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Original Article

Genetic diversity of *Paecilomyces variotii* isolates by SSR marker in Kerman province, Iran

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Abstract: Die-back disease caused by *Paecilomyces variotti* is one of the most destructive diseases of pistachio in Iran. In this study, genetic diversity of *Paecilomyces variotti* isolates was investigated with SSR marker, using six primers pairs. Sampling was done from pistachio gardens in different geographical zones of Kerman province during summer 2013. Various fungal isolates were isolated from infected twigs on PDA. Based on the morphological criteria, seventy isolates were identified as *P. variotii*. These identifications were confirmed by sequencing data analysis. Twenty isolates were selected for the genetic diversity assay. A total of 22 alleles was identified with an average of two alleles per each primer pair. The mean of Nei's coefficient among isolates was 1.53. The average of Polymorphic Information Contents (PIC) for the SSR markers in *P. variotti* was 0.68. UPGMA Clustering grouped all the tested isolates into eight groups with a high genetic diversity. Based on these results, we can conclude that SSR markers are suitable for illustrating levels of genetic diversity within the isolates, but it is not adequate for demonstrating the relationship between a selected set of isolates and their related geographical regions.

Key words: molecular marker, pathogen, pistachio, sequencing

INTRODUCTION

Pistachio (*Pistachio vera* L.) is one of the most important and highly economically valued garden-products in Iran. Kerman province in the south of Iran, with a farming land of approximately 300 thousand hectares and annual production of about 200 thousand tons, is one of the most important production regions of pistachio. Die-back disease of pistachio trees is one of the destructive disease causing yield loss during the recent years in Iran and other countries (Alizadeh et al. 2000). In Iran, different fungal species belonging to the genera *Paecilomyces*, *Stemphyllium*, *Alternaria*, *Nattrasia*, *Bipolaris*, *Trichoderma*, *Chaetomium*, *Fusarium*, and *Cytospora* have been reported from infected pistachio gardens (Ershad 1995; Khosravi-Moghadam et al. 2014; Sheibani 1994), among which *Paecilmyce variotii* has been recovered more frequently (Alizadeh et al. 2000; Khosravi et al. 2007). *Paecilomyces* species are filamentous fungi distributed throughout the world (Groll & Walsh 2001; Pastor & Guarro 2006). *Paecilomyces variotii* and *Paecilomyces lilacinus* are the members of the genus primarily implicated in invasive human diseases (Barker et al. 2014). *Paecilomyces variotii* has been reported for the first time on human from a patient referred to hospital in Faisalabad, Pakistan (Abbas et al. 2009). Also, *P. variotii* has been isolated from the pediatric lung Tx population (Das et al. 2000).

Recently, we have reported three species belonging to *Paecilomyces* genus from infected twigs of pistachio including *P. variotii*, *P. marquandii* and *P. viridis* among which *P. variotii* showed high frequency (Khosravi Moghadam et al. 2014). Molecular characterization and the population structure of pathogenic fungi is important for understanding the biological behavior and for development of disease-control strategies (Malvick & Percich 1998).

There is little information about the study of population structure of *P. variotii* isolates, particularly in respect of molecular characterization. Little information is available concerning molecular characterization of *P. variotii* and other species of *Paecilmyces* (Cantone & Vandenberg 1998; Fargues et al. 2002; Tigano-Milani et al. 1995; Yanna et al. 2006). Microsatellite bio-markers have been used as a

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powerful tool for genomic studies and evaluating the genetic variation and population structure (Goldstein & Schlotterer 1999; Queller et al. 1993). Microsatellite marker has advantages such as the wide distribution throughout the genome, high level of polymorphism, discriminating power and codominant transmission (Li 1991). This marker has been continually used by researchers in mycology (Fisher et al. 2002; Fournier et al. 2002; Zhou et al. 2003). The aim of this study was firstly to assess the genetic diversity among isolates of *P. variotii* collected from infected branches of pistachio in Kerman province gardens, as an economical center of pistachio production in Iran, and secondly to investigate the relationship between geographical sampling regions and genetic diversity of isolates.

MATERIALS AND METHODS

Sampling

Sampling was performed from the infected twigs with necrosis symptoms in pistachio gardens of Kerman province at four geographical regions, during summer 2013. The samples were surface disinfested with a 3% Chloramine T (Sigma, France) for 20 minute, followed by washing three times with sterile distilled water and drying with sterile filter paper under laminar flow.

Fungal isolates

In order to recover the isolates, the surface–sterilized twigs were cut, using flame-sterilized scalpel. Small pieces from the central core of tissues were transferred to the plates containing Potato Dextrose Agar (PDA; Difco, France). The Petri dishes were incubated at 22°C ± 2°C under alternate cycles of 12 h of light and darkness for 7 days. Malt Agar (2% malt extract) was used to obtain the purified cultures, using hyphal tip method. PDA was also used for conidia production and for determination of their morphological characters (Castelli et al. 2008). Identification of fungal isolates was carried out, using valid mycological keys (Brown & Smith, 1957; Hoog et al. 2000; Samson 1974). The isolates were kept in 4°C for further molecular studies. Molecular analysis was performed to confirm the morphological identification, using the sequence data of ITS1-ITS4 regions of some isolates.

Sequencing

To confirm the morphological identification, five selected isolates were sequenced. Two primers ITS1 [TW81; 5'-GTTCCGTAGGTGAACCTG-3' (Qiagen)] and ITS2 [AB28; 5'-ATATGCTTAAGTTCAGCGG GT-3' (Qiagen)] were used for the amplification of the DNA region encoding ITS18S–ITS28S of the DNA samples (White et al., 1990). Both strands of each PCR product were sequenced by Genome Express (Pishgam, South Korea). DNA sequences were queried, using NCBI stand-alone BlastAll program (Altschul et al., 1990) against the NCBI non-redundant (nr) protein reference library, Swissprot version 6, UniProt and UniRef100. Sequence similarities above 50% with an E value less than $1E^{-10}$ were considered as statistically significant positive matches. Deposited sequences were retrieved from GenBank. The obtained sequences were aligned with an rDNA-ITS sequence of *P. fumosoroseus* (AB2651 46.1), using Clustal W version 1.81.

PCR amplification and SSR analysis

In this research, ten SSR primer pairs were assayed on twenty selected *P. variotti* isolates. SSR markers were chosen from the genomic sequence of *P. fumosoroseus*, a closely related species to *P. variotti* (Dalleau-Clouet et al. 2005). The sequences of SSR primers are summarized in Table 2. Seven day-old isolates grown on Potato Dextrose Broth (PDB, Merck, Germany) were used for DNA extraction. The fungal hyphae were freezed in liquid nitrogen, followed by grinding in a mortar with a pestle. Then, the genomic DNA of *P. variotii* isolates was extracted, using Cetyl trimethylammonium bromide (CTAB) method (Zhang et al. 2010). Visual detection of DNA on 1% agarose gels showed high quality and no degradation. The quantity of recovered DNA was measured with Scandrop spectrometer (Analitika, Germany). Total DNA yield was diluted to 20 ng/µl, and kept at -20 °C for further use.PCR was carried out in a 25 µl volume, containing 1.5 µl 10× buffer (100 mM Tris–HCl, 15 mM $MgCl_2$, 500 Mm KCl, pH 8), 2 mM $MgCl_2$, 200 µM dNTPs, 0.24 µM of primers, 20–25 ng of template DNA and 1 unit of *Taq* polymerase with 35 cycles of the following program : denaturing at 94°C for 2 min, annealing at 48-54°C (table 1) for 1 min, elongation at 72°C for 1 min. A final extension step was performed at 72ºC for 5 min. PCR amplification was carried out in an Eppendrof PCR system (Germany). PCR products were separated in 2% agarose gels, using 1×TBE buffer. Each experiment included a negative control. (a PCR reaction with all reaction materials without template DNA). The gels were stained with ethidium bromide at a concentration of 0.5 µg/ml, and banding patterns were visualized by a UV-transluminator in Geldoc apparatus (Vilber, France).

Statistical Analysis

To determine the rate of similarity among the studied *P. variotti* isolates, clear bands were detected. The presence or absence of amplified fragments was considered as state 1 or 0, respectively. Finally, data were exported to the Excel software. Popgene (version 1.31) was used to calculate genetic similarity, genetic distance and parameters such as effective allele number, polymorphic loci, etc. (Yeh et al. 1997).

Cluster Analysis

Cluster analysis of data related to DNA fingerprinting was performed, using NTSYS pc version 2.10. Accordingly, cluster analysis of the data was performed using the Jaccard coefficient and the Unweighted Pair Group Mean Arithmetic Method (UPGMA) algorithms. Polymorphic Information Content (PIC) was calculated based on the bands frequency (Ghislain et al. 1999).

RESULTS

Identification of the fungus

Seventy isolates out of a total number of 100 isolates were identified as *P. variotii* by morphological and phenoipic characterestics. These isolates form powdery yellow-brownish colonies and have a high growth rate at 25°C and 37°C. (Fig 1: A-B).

Phialides are swollen at the base and gradually taper to a sharp point at the tip (Fig 1: C-D). Conidia are single-celled, hyaline, and are borne in chains with the youngest at the base (fig 1: E-F) (Houbraken et al., 2008). The alignment results of obtained sequences related to ITS1-ITS2 regions of morphological identified *P. variotii* isolates show a high homology with sequences deposited in data base (NCBI). These results confirmed our morphological identification (Table 1).

SSR polymorphism

SSR amplification products showed that although four primer pairs yielded no amplification products, the six other primer pairs, including PfrBtD11a, PfrBtD11b, PfrBtB04, PfrBtD05, PfrBtb07, PfrBtD01 were polymorphic and amplified products which were detected as clear electrophoresis bands on electrophoresis gels. PCR amplification by PfrBtD01 produced one electrophoresis band for each isolate, so these results indicate that this primer pair might amplify alleles at individual loci. However, PfrBtB04 and PfrBtD05 amplified 2-6 bands for each isolate. Based on these results, we could suggest that in our experimental conditions, two PfrBtB04 and PfrBtD05 SSR primers could amplify related alleles at numerous loci.

The characteristics of the six SSR loci are summarized in Table 2. The results showed that PfrBtD01 primer pair is more appropriate to study the genetic diversity than others, and the isolates of Ravar are more genetically variable comparing the isolates of other areas. The initial assessment of Nei coefficient from genetic similarity and genetic distance showed that the isolates from Zarand and Kerman have the most genetic similarity (0.9289), and the isolates collected from Ravar and Sirjan have the most genetic distance (0.321).

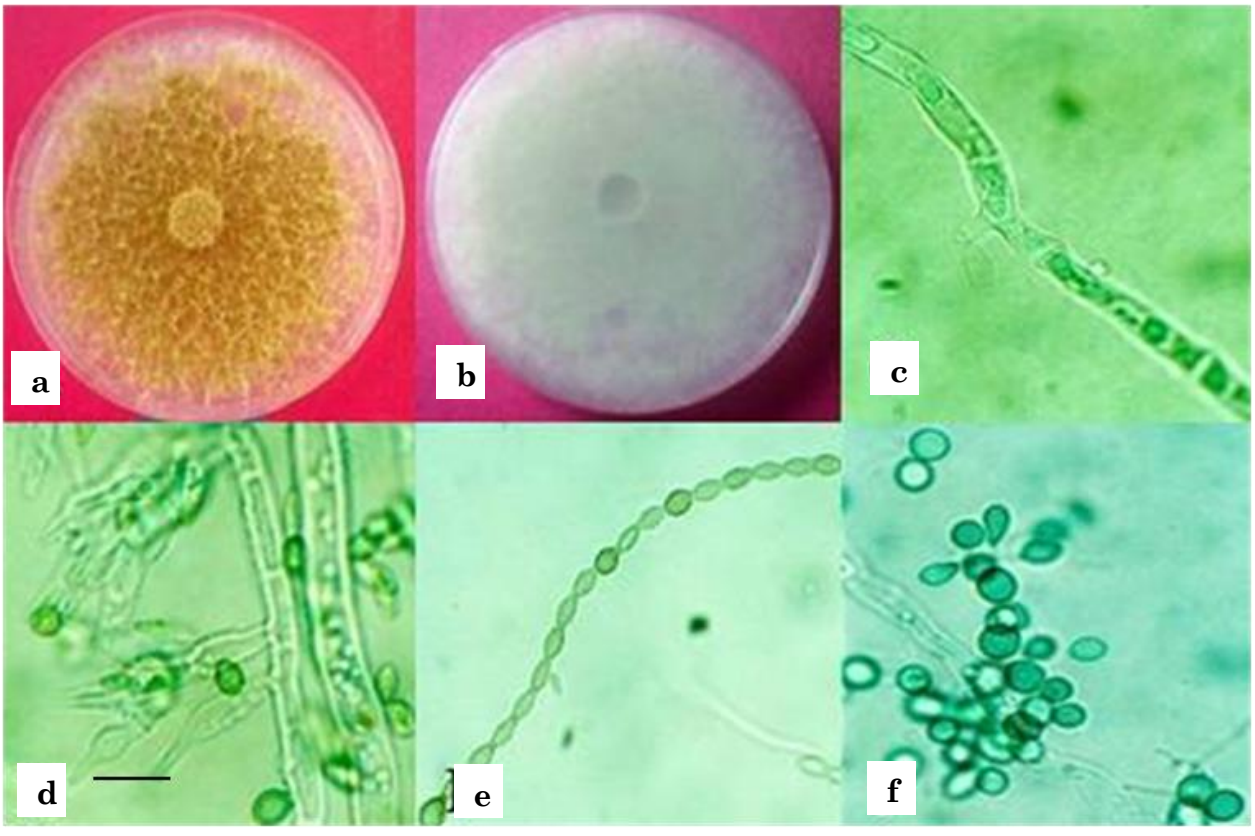

Fig. 1. *Paecilomyces variotii*. **a.** Colony surface, **b.** bottom, **c-d.** phialides (C-D), **e-f.** conidia on PDA medium. Scale bar (10µm).

Cluster analysis

The isolates were divided into eight groups (A-H) with 73% similarity level, using cluster analysis of the obtained data based on UPGMA algorithm and Jaccard's similarity coefficient. These groups (A-H) have 1, 6, 5, 1, 1, 1, 2 and 1 members, respectively (Fig. 2).

PCoA data analysis was performed, using NTSYS and Jaccard's similarity matrix. According to the results, out of the 20 components, 12 components had eigen values greater than one, and 96.36 percent of the variation was explained (Fig. 3). The polymorphism information content (PIC) values of single sequence repeat marker loci were arranged from 0/37 (PfrBtD11a) to 0/79 (*PfrBtD01*) with an average of 0/60. Twenty four unique allele was found at 6 SSR loci among the analyzed isolates (Table 2).

The plotting diagram of isolates was designed based on two first components. PCoA diagram showed that there was not a significant relationship between the geographical origin of the isolates and the groups obtained based on the genetic diversity analyses, and the isolates belonging to the same region were placed in different groups (Fig. 3).

Table 1: Homology of ITS1-ITS2 sequence of *Paecilomyces variotti* isolates with sequences deposited in data base (NCBI)

Isolate	Percentage of similarity in BLAST	Isolate in NCBI	Accession number
SK 20	96%	*Paecilomyces variotii* SUMS0303	FJ011547.1
ES 10	98%	*Paecilomyces variotii* BCC 14365	AY753332.1
SR7	97%	*Paecilomyces variotii* KUC5015	GQ241284.1
ZW 1	98%	*Paecilomyces variotii* isolate 15	FJ895878.1
KW 18	99%	*Paecilomyces variotii* SCSGAF0038	JN850996.1

Table 2.Characteristics of SSR primer pairs used in the study of genetic diversity of *Paecilomyces varietti* isolates in Kerman province (Dall eau-Clouet et al 2005).

Primer	Sequence (5'-3')	CG %	Total no. of loci	No. of polymorphic loci	Total no. of amplified fragments	Size Rang (bp)	Tm (°C)
PfrBtD11a	F: GCGAATCTCGTCTTCAAC	50%	2	2	25	109	65-55
	R: CACTGGCACACGACACTC	66%					
PfrBtD11b	F: GAGTGTCGTGTGCCAGTG	61%	4	3	50	83-93	65-55
	R:GCTATGTGCGTGCTAGAT	50%					
PfrBtB04	F: GTTGGTGCGGTGTGAGAG	61%	2	2	30	126-134	58-48
	R: ATGTAAGGCGATTGGCAG	50%					
PfrBtD05	F:GGCAAGTCACCAGATACGTC	55%	4	4	45	182-199	60-50
	R: GGATATGATGAAAGCCCTC	47%					
PfrBtA07	F: CTTGTAATCTGTGCGTATGTA	38%	4	4	70	254-259	60-50
	R: CTATTAGAAGAAGCGGGAG	47%					
PfrBtD01	F: CTCGTCATCCAAGGAGAGTC	55%	4	3	54	261-289	58-48
	R: CAATACAGGAACAAGATGC	42%					
Total			22	20	308		

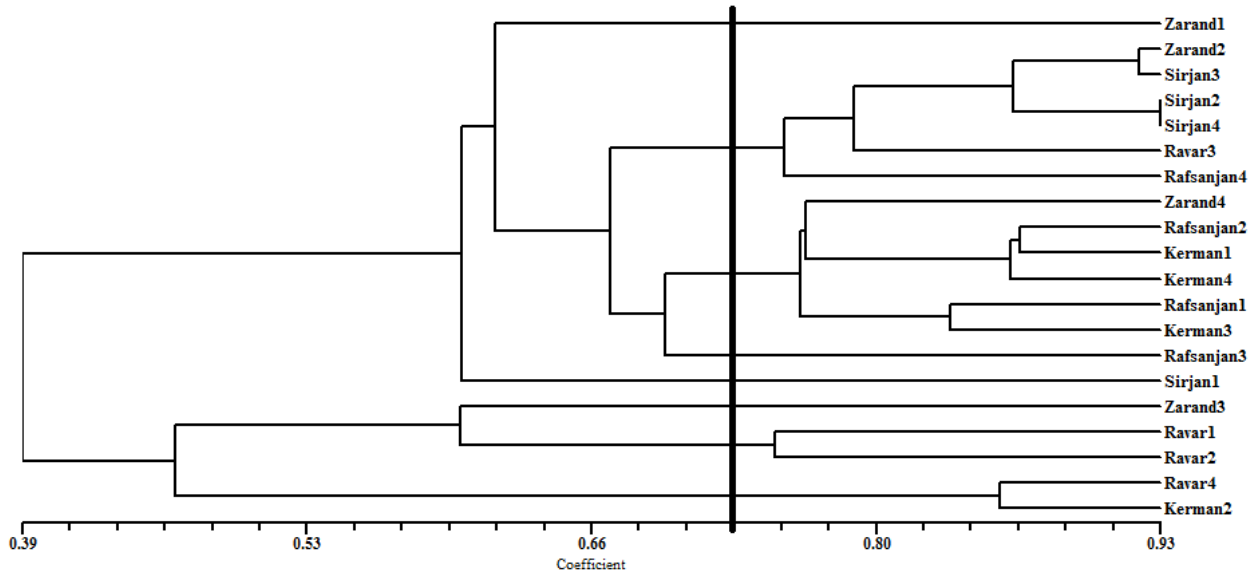

Fig. 2. UPGMA analysis of twenty *Paecilomyces variotti* isolates based on combined SSR data by UPGMA and using Nei and Li`s similarity coefficients

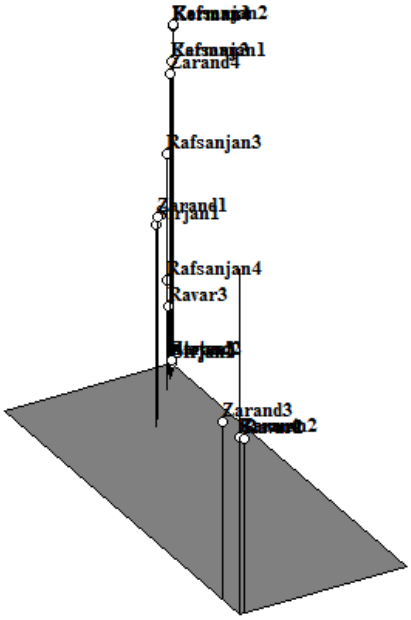

Fig. 3. Three-dimensional PCoA analysis of SSR amplification products from 20 isolates of *Paecilomyces variotti* belonged to different sampling regions of Kerman provine including Rafsanjan, Sirjan, Ravar, Kerman and Sirjan, Iran

DISCUSSION

Die-back disease caused by *P. variotti* is one of the most destructive diseases of pistachio in Iran. In this study, firstly, different fungi species were isolated from infected twigs and secondly, all *P. variotii* isolates were purified and then identified and finally, genetic diversity of twenty selected isolates from different geographical regions was investigated. Morphological identification confirmed that majority of the isolates belonged to *Paecilomyce variotii*. This result is in agreement with previous studies which introduced this species as the principal agent of die-back disease in pistachio gardens of Iran (Alizadeh et al. 2000; Aminaee 1993). Out of two hundred fugal isolates from the studied region, seventeen isolates were identified as *P. variotii* based on morphological characteristics, such as color and growth condition and type of conidium (Samson 1974). Morphological identifications are complicated by similarities among

the members of the *P. variotii* complex as well as some closely related species (Barker et al. 2014). So, to confirm the morphological identification, the sequence of region encoding ITS18S–ITS28S from five selected isolates was applied. These sequences were queried, using the NCBI stand-alone BlastAll program. A high homology of sequences was observed with submitted sequence of closely related species in GeneBank database. According to high frequency of *P. variotii* isolates from cultured tissue, we attempted to assay genetic diversity of this specie. Few studies have been performed to investigate the genetic diversity of the isolates of *P. variotti*. On the other hand, SSR marker has not been used to distinguish *P. variotti* genotype. Since the complete sequence of *P. variotii* was not available, all the SSR primer pairs were chosen from *P. fumosoroseus*, a closely related species to *P variotii* (Fargues et al. 2002). A total of 10 primer pairs were selected and designed to serve in SSR analysis, among which six tested primer pairs were able to amplify the desired fragments. The number of alleles in loci for each primer pair showed no significant difference in the whole genome. Longer SSRs in fungi are rare and the relative abundance of SSRs in fungi is low, compared with the human genome (Karaoglu et al. 2005). Our results are in accordance with the obtained results from other species of this genus, using various genetic markers (Cantone & Vandenberg 1998; Inglis & Tigano 2006; Tigano-Milani et al. 1995). Phylogenetic analysis based on the rDNA-ITS region and analysis of the ITS-RFLP patterns revealed a high level of polymorphism within *P. fumosoroseus* isolates *(*Fargues et al. 2002*)* and *P. variotti* (Khosravi-Moghadam et al. 2014), respectively.

This species has been reported as the principal causal agent of dieback disease in Iran. Nevertheless, *Xantomonas translucent* has been identified as the causal agent of dieback disease of pistachio in Australia (Facelli et al. 2005). Based on these information, this species could not be a unique causal agent of disease in worldwide and because of this dispersion this species has been excluded to more investigation.

Based on UPGMA cluster analysis, there is no correlation between geographical zones and genetic diversity, because the isolates belonging to the same sampling region were placed in different distance groups.

Based on these data, we can conclude that SSR markers could be suitable for illustrating levels of genetic diversity within a population, but it is not adequate for demonstrating the relationship between the isolates of a population and their related geographical regions. Recently, we compared two PCR-based marker assays including SSR and (PCR-RFLPs) to determine the genetic diversity of *Paecilomyces variotti* isolates. Our results showed that the feasibility of PCR-RFLP marker was higher than SSR markers (Rostami et al. 2015). According to spread of the disease in all pistachio gardens of Iran and introduction of *P. variettii* as one of the main causes of dieback disease in Iran, a comprehensive research on dieback agents over Iran can prevent future disease epidemies. Screening pistachio varieties with fungus species could be introduced as the best way to recognize the resistant varieties. However, attending to plant alimentation will be effective on dieback disease prevention and even other infectious diseases.

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مطالعه تنوع ژنتیکی جدایه های *Paecilomyces variotii* استان کرمان با استفاده از نشانگر SSR

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چکیده: بیماری مرگ سرشاخه ایجاد شده به وسیله قارچ *Paecilomyces variotti* یکی از مخربترین بیماری های پسته در ایران می باشد. در این مطالعه تنوع ژنتیکی جدایه های قارچ *P. variotti* با نشانگر SSR و با استفاده از شش جفت آغازگر مورد بررسی قرار گرفت. نمونه برداری از باغات پسته کرمان واقع در مناطق مختلف جغرافیایی طی تابستان ۱۳۹۱ انجام گردید. جدایه های مختلف قارچی از سرشاخه های آلوده بر روی محیط کشت PDA جداسازی گردید. براساس مشخصات ظاهری، ۷۰ جدایه به عنوان گونه *P. variotii* شناسایی شد. این شناسایی ها با استفاده از انالیز داده های توالی یابی مورد تایید قرار گرفتند. ابتدا تنوع ژنتیکی ۲۰ جدایه منتخب با استفاده از ۱۲ جفت آغازگر مورد ارزیابی قرار گرفت. سپس از بین آنها هفت جفت آغازگر انتخاب و تکثیر DNA به وسیله این آغازگرها انجام شد. مجموعه ای از ۲۲ آلل با متوسط دو آلل برای هر جفت آغازگر تعیین گردید. متوسط ضریب نی بین جدایه ها ۱/۵۳ تعیین شد. متوسط محتوای اطلاعات چند شکلی برای نشانگر های ریز ماهواره مربوط به جدایه های *P. variotii*، ۰/۶۸ تعیین شد. روابط ژنتیکی بین جدایه های مورد ارزیابی با استفاده از تجزیه خوشه ای به روش UPGMA بر اساس ماتریس ضرایب تشابه مورد بررسی قرار گرفت. آنالیز خوشه بندی، جدایه ها را به ۵ گروه عمده گروه بندی کرد و یک گستره وسیعی از تنوع در بین جدایه های ارزیابی شده مشخص شد. بیشترین فاصله ژنتیکی بین جدایه های زرند (var1) و رفسنجان (var15) تشخیص داده شد. یافته های این تحقیق نشان می دهد که بین جدایه های گروه بندی شده در یک گروه و منطقه جغرافیایی نمونه برداری شده رابطه ضعیفی وجود دارد. بر پایه این نتایج ما می توانیم چنین نتیه گیری کنیم که نشانگرهای SSR ابزار مناسبی برای درک سطوح تنوع ژنتیکی یک جمعیت می باشد ولی به تنهایی برای تعیین ساختار جمعیت ها و مناطق جغرافیایی مرتبط مناسب نیست

واژه‌های کلیدی: نشانگر مولکولی، بیمارگر، پسته، تعیین توالی

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