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Genetic variation of yellow pistachio hard scale, *Lepidosaphes pistaciae* Archangelskaya (Hem.: Diaspididae) populations in Kerman province, Iran revealed by ISSR markers

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Abstract: Yellow pistachio hard scale, *Lepidosaphes pistaciae* (Hem.: Coccoidea: Diaspididae) is one of the detrimental pests to pistachio trees. This pest is distributed throughout the pistachio producing regions of Iran. It is complex species, having distinct genetic variation. As genetically diversity awareness is essential for identification and management, the diaspidid samples selected from 10 infected region and used to test hypotheses about the genetic variability between and within its populations, during 2016. Inter simple sequence repeat (ISSR) molecular marker was used to assess genetic diversity. Extracted DNA of specimens amplified with nine ISSR primers, six of primers showed the best polymorphism. After observation and scoring bands patterns, data were analyzed with NTSYS ver. 2.02 and POPGENE ver. 1.31 software. Results showed that the bands are in the range between 100 and 2000 bp. The used ISSR primers generated 63 polymorphic fragments, and the average heterozygosity for each primer was 0.266 and the maximum number of bands were recorded for primer SMR7. A dendrogram based on the Unweighted Pair Group Method with Arithmetic mean (UPGMA) method placed them in three groups also, Anar and Baft populations were the most difference among populations. The dendrogram includes the group A (comprise populations collected from Baft, Bardsir, Zarand, Sirjan and Shahrbabak), group B (including populations collected from north Kerman, south Kerman, Kabootarkhan, and Rafsanjan) and group C (including populations collected from Anar). The results showed that ISSR markers technique is able to detect the genetic diversity among the yellow pistachio hard scale populations of various commercial pistachio cultivars within the pistachio orchards, in Kerman, Iran.

Key words: Genetic diversity; ISSR marker; Polymorphism; Scale insects.

Introduction

The pistachio's nut is the major agricultural product in Iran, as the first global pistachio producer with production of 300,000 MTs. Kerman province is the main center of commercial pistachio production in Iran, (for example, *Pistacia vera* L. (Sapindalis: Anacardiaceae)). All commercial and non-commercial pistachio trees attacked by yellow pistachio hard scale, *L. pistaciae* Archangelskaya (Hem: Diaspididae), which mainly restricted to pistachio (*Pistacia* spp). This is sap sucking insect and injurious to commercial pistachio trees and usually cause significant loss on pistachio yields (1). *L. pistaciae* is a subtropical species, originating from Asia, including Iran, Iraq, Afghanistan, Pakistan, Syria, Turkey, China and Japan (2-4); Europe (former USSR) including Armenia, Georgia, Kazakhstan, , Kirgizistan, west of Asia, Tadjikistan, Transcaucasia, Turkmenistan and Uzbekistan (2, 4). It has not been recorded from Africa, the Western Hemisphere, Australia, or from the Pacific islands (4).There is more problem with scale insect resistance to pesticide and their extensive spread. Therefore, genetically diversity awareness that is essential to identification and management of this pest. However, finding and conserving biodiversity is one of the important international challenges that scientists are facing (5), today, many molecular techniques are presented into diverse research fields (6). Actually, microsatellite markers have significant role in determining genetic diversity and phylogenetic relationships of animals, like insects. Inter-simple sequence repeats (ISSRs) is a technique of molecular markers based on PCR method, which was recommended by Zietkiewicz et al. (7). Recently, ISSR markers have been indicated as reliable, cheap, easy synthesis and higher stability, and has been widely enforced in researches on genetic diversity and family ties of animals, especially insects (6, 8). In insects, the use of ISSR marker has increased in recent years. ISSR marker were used by researchers in limited family such as Noctuidae (Lepidoptera) and Bombycidae (Diptera) more frequently than other families of insects (9-11).

However, most of phylogenetic studies have focused on the barcoding of armored scale insects, for example molecular and morphological study of yellow pistachio hard scale (12); similar researches have been studied in other coccids, like lac insects by using ISSR technique (13) and mealy bugs through DNA sequence variations of nuclear genes for analyzing phylogenetic relationships (14, 15). Saha et al. (13) studied the genetic diversity of commercially important *Kerria* spp. by using ISSR technique. The global literature review showed that no record was available relating to the genetic diversity of armored scale insects like yellow pistachio hard scale, *L. pistaciae.* Pistachio is one of the most important horticultural products in Iran and other parts of the world. Regard to the fundamental role of this insect in all pistachio-producing regions and access the slight-

ly results to better management of pistachio orchards scale insect pests. The main objective of this study is findings of ISSR-based genetic diversity analysis within local population of thirty lines of yellow pistachio hard scale, which collected from various commercial pistachio cultivars within the pistachio orchards, in Kerman, Iran.

Materials and Methods

Sampling

Thirty samples were collected across 30 locations of pistachio-producing areas in Kerman province, in Iran including Anar, Baft, Bardsir, Rafsanjan, Zarand, Sirjan, Shahrbabak, Kabootarkhan, north Kerman and south Kerman during the 2016 spring (Figure 1). For this purpose, samples with the same ages (15 to 20 years) were selected and the infested leaves and branches by pest colonies were cut from trees and were put in plastic container for transferring to the entomology laboratory, Razi University. Adults female were removed from infested parts and were preserved in 99.9% alcohol at -20ºC until use. A number of specimens were mounted on microscope slides using the methodology of Hodgson and Henderson (16) and identified based on Danzig (2) morphological keys.

Genetic diversity assessment using ISSR primers

DNA extraction

Total genomic DNA was extracted from *L. pistaciae* using the method (17) of a modified phenol/chloroform method with some modifications. In brief, individual specimens stored in 2 ml microfuge tubes in a -20 ºC freezer for a night and were homogenized in 60 µl of lysis buffer (TNES: 40 mM Tric-HCl, pH 7.5; 80 mM NaCl, 80 mM EDTA, pH 8; 05% sodium dodecyl sulfate). The samples were incubated with 150 µg/mL proteinase K at 70ºC for 45 min. Next, 200 µl of chloroform/isoamyl alcohol (24/1) solution was added to each microtube and was centrifuged, 8000 rpm, 5 minutes at 20°C. Then isoamilalcohol was added and centrifuged with 11000 rpm, 10 minutes at 20°C, again to reach the genomic DNA. Extracts were solved in 20 µl deionized water and used as a template for PCR.

Figure 1. Sampling localities and geographical positions of yellow pistachio hard scale used for ISSR analysis.

Table 1. Sequence and characteristics of the used ISSR primers (13, 18).

Primer code	Core sequence	Tm (°C)*
SMR1	$(AC)_8$ WT	51
SMR2	$(GA)_8$ C	53
SMR3	$(GA)_8$ YG	54
SMR4	$(AC)_8$ YG	54
SMR5	$(AG)_8$ TA	51
SMR6	$(AG)_8$ GC	56
SMR7	$(AC)_8$ AG	51
SMR8	$(CA)_8$ A	52
SMR9	$(GT)_8$ A	50
SMR10	$(AC)_8$ C	52
SMR11	$(GAG)_3$ GC	39
SMR12	$(ATG)_6$ C	51

*Tm: annealing temperature.

Primers used

Twelve ISSR primers were selected among 73 primers with global literature review and lab test and synthesized by SinaColon (www.sinaclon.com) (Table 1). The selected primers were chosen based on the previous reports and due to number of bands scored and polymorphic bands.

PCR amplification

PCR reaction mix

Each PCR amplification reaction mixture consisted of 12.5 µl master mix (Cat. No: MM2011; Sina Colon), 1 µl DNA templates, 1.5 µl primer (10M) and 10 µl deionized water.

PCR program

Amplification was performed under the following temperature program: 4 min at 94 ºC, followed by 35 cycles of 1 min at 94 ºC, 1 min annealing (temperature depending on primers used) (Table 2), and 2 min extension at 72 ºC, ending with 10 min at 72 ºC for a final extension

Analysis of PCR products

Amplification products were electrophoresed in a 1.2% agarose gel and was carried out with a constant voltage of 80 V for 90 min. Band size was compared with a 100 bp DNA ladder (Fermentas Inc., MD, USA). Finally the gel was stained with Ethidium Bromide (0.5 g/ml) and photographed with the gel documentation system visualized

Data analysis

Band patterns were recorded with a gel documentation system and Gel-Pro Analyzer (Media Cybernetics) software was used for analysis the gel pictures. The ISSR molecular data were analyzed by the NTSYS-pc

Table 2. Inter-simple sequence repeat (ISSR) primers list, sequences and polymorphism for *L. pictaciae* populations.

Primer name	Sequence	Annealing temperature(ºC)	Number of bands scored	Number of polymorphic bands	Polymorphism (%)	Range of fragment size (bp)	PIC**
SMR1	$(AC)_8$ WT*	51	9	9	100	200-2000	0.73
SMR2	$(GA)_8$ C	53	9	9	100	100-1200	0.74
SMR3	$(GA)_8$ YG*	54	8	8	100	100-800	0.81
SMR7	$(AC)_8$ AG	56	15	15	100	200-2000	0.86
SMR8	$(CA)_8$ A	51	12	12	100	200-2000	0.79
SMR9	$(GT)_8$ A	52	13	12	92.3	300-1800	0.83
Mean	-	-	11	10.83	98.71	183-1633	0.79

*W(A, G), Y(C, T);**PIC - polymorphic information content.

(Numerical Taxonomy System) version 2.02 (Applied Biostatistics, Inc., NY, USA) computer program (19) and the POPGENE software version 1.31 (20) was used for estimating heterozygosity. Amplified products were scored from the gel images for scoring as present (1) and absent (0) same other dominant molecular markers. Thus a matrix (1, 0) was obtained. Based on these matrices, dendrograms were constructed using the Neighbor-Joining (NJ) method and the unweighted pair-group method with arithmetic averages (UPGMA) was performed on the same data sets.

Results

Genetic diversity

Three ISSR primers among total of primers showed good reproducibility and polymorphism in amplified locis represented in Table 2. These primers produced a total of 66 recognizable and clear bands, with an average of 11 fragments per primer ranging from 8 to 15 fragments per primer which 98.71% (65 bands) of that were polymorphic (Table 2).

The size of the polymorphic bands ranged from 100 bp to 2000bp. The ISSR-PCR representative banding profiles are shown in figure 2. The measured results of each locus variability showed an average of 0.266 heterozygosity. For achieving this purpose each locus was selected basis of polymorphic fragments with two allelic classes, and 0.5 was considered as the highest heterozygosity value for a locus. Accordingly, the average heterozygosity ranged from 0.21 to 0.34 with an average of 0.26 which indicated that the studied insects were approximately diverse.

The UPGMA method used for cluster analysis to show the genetic relationships of the studied insects and was offered in figure 3. The dendrogram showed highly variable genetic distances among the populations. The populations were divided into three groups and the genetic distance was the highest (0.0187) between the northern population (Anar) and southern population (Baft). The dendrogram includes the group A, comprises populations collected from Baft, Bardsir, Zarand, Sirjan and Shahrbabak, group B, including populations collected from north Kerman, south Kerman, Kabootarkhan, and Rafsanjan group C, including populations collected from Anar. The insects gathered from the same geographic regions ordinarily grouped in similar cluster or near clusters.

Group B was divided into two subgroups, clearly. The first subgroup included north Kerman and Kabootarkhan, and the second subgroup included south Kerman and Rafsanjan. Also, group A was divided into two subgroups which Zarand is separated of other and located with population from north of Rafsanjan in a subgroup.

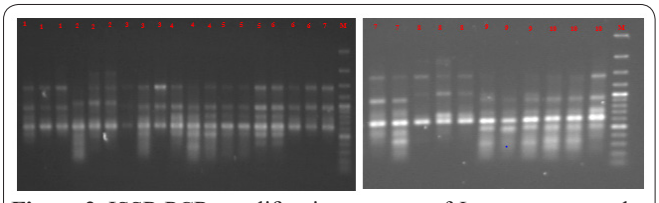

Figure 2. ISSR PCR amplification patterns of *L. pistaciae* populations using primer (AC)8WT (100 bp DNA ladder (M)). 1: Baft, 2: Zarand, 3: North Kerman, 4: South Kerman, 5: Kabootarkhan, 6: Anar, 7: Sirjan, 8: Rafsanjan, 9: Shahrbabak, 10: Bardsir.

Discussion

L. pistaciae Archangelskaya (Diaspididae) is an important pest of pistachio orchards in central part of Iran (Kerman). To the extent of our knowledge, we believe that this is the first report of employing DNA markers (ISSR) in understanding the molecular diversity present in the populations of yellow pistachio hard scale, *L. pistaciae* around the world.

The ISSR marker showed a bio-geographic relationships and genetic similarity within and among populations of *L. pistacie*. The scientists believe that ISSRs marker can show useful information at different levels of genetic differentiation (21), and these primers can use as beneficial items when time and material costs prevent the development of other markers (like SSRs) (22). The

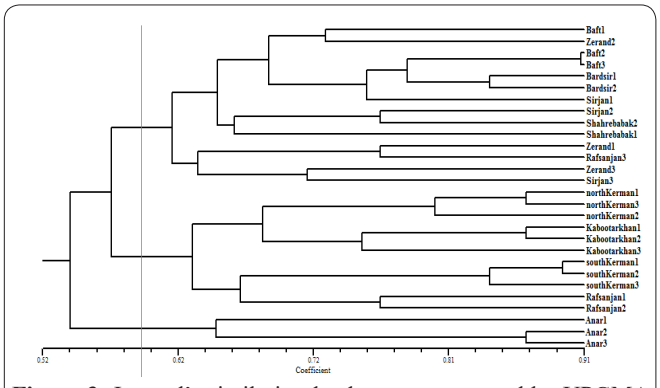

Figure 3. Jaccard's similarity dendrogram generated by UPGMA based on ISSR marker system ($(AC)_8$ WT, $(GA)_8$ C, $(GA)_8$ YG, $(AC)_8$ AG, $(CA)_8$ A and $(GT)_8$ A) of *L. pistaciae* populations which clustered into three groups.

dinucleotide ISSR primers were poly (AC) or poly (AG). It was according to other research results which are correspondent with animal groups where dinucleotide ISSR primers and poly (AC) and (AG) repeats are common repeat pattern across animal and especially insect (5, 22). As expected, dinucleotide primers with sequences (AG) and (AC) showed good performance and these primers were used in a high percentage of polymorphism. Liu et al. (22) and Saha et al. (13) reported same results. The dinucleotide ISSR primers and poly (AC) or poly (AG) were selected by Saha et al. (13) on a scale insect population (*Kerria lacca*) showed excellent polymorphism and number of scored bands. It has been showed that yellow pistachio hard scale genome sequences are A+G and A+C that are abundant and common sequences in insects such as scale insect (13), blackflies (23) ,plant hopper (22), honey bees (8) and other insects (24). Our results indicated 38 polymorphic bands with an average of 12.6 bands per primer. The results declared that most of the areas studied were polymorphic. These results are in accordance with the other researchers observation (13, 25). The fragments observed from (AC) and (AG) were in range 100-3000 for different insects. Saha et al. (13) observed fragments with range between 250-2000 (scale insect), and the fragments observed by Liu et al. (22) were in range 220-2000, and the fragments observed by Shouhani et al. (8) were in range from 150 bp to 1500 bp (honey bee). Our results are in this range (100 bp to 2000 bp) and we conclude that as we were successful to display polymorphism between yellow pistachio hard scale populations in spite of low number usage of primers with the appropriate selection, so ISSR primers should be measured as an effective and powerful marker to exhibit polymorphism in insect populations as a whole. However, there was limited number of publications about ISSR markers to differentiate of scale insect, the investigation of this microsatellite on insect group demonstrated that the ISSR marker was a good tool for discrimination and genetic structure analysis of insect populations, like scale insect (13), native to different geographical populations.

Inter-simple sequence repeat (ISSR) markers were used successfully for discrimination among and within species of *Gonatoerus* species (Hymenoptera: Mymaridae) (26); *Tospovirus* transmitting *Thrips* populations (27) and blackflies (Diptera, Simulidae) (23). Inter simple sequence repeats could make clear genetic variation between New Zealand and French specimens of *Medicago aethiopoides* (28).

The UPGMA dendrogram separated into three groups according to their origins (Figure 3). This dendrogram shows great variability in the yellow pistachio hard scale population and there was clear genetically diverse between North populations (Anar) and South populations (Baft). Interestingly, in a recent study Baft, Sirjan, Bardsir and Shahrbabak sampels (*L. pistaciae* populations) were inserted in a group and the second group include the samples of Kerman, Kabootarkhan and Rafsanjan. This genetic similarity could be expected in relation with the geographic distances between the lines under study. Also, the findings determined that Anar population are dividing from others. The clustering dendrogram showed the maximum distance between Anar and Baft populations that has the most geographical distance with quite different climate. The ability of the ISSR markers to recognize the genetic variability between and within the yellow pistachio hard scale populations is cleared evidently based on different levels of genetic distance. These results coincide Rahimi et al. (29) and Shouhani et al. (8).

The present study like Saha et al. (13) research demonstrates the potential use of ISSR markers in characterization of genetic diversity and phylogenetic relationships of scale insect. Existence the diversity between species, allow to access the slightly results in systematic, immigration and how to control the pest.

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