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МОЛЕКУЛЯРНАЯ КЛАССИФИКАЦИЯ ШТАММОВ *ECHINOCOCCUS GRANULOSUS* ОТ КРУПНОГО РОГАТОГО СКОТА В ЛИВИИ

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Реферат

В данном исследовании представлено распространение различных штаммов *E. Granulosus* овцы, крупного рогатого скота и верблюда. Обычный вид овечьего штамма G1 обнаруживают, в основном, у овец и крупного рогатого скота, однако верблюды также могут быть заражены этим штаммом.

Напротив, верблюжий штамм G6, чаще всего, находили у верблюдов и реже у овец и крупного рогатого скота.

Однако, в ходе исследований установлена вероятность наличия криптических видов, тесно связанных с обоими генотипами крупного рогатого скота в Ливии, что является подтверждением высокого уровня мутаций у некоторых видов.

Исходя из того, что на территории Ливии отмечается преобладание хозяев штамма *E. Granulosus*, необходимо провести дополнительные исследования циклов передачи инвазии и генотипов *E. Granulosus*. Кроме того, рекомендуется провести обследование потенциальных промежуточных хозяев, включая частных владельцев собак, являющихся дефинитивными хозяевами, используя при этом молекулярные устройства высокого разрешения, такие как микросателлитные маркеры.

Keywords: Echinococcus granulosus, печень, легкие, обычный вид овечьего штамма G1.

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MOLECULAR CLASSIFICATION OF *ECHINOCOCCUS GRANULOSUS* STRAINS FROM LIVESTOCK ANIMALS IN LIBYA

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Abstract

This study demonstrates the distribution of various *E. granulosus* strains in sheep, cattle and camel. The common sheep strain G1 is mainly found in sheep and cattle, but also parasitized camels. In contrast, the camel strain G6 is found mainly in camels and rarely in sheep and cattle. However, the study also revealed the possible presence of cryptic species that are closely related to both genotypes in livestock of Libya as evident by high mutations in several specimens. Based on the occurrence of overlapping hosts of *E. granulosus* in Libya, more research on the transmission cycles and genotypes of *E. granulosus* in Libya is required. In addition, it is suggested that surveys on potential intermediate hosts, including in humans with dogs as the major final host in Libya using higher resolution molecular tools such as microsatellite markers is recommended.

Keywords: *Echinococcus granulosus, liver, lung, common sheep strain G1*

Introduction

Through the past five decades, significant phenotypic and genetic variabilities have been recognized and identified in various strains of *E. granulosus* isolated from different regions (Van Herwerden *et al*., 2000; Thompson and McManus 2001, 2002; Pearson *et al*., 2002 and Huttner *et al*., 2008) . These studies have revealed that the different strains of *E. granulosus* consist of heterogeneous groups of genetic variants (McManus, 2002). Thompson (1995) illustrated that different strains may display variations in morphology, host specificity, development rate, pathogenicity and geographical distributions. Moreover, many studies have been conducted to determine the host and geographic ranges of these strains, and whether genetic variations were characteristic and specific to the different endemic areas throughout the world (Jenkins and Thompson, 2005). Thompson and Kumaralilake (1982) observed that some strains of *E. granulosus* share similar morphological characters but showed epidemiological differences; thus, this parasite showed high diversity. Identification of strain types of *E. granulosus* is very important in strategizing and implementing an *Echinococcosis* control and management programmer.

Until now, 10 strains or genotypes, namely G1- G10 have been recognized and described in *E. granulosus* based on mitochondrial and nuclear gene analyses (Bowles *et al*., 1992, 1994; Scott *et al*., 1997; Lavikainen *et al*., 2003; Thompson *et al*., 2008; Saarma *et al*., 2009). According to mitochondrial data, *E. granulosus* has been traditionally assigned to the various taxonomic species in relation to the G1- G10 genotypes; *E. granulosus sensu stricto* (G1, G2, and G3), *E. equinus* (G4), *E. ortleppi* (G5), and *E. canadensis* (G6-G10) (Nakao *et al*., 2007; Moks *et al*., 2008). However, recent studies based on nuclear data categorized the genotypes (G6- G10) into two strains; cervid genotypes G8 and G10 belonging to *E. canadensis*, whilst camel and pig genotypes G6, G7 belonged to *E. intermedius* (Saarma *et al*., 2009).

The aim of this study was to determine *E. granulosus* genotypes present in Libyan livestock by molecular genetic strain typing. For the analysis, the mitochondrial (ATP6) and nuclear (Act II) genes were utilized.

Materials and Methods

Parasite samples

Hydatid cyst samples were collected from the major slaughtered livestock namely sheep, camel and cattle from government abattoirs located in four regions in Libya from January to end of 2010. In total, 120 samples were used for molecular analysis, from liver, lung and other organs from the three major livestock. To obtain fertile cysts, the protoscoleces were collected from the sediment of hydatid sand by pipette under sterile conditions and rinsed three times with normal saline. Suspensions of protoscoleces were fixed in 90% (v/v) ethanol and then stored at - 20°C until DNA extraction. To obtain infertile cysts, the thin germinal layer from the wall cyst was cut into small pieces and washed with normal saline and then stored at - 20°C for further processing.

DNA extraction

The protoscoleces were washed several times in nucleic acid-free water to remove the alcohol preservation solution. Genomic DNA was extracted using a QIAamp DNeasy mini kit (Qiagen, Germany) according to the manufacturer`s protocol.

Electrophoresis was preceded on a 1% agarose gel with GelRed Nuclei Acid Gel Stain as marker, at 100 volt for 30 min to assess the success of DNA extraction. After that, the agarose gel was visualized in a gel documentation system (GENE Flash, Syngene Bio Imaging, USA) for the presence of the extracted DNA bands. To investigate the presence of sterile cyst (from cattle), DNA extraction was conducted on 25 mg of the cut pieces of infertile germinal layer and placed into a 1.5 ml Eppendrof tube. Then according to the manufacturer`s protocol.

Polymerase chain reaction (PCR) amplification

The partial fragments of the mitochondrial gene (ATP6) were PCR-amplified on the DNA extract using specific primers designed by Xiao *et al*. (2005).

ATP6 forward: 5`- GCA TCA ATT TGA AGA GTT GGG GAT AAC-3`

ATP6 reverse: 5`- CCA AAT AAT CTA TCA ACT ACA CAA CAC-3`

The PCR reaction contained 5.5 µL of 5X PCR buffer, 4 µL 25 mM MgCl2 solution, 0.3 µL of 5u/ µL Taq DNA polymerase, 0.7 µL of 10 mM dNTP (Promega, USA), 0.5 µL of each primer and 2.0 µL of the target DNA in a total volume of 25 µL. The PCR protocol consisted of an initial incubation at 94°C for 30 s, 35 cycles at 94 °C, 55 °C for 30 s and 72 °C for 1 min, 72 °C for 5 min in the final extension, The PCR amplification was conducted using the Mastercycler Gradient- Thermal cycler (Eppendorf, Germany).

The Act II fragment was PCR amplified using primers specifically designed by De Silva *et al*. (1993).

Act II forward: 5`- TCT TCC CCT CTA TCG TGG G-3`

Act II reverse: 5`- CTA ATG AAA TTA GTG CTT TGT GCG C-3`

The PCR was carried out in a 25 µL volume containing 2 µL target DNA, 5 µL of 5X PCR buffer, 5 µL 25 mM MgCl2 solution, 0.25 µL of 5u/ µL Taq DNA polymerase, 0.5 µL of 10 mM dNTP (Promega, USA), 0.5 µL of each primer. The PCR conditions were as follows: 94°C for 30 s, 40 cycles at 94 °C for 30 s, 60 °C for 30 s and 72 °C for 1.5 min, and then a final extension at 72 °C for 5 min, The PCR amplification used the Mastercycler Gradient- Thermal cycler (Eppendrof, Germany).

After the PCR amplification, PCR products were assessed by electrophoresis in a 1.5 % agarose gel using GelRed Nuclei Acid Gel Stain as marker at 100 volt for 25 min. finally the purified DNA samples were sent to the service provider for sequencing procedure (First

BASE Laboratories Sdn. Bhd. and Centre of Chemical Biology, CCB at Universiti Sains Malaysia).

Data analysis

The obtained sequences were edited using MEGA 5.05 program (Tamura *et al*., 2007). The genetic relationships between haplotypes were determined by constructing phylogeny trees based on Neighbour-Joining (NJ). GenBank sequences of *E. granulosus* were downloaded for comparisons with the current data for each gene. For ATP6 gene the following GenBank sequences were used- Acc. No AF 297617.1 sheep strain and AB208063.1 camel strain G6. For Act II gene the GenBank sequences AF 528499.1 sheep strain G1 and AF 528500.1 camel strain G6 were used to compare with the sequences in this study.

Results

A total of 120 *E. granulosus* samples were successfully amplified by using the optimized PCR conditions. Length of partial fragment of ATP6 mtDNA was 513bp and partial fragment of Act II DNA fragment was 267bp in length.

Multiple sequence alignments of ATP6 and Act II genes were carried out for 102 individuals from livestock hydatid cyst (60 sheep, 31 camels and 11 cattle) and 89 individuals from livestock hydatid cyst (54 sheep, 25 camels and 10 cattle) respectively using MEGA 5 Software ClustalW (1.6) DNA weight matrix. Blastn search showed that all the aligned sequences belonged to ATP6 and Act II genes with high similarities (96-100%).

Neighbour Joining (NJ) analysis

The Neighbour-Joining (NJ) analysis of ATP6 gene was carried out based on Kimura 2-parameter as presented in Fig 1. The same GenBank sequences of *E. granulosus* from the previous sheep population study (AB 208063.1; AB235847.1; AB031283.1) were included. Two main clusters were formed; the first cluster consisted of mixed populations (different organs from different animals) with a low bootstrap confidence level of 51% and a second cluster with high support (99%). This cluster was divided into seven internal subclusters with low to moderate support but with no obvious genetic relationships to host or organ types. The GenBank (AB031283.1) representing sheep strain grouped in this cluster. The second monophyletic cluster with 99% support consisted of four subclusters.

Unlike Cluster 1, the second cluster was generally made up of parasites of camel origin with GenBank taxon (AB 208063.1) and (AB235847.1) representing camel strain grouping in this cluster. However, there were two exceptions, namely of parasites from host sheep, SMT1 (KF255896) and SMT2 (KF255899) grouping into this cluster. This provided further evidence of the previous population analysis that these two sheep parasite sequences in the mesentery are more closely related to camel strain. The four subclusters were low to moderately support. These internal subclusters did not show any obvious pattern to specific organs.

The NJ tree formed with 1000 replicates of Act II gene is presented in Fig 2. Sheep strain (gi 22653316) and camel strain (gi 22653318) GenBank haplotypes. There was no phylogenetic structuring into major clusters. However, a strongly supported cluster (99%) was observed consisting of several haplotypes (KP843657 with groups of KP843653, KP843652, KP843647, KP843639, KP843626) from mixed populations and the GenBank G6 camel strain haplotype. Four weakly supported and a single moderately supported terminal clusters consisting of mixed populations (different organs from different regions) were also observed.

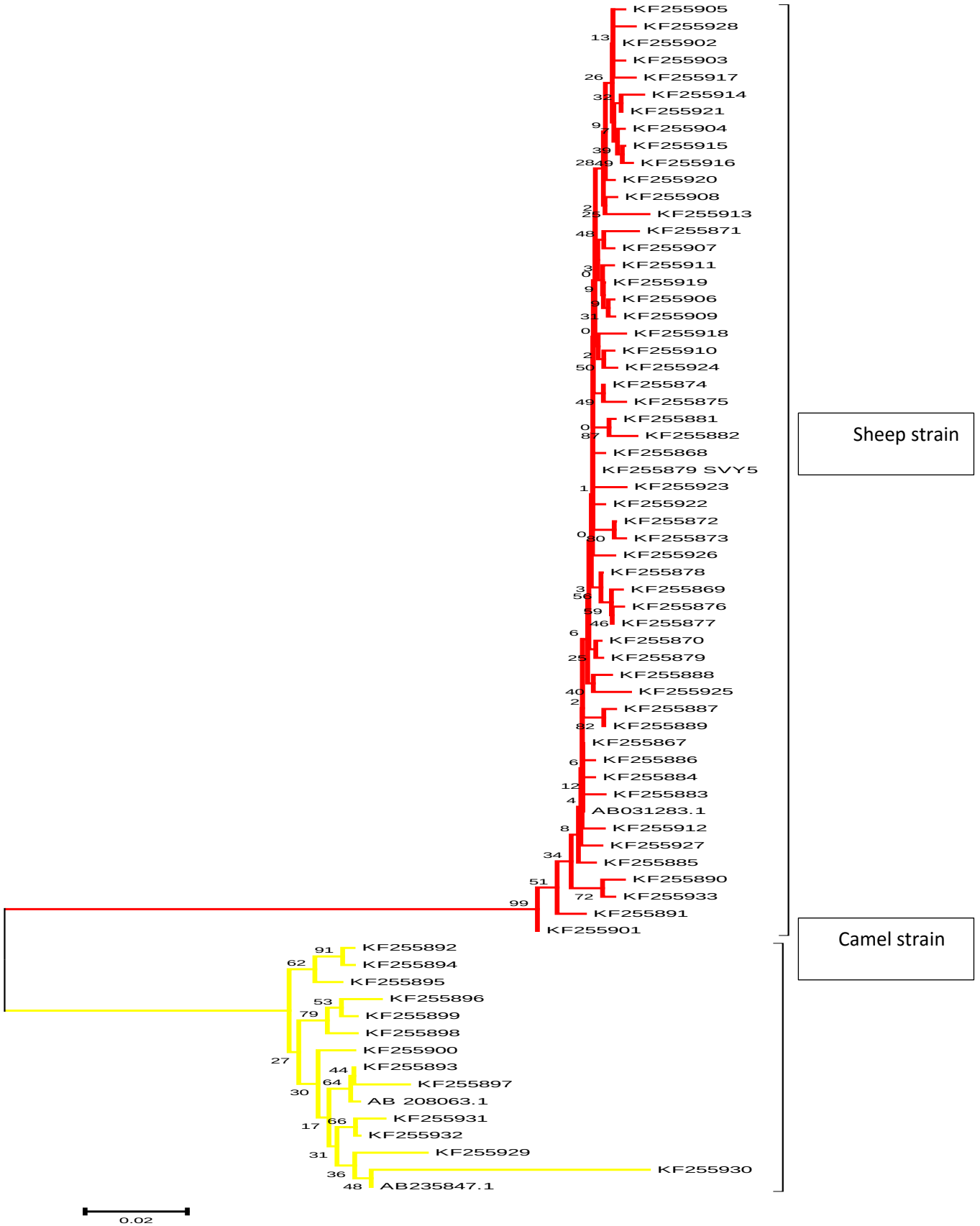
KF255905
KF255928
13
KF255902
KF255903
26
KF255917
32
KF255914
KF255921
9
KF255904
39
KF255915
2849
KF255916
KF255920
KF255908
2
25
KF255913
48
KF255871
KF255907
3
0
KF255911
KF255919
9
KF255906
9
31
KF255909
0
KF255918
2
KF255910
50
KF255924
KF255874
49
KF255875
0
KF255881
87
KF255882
KF255868
KF255879 SVY5
1
KF255923
KF255922
KF255872
0
30
KF255873
KF255926
KF255878
3
56
KF255869
59
KF255876
46
KF255877
6
KF255870
25
KF255879
KF255888
40
KF255925
2
KF255887
82
KF255889
KF255867
6
KF255886
KF255884
12
KF255883
4
AB031283.1
8
KF255912
KF255927
34
KF255885
51
KF255890
72
KF255933
99
KF255891
KF255901
91
KF255892
62
KF255894
KF255895
53
KF255896
79
KF255899
KF255898
KF255900
27
KF255893
44
64
KF255897
30
AB 208063.1
KF255931
17
66
KF255932
31
KF255929
36
KF255930
48
AB235847.1
0.02
Sheep strain
Camel strain

Fig 1: Neighbour joining phylogenetic tree of *E. granulosus* haplotypes from Libyan combined sheep, cattle and camel livestock of ATP6 gene.

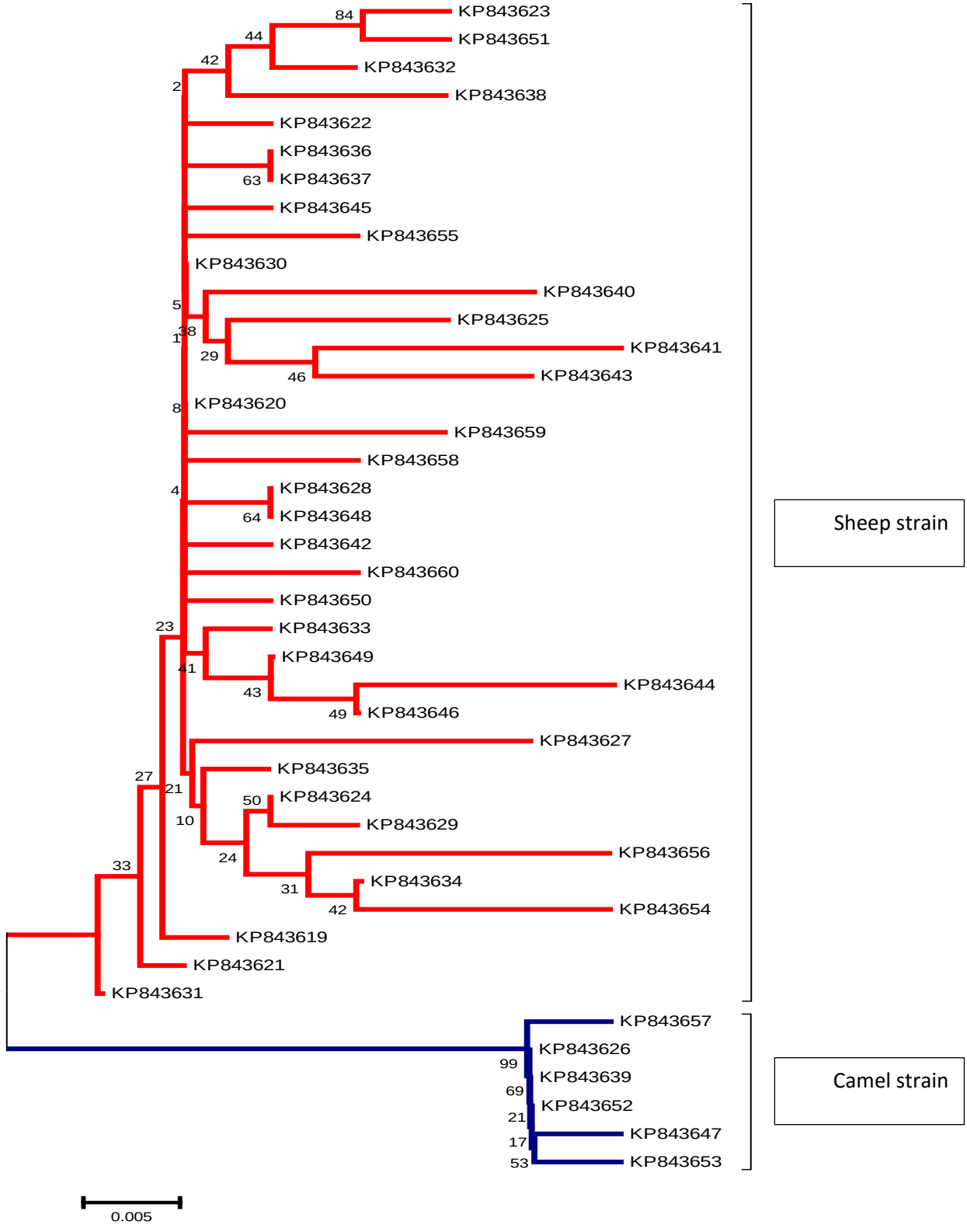

Fig 2: Neighbour joining phylogenetic tree of *E. granulosus* haplotypes from Libyan livestock of Act II gene.

Discussion

To date, six genotypes of *E. granulosus* complex have been identified in Africa; G1 (sheep strain), G2 (Tasmanian sheep strain), G4 (horse strain), G5 genotype (cattle strain) and the G6 (camel strain) genotypes (Thompson and McManus, 2002; Dinkel *et al*., 2004; Huttner *et al*., 2008 and Casulli *et al*., 2010). Limited studies in Libya, primarily conducted by Tashani *et al*. (2002) in eastern Libya reported that all livestock animals (sheep, cattle and camels) were infected with the same genotype of *E. granulosus* (G1, sheep strain). Another study by Abushhewa *et al*. (2010) from different areas in Libya recorded two groups; the first group belonging to G1-G3, and the second group belonging to G6-G10. Thus, the present study is a valuable contribution of data on the population genetics, diversity as well as complements existing taxonomic knowledge of this parasitic species from various organs in different hosts from Libya based on each of a mitochondrial and nuclear gene.

The DNA sequence variation of the partial mtDNA ATP6 of *E. granulosus* conducted in this investigation has indicated the transmission of two main strains in livestock animals (sheep, camels and cattle) in Libya. This study is the first documentation on ATP6 sequences of *E. granulosus* from livestock in Libya and has provided data on the common strain distributed in Libya of *E. granulosus*.

Various mutations in different populations of sheep, camels and cattle were observed. Of these, 80.5% and 19.4% haplotypes belonged to common sheep strain (G1) and camel strain (G6) respectively. These findings corresponded to the results by Eryildiz and Sakru (2012) in a Turkish study, who recorded that most of their isolates, belonged to G1-G3. Only one isolate belonged to G6-G10. Moreover, Hailemariam *et al*. (2012) recorded similar findings in an Ethiopian study which observed 87% of livestock samples were identified as G1 and 13% as the G6. On the other hand, Abushhewa *et al*. (2010) in a Libyan study reported that all isolates from camels belonged to camel strain G6. This indicated the all camels in their study lived in the private pastures with no connection to sheep farms.

Phylogenetic analysis of *E. granulosus* populations in livestock

Phylogenetic analysis based on NJ method generated trees with similar topologies, which appeared less efficient based on the clustering and bootstrap approaches. Generally, in the present study, the topology of the NJ tree from combined sheep, camel and cattle populations, showed that all sheep populations referred to as G1 genotype sheep strain combined together, while only two individuals belonged to G6 genotype camel strain. However, all cattle individuals belonged to sheep strain, while several individuals from camels belonged to the sheep strain while the rest belonged to camel strain. Previous studies conducted in Libya by Tashani *et al*. (2002) recorded that all *E. granulosus* isolates from different livestock (sheep, cattle and camels) belonged to the common sheep strain (G1). In addition, the present study showed that all hydatid cysts from cattle were sterile; due to the infection by the common sheep strain (G1). This was also observed by Tashani *et al*. (2002).

Abushhewa *et al*. (2010) in their study on Libya and Omer *et al*. (2011) in a study in Sudan reported that all *E. granulosus* isolates from camels (100%) belonged to the G6-G10 complex. Interestingly, 87% of cattle hydatid cysts investigated by Abushhewa *et al*. (2010) and 99% by Omer *et al*. (2011) belonged to the G6 genotype. More recent evidence by Abdel Aaty *et al*. (2012) and Omer *et al*. (2011) reported that all isolates from sheep, camels, pigs and cattle were identified as G6 camel strain in Egypt and Sudan. Thus their findings

suggested that the camel strain play the major role in the transmission cycle of *E. granulosus* in Egypt and Sudan.

Considering the overall livestock, high substitution rates were observed in haplotypes closely related to G6. Furthermore, a single sample (camel liver) from this group had unusually high nucleotide substitutions. This sample was closely related to GenBank G6 Acc No AB208063.1 (97%) and GenBank G7 Acc no. AB235847.1 G7 (96%). Farjallah *et al*. (2007) found G7 in slaughtered camels from Tunisia and Mauritania. This is the first record of a strain belonging to G7 or very closely related to it. Therefore, it is suggested that the prevalence reported in this study is due to the movement of livestock animals from neighboring countries such as Tunisia or due to the occurrence of high random mutation in this sample.

Furthermore, many related studies in North Africa correspond to the present findings; from Algeria, Bart *et al*. (2004) observed two distinct well supported clusters (G1 and G6) based on the mitochondrial (ND1, COX1) genes. The same situation was observed in Middle Africa, from Kenya and Sudan, where many genetic studies (Bowles *et al*., 1992; Wachira *et al*., 1993 and Dinkel *et al*., 2004) have demonstrated the importance of G1 and G6 in livestock. But Dinkel *et al*. (2004) also noted other strains in Kenya originating from pig and in Sudan from cattle (*E. ortleppi*).

Nuclear gene Act II

To further investigate the strain identity of *E. granulosus* from sheep, camels and cattle in Libya, a 262 bp of Act II gene sequence data was analyzed. This is attributed to the lower mutation rates of nuclear markers compared to mitochondrial DNA. According to the phylogenetic tree two main genotypes, G1 (common sheep strain) and G6 (camel strain) with 98-100% homology with GenBank (AF528499 and AF528500 respectively) were observed. These findings were in agreement with previous studies by Gudewar *et al*. (2009) in India and Maillard *et al*. (2007) in Africa using the same Act II gene. Bart *et al*. (2006) used BG1/3 nuclear gene to identify *E. granulosus* among sheep, cattle and pigs, and identified two genotypes, a sheep strain and the pig strain.

The present results showed that most of individuals from different sheep populations were placed in common sheep strain (G1), while only two samples (Misurata liver and mesentery) populations were placed in camel strain (G6). The results from mitochondrial and nuclear markers revealed that most of the sheep hydatid cysts from different organs in different areas belonged to the sheep strain, but were rarely infected by camel strain. This indicates that generally the camel strain was not effective in infecting the sheep host in Libya with several exceptions – in four sheep individuals, specifically 3 individuals from mesentery organ and another from liver organ infected with the camel strain. The G1 genotype is effective in infecting different organs in camels and cattle. In contrast, the camel strain is ineffective to infect the cattle host i.e. all cattle individuals were only infected with sheep strain. For the nuclear marker only two samples from liver and lung cattle were infected by camel strain G6, while the sheep strain G1 was more suitable to infect cattle host than the G6. However, all hydatid cysts in different organs of cattle were sterile. This indicates that sheep and camel strains are not the adapted for propagation in cattle in Libya. Furthermore, the G6 strain infection was also recorded in several individuals of sheep and cattle based on the nuclear gene, an observation also previously noted by Kamenetzky *et al*. (2002) in Argentina and Haag *et al*. (2004). Thus, both the mitochondrial and nuclear gene analyses generated two main clades (G1 and G6) of *E. granulosus* which represented the common intermediate hosts (sheep, cattle and camels) from Libya as identified based to the Genbank databases.

Why are there incidences of G1 and G6 being present in the same host? An explanation that could be put forward is the interaction between the camel-dog and sheep-dog cycles. Dog is the final host of this parasite. Due to the co-existence of camels with sheep and cattle

together in a close neighborhood, cross transmission of camel and sheep strains may occur in different livestock as a result of overlapping cycles. Clearly, more focused studies on the G1 and G6 in overlapping hosts of *E. granulosus* in Libya need to be conducted. In addition, there is a possibility of cross fertilization between genotypes of G1 and G6 in the dog which is the final host and thus, producing a hybrid genotype.

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