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Phylogeography, genetic variability and structure of *Acanthamoeba* metapopulations in Iran inferred by 18S ribosomal RNA sequences: A systematic review and meta-analysis

Adel Spotin[1✉], Hamid Reza Moslemzadeh[1], Mahmoud Mahami-Oskouei[2], Ehsan Ahmadpour[2], Maryam Niyyati[3], Seyed Hossein Hejazi[4], Fatemeh Memari[3], Jafar Noori[2]

[1]*Immunology Research Center, Tabriz University of Medical Sciences, Tabriz, Iran*

[2]*Department of Parasitology and Mycology, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran*

[3]*Department of Medical Parasitology and Mycology, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran*

[4]*Department of Parasitology and Mycology, School of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran*

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ABSTRACT

Objective: To verify phylogeography and genetic structure of *Acanthamoeba* populations among the Iranian clinical isolates and natural/artificial environments distributed in various regions of the country.

Methods: We searched electronic databases including Medline, PubMed, Science Direct, Scopus and Google Scholar from 2005 to 2016. To explore the genetic variability of *Acanthamoeba* sp, 205 sequences were retrieved from keratitis patients, immunosuppressed cases and environmental sources as of various geographies of Iran.

Results: T4 genotype was the predominant strain in Iran, and the rare genotypes belonged to T2, T3, T5 (*Acanthamoeba lenticulata*), T6, T9, T11, T13 and T15 (*Acanthamoeba jacobsi*). A total of 47 unique haplotypes of T4 were identified. A parsimonious network of the sequence haplotypes demonstrated star-like feature containing haplogroups IR6 (34.1%) and IR7 (31.2%) as the most common haplotypes. In accordance with the analysis of molecular variance, the high value of haplotype diversity (0.612–0.848) of *Acanthamoeba* T4 represented genetic variability within populations. Neutrality indices of the 18S ribosomal RNA demonstrated negative values in all populations which represented a considerable divergence from neutrality. The majority of genetic diversity belonged to the infected contact lens and dust samples in immunodeficiency and ophthalmology wards, which indicated potential routes for exposure to a pathogenic *Acanthamoeba* sp. in at-risk individuals. A pairwise fixation index (F_{ST}) was from low to high values (0.024 33–0.418 92). The statistically F_{ST} points out that T4 is genetically differentiated between north-west, north-south and central-south metapopulations, but not differentiated between west-central, west-south, central-south, and north-central isolates.

Conclusions: An occurrence of IR6 and IR7 displays that possibly a gene flow of *Acanthamoeba* T4 occurred after the founder effect or bottleneck experience through ecological changes or host mobility. This is the first systematic review and meta-analysis providing new approaches into gene migration and transmission patterns of *Acanthamoeba* sp, and targeting at the high-risk individuals/sources among the various regions of Iran.

1. Introduction

Free-living amoebae of the *Acanthamoeba* spp. are well-known as amphizoic ubiquitous protozoan parasites in the worldwide that are found in various clinical isolates from artificial/natural environments including soil, sea water, mineral water, feces, air, contact lenses, air conditioning units, biofilms, and clinical samples (immunosuppressed and keratitis patients) [1–3]. Clinically, *Acanthamoeba* spp. are the causative agents of *Acanthamoeba* keratitis (AK) and granulomatous amebic encephalitis and disseminated infections [4]. The phylogenetic analyses based on the nuclear 18S ribosomal RNA (*rRNA*) gene and 18S ribosomal DNA sequences have recently distinguished at least 20 genotypes (T1–T20 genotypes) and 3

✉First and corresponding author: Adel Spotin, Ph.D, Immunology Research Center, Tabriz University of Medical Sciences, Tabriz, Iran.
Tel: +98 04133344777
E-mails: Adelespotin@gmail.com, Spotina@tbzmed.ac.ir

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morphological groups of *Acanthamoeba* spp. [5,6]. The clinical isolates and environmental samples are dominantly infected to the genotype T4 and morphological group II [7,8].

However, the ecological changing, imported cases from neighboring countries, agricultural activities, immunological interactions between host-parasite and cross-transmission of *Acanthamoeba* spp. among the subpopulations have led to drifting parasite genome and the emergence of new *Acanthamoeba* haplotypes. Over the past few years, what the evolutionary history of the parasites has discussed was based on macroevolutionary and microevolutionary events [9–11]. Macroevolutionary discovery by using the next generation sequencing of neutral (non-adaptive) genomes provides a decisive sketch for perception of the origins of *Acanthamoeba* spp. lineages; furthermore, the genetic differentiation on microevolutionary scale confers valuable data regarding the gene flow (migration), speciation and mode of *Acanthamoeba* spreading in geographical districts of the world [12]. Exploring traits of homogeneity and heterogeneity in *Acanthamoeba* sp. (haplotypes diversity and nucleotide diversity) among the keratitis/immunosuppressed patients and natural sources can reflect a warning alarm for pathogenicity virulence infectivity. Amoebic keratitis due to *Acanthamoeba* parasites is dramatically rising in Iran [3]. In spite of the fact, a number of regional researchers have focused their molecular explorations on discrimination of *Acanthamoeba* sp. [13–16], but here is not an eligible comparative study on genetic population structure, groups at-risk, phylogeography and expansion patterns of *Acanthamoeba* spp. in various regions of Iran.

According to the clinical importance of acanthamoebiasis, a genetic analysis of parasite can be helpful to target at-risk populations and to explain how the parasite has extended in various geographical regions. In the current systematic review and meta-analysis, we will address papers on *Acanthamoeba* infection to show the phylogeography, genetic variability and structure of *Acanthamoeba* metapopulations in Iran inferred by *18S rRNA* sequences. In addition, the potential sources threatening susceptible populations will forecast based upon the genetic indices.

2. Materials and methods

2.1. Search strategy and study collection

We surveyed electronic database sources including Science Direct, PubMed, Scopus, Google Scholar and Proquest for articles in English and IranMedex, Magiran, SID and IranDoc for articles in Persian from 2005 to 2016. The following keywords were considered in our initial search strategy: *Acanthamoeba* spp., genetic variability, gene flow, molecular phylogeny, Iran, recreational areas, water, river, soil, biofilm, contact lens, keratitis patients, and immunosuppressed patients. The flowchart exhibits the investigation design route (Figure 1).

Inclusion criteria: *Acanthamoeba* articles published in the period of 2005–2016 in Iran related to genotyping and sequencing strategies were included.

Exclusion criteria: articles conducted by RFLP method, and written in a language except Persian/English. In addition, congress articles that had not released in suitable journals were excluded.

2.2. Data extraction and analysis

To analyze the population structure and genetic variability of *Acanthamoeba* sp. (principally T4 genotype), 205 sequences of *18S rRNA* gene [as a non-neutral (adaptive) marker] were directly retrieved from GenBank database for FASTA format. Sequences belonged to keratitis patients (corneal scarping and contact lens for cosmetic purposes or visual corrections), immunosuppressed cases (HIV, cancer, diabetes, hepatitis, splenectomy and steroid therapy after organ grafts), natural environmental (soil, fresh waters, mineral springs, sea, spas, and hot springs) and dust source (biofilm) in immunodeficiency/ophthalmology wards as of various geographical subpopulations of Iran (Central, North, South-Southwest and West-Northwest). Ambiguous sites of retrieved sequences were coded using the standard IUPAC codes for combinations of two or more bases. Sequence contigs (overlapped sequences) of all samples were aligned by Clustal W method and edited visually in consensus

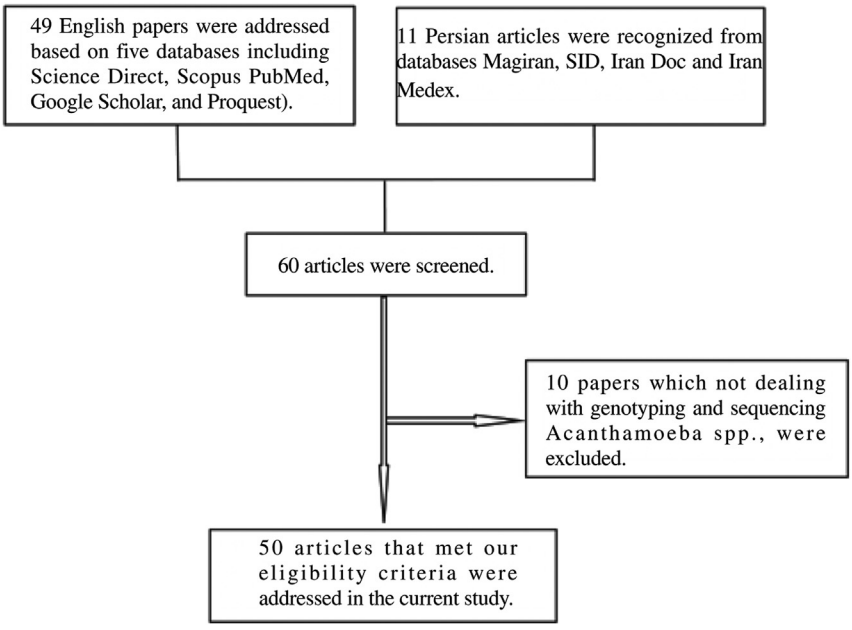

Figure 1. Flowchart describing the study design process.

Table 1

The sequences retrieved in this study from Iranian clinical isolates and natural/artificial environments distributed in various regions of the country.

Regions	Province	Contact lens	Immunosuppressed patients	Environmental sources	Dust sample
West and Northwest	- East Azerbaijan - West Azerbaijan - Ardabil	ND	ND	KT985962–78 JN585806–JN585817	ND
North	- Guilan - Mazandaran	ND	ND	JN399011–JN399023	ND
Central	- Tehran - Alborz	KU936102–KU93619	KJ786503–KJ786526	EU934046–EU934072, AB525818–AB525833	HQ833407–HQ833424, HQ833426–HQ833440, and HQ833442–HQ833445 FJ158844–FJ158856
South and Southwest	- Khuzestan (Ahvaz) - Ilam (Dehloran) - Hormozgan (Kish) - Fars (Shiraz)	ND	ND	KU356836–5, KP337292–KP337308, KP221677–KP221685	ND

Table 2

Diversity and neutrality indices of *Acanthamoeba* T4 type from different geographical foci of Iran inferred by *18S rRNA* gene.

Geographic regions of Iran	Province	Diversity indices					Neutrality indices	
		N	Hn	Hd ± SD	Nd(π) ± SD	No. of segregating sites	Tajima'*D*	Fu's *Fs* statistic
West and Northwest	- East Azerbaijan - West Azerbaijan - Ardabil	33	5	0.612 00 ± 0.005 33	0.002 76 ± 0.002 04	4	−0.598 54	−1.288
North	- Guilan - Mazandaran	12	8	0.848 00 ± 0.010 80	0.024 62 ± 0.008 58	22	−0.289 11	0.464
Central	- Tehran - Alborz	106	18	0.705 00 ± 0.001 47	0.006 05 ± 0.006 29	36	−2.308 56	−8.096
South and Southwest	- Khuzestan (Ahvaz) - Ilam (Dehloran) - Hormozgan (Kish) - Fars (Shiraz)	52	16	0.686 00 ± 0.052 10	0.005 54 ± 0.006 77	30	−2.469 27	−9.501
Total		205	47					

Hn: number of haplotypes; Hd: haplotype diversity; Nd: nucleotide diversity; ND: not determined.

string using Sequencher TMv.4.1.4 software; for PC Translation of nucleotide sequences to a protein sequence, it was performed by EXPASY online translation tool (http://web.expasy.org/translate/).

The number of segregating sites, diversity indices (Haplotype diversity: Hd and nucleotide diversity: π) and neutrality values (Tajima's *D* and Fu's *Fs* tests) were calculated by DnaSP software version 5.10 [17]. The degree of gene flow among the populations was evaluated using a pairwise fixation index (F_{ST}: F-statistics) and a number of migrants per generation (Nm) [18]. The haplotype network inferred by the common to new identified haplotypes of *18S rRNA* sequences was constructed by PopART software and median-joining algorithm [19].

Table 3

Determination of infected patients/sources to *Acanthamoeba* spp. in diverse regions of Iran based on their genotypes, haplotype numbers (Hn) and haplotype diversity (Hd).

Regions	Province	Contact lens (Hn = 9 Hd: 0.636)	Immunosuppressed patients (Hn = 1 Hd: 0.000)	Environmental sources (Hn = 9 Hd: 0.282)	Dust sample (Hn = 5 Hd: 0.562)	Reference
West and Northwest	- East Azerbaijan - West Azerbaijan - Ardabil	ND	ND	Soil: T3, T4, T5 and T11 Water: T3/T4, T4, T5, and T13	ND	[28–32]
North	- Guilan - Mazandaran	ND	ND	T4, T5, *Saccamoeba limax*	ND	[33,34]
Central	- Tehran - Alborz	T3, T4, T9 and T11/T4	T3, T4, T5	T4, T5, T6, T15 (*A. jacobsi*)	T4, T5, T11 *Hartmannella* and *Vahlkamfia*	[14,16,23,35–40]
South and Southwest	- Khuzestan (Ahvaz) - Ilam (Dehloran) - Hormozgan (Kish) - Fars (Shiraz)	ND	ND	T2, T3, T4, T5 (*A. lenticulata*) and T11, T15	ND	[23,41–43]

ND: Not determined.

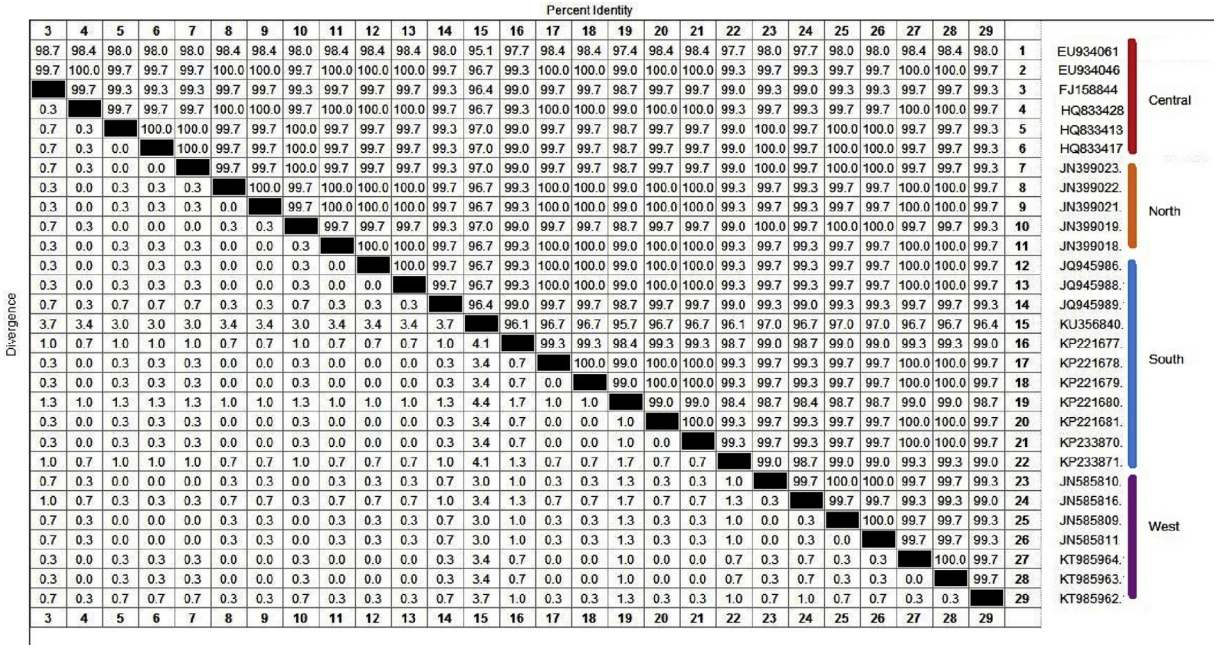

Figure 2. The sequence pairwise distances (divergence and percent identity) of *Acanthamoeba* T4 among various geographical isolates of Iran determined by the *18S rRNA* gene.

To demonstrate the demographic history of the *Acanthamoeba* population, the mismatch distribution was estimated by DnaSP software. Deviation values between the observed and expected mismatch distribution were Raggedness index, Ramos-Onsins and Rozas R2 statistics and estimate of Tau (τ; as a moment estimator in population expansion). Unimodal curves in populations indicated rapid population distribution [20].

The grade of genetic discrimination from a regional population (infrapopulation) to metapopulation annotates by F_{ST} value with ranging; 0 to 1 $F_{ST} < 0.05$ (insignificant differentiation), 0.05–0.15 (moderate differentiation), 0.15–0.25 (great differentiation) and $F_{ST} > 0.25$ (huge differentiation). To determine the definite taxonomic status of *Acanthamoeba* genotypes, a phylogenetic tree was constructed by MEGA 5.05 software based upon the maximum likelihood algorithm and Kimura 2-parameter model [21]. The accuracy of the phylogenetic tree was assessed by 1 000 bootstrap re-samplings. The pairwise distance including percent identity and divergence amongst aligned sequences were constructed using the MegAlign program (Laser Gene Bio Computing Software Package).

3. Results

The accession numbers retrieved in this study are given in Table 1. Multiple sequence alignment analysis identified 47 unique haplotypes (22.9%) (with both transversion and transition substitutions) of T4 genotype (Table 2). At *Acanthamoeba* DNA consensus string, neither insertion/deletion (Indel) mutations nor amino acid substitution (Codon position) was identified. The number of isolates, identified haplotypes and segregating sites in various geographical regions of Iran is given in Table 2. The most haplotype diversity (0.705) and identified haplotypes ($n = 18$) belonged to Central Iran (Tehran and Alborz cities). According to the analysis of molecular variance, a high value of Hd (0.612–0.848) of *Acanthamoeba* T4 represented genetic variability within all metapopulations, while nucleotide diversity was low (0.005 54 to 0.024 62) (Table 2). Neutrality indices of the *18S rRNA* gene were shown negative values (Tajima's *D* and Fu's *F*s statistic) in all populations except north isolates (Fu's *F*s: 0.464) which indicated significant divergence from neutrality (Table 2).

T4 genotype was the predominant *Acanthamoeba* strain in Iran, and rare genotypes belonged to T2, T3, T5 (*Acanthamoeba lenticulata*), T6, T9, T11, T13 and T15 (*Acanthamoeba jacobsi*) (Table 3). The sequence pairwise distances of *Acanthamoeba* T4 demonstrated a percent identity 96.1–100% and divergence 0.0–4.1% among different regions (Figure 2). The taxonomic status of *Acanthamoeba* genotypes is given in Figure 3. The majority of Hd (0.282–0.636) and haplotype number (Hn: 5 to 9) belonged to the infected contact lens, water source and dust samples in immunodeficiency/ophthalmology wards, which indicated potential routes for exposure to a pathogenic *Acanthamoeba* in at-risk individuals. Interestingly, no considerable genetic diversity of *Acanthamoeba* T4 was found in immunosuppressed patients (Hd: 0.000 and Hn: 1).

The statistical parsimony network was drawn to differentiate a genealogical correlation among the common and new haplotypes. A haplotype network displayed star-like features in the entire population containing two distinct geographical haplogroups IR6 (34.1%: Central Iran) and IR7 (31.2%: Northern, Western, Central, and Southern Iran) as the most common haplotypes (Figure 4).

The haplotypes IR75, IR133, IR137, IR140 and IR168 were shared between IR6 and IR7 (Figure 4). The observed and expected mismatch distribution, including Raggedness index (0.071 6), R2 statistic (0.023 0) and τ (0.000) for *Acanthamoeba* T4 are shown in Figure 5. An F_{ST} value as a degree of gene flow ranged from low to high values (0.024 33 to 0.418 92) (Table 4). The statistically F_{ST} indicated that T4 is genetically differentiated between north-west, north-south, and central-south metapopulations (F_{ST}: 0.114 87–0.418 92, Nm: 0.35–1.93) but not differentiated between west-central, west-south, central-south,

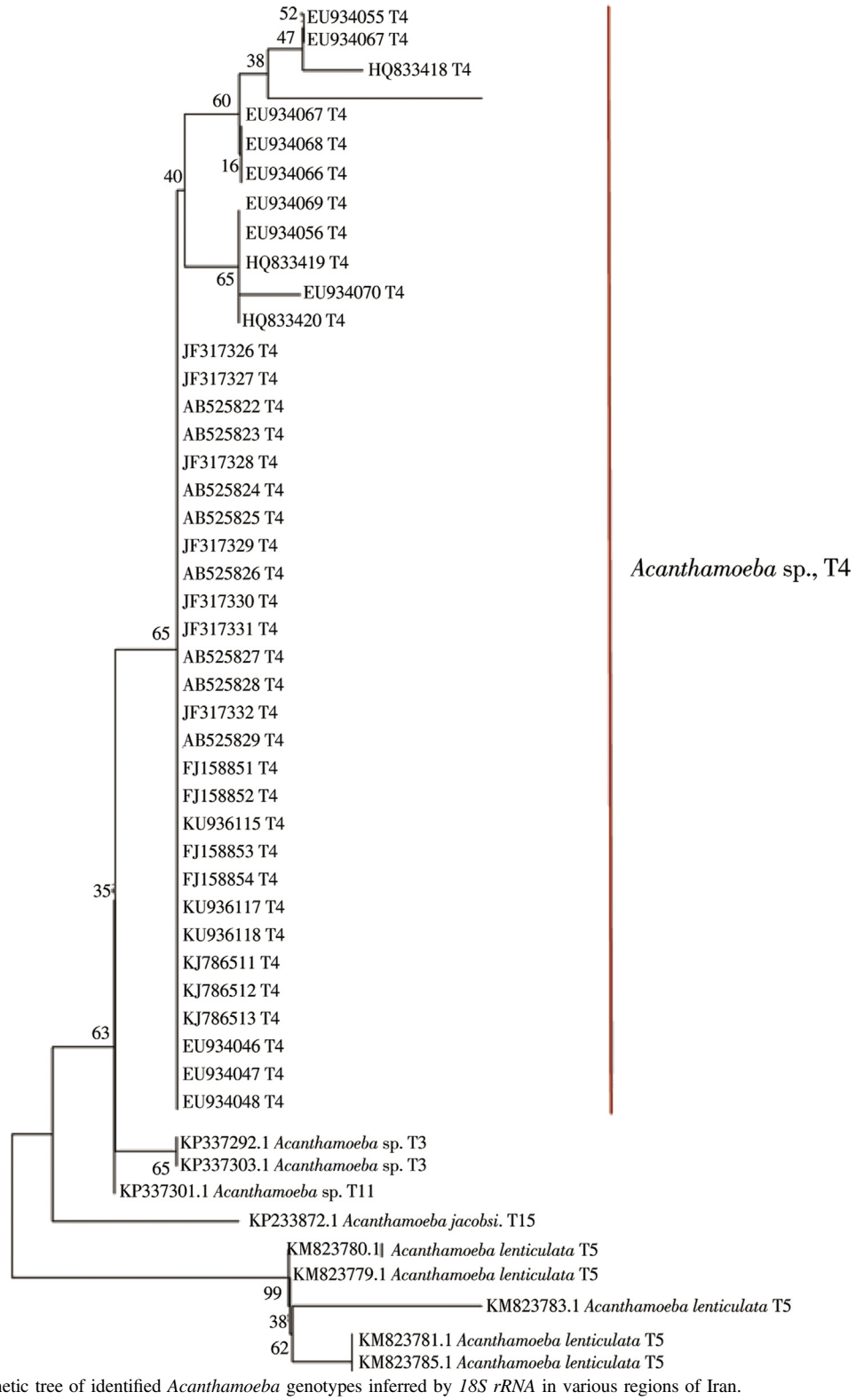

Figure 3. Phylogenetic tree of identified *Acanthamoeba* genotypes inferred by *18S rRNA* in various regions of Iran.

and north-central isolates (F_{ST}: 0.024 33–0.079 16, Nm: 2.91–10.02). The patterns of gene migrations in *Acanthamoeba* T4 among the various geographical of Iran are shown in Figure 6.

4. Discussion

The current phylo-molecular appraisal allows us to infer the genetic diversity and population structure of *Acanthamoeba* sp. on how the dominant genotype T4 has sympatrically flowed among the at-risk individuals and environmental sources in the various areas of Iran.

The meticulous knowledge on the genetic structure of *Acanthamoeba* metapopulations exemplifies the patterns of parasite sharing in consequence of ecological alterations, host mobility and imported cases from neighboring countries.

In this study a significant genetic variability of *Acanthamoeba* T4 was identified in contact lens infected to AK (Hd: 0.636) and dust samples in immunodeficiency/ophthalmology

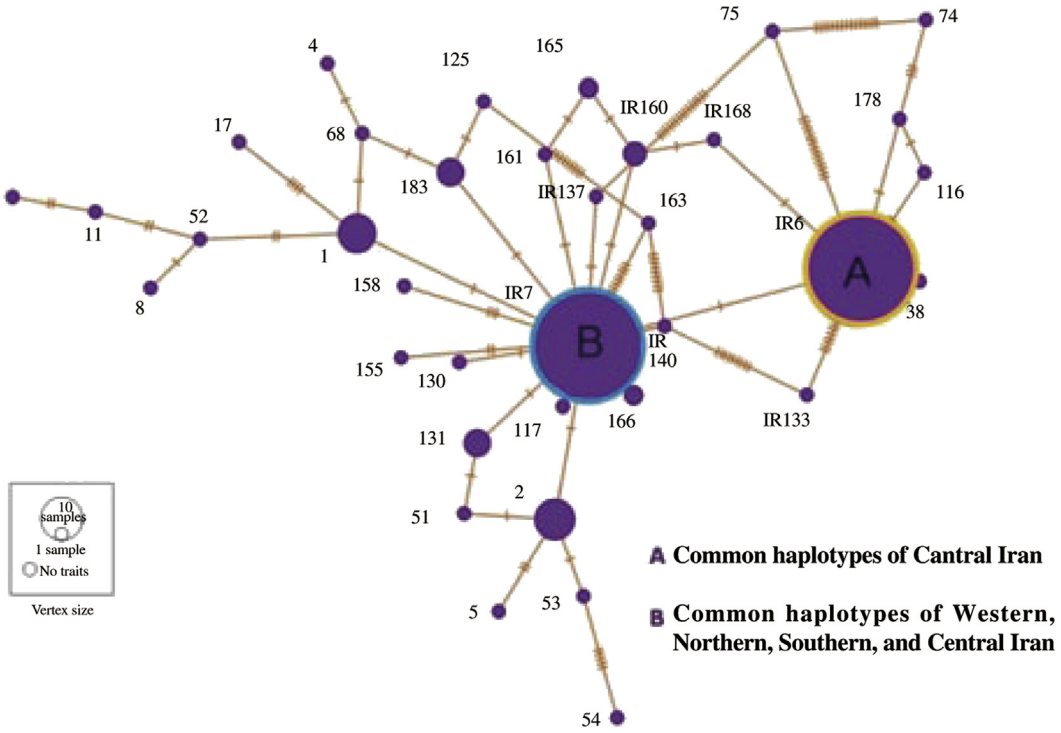

Figure 4. The haplotype network of *Acanthamoeba* T4 constructed based on median-joining algorithm from various geographical foci of Iran. The haplogroups IR6 and IR7 were addressed as common haplotypes.

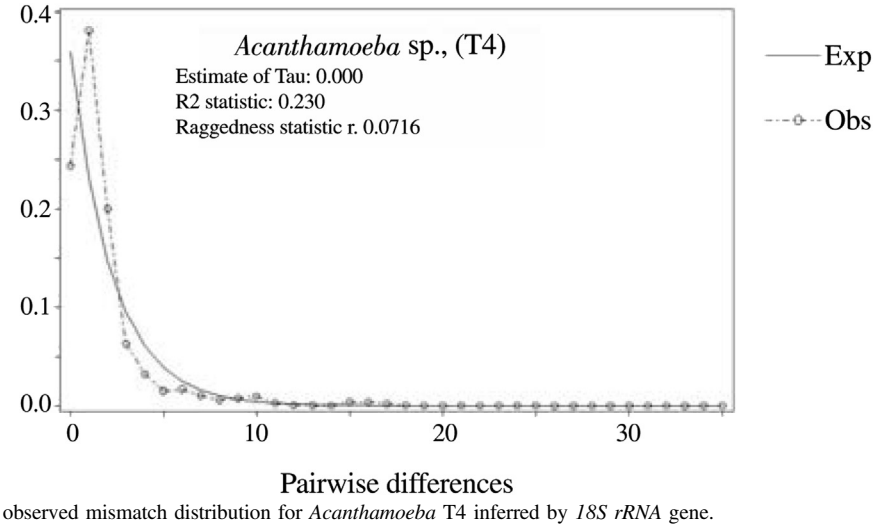

Figure 5. Expected and observed mismatch distribution for *Acanthamoeba* T4 inferred by *18S rRNA* gene.

Table 4

Pairwise fixation index (*F*st) (below diagonal) and a number of migrants per generation (Nm) (above diagonal) between *Acanthamoeba* T4 sub-populations of Iran based on *18S rRNA* gene.

Populations	Populations			
	West and Northwest	Central	South and Southwest	North
West and Northwest	–	2.91	6.80	0.35
Central	0.079 16	–	1.93	10.02
South and Southwest	0.035 46	0.114 87*	–	1.00
North	0.418 92*	0.024 33	0.200 40*	–

*Significant values at $P < 0.05$.

hospital wards (Hd: 0.562). However, no notable codon substitutions were identified in AK clinical isolates. Current results could be noticed as a healthy warning alarm for potential transmission-related pathogenic *Acanthamoeba* strains to contact lens wearers and patients with eye surgery (for example LASIC). These findings emphasize an apparent need for improved disinfection particularly at-risk residents who are with immunocompromised immune system or undergo eye operation.

Until now, the only reported case caused by *Acanthamoeba* in Iran is associated with AK [3]. Although encephalitis due to *Acanthamoeba* has not been definitely reported in Iran yet, nevertheless the occurrence of significant heterogeneity traits of parasite should not be neglected in the probable treatment failure and emergence of granulomatous amebic encephalitis among at-risk populations. Up to now, a total of 150 clinical cases of AK are identified in Iran [13,22], which dominantly belonged to *Acanthamoeba palesinensis*, *Acanthamoeba griffin* and *Acanthamoeba castellanii* [22]. A recent investigation by Niyyati *et al.* (2015) represented that the filtrated and tap waters could be the sources of *Acanthamoeba* strains for developing AK in contact lens wearers [23].

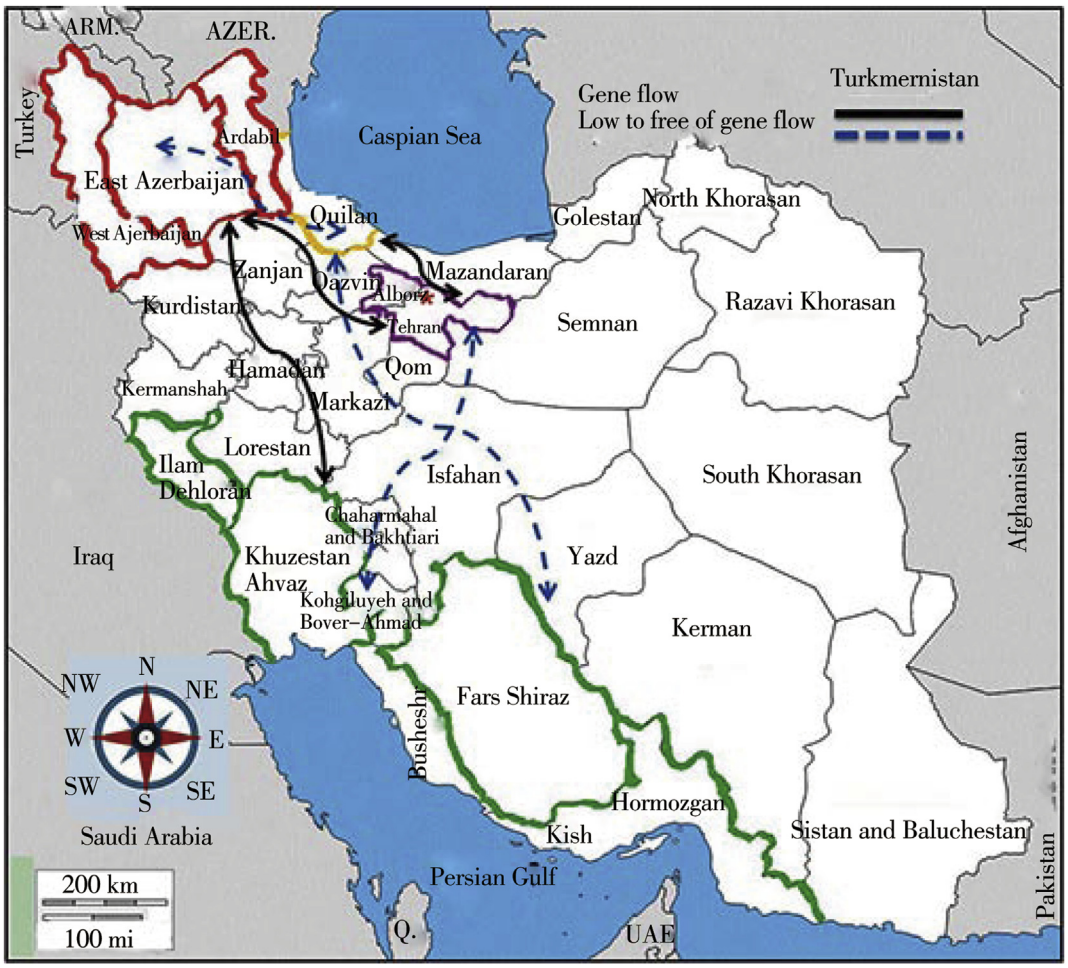

Figure 6. The map of distribution and gene flow of *Acanthamoeba* T4 among various geographical foci of the Iran. The dashed line indicating a lack of gene migration and solid line representing a gene flow.

It is well documented that the genetic diversity of parasites can regularly happen following the high gene migration which expands the effective population size in a variety of geographical regions, where the heterogeneity traits are potentially dominant [10,24].

In this study, no genetic variability of *Acanthamoeba* T4 (Hd: 0.000) was found in immunosuppressed patients (HIV, cancer, diabetes, hepatitis, splenectomy and steroid therapy after organ grafts). This may be due to the inhibitory interactions of chemotherapeutic procedure on emergence of parasite haplotypes; furthermore, the low level of allelic sequence heterozygosity of *Acanthamoeba* T4 among the mentioned patients appeared. However, there is no sufficient statement regarding the clinical intensity of acanthamebiasis in Iranian immunosuppressed patients. Memari *et al.* showed that the potentially pathogenic T3, T4 and T5 genotypes could reside in nasal mucosa of cancer patients, which could be a serious health concern for emerging granulomatous amebic encephalitis [16].

The occurrence of haplotypes IR75, IR133, IR137, IR140 and IR168 shared between common haplogroups IR6 and IR7 may probably support that these haplotypes appeared in various geographical regions, and extend into mentioned metapopulations (IR6 and IR7). As well, these haplotypes emerged in each region, deriving from their common haplotypes.

In the drawn haplotype network, the extension of IR5, IR8, IR11, IR14 (dust samples) and IR52, IR53 and IR54 (AK isolates) to distant positions indicated the high haplotype diversity.

In this investigation, no comparative molecular analysis is achieved on inter-variability ranges of *Acanthamoeba* genotypes T2, T3, T5, T6, T11 and T15 due to a limitation of the number of registered sequences in the GenBank database.

F_{ST} index as a scale of gene migration was shown low to high ranges (0.024 33–0.418 92). The statistically F_{ST} value indicates that T4 is genetically differentiated between north-west, north-south, and central-south metapopulations (F_{ST}: 0.114 87–0.418 92, Nm: 0.35–1.93). No significant genetic differences were identified between west-central, west-south, central-south, and north-central isolates (F_{ST}: 0.024 33–0.079 16); furthermore, the number of migrants per generation remained high (Nm: 2.91–10.02) between west-central, west-south, central-south, and north-central populations. This signifies that here is probably a high gene flow due to an occurrence of bottleneck events, inter-transregional of *Acanthamoeba*, ecological alterations, agricultural activities, and host mobility [10,25]. This can be also justified by founder effect that represents *Acanthamoeba* T4 isolates geographically separated from the original population and they form a new colony. Moreover, it is suggested the dynamic sexual cycle during recombination could lead to homogeneity of parasite genome [25]. Although no treatment failure of *Acanthamoeba* resistant cases has been reported in Iran, it was shown that in the population genetic study of some metazoan (nematodes), the infected host mobility and gene flow of parasite could play a key role in the virulent mutant alleles or drug resistant [26].

The existence of negative values of Tajima's *D* in support of *Acanthamoeba* populations implies evidence of some mechanisms including slippage and unequal crossing over/transposition, selective sweep hypothesis, the model of neutral mutation, population size equilibrium, genetic drift, purifying selection, and negative selection [9,12,27]. Additionally, in a unimodal applied mismatch distribution test, the Tau value as a divergence time for unequal population sizes revealed the *Acanthamoeba* T4 has lately experienced a population expansion among the various geographical regions of Iran.

One of the limitations of the current systematic review was that comprehensive investigations have not been done on genotyping of animal acanthamebiasis in Iran; therefore, employing concatenated sequences of different loci would provide sufficient evidence concerning zoonotic potential and dynamic of *Acanthamoeba* spp. Moreover, the conducting NGS technique with the great potential of neutral genetic markers (most commonly microsatellite) would reflect an accurate insight of the origins of *Acanthamoeba* spp. lineages and gene dispersal in Iran.

In conclusion, *Acanthamoeba* belonging to T4 genotype is the most prevalent strain in environmental and clinical samples in several regions of Iran. Findings show that various levels of gene migrations of *Acanthamoeba* T4 are unambiguously circulating among some regions of Iran. Trans-regional sharing of T4 genotype could be a threat as a health dilemma in the transmission of pathogenic alleles to at-risk populations. Based upon the genetic analyses and clinical observations, the AK infected by the contact lens, and dust samples in immunodeficiency and ophthalmology wards introduce as the at-risk individual/source for the *Acanthamoeba* involvement. This is the first systematic review and meta-analysis providing new approaches into *Acanthamoeba* genetic structure, highlighting at-risk population and transmission patterns of parasite from various regions of Iran.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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