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Molecular phylogenetic analysis of *Anopheles* and *Cellia* subgenus anophelines (Diptera: Culicidae) in temperate and tropical regions of Iran

Saber Gholizadeh[a,*], Navid Dinparast Djadid[b,*], Behzad Nouroozi[a], Mojtaba Bekmohammadi[a]

[a] *Medical Entomology Department, School of Public Health, Urmia University of Medical Sciences, Urmia, Iran*
[b] *Malaria and Vector Research Group (MVRG), Biotechnology Research Center (BRC), Pasteur Institute of Iran (PII), Tehran, Iran*

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ABSTRACT

Molecular studies on population genetics of speciation across Iran have recently started. Morphological and molecular studies have showed that 25 species of genus *Anopheles* are present in the country; however, relationships between vector and non-vector species as well as compatibility of morphological characters with molecular data have not been verified. Molecular phylogenetic analysis was undertaken on the *Anopheles* and *Cellia* subgenus members internal transcribed spacer 2 (ITS2) sequences submitted to GenBank among the Oriental and Palearctic members in north and southern Iran. rDNA-ITS2 sequences were extracted from the GenBank and analyzed using bioinformatics softwares: BLAST, ITS2 annotation tool (version 3.0.13), ClustalW, and MEGA5 in neighbor-joining and maximum likelihood algorithms. There are not any submitted sequences in GenBank from Iran for the following seven species: *Anopheles algeriensis*, *Anopheles marteri*, *Anopheles plumbeus*, *Anopheles peditaeniatus*, *Anopheles melanoon*, *Anopheles subpictus*, and *Anopheles mongolensis*; therefore, they have not been included in the study. Although these molecular-based phylogenetic trees match well enough with classical morphological taxonomy, the arrangement of species did not match with morphological classification in some cases. Correct species identification is essential for control of vector born disease such as malaria; therefore, phylogenetic methods will help to understand the relationship among the members of the target species within the genus *Anopheles*. It could also help us to design molecular markers for species differentiation particularly in cryptic species, which is difficult to classify them based on morphological features.

1. Introduction

More than half of the century ago, malaria was highly endemic in most parts of Iran (Sadrizadeh, 1999; Zakeri et al., 2004; Edrissian, 2006). Despite different control programs, malaria still remains a major public health problem in southern parts of the country, bordering with Afghanistan and Pakistan. About 15.99% of populations are at risk of disease, and 89.59% of cases are related to *Plasmodium vivax* (WHO, 2010, 2011).

Culicidae is a large family of order Diptera that occurs throughout temperate and tropical regions of the world (Harbach, 2007). Shahgudian (1960) reported 19 species and varieties in adult female *Anopheles* of Iran (Shahgudian, 1960). Sedaghat and Harbach (2005) reported 24 species in checklist of Iranian *Anopheles* (Sedaghat and Harbach, 2005), while the most recent checklist of Iranian anophelines includes 28 species (Azari-Hamidian, 2007). *Anopheles maculipennis*, *Anopheles sacharovi* and *Anopheles superpictus* have been recognized as malaria vectors in north of the Zagros mountain range. *Anopheles stephensi*, *Anopheles culicifacies*, *Anopheles fluviatilis*, *Anopheles dthali*, and *An. superpictus* are well known malaria vectors in south and southeast of Iran, bordering with Iraq, Afghanistan and Pakistan (Edrissian, 2006; Azari-Hamidian, 2007; Djadid et al., 2007). Eshghy (1977) observed *Plasmodium* oocyst in *Anopheles multicolor* midgut (Eshghy, 1977). By performing a 2-site immunoradiometric assay on the head and thorax of *An. culicifacies* s.l. and *Anopheles pulcherrimus* females, Zaim et al. (1993) reported the natural infection of *An. pulcherrimus* to malaria parasite in Ghassreghand district (Baluchistan, Iran) (Zaim et al., 1993). However, the most recent *Plasmodium falciparum* infection was reported in one pool of five *Anopheles hyrcanus* from Fuman district in northern Iran (Djadid et al., 2009).

Different techniques have been used for identification of *Anopheles* species complexes (Leoratti et al., 2012; Ngonghala et al., 2012). Species complex are often morphologically similar, causing difficulties in identifying mosquitoes responsible for disease transmission and potentially misleading control efforts (Marrelli et al., 1999; Torres et al., 2000; Van Bortel et al., 2000). Individuals

* Corresponding authors.
E-mail addresses: saber@umsu.ac.ir, sabergholizadeh@yahoo.com (S. Gholizadeh), navidmvrg@gmail.com (N.D. Djadid), behzadnorouzi66@yahoo.com (B. Nouroozi), saber@umsu.ac.ir (M. Bekmohammadi).

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within a species complex sometimes exhibit distinct differences in resting habitats, host preference, insecticide resistance, and susceptibility to malaria parasites (Leoratti et al., 2012). In recent decades, interests in re-examining the taxonomic status of *Anopheles* species have been increased. Advancements in DNA-based methods have facilitated the development of simple, sensitive and rapid molecular tools for identification of species complex (Collins et al., 2000; Norris, 2002; Krzywinski and Besansky, 2003; Leoratti et al., 2012). The intergenic spacer and internal transcribed spacers 1 and 2 (ITS1 and ITS2) within the nuclear ribosomal genome have become very popular targets for addressing taxonomic issues among anophelines. The nucleotide sequences of these spacer regions are often much more polymorphic among species than within them (Manonmani et al., 2001; Djadid et al., 2006). This characteristic makes this region of genome useful for delineating molecular differences among cryptic species by length or sequence polymorphism (Mechai et al., 2012).

Recently, different researchers have used widely the genomic DNA for phylogenetic analysis in several malaria vectors: *An. pulcherrimus* (Djadid et al., 2003; Abramova et al., 2005), *An. stephensi* (Djadid et al., 2006; Ali et al., 2007; Alam et al., 2008), *An. hyrcanus* (Poncon et al., 2008; Djadid et al., 2009; Paredes-Esquivel et al., 2011), *An. maculipennis* complex (Oshaghi et al., 2003; Sedaghat et al., 2003; Nicolescu et al., 2004; Djadid et al., 2007; Ghavami et al., 2008; Gordeev et al., 2010), *An. fluviatilis* (Naddaf et al., 2003, 2010; Chen et al., 2006; Mehravaran et al., 2011), *An. superpictus* (Abramova et al., 2005; Oshaghi et al., 2007b) and *An. culicifacies* (Dassanayake et al., 2008). 20 out of 28 species reported from Iran (Azari-Hamidian, 2007) had rDNA-ITS2 sequences in GenBank. There was no ITS2 sequence in the remaining seven species, including *Anopheles algeriensis*, *Anopheles subpictus*, *Anopheles peditaeniatus*, *Anopheles marteri*, *Anopheles plumbeus*, *Anopheles mongolensis*, and *Anopheles melanoon*. Despite all accumulated molecular systematic data originated from these studies, there has not been any combined survey on phylogenetic relationships among members of the Iranian anophelines.

Since many of the primary malaria vectors belong to species complex, accurate phylogenetic reconstruction and species identification are necessary for understanding malarial transmission. The objective of the current study was to examine phylogenetic relationships among members of the Anophelines of subgenus *Cellia* and *Anopheles*, prevalent in Iran, by using rDNA-ITS2 sequences.

2. Materials and methods

rDNA-ITS2 sequences ($n = 375$) used in the current study were extracted from GenBank using ITS2, *Anopheles*, "Iran" keywords. The details of extracted sequences, total number of sequences from each species, outgroup, and accession numbers of sequences used for phylogenetic analysis have been shown in Table 1. All obtained sequences include partial sequence of 5.8S, complete sequence of ITS2, and partial sequence of 28S. Using the ITS2 annotation tool (Koetschan et al., 2010), the ITS2 sequences were annotated. rDNA-ITS2 nucleotide sequences of the selected sequences were aligned using the multiple alignment program ClustalW (Thompson et al., 1994) and MEGA5 (Tamura et al., 2007). ITS2 sequences of *Aedes japonicus* (GenBank ID: FJ641870) (Versteirt et al., 2009) were used as outgroup.

The DNA sequence-based phylogenetic tree was constructed using distance, neighbor-joining method (Saitou and Nei, 1987). In addition, the ITS2 sequences were analyzed by using maximum likelihood method based on the Tamura-Nei model's model (Tamura and Nei, 1993). Evolutionary analysis was conducted using MEGA5 software (Tamura et al., 2007). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are indicated in branches (Felsenstein et al., 1985). The tree is scaled-base, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. All positions containing gaps and missing data were considered as complete deletion and numbers of nucleotide substitutions per site were predictable.

The transition/transversion ratio and nucleotide frequencies of 25 nucleotide sequences were estimated using the Kimura's 2-parameter model (Kimura, 1980). All positions containing gaps and missing data were eliminated in MEGA5 (Tamura et al., 2007).

Table 1
Details of different *Anopheles* species of subgenus *Anopheles* and *Cellia* submitted from Iran used for phylogenetic analysis by using rDNA-ITS2 gene.

Species	Submitted sequences from Iran	Accession number	Reference
An(*A*). *maculipennis*	92	AY137816	Sedaghat et al. (2003)
An(*A*). *persiensis*	40	DQ243834	Djadid et al. (2007)
An(*A*). *hycanus*	36	EF613291	Djadid et al. (2009)
An(*C*). *fluviatilis*	35	AF509353	Naddaf et al. (2003)
An(*C*). *superpictus*	34	AY941117	Oshaghi et al. (2005, Direct submission)
An(*A*). *sacharovi*	32	AY114210	Sedaghat et al. (2003)
An(*A*). *pseudopicus*	29	GU478907	Oshaghi et al. (2010, Direct submission)
An(*C*). *stephensi*	22	DQ662406	Djadid et al. (2006, Direct submission)
An(*C*). *fluviatilis* U	21	GQ926588	Oshaghi et al. (2009, Direct submission)
An(*C*). *pulcherrimus*	6	AY533854	Djadid et al. (2003)
An(*C*). *culicifacies* A	4	AF402297	Djadid (2001, Direct submission)
An(*C*). *superpictus* B	4	DQ487154	Djadid et al. (2006, Direct submission)
An(*C*). *dthali*	3	AY445827	Djadid et al. (2003, Direct submission)
An(*A*). *atroparvus*	2	AF436064	Djadid et al. (2001, Direct submission)
An(*A*). *claviger*	2	DQ229313	Djadid et al. (2005, Direct submission)
An(*A*). cf. *hyrcanus* DDN-2007	2	EF613309	Djadid et al. (2009)
An(*C*). *fluviatilis T*	2	GQ926591	Oshaghi et al. (2009, Direct submission)
An(*C*). *multicolor*	2	AY564228	Djadid et al. (2004, Direct submission)
An(*C*). *fluviatilis*	1	AF333384	Djadid et al. (2001, Direct submission)
An(*A*). *messeae*	1	AY050639	Djadid et al. (2001, Direct submission)
An(*C*). *sergenti*	1	AY533851	Djadid et al. (2004, Direct submission)
An(*C*). cf. *dthali*	1	AF402296	Djadid et al. (2001, Direct submission)
An(*C*). *apoci*	1	AY445826	Djadid et al. (2003, Direct submission)
An(*C*). *subpictus*	1	AY049004	Djadid et al. (2001, Direct submission)
An(*C*). *turkhudi*	1	AY456391	Djadid et al. (2003, Direct submission)
Aedes japonicus	1	FJ641870	Versteirt et al. (2009)

(*A*): *Anopheles*; (*C*): *Cellia*.