulosus* G1 and G2 sheep strain.**

**Key words:** *Echinococcus granulosus* ; Genotypes; Mitochondrial DNA genes.

Cystic echinococcosis (CE) is a zoonotic parasitic infection of many mammalian species caused by the larvae of *Echinococcus granulosus* which is a small tapeworm. The lifecycle involves, dogs and other canids are typical definitivehosts and ungulates especially cattle, buffalo, sheep, goat, pig and horse are intermediate hosts in which the hydatid cysts occur (Moro and Schantz, 2009). It causes severe economic loss and public health problem to both human beings and livestock in many temperate and tropical areas including India. The most tangible economic effects of this are the loss of offal from food animals. This may result in the entire loss of an infected organ or at least the trimming and down grading of that organ (Irsadullah *et al*., 1989).

The body weight of infected animal will be 1 per centless than uninfected animals (Torgerson and Macpherson,2011). CE often leads to a decline of health status that in turn translates into serious production losses to livestock industries. Economic losses arise not only from the condemnation of infected viscera, but also from decreases in yield and quality of meat, milk and value, reduced birth rate and fecundity, and delayed performance and growth. Other CE-associated costs that should be considered include the elimination of the infected viscera and dead animals or the potential bans on export of infected animals and their products (Battelli, 2009). Therefore, productivity losses due to CE in different livestock species with CE have been estimated to be in the range of 2.5–20 per cent for reduction in carcass weight, 2.5–12per cent for reduction in milk production, 3–12 per cen tfor decrease in fecundity, and 0.2 per cent for decrease in hidevalue (Cardona and Carmena, 2013).

The global annual live-stock production loss due to CE is estimated to be US$141, 605, 195 (Budke, 2006). In addition, this disease is of utmost zoonotic importance in human beings and requires

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expensive and prolonged medical treatment, often surgical interventions and the mortality may go up to 2–4 percent (Eckert *et al*., 2001). The global annual monetary loss due to CE in man has accounted for US$ 193, 529, 740(Budke, 2006). The cases of CE in humans and domesticated animals such as sheep, cattle, buffaloes, pigs and wild animals are being increasingly reported from India (Bhatacharya *et al*., 2007; Sunil Kumar, 2007) and from different parts of Tamilnadu,India (Parija and Sheela Devi,1999; Raman and Lalitha John, 2003; Sangaran, 2013 and Jeyathilakan, 2007).

A high degree of intra-specific variation has been detected within *E. granulosus* and a number of well characterized strains are now recognized. They display significant differences in life cycle patterns and host preferences (Thompson andMcManus, 2002;McManus, 2002). To date, molecular genotyping, using mitochondrial DNA (mtDNA) sequences, have identified 10 distinct genetic variants or strains (genotypes G1–G10) within *E. granulosus* (Bowles and McManus, 1993; Bowles *et al*., 1994,; Zhang *et al*., 1998; Snabel *et al*., 2000; McManus, 2002; Lavikainen *et al*., 2003; McManus and Thompson, 2003; Turcekova *et al*., 2003; Capuano *et al*., 2006;Helier *et al*.,2012). Two former strains (horse and cattle strains) have been recently recognized as distinct species: E. equinus and E. ortleppi, respectively (Thompson and McManus, 2002; Jenkins *et al*., 2005; Nakao *et al*., 2006; Naidich *et al*., 2006; Rinaldi.,2007 ; Simsek *et al*., 2011).

However, the taxonomy of *E. granulosus* is still in a state of flux. Only few studies aimed to the genetic characterization of *E. granulosus* strains have been carried out in the European Mediterranean region, with the exception of recent investigations in Spain, where the G1 (common sheep strain), the G4 (horse strain) and the G7 (pig strain) genotypes were recorded (Gonzalez *et al*., 2002; Daniel Mwambete *et al*., 2004;). In the Greek region of Peloponnesus, the G1, the G3 (Indian buffalo strain) and the G7 strains were found to occur (Varcasia *et al*., 2006). In India , cystic echinococcosis represents one of the most important zoonosis, with relevant economic losses and a significant public health impact. Its occurrence is primarily linked to the sheep–dog cycle.

The highest prevalence values in dogs and livestock were reported from Sardinia (Gabriele *et al*., 2006; Garippa, 2006; Scala *et al*., 2006a) and Sicily (Battelli *et al*., 2002; Giannetto *et al*., 2004). On the basis of their occurrence in intermediate hosts, mainly sheep and, to a lesser extent, cattle, it has been supposed that the dominant strain occurring in Italy is the common sheep strain (G1 genotype).

The aim of the present paper is to identify the *E. granulosus* strains occurring in different regions of Chennai, by sequence analysis of three mitochondrial genes (cytochrome c oxidase I gene, NADH dehydrogenase subunit I gene, small subunit ribosomal mitochondrial DNA), which will be of immense value in the control of Cystic echinococcosis (CE).

## MATERIALS AND MATHODS

### Samples and DNA extraction

In total 90 hydatid cysts were collected in buffalo from perambur slaughter house Chennai. Recovered isolates refer to protoscolices were accepted for molecular analysis. DNA was extracted from ethanol preserved or frozen samples. Genomic DNA was extracted using the QIAamp DNA mini kit (Qiagen, Germany) following manufacturer's instructions.The obtained DNA was stored at -20°C until used for PCR amplification.

### PCR amplification

Three target sequence of mitochondrial DNA NADH dehydrogenase (NADH), Cytochrome oxidase (COX 1), and small subunit of ribosomal RNA (RRNS) were amplified according to the following protocol: PCR was performed using 10 µl Amplicon Master Mix (Amplicon, Denmark), 1.0 µl Forward primer (10pm/ µl), 1.0 µl Reverse Primer (10pm/ µl), 1.0 µl DNA Template, 7.0 µl Nuclease free water(Qiagen® Germany) in 20 µl volume of reaction.

Two conserver primers COX1(Forward) 5'TTTTTTGGCCATCC TGAGGTTTAT – 3' and COX1 (Reverse) 5' TAA CGA CATA ACAT AAT GAAATG-3'(Bowles *et al.,* 1992) were used to amplify the mtDNA region corresponding to the part of COX1 gene, under following conditions: an initial denaturation step at 94°C for 1 min, followed by 30 cycles at 94°C for30 s (denaturation), 50°C for 30 s (annealing), 72°C for 30 s (extension), and a final extension step at 72°C for 7 min.

Two conserver primers NADH (Forward) 5'AGGTTTGCC GAT TTC TTG AAG 3' and NADH ( Reverse) 5' CAA CAG CAT AAA GCG CAA AAA ATAA 3'(Busi *et al*., 2007) were used to amplify the mtDNA region corresponding to the part of NADH gene, under following conditions: an initial denaturation step at 94°C for 2 min, followed by 30 cycles at 94°C for30 s (denaturation), 45°C for 30 s (annealing), 72°Cfor 40 s (extension), and a final extension step at 72°Cfor 7 min.

Two conserver primers RRNS(Forward) 5' TTA AGA TATATG TGG TAC AGG ATT AGA TACCC 3' and RRNS (Reverse) 5' AACCGAGGGTG ACGGGCGGTGTGTACC 3'(Busi *et al*., 2007) were used to amplify the mtDNA region corresponding to the part of RRNS gene, under following conditions: an initial denaturation step at94ÚC for 2 min, followed by 30 cycles at 94ÚC for30 s (denaturation), 55ÚC for 30 s (annealing), 72ÚCfor 40 s (extension), and a final extension step at 72ÚCfor 7 min. PCR products were detected on a 1.5% ethidium bromide stained low melting agarose gel then bands were cut from the gel and the amplified DNAs were purified by a QIAquick GelExtraction Kit (Qiagen).

**Sequence Analysis**

Sequencing of COX1, NADH and RRNS amplicons was carried out through amnion sequence services, Bengaluru. DNA sequence comparison was achieved using Gen Bank (http://www.pubmed.com ) with the BLAST system to find out the best region of similarity between sequences. Multiple alignment of sequence information was done using Clustal W parameter considering default gap opening, gap extension penalty and transition weight .The extent of variation among detected mitochondrial genotype was estimated by pair wise comparisons of nucleotide sequence with available sequence information from public data base.

**Phylogenetic analysis**

Phylogenies were constructed by maximum parsimony method using homogenous pattern among lineages and tested by boostrap with 1000 replicates using MEGA version.

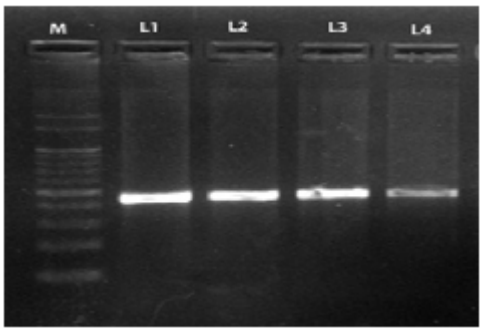
## RESULTS

**PCR Amplification**

PCR Amplification on COX1, NADH, RRNS gene showed amplicon size of 444bp(Fig-1), 323bp ( Fig-2), 370bp (Fig-3).

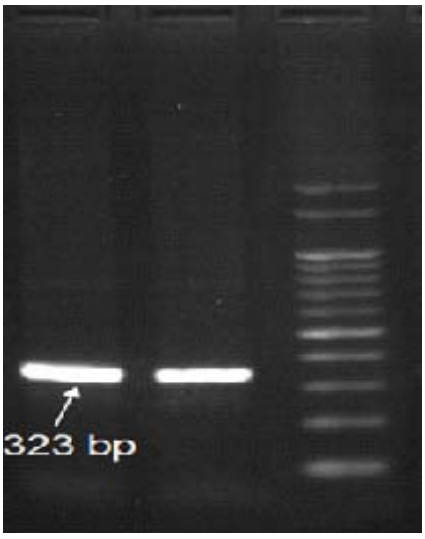
**Analysis of COX1 gene**

Nine buffalo isolates of *Echinococcus granulosus* were sequenced for COX1 gene. The COX1 gene sequence of different genotypes (G1, G2, G3, G4, G5, G6, G7, G8, G10) available in the GenBank was used along with the sequence of COX1 gene in the present study to do the multiple sequence alignments. By multiple sequence alignment of COX1 gene nucleotide of *E. granulosus*, the nine isolates of the study showed 99% homology with G1(common sheep strain DQ062857 from Italy),G2(Tasmanian sheep strain



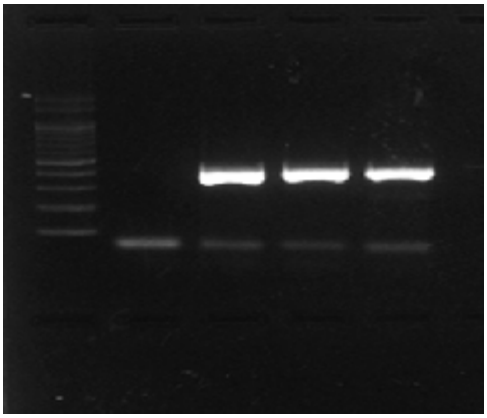

M: Marker (100bp ladder); L1-L4( Lane): Isolates with amplicon size of 444bp

**Fig. 1.** PCR Amplification of COX1 gene




L1,L2( Lane): Isolates with amplicon size of 323bp; M: Marker (100bp ladder);

**Fig. 2.** PCR Amplification of NADH gene



M: Marker (100bp ladder); L2-L4( Lane): Isolates with amplicon size of 370bp

**Fig. 3.** PCR Amplification of RRNS gene

M84662 ) and G3 (buffalo strain M84663). Further among the nine isolates, buffalo 2 isolate alone showed nucleotide change at 56 (T→C) and at 90 C→T) when compared with G1 sequence (Fig-4). However, except buffalo, all the other isolates had nucleotide change at 349(G →A ) when compared with G1 sequence. Phylogenetic analysis revealed that nine isolates clade with G1, G2 and G3 genotypes (Fig-5).

**Analysis of NADH gene**

Nine buffalo isolates of *E.granulosus* were sequenced for NADH gene. The NADH gene sequence of different genotypes (G1, G2, G3, G4, G5, G6, G7, and G10) available in the GenBank was used along with the sequence of NADH gene in the present study to do the multiple sequence

```
G2-M84662              CCTGGATTTGGTATAATTAGTCATATTTGTTTGAGTATTAGTGCTAATTTTGATGTGTTT 60
G3-M84663              CCTGGATTTGGTATAATTAGTCATATTTGTTTGAGTATTAGTGCTAATTTTGATGCGTTT 60
Buff6                  -----------------------CATATTTGTTTGAGTATTAGTGCTAATTTTGATGCGTTT 39
Buff8                  -----------------------CATATTTGTTTGAGTATTAGTGCTAATTTTGATGCGTTT 39
Buff9                  -----------------------CATATTTGTTTGAGTATTAGTGCTAATTTTGATGCGTTT 39
Buff7                  -----------------------CATATTTGTTTGAGTATTAGTGCTAATTTTGATGCGTTT 39
Buff2                  -----------------------CATATTTGTTTGAGTATTAGTGCTAATTTTGATGCGTTT 39
Buff3                  -----------------------CATATTTGTTTGAGTATTAGTGCTAATTTTGATGCGTTT 39
Buff4                  -----------------------CATATTTGTTTGAGTATTAGTGCTAATTTTGATGCGTTT 39
G1-DQ062857            -----------------------CATATTTGTTTGAGTATTAGTGCTAATTTTGATGCGTTT 39
Buff10                 -----------------------CATATTTGTTTGAGTATTAGTGCTAATTTTGATGCGTTT 39
Buff1                  -----------------------CATATTTGTTTGAGTATTAGTGCTAATTTTGATGCGTTT 39
G6-M84666              CCTGGATTTGGTGTTATTAGTCATATTTGTTTGAGGATTAGTTCTAATTTGGATGTTTTT 60
G7-84667               CCCGGATTTGGTGTTATTAGTCATATTTGTTTGAGGATTAGTTCTAATTTGGATGTTTTT 60
G10-DQ144022           ----GATTTGGTGTTATTAGTCATATTTGTTTGAGGATTAGGGCTAATTTGGATGTTTTT 56
G8-M84668-DQ144021     ----GATTTGGTGTTATTAGTCATATTTGTTTGAGGATTAGTTCTAATTTGGATGTTTTT 56
G5-M84665              CCTGGGTTTGGTGTTATTAGTCATATTTGTTTGAGTATCAGTGCTAATTTGGATGTTTTT 60
G4-M84664              CCTGGGTTTGGTATAATTAGTCATATTTGTTTAAGGATTAGTGCTAATTTGGATGTGTTT 60
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G2-M84662              GGGTTTTATGGGTTGTTGTTTGCTATGTTTTCTATAGTGTGTTTGGGTAGCAGGGTTTGG 120
G3-M84663              GGGTTTTATGGGTTGTTGTTTGCTATGTTTTCTATAGTGTGTTTGGGTAGCAGGGTTTGG 120
Buff6                  GGGTTTTATGGGTTGTTGTTTGCTATGTTTTCTATAGTGTGTTTGGGTAGCAGGGTTTGG 99
Buff8                  GGGTTTTATGGGTTGTTGTTTGCTATGTTTTCTATAGTGTGTTTGGGTAGCAGGGTTTGG 99
Buff9                  GGGTTTTATGGGTTGTTGTTTGCTATGTTTTCTATAGTGTGTTTGGGTAGCAGGGTTTGG 99
Buff7                  GGGTTTTATGGGTTGTTGTTTGCTATGTTTTCTATAGTGTGTTTGGGTAGCAGGGTTTGG 99
Buff2                  GGGTTCTATGGGTTGTTGTTTGCTATGTTTTCTATAGTGTGTTTGGGTAGTAGGGTTTGG 99
Buff3                  GGGTTTTATGGGTTGTTGTTTGCTATGTTTTCTATAGTGTGTTTGGGTAGCAGGGTTTGG 99
Buff4                  GGGTTTTATGGGTTGTTGTTTGCTATGTTTTCTATAGTGTGTTTGGGTAGCAGGGTTTGG 99
G1-DQ062857            GGGTTTTATGGGTTGTTGTTTGCTATGTTTTCTATAGTGTGTTTGGGTAGCAGGGTTTGG 99
Buff10                 GGGTTTTATGGGTTGTTGTTTGCTATGTTTTCTATAGTGTGTTTGGGTAGCAGGGTTTGG 99
Buff1                  GGGTTTTATGGGTTGTTGTTTGCTATGTTTTCTATAGTGTGTTTGGGTAGCAGGGTTTGG 99
G6-M84666              GGGTTTTATGGGTTGTTGTTTGCTATGTTTTCTATAGTGTGTTTAGGTAGTAGTGTTTGG 120
G7-84667               GGGTTTTATGGGTTGTTGTTTGCTATGTTTTCTATAGTGTGTTTAGGTAGTAGTGTTTGG 120
G10-DQ144022           GGGTTTTATGGGTTGTTGTTTGCTATGTTTTCTATAGTGTGTTTAGGTAGTAGTGTTTGG 116
G8-M84668-DQ144021     GGGTTTTATGGGTTGTTGTTTGCTATGTTTTCTATAGTGTGTTTAGGTAGTAGTGTTTGG 116
G5-M84665              GGGTTTTATGGGTTGTTATTTGCTATGTTTTCTATAGTGTGTTTAGGTAGTAGTGTTTGG 120
G4-M84664              GGGTTTTATGGGTTGTTGTTTGCTATGTTTTCTATAGTGTGTTTAGGAAGTAGGGTTTGG 120
                       ***** **********.**************************.**:** ** ******

G2-M84662              TTTACGTTTGGGGGAGTTACGGGTATAGTTTTGTCTGCTTGTGTGTTAGATAATATTTTG 360
G3-M84663              TTTACGTTTGGGGGAGTTACGGGTATAGTTTTGTCTGCTTGTGTGTTAGATAATATTTTG 360
Buff6                  TTTACGTTTGGGGGAGTTACGGGTATAGTTTTGTCTGCTTGTGTGTTAAATAATATTTTG 336
Buff8                  TTTACGTTTGGGGGAGTTACGGGTATAGTTTTGTCTGCTTGTGTGTTAAATAATATTTTG 336
Buff9                  TTTACGTTTGGGGGAGTTACGGGTATAGTTTTGTCTGCTTGTGTGTTAAATAATATTTTG 336
Buff7                  TTTACGTTTGGGGGAGTTACGGGTATAGTTTTGTCTGCTTGTGTGTTAAATAATATTTTG 336
Buff2                  TTTACGTTTGGGGGAGTTACGGGTATAGTTTTGTCTGCTTGTGTGTTAGATAATATTTTG 336
Buff3                  TTTACGTTTGGGGGAGTTACGGGTATAGTTTTGTCTGCTTGTGTGTTAAATAATATTTTG 336
Buff4                  TTTACGTTTGGGGGAGTTACGGGTATAGTTTTGTCTGCTTGTGTGTTAAATAATATTTTG 336
G1-DQ062857            TTTACGTTTGGGGGAGTTACGGGTATAGTTTTGTCTGCTTGTGTGTTAGATAATATTTTG 336
Buff10                 TTTACGTTTGGGGGAGTTACGGGTATAGTTTTGTCTGCTTGTGTGTTAGATAATATTTTG 336
Buff1                  TTTACGTTTGGGGGAGTTACGGGTATAGTTTTGTCTGCTTGTGTGTTAGATAATATTTTG 336
G6-M84666              TTTACGTTTGGGGGCGTCACTGGTATAGTTTTGTCTGCTTGTGTGTTGGATAATGTTTTA 360
G7-84667               TTTACGTTTGGGGGCGTCACTGGTATAGTTTTGTCTGCTTGTGTGTTGGATAATGTTTTA 360
G10-DQ144022           TTTACGTTTGGGGGCG---------------------------------------------- 312
G8-M84668-DQ144021     TTTACCTTTGGGGGCG---------------------------------------------- 312
G5-M84665              TTCACGTTTGGTGGTGTTACTGGTATAGTTTTGTCGGCTTGTGTGTTGGATAATGTTTTG 360
G4-M84664              TTTACTTTTGGAGGAGTTACTGGTATAGTTTTGTCTGCTTGTGTATTGGATAATGTTTTA 360
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**Fig. 4.** Multiple alignment of COX1 gene in comparision with available sequences for other genotypes of *E.granulosus*

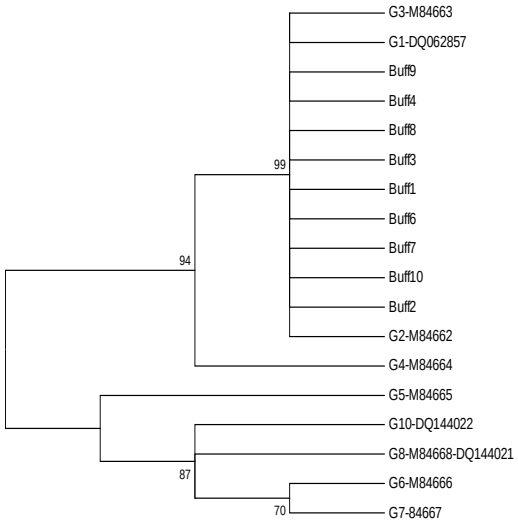


**Fig. 5.** Phylogenetic analysis of COX1 gene nucleotide sequence of *E.granulosus* field isolates of Maximum parsimony method using MEGA6.0 programme

alignments. By multiple alignment, eight isolates showed 100 per cent homology with G1 (common sheep strain AJ237632) , G2 (Tasmanian sheep strain AJ237633) and G3 (buffalo strain AJ237634) as was observed from Poland as per the GenBank accession (Fig-6) .Phylogenetic analysis revealed that these eight isolates clade with G1,G2 and G3 genotypes (Fig-7).

**Analysis of RRNS**

Eight buffalo isolates of *E. granulosus* were sequenced for RRNS gene. The RRNS gene sequence of different genotypes (G1, G5, G6, G7) available in the GenBank was used along with the sequence of RRNS gene in the present study to do the multiple sequence alignments. Using multiple sequence of alignment eight isolates showed 100 per cent homology with (G1 sheep strain AY462127) as observed in Switzerland as per the GenBank accession ( Fig-8). Phylogenetic analysis revealed that eight isolates clade with G1 genotype (Fig-9).

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G2-AJ327633       TTGGTGATTGTTTATTCATTTATTTATGGTAGATATTATAGAGCTAGTTATAGAGGCCTC 174
G3-AJ327634       TTGGTGATTGTTTATTCATTTATTTATGGTAGATATTATAGAGCTAGTTATAGAGGCCTC 174
G1-AJ237632       TTGGTGATTGTTTATTCATTTATTTATGGTAGATATTATAGAGCTAGTTATAGAGGCCTC 174
Buff5             TTGGTGATTGTTTATTCATTTATTTATGGTAGATATTATAGAGCTAGTTATAGAGGCCTC 150
Buff6             TTGGTGATTGTTTATTCATTTATTTATGGTAGATATTATAGAGCTAGTTATAGAGGCCTC 153
Buff2             TTGGTGATTGTTTATTCATTTATTTATGGTAGATATTATAGAGCTAGTTATAGAGGCCTC 156
Buff4             TTGGTGATTGTTTATTCATTTATTTATGGTAGATATTATAGAGCTAGTTATAGAGGCCTC 156
Buff10            TTGGTGATTGTTTATTCATTTATTTATGGTAGATATTATAGAGCTAGTTATAGAGGCCTC 147
Buff1             TTGGTGATTGTTTATTCATTTATTTATGGTAGATATTATAGAGCTAGTTATAGAGGCCTC 162
Buff3             TTGGTGATTGTTTATTCATTTATTTATGGTAGATATTATAGAGCTAGTTATAGAGGCCTC 144
Buff8             TTGGTGAT-GTTTATTCATTTATTTATGGTAGATATTATAGAGCTAGTTATAGAGGCCTC 131
G7-AJ327638       TTGGTTGTGGTGTATTCGTTTATTTATGGTAGATATTATAGAGTTAGTTATAGTATGCTT 174
G10-DQ144041      TTGGTTGTGGTGTATTCGTTTATTTATGGTAGATATTATAGAGTTAGTTATAGTAGGCTT 164
G6-AJ327637       TTGGTTGTGGTGTATTCGTTTATTTATGGTAGATATTATAGAGTTAGTTATAGTATGCTT 174
G5-AJ327636       TTGGTTGTGATGTATTCGTTTGTTTATGGTAGATATTATAGAGTTAGGTATAGTGGTCTT 174
G4-AJ327635       TTAGTAGTTATATATTCGTTTGTTTACGGTAGATATTATAGAATTAGTTATAGTGGTCTT 174
                  **.** .* .* *****.***.**** *************** *** *****:. **

G2-AJ327633       TCCGTGTTGTGGTTTTTGGCTGCCGCCAGAACATCTAGGTATTCTTTGTTGTGTACTGGT 234
G3-AJ327634       TCCGTGTTGTGGTTTTTGGCTGCCGCCAGAACATCTAGGTATTCTTTGTTGTGTACTGGT 234
G1-AJ237632       TCCGTGTTGTGGTTTTTGGCTGCCGCCAGAACATCTAGGTATTCTTTGTTGTGTACTGGT 234
Buff5             TCCGTGTTGTGGTTTTTGGCTGCCGCCAGAACATCTAGGTATTCTTTGTTGTGTACTGGT 210
Buff6             TCCGTGTTGTGGTTTTTGGCTGCCGCCAGAACATCTAGGTATTCTTTGTTGTGTACTGGT 213
Buff2             TCCGTGTTGTGGTTTTTGGCTGCCGCCAGAACATCTAGGTATTCTTTGTTGTGTACTGGT 216
Buff4             TCCGTGTTGTGGTTTTTGGCTGCCGCCAGAACATCTAGGTATTCTTTGTTGTGTACTGGT 216
Buff10            TCCGTGTTGTGGTTTTTGGCTGCCGCCAGAACATCTAGGTATTCTTTGTTGTGTACTGGT 207
Buff1             TCCGTGTTGTGGTTTTTGGCTGCCGCCAGAACATCTAGGTATTCTTTGTTGTGTACTGGT 222
Buff3             TCCGTGTTGTGGTTTTTGGCTGCCGCCAGAACATCTAGGTATTCTTTGTTGTGTACTGGT 204
Buff8             TCCGTGTTGTGGTTTTTGGCTGCCGCCAGAACATCTAGGTATTCTTTGTTGTGTACTGGT 191
G7-AJ327638       TCTGTGTTATGATTTTTAGCTGCTTCTAGAATTTCTAGGTATTCCTTGTTGTGTACTGGT 234
G10-DQ144041      TCTGTGTTATGATTTTTAGCTGCTTCTAGAATTTCTAGGTATTCTTTGTTGTGTGCTGGT 224
G6-AJ327637       TCTGTGTTATGATTTTTAGCTGCTTCTAGAATTTCTAGGTATTCCTTGTTGTGTACTGGT 234
G5-AJ327636       TCTGTATTATGATTTTTAGCTGCTGCTAGAATTTCTAGATATTCTTTGTTGTGCGCAGGT 234
G4-AJ327635       TCTGTATTATGGTTTTTGGCTGTTGCTAGAGTTTCTAGATATTCTTTGTTGTGTGCGGGT 234
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**Fig. 6.** Multiple alignment of NADH gene in comparision with available sequences for other genotypes of *E.granulosus*

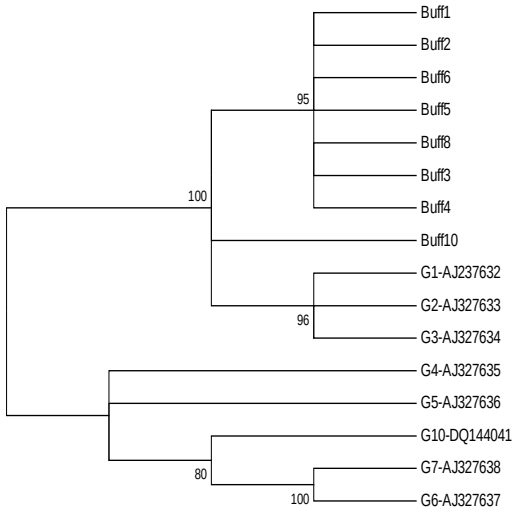


**Fig. 7.** Phylogenetic analysis of NADH gene nucleotide sequence of *E.granulosus* field isolates of Maximum parsimony method using MEGA6.0 programme

## DISCUSSION

The results obtained indicate the prominent circulation of three strains of *E. granulosus* in Chennai, the common sheep strain (G1 genotype),Tasmanian sheep strain(G2 genotype) and the Indian buffalo strain (G3 genotype). Isolates possessing G2 genotypes were detected in buffaloes from Kolkata by Bhattacharya *et al*., 2007 . The analysis of the obtained cox1 sequences confirms the discrimination of the G1,G2, G3 genotypes as two distinct haplogroups, each composed of several variant haplotypes. In the haplogroup corresponding to the G1 genotype, the majority of isolates corresponded to the common haplotype described for the common sheep strain, whereas several G1 isolates from Chennai showed distinct haplotypes with respect to the sequence chosen as reference.

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RRNS_sample-1     TAGTGAAGGATGGTCCACCTATTAGTTTACTCTTTTTATGTTGGTGTATGTCTGGTTTGA155
RRNS_Sample-8     TAGTGAAGGATGGTCCACCTATTAGTTTACTCTTTTTATGTTGGTGTATGTCTGGTTTGA179
RRNS_Sample-7     TAGTGAAGGATGGTCCACCTATTAGTTTACTCTTTTTATGTTGGTGTATGTCTGGTTTGA155
RRNS_Sample-4     TAGTGAAGGATGGTCCACCTATTAGTTTACTCTTTTTATGTTGGTGTATGTCTGGTTTGA155
RRNS_Sample-2     TAGTGAAGGATGGTCCACCTATTAGTTTACTCTTTTTATGTTGGTGTATGTCTGGTTTGA155
RRNS_Sample-5     TAGTGAAGGATGGTCCACCTATTAGTTTACTCTTTTTATGTTGGTGTATGTCTGGTTTGA155
RRNS_Sample-3     TAGTGAAGGATGGTCCACCTATTAGTTTACTCTTTTTATGTTGGTGTATGTCTGGTTTGA155
RRNS_Sample-6     TAGTGAAGGATGGTCCACCTATTAGTTTACTCTTTTTATGTTGGTGTATGTCTGGTTTGA155
G1-AY462129       TAGTGAAGGATGGTCCACCTATTAGTTTACTCTTTTTATGTTGGTGTATGTCTGGTTTGA117
G6-AY462126       TAGTAAAGGATGGTCCACCTATTATTTCACTCTTGTTATGTTGGTGTATATCTGGTTTGA144
G7-AY462128       TAGTAAAGGATGGTCCACCTATTATTTCACTCTTGTTATGTTGGTGTATATCTGGTTTGA141
G5-AY462127       TAGTAAAGGATGGTCCACCTATTATTTTGCTCTTGTTATGTTGGTGTATATCTGGTTTGA131
                  ****.******************** **  .***** **************.**********

RRNS_sample-1     TATTATTGTTGAATAATTTAAGTTTGTGTAGTTTTAGTTAAGCTAAGTCTATGTGCTGCT215
RRNS_Sample-8     TATTATTGTTGAATAATTTAAGTTTGTGTAGTTTTAGTTAAGCTAAGTCTATGTGCTGCT239
RRNS_Sample-7     TATTATTGTTGAATAATTTAAGTTTGTGTAGTTTTAGTTAAGCTAAGTCTATGTGCTGCT215
RRNS_Sample-4     TATTATTGTTGAATAATTTAAGTTTGTGTAGTTTTAGTTAAGCTAAGTCTATGTGCTGCT215
RRNS_Sample-2     TATTATTGTTGAATAATTTAAGTTTGTGTAGTTTTAGTTAAGCTAAGTCTATGTGCTGCT215
RRNS_Sample-5     TATTATTGTTGAATAATTTAAGTTTGTGTAGTTTTAGTTAAGCTAAGTCTATGTGCTGCT215
RRNS_Sample-3     TATTATTGTTGAATAATTTAAGTTTGTGTAGTTTTAGTTAAGCTAAGTCTATGTGCTGCT215
RRNS_Sample-6     TATTATTGTTGAATAATTTAAGTTTGTGTAGTTTTAGTTAAGCTAAGTCTATGTGCTGCT215
G1-AY462129       TATTATTGTTGAATAATTTAAGTTTGTGTAGTTTTAGTTAAGCTAAGTCTATGTGCTGCT177
G6-AY462126       TATTATCGTTTAATGGTTTGAGTTTGTGTAGTTTTAGTTAAGCCAAGTCTATGTGCTGCT204
G7-AY462128       TATTATCGTTTAATGGTTTGAGTTTGTGTAGTTTTAGTTAAGCCAAGTCTATGTGCTGCT201
G5-AY462127       TATTATCGTTTAATGGTTTGAGTTTGTGTAGTTTTAGTTAAGCCAAGTCTATGTGCTGCT191
                  ****** *** ***.**.***.********************** **************
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**Fig. 8.** Multiple alignment of RRNS gene in comparision with available sequences for other genotypes of *E.granulosus*

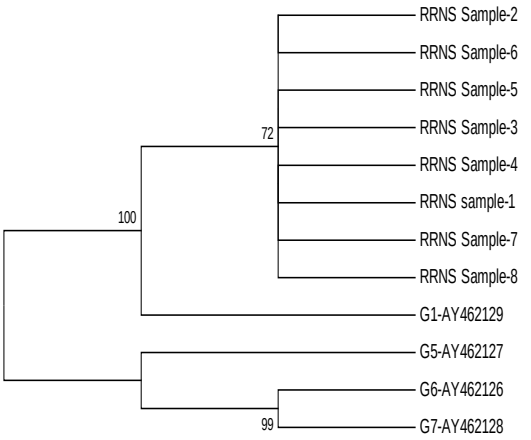


**Fig. 9.** Phylogenetic analysis of RRNS gene nucleotide sequence of *E.granulosus* field isolates of Maximum parsimony method using MEGA6.0 programme

In the present study variation was observed at T56c position (C90T), (G349A). The observation in the present study is in accordance with the result of Busi *et al*., (2007 The observation on the occurrence of sheep strain in buffalo isolates in the present study correlates with the earlier report by Bowles *et al*., (1992). The possibility of the sheep strain in buffalo could be due to the life cycle of the tapeworm *E. granulosus* involving dog – sheep. Sheep is a natural intermediate host for the tapeworm because of harbouring more fertile cysts as compared to any other herbivorous intermediate host. From this type of life cycle, the tapeworm eggs could have been transmitted to buffaloes via contaminated environment, which explain the occurrence of G1, G2 genotypes in Chennai, India. It is noteworthy that the same haplotype has been recently recorded in Tunisia (M'rad *et al*., 2005),in China (Bart *et al*., 2006a), in Romania (Bart *et al*., 2006b), in Turkey and in Austria (Obwaller *et al*., 2004).

This T - C mutation at position 56 is responsible for the substitution of alanine with valine based on the translation to the corresponding peptide sequence by EMBOSS software (Rice *et al*., 2000). Given that the above microvariant of G1 was found in several parts of the world we interpret its occurrence as a result of retention of an ancestral shared polymorphism between G1 and G2 gene pools rather than independent mutation events, although mitochondrial introgression, even if rare, cannot be excluded. In G3 isolates, intra-strain variation in cox1 was observed only in samples from Chennai, with one major haplotype within the haplogroup, fully corresponding to the available sequence in GenBank, that was observed in 27 isolates and three additional haplotypes being detected in 3–5 isolates. One of the additional haplotypes was affiliated exclusively with the cattle host (observed in three isolates), and displayed two transitions ( C = T substitutions) compared with the G3 reference sequence.According to these authors, import/export of sheep may explain the occurrence of the G2 genotype in both India .

The use of three different mitochondrial genes as markers for the identification of the genotypes allows to avoid pitfalls related to the limited usefulness of the NADH marker in distinguishing G1, G2 and G3 genotypes, given the position of one variable site between the G1 and the G2/G3 genotypes at the beginning of the sequence and the identity of nad1 sequences in the G2 and G3 genotypes. A lower rate of interpretable sequences in the NADH gene was previously observed also by Bart *et al*. (2004).Similarly, the rrnS marker was shown to be useful for the G1 and G3 differentiation. Unfortunately it was not possible to characterize the single G2 isolate for the RRNS gene. The total homogeneity of rrns sequences within G1 facilitates a correct diagnosis.

The occurrence of intra-strain variation may jeopardize the correct identification of hydatid cysts in some cases, thus advocating the need of using a multiple gene approach in the strain determination of *E. granulosus* complex. The present study provides the first characterization of the partial rrnS mitochondrial gene towards the identification of the Indian buffalo strain. Obwaller *et al.* (2004) argued that sequence variation within the genotype cluster G1–G3 can be partially explained by variability within individual isolates (mainly due to heteroplasmy), thus implicating identity of these closely related genotypes questionable.

However, fixed nucleotide differences between G1 and G2 across the rrns and cox1 genes detected in this study substantiate the conserved variation between these close related genotypes. Complementary studies will be undertaken to assess in more detail a possibility of genetic

recombination within the G1–G3 cluster, and to more precisely confirm infectivity of the G3 genotype (even compared with the G1 genotype) for buffaloes as indicated in this report.

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