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Original Article

Phylogeography, Genetic Diversity and Population Structure of *Echinococcus granulosus* Sensu Stricto Inferred by Mitochondrial DNA Markers between Southeast of Iran and Pakistan

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Abstract

Background: Current study was designed to provide a better insight into the circulating genotypes, genetic diversity, and population structure of *Echinococcus* spp. between southeast of Iran and Pakistan**.**

Methods: From Jun 2020 to Dec 2020, 46 hydatid cysts were taken from human (n: 6), camel (n: 10), goat (n: 10), cattle (n: 10) and sheep (n: 10) in various cities of Sistan and Baluchestan Province of Iran, located at the neighborhood of Pakistan. DNA samples were extracted, amplified, and subjected to sequence analysis of *cox1* and *nad1* genes.

Results: The phylogeny inferred by the Maximum Likelihood algorithm indicated that G1 genotype (n: 19), G3 genotype (n: 14) and G6 genotype (n: 13) assigned into their specific clades. The diversity indices showed a moderate (*nad1*: Hd: 0.485) to high haplotype diversity (*cox1:* Hd: 0.867) of *E. granulosus* s.s. (G1/G3) and low nucleotide diversity. The negative value of Tajima's *D* and Fu's Fs test displayed deviation from neutrality indicating a recent population expansion. A parsimonious network of the haplotypes of *cox1* displayed star-like features in the overall population containing IR9/PAK1/G1, IR2/PAK2/G3 and IR18/G6 as the most common haplotypes. A pairwise fixation index (*F*st) indicated that *E. granulosus* s.s. populations are genetically moderate differentiated between southeast of Iran and Pakistan. The extension of haplotypes PAK18/G1 (sheep) and PAK26/G1 (cattle) toward Iranian haplogroup revealed that there is dawn of *Echinococcus* flow due to a transfer of alleles between mentioned populations through transport of livestock or their domestication.

Conclusion: The current findings strengthen our knowledge concerning the evolutionary paradigms of *E. granulosus* in southeastern borders of Iran and is effective in controlling of hydatidosis.

Introduction

Cystic echinococcosis (CE) caused by metacestode of tapeworm *Echinococcus granulosus* sensu lato (s.l.) is addressed to be neglected tropical disease in the globe (1), that leads to significant economic losses of over 3 billion $ annually (2). Current phylomolecular investigations based on mitochondrial DNA markers disclosed that *E. granulosus* s.l. consists at least four valid clades including *E. granulosus* sensu stricto (s.s.) (G1/G3 genotype), *E. equinus* (G4), *E. ortleppi* (G5), *E. canadensis* (Syn~*E. intermedius*) (G6/G7), sylvatic genotypes of *E. canadensis* (G8/G9/G10) and *E. felidis* (lion strain) (3–5). Although, G2 has extensive been discussed to be a distinct strain but is recognized now to be a microvariant of G3 (3,6,7).

Since, genotypes of *E. granulosus* s.l. in their intermediate hosts are unequivocally characterized by diverse transmission patterns, clinical complexity, genomic traits and various immunological responses (8–11), the emerging new single nucleotide polymorphism of *Echinococcus* species will be more likely (12–14).

Therefore, utilizing reliable mitochondrial DNA (mtDNA) markers such as cytochrome oxidase subunit 1 (cox1) and NADH dehydrogenase subunit 1 (nad1) may well be unambiguously identified the new subspecies/haplotypes (12,15,16).

In the past few years, import of livestock from Pakistan toward southeast of Iran has been increasing. According to research conducted in Iran and Pakistan on the phylogenetic pattern *of E. granulosus* in different hosts, the genotypes of *E. granulosus* G1/G3 complex are sympatrically circulating with different level of genetic diversity (17–27). Regarding the genetic structure of parasite populations, computation of F-statistics (as a degree of gene migration) among adjoining endemic countries be able to provide precious information about epidemiological drift of cestodes, speciation and allele occurrences (8,28,29).

Given the scarcity of comparative phylogenetic knowledge on the genetic variability of *E. granulosus* s.s. between Sistan and Baluchestan province (southeastern of Iran) and Pakistan, current investigation was designed to provide a comprehensive understanding of the circulating genotypes, genetic diversity and population structure of *Echinococcus* spp. inferred by mtDNA markers intended for propose a microevolutionary scale on how the *Echinococcus* haplotypes have extended amongst two different metapopulations (regional population).

Materials and Methods

Sampling

From June 2020 to December 2020, 46 hydatid cysts were taken from human (n: 6), camel (n: 10), goat (n: 10), cattle (n: 10) and sheep (n: 10) in various cities (Zabol, Pishin, Saravan, Zahedan, Iranshahr and Chabahar) of Sistan and Baluchestan Province (southeast of Iran), where located at the neighborhood of Pakistan.(Code of ethics: IR.MAZUMS..REC.1398.4712) (Fig. 1).

Fig. 1: Iran map presenting study locations in Sistan and Baluchestan province (southeast of Iran) and Pakistan. The migrated haplotypes have marked by asterisk (*)

Human hydatid cysts were surgically obtained from CE patients. Animal hydatid cysts were also collected in slaughterhouses and from livestock from different regions within Sistan and Baluchestan province. In order to obtain protoscoleces (PSCs), cyst fluid was aspirated under sterile conditions, then washed three times with normal saline, and finally stored at -20°C.

DNA extraction and PCR amplification

The total genomic DNA was extracted from PSCs and germinal layers using preparation kit (WizPrep™ gDNA Mini Kit (Cell/Tissue), Wizbiosolutions, South Korea) according to the protocol recommended by the manufacturers and stored at -20 °C up to PCR amplification. with the aim of amplification of cytochrome oxidase subunit 1 gene fragment, JB3 (5'-TTTTTTGGGCATCCTGAGGTTTAT-3') and JB4.5 (5'-TAAAGAAAGAACATAATGAAAATG-3') (12) primers; and for amplification of the NADH dehydrogenase subunit 1 gene, MS1 (5'-CGTAGGTATGTTGGTTTGTTTGGT-3') and MS2 (5'-CCATAATCAAATGGCGTACGAT-3') primers were used, respectively (12,30).

The single round-PCR amplification was carried out in 25 µL reaction volumes containing 0.3 µL (5u/µL) of Taq DNA polymerase (Cinnagen, Iran), 2.5 µL of 10×PCR buffer (Cinnagen, Iran), 0.7 µL (1.5 mM) MgCl2 (Cinnagen, Iran), 0.5 µL (10 mM) of dNTP Mix (Cinnagen, Iran), 13 µL deionized distilled water, 1.5 µL of each forward and reverse primers (10 pmol) and 5 µL of DNA template (50 ng). The details of thermal cycling conditions for amplification of *cox1* and *nad1* genes were described earlier (13,30). PCR products were electrophoresed on 1.5% agarose gel after staining with safe stain and then fragment 444 bp for *cox1* gene and 400 bp for *nad1* gene were investigated under ultraviolet light utilizing transilluminator.

Sequencing, phylogenetic analysis and haplotype network

Forty-six PCR products of *cox1* (n: 23) and *nad1* (n: 23) genes were purified and sequenced by Bioneer Corporation (South Korea). Furthermore, with the aim of compare the analyzed sequences of current survey with isolates of *E. granulosus* s.s. populations of Pakistan country, 32 sequences were recovered from GenBank (MN604237, MN640726 to MN640735, MN886275 to MN886286, MW405495 to MW405502 and MW407999). Unclear sites of nucleotide sequences were coded by the standard IUPAC codes for composition of two or more bases. Contigs (overlapped sequences) from all isolates were aligned and edited with reference sequences of each genotype within *E. granulosus* (s.l.) retrieved from GenBank using Sequencher Tmv.4.1.4 software. Moreover, haplotype network was created using PopART software determined by the Median-joining algorithm for inferring intra-specific phylogenies to ascertain the genealogical relationships of *E. granulosus* s.s. (31). To authenticate taxonomic status and cladistic relationship between the identified genotypes of *E. granulosus* s.s., a phylogenetic tree based on Maximum Likelihood algorithm with kimura two-parameter model was constructed using MEGA software (version 5.05). The topology of the constructed tree was supported by bootstrap values higher than 70%. The accuracy of phylogenetic tree was evaluated by 1000 bootstrap re-sampling. Distance scale was 0.02 indicating the number of base substitutions per site.

Analysis of molecular variance, population structure and pairwise distance matrix

In this study, to estimation of genetic diversity degree of *cox1* and *nad1* genes among nucleotide sequences of *E. granulosus* s.s., the analysis of molecular variance (AMOVA) including diversity (Haplotype diversity: Hd, haplotype number: Hn and Nucleotide diversity: π), neutrality indices (Tajima's *D* and Fu's Fs statistic) were carried out using DnaSP

software version 5.10 (32). As well, to compute the gene flow and *E. granulosus* (G1/G3) population structure between Iranian and Pakistani isolates, number of migrants per generation (Nm) and pairwise fixation index (*F*-statistics; *F*st) were performed using DnaSP software version 5.10. The range of *F*st in population structures is graded as follows; *F*st < 0.05 (non-meaningful differentiation), 0.05–0.15 (moderate differentiation), 0.15–0.25 (great differentiation) and *F*st > 0.25 (immense differentiation) (33). To demonstrate the inter-intra divergence and identity among identified G1, G3 and G6 genotypes, a pairwise sequence distance matrix was generated by Meg Align program (Lasergene Bio Computing Software Package).

Results

Nucleotide sequence analysis

Amplification of the partial length *nad1* and *cox1* genes yielded PCR products of approximately 444 bp and 400 bp, respectively. Nucleotide sequences of all 46 isolates analyzed in current study were aligned with reference sequences of each genotype within *E. granulosus* s.l. A total of 46 edited sequences, 41.3% (n: 19), 30.4% (n: 14) and 28.2% (n: 13) of genotypes were explicitly belonged to G1, G3 and G6, respectively. No mixed infection of *Echinococcus* genotypes was found during analysis of overlapped chromatograms. Sequence analysis of *cox1* gene (n: 23) indicated that 39.13% and 30.43% of isolates belonging to G1 (Accession numbers: MW315450 to MW315454: sheep, MW315470 to MW315472: human, MW315458: goat) and G3 (Accession numbers: MW315465 to MW315469: cattle, MW315460: camel, MW315457: goat) genotypes of *E. granulosus* s.s., respectively. Moreover, 30.43% of isolates belonging to G6 genotype (Accession numbers: MW315461 to MW315464: camel, MW315455, MW315456 and MW315459: goat) of *E. canadensis*. Sequence analysis of *nad1* gene (n: 23) indicated that 43.47% and 30.43% of isolates belonging to G1 (Accession numbers: MW321648 and MW321649: cattle, MW321650 to MW321652: human, MW321630 to MW321634: sheep) and G3 (Accession numbers: MW321645 to MW321647: cattle, MW321640: camel, MW321636 and MW321638: goat) genotypes of *E. granulosus* s.s. and 26.08% of isolates belonging to G6 genotype (Accession numbers: MW321641 to MW321644: camel, MW321635 and MW321639: goat) of *E. canadensis* in southeast of Iran.

Phylogenetic tree and haplotype network

Distance-based Maximum Likelihood cladistic trees generated by *cox1* and *nad1* sequences demonstrated that *E. granulosus* s.l. G1, G3 and G6 genotypes assigned into their specific clades (Fig. 2 and 3). A statistical parsimony of the sequence haplotypes of *cox1* and *nad1* displayed star-like features in the overall population including IR9/PAK1/G1, IR2/PAK2/G3 and IR18/G6 for *cox1* (Fig. 4) and IR1-PAK24/G1/G3, IR4-PAK25/G1/G3 and IR11/G6 as the most common haplogroups for *nad 1* (Fig. 5). The extension (drifting) of haplotypes PAK18/G1 (sheep) and PAK26/G1 (cattle) toward Iranian haplogroup reveal that there is dawn of *Echinococcus* flow between two distinct populations (Figs. 1, 4 and 5; marked by asterisk*). In the haplotype network generated by *cox1* gene, a significant haplotype diversity of *E. granulosus* G1 and G3 genotypes was identified (including haplotypes IR1, IR2, IR4, IR8, IR9, IR11 and IR13) compared to *nad1* gene (haplotypes IR1 and IR4) (Table 1 and 2).

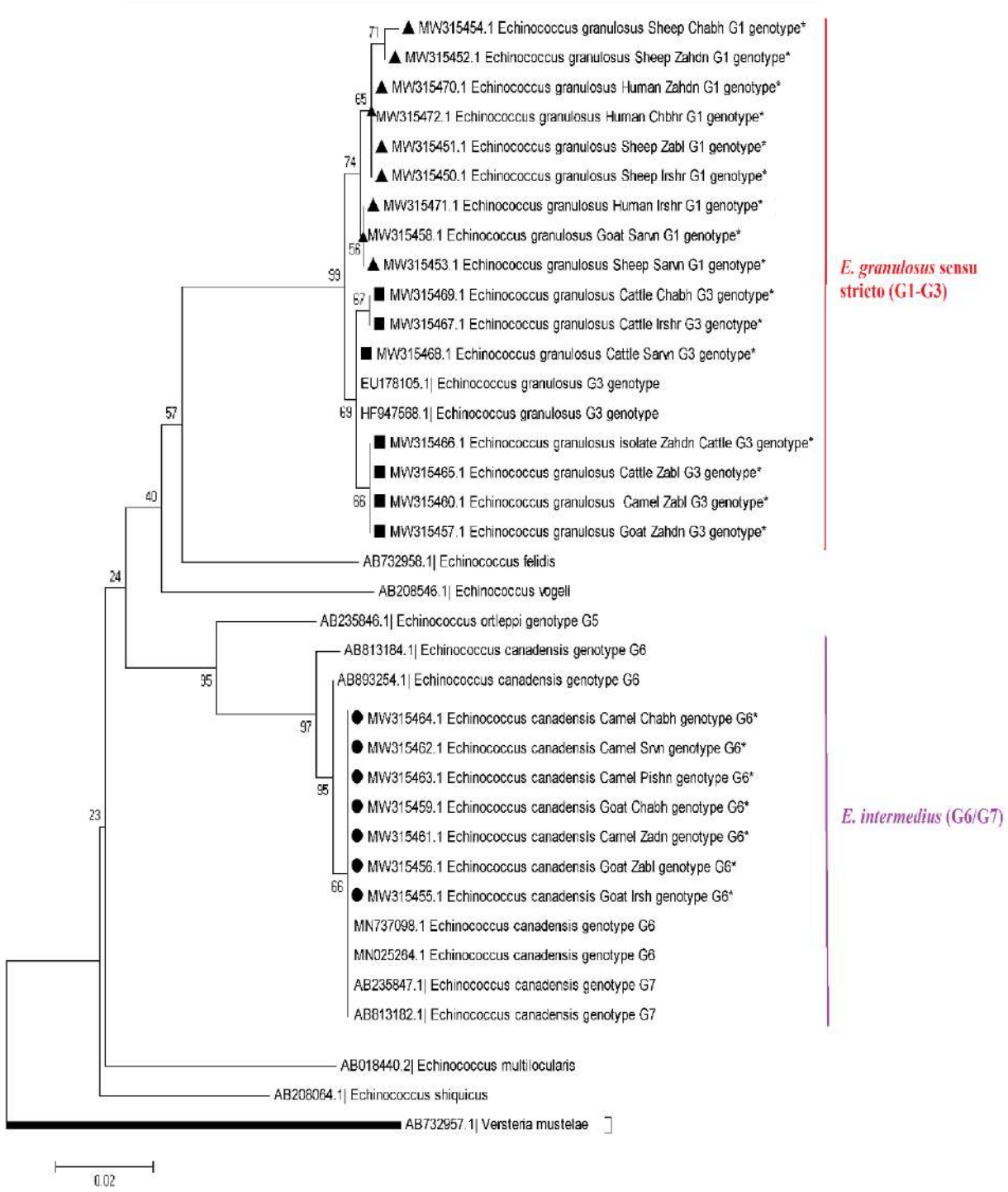

Fig. 2: A distance-based Maximum Likelihood cladistic tree of *E. granulosus* sensu lato based on the *nad1* gene. Values on the tree nodes are bootstrap proportions (%). Only bootstrap values higher than 70% are indicated on each branch. The collected location characterized genotypes (G1/G3/G6) and their accession numbers marked by asterisk (*) and geometric shapes. *Taenia (Versteria) mustelae* was considered as an out-group branch (Accession No: AB732957)

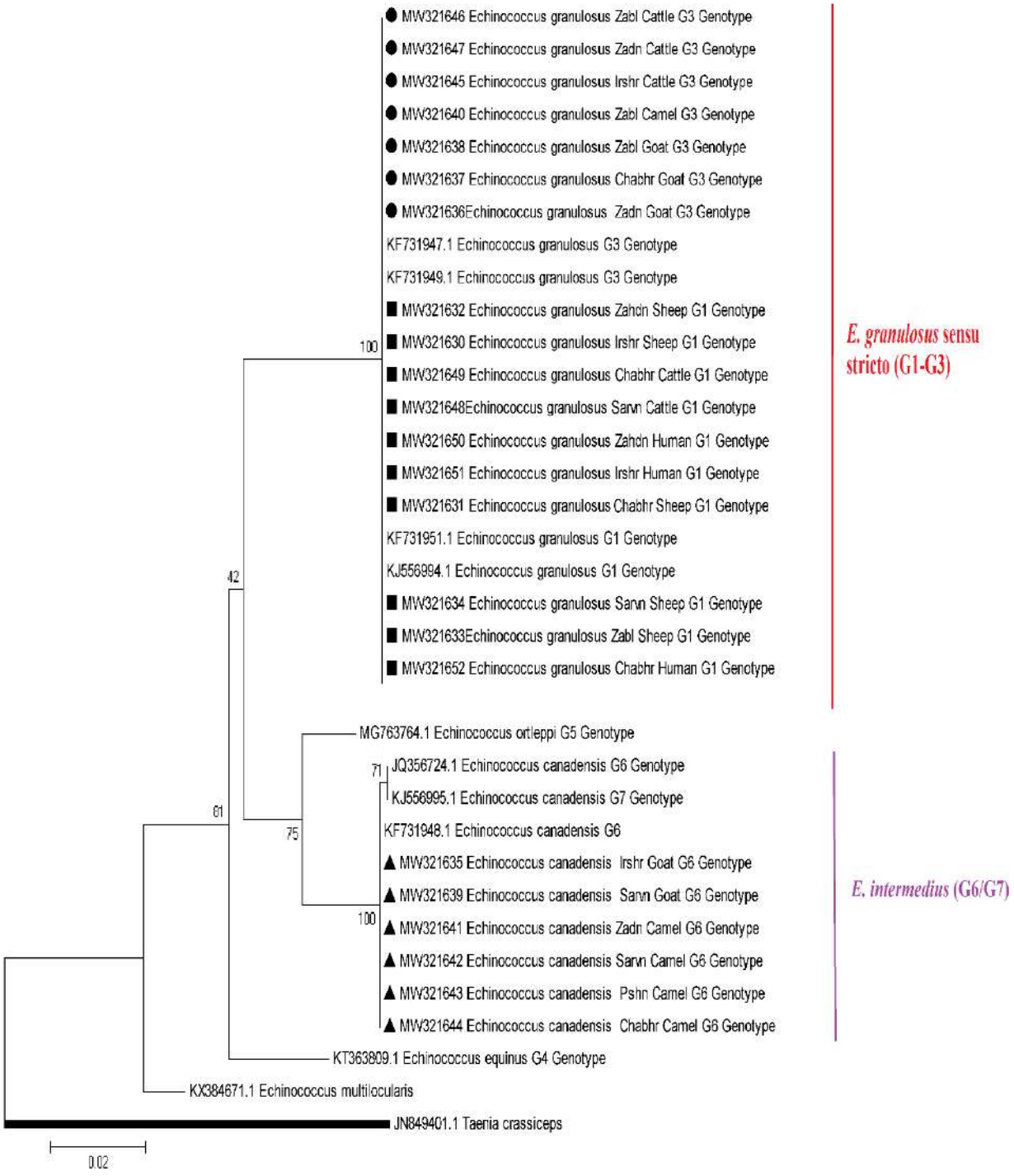

Fig. 3: A distance-based Maximum Likelihood cladistic tree of *E. granulosus* s.l. based on the *cox1* gene. Values on the tree nodes are bootstrap proportions (%). Only bootstrap values higher than 70% are indicated on each branch. The collected location characterized genotypes (G1/G3/G6) and their accession numbers marked by asterisk (*) and geometric shapes. *Taenia crassiceps* was considered as an out-group branch (Accession No: JN849401)

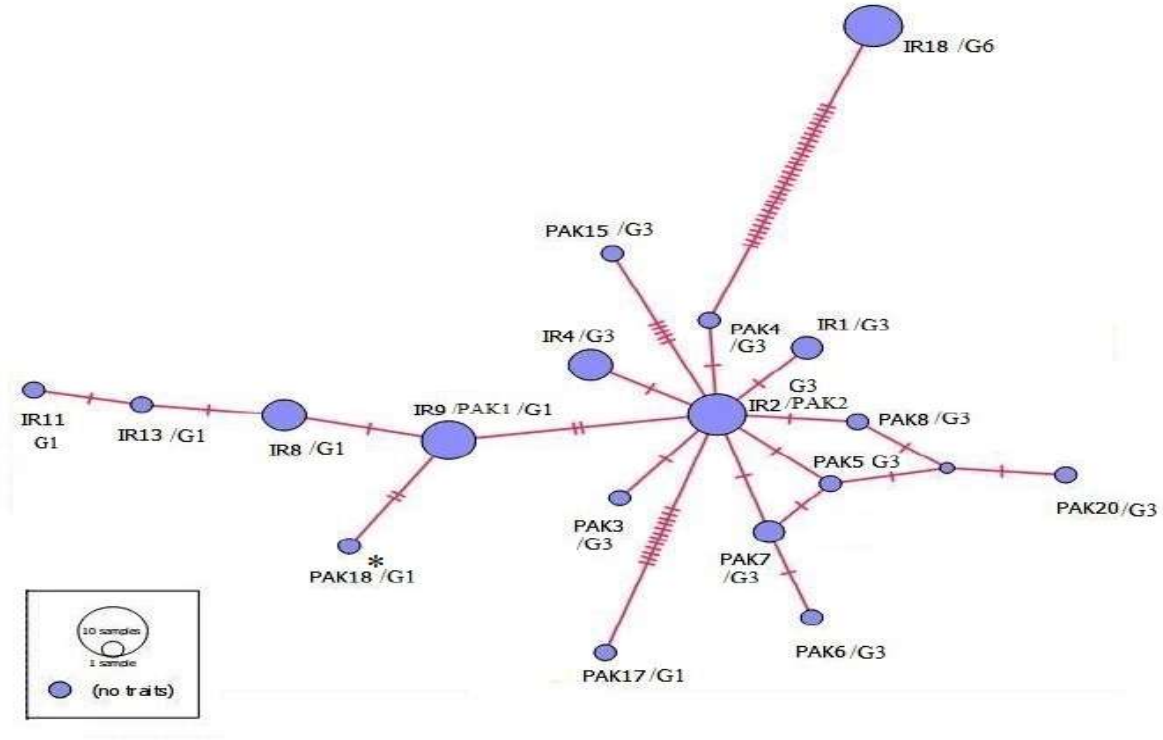

Fig. 4: The Median-joining haplotype network of *E. granulosus* G1/G3/G6 genotypes based on the *cox1* gene. Haplogroups were designated as IR9/PAK1/G1, IR2/PAK2/G3 and IR18/G6 as the most common haplotypes. Abbreviations: IR: Iranian haplotype, PAK: Pakistani haplotype

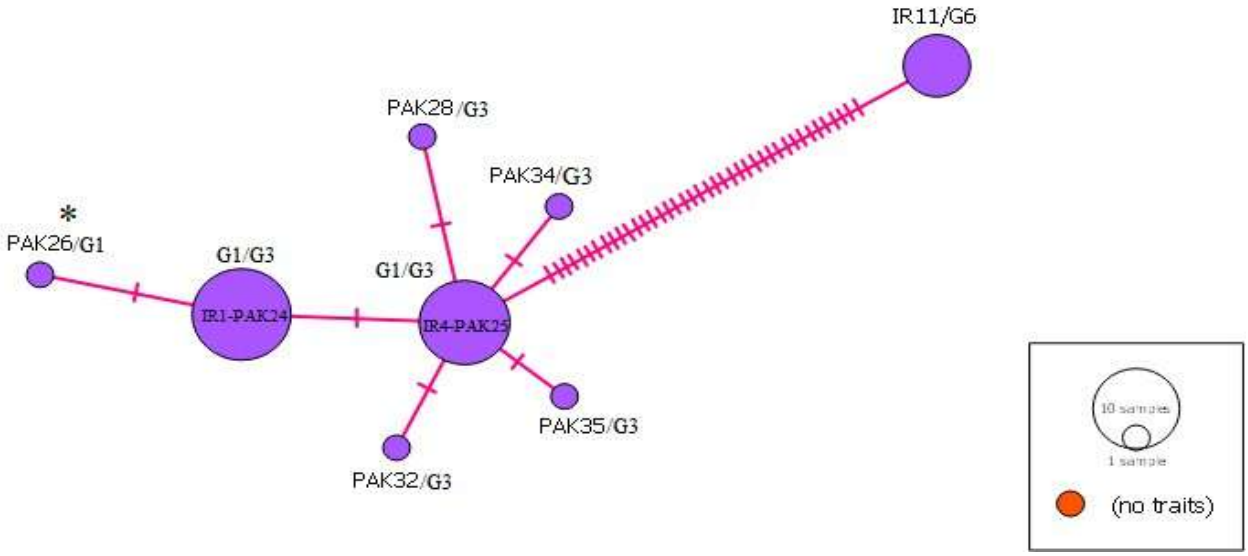

Fig. 5: The Median-joining haplotype network of *E. granulosus* G1/G3/G6 genotypes based on the *nad1* gene. Haplogroups were designated as IR1/PAK24/G1/G3, IR4/PAK25/G1/G3 and IR11/G6 as the most common haplotypes. Abbreviations: IR: Iranian haplotype, PAK: Pakistani haplotype

Table 1: Diversity and neutrality indices of *E. granulosus* sensu stricto (G1/G3) and *E. canadensis* (G6) isolates obtained from this study based on nucleotide sequences of *cox1* gene. N: number of isolates; Hn: number of haplotypes; Hd: haplotype diversity; Nd: nucleotide diversity.

Country (Province)/Genotype	***Host (n; genotype)***	***Diversity indices***					***Neutrality indices***	
		N	**Hn**	**Hd± SD**	**Number of segregating sites**	**Nd (π)**	**Tajima's D***	**Fu's Fs statistic****
Iran (Sistan and Baluchestan)/*E. granulosus* sensu stricto (G1-G3)	Human (3; G1), Sheep (5; G1), Cattle (3;G1, 2;G3), Camel (1; G3) and Goat (1; G1, 1;G3	16	7	0.867 ± 0.050	7	0.00694	-0.00694	-1.002
Iran (Sistan and Baluchestan) *E.canadensis* (G6)	Camel (4) and Goat (3)	7	1 (Common haplotype;IR18)	0.000	0.000	0.000	0.000	0.000
Total		23	8					

*$P < 0.01$
**$P < 0.02$

Table 2: Diversity and neutrality indices of *E. granulosus* sensu stricto (G1/G3) and *E. canadensis* (G6) isolates obtained from this study based on nucleotide sequences of *nad1* gene. N: number of isolates; Hn: number of haplotypes; Hd: haplotype diversity; Nd: nucleotide diversity

Country (Province)/Genotype	***Host (n; genotype)***	***Diversity indices***					***Neutrality indices***	
		N	**Hn**	**Hd± SD**	**Number of polymorphic sites**	**Nd (π)**	**Tajima's D***	**Fu's Fs statistic****
Iran (Sistan and Baluchestan)/*E. granulosus* sensu stricto (G1-G3)	Human (3; G1), Sheep (5; G1), Cattle (2;G1, 3;G3), Camel (1; G3) and Goat (3;G3)	17	2	0.485± 0.00618	1	0.00149	1.23804	1.233
Iran (Sistan and Baluchestan) *E. canadensis* (G6)	Camel (4) and Goat (3)	6	1 (Common haplotype;IR 11)	0.000	0.000	0.000	0.000	0.000
Total		23	3					

* $P < 0.01$
** $P < 0.02$

Diversity, neutrality, fixation indices and pairwise distance matrix

In current study, the diversity and neutrality indices were estimated for *cox1* and *nad1* nucleotide sequences of *E. granulosus* s.s. in southeast of Iran (Tables 1 and 2). Based on AMOVA test, a moderate (*nad1*, Hd: 0.485; Hn: 2) to high (*cox1,* Hd: 0.867; Hn: 7) haplotype (genetic) diversity of *E. granulosus* s.s. (G1/G3) and a low nucleotide diversity (π: 0.00149-0.00694) were observed in human and animal intermediate hosts (Tables 1 and 2). However, no genetic diversity (Hd: 0.00; Hn: 1) was observed among isolates belonging to G6 genotype. The negative value of neutrality test (for *cox1*: Tajima's *D*: -0.00694; Fu's Fs statistic: -1.002) for *E. granulosus* s.s. (G1/G3) displayed deviation from neutrality.

Moreover, the pairwise fixation index (*F*st: 0.11144-0.12759) indicated *E. granulosus* s.s. (G1/G3) populations are genetically moderate differentiated between southeast of Iran and Pakistan (Table 3), while number of migrants for *E. granulosus* s.s., populations was 1.71-1.99. The findings of pairwise *cox1* sequence distance matrix G1 genotype displayed an intra-identity 99.2%-100% and intra-divergence 0%-0.8%. In addition, an intra-identity 99.5%-100% and intra-divergence 0%-0.5% was identified for G3 genotype. Inter-species divergence between G1-G3, G1-G6 and G3-G6 was 0.5%-1.6%, 8.7%-9.2% and 9.4%-9.7%, respectively (Fig. 6). However, no intra-divergence (identity 100%) was observed among sequences of G6 genotype (Fig. 6).

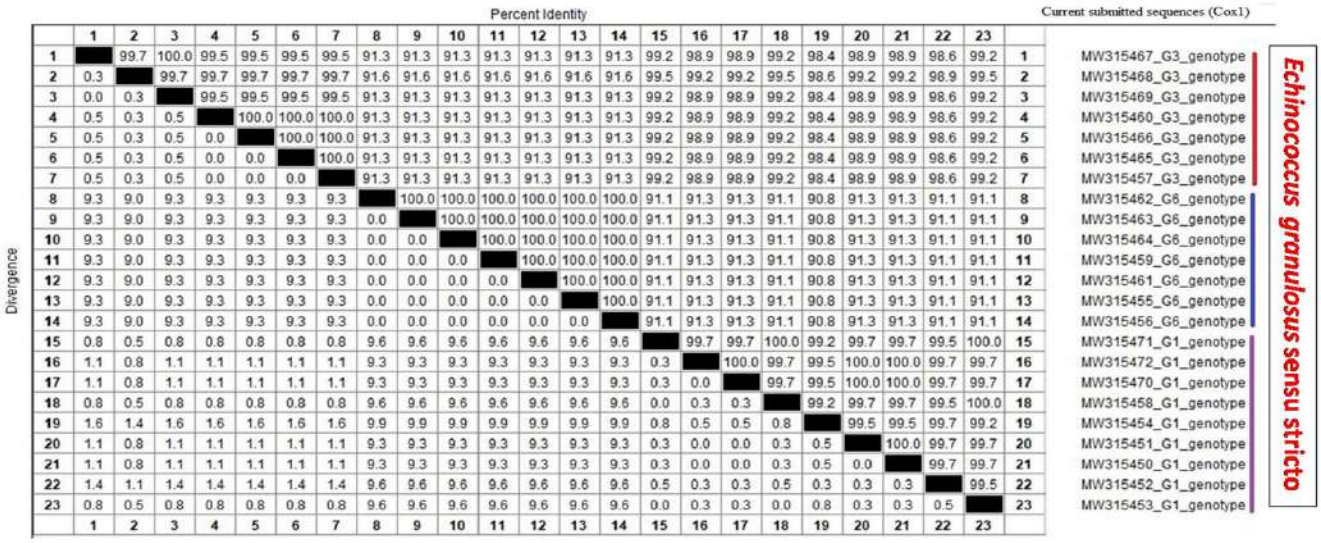

Fig. 6: The pairwise sequence distance matrix (divergence and percent identity) of *E. granulosus* genotypes (G1/G3/G6) based on *cox1* gene

Table 3: Pairwise Fst (below the diagonal) and estimated number of migrants per generation (Nm) between *E. granulosus* s.s., populations calculated from the nucleotide data set of *cox1* and *nad1* genes at southeast of Iran (Sistan and Baluchestan province) and Pakistan

Populations (Cox1)			***Populations (Nad1)***		
Country	Iran (Sistan and Baluchestan)	Pakistan	Country	Iran (Sistan and Baluchestan)	Pakistan
Iran (Sistan and Baluchestan)	-	1.99	Iran (Sistan and Baluchestan)	-	1.71
Pakistan	0.11144	-	Pakistan	0.12759	-

Discussion

Knowledge on the circulating CE genotypes and their genetic traits in various ranges of intermediate hosts in adjoining countries can provide a keystone for upcoming epidemiological study on the transmission paradigms of *E. granulosus* to implement preventive policies for monitoring, surveillance and CE control procedures (8,13,34).

Sequence analysis of *cox1-nad1* genes illustrated that G1 (isolated from human, cattle, sheep and goat), G3 (isolated from goat, camel and cattle) and G6 (isolated from camel and goat) genotypes of CE with a moderate (*nad1*, Hd: 0.485) to high (*cox1*, Hd: 0.867) genetic diversity are being unambiguously circulated in southeast of Iran, where located at the neighborhood of Pakistan. Our molecular epidemiology results indicate that the goat as a susceptible intermediate host can potentially play in the maintenance and diverse transmission of *E. granulosus* G1/G3/G6 genotypes in the interaction with canids in Sistan and Baluchestan province.

In current study, the mitochondrial DNA markers were employed to explore intraspecific variations, because of semi-conserved nature, maternal inheritance, high mutation rate, do not recombine and high evolutionary rate (16,35). However, the current Hd findings specified that the partial length *cox1* (Hn: 7, Hd: 0.867) gene can conspicuously discriminate the new mutants compared to *nad1* (Hn: 2, Hd: 0.485), which may be attributed to hyper variation in the copy number/per cell of *cox1* and/or the presence of considerable polymorphic coding regions of *cox1* (16,36).

The negative neutrality indices (for *cox1*: Tajima's *D*: -0.00694; Fu's Fs statistic: -1.002) for *E. granulosus* s.s. (G1/G3) displayed variation from neutrality suggesting a new population structure expansion or negative selection. The occurrence of these negative values implies evidence of some probable mechanisms in the southeast of Iran containing population size equilibrium, selective sweep hypothesis, and pattern of neutral mutation (14,28,29).

In this study, the Median-joining networks of *cox1* and *nad1* and phylogeographic analysis demonstrated that haplotypes of *E. granulosus* s.l. (G1/G3/G6) were grouped into their specific haplogroups. However, the haplotypes PAK18/G1 (Accession no: MN640735, sheep) and PAK26/G1 (Accession no: MN886277, cattle) extended toward Iranian haplogroup (IR9/PAK1 and IR1-PAK24). The fact that the haplotypes PAK18/G1 and PAK26/G1 extended into Iranian haplogroup, reveals that there is dawn of *Echinococcus* flow owing to alleles shift of between mentioned populations via transport of livestock or their domestication.

In this exploration, the amounts of *F*st value (0.11144-0.12759) for both *cox1* and *nad1* genes revealed that *E. granulosus* s.s. populations are genetically moderate differentiated between Iranian and Pakistani populations which is strongly confirmed by migration of haplotypes PAK18/G1 and PAK26/G1 toward Iranian haplogroup. In general, diploid helminthes e. g. *Echinococcus* with sexual and asexual reproduction methods reveal various patterns of hypothetical genetic diversity (37).

In a recent study, Alvi et al., by employing the complete *nad1* and *cox1* gene sequences of *E. granulosus* s.s. demonstrated a higher genetic diversity in cattle and buffalo hydatid cysts isolated in Pakistan (21).

In concurrence with our results, Mahami-Oskouei et al., by targeting concatenated nucleotide sequences of *cox1* and *nad1* characterized that *E. granulosus* s.s. (G1/G3) populations are genetically moderate differentiated among northwest of Iran and Turkey (13).

On the one hand, it has been demonstrated that *Echinococcus* s.s. (G1/G3) with high degree of genetic diversity (*cox1*), are not genetically differentiated among north, south, west and center of populations of Iran due to extensive gene sharing of *Echinococcus* haplotypes (8).

Also, Yan et al., by utilizing *nad1* and *atp*6 markers, were demonstrated that the variation of *E. granulosus* in populations of Tibetan plateau which was the main pattern of the total genetic diversity, as well as based on *F*st and Nm indices were demonstrated that the *Echinococcus* populations not differentiated in genetic pattern (38).

Conclusion

Current results indicate that a various range of genetic variation of *E. granulosus* G1, G3 and G6 as dominant genotypes of CE are unequivocally circulating among human and domestic livestock in the southeastern Iran. We indicated that the genetic structure of *E. granulosus* s.s. population was genetically moderate differentiated due to gene migration of G1 haplotypes between Iranian and Pakistani populations; also, it will become the basis of public health strategy for hydatidosis control. To evaluate future studies on a macroevolutionary scale, employing full-length concatenated mitochondrial genes based on next-generation sequencing and/or multilocus microsatellite typing can provide a principal framework for perceiving the origins of *Echinococcus* lineages and their patterns of diversification in other border areas of Iran.

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Conflicts of interest

The authors declare that there is no conflict of interests.

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