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Genetic variability of *Paecilomyces variotii* isolates, the causal agent of die–back disease in pistachio, using ITS–RFLP analysis

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Abstract: *Paecilomyces variotii* is one of the most important causal agents of dieback disease in pistachio (*Pistacia vera*) gardens. The disease affects different parts of the tree, such as branch and trunk. Assessment of genetic structure in different populations of this species will lead to more useful management of pistachio dieback disease. In this study, genetic variation within samples of *P. variotii* isolates from different geo–climatic origins of Kerman province was studied using ITS– RFLP analysis. Universal primer pairs AB28 and TW81 were used for ITS region amplification. Thirteen restriction enzymes were subjected to digest PCR products. Seven out of the 13 restriction enzyme including: EcoR I, Hpyf 3I, Apa I, Hinf I, Mbo I, Msp I, Rsa I showed restriction pattern. Jaccard's similarity coefficient used to determine of genetics similarity and cluster analysis dendrog was designed by using UPGMA algorithm method. Data analysis showed a high similarity at the level of 70% between isolates and all isolates were divided into 9 distinct groups. Analysis of molecular variance (AMOVA) showed a variation of 85% and 15% among of within isolates, respectively. Based on these results we can conclude that ITS–RFLP is useful for wider genetic diversity assessment and epidemiological studies of distantly related isolates. The future studies could be performed to develop new molecular markers to detect this fungus in field.

Key words: Phytopathogen, population, restriction enzyme, molecular marker

INTRODUCTION

Dieback of pistachio (*Pistacia vera* L.) is one of the most important, destructive and threatening diseases of Iran pistachio orchards. The disease affects different parts of the tree such as branch and trunk. Dieback of pistachios was first reported in 1987 in Iran (Aminaei 1987). Beside its plant pathogenic activity, it is also associated with many types of human infections (Abbas et al. 2009). Hyphomycosis disease in human caused by two species of *Paecilomyces lilacinus* and *P. variotii* (Houbraken et al. 2008).

At this time, the majority of studies on phylogeny of *Paecilomyces* species using molecular markers have been performed on entomopathogenic species (Dalleau–Clouet et al. 2005; Luangsa–ard et al. 2004). Recently, researchers have attempted to find out more information about the relationships between the different species of *Paecilomyces,* especially insect–pathogen species. Genetic similarities in unidentified isolates of *P. fumosoroseus* and some selected strains were observed using ITS and RAPD markers (Azevedo et al. 2000). Arbitrarily primed PCR and PCR with tRNA consensus primers have been used to analyse genetic variability among *P. fumosoroseus* isolates (Tigano–Milani et al. 1995).

The conserved sequence of rDNA–ITS regions has been used for molecular phylogenetic analysis of fungi (Kiss 1997; Nilsson et al. 2008). Sequence variation within the ribosomal DNA region has been used extensively for the phylogenetic analysis of both closely related and distantly related organisms (White et al. 1990). This can also provide an alternative approach to RAPD–PCR and tRNA–PCR for both the estimation of genetic diversity and the determination of phylogenetic relationships. Furthermore, the fast–evolving ITS region has been found to be a powerful tool for characterization of most fungal bio–control agents (Avis et al. 2001).

Ribosomal genes evolve cohesively within a single species and exhibit only limited sequence divergence between rDNA copies. In contrast, comparison between species showed normal levels of sequence divergence (Arnheim et al. 1980). There is not enough information about the genetic variability of this species in the literatures.

The aim of the present study was to investigate the genetic diversity among *P. variotii* isolates of different geo–climatic regions from Kerman province, using ITS and RFLP analysis.

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MATERIALS AND METHODS

Sampling

Samples were collected from different pistachio farms of Kerman province in Iran during 2011–2012 (Fig. 1). Sampling area were divided to seven geographical zone based on GPS information (Table1). The infected branches showing necrosis symptom were cut, kept in nylon pockets and transferred immediately to the refrigerator at 20 °C.

Fig. 1. The location of sampling regions on map of Karman province, Iran. Sampling regions are indicated by black filled circle.

Isolation and purification of isolates

The small pieces from the central core of infected barks of pistachio branches were surface–sterilized with 3% chloramine T (Sigma Co., Germany) and were placed on PDA (potato dextrose agar; Merck, Germany) culture medium for fungal growth at 22–25 °C for one week (Ebrahimi et al. 2015). Purification of fungal isolates was conducted by the hyphal–tip method and fungal identification at the genus/species level was carried out by morphological criteria (Brown & Smith 1957; Hoog et al. 2000; Samson 1974). Out of 116 *P. variotii* isolates, 28 selected isolates were recovered from all sampling region (four isolates from each region) which showed that typical species characters were selected to assess genetic diversity for further analysis.

DNA extraction

A piece of ten–day–old fungal colony on PDA medium was transferred to 100 mL Erlenmeyer flasks containing 200 mL of PDB liquid medium (Merck, Germany). The flasks were placed on a rotary shaker (120 rpm min^{-1}) for eight days at 25 °C and then the mycelia were harvested by filtering. Total genomic DNA was extracted from dried mycelium using the CTAB method (Nicholson et al. 1997). Total DNA was quantified using a Scanodrop 200 (Analytik Jena, Germany) spectrophotometer and the concentration of DNA was adjusted to 25 ng.µL^{-1} for use in PCR assay. DNA quality was assessed by 1% agarose gel electrophoresis stained by ethidium bromide.

Table 1. Code number, Location and geographic position calculated by GPS of *Paecilomyces variotii* isolates used in this study.

Isolate	Sampling Region	GPS	
		N	E
Z_1	Zarand	30 39' 42.14"	57 01' 46.25"
Z_2	Zarand	30 57' 07.39"	56 35' 40.68"
Z_3	Zarand	30 57' 07.39"	56 35' 40.68"
Z_4	Zarand	30 38' 33.35"	56 20' 10.92"
X_1	Ravar	31 15' 30.83"	56 50' 09.42"
X_2	Ravar	31 16' 05.86"	56 46' 59.4279"
X_3	Ravar	31 18' 27.95"	56 48' 07.77"
X_4	Ravar	31 18' 59.72"	56 50' 24.26"
S_1	Sirjan	31 32' 05.23"	55 36' 08.30"
S_2	Sirjan	31 37' 54.37"	55 27 20.46"
S_3	Sirjan	31 25' 50.97"	55 40' 24.03"
S_4	Sirjan	29 35' 53.51"	55 31' 18.85"
R_1	Rafsanjan	30 25' 57.05"	55 57' 05.44"
R_2	Rafsanjan	30 26' 32.12"	55 32' 58.26"
R_3	Rafsanjan	30 26' 32.12"	55 32' 58.26"
R_4	Kerman	30 11'40.46"	56°45'55.95"
K_1	Kerman	3011'17.15"	56°48'55.96"
K_2	Kerman	30 09'49.73"	56°45'19.07"
K_3	Kerman	30 11'34.71"	56°42'09.63"
K_4	Kerman	30 14' 10.91"	56 37' 42.42"
B_1	Bardsir	29 57' 57.37"	56 30' 27.46"
B_2	Bardsir	29 51' 10. 76"	56 37' 11.18"
B_3	Bardsir	29 47' 52. 57"	56 41' 44.67"
B_4	Bardsir	29 51' 12. 22"	56 33' 08.89"
T_1	Tahrood	28 41' 24. 25"	59 02' 47.30"
T_2	Tahrood	28 41' 23. 84"	59 01' 59.44"
T_3	Tahrood	28 41' 11. 57"	59 02' 20.84"
T_4	Tahrood	28 40' 46. 57"	59 05' 28.77"

DNA amplification

Primers TW81 (5′–GTTCCGTAGGTGAACCTG C–3′) and AB28 (5′–TATGCTTAAGTTCAGCGG GT–3′) were used to amplify the ITS–rDNA region (White et al. 1990). Amplification were carried out in volumes of 25 µL containing: 1 µL of genomic DNA (25 ng), 1.5 µL of 10×buffer PCR (100 mM Tris–HCl, 15 mM $MgCl_2$, 500 mM KCl, pH 8), 1 µL of $MgCl_2$ (50 mM), 0.25 µl of dNTPs (100 mM), 5U Master *Taq* DNA polymerase (Genall, Sout Korea), and 25 µL of each primer (20 mM). The PCR reaction was performed with the following steps: an initial denaturation step at 95 °C for 5 min, 35 cycles at 95 °C (30 s)/56 °C (60 s)/72 °C (60 s), and a final extension step at 72 °C for 10 min. A negative control deleting DNA template was used in every set of reactions. PCR products were separated by electrophoresis on 1.2% agarose gels stained with ethidium bromide (0.5 µg.mL^{-1}) and photographed under UV light.

PCR–RFLP analysis

The PCR products were purified using the PCR purification kit (Genall, South Korea,) for the PCR–RFLP analysis. Thirteen restriction enzymes including *Eco*R I, *Hpyf*3I, *Hinf* I, *Msp* I, *Apa* I, *Mbo* I, *Pst* I, *Not* I, *Rsa* I, *Dra* I, *BamH* I, *Hind* III, and *Mse* I (SinaClon, Iran) were used to digest ITS–rDNA PCR products. Ten units of each enzyme, with a total volume of 15 µL were used in the reaction. The reaction was incubated for 18 h at 37 °C.

Genetic diversity

ITS–RFLP patterns were used to estimate similarities among the isolates. Restriction–enzyme digests were used to generate ITS–RFLPs. For this purpose, each DNA band formed by the digestion in RFLP analysis was considered to be a character, and only the presence or absence of RFLPs fragments was recorded. A dendrogram was constructed from the resulting distance matrix using the Unweighted Pair Group Method with Arithmetic Mean Algorithms (UPGMA) and genetics similarity determined using Jaccard's similarity coefficient (Sneath & Sokal 1973). The software PopGene 32 was used to perform the distance analysis (Kumar et al. 2008). The PAUP version 0.4.0 beta program was used for phylogenetic analysis of the various data sets (Swofford 2003). Genetic variations within and between populations was estimated by analysis of molecular variance (AMOVA) performed with GenALEX version 6.1.

Sequencing

Both strands of each PCR products were sequenced by PishgamBiotech Company (Tehran, Iran). DNA sequences were queried using the NCBI stand–alone BlastAll program (Altschul 1990) against the NCBI non–redundant (nr) protein reference library, Swissprot version 6, UniProt and UniRef100. Sequence similarities above 90% 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 a rDNA–ITS sequence of *P. variotti* isolates in gene bank using the Clustal W program, version 1.81 (Thompson et al. 2002).

RESULTS

Identification of fungal isolates

All recovered fungal isolates from infected twigs were identified by morphological criteria using valid mycology keys. One hundred sixteen isolates out of 180 were identified as *Paecilomyces variotii*. After two weeks growth, the isolates showed a brown or yellow–brownish colour on the surface of solid medium. A powdery yellow–brownish colony with a high growth rate at 25 °C and 37 °C was observed on PDA medium. Single–celled and hyaline conidia were born in chains with the youngest cell at the base of conidiophores. The phialides were swollen at the base and gradually taper to a sharp point at the tip. To confirm morphological diagnosis, the sequences of five represented isolates from different geographic regions were queried against data base. Analysis of alignment showed a high similarity of our sequences (96–99%) with deposited sequences of *P.variotii* in geneBank (Table 2, Fig. 2).

Polymorphism of ITS–RFLP patterns

Amplification of the region from the 3´ end of the 18S rDNA to the 5´ end of the 28S of rDNA resulted in an approximately 600–800 base pair (bp) fragment (Fig. 2). The ITS1–ITS2 amplicons were subjected to digestion with thirteen different restriction enzymes. Seven out of the 13 restriction endonuclease (*EcoR* I, *Hpyf* 3I, *Apa* I, *Hinf* I, *Mbo* I, *Msp* I, showed restriction pattern. No restriction sites were found when DNA was treated with *Rsa* I, *Not* I, *Pst* I, *BamH* I and *Hind* III. The banding patterns obtained with restriction endonuclease digestion, the number and the size of the fragments from 28 *P. variotii* isolates are characterized in Table 3. Based on resulted patterns of digested PCR products, all isolates were divided into three distinct groups. The sixteen isolates from various graphic regions (Ravar, Sirjan, Rafsanjan and Kerman) were clustered in group 1 based on ITS–RFLP patterns. Group 2 consisted of 8 isolates originated from diverse geographic locations representing four isolates from Zarand, two isolates from Bardsir and two isolates from Tahroud origins and group 3 contain three isolates from two different geographical regions including Bardsir (2 isolates) and Tahroud (one isolates) isolates (Table 3).

The enzyme *Bam*H1 digested the fragment, but showed no polymorphisms among isolates. The highest number of restricted fragment was obtained for the *Aps* I enzyme, whereas the *Eco*R I and *Msp* I showed the lowest digestion. The *Mbo* I enzyme revealed a higher variety and the *Msp* I enzyme showed a low diversity among isolates. The maximum number of nucleic acid band ranged from 45 – 325 was obtained for *Aps* I pattern (Table 3).

The *Mbo* I and *Msp* I enzymes revealed the highest and lowest values for H^c (0.453 and 0.347 respectively. The highest (0.644) and lowest (0.525) I^d values were obtained for *Mbo* I and *Msp* 1 (Table 4). Cluster analysis using NTSYSpc software (version 2.2) based on the Jaccard's coefficient showed that all isolates were divided to nine separate groups with a high similarity value of 70%. Isolates were grouped into nine clusters designated from A to I. Isolates of group A–B and group D–E contain isolates from Zarand and Rafsanjan regions with similarity value of 66% and 50% respectively. Other isolates were placed in a distinct group (C, D, E, F, I) (Fig. 4).

Table 2. Similarity percentage of studied isolates of *Paecilomyces variotii* with deposited sequences in GeneBank

Isolate	Percent of BLAST	Isolate in NCBI	Accssion number
Z_2	96%	*Paecilomyces variotii* SUMS0303	FJ011547.1
X_3	98%	*Paecilomyces variotii* BCC 14365	AY753332.1
R_1	97%	*Paecilomyces variotii* KUC5015	GQ241284.1
K_2	98%	*Paecilomyces variotii* isolate 15	FJ895878.1
B_3	99%	*Paecilomyces variotii* SCSGAF0038	JN850996.1

Analysis of molecular variance showed a high proportion of total variation is supported by variability (85%) among isolates and less proportionately (15%) within isolates (Table 5).

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SUMS0303      TGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAA-GGATCATTACCGA 59
KUC5015       ------------TCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAA-GGATCATTACCGA 47
BCC14365      -------------------------------------------GAA-GGATCATTACCGA 16
Z2            -----------------------GTTCCGTAGGTGAACCTGCGGAA-GGATCATTGCAGC 36
B3            -----------------------GTTCCGTAGGTGAACCTGCGGAA-GGATCGTAAACCT 36
K2            -----------------------GTTCCGTAGGTGAACCTGCGGAA-GGATCATTACCAC 36
X3            -----------------------GTTCCGTAGGTGAACCTGCGGAAAGGATCATTACCGA 37
R1            -----------------------GTTCCGTAGGTGAACCTGCGGAA-GGATCATTACCGA 36
Isolate15     --------------------------TCCGTAGGTGAACCTGCGGAAGGATCATTACCGA 34
SCSGAF0038    ------------------------------------------------------TACCGG 6

SUMS0303      GTGAGGGTCC-CACGAGGCCCAACCTCCCATCCGTGTTG-AACTACACCTGTTGCTTCGG 117
KUC5015       GTGAGGGTCC-CACGAGGCCCAACCTCCCATCCGTGTTG-AACTACACCTGTTGCTTCGG 105
BCC14365      GTGAGGGTCC--ACGAGGCCCAACCTCCCATCCGTGTTG-AACTACACCTGTTGCTTCGG 73
Z2            GTGCGGGACC-CACGCAGATACACCCTCCACCCGTGTTATAACTACACCTGTTGCTTCGG 95
B3            GCGTGGGTCT-CATGAGTGACAATGCTGCATCCGTGTTG-AACTACACCTGTTGCTTCGG 94
K2            GGGCTGGTCCACGCAGAGAAGAACCTCCCATCCGTGTTG-AACTACACCTGTTGCTTCGG 95
X3            GTGAGGGTCA-CGCATATACCAACCTCCCATCCGTGTTG-AACTACACCTGTTGCTTCGG 95
R1            GTGAGGGTCC-CACGAGGCCCAACCTCCCATCCGTGTTGGAACTACACCTGTTGCTTCGG 95
Isolate15     GTGAGGGTCC-CTCGAGGCCCAACCTCCCATCCGTGTTG-AACTACACCTGTTGCTTCGG 92
SCSGAF0038    ATTAGA--TC-CACGAG--CTAACCTCC-ATCCGTGTTG-AACTACACCTGTTGCTTCGG 59

SUMS0303      CGGGCCCGCCGTGGTTCACGCCCGGCCGCCGGGGGGCCTTGTGCTCCCGGGCCCGCGCCC 177
KUC5015       CGGGCCCGCCGTGGTTCACGCCCGGCCGCCGGGGGGCCTTGTGCTCCCGGGTCCGCGCCC 165
BCC14365      CGGGCCCGCCGTGGTTCACGCCCGGCCGCCGGGGGGCCTTGTGCCCCCGGGCCCGCGCTC 133
Z2            CGGGCCCGTCGAGGTTCACGCCCGGCCGCCGGGGGGCCTTGTGCCCCCGGGCCCGCGCCC 155
B3            CGGGCCCGCCGTGGTTCACGCCCGGCCGCCGGGGGGCCTTGTGCCCCCGGGCCCGCGCCC 154
K2            CGGGCCCGCCGTGGTTCACGCCCGGCCGCCGGGGGGCCTTGTGCCCCCGGGCCCGCGCCC 155
X3            CGGGCCCGCCGTGGTTCACGCCCGGCCGCCGGGGGGCCTTGTGCCCCCGGGCCCGCGCCC 155
R1            CGGGCCCGCCGTGGTTCACGCCCGGCCGCCGGGGGGCCTTGTGCCCCCGGGCCCGCGCCC 155
Isolate15     CGGGCCCGCCGTGGTTCACGCCCGGCCGCCGGGGGGCCTTGTGCTCCCGGGCCCGCGCCC 152
SCSGAF0038    CGGGCCCGCCGTGGTTCACGCCCGGCCGCCGGGGGGCCTTGTGCCCCCGGGCCCGCGCCC 119

SUMS0303      GCCGAAGACCCCTCGAACGCTGCCCTGAAGGTTGCCGTCTGAGTATAAAATCAATCATTA 237
KUC5015       GCCGAAGACCCCTCGAACGCTGCCCTGAAGGTTGCCGTCTGAGTATAAAATCAATCATTA 225
BCC14365      GCCGAAGACCCCTCGAACGCTGCCCTGAAGGTTGCCGTCTGAGTATAAAATCAATCATTA 193
Z2            GCCGAAGACCCCTCGAACGCTGCCCTGAAGGTTGCCGTCTGAGTATAAAATCAATCATTA 215
B3            GCCGAAGACCCCTCGAACGCTGCCCTGAAGGTTGCCGTCTGAGTATAAAATCAATCATTA 214
K2            GCCGAAGACCCCTCGAACGCTGCCCTGAAGGTTGCCGTCTGAGTATAAAATCAATCATTA 215
X3            GCCGAAGACCCCTCGAACGCTGCCCTGAAGGTTGCCGTCTGAGTATAAAATCAATCATTA 215
R1            GCCGAAGACCCCTCGAACGCTGCCCTGAAGGTTGCCGTCTGAGTATAAAATCAATCATTA 215
Isolate15     GCCGAAGACCCCTCGAACGCTGCCCTGAAGGTTGCCGTCTGAGTATAAAATCAATCATTA 212
SCSGAF0038    GCCGAAGACCCCTCGAACGCTGCCCTGAAGGTTGCCGTCTGAGTATAAAATCAATCGTTA 179

SUMS0303      AAACTTTCAACAACGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAAATGCGAT 297
KUC5015       AAACTTTCAACAACGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAAATGCGAT 285
BCC14365      AAACTTTCAACAACGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAAATGCGAT 253
Z2            AAACTTTCAACAACGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAAATGCGAT 275
B3            AAACTTTCAACAACGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAAATGCGAT 274
K2            AAACTTTCAACAACGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAAATGCGAT 275
X3            AAACTTTCAACAACGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAAATGCGAT 275
R1            AAACTTTCAACAACGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAAATGCGAT 275
Isolate15     AAACTTTCAACAACGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAAATGCGAT 272
SCSGAF0038    AAACTTTCAACAACGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAAATGCGAT 239

SUMS0303      AAGTAATGTGAATTGCAGAATTCCGTGAATCATCGAATCTTTGAACGCACATTGCGCCCC 357
KUC5015       AAGTAATGTGAATTGCAGAATTCCGTGAATCATCGAATCTTTGAACGCACATTGCGCCCC 345
BCC14365      AAGTAATGTGAATTGCAGAATTCCGTGAATCATCGAATCTTTGAACGCACATTGCGCCCC 313
Z2            AAGTAATGTGAATTGCAGAATTCCGTGAATCATCGAATCTTTGAACGCACATTGCGCCCC 335
B3            AAGTAATGTGAATTGCAGAATTCCGTGAATCATCGAATCTTTGAACGCACATTGCGCCCC 334
K2            AAGTAATGTGAATTGCAGAATTCCGTGAATCATCGAATCTTTGAACGCACATTGCGCCCC 335
X3            AAGTAATGTGAATTGCAGAATTCCGTGAATCATCGAATCTTTGAACGCACATTGCGCCCC 335
R1            AAGTAATGTGAATTGCAGAATTCCGTGAATCATCGAATCTTTGAACGCACATTGCGCCCC 335
Isolate15     AAGTAATGTGAATTGCAGAATTCCGTGAATCATCGAATCTTTGAACGCACATTGCGCCCC 332
SCSGAF0038    AAGTAATGTGAATTGCAGAATTCCGTGAATCATCGAATCTTTGAACGCACATTGCGCCCC 299
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Fig. 2. A part of sequence alignment showing high similarity between the studied sequences of *Paecilomyces variotii* isolates and deposited sequences of this specie in GeneBank.

Table 3. Patterns within the *Paecilomyces variotii* rDNA–ITS–rDNA region after digestion with *Eco*R I, *Hpyf* 3 I, *Apa* I, *Hinf* I, *Mbo* I, *Msp* I, *Ms*e I restriction endonucleases.

Isolate	Restriction fragment length (bp)						
	***Eco*R I**	***Hpyf* 3I**	***Apa* I**	***Hinf* I**	***Mbo* I**	***Msp* I**	***Ms*eI**
ZARAND $_{1-1}$	295	190,350	45,90,165,285	280,305	95,210,230	105	210,380
ZARAND$_{2-2}$	295	190,350	45,90,165,285	280,305	95,210,230	105	210,380
ZARAND$_{3-3}$	295	190,350	45,90,165,285	280,305	95,210,230	105	210,380
ZARAND$_{4-4}$	295	190,350	45,90,165,285	280,305	95,210,230	105	210,380
RAVAR$_{1-5}$	290	195,345	55,100,160,290	285,305	105,205,225	120	210,385
RAVAR$_{2-6}$	290	195,345	55,100,160,290	285,305	105,205,225	120	210,385
RAVAR$_{3-7}$	290	195,345	55,100,160,290	285,305	105,205,225	120	210,385
RAVAR$_{4-8}$	290	195,345	55,100,160,290	285,305	105,205,225	120	210,385
SIRJAN$_{1-9}$	290	195,345	55,100,160,290	285,305	105,205,225	120	210,385
SIRJAN$_{2-10}$	290	195,345	55,100,160,290	285,305	105,205,225	120	210,385
SIRJAN$_{3-11}$	290	195,345	55,100,160,290	285,305	105,205,225	120	210,385
SIRJAN$_{4-12}$	290	195,345	55,100,160,290	285,305	105,205,225	120	210,385
RAFSANJAN$_{1-13}$	290	195,345	55,100,160,290	285,305	105,205,225	120	210,385
RAFSANJAN$_{2-14}$	290	195,345	55,100,160,290	285,305	105,205,225	120	210,385
RAFSANJAN$_{3-15}$	290	195,345	55,100,160,290	285,305	105,205,225	120	210,385
RAFSANJAN$_{4-16}$	290	195,345	55,100,160,290	285,305	105,205,225	120	210,385
KERMAN$_{1-17}$	290	195,345	55,100,160,290	285,305	105,205,225	120	210,385
KERMAN$_{2-18}$	290	195,345	55,100,160,290	285,305	105,205,225	120	210,385
KERMAN$_{3-19}$	290	195,345	55,100,160,290	285,305	105,205,225	120	210,385
KERMAN$_{4-20}$	290	195,345	55,100,160,290	285,305	105,205,225	120	210,385
BARDSIR$_{1-21}$	290,295	190,350	50,100,160, ,325	275,300,425	95,205,225	120	205,370
BARDSIR$_{2-22}$	290,295	190,350	50,100,160,290	275,300	95,205,225	120	205,370
BARDSIR$_{3-23}$	290,295	190,350	45,100,160,290	275,300	95,205,225	120	205,370
BARDSIR$_{4-24}$	290,295	190,350	45,100,160,290	275,300	95,205,225	120	205,370
TAHROD$_{1-25}$	290,295	190,350	50,100,160, ,325	275,300,425	95,205,225	120	205,370
TAHROUD$_{2-26}$	290,295	190,350	50,100,160,290	275,300	95,205,225	120	205,370
TAHROUD$_{3-27}$	290,295	190,350	50,100,160,290	275,300	95,205,225	120	205,370
TAHROD$_{4-28}$	290,295	190,350	50,100,160,290,325	275,300,425	95,205,225	120	205,370

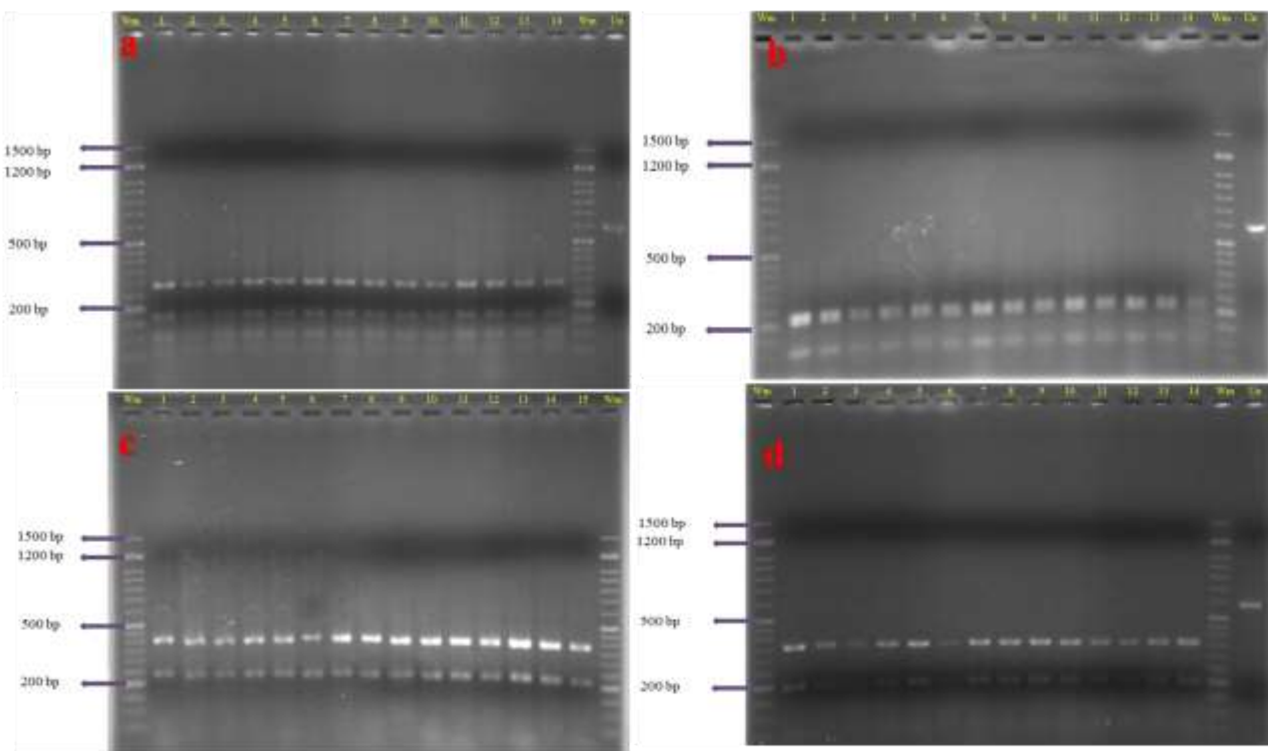

Fig. 3. ITS–RFLP pattern of represented *Paecilomyces variotti* isolates using restriction enzymes. Apa I (a); Mbo I (b); Mse I (c) and Hpyf3 I (d). Lin 1: R3; Line 2; R4; Line 3:K1; Line 4: K2; Line 5: K3; Line 6: K4; Line 7: B1; Line 8: B2; Line 9: B3; Line 10: B4; Line 11: T1; Line 12: T2; Line 13: T3; Line: 14:T4; wm, Molecular sizes in Kilobases are indicated on the right and left; Un, Negative control.

Table 4. Genetic diversity indices of *Paecilomyces variotii* isolates.

Enzyme	N	Na	Ne	He	Id
*Eco*R 1	28	2	1.696	0.393	0.576
Hinf 1	28	2	1.847	0.453	0.643
Hpyf 31	28	2	1.766	0.428	0.618
Mbo 1	28	2	1.867	0.454	0.644
Msp 1	28	2	1.575	0.347	0.525
Apa 1	28	2	1.737	0.416	0.605
Mse 1	28	2	1.717	0.401	0.586
Mean	28	2	1.755	0.418	0.605

Na: Number of different alleles; *Ne*: Number of effective alleles; *He:* Nei's Unbiased Expected Heterozygosity. *Id*: Shannon Index

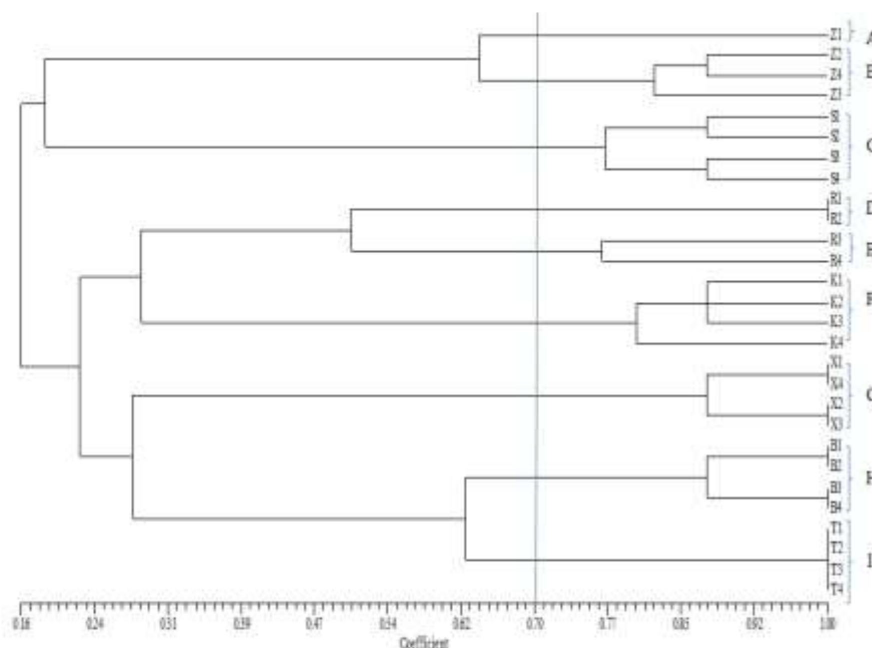

Fig. 4. Dendrogram constructed from analysis of DNA fragments 28 *Paecilomyces variotii* isolates amplified by PCR–RFLPP. The matrix was created with the Jacard similarity coefficient, and clustering was performed with UPGMA algorithm.

Table 5. Analysis of molecular variance *of P. variotii* isolates.

Source	**Df**	**SS**	**MS**	**Est. Var.**	**%**
Among Pops	6	209.071	34.845	8.342	85%
Within Pops	21	31.000	1.476	1.476	15%
Total	27	240.71		9.817	100%

DISCUSSION

The genus *Paecilomyces* represents a wide spread species reported as a pathogen of many different insects, plants and human. This genus has been divided in two sections: *Paecilomyces* and *Isarioidea* (Samson 1974). Classification of the genus *Paecilomyces* was based on morphological characteristics, such as conidial and chain of conidiophores form, however was often highly subjective and lead to obscure identifications at the level of species (D'Alessandro et al. 2014). Using molecular markers such as ITS–rDNA, *B–tubulin* gene and the elongation factor 1–alpha (EF1–a) combined to morphological criteria have been used for the molecular characterization at the level of species (Kis et al. 1997; Tanabe et al. 2004; Rostami et al. 2015). In this study, we firstly isolated different fungal genera from infected pistachio trees included *Paecilomyces*، *Stemphyllium, Alternaria, Nattrasia, Bipolaris, Trichoderma, Chaetomium, Fusarium and Cytospora.* Of 180 fungal isolates, 166 isolates were morphologically identified as *Paecilomyes varioti* species. Secondly, the genetic diversity of some selected isolates from different regions sampling was assayed to illustrate the genetic relation between different populations.

Analysis of ITS–RFLP patterns revealed a high level of polymorphism within isolates morphologically classed as *Paecilomyces variotii*. The analysis of ITS–RFLP profiles generated by restriction endonucleases enzymes enabled a clustering of *Paecilomyces variotii* isolates. Furthermore, the sequence data and the resulting phylogenetic dendrogram using the maximum of parsimony method strongly supported the conclusions of the ITS–RFLP analysis (data non–showed).

Fargus et al. (2002) found that *Hae* III alone could be used in polymorphism detection and discrimination

of all isolates of *Paecilomyces* spp, *P. fumosoroseus* and *P. tenuipes*; however, in our study this enzyme did not allow to restrict genome of studied isolates. The patterns within the rDNA–ITS region of *P. variotii* after digestion with seven enzymes showed different restricted–fragment ranges. For the *EcoR* I and *Msp* I enzymes, we observed only one band, while other enzymes were able to restrict PCR products with more than one band (Table 3).The analysis of ITS–RFLP profiles generated by a limited number of endonucleases enabled a clustering of *P. variotii* isolates. ITS–RFLP and RAPD marker have been used to molecular characterization of 7 *Paecilomyces fumosoroseus*, 5 *Paecilomyces sp.* and 5 *Paecilomyces tenuipes* isolates from different countries (Azevedo et al., 2000). Molecular analysis showed that similarity among five unidentified isolates and strains of *P. fumosoroseus* was higher than other reference species as *P. tenuipes.* These results were expected because similar isolates were isolated from the same pathogenesis phase in studied area. Our results are in agreement with those showing a closely related genetic similarity among isolates from same geographical regions. In this study, the amplified band resulting from the PCR was determined in 600 base pair (bp) long and as a single band. Our result is approximately in accordance with the results of Fargus et al. (2002), who showed that the multiplication of the same fragment in *P. fumosoroseus* isolates in the range of 670 bp produced a single band. Amplication of RDNA–ITSregions was done by using the same primers in study of Fargus et al. (2002). Genetic variability within 48 *P. fumosoroseus* isolates collected from different geographical origins was evaluated using rDNA–ITS marker.

Genetic variability among *Paecilomyces fumosoroseus* isolates from various geographical and host insect origins based on the rDNA–ITS regions showed a high level of polymorphism within the *P. fumosoroseus* isolates (Fargues et al. 2002). The genetic diversity of 20 isolates of *P. variotii* in Kerman province was investigated based on pathogenicity tests, sampling area, and genetic diversity using microsatellite marker (SSR) and the results, showed that there is no special relationship between the genetic groups and origin of the isolates (Ebrahimi & Sabbagh, 2012). Our results are not in concordance with these results. This disagreement could be caused by the different markers used and the lack of information on the whole genome of fungi genera. DNA restriction fragment polymorphispm (RFLP) has been widely used in human and some plant genetic (Michelmore & Hulbert 1987) and is the most common DNA technique to define multilocus genotypes for population studies of fungi (Rosendahl & Taylor 1997).

Study of population structure of Mycosphaerella was thoroughly done by McDonald and Martinez (1990). Their results encouraged other researchers to use of RFLP in thorough studies of other plant pathogenic fungi, such as *Fusarium* (Gordon et al. 1992), *Sclerotinia* (Kohn 1995), and *Crypphonectria* (Milgroom et al. 1996).

Using molecular marker; PCR–RFLP and RAPD to genetic diversity study of some isolates of *Macrophomina phaseolina* showed that RAPD marker is more efficient than RFLP marker (Bakhshi et al., 2010). However, investigation of genetic diversity of *Macrophomina phaseolina* isolates causal agent of root rot of cluster bean by Purkayastha et al. (2008) showed that RFLP marker is an enforceable marker to assay genetic diversity in these isolates. Occurrence of parasexual phenomen could increase reliability of this marker to study of genetic variety in fungi with this phenomen. Dispersion of fungi units to new ecological niches could influence biological cycles and adaptation to new hosts. In entomopathogenic *Paecilomyces*, it has been suggested that the mobility of dispersion units (spores) has a major influence on the life strategy of species of this genera; so host range, geographical distribution and genetic variability deriving could be affected (Oborník et al. 2000). Our results also suggest no relationship between genetic diversity and transmittance of fungal isolates and the distance of different geographical regions of Kerman province. In other works, increasing or decreasing the distance between two regions did not influence the similarity rate or genetic diversity of the studied isolates (Ebrahimi et al. 2015).

The elongation factor 1–alpha (*EF1–α*) and ITS1–5.8S–ITS2 regions have been used to molecular phylogeny study of *Isaria* spp. strains (Ascomycota: Hypocreales). Based on obtained results, these markers were found to be powerful tools to improve the characterization, identification, and phylogenetic relationship of the *Isaria* strains and other entomopathogenic fungi (D'Alessandro et al. 2011). Based on our results, it can be concluded that beside of using ITS –RFLP marker for molecular phylogeny and genetic diversity studies, diagnostics of group level using these marker could be easily developed for epidemiological and ecological studies of distantly related isolates of *P. variotii*, as has been done for *P. fumosoroseus* isolates (Fargues et al. 2002).

High genetic diversity of isolates from different region could be resulted to increase risk of compatibility of isolates to change of environmental condition and so, affect the disease controlling methods. Knowledge of structural genetics of plant pathogenic fungal will be a useful tool for plant breeding programs and prevent of new isolates from other regions or countries. Regarding to prevalent of Dieback disease of pistachio in Iran, and little information about structural genetic of this fungus, we propose a range–wide genetic assessment of *Paecilomyces* species in different pistachio cultured zones. By the future studies could develop new molecular markers to detect this fungus in field.

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بررسی تنوع ژنتیکی جدایه های *Paecilomyces variotti* عامل سرخشیدگی درختان پسته با استفاده از آنالیز ITS–RFLP

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چکیده: قارچ *Paecilomyces variotti* یکی از مهمترین عوامل بیماری زوال پسته (*Pistacia vera* L.) می باشد. بیماری قسمت-های مختلف درخت نظیر شاخه و تنه را مورد حمله قرار می دهد. ارزیابی ساختار ژنتیکی جمعیت های این گونه در مدیریت بیماری زوال پسته دارای اهمیت می باشد. در این مطالعه تنوع ژنتیکی جدایه های *P. variotti* از مناطق جغرافیایی مختلف استان کرمان با استفاده از آنالیز ITS–RFLP مورد مطالعه قرار گرفت. از آغازگرهای عمومی AB28 و TW81 برای تکثیر نواحی ITS استفاده شد. از ۱۳ آنزیم برشی جهت هضم آنزیمی محصولات واکنش زنجیره ای پلی مراز استفاده شد. ۷ از ۱۳ آنزیم شامل EcoR I, Hpyf 3I, Apa I, Hinf I, Mbo I, Msp I, Rsa I الگوی برشی را نشان دادند. از ضریب تشابه جاکارد برای تعیین میزان تشابه ژنتیکی جدایه ها استفاده و دندروگرام تجزیه خوشه ای با استفاده از روش الگوریتم UPGMA رسم گردید. آنالیز داده های حاصل یک تشابه بالایی در سطح ۷۰ درصد را نشان داده و جدایه ها در نه گروه مختلف قرار گرفتند. آنالیز تنوع مولکولی نشان داد که بین جدایه ها و درون جدایه ها به ترتیب ۸۵ و ۱۵ درصد تنوع وجود دارد. بر اساس نتایج بدست آمده ما چنین نتیجه گیری می کنیم که نشانگر ITS–RFLP یک ابزار مفیدی بری ارزیابی وسیع ژنتیک جمعیت و مطالعات همه گیر شناسی جدایه های مرتبط با هم می باشد. مطالعات بعدی می تواند برای توسعه نشانگرهای مولکولی جدید در جهت تشخیص این قارچ در مزرعه انجام گیرد.

کلمات کلیدی: بیمارگر گیاهی، جمعیت، آنزیم برشی، نشانگر مولکولی

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