The Mediterranean fruit fly (*Ceratitis capitata*) in Iran: genetic diversity and comparison with other countries

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Abstract

The Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann) is an economically important pest on fruits all over the world. The origin of this fly is thought to be from Africa, but it has recently expanded its distribution in many geographic regions including Iran. Due to the wide spread of this pest in Iran and its serious damage to fruit on trees, including citrus orchards of northern Iran, the present study was conducted firstly to investigate genetic diversity within populations of *C. capitata* based on the sequences of three mitochondrial DNA (mtDNA) genes including cytochrome C oxidase I (*COI*), NAHD dehydrogenase subunits 4 and 5 (*ND4* and *ND5*) and secondly to compare the Iranian haplotypes with those found in other countries. Results of this study indicated low levels of genetic diversity (four, four and three haplotypes among different populations of this pest, respectively for the *COI*, *ND4* and *ND5* genes) in northern Iranian populations. The genetic similarity and very low levels of genetic diversity of northern Iranian populations suggest that the pest colonisation occurred relatively recently. In addition, haplotypes of Mazandaran province are similar to haplotypes of those countries that have recently been infected by this pest.

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Key words: Mediterranean fruit fly; mitochondrial DNA; genetic variation; haplotypes; northern Iran.

Acknowledgements: we would like to thank the Sari Agricultural Sciences and Natural Resources University for financial support of this research. Also we are appreciated to Dr. Antonio Carapelli from Italy for kind help during the work.

Received for publication: 16 May 2014.
Revision received: 22 October 2014.
Accepted for publication: 16 January 2015.

Journal of Entomological and Acarological Research 2015; 47:4055
doi:10.4081/jear.2015.4055

Introduction

The Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae) is one of the world's most economically important pest species of fruit trees (Sheppard *et al*., 1992). This pest has spread rapidly from its putative source area in central Africa to north and South Africa in 1842 (Hagen *et al.,* 1981). The medfly is a major reason for direct economic losses in fruit production, and eradication programs are routinely carried out in several countries where this pest causes considerable damage (Dowell & Krass, 1992). For these reasons, several research projects have focused on the genetic diversity of the medfly populations in many regions worldwide (Sheppard *et al*., 1992; Elfekih *et al*., 2010a, 2010b). Different kinds of genetic markers have been used for analysis of diversity in medfly populations (Reyes & Ochando, 2004).

C. capitata was originally recorded from Iran in 1958 (southern Iran), and subsequently in 1980 (northern Iran) (Mirsardo *et al*., 2010). Given the wide distribution, high invasiveness and serious economic impact of this pest on fruit production, it is crucial to conduct a survey of the genetic diversity of medfly populations where this pest occurs (Elfekih *et al*., 2010b). Previous studies used several markers including mitochondrial DNA, which proved to be very useful given its haploid nature and maternal inheritance as well as its lack of recombination (Hewitt, 2004).

Despite the extensive research work done on medfly genetic diversity in several areas worldwide (Reyes & Ochando, 2004; Barr, 2009; Elfekih *et al*., 2010b), very limited information is available on the genetic variability of medfly populations in Iran.

The main objective of the present study is to characterise the genetic diversity among medfly populations in Iran using mitochondrial DNA markers. The sequences of mtDNAs including the cytochrome oxidase I (*COI*), NADH dehydrogenase subunits 4 and 5 (*NADH4* and *NADH5*) genes are used for characterisation of genetic diversity in medfly populations in different areas of Mazandaran province (northern Iran). Furthermore, the sequences of mitochondrial DNA from other countries, recorded in National Centre for Biotechnology Information (NCBI), were employed for comparisons.

Materials and methods

Adult specimens of *C. capitata* were collected from different areas of Mazandaran province in northern Iran (Figure 1). The specimens

were captured using pheromone traps (Xlure-cec), placed in citrus orchards. All samples were preserved in 90% ethanol (Table 1).

Total genomic DNA was extracted from individual specimens using the DNeasy Blood and Tissue DNA extraction Kit (Qiagen, Hilden, Germany). DNA was extracted by placing single individuals in an Eppendorf tube and the wings were removed.

The extracted DNA from medfly specimens was amplified using three pair primers (Table 2). The COI primer pair amplifies a 631 bp fragment of the *COI* gene. Amplifications were performed in 25 μL microtubes containing 1 μL $MgCl_2$, 1.5 μL primers (10 pm/μL), 0.5 μL dNTPs, 2.5 μL polymerase chain reaction (PCR) buffer, 0.2 Taq Polymerase, 16.8 μL ddH_2O, and 1 μL DNA. The amplification program has an initial denaturation step of 5 min at 94°C, followed by 35 cycles of 60 s in 94°C, 60 s in 43°C, 90 s at 72°C, and a final extension of 7 min at 72°C. The ND4 and ND5 primer pairs are used to amplify a 350 and 950 bp fragment of the *NADH4* and *NADH5* genes, respectively. The PCR mix for these primers contain 16.8 μL ddH_2O, 2.5 μL PCR buffer, 3 μL $MgCl_2$, 0.5 μL dNTPs, 0.5 μL primers, 1 μL DNA and 0.2 μL Taq

Table 1. Information on sampling sites in Mazadaran province.

Sampling site	Coordinate		Number of individuals from each gene		
Locality	Longitude (N)	Latitude (E)	*COI*	*ND4*	*ND5*
Amol	36° 28' 14"	52° 21' 47"	1	2	3
Behshahr	36° 41' 39"	53° 32' 33"	1	1	3
Tonekabon	36° 48' 31"	50° 52' 54"	1	1	2
Juybar	36° 38' 17"	52° 54' 42"	1	2	3
Sari	36° 34' 4"	53° 03' 31"	1	2	3
Qaemshahr	36° 27' 47"	52° 51' 36"	1	2	3
Mahmudabad	36° 36' 47"	53° 51' 36"	0	1	3
Neka	36° 39' 5"	53° 17' 48"	1	2	3
Nur	36° 33' 45"	52° 01' 54"	1	2	3

Table 2. Name and sequence of mtDNA primers used for polymerase chain reaction reaction.

Primer	Sequence of primer (5 -3)	Gene	Size (bp)	Reference
LCO1490	GGTCAACAAATCATAAAGATATTGG	*COI*	360	Folmer *et al.*, 1994
HCO2198	TAAACTTCAGGGTGACCAAAAAATC			
N-4-J-8883	TAATAATCCATATCCTCCTA	*ND4*	350	Elfekih *et al.*, 2010a
N-4-N-9243	TTAGTTTTAACATTTAGAAG			
N-5-J-7991	TAATAAACTCATTCAATCAA	*ND5*	950	Elfekih *et al.*, 2010b
N-4-N-8916	ATAGAAGCTCCTGTATCTGG			

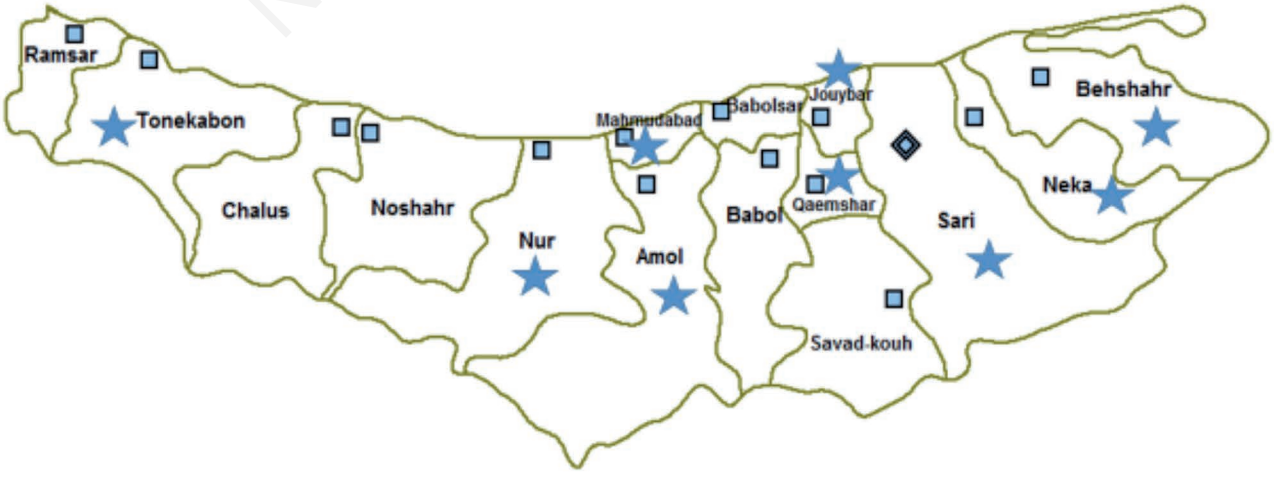

Figure 1. Map of Mazandaran province (northern Iran) indicating the sampling localities.

Polymerase. The amplification program has an initial denaturation step of 2 min at 94°C, followed by 40 cycles of 30 s in 94°C, 30 s in 46°C, 2 min at 66°C, and a final extension of 2 min at 72°C (Elfekih *et al*., 2010a). Amplification products (5 μL) were visualised after electrophoresis in 2% agarose gels in TAE buffer (24.2 gr Tris *Acetic acid 71 mL* EDTA 10 mL *ddH2O 60 mL*) with the 1-log ladder as a molecular weight marker. Purification and sequencing of PCR products for forward primer from individual specimens performed by Takapu-zist Company. The accession numbers in GenBank for COI sequences of *C. capitata* obtained here are KM660641-KM660652.

A Blast search of GenBank sequences using the sequence obtained from the PCR product was conducted through the NCBI website (http://www.ncbi.nlm.gov). Sequences were aligned using Clustal X with defaults/parameters (Thompson *et al.,* 1997). Phylogenetic trees were constructed using the maximum likelihood algorithm in MEGA5 (Tamura *et al.,* 2011). Numbers of haplotypes and numbers of mutations were identified using DnaSP (Librado & Rozas, 2009).

Results

Intraspecific genetic variation was investigated using molecular marker obtained from COI, ND4 and ND5 sequences in nine populations of the Mediterranean fruit fly, *C. capitata* from northern Iran. In sum, genetic relationships were constructed for mitochondrial DNA sequences generated from 48 individuals. The total length of COI, ND4 and ND5 products were 650, 372 and 950 bp, respectively (Figure 2). In order to eliminate the errors due to sequencing artifacts, we considered only fragments of 631 bp of COI, 350 bp of ND4 and 899 bp of ND5 for all sequences by deleting the beginning and end of the sequences.

The number of haplotypes, number of mutations and average frequency of bases for the different markers are shown in Table 3. The A-T rich sequences were observed in nucleotide composition, which is a pattern that has been repeatedly seen in the mtDNA of Dipteran species (Bajpai & Tewari, 2010).

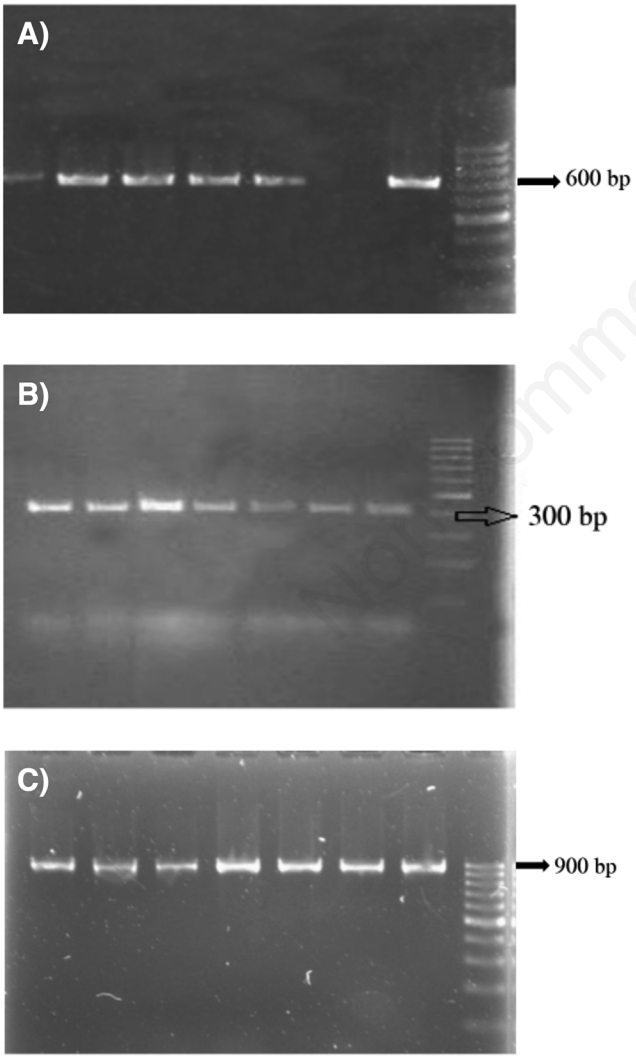

Figure 2. A) *COI*- ; B) *ND4*- ; C) *ND5*-DNA fragment amplified of Mediterranean fruit fly from different cities of Mazandaran province.

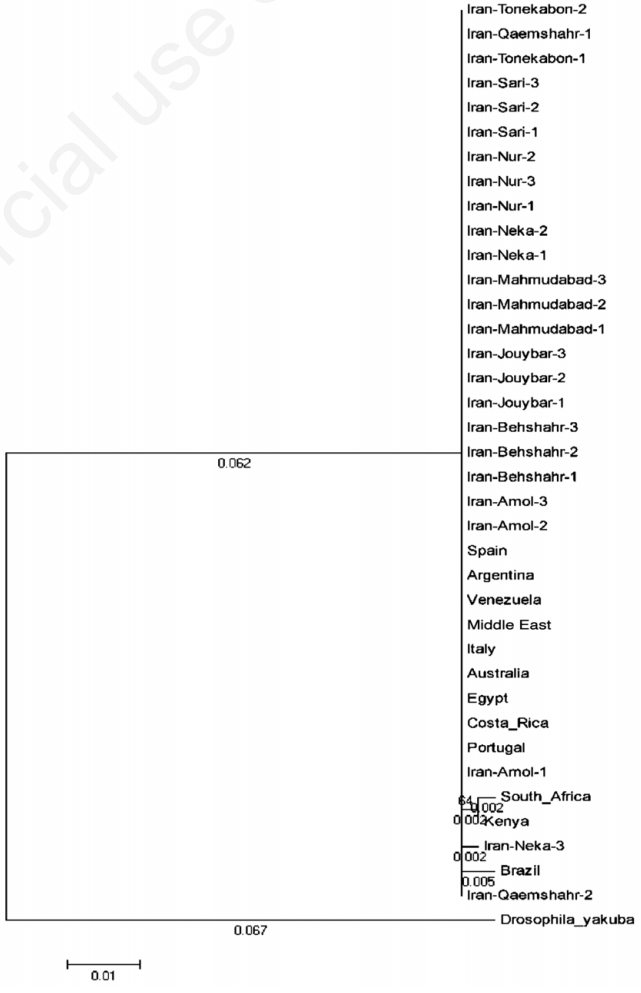

Figure 3. Maximum likelihood tree of 25 specimens of *Ceratitis capitata* from Mazandaran province and twelve other countries for *COI*, calculated in MEGA5. *Drosophila yakuba* used as outgroup. Numbers above the branches indicate the bootstrap values for nodes (1000 replications).

Genetic distances were calculated among the nine populations using the binary data obtained for each primer pair. Very low genetic distance values were found, indicating a high genetic similarity among the derived populations in general. The largest genetic distance values are observed between $Neka_1$ and $Neka_3$ (0.008) for COI, Behshahr and $Neka_2$ (0.022) for ND5, Qaemshahr and Sari (0.002) for ND5.

Phylogenetic trees were constructed using likelihood based (ML from 1000 bootstrap replicates) algorithms in MEGA5 (Tamura *et al.,* 2011). A set of medfly mitochondrial DNA sequences, available on Genbank, was used in order to compare the Iranian populations with medfly populations from other geographical regions (Figures 3-5). The species, *Drosophila yakuba* (Diptera: Drosophilidae) was used as an out-group for construction of phylogenetic trees. Additionally, statistical parsimony (Templeton, 2004) implemented in the TCS software (Clement *et al.,* 2000) with a 150-step connection limit, was used to construct parsimonious networks of COI haplotypes for populations of *C. capitata*.

The phylogenetic tree (Figure 3) shows that different populations of the medfly are similar in *COI* gene sequences in that they are placed in one branch separated from the out-group. Three populations, including Neka-1, Neka-3 and Tonekabon-2, appear to be a little bit different from the others. The presence of four haplotypes among the nine populations of the medfly is confirmed by the TCS plot as well for *COI* gene (Figure 6). These medfly populations have a bias toward haplotype A. The phylogenetic tree also affirms a very close genetic similarity among populations of medfly in northern Iran and some other countries such Spain, Middle East, and Venezuela. However, populations of South Africa and Kenya show less similarity with the population in northern Iran.

The phylogenetic tree constructed for the *ND4* gene (Figure 4) shows populations of medfly from different cities are genetically similar to each other. Also the maximum likelihood tree shows the medfly populations come from Middle East and Tunisia are relatively close to Iranian population. But in compare to the phylogenetic tree constructed by *COI* gene Middle East population showed a bit different from Iranian population.

The phylogenetic tree constructed based on *ND5* sequences (Figure 5) also reveals genetic similarity among the populations of northern Iran. Populations of Neka, Behshahr, jouybar and Tonekabon are mostly close to each other and form one haplotype. Such genetic similarity is also repeated between populations of Nur, Sari and Amol. The populations of the other countries (USA and Argentina) are closely related to the populations of northern Iran. However Tunisia population showed again a bit deviation from others.

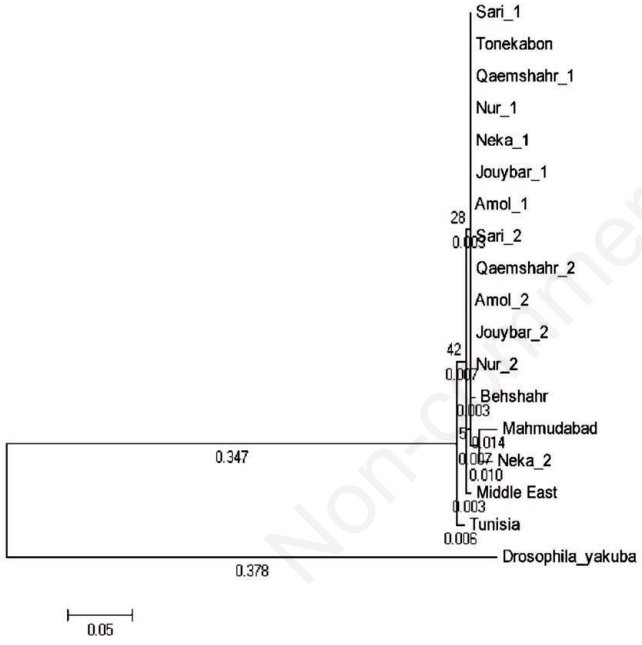

Figure 4. Maximum likelihood tree of sequences of 15 specimens of *Ceratitis capitata* from Mazandaran province and two other countries for *ND4*, with *Drosophila yakuba* as an out-group, calculated in MEGA5. Numbers indicate bootstrap values for nodes (1000 replications).

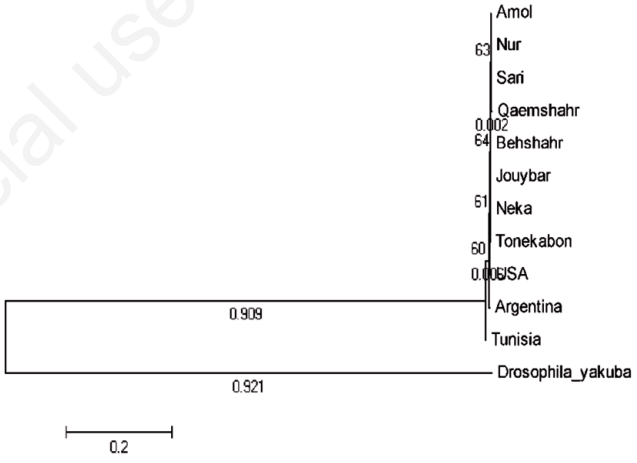

Figure 5. Maximum likelihood tree of sequences of 8 specimens of *Ceratitis capitata* from Mazandaran province and three other countries for *ND5*, with *Drosophila yakuba* as an out-group, calculated in MEGA5. Numbers indicate bootstrap values for nodes (1000 replications).

Table 3. The haplotypes of medfly populations according to sequences of the *COI*, *ND4* and *ND5* genes.

	No. of haplotypes	No. of mutations	Average frequency of bases			
			A (%)	T (%)	C (%)	G (%)
COI	4	6	28.7	39.3	16	16
ND4	4	10	47.1	33	10.9	8.9
ND5	3	3	44.2	31.9	16.1	7.8

Discussion and conclusions

DNA markers, such as those derived from nuclear genes, RAPDs and intron sequences, microsatellites, as well as those derived from mitochondrial DNA, have been used previously to study Mediterranean fruit fly populations (Elfekih *et al.,* 2010b). Despite the wide spread of the *C. capitata* in northern Iran, no research has been done on previously on genetic diversity and evolutionary relationships of different populations of this pest in Iran.

According to Nei's estimates (1975) of the genetic distance ranges for local race of a species, low genetic distances among populations of different areas show genetically close relationships among them (Menezes, 1990). The low genetic distances found among populations of the medfly in Mazandaran province in this study indicate that these populations are genetically similar and that colonisation in citrus orchards by this pest took place once and relatively recently. The similarity among the medfly populations can be for different reasons. One reason could be the close distance of cities in Mazandaran province. Secondly, the spaces between the sampling localities have been filled with vast citrus orchards. Also the products of the orchards are exchanged between the cities, and transmission of infested fruit is one of the most important paths for distribution of the medfly in northern Iran (Mirsardo *et al*., 2010).

High genetic diversity is generally used as evidence that a species is native, or that it has been established for a long period of time (Solorzano *et al.,* 2010). According to the haplotype diversity calculated in this study, it can be concluded that in northern Iran the medfly has not been present for a long time. Also, it is a new pest recorded for the first time in 1980 in northern Iran. Additionally, according to the assumptions of Malacrida *et al*., *C. capitata* populations can be divided into three main categories according to their colonisation pattern: ancestral, ancient and new populations, corresponding to populations from Africa, the Mediterranean basin and America, respectively (Reyes & Ochando, 2004). Finding of this study indicated there are few haplotypes in medfly population in Mazndaran province, so the population of medfly in northern Iran can be placed in the third category of Malacrida's classification. In other words, the colonisation of the pest has happened more recently and the pest has not had enough time for genetic divergence (Reyes & Ochando, 2004). The phylogenic trees for *COI* gene sequences also confirm Malacrida's classification very well. The population from Africa (Kenya and South Africa) showed older colonisation than populations from Europe such as Spain and Italy, Middle East countries and even countries of the Americas such as Argentina and Venezuela. The close relations of the population of Iran with other countries show short history of existence of the medfly in these countries.

The phylogenic tree for ND4 showed also the African population (Tunisia) colonised older than Middle East countries such as Iran. Low genetic diversity in populations from Middle East (constructed by COI and ND4) indicated recent colonisation of pest in this area. The phylogenetic tree for ND5 also indicated older colonisation in Tunisia (African population) than American and Iranian populations.

The results of this study indicate that genetic analyses based on the use mitochondrial genes can provide useful tools for unravelling genetic and phylogenetic relationships in *C. capitata* populations in northern Iran. The variability identified by the direct analysis of such sequences will allow for answering a range of questions relevant to pest population dynamics such as the origin of new infestations, the relationships of existing populations and will allow pest management controls to be examined in greater detail than had been possible previously.

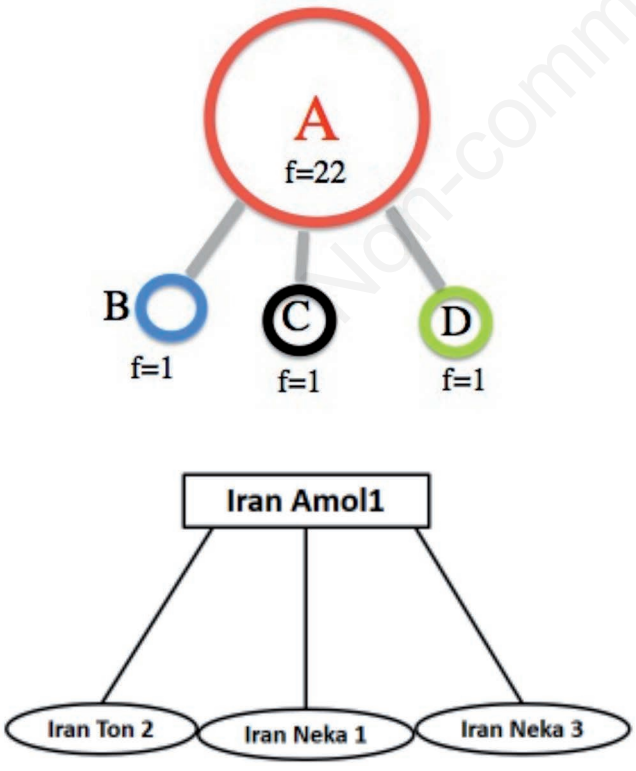

Figure 6. Reconstructed TCS plot of 4 cytochrome *COI* haplotypes of *Ceratitis capitata*; A to B-C-D = one substitution.

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