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Genetic structure and diversity of the common pistachio psylla, *Agonoscena pistaciae* Burckhardt & Lauterer, (Hemiptera: Aphalaridae) in Iran

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Abstract. *Agonoscena pistaciae* Burckhardt & Lauterer is a serious global economic pest of pistachio. Despite its economic importance, little is understood of its genetic diversity and population relatedness. We used two mitochondrial genes (COI and Cyt-*b*) and one nuclear sequence (ITS2) to investigate the genetic structure and diversity of 5 populations spanning the distribution of this pest in Iran. High levels of genetic diversity was found for all three genes due to the existence of two genetically divergent lineages. Haplotype network and phylogenetic analyses separated populations into two major clades, the central – southeastern and the northeastern clades. Because these lineages are genetically highly different with the genetic divergence of 4.57% – 4.99% and 3.61%-4.58% based on cytochrome c oxidase I and Cyt-*b* and 2.88% - 4.91% for nuclear 5.8S/ITS2 sequences suggesting the possibility of existence of cryptic *A. pistaciae* species in Iran. Genetic structure analysis revealed that geographic adjacent populations share haplotypes, while distant populations exhibit distinct haplotypes, indicating geographic isolation and limited gene flow among populations. This finding is consistent with population pairwise F_{ST} analysis which found that most populations were genetically distinct. The high level of population genetic structuring is most probably related to dispersal capacity, life history variation and the geographic isolation among the populations.

Key words: *Agonoscena pistaciae*, genetic diversity, genetic variation, gene flow, population genetics.

Introduction

Pistachio, *Pistacia vera* L., is a crucial economic horticultural product presently cultivated across Asia, Americas, Europe, Africa and Oceania. Currently, Iran, the United States and Turkey are ranked as the three major pistachio producers, supplying about 92% of the global pistachio demand (FAOSTAT, 2019). The common pistachio psyllid, *Agonoscena pistaciae* Burckhardt & Lauterer, 1989, is one of the most destructive pests of cultivated pistachio trees in Iran and is distributed throughout all the country's pistachio-producing areas in the country (Mehrnejad 2003, Mostafavi et al. 2017). *Agonoscena pistaciae* is an indigenous insect in Iran, firstly reported on this country's wild and cultivated pistachio trees by Kiriukhin (1946). High population density of pysllid nymphs and adults can drastically reduce kernel development, leading to bud drop and defoliation (Mehrnejad 2001a,b). This damage leads to tremendous yield losses in the immediate term and yields are unable to rebound over successive years (Mehrnejad 2003). Moreover, a combination of multivoltine life-history and high reproductive rate often leads to outbreaks, resulting in significant levels of damage and astounding crop losses (Mehrnejad 2001a).

Although the control of *A. pistaciae* is virtually limited to insecticides, the propensity of the species to evolve resistance against pesticides has been documented since nearly 70 years ago (Davatchi 1958, Mehrnejad 2001b). For identifying alternative management programs, especially with potential for evolution of pesticide resistance, it is important to establish the extent of existing population genetic diversity, genetic structure and gene flow (Roderick 1996, Estoup & Guillemaud 2010, Qin et al. 2016).

Well conserved, maternally inherited mitochondrial haplotypes are reliable evolutionary markers for determining both intra and inter-specific relationships and phylogeographical structures in sundry insects (Brower 1994a,b, Mun et al. 2003, Nardi et al. 2005, Shi et al. 2005, 2010, Guidolin et al. 2014, Koohkanzadeh et al. 2019). Several studies have established that mitochondrial DNA sequences can be employed for psyllids population genetic studies (Boykin et al. 2012, Katoh et al. 2014, Guidolin et al. 2014). Moreover, the nuclear ribosomal internal transcribed spacer 2 (ITS2) as a noncoding fast-evolving region has been another candidate utilized for studying phylogeographic structure or population genetics of various insect species (Fenton et al. 1998, Karimi et al. 2014, Zhang et al. 2015, Zhang et al. 2017, Lashkari et al. 2020).

In this study, we assessed the genetic structure, diversity and demographic history of five populations of *A. pistaciae* collected across the major pistachio growing regions in Iran. These data would allow a better understanding of the interconnectedness of the different populations and therefore infer population resilience to the management tools.

Material and Methods

Psyllid samples

Five geographical distinct populations of *A. pistaciae* were collected from pistachio trees from orchards in the major pistachio producing regions in Iran (Table 1). For each population, at least ten psyllid individuals were collected and stored in 90% ethanol at -20°C until processing.

Table 1. Details of the sampling sites of pistachio psyllid in Iran.

Location	Geographic region	Latitude / Longitude	Code
Feizabad	Northeast	36.7736° N, 56.7720° E	Pop1
Damghan	Center	36.1646° N, 54.3576° E	Pop2
Neishabour	Northeast	36.2141° N, 58.7961° E	Pop3
Rafsanjan	Southeast	30.3549° N, 56.0027° E	Pop4
Sirjan	Southeast	29.4587° N, 55.6714° E	Pop5

DNA extraction, amplification and sequencing

Genomic DNA was extracted from individual adult psyllids by grinding in 50µl 5% Chelex-100 (Sigma-Aldrich Chemie GmbH, Germany) and 2µl proteinase K and incubating the homogenized mixture at 60°C for 2 h, followed by a 10 min incubation at 95°C to denature the proteinase K. The extracted DNA was stored at –20°C. Two mitochondrial genes, cytochrome c oxidase subunit 1 (COI) and cytochrome b (Cyt-*b*), were amplified using the primer pairs LCO1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) (Folmer et al., 1994) and cytBF (5′-TGAGGNCAAATATCHTTYTGA-3′) and cytBR (5′-GCAAATARRAARTATCATTCDG-3′) (Simon et al. 1994, Percy et al. 2016), respectively. The polymerase chain reaction amplifications were performed in a total volume of 25 µl containing 12.5 µl PCR master mix (Amplicon), 1 µl of each of primers (10 pmol/µl) and 2.5 µl of template DNA. The PCR programs for two genes were as follows: 94°C for 3 min, 35 cycles of 92°C for 30 s, 50°C (mtCOI gene) or 56°C (Cyt-*b* gene) for 40 s, 72°C for 1 min followed by 72°C for 5 min. The ITS2 and part of 5.8S gene were amplified with primers CAS5p8sFcm (5′-CGAACATCGACAAGTCGAACGCACA-3′) and CAS28sB1d (ID: S50426), (5′- TTGTTTTCCTCCGCTTATTAATATGCTTAA-3′) (Ji et al. 2003). The PCR amplification conditions of Peccoud *et al*. (2013) were used in our study. A control PCR tube containing all components without genomic DNA was also prepared and run for all the genes. The PCR products were checked using a 1% agarose gel and sequenced in both directions using the same primers as in the PCR by Macrogen sequencing service (Seoul, Korea).

Data analysis

The sequences were manually edited with the BioEdit sequence editor (Hall 1999). To obtain single consensus sequences from the bidirectional reads, the DNA Baser software package (version 3.4) was utilized. The accuracy of the COI and Cyt-*b* nucleotide sequences were checked by confirming that they could be translated into proteins by using MEGA ver 10.05 (Kumar et al. 2018). BLAST of the specimens was conducted by inputting the sequence in nBlast tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The alignment of DNA sequences was subsequently carried out by ClustalW program implemented in MEGA ver 10.05. The number of haplotypes (H), haplotype diversity (Hd) and nucleotide diversity (π) (Nei 1987) were calculated in DnaSP v6 (Rozas et al. 2017). The median joining (MJ) network (Bandelt et al. 1999) based on individual gene and concatenated sequences were constructed using PopART (Leigh & Bryant 2015).

Congruence between separate genes was tested using the partition homogeneity test (Farris et al., 1995) with 1,000 replicates implemented in PAUP* v. 4.10b (Swofford 2002). The results indicated significant differences between the separate gene regions ($P < 0.001$). Therefore, phylogenetic relationships using three methods, i.e. neighbor joining (NJ), Bayesian analysis (BA) and maximum likelihood (ML) were inferred separately for each gene. NJ analysis was performed using MEGA X (Kumar et al. 2018). Branch support for NJ tree was calculated using the bootstrapping method with 1,000 replications. The BA analysis was performed in MrBayes 3.2.7a (Ronquist et al. 2012) with 2,000,000 generations and sampling frequency of 100 generations. ML analysis was implemented in PhyML 3.0 (Guindon et al. 2010). Branch support was calculated using approximate likelihood ratio tests (Anisimova & Gascuel 2006). For all phylogenetic analyses, a closely related species, *Agonoscena bimaculata*, was used as outgroup. The exception is for 5.8S/ITS2 sequence because it is not available for this species.

Genetic differentiation between populations was estimated using population pair- wise F_{ST}. The significance statistic test was obtained based on 1023 permutations. Analysis of molecular variance (AMOVA) was performed to test the genetic differentiation among groups of populations from different geographic regions: northeastern populations (Neishabour and Feizabad), central population (Damghan) and southeastern populations (Rafsanjan and Sirjan). Both population pairwise F_{ST} and AMOVA analyses were performed in Arlequin (Excoffier & Lischer 2010) using the Kimura 2-parameter model (K2P). The results of molecular variation analysis (AMOVA) were visualized utilizing an R program script integrated in Arlequin. To assess an isolation-by-distance (IBD) model, the Mantel test (Mantel 1967) was carried out to determine the relationship between genetic distance (F_{ST} from Arlequin) and geographic distance (km). The Mantel test was performed in IBD v1.52 (Bohonak 2002) using 1000 randomizations. The geographic distance between each pair of populations was estimated utilizing Google Maps Distance Calculator (http://www.daftlogic.com/projects-google-maps-distance-calculator.htm).

Results

Mitochondrial DNA sequence variation

A total of 58, 46 and 42 specimens of *A. pistaciae* were sequenced for COI (481 bp), Cyt-*b* (591bp) and 5.8S/ITS2 (591 bp) sequences, respectively. Sequences were deposited in GenBank with the accession numbers MT111653-MT111710 (COI), MT119099-MT119144 (Cyt-*b*) and MT133693-MT133734 (5.8S+ITS2).

The COI fragment contained 25 variable sites that delineated 7 haplotypes. Three common haplotypes (haplotype 2, 3 and 4) are shared by 3, 14 and 10 specimens, respectively. These haplotypes are found in three populations, namely pop1, pop4 and pop5 (Table 2, Appendix 1a). Haplotype 1 and 5 are found in two geographically adjacent populations, pop1 and pop3. Haplotype 6 is unique, occuring only in population 2 (Table 2 and Appendix 1a). Haplotype and nucleotide diversities are highest in Feizabad (Pop1). This population show very high diversity (>10 times) compared to others (Table 2), due mainly to the possession of divergent haplotypes within population (Fig. 1a). Pop2 possess no genetic diversity as only one haplotype occurs in this population (Fig. 1a).

The Cyt-*b* sequences contained 19 variable sites. There are 7 haplotypes with haplotypes 1, 2 and 5 being shared between multiple locations (Table 2, Appendix 1b). The greatest nucleotide and haplotype diversities were recorded in Feizabad (pop1) and Sirjan (pop5), while the lowest were observed in Damghan (pop2) and Rafsanjan (pop 4) for COI and Cyt-*b*, respectively (Table 2).

Lower diversity for this nuclear DNA sequences compared to the mitochondrial DNA (Table 2). Compared to the results of the combined mitochondrial dataset, fewer haplotypes (HN = 6) and lower haplotype diversity (0.5738) were observed in the nuclear data (Table 2). Of the 6 examined haplotypes, 4 were unique (almost 67%) (Appendix 1c). In addition, of the 14 observed polymorphic sites, 12 were parsimony-informative sites (2.03%) and 2 were singleton-variable sites (0.34%). The most common haplotype (haplotype 1) was recorded at all of the sampled locations. Nucleotide diversity varied across populations, ranging from 0.004360 to 0.013630,

Table 2. Number of haplotypes in each population (H), haplotypic diversity h (mean±SD) and nucleotide diversity (π) (mean±SD) observed for the COI, Cyt-*b*, combined mitochondrial sequence data and 5.8S/ITS2 of the studied populations of *A. pistaciae*.

Marker		H	Haplotype diversity (h)	Nucleotide diversity (π)
COI	Pop1	5	0.7750 ± 0.0678	0.025797 ± 0.013774
	Pop2	1	0.0000 ± 0.0000	0.000000 ± 0.000000
	Pop3	2	0.4667± 0.1318	0.000970 ± 0.001041
	Pop4	3	0.6182± 0.1038	0.001814 ± 0.001551
	Pop5	4	0.7333± 0.1199	0.002726 ± 0.002093
Cyt-*b*	Pop1	2	0.5556± 0.0745	0.001339 ± 0.001347
	Pop2	2	0.5556± 0.0902	0.001339± 0.001363
	Pop3	2	0.5556± 0.0902	0.001339 ± 0.001363
	Pop4	1	0.0000 ± 0.0000	0.000000 ± 0.000000
	Pop5	3	0.6667± 0.1318	0.002811± 0.002256
5.8S/ITS2	Pop1	3	0.2857± 0.1964	0.010708± 0.006626
	Pop2	8	0.4554± 0.1701	0.008691± 0.005100
	Pop3	6	0.4167± 0.1907	0.011186 ± 0.006619
	Pop4	5	0.2500± 0.1802	0.001295± 0.001208
	Pop5	4	0.0000± 0.0000	0.00000 ± 0.000000

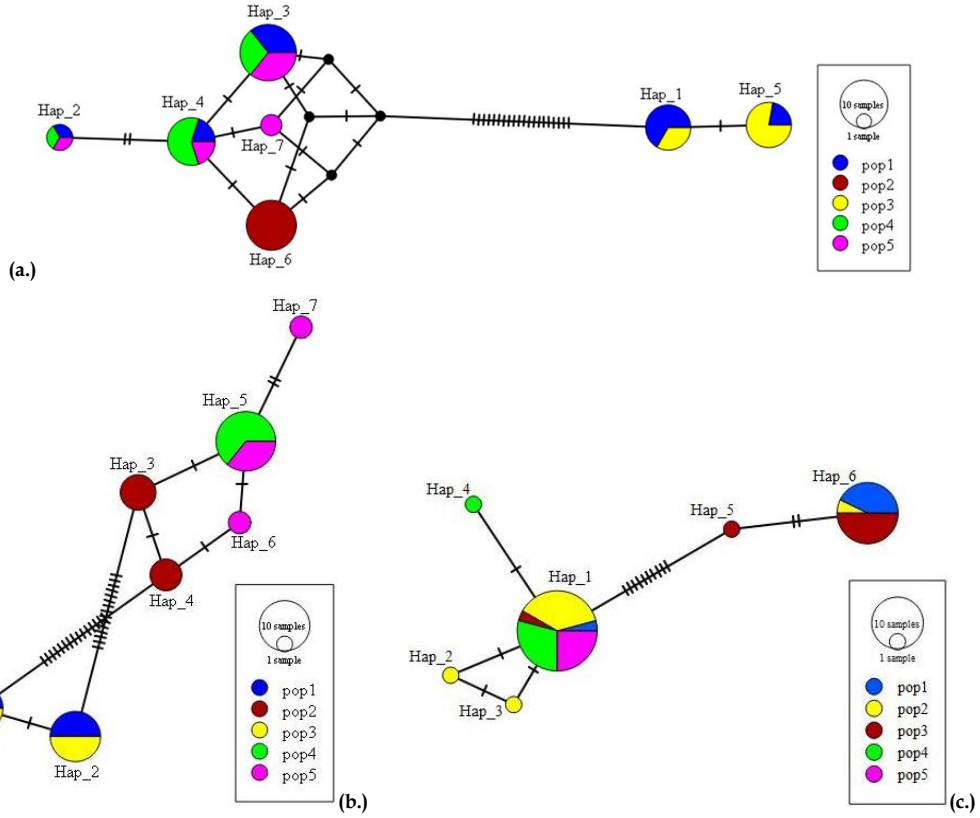

Figure 1. Haplotype network of populations of *A. pistaciae* based on COI (a), Cyt-*b* (b) and 5.8s/ITS2 sequences (c).

the neighbor populations having the highest nucleotide diversity.

Genealogy

The median-joining networks of COI, Cyt-*b* and combined data for these two genes and 5.8S/ ITS2 sequences were showed in Figure 1a, b, c and d.

The MJ network based on COI sequences revealed two distinct genetic lineages (I and II) among 58 specimens of *A. pistaciae* in Iran (Fig. 1a). These two lineages are separated by 20 mutation steps or 4.57% - 4.99% genetic divergence based on Kimura 2-parameter (K2P) model. This level of genetic divergence is much higher than of those within the lineage. The K2P genetic divergence within lineage I is 0 – 0.62% and for lineage II is 0 – 0.21%. Lineage I comprised of five haplotypes, three of these shared by population 1(Feizabad), 4 (Rafsanjan) and 5 (Sirjan). Two haplotypes are unique. Haplotype 6 was found only in population 2 (Damghan) and haplotype 7 was found only in population 5 (Sirjan). However, these haplotypes are separated by short mutation steps (<2). Lineage II is characterized by two haplotypes (1 and 5) that are found only in two populations, population 1 (Feizabad) and 5 (Sirjan).

In lineage II, population 2 (Damghan) with its unique haplotype (haplotype 6) is completely distinct from population 4 Rafsanjan) and 5 (Sirjan) with 3 shared haplotypes (Fig. 1a). The haplotype cluster in the MJ network was associated with geographical regions; population 1 and 3 are located in northeastern (cluster I), population 2 is located in central and population 4 and 5 are located in south-eastern regions of Iran (Fig. 1a). Overall, the network does not have a distinct star-like shape, of all 7 haplotypes, only haplotypes 3 and 4 are shared between three populations (pop1 (Feizabad), pop4 (Rafsanjan) and pop5 (Sirjan)) (Fig. 1a).

The MJ network based on Cyt-*b* sequences revealed similar topologies with those of the COI gene. Based on the Cyt-*b* sequences, two genetically divergent lineages were found among 42 specimens of *A. pistaciae* in Iran (Fig. 1b). These two lineages were separated by 25 mutation steps or 3.61% - 4.58% based on K2P model. Levels of genetic differentiations between the two lineages are much higher than those within lineages. Genetic divergent within lineage I is 0 – 0.96% and for lineage II is 0 – 0.24%. Lineage I comprises five haplotypes, one haplotype shared by population 4 (Rafsanjan) and 5 (Sirjan) and remaining are unique to population 2 (Damghan) (two haplotypes) and population 5 (Sirjan) (two haplotypes). Lineage II comprises only two haplotypes, one found in population 1 (Feizabad) and 3 (Neishabour) and another is unique to population 3 (Neishabour). However, there is only a single mutation step separating these haplotypes.

The MJ network based on 5.8/ITS2 sequences revealed two genetically distinct lineages similar to those of COI and Cyt-*b* sequences (Fig. 1c). Based on K2P model, these two lineages separated by 10 mutation steps.

Lineage I comprises one common haplotype, being shared by all populations and three unique haplotypes. Lineage II comprises two haplotypes, one is common haplotype shared by three populations and another is unique to population 3.

In addition to the haplotype network, phylogenetic trees (Fig. 2) provided further confirmation of existence of the two clades among our haplotypes with high values for bootstrap of neighbor joining, posterior probability of Bayesian analysis and also maximum likelihood branch supports (Figs 2 a, b, c). The populations clustering comply well with the results from the haplotype networks (Fig. 2).

By applying species delimitation methods, including Kimura 2-parameter genetic divergence values within and between two lineages of *A. pistaciae* found in Iran, it was revealed that these lineages are genetically highly different, with the genetic divergece of 4.57% – 4.99%, 3.61%-4.58% and 2.88% - 4.91% based on cytochrome c oxidase I, Cyt-*b* and 5.8S/ITS2 sequences, respectively, suggesting the possibility of existece of incipient cryptic *A. pistaciae* species in Iran (Table 3).

Table 3. Kimura 2-parameter genetic divergence values within and between lineages of *A. pistaciae* found in Iran based on mitochondrial cytochrome oxidase I (COI), Cyt-*b* and 5.8S/ITS2 sequences.

Marker	Lineage (n)	Within lineage	Between lineage (mean)
COI	A (40)	0 - 0.62% (0.26%)	4.57% – 4.99% (4.75%)
	B (18)	0 – 0.21% (0.11%)	
	All (58)	0 – 4.99% (2.20%)	
Cyt-*b*	A (27)	0 – 0.96% (0.27%)	3.61% - 4.58% (3.93%)
	B (19)	0 – 0.24% (0.13%)	
	All (46)	0 – 4.58% (2.06%)	
5.8S/ITS2	A (27)	0 – 1.35% (0.50%)	2.88% - 4.91% (3.97%)
	B (15)	0 – 1.86% (0.44%)	
	All (42)	0 – 4.91% (2.12%)	

Population genetic structure

Population pairwise F_{ST} values for COI, Cyt-*b* genes and concatenated data revealed that most populations were genetically distinct (Table 4, Appendix 2). For COI, the exception was the difference between populations 4 and 5, where F_{ST} value was statistically unsignificantly different (-0.04308), indicating low genetic differentiation among them. For Cyt-*b*, populations 1 and 3 (-0.11111) and populations 4 and 5 (0.22773) were not significantly different (Table 4). In total, the genetic differentiation among populations 4 and 5 was relatively low and unsignificant, indicating that existing gene flow between these two populations. These results are consistent with the results of the clustering analysis based on a Median-Joining network and NJ tree.

Nuclear sequences yielded an $F_{ST} = 0.68530$ ($p < 0.001$) for all populations. Overall, genetic differentiation among populations was high and the defined groups showed strong genetic structure.

AMOVA analysis by grouping populations according to the geographic regions found no significant differences among groups; although, significant genetic differentiation among the populations for COI was revealed (Table 5). Mantel's test revealed weak but significant relationships between genetic (pairwise F_{ST}) and geographic distances for COI ($r^2 = 0.3262$, $P = 0.049000$). However, for Cyt-*b* gene (r^2=0.5215, p= 0.102) and 5.8S/ITS2 sequences (r^2= 0.0136, p=0.329) the results of Mantel's test were insignificant (Table 5).

Discussion

Understanding genetic structure and diversity among populations of important insect pests are salient prerequisites for implementing management strategies against them. The genetic variation in natural populations of any organism is the consequence of a balance between evolutionary and demographic processes and hence supplies devices to elucidate the evolutionary potential of a species (Li et al. 2013, Choudhary et al. 2017).

The genetic diversity and structure of *A. pistaciae* populations sampled throughout key distribution regions in Iran were investigated using two mitochondrial and one nuclear s. Our results indicate a relatively high *A. pistaciae* genetic diversity in the sampled areas. Intraspecific genetic diversity (max. 4.99%) based on COI and Cyt-*b* (max. = 4.58%) sequences is much higher than a previous report of this species, also from Iran (0%) (Lashkari et al. 2020). Although, it is necessary to mention that Lashkari et al. (2020) only studied *A. pistaciae* populations in one province, Kerman. In our study, several populations from different provinces with long distances were examined and significant differences were only observed between populations which belonged to different provinces. In our analysis, low genetic differentiation was also observed among Kerman populations (Population 4 and 5), which was in accordance with Lashkari et al. (2020) results.

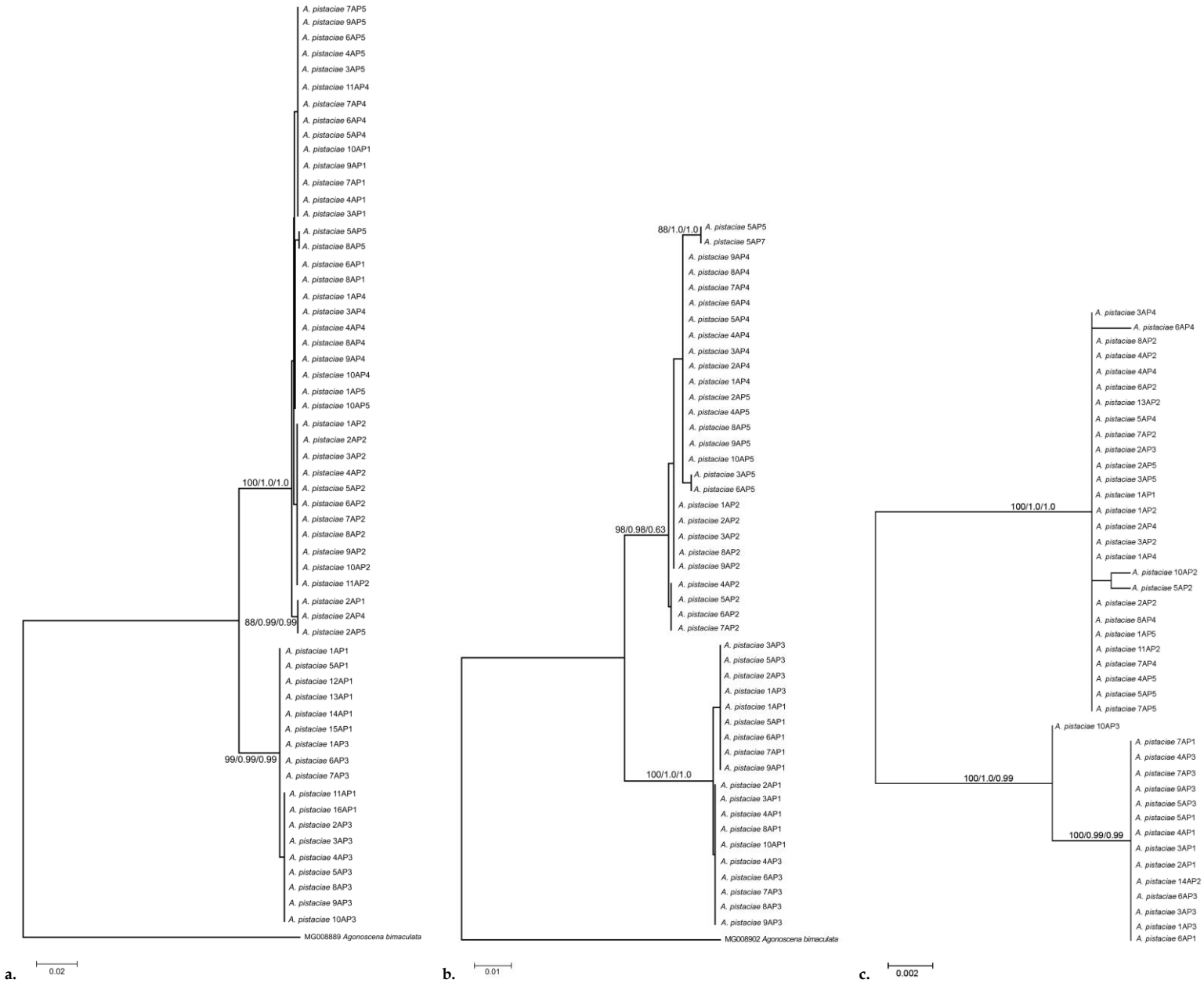

Figure 2. Neighbor-joining trees constructed based on mitochondrial COI (a), Cyt-*b* (b) and (5.8s/ITS2) (c) sequences for 58, 46 and 42 sequences of *A. pistaciae*, respectively, Bootstrap values for neighbor joining and posterior probability of Bayesian analysis and maximum likelihood are shown above or near the branches. The characters following the species name represent the individual number from a given population (e.g. 8AP2 is for individual 8 of population 2).

Table 4. Population Fst values based on 5 populations of *A. pistaciae* in Iran. The values in bold indicate statistical significance at p<0.05.

Marker		Pop1	Pop2	Pop3	Pop4	Pop5
COI	Pop1	0.00000				
	Pop2	**0.43114**	0.00000			
	Pop3	**0.39567**	**0.99065**	0.00000		
	Pop4	**0.39640**	**0.71769**	**0.97204**	0.00000	
	Pop5	**0.36655**	**0.66815**	**0.96287**	-0.04308	0.00000
Cyt-*b*	Pop1	0.00000				
	Pop2	**0.96514**	0.00000			
	Pop3	-0.11111	**0.96513**	0.00000		
	Pop4	**0.98272**	**0.80792**	**0.98361**	0.00000	
	Pop5	**0.95167**	**0.55061**	**0.95059**	0.22773	0.00000
5.8S/ITS2	Pop1	0.00000				
	Pop2	**0.68648**	0.00000			
	Pop3	-0.12592	**0.68465**	0.00000		
	Pop4	**0.82273**	-0.03718	**0.80207**	0.00000	
	Pop5	**0.82013**	-0.03789	**0.79668**	-0.04025	0.00000

Table 5. Results of the AMOVA analysis of 5 populations of *A. pistaciae* from Iran, grouped by geographic regions, P<0.05.

Marker	Source of variation	d.f.	SSD	Variance components	Percentage of variation	F-statistic
COI	Among groups	2	167.137	3.51430	49.82	0.49816
	Among populations within groups	2	37.734	1.47485	20.91	0.41659*
	Within populations	53	109.468	2.06544	29.28	0.70722*
	Total	57	314.340	7.05459		
Cyt-*b*	Among groups	2	179.882	6.10612	95.47	0.95475
	Among populations within groups	2	0.681	0.00622	0.10	0.02149
	Within populations	41	11.611	0.28320	4.43	0.95572*
	Total	45	192.174	6.39553		
5.8S/ITS2	Among groups	2	145.967	5.26082	74.33	0.74334
	Among populations within groups	2	0.547	-0.24240	-3.43	-0.13345
	within populations	37	76.177	2.05883	29.09	0.70909*
	Total	41	257.214	7.91537		

Based on the 3% interspecific genetic divergent threshold use for many psyllids species (Martoni et al. 2018), high genetic diversity observed in this study most likely leads to significant differences in behavioral and physiological characteristics and might indicate the presence of cryptic diversity in this species, which needs to be further studied in the future. Both COI and Cyt-*b* gene and nuclear DNA (5.8s/ITS2) sequences revealed two divergent lineages according to the MJ networks and phylogenetic analysis. These lineages are associated to geographic origins in some extents. All specimens from population 3 and many from population 1 (northeastern region) belong to lineage 2. It seems probable that the initial divergence occurred in allopatry and physical barriers prevented gene flow between these putative cryptic species. In other words, mating isolation and, consequently, prevention of gene flow could occur if adults from different lineages do not meet during the mating season due to various reasons including locality differences. As there is not any information about ecology and possibility of mating among these lineages, a salient next step could be assessing these issues.

Numerous species of insects are composed of evolutionarily distinct groups which are genetically desperately differentiated, despite being morphologically indistinguishable (cryptic species). Cryptic species existence could give rise to serious challenges in assessing biodiversity and, if remained unidentified, incorrect inferences in biological and conservational studies may result (Leys et al. 2016). In our study, two genetically high differentiated lineages (A and B) without any obvious morphological differences characters occurred in various regions of Iran i.e. northeast (lineage A) and center+southeast (lineage B). Despite assigning these lineages to the same morphospecies regarding taxonomic characteristics, the results of current study evidently demonstrated that they are genetically distinct. According to our data, it is quite likely that common and highly-studied species are composed of reproductively isolated groups with distinct evolutionary histories and unlike evolutionary potential (Leys et al. 2016). Taken together, studying hidden diversity, even in seemingly highly-studied species, is prerequisite for evaluation of biological diversity and implementing any conservational procedures.

Nearly all populations exhibited few mitochondrial haplotypes, of which several were shared across the populations. From the 18 examined haplotypes based on combined genes (COI and Cyt-*b*), 15 were unique and did not share the same ancestral haplotype. For 5.8S/ ITS2, 9 out of 16 haplotypes were unique. High numbers of unique haplotypes and lack of ancestral haplotype could be considered as indicators for endemism, especially for native species with a low dispersal capacity (Zheng et al. 2013). Both of these features existed

among *A. pistaciae* haplotypes demonstrating that this species is native to Iran. This is in accordance with literature that regarded this species as an indigenous pest in Iran. Although the origin of *A. pistaciae* remains uncertain, it is considered native to the Middle Eastern region (Lashkari et al. 2020). Within this context, the high genetic diversity reported in this study supports the assumption that Iran is part of the original range of *A. pistaciae*.

Based on the results of two mitochondrial genes (combined) and also 5.8S/ITS2, including AMOVA analysis, haplotype network and phylogenetic analysis, we conclude that *A. pistaciae* populations were geographically structured in three regions: northeastern, central and southern Iran. The significant genetic differentiations between populations suggests a gene flow limitation. The geographical barriers like mountains and habitat fragmentation might have acted as substantial barriers to gene flow. Such natural barriers that delineate the sampled populations may limit the gene flow in *A. pistaciae*, as has been described in many other species (Meeyen et al. 2014, Kunprom et al. 2015). Populations from northeastern Iran (Feizabad - pop1 and Neishabour - pop3), and southeastern Iran (Rafsanjan and Damghan) have some shared haplotypes, likely due to the relatively proximity of these populations compared to other locations. The lack of shared haplotypes between geographically isolated populations suggests the major role of the physical barriers. As *A. pistaciae* is a monophagous species that utilizes 6 plant species (Ouvard 2019) and, considering its habitat range (most of its host plants are distributed in northeastern, southeastern and central areas of Iran), physical barriers appear to be likely explanations for the genetic structure detected in pistachio psyllid populations in Iran. Moreover, the discrete geographical presence of this species hosts might be another probable reason of low gene flow and the geographic isolation of this species populations. The limited ability for active dispersal might be another factor that prevents gene flow. The flight capacity of *A. pistaciae* could also contribute to geographically distinct populations, although considering that, with the exception of some crucial vector psyllid species transmitting important plant diseases (Čermák & Lauterer 2008, Grafton-Cardwell et al. 2013), little is known about the psyllid dispersal ability (Burckhardt et al. 2014), therefore, the flight behavior of this species should be further studied. The IBD relationship in the present study supports this hypothesis. Moreover, variations in life history of pistachio psyllids in these regions, due to various climatic conditions, might be another reason contributing to the significant genetic differentiation.

In conclusion, high level of genetic differentiation due to the existence of two divergent lineages in *A. pistaciae* in Iran necessitates further studies on this species. Future population genetic research on *A. pistaciae* in Iran should cover a larger area and a greater number of individuals to further detail population genetic structure, especially fine-scale population structure. Additional research needs to be done to detail the geographic origins of *A. pistaciae* within Iran and describe its spread throughout the Middle East.

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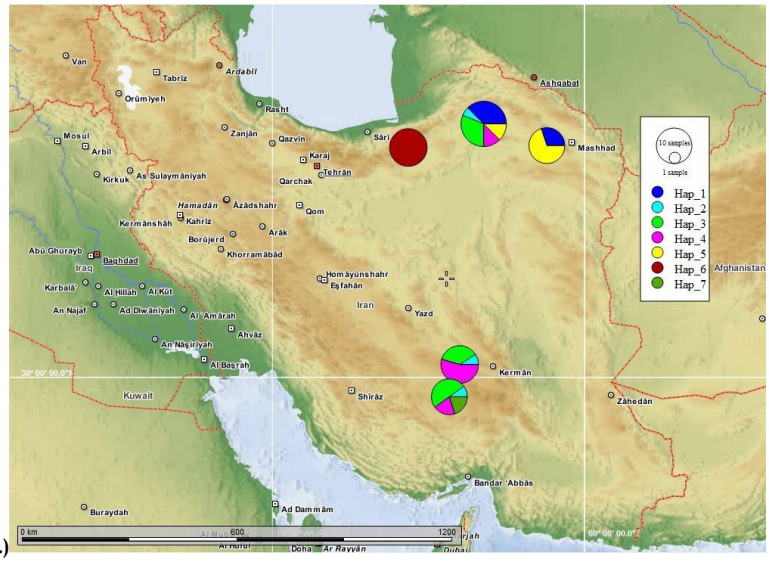
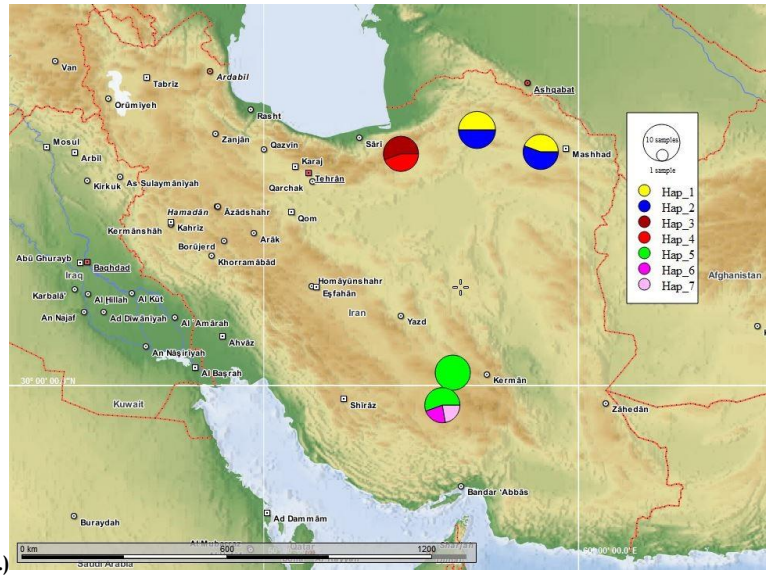
+ Appendix 1 and 2 – 2 printed pages

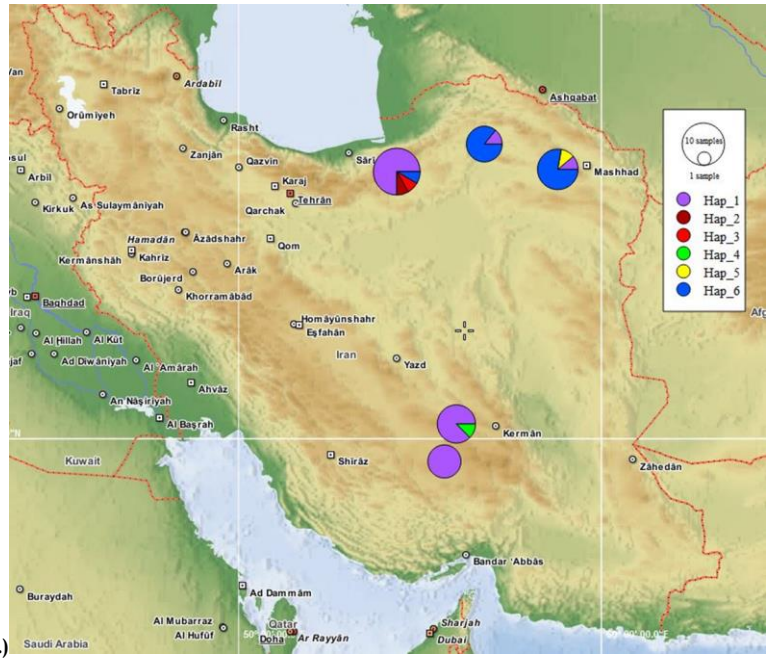
Appendix 1. Mapping haplotypes: a) COI, b) Cytb and c) ITS2.



a.)

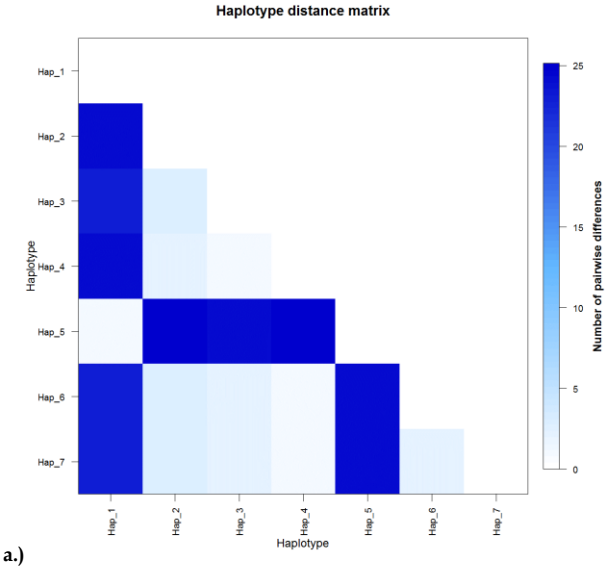


b.)

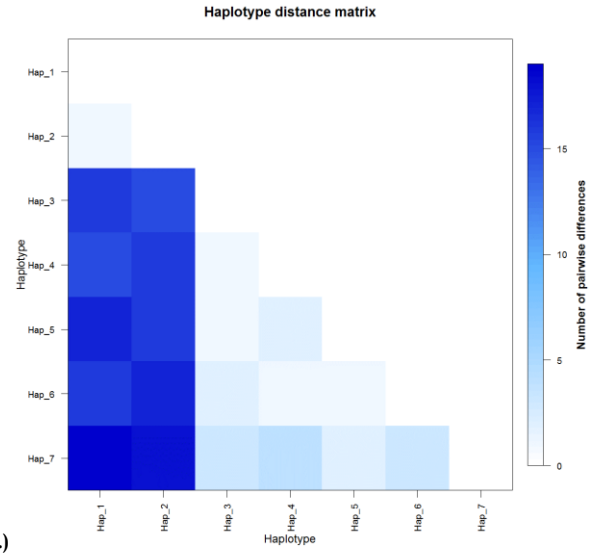


c.)

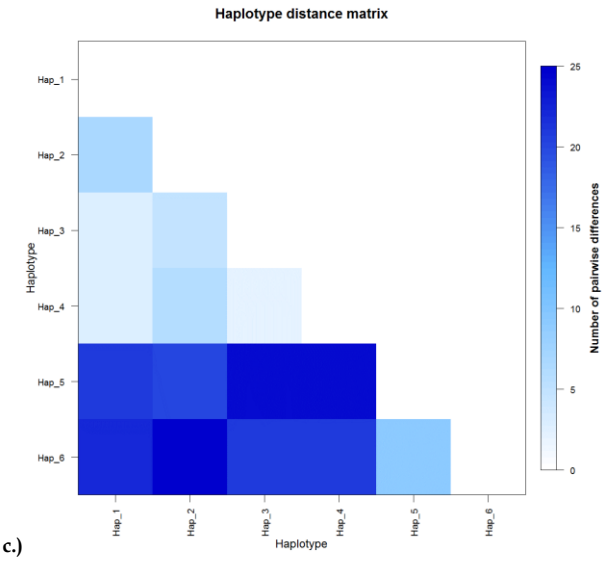
Appendix 2. Results of AMOVA for COI, Cytb and ITS_2 of *Agonoscena pistaciae*. a, b, c: Haplotype distance matrix; d, e and f: Nei`s within and between population distances.



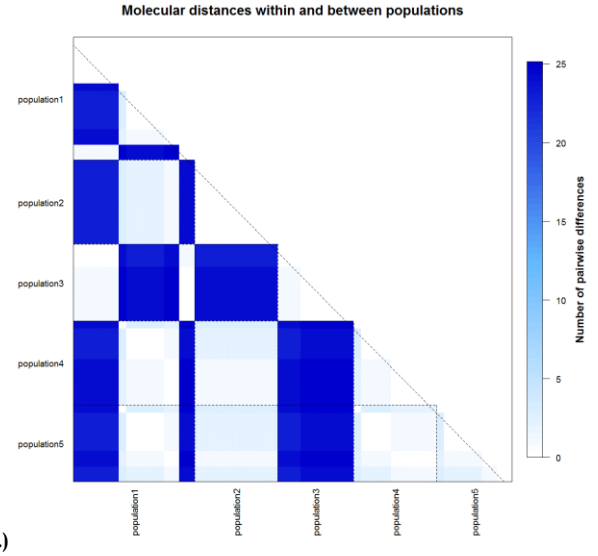
a.)



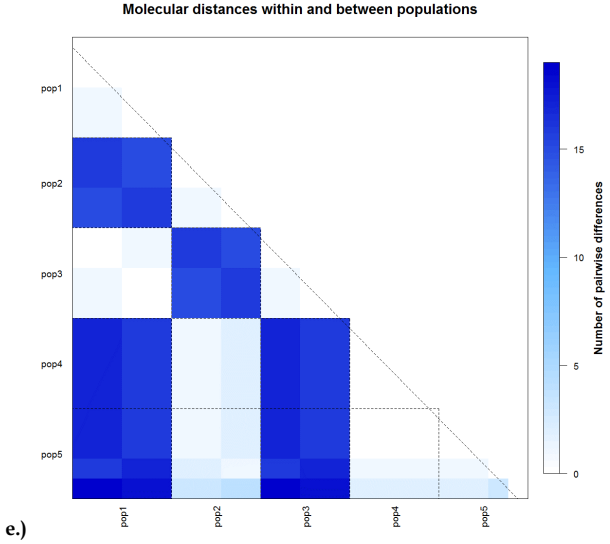
b.)



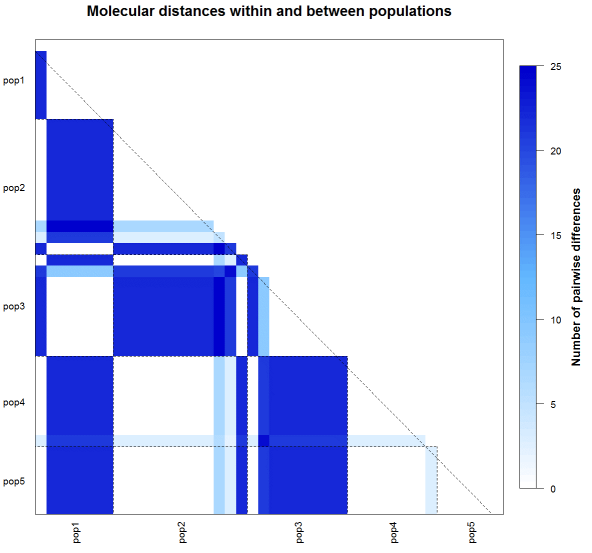
c.)



d.)



e.)



f.)