



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

S. K. Sabbagh ✉

Department of Biology, College of Science, Yazd University, Yazd, Iran

F. Khosravi Moghaddam

Department of Plant Protection, Faculty of Agriculture, University of Zabol, Zabol, Iran

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 dendrolog 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.

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 (Table 1). 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	30 11' 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 MgCl₂, 500 mM KCl, pH 8), 1 µL of MgCl₂ (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 *EcoR* 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. variotii* 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 geographic 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 *BamH*I digested the fragment, but showed no polymorphisms among isolates. The highest number of restricted fragment was obtained for the *Aps* I enzyme, whereas the *EcoR* 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' (0.453 and 0.347 respectively). The highest (0.644) and lowest (0.525) I' values were obtained for *Mbo* I and *Msp* I (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	Accession number
Z ₂	96%	<i>Paecilomyces variotii</i> SUMS0303	FJ011547.1
X ₃	98%	<i>Paecilomyces variotii</i> BCC 14365	AY753332.1
R ₁	97%	<i>Paecilomyces variotii</i> KUC5015	GQ241284.1
K ₂	98%	<i>Paecilomyces variotii</i> isolate 15	FJ895878.1
B ₃	99%	<i>Paecilomyces variotii</i> 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).

SUMS0303	TGGAAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAA-GGATCATTACCGA	59
KUC5015	-----TCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAA-GGATCATTACCGA	47
BCC14365	-----GAA-GGATCATTACCGA	16
Z2	-----GTTCCGTAGGTGAACCTGCGGAA-GGATCATTGCGAGC	36
B3	-----GTTCCGTAGGTGAACCTGCGGAA-GGATCGTAAACCT	36
K2	-----GTTCCGTAGGTGAACCTGCGGAA-GGATCATTACCAC	36
X3	-----GTTCCGTAGGTGAACCTGCGGAAAGGATCATTACCGA	37
R1	-----GTTCCGTAGGTGAACCTGCGGAA-GGATCATTACCGA	36
Isolate15	-----TCCGTAGGTGAACCTGCGGAAAGGATCATTACCGA	34
SCSGAF0038	-----TACCGG	6
SUMS0303	GTGAGGGTCC-CACGAGGCCAACCTCCCATCCGTGTTG-AACTACACCTGTTGCTTCGG	117
KUC5015	GTGAGGGTCC-CACGAGGCCAACCTCCCATCCGTGTTG-AACTACACCTGTTGCTTCGG	105
BCC14365	GTGAGGGTCC--ACGAGGCCAACCTCCCATCCGTGTTG-AACTACACCTGTTGCTTCGG	73
Z2	GTGCGGGACC-CACGCAGATACACCCTCCACCCTGTTATAACTACACCTGTTGCTTCGG	95
B3	CCGTGGGTCT-CATGAGTGACAATGCTGCATCCGTGTTG-AACTACACCTGTTGCTTCGG	94
K2	GGGTGGTCCACGCAGAGAAGAACCTCCCATCCGTGTTG-AACTACACCTGTTGCTTCGG	95
X3	GTGAGGGTCA-CGCATATACCAACCTCCCATCCGTGTTG-AACTACACCTGTTGCTTCGG	95
R1	GTGAGGGTCC-CACGAGGCCAACCTCCCATCCGTGTTGAACTACACCTGTTGCTTCGG	95
Isolate15	GTGAGGGTCC-CTCGAGGCCAACCTCCCATCCGTGTTG-AACTACACCTGTTGCTTCGG	92
SCSGAF0038	ATTAGA--TC-CACGAG--CTAACCTCC-ATCCGTGTTG-AACTACACCTGTTGCTTCGG	59
SUMS0303	CGGGCCCGCGTGGTTCACGCCCGCCCGCGGGGGCCTTGTGCTCCCGGGCCCGCGCC	177
KUC5015	CGGGCCCGCGTGGTTCACGCCCGCCCGCGGGGGCCTTGTGCTCCCGGGTCCCGCGCC	165
BCC14365	CGGGCCCGCGTGGTTCACGCCCGCCCGCGGGGGCCTTGTGCTCCCGGGCCCGCGCC	133
Z2	CGGGCCCGTCCGAGTTCACGCCCGCCCGCGGGGGCCTTGTGCTCCCGGGCCCGCGCC	155
B3	CGGGCCCGCGTGGTTCACGCCCGCCCGCGGGGGCCTTGTGCTCCCGGGCCCGCGCC	154
K2	CGGGCCCGCGTGGTTCACGCCCGCCCGCGGGGGCCTTGTGCTCCCGGGCCCGCGCC	155
X3	CGGGCCCGCGTGGTTCACGCCCGCCCGCGGGGGCCTTGTGCTCCCGGGCCCGCGCC	155
R1	CGGGCCCGCGTGGTTCACGCCCGCCCGCGGGGGCCTTGTGCTCCCGGGCCCGCGCC	155
Isolate15	CGGGCCCGCGTGGTTCACGCCCGCCCGCGGGGGCCTTGTGCTCCCGGGCCCGCGCC	152
SCSGAF0038	CGGGCCCGCGTGGTTCACGCCCGCCCGCGGGGGCCTTGTGCTCCCGGGCCCGCGCC	119
SUMS0303	GCCGAAGACCCCTCGAACGCTGCCCTGAAGGTTGCCGTCTGAGTATAAAATCAATCATT	237
KUC5015	GCCGAAGACCCCTCGAACGCTGCCCTGAAGGTTGCCGTCTGAGTATAAAATCAATCATT	225
BCC14365	GCCGAAGACCCCTCGAACGCTGCCCTGAAGGTTGCCGTCTGAGTATAAAATCAATCATT	193
Z2	GCCGAAGACCCCTCGAACGCTGCCCTGAAGGTTGCCGTCTGAGTATAAAATCAATCATT	215
B3	GCCGAAGACCCCTCGAACGCTGCCCTGAAGGTTGCCGTCTGAGTATAAAATCAATCATT	214
K2	GCCGAAGACCCCTCGAACGCTGCCCTGAAGGTTGCCGTCTGAGTATAAAATCAATCATT	215
X3	GCCGAAGACCCCTCGAACGCTGCCCTGAAGGTTGCCGTCTGAGTATAAAATCAATCATT	215
R1	GCCGAAGACCCCTCGAACGCTGCCCTGAAGGTTGCCGTCTGAGTATAAAATCAATCATT	215
Isolate15	GCCGAAGACCCCTCGAACGCTGCCCTGAAGGTTGCCGTCTGAGTATAAAATCAATCATT	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

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 *EcoR* I, *Hpyf* 3 I, *Apa* I, *Hinf* I, *Mbo* I, *Msp* I, *Mse* I restriction endonucleases.

Isolate	Restriction fragment length (bp)						
	<i>EcoR</i> I	<i>Hpyf</i> 3 I	<i>Apa</i> I	<i>Hinf</i> I	<i>Mbo</i> I	<i>Msp</i> I	<i>Mse</i> I
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

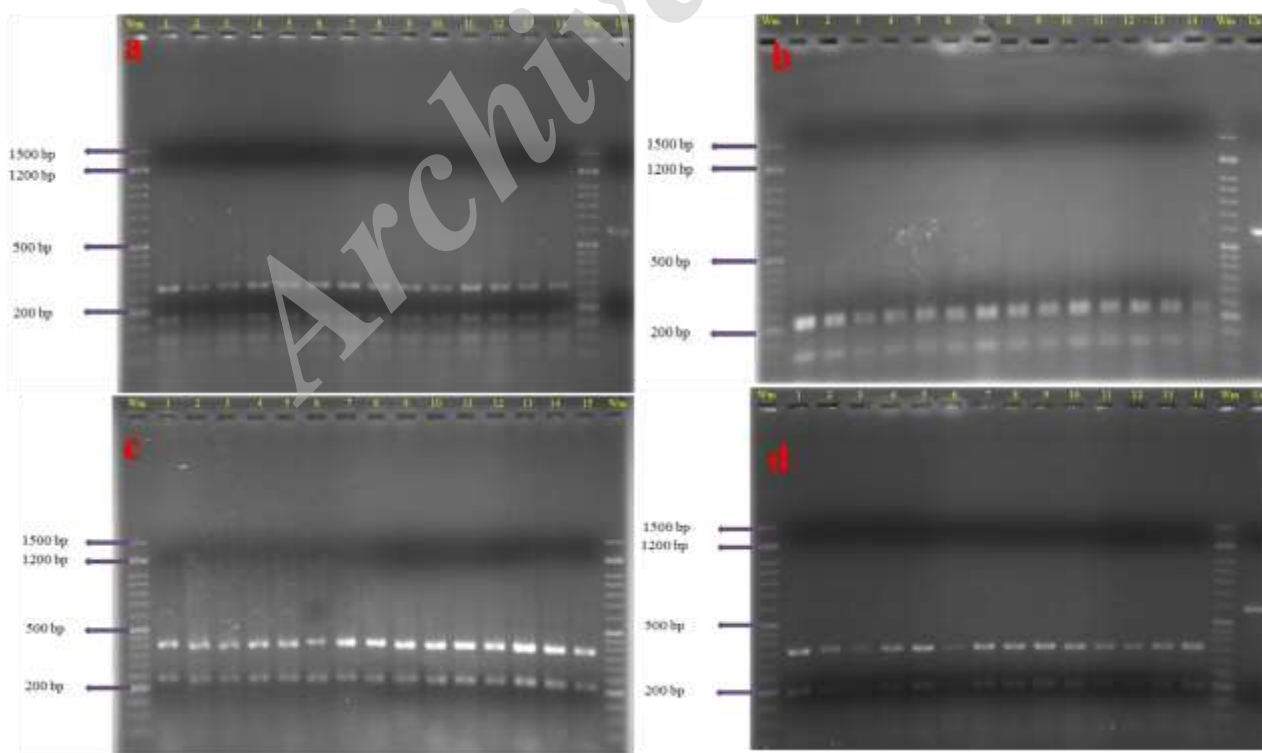


Fig. 3. ITS-RFLP pattern of represented *Paecilomyces variotii* isolates using restriction enzymes. *Apa* I (a); *Mbo* I (b); *Mse* I (c) and *Hpyf*3 I (d). Lin 1: R3; Line 2: R4; Line 3:K1; Line 4: K2; Line 5: K3; Line 6: K4; Line 7: B1; Line 8: B2; Line 9: B3; Line 10: B4; Line 11: T1; Line 12: T2; 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
<i>EcoR</i> 1	28	2	1.696	0.393	0.576
<i>Hinf</i> 1	28	2	1.847	0.453	0.643
<i>Hpyf</i> 31	28	2	1.766	0.428	0.618
<i>Mbo</i> 1	28	2	1.867	0.454	0.644
<i>Msp</i> 1	28	2	1.575	0.347	0.525
<i>Apa</i> 1	28	2	1.737	0.416	0.605
<i>Mse</i> 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

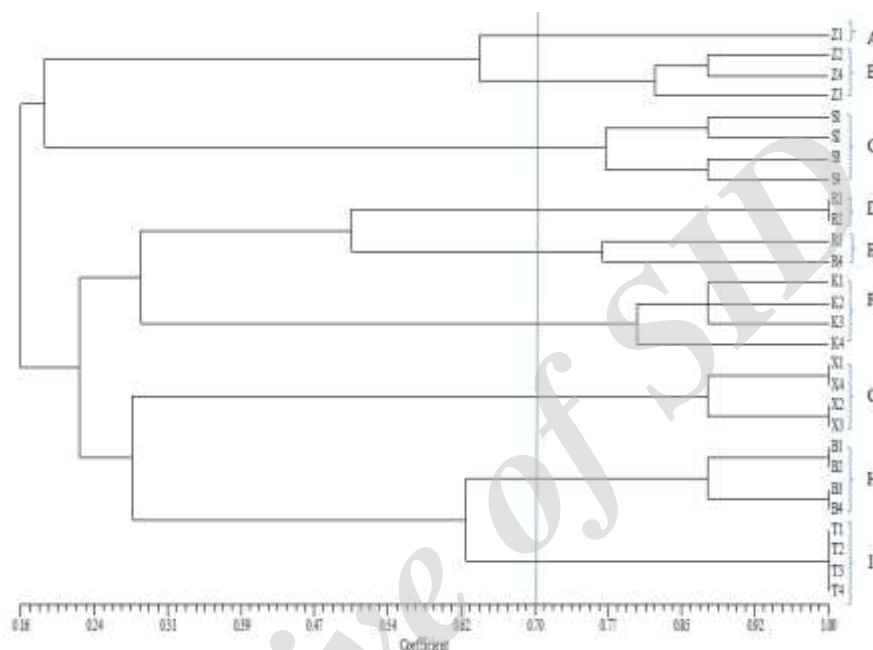


Fig. 4. Dendrogram constructed from analysis of DNA fragments 28 *Paecilomyces variotii* isolates amplified by PCR-RFLP. The matrix was created with the Jacard similarity coefficient, and clustering was performed with UPGMA algorithm.

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 α (EF1 α) 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*,

Natrasia, *Bipolaris*, *Trichoderma*, *Chaetomium*, *Fusarium* and *Cytospora*. Of 180 fungal isolates, 166 isolates were morphologically identified as *Paecilomyces 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-shown).

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. Amplification of RDNA–ITS regions 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 polymorphism (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 *Cryphonectria* (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 phenomenon could increase reliability of this marker to study of genetic variety in fungi with this phenomenon. 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.

ACKNOWLEDGEMENTS

This work was done in institute of plant biotechnology in university of Zabol, Iran. Here, we thank Mrs. Hamideh Khajeh to help us for technical support. Authors declare that the experiments comply with the current laws of Iranian ministry of Science, Research and Technology.

REFERENCES

- Abbas SQ, Maan A, Iqbq J, Niaz M. 2009. A Report of *Paecilomyces variotii* on Human from Pakistan. *Pakistan Journal of Botany* 41: 467–472.
- Aminae MM, Ershad D. 1987. Die-back of young shoots of pistachio trees in Kerman province. *Proceeding of 11th Plant protection Congress., Iran, Rasht, Iran, Pp.* 216.
- Arnheim N, Seperack P, Banerji J, Lang RB, Miesfeld R, Marcu KB. 1980. Mouse rDNA nontranscribed spacer sequences are found flanking immunoglobulin genes and elsewhere throughout the genome. *Cell* 22: 179–185.
- Avis TJ, Hamelin RC, Bélanger RR. 2001. Approaches to molecular characterization of fungal biocontrol agents: some case studies. *Canadian Journal of Plant Pathology* 23: 8–12.
- Azevedo A, Furlaneto MC, Sosa-Gómez DR, Fungaro MHP. 2000. Molecular characterization of *Paecilomyces fumosoroseus* (Deuteromycotina: Hyphomycetes) isolates. *Scientia Agricola* 57:729–732.
- Brown HS, Smith G. 1957. The Genus *Paecilomyces* Bainier and its Perfect Stage *Byssoschlamys* Westlmg. *Transactions of the British Mycological Society* 40:17–59.
- Milgroom M, Lipari S. 1995. Spatial analysis of nuclear and mitochondrial RFLP genotypes in populations of the chestnut blight fungus, *Cryphonectria parasitica*. *Molecular Ecology* 4: 633–642.
- D'Alessandro C, Padin S, Urrutia M, López Lastra C. 2011. Interaction of fungicides with the entomopathogenic fungus *Isaria fumosorosea*. *Biocontrol Science and Technology* 21:189–197
- D'Alessandro CP, Jones LR, Humber RA, López Lastra CC, Sosa - Gomez DR. 2014. Characterization and phylogeny of *Isaria* spp. strains (Ascomycota: Hypocreales) using ITS1 - 5.8 S - ITS2 and elongation factor 1 - alpha sequences. *Journal of Basic Microbiology* 54 (S1).
- Dalleau-Clouet C, Gauthier N, Risterucci AM, Bon M, Fargues J. 2005. Isolation and characterization of microsatellite loci from the entomopathogenic hyphomycete, *Paecilomyces fumosoroseus*. *Molecular Ecology Notes* 5: 496–498.
- Ebrahimi S, Sabbagh SK. 2012. Study on genetic diversity and Pathogenic Progression of *Paecilomyces variotii* isolates in Kerman province, University of Zabol, Zabol. pp. 98.
- Ebrahimi S, Sabbagh SK, Aminaei M. 2015. Genetic diversity of *Paecilomyces variotii* isolates by SSR marker in Kerman province, Iran. *Mycologia Iranica* 2: 45–38.
- Fargues J, Bon M-C, Manguin S, Couteaudier Y. 2002. Genetic variability among *Paecilomyces fumosoroseus* isolates from various geographical and host insect origins based on the rDNA-ITS regions. *Mycological Research* 106: 1066–1074.
- Felsenstein J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39: 783–791.
- Gordon T, Okamoto D, Milgroom M. 1992. The structure and interrelationship of fungal populations in native and cultivated soils. *Molecular Ecology*. 1: 241–249.
- Hoog GS, Guarro J, Gene J, Figueras MJ. 2000. *Atlas of Clinical Fungi*. Centraalbureau voor schimmelcultuur Utrecht, The Netherlands:Pp. 794–811.
- Houbraken J, Varga J, Rico-Munoz E, Johnson S, Samson RA. 2008. Sexual reproduction as the cause of heat resistance in the food spoilage fungus *Byssoschlamys spectabilis* (anamorph *Paecilomyces variotii*). *Applied and Environmental Microbiology* 74: 1613–1619.
- Kiss L. 1997. Genetic diversity in *Ampelomyces* isolates, hyperparasites of powdery mildew fungi, inferred from RFLP analysis of the rDNA ITS region. *Mycological Research* 101: 1073–1080.
- Kohn L. 1995. The clonal dynamic in wild and agricultural plant-pathogen populations. *Canadian Journal of Botany*. 73: 1231–1240.
- Kumar S, Nei M, Dudley J, Tamura K. 2008. MEGA: a biologist-centric software for evolutionary analysis of DNA and protein sequences. *Briefings in Bioinformatics* 9: 299–306.
- Luangsa-ard JJ, Hywel-Jones NL, Samson RA. 2004. The polyphyletic nature of *Paecilomyces sensu lato* based on 18S-generated rDNA phylogeny. *Mycologia* 96: 773–780.
- Michelmore R, and Hulbert S. 1987. Molecular markers for genetic analysis of phytopathogenic fungi. *Annual Review of Phytopathology* 25: 383–404.
- Nicholson P, Rezanoor H, Simpson D, Joyce D. 1997. Differentiation and quantification of the cereal eyespot fungi *Tapesia yallundae* and *Tapesia acuformis* using a PCR assay. *Plant Pathology* 46: 842–856.
- Nilsson RH, Kristiansson E, Ryberg M, Hallenberg N, Larsson K-H. 2008. Intraspecific ITS variability in the kingdom Fungi as expressed in the international sequence databases and its implications for molecular species identification. *Evolutionary Bioinformatics Online* 4: 193.
- Oborník M, Klíč M, Zizka L. 2000. Genetic variability and phylogeny inferred from random amplified polymorphic DNA data reflect life strategy of entomopathogenic fungi. *Canadian Journal of Botany* 78: 1150–1155.

- Purkayastha S. 2005. Race identification of *Macrophomina Phaseolina*, Causal agent of root rot of cluster bean using molecular markers. Ph. D. dissertation. Guru Jambheshwar University, Hisar, India.
- Rostami F, Khosravi Moghaddam F, Sabbagh SK, Saeidi S. 2015. Comparison of PCR-RFLP Based on Ribosomal Regions and SSR Markers in Genetic Diversity of Pistachio Die-Back Caused by *Paecilomyces variotii* Gene, Cell and Tissue 2: e24340.
- Samson RA. 1974. *Paecilomyces* and some allied Hyphomycetes. *Studies in Mycology* 6: 1-119.
- Sneath P, Sokal R. 1973. *Numerical Taxonomy*. Freeman. San Francisco: W. H. Freeman and Company.: 573.
- Swofford DL. 2003. "PAUP* ver 4.0. b10." *Phylogenetic Analysis Using Parsimony and Other Methods*. Sunderland, MA: Sinauer Associates, Sunderland.
- Thompson JD, Gibson T, Higgins DG. 2002. Multiple sequence alignment using ClustalW and ClustalX. *Current Protocols in Bioinformatic*, Chapter 2: Unit 2.3.
- Tigano-Milani MS, Honeycutt RJ, Lacey LA, Assis R, McClelland M, Sobral BW. 1995. Genetic variability of *Paecilomyces fumosoroseus* isolates revealed by molecular markers. *Journal of Invertebrate Pathology* 65: 274-282.
- Tanabe Y, Saikaza M, Watanabe MM, Sugiyama J. 2004. Molecular phylogeny of Zygomycota based on EF-1 [alpha] and RPB1 sequences: limitations and utility of alternative markers to rDNA *Molecular Phylogenetics and Evolution* 30: 438-449.
- White TJ, Bruns T, Lee S, Taylor J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR protocols: a guide to methods and applications*. (MA Innis, DH Gelfand, sninsky J.J. , eds): 315-322. Academic Press, San Diego, USA.

Archive of SID

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

سید کاظم صباغ ✉ و فاطمه خسروی مقدم

۱- گروه زیست شناسی، دانشکده علوم، دانشگاه یزد، یزد

۲- گروه گیاهپزشکی، دانشکده کشاورزی، دانشگاه زابل، زابل

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 شامل 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 برای 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