



# Molecular and morphometric identification of pistachio psyllids with niche modeling of *Agonoscena pistaciae* (Hemiptera: Aphalaridae)

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## Research Paper

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### Abstract

Species of *Agonoscena* (Hemiptera: Aphalaridae) are key pests of pistachio in all of the most important pistachio producing countries in the Old World. The efficiency and accuracy of DNA barcoding for the identification of *Agonoscena* species were tested using mitochondrial cytochrome c oxidase subunit 1 (*mtCOI*) and cytochrome b (*cytb*) gene sequences. Moreover, morphometric sexual dimorphism was studied. Finally, the potential geographical distribution of *Agonoscena pistaciae*, the most important pistachio pest, was calculated using the MaxEnt model. Similar relationships of clustering were found in the morphometric analysis and the molecular analyses with *mtCOI* and *cytb* genes, with *A. bimaculata* and *A. pistaciae* being closely related, and *A. pegani* constituting their sister group. Although the results showed that the *cytb* gene is a better marker for barcoding in this group, the *mtCOI* gene clearly separates the three psyllid species making *mtCOI* suitable for diagnostic purposes. A geometric morphometric analysis showed that the distance between landmark number 7 (bifurcation of vein M) to the fore margin of the forewing, and the distance between landmarks number 6 (apex of vein Cu<sub>1b</sub>) and 11 (wing base), are the most important geometric characters for diagnosing the studied species. Moreover, the forewing shape of males vs females is similar in *A. pistaciae* and *A. bimaculata* but differs significantly in *A. pegani*. In the ecological niche modeling of the distribution of *A. pistaciae*, the most important contribution was made by the variable ‘minimum temperature of coldest period’. The most suitable areas for *A. pistaciae* are restricted to Eastern, Southern and some parts of Central Iran.

## Introduction

Jumping plant-lice (Hemiptera: Psylloidea) comprise some economically important pests in agriculture, forestry and horticulture. The damage to the crop is inflicted directly by removal of plant sap or indirectly by acting as a vector of plant diseases (Burckhardt and Ouvrard, 2012). Psyllids are pests on pistachio (*Pistacia vera* L.), e.g. *Agonoscena pistaciae* Burckhardt and Lauterer, 1989, native to the Middle East. In Iran, this is the most serious pistachio pest today (Mehrnejad, 2000, 2003). It is also a major pest in neighboring countries of Iran, such as Armenia, Iraq, Turkey, and Turkmenistan, and in the Mediterranean as e.g. in Greece and Syria (Burckhardt and Lauterer, 1989, 1993; Mart *et al.*, 1995; Bolu, 2002; Souliotis *et al.*, 2002).

*Agonoscena* Enderlein, 1914 comprises 14 extant and one fossil described species in the Palaearctic, Oriental, and Afrotropical regions (Burckhardt and Lauterer, 1989; Malenovsky *et al.*, 2012). One species, *Agonoscena succincta* (Heeger, 1856), has been introduced into the New World on *Ruta graveolens* L. (Rutaceae). The following three species are known from Iran: *Agonoscena bimaculata* Mathur, 1973 developing on *Pistacia atlantica* Desf. and *P. khinjuk* Stocks (Anacardiaceae), *A. pegani* Loginova, 1960 on *Peganum harmala* L. (Nitrariaceae), and *A. pistaciae* on *Pistacia atlantica*, *P. palaestina* Boiss., *P. terebinthus* L. and *P. vera* (Burckhardt and Lauterer, 1989, 1993). Within the subfamily Rhinocolinae, *Agonoscena* is diagnosed by the presence of a subapical rhinarium on each of antennal segments 4–9, the presence of a dark forewing pattern consisting of spots or transverse bands often forming a zig-zag pattern along the apical wing margin, parameres bearing a posterior lobe and the ventral valvulae of the female ovipositor being ventrally serrate (Burckhardt and Lauterer, 1989; Burckhardt and Basset, 2000). The species can be recognized by the forewing pattern and size, the structure of the male and female terminalia and the morphology of the last instar (Burckhardt and Lauterer, 1989). The morphological differences between species are often small and subtle making species identification difficult for non-specialists. It is not surprising that in the past, before the revisions of Hodkinson and Hollis (1981) and Burckhardt and Lauterer (1989), species have often been confused: e.g. old records of

**Table 1.** Number of specimens used for molecular and morphometric analyses (No.), geographic information of samples (locality name, geographical coordinates and altitude in m above sea level) and host name for three *Agonoscena* species, *A. pistaciae*, *A. pegani*, and *A. bimaculata*

Species	No. for molecular study	No. for morphometric study	Locality	N	E	Altitude	Host
<i>A. bimaculata</i>	5		Bardsir, Sang Bur	29°59'51"	56°44'17"	2383	<i>Pistacia khinjuk</i>
<i>A. bimaculata</i>		30	Sirjan	29°02'25"	55°22'05"	1748	<i>Pistacia khinjuk</i>
<i>A. pistaciae</i>	4		Bardsir	29°53'55"	56°40'28"	2076	<i>Pistacia vera</i>
<i>A. pistaciae</i>		30	Sirjan	29°23'26"	55°41'29"	1718	<i>Pistacia vera</i>
<i>A. pegani</i>	4		Bardsir	29°32'35"	56°40'53"	2577	<i>Peganum harmala</i>
<i>A. pegani</i>		30	Sirjan	29°23'09"	55°47'32"	1737	<i>Peganum harmala</i>



**Figure 1.** Collection sites of three *Agonoscena* species: *A. pistaciae*, *A. pegani*, and *A. bimaculata*. See table 1 for details.

*A. succincta* from Armenia, Tadjikistan, and Turkey, and those of *A. targionii* from Iran concern, in fact, *A. pistaciae* (Burckhardt and Lauterer, 1989).

Deoxyribonucleic acid (DNA) barcoding is often an effective method for species identification (Hebert et al., 2003a). Usually, the 5'-end of the mitochondrial cytochrome c oxidase subunit I gene, *mtCO1*, is used as a standard in DNA barcoding (Hebert et al., 2003b), though it does not work properly in some taxa (Park et al., 2011). The mtDNA has been widely used for barcoding psyllids (Percy et al., 2016; Taylor et al., 2016; Percy, 2017; Martoni et al., 2018). As the barcoding areas of mtDNA, *mtCO1*, and cytochrome b (*cytb*) are short (typically 472 bp for *mtCO1* and 385 bp for *cytb*), they are easy to amplify and sequence successfully even when the DNA is fairly degraded.

Morphometrics is another useful technique for separating morphologically similar taxa (Burckhardt and Basset, 2000; Serbina et al., 2015). In geometric morphometrics, prior to the analysis of shape, the non-shape variation must be removed from the analysis and then graphical illustrations of the shape can be generated. Although most studies have focused on separating populations

within a species, some have analyzed morphological similarities and differences between species (Gómez et al., 2014; Mitrovski-Bogdanović et al., 2014; Shamsi Gushki et al., 2018).

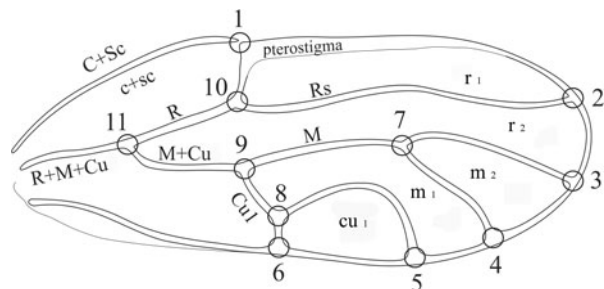
Modeling of the potential geographical distribution of species based on environmental factors of sites using predictive models is an important tool in conservation biology (Corsi et al., 1999), biogeography (Peterson and Holt, 2003; Solhjoui-Fard et al., 2013; Erfanfar et al., 2014), epidemiology (Peterson and Shaw, 2003) as well as management of pests and diseases (Roura-Pascual et al., 2009; Lashkari et al., 2013; Queiroz et al., 2013; Shabani et al., 2013; Kumar et al., 2014; Bosso et al., 2016). Phillips et al. (2006) introduced the maximum entropy method (Maxent) as a predictive model for predicting the geographic distribution of species, suitable for presence-only data sets.

As *A. pistaciae* is the economically most important pistachio pest in Iran, in addition to rapid and easy identification of specimens to control the species, biogeographical considerations are required for designing management strategies.

The aim of our study is (1) to use *mtCO1* and *cytb* as DNA barcodes to provide an estimation of mitochondrial genetic

**Table 2.** The primers used in PCR amplifications of *mtCO1* and *cytb* genes

Gene	Primer name	Primer sequence (5'-3')	Product size (bp)
<i>mtCO1</i>	C1-J-1718	GGAGGATTTGGAATTGATTAGTTCC	472
	C1-N-2191	CCCGGTAAAATTAATAAATAAAGTTC	
<i>cytb</i>	Cytb-F	TGAGGNCAAATATCHTTYTGA	385
	Cytb-R	GCAAATARRAARTATCATTCDG	

**Figure 2.** Position of landmarks (circles) on the right forewing of *A. pistaciae*. Positions of landmarks follow Lashkari *et al.* (2013).

distances between the Iranian *Agonoscena* species; (2) to use geometric morphometrics to find geometric characters diagnostic for each species and to detect sexual dimorphism in the three studied species; (3) to estimate the present and future potential geographical distributions of *A. pistaciae* in Iran; and (4) to determine the environmental factors affecting the potential distribution of this pest in Iran.

## Materials and methods

### Collection of specimens

For the molecular study, psyllids were collected in two regions of the Kerman province, the major pistachio producing area in Iran and the world, during 2016 (table 1). Adults were collected with net and aspirator, transferred to 95% ethanol and stored at  $-20^{\circ}\text{C}$ . GPS coordinates were recorded (table 1), and collection sites were mapped (fig. 1) using <http://www.simplemappr.net>. Four or five specimens were randomly chosen for each species for the molecular study (table 1).

For the morphometric study, adults of three *Agonoscena* species were collected in Sirjan (Kerman province, Iran) in 2015 (table 1). Thirty males and 30 females from each species were randomly selected, which is more than the number of variables in W matrix (Zelditch *et al.*, 2004).

Species identification using characters of the male and female terminalia was confirmed by the second author.

### DNA extraction and amplification

Genomic DNA was extracted from the material preserved in ethanol (from single specimens) using a DNA Extraction Kit DNP™ (SinaClon, Iran) according to the manufacturer's protocol.

Two mitochondrial gene regions, *mtCO1* and *cytb*, were amplified as DNA barcodes (Percy *et al.*, 2016). The polymerase chain reaction for *mtCO1* and *cytb* genes follows those described in

Percy *et al.* (2016) under the following conditions: denaturation step of 3 min at  $94^{\circ}\text{C}$ , followed by 40 cycles of denaturation for 30 s at  $92^{\circ}\text{C}$ , annealing for 40 s at  $50^{\circ}\text{C}$  (*mtCO1* gene) or  $56^{\circ}\text{C}$  (for *cytb* gene), and extension for 1 min at  $72^{\circ}\text{C}$ , followed by a final extension step of 10 min at  $72^{\circ}\text{C}$ . The primers were obtained to amplify *mtCO1* and *cytb* genes in Simon *et al.* (1994) and Percy *et al.* (2016), respectively (table 2). The primers used in polymerase chain reaction (PCR) amplifications were synthesized by Macrogen Inc. (Seoul, South Korea). Positive and negative controls were included in each run. All PCR amplifications of all genes were carried out using a TAdvanced 96 SG thermocycler (Biometra GmbH, Germany).

### Purification and sequencing

All amplified fragments were electrophoresed through a 1.2% agarose gel in TAE buffer (pH 8.0) at 80 V for 1 h. The DNA was then stained with ethidium bromide and visualized under UV light. The PCR products were purified and sequenced by Macrogen Inc. (Seoul, South Korea). Sequences of *mtCO1* and *cytb* are deposited in the GenBank database under accession numbers MG008889–MG008901 and MG008902–MG008907, respectively.

### Analysis of molecular data

The sequences of *mtCO1* and *cytb* genes of the studied species were edited manually to remove primers using software BioEdit v.7.2.6. All nucleotide sequences were aligned using ClustalW in software MEGA X (Kumar *et al.*, 2018). All sequences were compared with sequences on GenBank using BLASTn (<http://www.ncbi.nlm.nih.gov/blast/>). Genetic distances were calculated using the maximum likelihood algorithm in software MEGA X (Kumar *et al.*, 2018). Using both genes, the species tree was calculated with software package MrBayes v3.2.2. Jmodeltest version 2.1.4 (Darriba *et al.*, 2012) was used to determine the best fitting substitution model of evolution for each data set; Bayesian information criterion was used to select models (Schwarz, 1978). Data were analyzed using Bayesian inference based on a Markov chain Monte Carlo approach in the software package MrBayes v3.2.2 (Ronquist *et al.*, 2012). Analyses was run for 10 million generations with sampling every 1000 generations and the first 25% of each analysis were discarded as burn-in. The run convergence was monitored by finding the plateau in the likelihood scores (standard deviation of split frequencies  $<0.0015$ ) and the potential scale reduction factor approaching one.

Sequences of *Psyllopsis fraxini* (Linnaeus, 1758) (Hemiptera: Liviidae) (accession number: KU517187.1) and *Pariaconus ohiaicola* (Crawford, 1918) (Hemiptera: Trioziidae) (accession number: KY294469.1) were chosen as the out-groups for the analysis of *mtCO1* and *cytb* genes, respectively.

**Table 3.** Collection sites of *A. pistaciae* in Iran with geographic coordinates and data source (reference) used in ecological niche modeling.

Province	Region	N	E	Reference
Kerman	Zangi abad	30°24'42"	56°55'25	Karimi Darabi et al. (2014)
Kerman	Haft Bagh	30°12'18"	57°06'56	Karimi Darabi et al. (2014)
Kerman	Baft-Dashtab	28°58'37"	56°35'47	Karimi Darabi et al. (2014)
Kerman	Jiroft	28°39'31"	57°9'15	Karimi Darabi et al. (2014)
Kerman	Rafsanjan	30°23'42"	55°56'51	Alizadeh et al. (2011)
Kerman	Anar	30°45'30"	55°20'32	Alizadeh et al. (2011)
Kerman	Bam	29°20'13"	57°56'36	Alizadeh et al. (2011)
Kerman	Kerman	30°9'31"	56°47'32	Alizadeh et al. (2011)
Kerman	Shahr Babak	30°3'14"	55°7'51	Alizadeh et al. (2011)
Kerman	Harat	30°3'45"	54°26'29	Alizadeh et al. (2011)
Kerman	Pariz	29°51'42"	55°44'54	Alizadeh et al. (2011)
Kerman	Sirjan	29°22'47"	55°40'46	Alizadeh et al. (2011)
Kerman	Paghaleh	30°18'24"	55°25'44	Alizadeh et al. (2011)
Kerman	Bam	36°58'	57°59'	Burckhardt and Lauterer (1993)
Khorasan-e-Razavi	Mahvelat	34°40'	58°25'	Fazeli Salmani et al. (2012)
Khorasan-e-Razavi	Feyz Abad	34°54'	58°50'	Nadi (2014)
Khorasan-e-jonubi	Birjand	32°53'	59°13'	Nadi (2014)
Esfahan	Kashan	33°58'	51°26'	Nadi (2014)
Semnan	Damghan	36°10'	54°20'	Nadi (2014)
Fars	Neyriz	29°12'	54°20'	Nadi (2014)
Qazvin	Qazvin	36°15'	50°1'	Nadi (2014)
Markazi	Saveh	35°1'	50°19'	Nadi (2014)
Yazd	Meybod	32°14'	54°1'	Nadi (2014)
Guilan	Jirandeh	36°40'	49°43'	Lashkari (2013)
Khuzestan	Gazin	49°23'	31°33'	Burckhardt and Lauterer (1993)
Sanandaj	–	47°0'	35°19'	Burckhardt and Lauterer (1993)

### Analysis of morphometric data

The right forewing was used for the geometric morphometric analysis. Microscopic slides were prepared and the photos were captured at 40 times magnification with a digital camera mounted on a microscope. In the analysis, a total of 11 homologous landmarks (Type 1) were digitized on the forewing photos by the tpsDig2 programme (fig. 2) (Rohlf, 2016a). Then, the landmarks were aligned and analyzed using the tpsRelw programme (Rohlf, 2010). After this, the shape variables, partial warp scores or PWs, were extracted and used to compare shapes among the forewings of the three species. Centroid sizes, as a size measure of forewings, were calculated by the tpsRelw programme (Rohlf, 2010) and used to compare the wing size between the three species. One-way MANOVAs and ANOVA (with Tukey pairwise comparisons) procedures were designed in the SAS statistical programme to detect any significant differences in the wing shape and wing size among the three species. A regression of shape on size variables was designed to detect any allometric growth using the tpsReg ver.1.45 programme (Rohlf, 2016b). Relationships among the three studied species were shown by the UPGMA (unweighted pair group method with arithmetic mean) clustering method using the NTSYSpc ver.2.10e programme (Rohlf, 2000).

### Ecological niche modeling

Distribution data of *A. pistaciae* in Iran were taken from the literature and from material collected by the authors. The 26 localities represent different climatic regions (table 3).

Ecological niche modeling using the software MaxEnt ver. 3.4.1 (Phillips et al., 2018) was applied to estimate the potential distribution of *A. pistaciae* in Iran for both current and future climatic scenarios. WorldClim with 19 variables of temperature and precipitation as well as altitude at 30' (1 km<sup>2</sup>) resolution was used (table 4) (Hijmans et al., 2005). The same variables were also used to determine the effect of global climate change on the potential distribution of *A. pistaciae* in 2050, based on Global Climate Models, CSIRO-Mk3.0 with the A1B scenarios (Gordon et al., 2002). Finally, the model was evaluated with the area under the receiver operating characteristic curve (Graham and Hijmans, 2006; Phillips et al., 2006). The area under the curve statistic (AUC) with a range from 0.5 to 1.0 was used to indicate model prediction occurrences and perfect predictions, respectively (Phillips et al., 2006). The contribution of each variable on the performance of the model was estimated (in percent) by Jackknife analysis (Peterson and Cohoon, 1999).

**Table 4.** Environmental variables used in ecological niche modeling of *A. pistaciae* in Iran

Code	Bioclimatic variables
bio1	Annual mean temperature
bio2	Mean diurnal range
bio3	Isothermality
bio4	Temperature seasonality
bio5	Max temperature of warmest period
bio6	Min temperature of coldest period
bio7	Temperature annual range
bio8	Mean temperature of wettest quarter
bio9	Mean temperature of driest quarter
bio10	Mean temperature of warmest quarter
bio11	Mean temperature of coldest quarter
bio12	Annual precipitation
bio13	Precipitation of wettest period
bio14	Precipitation of driest period
bio15	Precipitation seasonality
bio16	Precipitation of wettest quarter
bio17	Precipitation of driest quarter
bio18	Precipitation of warmest quarter
bio19	Precipitation of coldest quarter
alt	Altitude

The map with the potential distribution was prepared with ArcGIS 9.3.

## Results

### Molecular study

Both the genes studied were successfully amplified. Comparison of the sequences with those in GenBank revealed close sequence matches for all studied genes. The pairwise genetic distance based on the maximum composite likelihood showed that the mean intra-specific distance was 0.0000 within each species for all genes, while the mean inter-specific distance was greater between species, which is in agreement with the requirement for ideal DNA barcodes. In the *mtCOI* gene, the maximum inter-specific distance was 0.0077 between *A. pistaciae* and *A. pegani*, while the minimum distance was 0.0025 between *A. pistaciae* and *A. bimaculata*. In the *cytb* gene, the maximum inter-specific distance (0.7091) was between *A. pistaciae* and *A. pegani*, and the minimum distance (0.3073) was between *A. pistaciae* and *A. bimaculata*. The tree of the clustering analyses obtained from MrBayes v3.2.2 is shown in [fig. 3](#).

### Morphometric study

The superimposed landmarks on the forewing of the three *Agonoscena* species of the analyzed specimens showed considerable variation especially in landmarks 1, 2, 5, 6, and 7 ([fig. 4](#)). The plots of the partial warp score matrix are shown in [fig. 5](#).

The position of each specimen is shown along the relative warps axis. The superimposed forewings of the three *Agonoscena* species ([fig. 6](#)) showed that the forewings of *A. pistaciae* are narrower and longer than those of the other species, as well as vein Rs which is longer (landmark 1). *A. pegani* and *A. bimaculata* share a similar forewing width, but the forewing of *A. bimaculata* is longer.

In the relative warp visualization plot ([fig. 6](#)), in *A. pistaciae*, landmark 7 (junction of veins  $M_{1+2}$  and  $M_{3+4}$ ) is closer to the fore than to the hind margin of the wing; and the distance between landmark 7 to the fore margin of the wing is about half the distance between landmark 7 and the hind margin of the wing. In the other two species, *A. pegani* and *A. bimaculata*, landmark 7 is located more to the middle of the wing, i.e. the distance between landmark number 7 and the fore margin of the wing is only slightly less than of distance between landmark number 7 and the hind margin. Comparing *A. pegani* and *A. bimaculata*, in the former, the distance between landmark 11 and 6 is longer than the distance between landmark number 11 and 1, whereas this distance is similar in the latter.

MANOVA showed a significant difference in the mean wing shape of the three species ([table 5](#)). In the UPGMA analysis, *A. pistaciae* and *A. bimaculata* clustered together and were placed distinctly from *P. pegani* ([fig. 7](#)).

Wing size comparisons between the three studied species showed significant differences ([table 5](#)). Pairwise comparisons between the three species (using HSD post-hoc test,  $\alpha = 0.01$ ) showed that *A. pistaciae* had the largest and *A. bimaculata* had the smallest wings, whereas *A. pegani* was intermediate ([fig. 8](#)). In all species, females have larger wings than males ([fig. 8](#)).

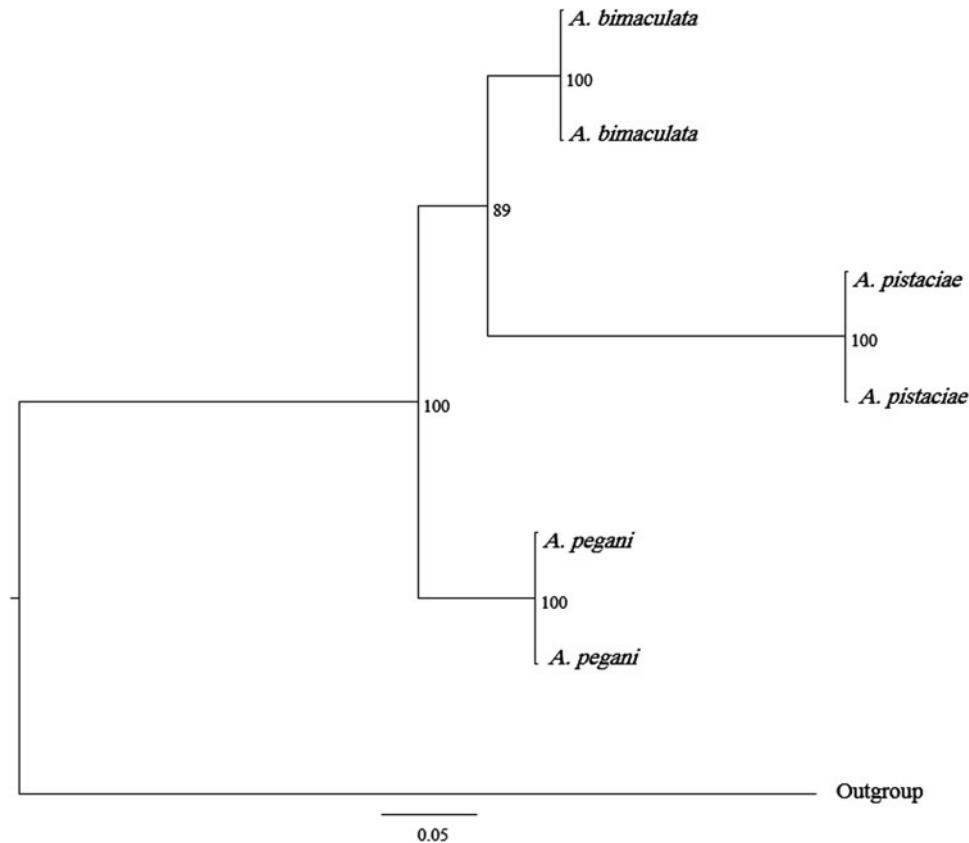
The results showed isometric growth among the studied species ([table 5](#)). Therefore, the variation in the wing shape among the three species is not related to the size.

### Key to species

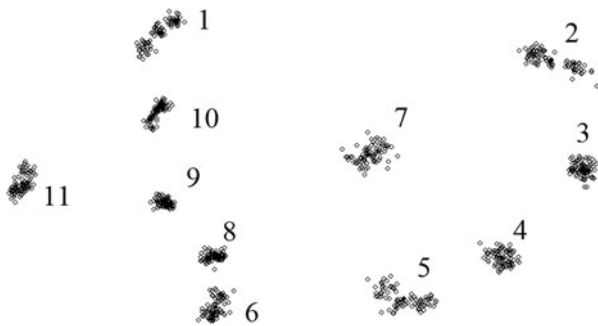
- Distance between landmark 7 (bifurcation of vein M) to fore margin about half as long as distance between landmark 7 to hind margin of forewing ([fig. 6](#)) *A. pistaciae*
  - Distance between landmark number 7 (bifurcation of vein M) to fore margin slightly shorter than distance between landmark 7 to hind margin of forewing ([fig. 6](#)) 2
- Distance between landmarks 11 (wing base) and 6 (apex of vein  $Cu_{1b}$ ) longer than distance between landmarks 11 and 1 (apex of vein  $R_1$ ) of forewing ([fig. 6](#)) *A. pegani*
  - Distance between landmarks 11 (wing base) and 6 (apex of vein  $Cu_{1b}$ ) similar to distance between landmarks 11 and 1 (apex of vein  $R_1$ ) of forewing ([fig. 6](#)) *A. bimaculata*

### Sexual dimorphism

The forewing shapes in male and in female are similar in *A. pistaciae* and *A. bimaculata* but differ significantly in *A. pegani* ([table 5](#)). The forewings in females of *A. pegani* are longer than those in the males. The most significant variations in the wing shape are related to the veins  $R_1$  (landmark 1), Rs (landmark 2) and, to a lesser extent,  $M_{1+2}$  (landmark 3), which are longer in females ([fig. 9](#)). The forewing size differs significantly between sexes in all studied species ([table 5](#)). A regression showed no allometric growth between females and males of *A. pegani* ([table 5](#), [fig. 9](#)). Therefore, the shape



**Figure 3.** Bayesian gene tree of *Agonoscena* species based on *mtCO1* and *cytb* genes sequences. Bayesian posterior probabilities (in %) are shown next to the node points. The scale bar represents the number of substitution per sites.



**Figure 4.** Superimposed landmarks on forewing of the three *Agonoscena* species: *A. bimaculata*, *A. pegani*, and *A. pistaciae*.

differences observed between the male and the female of *A. pegani* cannot be attributed to the size.

### Ecological niche modeling

The potential distribution of *A. pistaciae* in Iran in the present predicted by MaxEnt is shown in [fig. 10](#). According to this model, Eastern, Southern, and some parts of Central Iran provide the most suitable habitats for *A. pistaciae*, whereas Dasht-e-Kavir, Dasht-e-Lut, the coastal areas of the Caspian Sea, Oman Sea and Persian Gulf, the Alborz and Zagros Mountains and neighbouring regions are considered unsuitable. In other areas the potential occurrence is moderately likely ([fig. 10](#)).

Based on the global climate change models (including CSIRO-Mk3.0 emission scenarios) for the potential distribution of *A. pistaciae* in 2050, the greatest difference appears in the central region, with the disappearance of a red area (high potential). The model also predicts a shift of the most suitable habitats from the Southeast and Centre westwards to the slopes of the Zagros Mountains ([fig. 11](#)).

The most important variables influencing the species distribution are 'min temperature of coldest period (Bio6)', 'mean temperature of coldest quarter (Bio 11)' and 'mean temperature of wettest quarter (Bio 8)', contributing 21.97, 21.15, and 9.76%, respectively, to the model output.

The response curves for 'min temperature of coldest period' showed that areas with min temperature of about  $-2^{\circ}\text{C}$  during the coldest period, mean temperature of about  $8-9^{\circ}\text{C}$  during the coldest period, and mean temperature of about  $6.5^{\circ}\text{C}$  during the wettest quarter, had the highest predicted suitability. The AUC value was 0.898 that indicated a high level of accuracy for the model predictions.

### Discussion

In both the molecular and morphometric studies, *A. pistaciae* and *A. bimaculata* cluster together supporting the morphology based phylogeny by Burckhardt and Lauterer (1989).

The 5' region of the *mtCO1* gene serves as a good standard for barcodes in many taxa such as some insects (Hebert *et al.*, 2004b; Hajibabaei *et al.*, 2006), birds (Hebert *et al.*, 2004a), and fishes

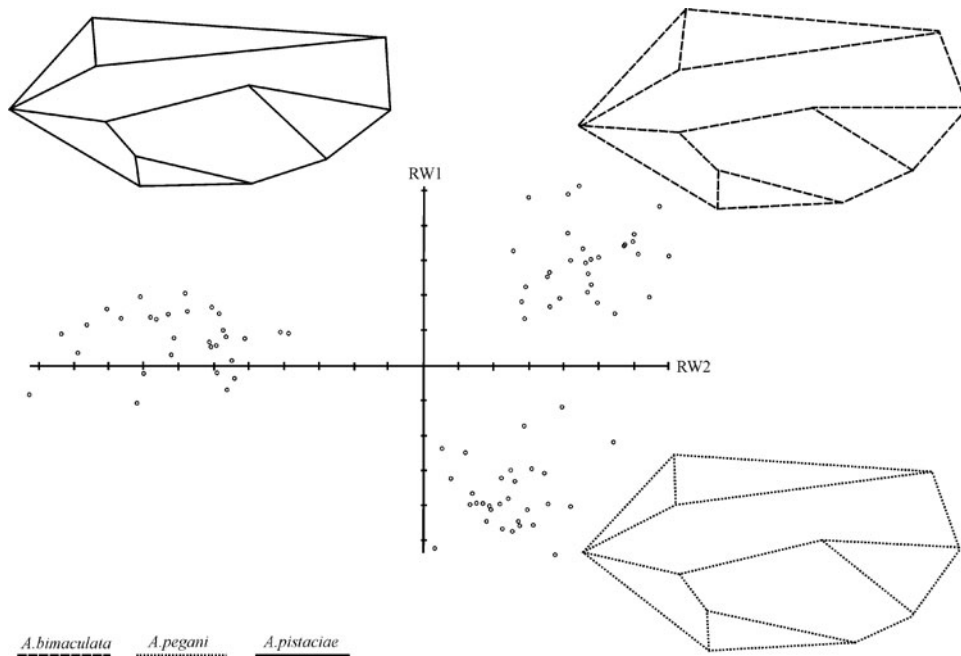


Fig. 5. Plots of the partial warp score matrix for the three *Agonoscena* species: *A. bimaculata*, *A. pegani*, and *A. pistaciae*.

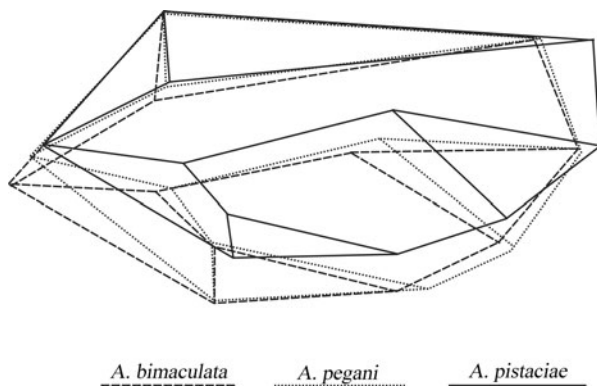


Fig. 6. Superimposed forewings of the three *Agonoscena* species: *A. pegani*, *A. bimaculata*, and *A. pistaciae*.

and amphibians (Ivanova *et al.*, 2007). In mammals, however, the *cytb* gene provides better results for separating species (Tobe *et al.*, 2010). A suitable genetic marker for species identification must be (1) a conserved region, (2) short enough to be sequenced in a single reaction, and (3) contain enough variability to be informative for identification (Nicolas *et al.*, 2012). The primers used in this study, which are effective for the studied species, were also used to sequence other psyllid taxa (Percy *et al.*, 2016). Both studied genes, *cytb* and *mtCO1*, could be easily identified due to the presence of the above mentioned characters. Regarding the length of the sequences in this study (472 bp for *mtCO1* and 385 bp for *cytb*), both genes could be sequenced in a single reaction. In our analyses, the *cytb* gene had a higher inter-specific variability. In the *mtCO1* gene tree, *A. pistaciae* is paraphyletic with respect to *A. bimaculata* but in the *cytb* gene tree all three species are monophyletic. This means that *cytb* is suitable for diagnosing *A. pistaciae*. Although the *mtCO1* appears to be less divergent, it still shows a very high support at the nodes and a different tree

topology; so it can also separate the three psyllid species. Most studies on barcoding insect use *mtCO1* as the DNA barcode (Hebert *et al.*, 2004b; Hajibabaei *et al.*, 2006; Ashfaq *et al.*, 2014a, 2014b, 2015). Other genes such as *cytb* have not been considered in these studies.

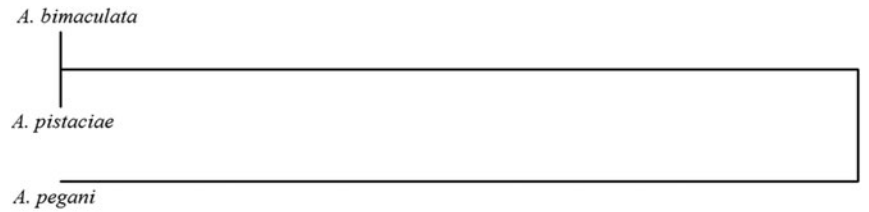
The forewing shape in males and females is similar in *A. pistaciae* and *A. bimaculata*, but differs in *A. pegani*, where the wings are longer in females. Allometric tests showed that this difference is not related to size. Sexual dimorphism in the wing shape is also known from other psyllids, such as two species associated with ash, viz. *Psyllopsis machinosus* Loginova and *P. repens* Loginova (Shamsi Gushki *et al.*, 2018). The more elongate forewings in the females of *A. pegani* may have better flight properties helping an efficient dispersal in search of new food sources. While *A. pistaciae* and *A. bimaculata* live on the perennial *P. vera* and *P. khinjuk*, *Peganum harmala*, the host of *A. pegani*, grows in Kerman only from early May to late July. The females of *A. pegani* have to migrate to other regions where the host is available. Bai *et al.* (2016) showed that populations of grasshoppers with longer wings display a better flight performance. Similarly, the more elongated wings in butterflies have a positive impact on longer spatial movements (Betts and Wootton, 1988). More biological studies on *A. pegani* are needed to test this hypothesis.

In their study of three *Psyllopsis* species using geometric morphometrics, Shamsi Gushki *et al.* (2018) showed that the best characters to separate the three species are found in the apical half of the forewing, related to the veins Rs, M, M<sub>1+2</sub> and M<sub>3+4</sub>. Similar results were obtained in the present study, where the differences concern mainly vein Rs as well as the distances between landmark 7 to fore and hind margin, and between landmarks 11 to 6 and 1. Geometric morphometrics has also been used to discriminate species in other insect orders, including Hemiptera (Aghagoli *et al.*, 2013), Hymenoptera (Mitrovski-Bogdanović *et al.*, 2014), and Coleoptera (Zúñiga-Reinoso and Benitez, 2015; Eldred *et al.*, 2016).

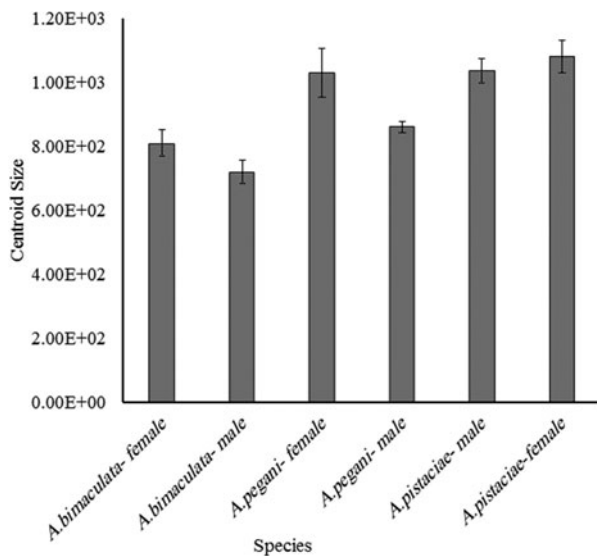
**Table 5.** The forewing shape and size with allometric growth test between sexes and species

Species	Shape		Size		Allometry	
	Wilks' lambda	Prob.	F	Prob.	Wilks' lambda	Prob.
<i>A. bimaculata</i>	0.4975	0.0610	47.59	<0.0001**	–	–
<i>A. pistaciae</i>	0.7336	0.6602	5.48	0.0226*	–	–
<i>A. pegani</i>	0.2413	<0.0001**	114.94	<0.0001**	0.6284	0.0525
Between species	0.0013	<0.0001**	690.35	<0.0001**	0.9532	0.9962

\*\*Significant at  $\alpha = 0.01$ , \*Significant at  $\alpha = 0.05$ .

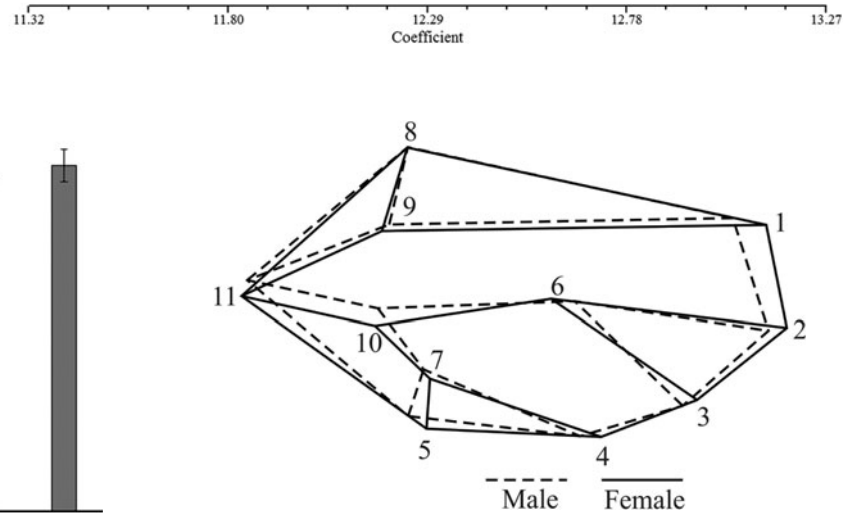


**Fig. 7.** Phenogram plotted by the UPGMA method based on the generalized distance matrix in the three *Agonosceca* species: *A. pegani*, *A. bimaculata*, and *A. pistaciae*.



**Fig. 8.** Centroid size comparison of three *Agonosceca* species: *A. pegani*, *A. bimaculata*, and *A. pistaciae*.

Characterizing environmental conditions is the most common strategy for determining the potential geographic distribution of a species. According to our model, within Iran, some area in the East and South provide the most suitable climate for *A. pistaciae* (fig. 10). These areas are associated with arid climate, cold winters and warm summers. The results show that the temperature, especially cold periods of the year, is the most important factors for the occurrence of the pest. The potential presence in the coastal area of the Caspian Sea is low, due to high humidity and low



**Fig. 9.** Superimposed forewings of male and female of *A. pegani*.

winter temperatures, going down to  $-7$  to  $13^{\circ}\text{C}$  in some years. Our results are consistent with Mehrnejad's study (2003) listing temperature and moisture as two important factors in the biology of pistachio psyllids. He showed that they start to develop at  $10^{\circ}\text{C}$  and that they tolerate high temperatures ( $35^{\circ}\text{C}$ ) reflected in a sharp increase of the population. High-relative humidity has a negative influence on the development of pistachio psyllids. Relative humidity above 65% can reduce the growth rate of this pest (Mehrnejad, 2000). A similar potential distribution in the south of Iran has the Asian citrus psyllid, *Diaphorina citri* Kuwayama, in the Maxent model (Lashkari *et al.*, 2013).

Other limiting factors may affect the distribution of this pest in the newly predicted areas. The species probably does not colonize areas where (1) the host plants are in low density or (2) the preferred host plants are absent. Three *Pistacia* species, *P. vera*,



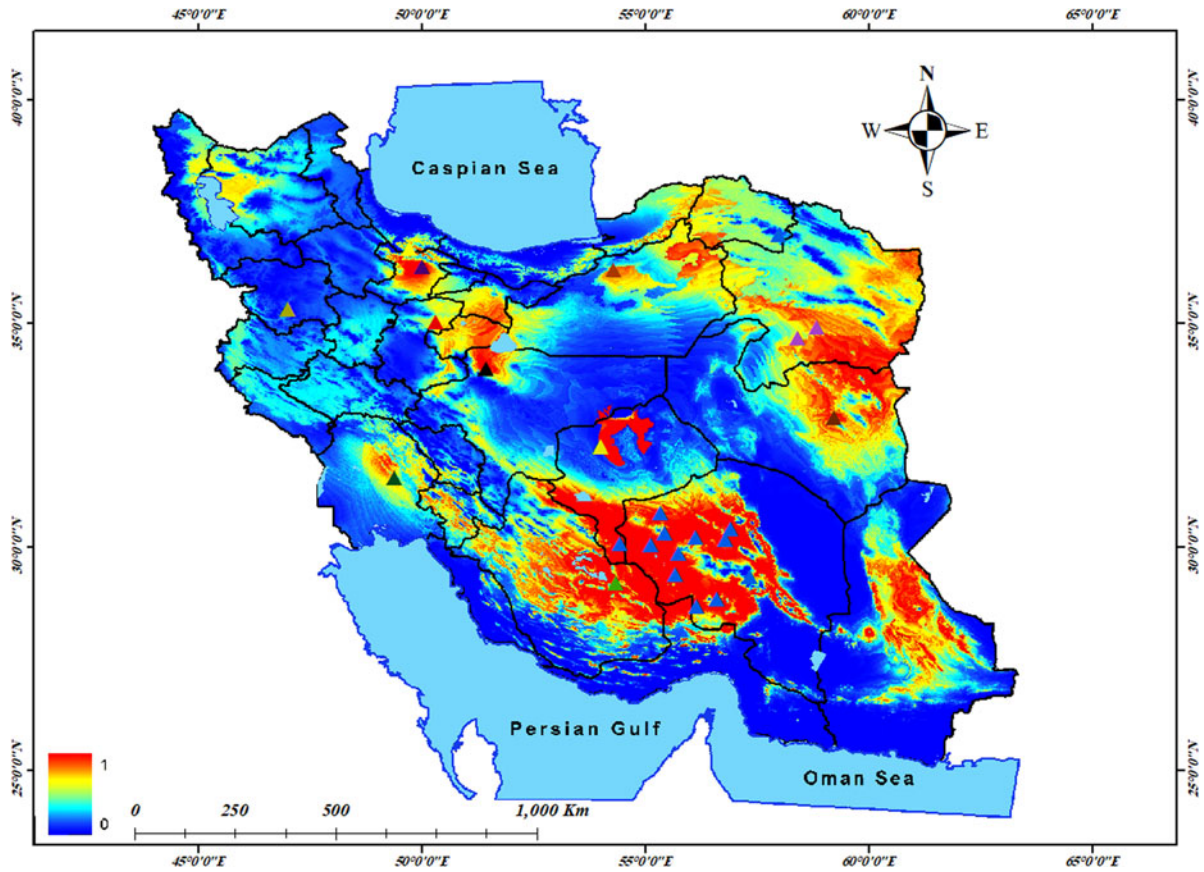


Fig. 10. Predicted potential distribution of *A. pistaciae* for the present. Probabilities 1 = high, 0 = low.

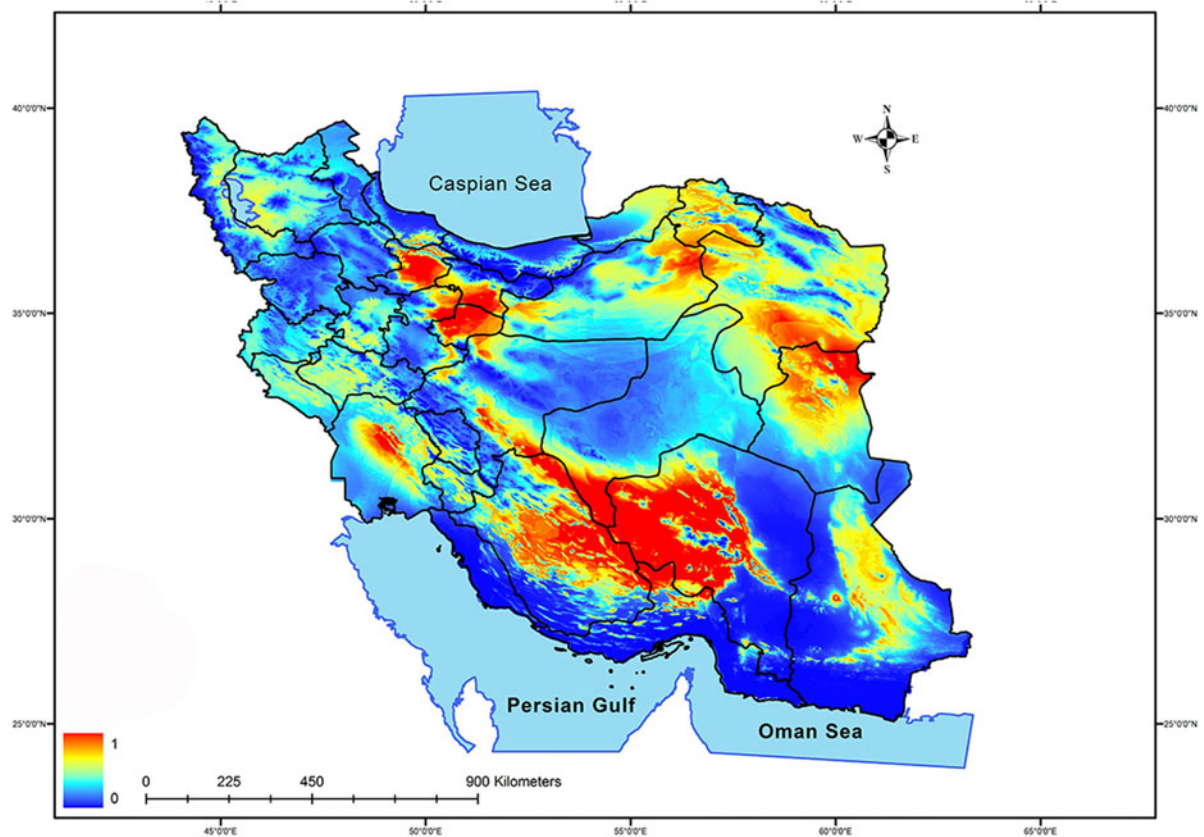


Fig. 11. Predicted potential distribution of *A. pistaciae* for the future. Probabilities 1 = high, 0 = low.

*P. mutica*, and *P. khinjuk*, are present in Iran (Khatamsaz, 1989) and long and warm summers are suitable for them (Esmail-pour, 1998). The main growing areas of *P. vera* are located in the east and south-east. Interconnected growing areas have suffered a large increase in the pest populations, furthering dispersal of the pest. In the newly predicted areas, especially in the west, *P. mutica* and *P. khinjuk* are widely distributed. Although, the pest sometimes occurs on *P. mutica* in the Kerman province (e.g. Meymand), it does not seem to build up large populations, due to the predominance of the less-preferred host. The monitoring of the population density of *A. pistaciae* is, therefore, necessary in the newly predicted areas.

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