Article

Genetic diversity of six isolated populations of the leopard moth, *Zeuzera pyrina* (Lep: Zeuzeridae)

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Received 22 September 2016; *Accepted 12 October 2016*; *Published online 1 March 2017*

Abstract

The leopard moth, *Zeuzera pyrina* (Lep: Zeuzeridae), is an important pest of a wide range of trees and shrubs including walnut and apple across the world. The natural populations of the leopard moth in different geographical areas of Iran show significant differences in some of their biological characteristics such as time of emergence, generation time and host specificity. So, we hypothesized that these populations may represent different subspecies that move toward a speciation event in their evolutionary route. In this study, we evaluated the genetic diversity of six different geographically isolated populations of the leopard moth using the sequence alignment of cytochrome oxidase c subunit one (COI). A fragment of 642 base pairs was amplified in all six populations and the phylogenetic tree was created based on sequenced fragments. Our results revealed significant differences in the nucleotide sequence of COI gene in these populations. Differences in climatic conditions of these regions seem to be the most powerful force driving this diversity among the studied populations.

Keywords COI; genetic diversity; Iran; leopard moth; *Zeuzera pyrina*.

Arthropods
ISSN 2224-4255
URL: http://www.iaees.org/publications/journals/arthropods/online-version.asp
RSS: http://www.iaees.org/publications/journals/arthropods/rss.xml
E-mail: arthropods@iaees.org
Editor-in-Chief: WenJun Zhang
Publisher: International Academy of Ecology and Environmental Sciences

1 Introduction

A major force in the course of speciation has been proposed to be geographical isolation of populations such that evolution within the isolated populations would lead to enough differences between them and speciation would be an eventual outcome (Mayr, 1963; Templeton, 1981). These differences may occur by the interplay between natural selection and random chance leading to reproductive isolation of the separated populations (Lemey et al., 2009). Given such high frequencies of ongoing speciation in all taxa, it seems critical to look for modern technological assistances for studying speciation and descripting newly derived and closely related species (Blaxter, 2003; Hebert et al., 2003). Recent studies on different groups of animals have suggested the feasibility of creating identification systems reliant on the analysis of sequence diversity in small segments of

DNA (Tautz et al., 2003). Among the DNA sequences that have been already used as molecular markers in biological studies, the predominant classes are mitochondrial DNA (mtDNA) and microsatellite nuclear DNA. Although, the later ones have been proved to be useful for the study of the interaction between the dynamics of population and its genetic structure, mtDNA is most used in animal population evolution and phylogenetic studies (Lunt et al., 1998). Hebert et al. (2003) proposed that a DNA barcoding system for animal taxa can be relied on the sequence diversity in cytochrome c oxidase subunit one (COI). The mitochondrial gene encoding COI possesses some unique characteristics, which make it a suitable molecular marker in evolutionary studies (Lunt et al., 1998). These characteristics include ease of isolation, high copy numbers per cell, inheritance from the maternal line, lack of recombination, conservation of sequence and structure across all aerobic organisms studied so far, and a range of mutation rates in different regions of the molecule (Moritz et al., 1987; Rokas et al., 2003; San Mauro et al., 2006; Galtier et al., 2009). Involved in the mitochondrial respiratory chain, COI is a relatively well-studied gene at the biochemical level and a large body of information related to different aspects of this sequence is present in the literatures. Additionally, COI is one of the largest protein-coding genes in mitochondrial genome that enable the researchers to amplify many more nucleotides with the same functional complex compared to any other mitochondrial gene (Lunt et al., 1998). Studying the COI gene enables researchers to resolve relationships between closely related taxa and populations as well as to construct higher levels of phylogeny because the synonymous third codon positions evolve fast, whereas the overall protein sequence evolves relatively slow (Tautz et al., 2003). There are now increasing numbers of studies relying on the sequence diversity of COI gene for differentiation of animal taxa at different taxonomic levels especially between closely related species and below the species level (for examples in insects see Maus et al., 2001; Otranto and Stevens, 2002; Harvey et al., 2003; Hebert et al., 2003a; Ames et al., 2006; Park et al., 2009; Raupach et al., 2010; Nagoshi et al., 2011; Park et al., 2011). For example, Park et al. (2011) have demonstrated that the analysis of COI gene is helpful in identification of 344 species of true bug belonging to 178 genera. Similarly, use of COI sequence has been shown to efficiently differentiate two morphologically undistinguishable strains of the armyworm, *Spodoptera frugiperda* in Florida (Levy et al., 2002).

In this study, the genetic diversity of the leopard moth, *Zeuzera pyrina* L. (Lep: Zeuzeridae) was investigated by amplification and alignment of COI sequence of specimens collected from different geographical regions of Iran. The leopard moth is an important polyphagous pest of a wide range of trees and shrubs, including apple, pear, quince, cherry, plum, grapevine, and walnut across Europe and Asia. The larvae of leopard moth feed inside the stems and branches of young trees causing die-backing of shoots, whitening of leaves and ultimately killing of the whole tree (Alford, 2007). Since the leopard moth is adapted to live on a wide range of host plants and in different geographical areas, we hypothesized that different populations of this species should represent a significant variation in their mitochondrial DNA sequence. So, analysis of COI sequence may provide a reliable tool for identification of various populations that live in different geographical regions or different host plants.

2 Materials and Methods

2.1 Study area

During 2010-2011, three sites in Alburz province (located at center of Iran, longitude from 53º26´ to 59º29´E and latitude from 25º55´ to 32º N) including Kamal shahr, Shahriar, and research garden of the college of Agriculture and Natural Resources located at Karaj (hereafter called Karaj) and three sites in Kerman province (located at center of Iran, longitude from 53º26´ to 59º29´E and latitude from 25º55´ to 32º N) including Baft, Rabor, and Dare Morid) were selected for evaluation of genetic diversity among populations of the leopard moth. The precise locations of the studied sites are shown in Fig. 1. The selected sites in Alburz and Kerman

province were among the most important regions for production of walnut in the country. The average annual precipitations of Kerman and Alburz provinces are 350-400 and more than 400 mm, respectively. The minimum and maximum monthly temperatures of Kerman province are 7 and 39, while those of Alburz province are 1ºC (January) and 30ºC (August).

Fig. 1 Geographical map of Iran and six studied area located at Alburz (1, Kamal Shahr, 2, Shahriar, 3, Karaj) and Kerman (4, Rabor, 5, Baft, 6, Dare Morid) provinces.

2.2 Insect collection

The larvae of leopard moth were collected by gathering the infected branches and twigs during February to June and dissecting them in laboratory. The collected larvae were placed in ethanol 96% and frozen at refrigerator with a temperature of -20ºC.

2.3 DNA extraction

DNA was extracted based on the method of Suzuki (1972). After twice washing of the samples by distilled water, individual larvae were homogenized in 1 ml micro-tubes containing 400 µl of the Lysis buffer [100 mM Tris-HCl (pH=8), 100 mM EDTA, 1% SDS (pH=8)]. Then, 55.5 µl proteinase K was added to the homogenate and the resultant mixture was incubated at 56 ºC for 90 min. After that, 250 µl NaCl (4.5 M) and 400 µl chloroform-isoamylalchol were added to the complex. The suspension was centrifuged at 13000 rpm for 5 min at 25 ºC and the supernatant was retained. The supernatant was re-suspended in an ethanol solution (50%) and centrifuged again like the previous conditions. The pellet was washed by methanol (70%) and the methanol was then evaporated by placing the tube in a desiccator for two min. The sample containing DNA was stored at -20 ºC until used.

2.4 PCR amplification and sequencing

Twenty-five µl of PCR buffer, 2.5 µl of each R- and F-primers, 2 µl *Taq*DNA polymerase, 3 µl DNA template, and 15 µl distilled water were mixed in PCR tubes. The PCR buffer was prepared by mixing 100 µl of 10 × Gold buffer, 20 µl dNTP mix, 30 µl $MgCl_2$ (50mM), 5 µl BSA (10 mg/ml), and 345 µl distilled water. The primers used in this study were 5'-CAC-ATA-TTA-TTA-CTC-AGG-3' and 5'-GAG-GGA-AAA-ATG-TTA-AGT-T-3'. The PCR temperature profile for the mitochondrial COI fragment consisted of an initial denaturation at 94ºC for 3 min followed by 35 cycles at 94 ºC (denaturation, 60 s), 54 ºC (annealing, 60 s), and 72 ºC (elongation, 60 s) and a final extension at 72 ºC for 5 min. The amplified DNA was incubated at 4ºC. The volume of 5.5 µl of PCR amplified product was detected by use of horizontal electrophoresis on 1%

agarose gel. Forty µl of the purified DNA fragment was sent to Bioneer Laboratory (South Korea) for sequencing. The reverse and forward sequences were manually edited by use of Bio Edite software and compared to other similar sequences present in BLAST information center for accuracy of amplification and sequencing. The consensus sequences were aligned using ClustalX software with the gap open penalty and gap extension penalty defaults. The ambiguous fragments were removed from the sequences. After UPGMA analysis of the resultant sequences in PAUP*4.8b with the distance default, the phylogenetic tree was drawn for the studied populations.

3 Results and Discussion

The results of nucleotide sequencing for the six studied populations of *Z. pyrina* have been summarized as below. The nucleotide data including reverse and forward sequences were aligned in BLAST system present in Gene Bank (NCBI). The resultant phylogenetic tree drawn using PAUP*4.8 software is presented in Fig. 2. As the figure shows, the six studied populations were divided into two separate clades with the Darremorid population constitute one branch and the five other populations one separate branch.

Shahriar (Accession KC595670)

```
      1 tttgtgtcac agctgaattt tgaaaccccg gatctttaat tggagatgat caaatctata
     61 atactattgt aacagctcat gctttcatta taatttttt catagtaata ccaatcataa
    121 ttggaggatt tggaaattga ctagtacctt taatacttgg agctcctgat atagcttttc
    181 cacgaataaa taatataaga ttttgactac tacccccatc attaacttta ttaatttcaa
    241 gaagaatcgt agaaaatgga gctggaactg gatgaactgt atacccccct ttatcatcta
    301 atattgccca tagaggaagt tctgttgacc tagcaatttt ctctcttcac ttagctggta
    361 tttcttctat tttaggagca attaatttta ttactacaat tattaatata cgtcctaata
    421 atataatatt tgatcaaata cccttatttg tctgagctgt aggtattaca gcactacttt
    481 tacttctctc cctccctgtt ttagctggag ctattactat acttttaaca gatcgaaatc
    541 taaatacatc tttttttgac cctgctggag ggggagaccc tattttatat caacatttat
    601 tctgattttt t
```

Kamal Shahr (Accession KC595668)

```
      1 ggtatactag gaacctctct aagattactt attcgagcag aattaggaaa ccccggatct
     61 ttaattggag atgatcaaat ctataatact attgtaacag ctcatgcttt cattataatt
    121 tttttcatag taataccaat cataattgga ggatttggaa attgactagt acctttaata
    181 cttggagctc ctgatatagc ttttccacga ataaataata taagattttg actactaccc
    241 ccatcattaa ctttattaat ttcaagaaga atcgtagaaa atggagctgg aactggatga
    301 actgtatacc ccccttttatc atctaatatt gcccatagag gaagttctgt tgacctagca
    361 attttctctc ttcacttagc tggtatttct tctattttag gagcaattaa ttttattact
    421 acaattatta atatacgtcc taataatata atatttgatc aaataccctt atttgtctga
    481 gctgtaggta ttacagcact acttttactt ctctccctcc ctgttttagc tggagctatt
    541 actatacttt taacagatcg aaatctaaat acatcttttt ttgaccctgc tggaggggga
    601 gaccctattt tatatcaaca tttattctga tttttt
```

Karaj (Bagh Daneshkadeh)(Accession KC595665)

```
      1 gtatactagg aacctctcta agattactta ttcgagcaga attaggaaac cccggatctt
     61 taattggaga tgatcaaatc tataatacta ttgtaacagc tcatgctttc attataattt
    121 ttttcatagt aataccaatc ataattggag gatttggaaa ttgactagta cctttaatac
    181 ttggagctcc tgatatagct tttccacgaa taaataatat aagattttga ctactacccc
    241 catcattaac tttattaatt tcaagaagaa tcgtagaaaa tggagctgga actggatgaa
    301 ctgtataccc ccctttatca tctaatattg cccatagagg aagttctgtt gacctagcaa
    361 ttttctctct tcacttagct ggtatttctt ctattttagg agcaattaat tttattacta
    421 caattattaa tatacgtcct aataatataa tatttgatca aataccctta tttgtctgag
    481 ctgtaggtat tacagcacta cttttacttc tctccctccc tgttttagct ggagctatta
    541 ctatactttt aacagatcga aatctaaata catcttttt tgaccctgct ggagggggag
    601 accctatttt atatcaacat ttattctgat ttttt
```

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Rabor (Accession KC595669)
    1 ggtatactag gaacctctct aagattactt attcgagcag aattaggaaa ccccggatct
   61 ttaattggag atgatcaaat ctataatact attgtaacag ctcatgcttt cattataatt
  121 tttttcatag taataccaat cataattgga ggatttggaa attgactagt acctttaata
  181 cttggagctc ctgatatagc ttttccacga ataaataata taagattttg actactaccc
  241 ccatcattaa ctttattaat ttcaagaaga atcgtagaaa atggagctgg aactggatga
  301 actgtatacc ccccttttatc atctaatatt gcccatagag gaagttctgt tgacctagca
  361 attttctctc ttcacttagc tggtatttct tctattttag gagcaattaa ttttattact
  421 acaattatta atatacgtcc taataatata atatttgatc aaatacccct atttgtctga
  481 gctgtaggta ttacagcact acttttactt ctctccctcc ctgttttagc tggagctatt
  541 actatacttt taacagatcg aaatctaaat acatcttttt ttgaccctgc tggaggggga
  601 gaccctattt tatatcaaca tttattctga tttttt

Baft (Accession KC595666)
    1 atctggtata ctaggaacct ctctaagatt acttattcga gcagaattag gaaaccccgg
   61 atctttaatt ggagatgatc aaatctataa tactattgta acagctcatg ctttcattat
  121 aatttttttc atagtaatac caatcataat tggaggattt ggaaattgac tagtaccttt
  181 aatacttgga gctcctgata tagcttttcc acgaataaat aatataagat tttgactact
  241 acccccatca ttaactttat taatttcaag aagaatcgta gaaaatggag ctggaactgg
  301 atgaactgta taccccccct tatcatctaa tattgcccat agaggaagtt ctgttgacct
  361 agcaattttc tctcttcact tagctggtat ttcttctatt ttaggagcaa ttaattttat
  421 tactacaatt attaatatac gtcctaataa tataatattt gatcaaatac ccttatttgt
  481 ctgagctgta ggtattacag cactactttt acttctctcc ctccctgttt tagctggagc
  541 tattactata cttttaacag atcgaaatct aaatacatct ttttttgacc ctgctggagg
  601 gggagaccct attttatatc aacatttatt ctgatttttt

Dare Morid (Accession KC595667)
    1 cagaattagg aaacccccgga tctttaattg gagatgatca aatctataat actattgtaa
   61 cagctcatgc tttcattata atttttttca tagtaatacc aatcataatt ggaggatttg
  121 gaaattgact agtaccttta atacttggag ctcctgatat agcttttcca cgaataaata
  181 atataagatt ttgactacta cccccatcat taactttatt aatttcaaga agaatcgtag
  241 aaaatggagc tggaactgga tgaactgtat acccccctttt atcatctaat attgcccata
  301 gaggaagttc tgttgaccta gcaattttct ctcttcactt agctggtatt tcttctattt
  361 taggagcaat taattttatt actacaatta ttaatatacg tcctaataat ataatatttg
  421 atcaaatacc cttatttgtc tgagctgtag gtattacagc actactttta cttctctccc
  481 tccctgtttt agctggagct attactatac ttttaacaga tcgaaatcta aatacatctt
  541 tttttgaccc tgctggaggg ggagacccta ttttatatca acatttattc tgatttttg
  601 g
```

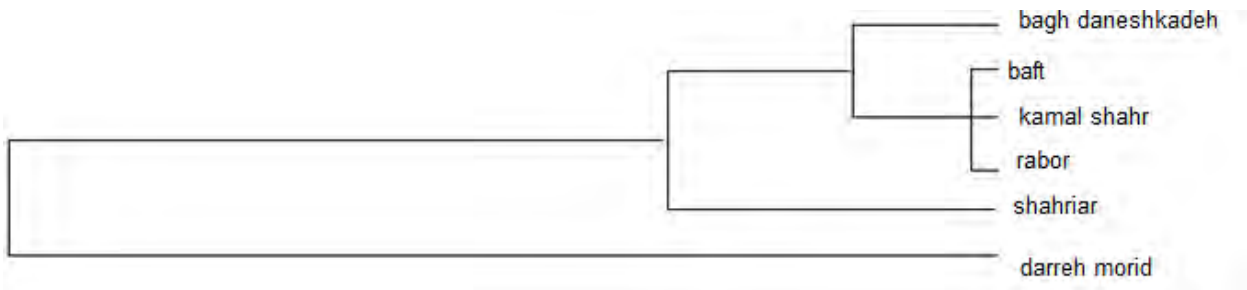

Fig. 2 The phylogenetic tree based on analysis of COI gene sequences, constructed for different populations of Iranian populations of *Zeuzera pyrina* using Maximum parsimony method in PAUP*4.8b software.

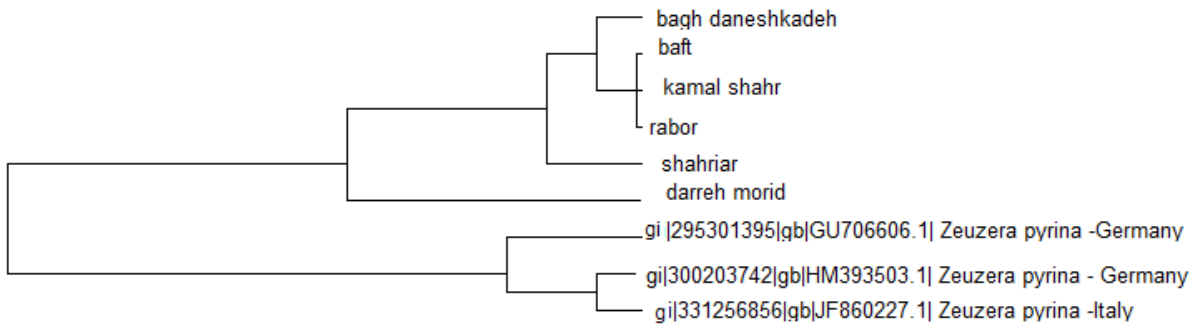

Fig. 3 The phylogenetic tree constructed for different populations of *Zeuzera pyrina* whose sequence data are present in Gene Bank.

In the former branch, two populations of Shahriar and Karaj were subsequently diverged from others whereas the three populations, Baft, Rabor, and Kamal Shahr showed the most similarities in the studied sequence and so, placed in a monophyletic group. This may be explained by the fact that the Kamal Shahr region is among the main centers for distribution of walnut seedlings across the country and the populations that are now present in Rabor and Baft regions may be developed from seedlings transferred from Kamal Shahr to these regions. Subsequently, some divergence force such as natural selection and random mutations may lead to slight variation of COI sequence of these three populations. The Darre Morid region has a significantly different climatic conditions compared to the two other regions in Kerman province. It is a relatively low-land region with tropical climatic conditions in contrast to Rabor and Baft that have a height of about 2000 m from sea level and a relatively cold weather. Indeed, it seems that the climatic conditions of Darre Morid region is more similar to those of studied regions in Alburz province rather than those of Baft and Rabor regions in Kerman province. So, it is likely that similar climatic conditions have led to relatively less differences in COI sequence of the studied populations of the leopard moth. The sequences achieved in the current study were compared to those present in the Gene Bank. We found only three sequences belonging to *Z. pyrina* (Fig. 3). The phylogenetic tree drawn by use of UPGMA method in PAUP*4.8 software revealed that the non-native populations constitute a completely separated group from Iranian populations. This may be simply justified by the geographical distances present among these populations.

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