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Genetic variation in populations of *Allothrombium pulvinum* (Acari: Trombidiidae) from Northern Iran revealed by mitochondrial *cox*I and nuclear rDNA *ITS*2 sequences

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Abstract *Allothrombium pulvinum* Ewing is a common natural enemy of aphids and some other arthropods. So far, there are no studies that have addressed genetic variation of this predatory mite. We investigated genetic variation of *A. pulvinum* across its whole known range in Iran. A 410 bp portion of the mitochondrial cytochrome *c* oxidase subunit I gene (*cox*I) and 797–802 bp portion of the internal transcribed spacer 2 of rDNA (*ITS*2) were sequenced for 55 individuals from 11 populations, resulting in 12 and 26 haplotypes, respectively. In the *cox*I region, haplotype and nucleotide diversities varied among populations from 0.00 to 0.90 and from 0.0000 to 0.0110, respectively. In the *ITS*2 region they varied from 0.20 to 0.91 and from 0.0006 to 0.0023, respectively. For both gene regions the highest haplotype and nucleotide diversities were detected in population Mahmoud Abad from northern Iran. Statistically significant population differentiation (F_{ST}) was detected in most pair-wise population comparisons. The results of population differentiation for both gene regions were generally congruent indicating that *A. pulvinum* from Iran consists of genetically different populations. This suggests that *A. pulvinum* comprises at least two geographically distinct populations or even more than one species. This study is an initial

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step towards understanding genetic variation of *A. pulvinum*, a taxon for which little molecular information is available. More intensive sampling and analysis of additional DNA regions are necessary for more detailed classification of this taxon.

Keywords Trombidiidae · Genetic variation · mtDNA cytochrome oxidase I · Nuclear rDNA *ITS*2 · *Allothrombium pulvinum* · Nucleotide

Introduction

Mites of the family Trombidiidae are ectoparasites in their larval stage and free-living predators in their post-larval stages of a variety of arthropods that are serious pest species (Zhang 1998; Chen and Zhang 1991). Members of the genus *Allothrombium* are the most common and best known species in the family (Zhang 1991, 1998; Saboori and Zhang 1996). The cosmopolitan species *Allothrombium pulvinum* Ewing is a natural enemy of a variety of pest arthropods in Iran (Saboori and Zhang 1996). Their larvae are ectoparasites of aphids, whereas the deutonymphs and adults are generalist predators of aphids, spider mites and various other small arthropods (Zhang 1992b, c, 1998). In Iran, *A. pulvinum* was reported only from the northern part of the country (Zhang and Faraji 1994; Saboori et al. 2003, 2007). Saboori and Zhang (1996) studied the field and laboratory biology of *A. pulvinum*. They showed that it is the most abundant species of Trombidiidae in West Mazandaran, Northern Iran. Because of the potential of the species as a biological control agent against pest aphids and mites, a series of studies has been undertaken on its systematics, morphology, seasonal distribution, life history, ecology, behavior, host preference, parasite-host, and predator–prey interactions (Zhang and Xin 1989; Zhang 1991, 1992a, b, c, d; Zhang and Chen 1993; Hosseini et al. 2002, 2005). On the other hand, there are still no studies on the genetic variation of natural populations of *A. pulvinum*.

The species belonging to the genus *Allothrombium* are impossible to distinguish by adult morphological characters and their identification still largely depends on morphology of the larvae (Krantz 1978; Moss 1962). Molecular studies of mites are becoming increasingly important in resolving taxonomic relationships (for review, see Navajas and Fenton 2000) as well as phylogenetic studies (Cruickshank 2002; Ros and Breeuwer 2007). However, there are relatively few studies on genetic variation in populations of predatory mites, despite their ecological and economic importance. This is in contrast to the phytophagous mites, where significant genetic differences among populations from different location have been reported (Navajas and Fenton 2000, and references therein).

The objectives of the present study were: (1) to examine the levels and patterns of nucleotide variation in natural populations of *A. pulvinum* from Iran, (2) to determine whether these populations are genetically differentiated, and (3) to evaluate the usefulness of DNA sequence data for population genetic studies on *A. pulvinum*. To address these issues, we determined DNA sequences of the mitochondrial cytochrome oxidase subunit I (*cox*I) gene and the nuclear internal transcribed spacer (*ITS*2) of the rDNA gene in 11 natural populations of *A. pulvinum*. These 2 regions are the most commonly analyzed DNA sequences in genetic studies on mites (e.g., Navajas et al. 1992, 1994, 1996a, b, 1998, 1999a, b; Nishimura et al. 2007; Xie et al. 2008).

Results of this analysis gave the first information regarding genetic variation of *A. pulvinum* populations across its known geographical range in Iran and will serve as a foundation for studies of regional, behavioral, and ecological differences within this species.

Materials and methods

Specimens

At the outset of the present study, we searched for the *A. pulvinum* in all parts of the country including northern, northwestern, central, southeastern and southern provinces (Mazandaran, Guilan, Ardebil, East and West Azerbaijan, Isfahan, Charmahal Bakhtiari, Kerman and Hormozgan), but we could find it only in northern and northwestern provinces. We therefore believe that the distribution of *A. pulvinum* in Iran is limited to these two parts of the country. Adult individuals of *A. pulvinum* were collected from 11 localities spanning North and Northwest of Iran during October to November 2006 (Fig. 1). Samples were collected from the following localities: Qaemshahr (Qs), Sari (Sr), Babol (Bl), Mahmoud Abad (Ma), and Nowshahr (Ns) in Mazandaran province; Rasht (Rt), Langeroud (Ld), and Lahijan (Ln) in Guilan province; Nir (Nr) in Ardebil province; Tabriz (Tz) in East Azerbaijan, and Orumie (Or) in West Azerbaijan province (Fig. 1). We also tried to obtain samples from other countries but we did not receive any replies to our requests.

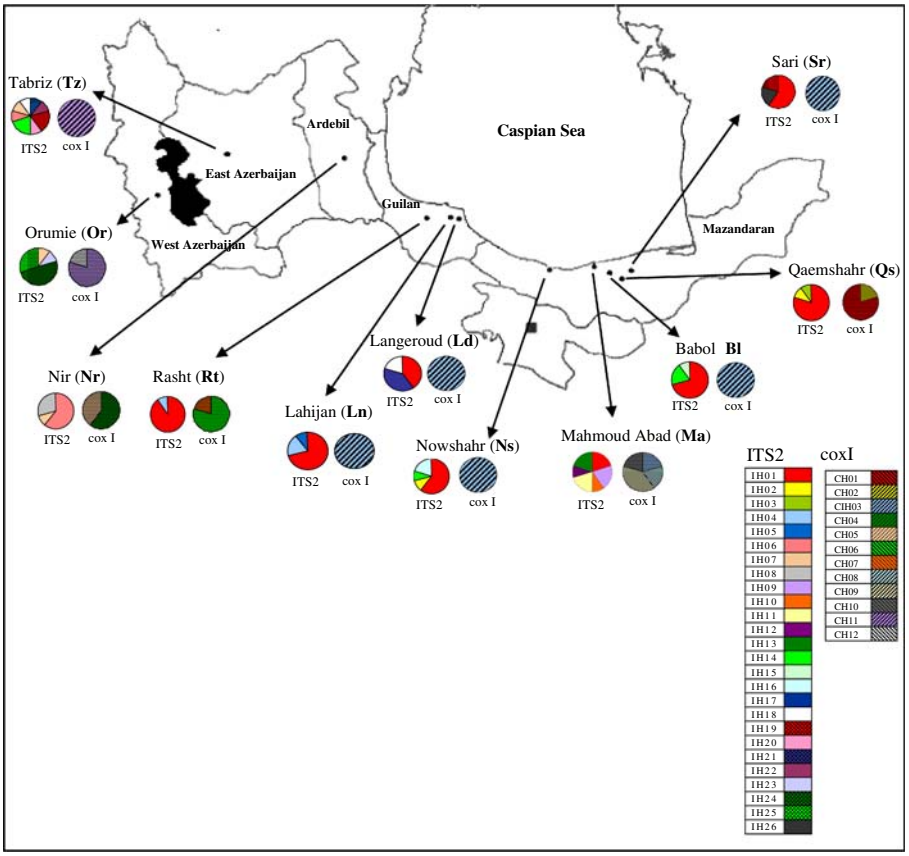

Fig. 1 Locations from which *Allothrombium pulvinum* were sampled are indicated with *black circles* and distribution of *coxI* and *ITS*2 haplotypes among populations. The *black square* indicates Tehran, the capital city of Iran

Depending on the locality, samples were collected from different tree species. Samples from the northern populations were collected from citrus and forest trees. On the other hand, samples from the northwestern part of the country were collected from apple, plum, and walnut trees. Furthermore, populations Nir, Tabriz, and Orumie were located in areas with harsh winter climate, while the remaining populations came from much warmer and more humid areas. Specimens were kept alive in soil medium for DNA extraction at 4°C. To confirm species identification, 20 adult mites from each population were selected and placed separately in a plastic Petri dish containing moist autoclaved soil as the substrate at 4 ± 1°C, a relative humidity of 80–90% and photoperiod 15L:9D during reproductive diapause. After 2 months, the temperature was gradually raised to 25°C and aphids were applied as food. Once eggs from each Petri dish hatched, larvae were transferred to lactophenol to make a permanent slide for species identification using larval morphological characters (Zhang and Norbakhsh 1995). Then, the corresponding adult mites were used for DNA extraction. The voucher specimens used in our study were deposited at the Jalal Afshar Zoological Museum, College of Agriculture, University of Tehran, Karaj, Iran.

DNA extraction, amplification and sequencing

DNA was extracted from individual mites using protocols described in Navajas et al. (1998). In brief, individual adult mites were crushed at 60°C in a 1.5-ml microcentrifuge tube containing 200 µl extraction buffer (2% CTAB, 1.4 M NaCl, 0.2% 2-β-mercaptoethanol, 20 mM EDTA, 100 mM Tris–HCl pH 8.0). The tube was incubated at 60°C for 60 min and proteins were removed with one volume chloroform isoamyl alcohol (24:1). DNA was precipitated with isopropanol and resuspended in 20 µl of distilled water. This DNA solution was used as a polymerase chain reaction (PCR) template.

The *ITS*2 region was amplified using the following primers, LC1: 5′-CGAGTA TCGATGAAGAACGCAGC-3′ and HC2: 5′-ATATGCTTAAGTTCAGCGGG-3′, located in conserved regions of the 5.8S and 28S rDNA flanking regions, respectively (Navajas et al. 1992, 1994). Each reaction mixture contained 2 µl of the extracted DNA, 0.5 µM of each primer, 1 U *Taq* polymerase (Takara Bio Inc., Japan), 50 mM KCl, 10 µM Tris–HCl pH 8.3, 1.5 mM $MgCl_2$), 0.2 mM of each dNTP in a total volume of 50 µl. PCR conditions for the *ITS*2 fragment were as follows: 4 min initial denaturation at 94°C, followed by 30 cycles of 1 min at 92°C, 1 min at 51°C, and 1 min at 72°C, with a final extension of 5 min (72°C). Two internal primers (5′-CTGATGGTGGTGGTTGAAAA-3′ and 5′-TT TTCAACCACCACCATCAG-3′) were designed for sequencing on the basis of direct sequence data of *A. pulvinum* using the Primer 3 program (http://fokker.wi.mit.edu/primer3/input-030.htm; Rozen and Skaletsky 2000). When the sequences obtained by direct sequencing had no or only one heterozygous site, sequence of both haplotypes of an individual were inferred. On the other hand, when two or more heterozygous sites or indels were detected by direct sequencing, PCR products were cloned into the PGEMT-easy vector (Promega, USA). Individual clones were sequenced using primers designed for the promoter sites of the vector, T7: 5′-TAATACGACTCACTATAGGG-3′ and SP6: 5′-TA TTTAGGTGACACTATAG-3′. To obtain the sequence of a single haplotype of a heterozygous individual, sequencing of individual clones was carried out until two identical sequences were obtained for each allele.

A portion of the *cox*I gene was amplified using the following primers, 772: 5′-TGA TTTTTTGGTCACCCAGAAG-3′ and 773: 5′-TACAGCTCCTATAGATAAAAC-3′. These primers were designed previously using the sequence of a cloned fragment of the central region of the *cox*I gene of *Tetranychus urticae* Koch (Navajas et al. 1994, 1996a).

PCR amplification was carried out in a total volume of 50 µl (consisting of 2 µl DNA as template, 0.2 mM dNTP, 0.2 µM of each primer, 50 µM KCl, 10 mM Tris–HCl pH 8.3, 1.5 mM $MgCl_2$ and 1 U *Taq* polymerase). PCR conditions were as follows: 5 min initial denaturation at 94°C, followed by 5 cycles of 50 s at 93°C, 50 s at 45°C, 1 min at 72°C, 28 cycles of 50 s at 93°C, 30 s at 50°C, and 1 min at 72°C, with final extension of 5 min at 72°C.

For both *ITS*2 and *cox*I regions, amplification products were separated by electrophoresis on a 0.8% agarose gel stained with ethidium bromide for DNA visualization under UV light. PCR products were excised from the agarose gel and purified using the Wizard SV Gel and PCR clean up system (Promega, USA). The purified products were directly used as template for sequencing reaction using the Big Dye™ Terminator Cycle Sequencing Kit v. 3.1 (Applied Biosystems, USA). Sequences were determined using ABI prism 3100 automatic sequencer (Applied Biosystems, USA). A negative control (no template DNA) was included in all experiments. For each population, PCR products from five individuals were sequenced using the same primers used for the PCR amplification (*cox*I and *ITS*2) and additional internal primers (*ITS*2).

Data analysis

All sequences were checked and assembled using the ATGC program v. 4 (Genetyx Corp.). Sequences were edited and aligned using the BioEdit program (Hall 1999). The MEGA4 program (Tamura et al. 2007) was used to calculate the number of variable sites, AT-content and the number of transition/transversion substitutions. Genetic differences between populations were estimated as fixation indices (F_{ST}) (Hudson et al. 1992) and their significance was tested by 10,000 permutations using the Proseq program v. 2.9 (Filatov 2002). Other inter- and intra-population diversity statistics, such as nucleotide diversity (π, without Jukes–Cantor correction), haplotype diversity (h_d), and the number of nucleotide substitutions between populations (d_A, Nei 1987), were estimated using the DnaSP program v. 4.20.2 (Rozas et al. 2003). Tajima's test (Tajima 1989) was performed for each population to check for departures from neutrality. Haplotype networks were constructed using median-joining method as implemented in the NETWORK program v. 4.5.0.0 (Bandelt et al. 1999) to visualize relationship and frequencies of individual haplotypes (indels were considered in the analysis). Isolation by distance was tested statistically by determining the significance of the correlation between F_{ST} estimates and geographical distance using the Mantel test where *P* values are determined by permutation procedures (Smouse et al. 1986) using the PAST v. 1.77 program (Hammer et al. 2001). To determine the congruence between the *cox*I and *ITS*2 data sets we also determined correlation between their corresponding F_{ST} values using the Mantel test and the PAST program. Furthermore, using the d_A values we constructed phylogenetic trees based on the *cox*I and *ITS*2 sequences. The trees were constructed using the neighbor-joining method (Saitou and Nei 1987), with 1,000 bootstrap replications. The trees were constructed using the MEGA4 program (Tamura et al. 2007).

Results

Sequence variation

The complete *ITS*2 region with portions of the flanking 5.8S and 28S rDNA genes (920–925 bp following alignment) was successfully sequenced for 55 individuals. The boundaries

of the *ITS*2 were determined using the conserved sequence of the flanking regions by comparison with the sequence of *T. urticae* (Navajas et al. 1997, 1998). The 3′ portion of the 5.8S region was 101 bp, while the 5′ portion of the 28S region was 22 bp for all sequences. There were no substitutions in either flanking region. The length of the entire *ITS*2 varied from 797 to 802 bp. The *ITS*2 region had 13 parsimony informative segregating sites of which 5 were transitions, 3 transversions and 5 indels. In total, we found 26 haplotypes (Table 1).

In the *ITS*2 region, the nucleotide diversity (π) within populations ranged between 0.0006 and 0.0023 (Table 2). Populations Babol, Rasht, and Nowshahr had lower diversity than the remaining populations. Haplotype diversity (h_d) ranged from 0.20 to 0.91. The highest haplotype and nucleotide diversity were observed in populations Mahmoud Abad and Tabriz (Table 2).

From the 11 populations examined (a total of 55 individuals) a consensus length of 410 bp excluding the primer region was directly sequenced for the *cox*I gene region. No

Table 1 Polymorphic sites in 925 bp region of the 26 *ITS*2 haplotypes of *Allothrombium pulvinum*

Haplotype	Position												
	237	259	507	536	627	638	639	640	691	747	748	803	886
IH1	G	G	T	C	C	C	T	C	A	T	G	T	T
IH2	.	A	.	.	.	.	.	.	.	.	.	.	.
IH3	.	.	A	.	.	–	–	–	.	–	–	.	.
IH4	A	.	.	.	.	.	.	.	.	.	.	.	.
IH5	.	.	A	T	A	.	.	.	.	.	.	.	.
IH6	.	.	A	.	.	–	–	–	.	–	–	A	.
IH7	.	.	A	.	.	–	–	–	.	.	.	.	.
IH8	.	.	A	.	.	–	–	–	.	.	.	A	.
IH9	.	.	A	.	.	.	.	.	.	.	.	.	C
IH10	.	.	A	T	.	.	.	.	.	.	.	.	C
IH11	.	.	.	.	.	.	.	.	.	.	.	A	C
IH12	.	A	A	.	.	.	.	.	.	.	.	.	C
IH13	.	.	A	.	.	.	.	.	.	.	.	A	C
IH14	.	.	A	.	.	.	.	.	.	.	.	.	.
IH15	.	.	.	.	.	.	.	.	.	.	.	A	.
IH16	.	.	.	.	A	.	.	.	.	.	.	.	.
IH17	.	.	.	T	.	.	.	.	.	.	.	.	.
IH18	.	.	A	T	.	.	.	.	.	.	.	.	.
IH19	.	.	A	T	.	.	.	.	.	–	–	.	.
IH20	.	.	A	T	.	–	–	–	.	–	–	A	.
IH21	.	.	A	.	.	.	.	.	.	.	.	A	.
IH22	.	.	A	T	.	–	–	–	.	.	.	.	.
IH23	.	.	A	.	.	–	–	–	G	.	.	.	.
IH24	.	.	A	.	.	–	–	–	G	–	–	.	.
IH25	.	.	A	T	.	–	–	–	G	–	–	.	.
IH26	.	.	A	T	.	.	.	.	G	–	–	.	.

– indicates Indels

Table 2 Mean (±SD) nucleotide (π) and haplotype (h_d) diversity (Nei 1987) in the investigated gene regions

Population	*ITS*2		*cox*I	
	π	h_d	π	h_d
Qaemshahr	0.0015 ± 0.0010	0.37 ± 0.18	0.0019 ± 0.0010	0.40 ± 0.23
Sari	0.0020 ± 0.0010	0.62 ± 0.13	0	0
Mahmoud Abad	0.0023 ± 0.0009	0.91 ± 0.06	0.0110 ± 0.0080	0.90 ± 0.16
Babol	0.0006 ± 0.0006	0.51 ± 0.16	0	0
Nowshahr	0.0008 ± 0.0007	0.64 ± 0.15	0	0
Lahijan	0.0010 ± 0.0008	0.51 ± 0.16	0	0
Langeroud	0.0012 ± 0.0008	0.71 ± 0.08	0	0
Rasht	0.0006 ± 0.0005	0.20 ± 0.15	0.0039 ± 0.0030	0.40 ± 0.23
Nir	0.0013 ± 0.0010	0.60 ± 0.13	0.0058 ± 0.0040	0.60 ± 0.17
Tabriz	0.0023 ± 0.0013	0.91 ± 0.09	0	0
Orumie	0.0010 ± 0.0010	0.71 ± 0.01	0.0029 ± 0.0020	0.40 ± 0.23
Total	0.0017 ± 0.0010	0.77 ± 0.15	0.0116 ± 0.0060	0.75 ± 0.18

indel (insertion or deletion) polymorphisms were observed among the obtained *cox*I sequences. Segregating nucleotide sites among the 12 *cox*I haplotypes detected in our study are shown in Table 3. Nucleotide substitutions were found at 19 sites, of which 18 were synonymous and parsimony informative. These included 16 transitions and three transversions. We did not detect any stop codons in the *cox*I sequences obtained in this study. Similar to the other mites and insects there was an AT bias ranging from 61.8 to 63.6% among populations. Such high AT content appears to be a general feature of the *cox*I region in arthropods (Navajas et al. 1996a; Lunt et al. 1996).

For the *cox*I gene region, haplotype (h_d) and (π) nucleotide diversities varied from 0.00 to 0.90 and from 0.0000 to 0.0110, respectively. The highest values were found in population Mahmoud Abad ($h_d = 0.90$ and $\pi = 0.0110$).

Haplotype sequences for both regions have been deposited in the GenBank database under accession numbers FJ358522–33 and FJ263064–89 for the *cox*I and *ITS*2 gene regions, respectively.

Haplotype frequencies

ITS2

Hereafter, we use the following haplotype IDs: IHXX and CHXX for the *ITS*2 and *cox*I regions, respectively, (where XX refers to the consecutive haplotype number). In the *ITS*2 region, all populations except Nir, Tabriz, and Orumie shared the haplotype IH01 at high frequency. The haplotype IH07 was shared by populations Tabriz, Orumie, and Nir. There were several haplotypes that were found in only one population (Fig. 1, Table 4). For instance, the haplotype IH08 was unique to population Nir. Tabriz population was the most heterogeneous population with eight haplotypes followed by Mahmoud Abad with six haplotypes. The constructed haplotype network is shown in Fig. 2a. Some haplotypes differed from each other by only one mutational step (e.g., IH09 and IH12), while others were several mutational steps apart (e.g., IH07 and IH14).

Table 3 Polymorphic sites in 410 bp region of the 12 *cox*I haplotypes of *Allothrombium pulvinum*

Haplotype	Position																		
	74	95	98	104	125	134	137	140	152	173	185	203	227	236	329	353	377	392	410
CH1	G	A	C	A	G	C	A	G	C	C	C	A	G	C	T	C	T	G	C
CH2	.	.	.	.	.	A	.	.	T	.	.	.	.	.	.	.	.	.	.
CH3	.	.	T	.	A	.	.	.	.	T	T	.	.	.	.	.	.	.	T
CH4	.	C	.	G	.	.	G	A	.	.	.	.	.	.	.	T	.	A	T
CH5	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	T
CH6	A	.	T	.	A	.	.	.	.	T	T	.	.	.	.	.	.	.	T
CH7	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	T
CH8	.	.	.	.	.	.	.	.	T	.	.	.	A	.	G	.	.	.	T
CH9	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	C	.	T
CH10	.	C	.	.	.	.	.	.	.	.	.	G	.	T	.	.	.	.	T
CH11	.	.	.	.	.	A	.	.	.	.	.	G	.	T	G	.	.	.	T
CH12	.	.	.	.	.	A	.	.	T	.	.	.	.	.	G	.	.	.	T

Table 4 Haplotype IDs and their frequencies (in brackets) in the investigated populations of *Allothrombium pulvinum* for the *ITS*2 region

Qaemshahr	Sari	Mahmoud Abad	Babol	Nowshahr	Lahijan	Langeroud	Rasht	Nir	Tabriz	Orumie
IH01 (0.8)	IH01 (0.6)	IH01 (0.2)	IH01 (0.7)	IH01 (0.6)	IH01 (0.7)	IH01 (0.4)	IH01 (0.9)	–	–	–
IH02 (0.1)	–	–	–	IH02 (0.1)	–	–	–	–	–	–
IH03 (0.1)	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	IH04 (0.2)	–	–	–	–	–
–	–	–	–	–	IH05 (0.1)	–	IH05 (0.1)	–	–	–
–	–	–	–	–	–	–	–	IH06 (0.6)	IH06 (0.1)	–
–	–	–	–	–	–	–	–	IH07 (0.1)	IH07 (0.1)	IH07 (0.1)
–	–	–	–	–	–	–	–	IH08 (0.3)	–	–
–	–	IH09 (0.2)	–	–	–	–	–	–	–	–
–	–	IH10 (0.1)	–	–	–	–	–	–	–	–
–	–	IH11 (0.2)	–	–	–	–	–	–	–	–
–	–	IH12 (0.1)	–	–	–	–	–	–	–	–
–	–	IH13 (0.2)	–	–	–	–	–	–	–	–
–	–	–	IH14 (0.2)	IH14 (0.1)	–	–	–	–	IH14 (0.2)	–
–	–	–	IH15 (0.1)	–	–	–	–	–	–	–
–	–	–	–	IH16 (0.2)	–	–	–	–	–	–
–	–	–	–	–	–	IH17 (0.4)	–	–	–	-
–	–	–	–	–	–	IH18 (0.2)	–	–	IH18 (0.1)	–
–	IH19 (0.2)	–	–	–	–	–	–	–	IH19 (0.2)	–
–	–	–	–	–	–	–	–	–	IH20 (0.1)	–
–	–	–	–	–	–	–	–	–	IH21 (0.1)	–
–	–	–	–	–	–	–	–	–	IH22 (0.1)	–
–	–	–	–	–	–	–	–	–	–	IH23 (0.1)
–	–	–	–	–	–	–	–	–	–	IH24 (0.5)
–	–	–	–	–	–	–	–	–	–	IH25 (0.3)
–	IH26 (0.2)	–	–	–	–	–	–	–	–	–

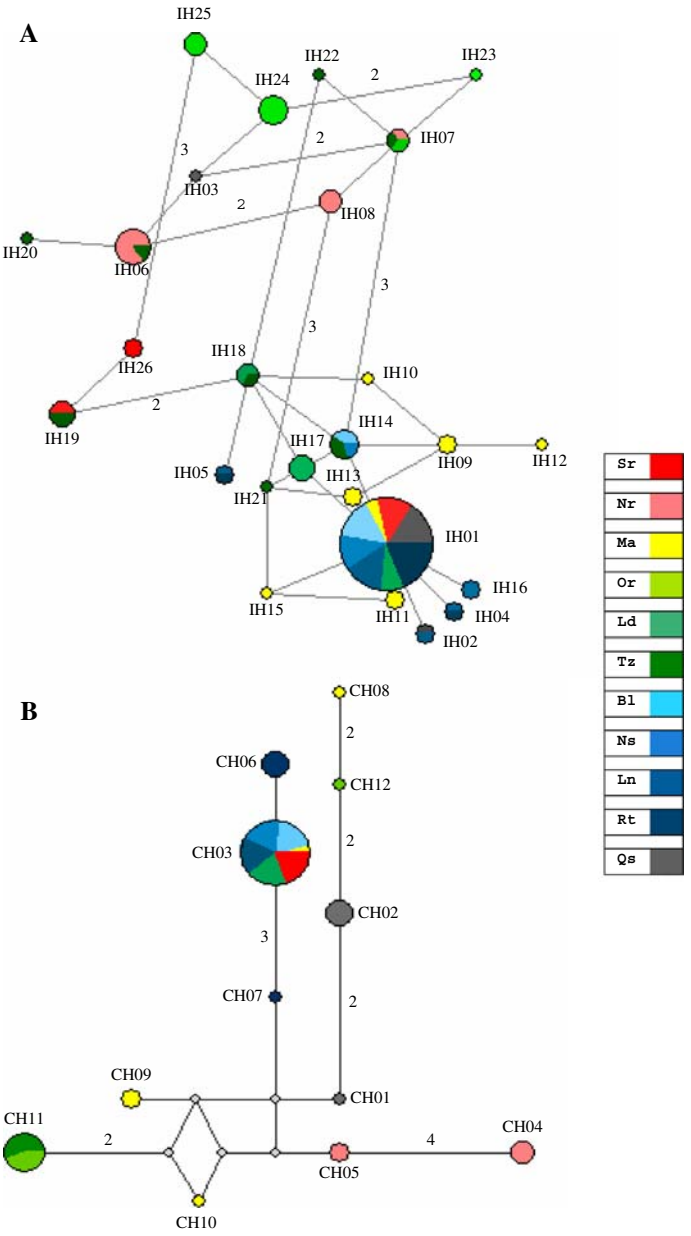

Fig. 2 Haplotype networks (unrooted minimum spanning trees), based on **a** the *ITS2* region and **b** the *cox* I region. *Small gray circles* in **b** represent nodes. *All other circles* represent haplotypes. The size of circles is proportional to the haplotype frequency. Branch lengths longer than one mutational step are marked with *numbers*

coxI

Except for the populations Sari, Babol, Nowshahr, Lahijan, Langeroud, and Tabriz that contained only a single haplotype, all other populations harbored more than one haplotype

(Fig. 1; Table 5). Population Mahmoud Abad had four haplotypes. The haplotype CH03 was found in most populations with high frequency. This haplotype was shared by Sari, Mahmoud Abad, Babol, Nowshahr, Lahijan, and Langeroud but was absent in the remaining populations. Haplotypes that were specific to individual populations were also observed (haplotypes CH01 and CH02 in Qaemshahr, CH04 and CH05 in Nir, CH06 and CH07 in Rasht). Haplotype CH11 was shared only by Orumie and Tabriz populations. Haplotype network of the *cox*I region (Fig. 2b) was simpler than that of the *ITS*2 region. Some haplotypes differed from each other by only one mutational step (e.g., CH03 and CH06), others were up to four mutational steps apart from each other (e.g., CH04 and CH05).

Population differentiation

The values of F_{ST} for all pairs of populations were estimated by the method of Hudson et al. (1992) to provide information about population differentiation (Table 6). In the *cox*I region, the F_{ST} values among pairs of populations ranged between 0.000 (e.g., population pair Babol–Sari) and 1.000 (e.g., populations Tabriz–Sari). In the *ITS*2 gene region, the values ranged between −0.041 (populations Qaemshahr–Rasht) and 0.842 (populations Nir–Qaemshahr). For both *cox*I and *ITS*2 regions populations Qaemshahr, Mahmoud Abad, Nir, Tabriz, and Orumie [except for Orumie–Tabriz pair (*cox*I), and Qaemshahr–Sari, Qaemshahr–Babol, Qaemshahr–Nowshahr, Qaemshahr–Lahijan, and Qaemshahr–Rasht pairs (*ITS*2)] had statistically significant F_{ST} values in comparisons with the remaining populations. On the other hand, no differentiation was observed among Sari, Babol, Nowshahr, Langeroud, Lahijan, and Rasht populations for both *cox*I and *ITS*2 regions. However, F_{ST} results were not congruent between the two loci in comparisons involving population Qaemshahr. As mentioned above, for the *cox*I region it showed significant F_{ST} values in all pair-wise population comparisons. On the other hand, for the *ITS*2 region it showed low and non-significant F_{ST} values in comparisons with populations Sari, Babol, Nowshahr, Lahijan, and Rasht.

The relationship between population differentiation and geographic distance was analyzed by computing the correlation between F_{ST} estimates and geographic distance. Significance of the relationship was tested using the Mantel test. In both regions, F_{ST} was significantly and positively correlated with geographical distance (*cox*I: $r = 0.41$, $P = 0.016$; *ITS*2: $r = 0.58$, $P = 0.007$), indicating that genetic differentiation significantly increases as distance between populations increases. Three adjacent populations Sari, Nowshahr, and Babol did not differ significantly from each other. However, we found significant F_{ST} values for pairs of some other geographically adjacent populations (Table 6).

As revealed by the Mantel test, there was no significant correlation between F_{ST} values for the *ITS*2 and *cox*I regions (data not shown). The topology of the neighbor-joining tree for the *ITS*2 sequences had poor bootstrap support (data not shown). We therefore did not compare it with the topology of the tree based on the *cox*I sequences.

Test of neutrality

No statistically significant deviation from neutrality was found in any of the investigated populations using Tajima's test (data not shown).

Table 5 Haplotype IDs and their frequencies (in brackets) in the investigated populations of *Allothrombium pulvinum* for the *cox*I region

Qaemshahr	Sari	Mahmoud Abad	Babol	Nowshahr	Lahijan	Langeroud	Rasht	Nir	Tabriz	Orumie
CH01 (0.2)	–	–	–	–	–	–	–	–	–	–
CH02 (0.8)	–	–	–	–	–	–	–	–	–	–
–	CH03 (1.0)	CH03 (0.2)	CH03 (1.0)	CH03 (1.0)	CH03 (1.0)	CH03 (1.0)	–	–	–	–
–	–	–	–	–	–	–	–	CH04 (0.6)	–	–
–	–	–	–	–	–	–	–	CH05 (0.4)	–	–
–	–	–	–	–	–	–	CH06 (0.8)	–	–	–
–	–	–	–	–	–	–	CH07 (0.2)	–	–	–
–	–	CH08 (0.2)	–	–	–	–	–	–	–	–
–	–	CH09 (0.4)	–	–	–	–	–	–	–	–
–	–	CH10 (0.2)	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	CH11 (1.0)	CH11 (0.8)
–	–	–	–	–	–	–	–	–	–	CH12 (0.2)

Table 6 Fixation index (F_{ST}) values for the two investigated gene regions, *cox*I (below diagonal) and the *ITS*2 (above diagonal) for all pair-wise population comparisons

Pop.	Sr	Qs	Bl	Ma	Ns	Ld	Ln	Rt	Nr	Tz	Or
Sr		0.186	0.132	0.335**	0.175	0.004	0.112	0.113	0.613**	0.236*	0.419**
Qs	0.939**		−0.038	0.460**	−0.031	0.328**	0.003	−0.041	0.842**	0.605**	0.757**
Bl	0.000	0.939**		0.399**	0.006	0.220	0.005	−0.031	0.766**	0.519**	0.769**
Ma	0.538*	0.555*	0.538*		0.429**	0.444**	0.411**	0.439**	0.489**	0.378**	0.576**
Ns	0.000	0.939**	0.000	0.538*		0.210	−0.018	−0.050	0.772**	0.555**	0.703**
Ld	0.000	0.939**	0.000	0.538*	0.000		0.191	0.208	0.763**	0.402**	0.652**
Ln	0.000	0.939**	0.000	0.538*	0.000	0.000		−0.051	0.737**	0.505**	0.667**
Rt	0.428	0.825**	0.428	0.433*	0.428	0.428	0.428		0.814**	0.549**	0.717**
Nr	0.857*	0.771*	0.857**	0.470*	0.857**	0.857**	0.857**	0.767*		0.473**	0.793**
Tz	1.000**	0.920**	1.000**	0.500*	1.000**	1.000**	1.000**	0.902**	0.857**		0.503**
Or	0.923**	0.776**	0.923**	0.380*	0.923**	0.923**	0.923**	0.825*	0.780*	0.000	

* $0.01 < P < 0.05$

** $P < 0.01$

Discussion

Levels of haplotype and nucleotide diversity

Both gene regions included in our study were highly variable with respect to the number of haplotypes: 12 (*cox*I) and 26 (*ITS*2) haplotypes were found in the 55 individuals sampled. This was reflected in the high levels of haplotypic diversity (h_d) in some populations (Table 2). Taking into account that only five individuals were sampled per population, the true number of *cox*I and *ITS*2 haplotypes is probably higher. The levels of nucleotide diversity (π) were highly variable among the investigated populations especially for the *cox*I region, which was fairly high in some populations, e.g., Mahmoud Abad: $\pi = 0.0110$ (Table 2). On the other hand, however, 6 of the 11 investigated populations showed no *cox*I polymorphism. In the ribosomal *ITS*2 region, the π values ranged between 0.0006 and 0.0023. It therefore appears that although both regions are useful for population genetic studies on *A. pulvinum*, the *ITS*2 may be more informative in this respect.

Population differentiation

The present study of *A. pulvinum* revealed significant levels of genetic differentiation among the investigated populations. We found statistically significant estimates of F_{ST} and relatively few shared haplotypes among the 11 populations included in our study. Although the F_{ST} values for the *cox*I and *ITS*2 were not significantly correlated, in general, estimates of population differentiation based on these two DNA regions were similar. We believe that the high levels of population differentiation detected in our study were caused by several different factors.

The significant positive correlation between population differentiation (measured by F_{ST} estimates) and geographic distance indicates that gene flow between geographically distant populations is very limited. Furthermore, significant F_{ST} values were also found for pairs of geographically adjacent populations in both *ITS*2 and *cox*I regions, e.g., in comparisons involving populations Qaemshahr and Mahmoud Abad. This suggests that gene flow may be restricted even on a fine geographical scale. Similar results have been obtained by Osakabe and Sakagami (1993) for the citrus mite *Panonychus citri* McGregor and by Tsagkarakou et al. (1997) for *T. urticae*.

Apart from geographic isolation, several additional factors could have contributed to the observed high levels of population differentiation. The presence of distinct haplotypes for both mitochondrial *cox*I and nuclear *ITS*2 sequences in the northwestern populations Nir, Tabriz, and Orumie, suggests that they were founded by mites with different genotypes, possibly due to specific ecological constraints or past isolation from other populations. Therefore, in addition to the geographic distance factor, the climatic differences probably also contributed to division of the investigated populations of *A. pulvinum* into at least two geographically distinct groups, roughly corresponding to northern and northwestern parts of Iran. These two geographical regions are separated by Alborz mountain range and have different climates. The northwestern region is one of the coldest parts of Iran with significantly lower humidity compared with the northern region. These discontinuous climatic conditions between the two regions could facilitate local adaptation and high genetic differentiation between northern and northwestern populations of the mite.

Furthermore, the level of genetic differentiation revealed in comparisons of populations Qaemshahr and Mahmoud Abad with other northern populations was quite high. This observation suggests that still more factors may be responsible for the genetically distinct

character of these populations. Most samples of populations from the northern group (Mazandaran and Guilan province) with the exception of Qaemshahr, were collected from citrus trees where the larvae mostly parasitise the citrus aphids (*Aphis spiraecola* Patch and *Toxoptera aurantii* Fonscolombe). On the other hand, in Qaemshahr the samples were collected from forest trees due to the lack of citrus gardens in this city. Therefore, it is possible that local geographical and/or ecological factors also contributed to differentiation of the northern group of populations.

The lack of population differentiation within the northern region is not surprising given the dispersal capabilities of *A. pulvinum* larvae. The mites of the family Trombidiidae in adult stage are more or less edaphic (Wohltmann 2000), and their dispersal capabilities strictly depend on the soil habitat. Therefore, even small and temporary geographic or environmental barriers may be sufficient to block adult's dispersal. On the other hand, the larvae are commonly the main dispersal agent via its host (Zhang 1998). *Allothrombium pulvinum* larvae are more or less restricted by the host range and they can only parasitize aphids, so the factors affecting migration of aphids also affect dispersal of the mites (Zhang 1998). Wind has been considered as the main agent of aphids spreading for relatively short distances. On the other hand, mite colonies can be transported together with citrus seedlings between the northern cities, which can stimulate gene flow between *A. pulvinum* populations occurring in the north.

In the case of inconspicuous organisms whose taxonomy relies on a few morphological characters, the use of molecular tools to estimate genetic variation might detect the presence of different genotypes, which cluster in distinct monophyletic groups. This has been taken as evidence of the presence of cryptic species (Canterino et al. 2000; Navia et al. 2005). In this regard, due to considerably high levels of population differentiation detected in our study, we suggest that *A. pulvinum* from Iran comprises at least two geographically distinct populations or even more than one cryptic species. This study is an initial step towards understanding genetic variation of *A. pulvinum*, a taxon for which little molecular information is available. Unfortunately, no additional information on DNA sequences of *A. pulvinum* is available at present, which could corroborate our present suggestion. Therefore, more studies including populations from other countries and additional DNA regions are necessary to elucidate in detail the taxonomic status of this species.

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