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Genetic variability of *Echinococcus granulosus* complex in various geographical populations of Iran inferred by mitochondrial DNA sequences

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ABSTRACT

To investigate the genetic variability and population structure of *Echinococcus granulosus* complex, 79 isolates were sequenced from different host species covering human, dog, camel, goat, sheep and cattle as of various geographical sub-populations of Iran (Northwestern, Northern, and Southeastern). In addition, 36 sequences of other geographical populations (Western, Southeastern and Central Iran), were directly retrieved from GenBank database for the mitochondrial cytochrome *c* oxidase subunit 1 (*cox1*) gene. The confirmed isolates were grouped as G1 genotype (n = 92), G6 genotype (n = 14), G3 genotype (n = 8) and G2 genotype (n = 1). 50 unique haplotypes were identified based on the analyzed sequences of *cox*1. A parsimonious network of the sequence haplotypes displayed star-like features in the overall population containing IR23 (22: 19.1%) as the most common haplotype. According to the analysis of molecular variance (AMOVA) test, the high value of haplotype diversity of *E. granulosus* complex was shown the total genetic variability within populations while nucleotide diversity was low in all populations. Neutrality indices of the *cox1* (Tajima's D and Fu's Fs tests) were shown negative values in Western-Northwestern, Northern and Southeastern populations which indicating significant divergence from neutrality and positive but not significant in Central isolates. A pairwise fixation index (*F*st) as a degree of gene flow was generally low value for all populations (0.00647–0.15198). The statistically *F*st values indicate that *Echinococcus* sensu stricto (genotype G1–G3) populations are not genetically well differentiated in various geographical regions of Iran. To appraise the hypothetical evolutionary scenario, further study is needed to analyze concatenated mitogenomes and as well a panel of single locus nuclear markers should be considered in wider areas of Iran and neighboring countries.

1. Introduction

Current phylogeographic exploration based on both mitochondrial and nuclear DNA genes disclosed that *Echinococcus granulosus* complex consists at least four valid clades; *E. granulosus* sensu stricto (genotype G1–G3), *E. equinus* (G4), *E. ortleppi* (G5) and *E. canadensis* (genotype G6–G10) (Thompson and McManus, 2002; Nakao et al., 2007, 2010a; Knapp et al., 2011), whereas the validity of the G9 genotype has been questioned (Nakao et al., 2007; Lavikainen et al., 2008; Thompson, 2008). Among mentioned clades, *E. granulosus* s.s. is dominantly regarded as extensive geographical distribution in Middle East countries particularly in Iran where the various definitive hosts (predatory canids) harbor adult tapeworms in their intestine and the larval stage of *E. granulosus* complex serve by intermediate hosts (sheep, camel, cattle, goat, horse and etc.,) (Craig et al., 2007; Spotin et al., 2012; Youssefi et al., 2013).

The overall annual cost of hydatidosis was estimated at US$232.3 million in Iran (Harandi et al., 2012). The creation of re-emergent and emergent *Echinococcus* strains containing various genetic diversity can be related to cross transmission patterns of

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parasite among canid-intermediate hosts and immunological interactions between host-parasite (Bowles et al., 1992; Amirmajdi et al. 2011; Spotin et al., 2012; Thompson 2013; Youssefi et al., 2013; Thompson and Jenkins 2014).

A number of Iranian researchers have been locally focused their molecular experiments on inter-intra divergence levels of *E. granulosus* strains in different definitive/intermediate host species and their findings have shown that the new haplotypes with different ranges of diversity indices are sympatrically circulating (Zhang et al., 1998; Harandi et al., 2002; Ahmadi and Dalimi, 2006; Amin Pour et al., 2011; Shahnazi et al., 2011; Gholami et al., 2012; Hajialilo et al., 2012; Parsa et al., 2012; Rajabloo et al., 2012; Pezeshki et al., 2013; Mahami-Oskouei et al., 2014, 2015; Rostami et al., 2015; Spotin et al., 2015). However, according to our current phylogenetic knowledge, here is not a comparative study on genetic variability and population structures of *E. granulosus* complex among different geographical foci of Iran (Yanagida et al., 2012).

Amongst mitogenome markers tested, the cytochrome *c* oxidase subunit 1 (*cox1*) because of having some exceptional traits including low repetitive sequences in length genome, to be haploid and its semi-conserved structure is extensively considered as one of the well known evolutionary marker for discriminating genetic structure of *Echinococcus* populations (Nakao et al., 2010a; Casulli et al., 2012; Yanagida et al., 2012).

Exploring genetic traits of *E. granulosus* populations has notable implications for controlling strategies such as efficacy of anti-helminthes treatment in dogs and their relationship with drug resistance/susceptibility; also it is of great importance to genetically characterize the population structures (Thompson and McManus, 2002). The main purpose of this study was to evaluate genetic variability of *E. granulosus* complex as of different geographical foci of Iran inferred by mitochondrial DNA sequences in order to get the parasitic gene flow among different populations.

2. Materials and methods

2.1. Sample collection

From August 2012 to February 2015, 79 hydatid cysts and adult *E. granulosus* were taken from several definitive/intermediate hosts (dog, human, camel, goat, sheep and cattle) at various sites of Iran including; Northwestern (East Azerbaijan province), Northern (Mazandaran and Semnan provinces) and Southeastern (Kerman province) populations (Table 1) (Fig. 1).

Moreover, to compare the analyzed sequences of current exhaustive investigation with other geographical populations of Iran, 36 sequences including Northwestern (Ardabil province), Central (Isfahan), Northern (Tehran provinces) and Western (Lorestan province) populations were directly retrieved from GenBank sequence database for FASTA format (Table 1). All animal samples were collected from slaughterhouses following the local laws for the preservation of domestic animals. Human isolates were surgically removed from cystic echinococcosis human cases and also some paraffin-embedded tissue samples were obtained for DNA extraction. Each isolate consisted of adult worm or germinal layer/protoscolices were removed from an individual adult/hydatid cyst sample. Taken samples were extensively washed in normal saline, fixed in 70% v/v ethanol and frozen at −20 °C until molecular activities.

2.2. Total genomic DNA extraction and PCR amplification

Genomic DNA was extracted using a High Pure PCR Template Preparation (Roche, Mannheim, Germany) kit according to the manufacturers' instructions. The standard PCR was employed to detect *Echinococcus* parasites by targeting the partial *cox*1 ORF (open reading frame) gene using the primer sets of JB3/JB4.5 (Bowles et al., 1992, 1993). PCR amplifications were performed under following PCR conditions: 94 °C for 5 min as an initial denaturation, 94 °C for 30 s, 56 °C for 45 s, 72 °C for 35 s in 35 cycles and a final extension at 72 °C for 10 min. PCR products (amplicons) were subjected to electrophoresis in 1.5% agarose gel and were observed under ultraviolet light after staining by safe stain for 30 min.

2.3. Sequencing, haplotype network, phylogenetic analyses

Some amplicons were purified with the Wizard SV Clean-up System (Promega). The final DNA concentration was estimated by comparing with DNA Ladder Marker (Promega) in 1.5% agarose gel. Amplicons were directly sequenced by targeting *cox1* gene in both directions by ABIPRISMTM 3130 Genetic Analyzer automated sequencer (Applieled Biosystem, USA). Ambiguous sites were coded using the standard IUPAC codes for combinations of two or more bases. Sequence contigs of all samples were aligned and edited visually in consensus positions compared to GenBank sequences from all regional species using Sequencher ™v.4.1.4 Software for PC (Gene Codes Corporation).

The number of segregating sites, diversity indices (Haplotype diversity; Hd and Nucleotide diversity: π) and neutrality indices (Tajima's D and Fu's Fs tests) were estimated by DnaSP software version 5.10 (Rozas et al., 2010). The population genetic structure was analyzed by Arlequin version 3.11 (Excoffier and Lischer, 2010). The degree of gene flow (gene migration) among the populations was evaluated using a pairwise fixation index (*F*st) (Reynolds et al., 1983). A network of mitochondrial haplotypes based on the sequences of *cox1* was drawn by PopART software using statistical parsimony (Bandelt et al., 1999).

3. Results

3.1. Nucleotide sequence analysis

The *cox1* gene (nearly 450 bp) was successfully amplified from all 79 isolates. The analyzed sequences were compared and processed with retrieved sequences (n = 36) from GenBank database.

Overall, the vast majority of isolates were belonged to G1 genotype (n = 92) (obtained from sheep, cattle, goat, human and dog cases in all areas), 14 isolates as G6 genotype (obtained from camel and dog cases in Northwestern, Northern and Southeastern areas), eight isolates as G3 genotype (obtained from sheep and camel cases in Northwestern, Central and Southeastern areas) and a isolate as G2 genotype (from dog case obtained from western Iran).

The geographical localization, identified genotypes and host origins are shown in Table 2.

The nucleotide sequences reported in this study were deposited at the GenBank under accession numbers: KT153995-KT154000, HM563012-HM563019, KP723336-KP723340 and KJ540227-KJ540231. The retrieved accession numbers of sequences from Western (Lorestan), Northern (Mazandaran and Tehran), Northwestern (Ardabil), Central (Isfahan), and Southeastern (Kerman) populations were: JN604097-JN604104, JN792923-JN792930, KF612376-KF612400, AB677806-AB677814, JN048505-JN048513, and KF443160-KF443187.

Although deletions or insertion mutations (*I*ndel) were not detected, however transition and transversion mutations were distinguished amongst our analyzed sequences. Within 280 bp consensus position, 56 point mutations were observed. 37 of these were parsimony-informative sites and 19 of these were singleton variable sites.