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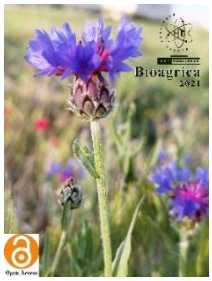

Diversity of *some* Iranian isolates of entemopathogenic fungi, *Beauveria bassiana* by PCR-RFLP *rpb* 1 & 2

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Abstract

Entomopathogenic fungi, especially the genus *Beauveria*, are naturally the cause of disease and death in various insects. Molecular markers provide a valid method for studying the genetic diversity and population genetic structure of most living organisms, especially entomopathogenic fungi such as *B. bassiana*. Twelve isolates of *Beauveria bassiana* were prepared from the soil of 3 different climates of Iran (hot and humid, hot and dry, cold and dry). Grouping of *B. bassiana* isolates was performed using PCR-RFLP markers in *rpb1* and *rpb2* regions. To determine the genetic diversity of isolates after extraction of genomic DNA, the amplified DNA was cut with specific primers *rpb1* and *rpb2* using Hind III restriction enzyme and the pattern of polymorphic bands in agarose gels was 0 And 1 were drawn using MVSP software version 3.22. Trees obtained from *rpb1* and *rpb2* amplified fragments were plotted using the UPGMA clustering method using Simpson's Coefficient. In clustering obtained from *rpb1*, except for Ahvaz isolates, all isolates in the hot and humid climate in the south of the country; Andimeshk, Poldakhtar, and Khorramabad, Yazd, and Kerman from Hot and dry climate were grouped. In this study, a pattern was not obtained under the geographical pattern, nevertheless, special results were obtained. For example, the isolates of Kerman and Yazd in both genes were placed next to each other and Boroujerd, Robatkarim, and Poldakhtar were placed in the same procedure.

1. Introduction

Entomopathogenic fungi infect their hosts through the external cuticle and are pathogenic to insects. They have been widely evaluated as control agents for a diverse variety of noxious arthropods of agricultural (including forestry and livestock) and horticultural importance (Glare *et al*., 2010; Goettel *et al*., 2010). Fungus *Beauveria bassiana* is commonly found in both cultivated and non-cultivated soils, although their natural distribution appears to be related to habitat (Meyling *et al*., 2009). A variety of molecular techniques such as RFLP, T-RFLP, AFLP, and strain-specific microsatellite markers have been used as diagnostic tools allowing fungi to be tracked in the environment (Liu *et al*., 1999; Matheny *et al*., 2002; Schwarzenbach *et al*., 2007; Inglis *et al*.,

2012; Enkerli and Widmer, 2010). A commonly used method is to digest PCR products of specific DNA regions, such as genes or intergenic regions, with restriction enzymes that produce fragments of variable size that can be isolated on the agarose gel. These PCR-RFLPs (finite fragment length polymorphisms) have been used to characterize both Beauveria and Metarhizium species (Rehner *et al*., 2011; Imoulan, *et al*., 2016).

Advances in the use of PCR techniques provide highly specific methods of monitoring fungal populations in 'real time' and in a quantitative manner, in soils, insects, and plants (Wang *et al*., 2004; Entz *et al*., 2005; Castrillo *et al*., 2008; Meyling *et al*., 2009; Enkerli and Widmer, 2010; Sedaghatfar *et al*., 2012; Inglis *et al*., 2012; Seddigh and Darabi, 2018). A multilocus phylogeny of Beauveria based on partial sequences of *rpb1*, *rpb2*, TEF, and the nuclear intergenic region, Bloc, was used by Rehner *et al*. (2011) to assess diversity within the genus Beauveria and to evaluate species concepts and their taxonomic status. Molecular studies based on multilocus phylogenetic analysis that included the Bloc nuclear intergenic region, internal transcribed spacer (ITS), translation elongation factor-1α (TEF), and RNA polymerase II largest subunit (*rpb1*) and second largest subunit (*rpb2*) demonstrated that Beauveria is composed of 26 species (Rehner *et al*. 2011, Sanjuan *et al*. 2014, Kepler *et al*. 2017, Chen *et al*. 2017). Multilocus phylogeny and five DNA-based methods to delimit species in Beauveria using three genes were sequenced, i.e., Bloc, *rpb1*, and tef1 (Bustamante *et al*., 2019). Phylogenetic analyses based on partial sequence of ITS, TEF, RBP1, *rpb2*, and the nuclear intergenic region Bloc, were realized and led to the description of a new species, *B. mimosiformis* sp. nov., isolated from Coleoptera larva in the North of Thailand (Khonsanit *et al*., 2020).

Costa *et al*. (2011) in the process of analyzing the genetic diversity of *Beauveria bassiana* used of internal-transcribed-spacer ribosomal region restriction fragment length polymorphism (ITS-RFLP) but they did not reveal characteristic markers to differentiate isolates. Zhang *et al*. Also, the *rpb2* and tef1 sections were used for the taxonomy of this group (Zhang *et al*. 2006). Agrawal *et al*. (2014) Using four genomic fragments in *rpb1*, *rpb2*, TEF, Bloc and, ITS regions, were able to introduce a new species of *Beauveria rudraprayagi* from sugarcane fields to control *Hoplochelus marginalis* (Agrawal *et al*. 2014). Robène-Soustrade *et al*. (2015) also introduced four new genera *Beauveria hoplocheli* from sugarcane fields to control *Hoplochelus marginalis* using four genomic fragments from *rpb1*, *rpb2*, TEF, Bloc, and ITS (Robène-Soustrade *et al*. 2015).

2. Materials and methods

2.1. Soil and specimen collection and fungus isolation

Isolates of B. were collected from the soil of different regions in Iran, including: 1-Quchan, 2-Arak, 3-Qom, 4-Astaneh, 5-L, 6-e, 7-salafchegan, 8-Kerman, 9-Mashhad, 10-Robatkarim, 11-Isfahan, 12-Shiraz, 13-P, 14-Poldokhtar, 15-Yazd, 16-Yassuj, 17-Shahryar, 18-Farahan, 19-Borojerd, 20- Andimeshk, 21-khoramabad.

2.2. Isolating the fungi from the soil

Soil samples were collected from 12 regions, including three different climates; Warm and humid climate; locations within 1- ahvaz, 2- Andimeshk, 3- Khoramabad, 4- Borojerd, Hot and dry climate: 5- Kerman, 6- Mashhad, 7- Yazd, and Cold and dry climate locations within 8-Robatkarim,

9- Esfahan, 10, Poldokhtar, 11- Farahan and 12-Astaneh. Beauveria strains were isolated from the soil samples using the former study's baiting method (Zimmermann, 1986) from formerly study (Arshadi *et al*., 2020). Conidia developing on insect cadavers were transplanted onto plates of sabouraud dextrose agar and cultured at 25 °C. Colonies of the isolated filamentous fungi appearing in the culture were transferred onto fresh PDA media.

2.3. DNA extraction

The fungal strains were cultured on sabouraud dextrose agar (SDA) medium and incubated at 25 ± 1 °C with a 12 h light and 12 h dark cycle. A small portion of mycelium was harvested with a sterile loop from one-week-old cultures and placed in a 1.5 mL microcentrifuge tube. DNA was extracted using Iraizol –DNA extraction buffer (Zist Fanavaran® RENA-Esfahan Kit-Iran) according to the manufacturer's instructions, and the supernatants that contained the genetic material were transferred to a 1.5 mL microcentrifuge tube. DNA samples were stored at -20 °C until use. Partial sequences of two nuclear protein-coding genes were amplified as follows: *rpb1* and *rpb2*, using primer pairs *rpb1*Af and *rpb1*A_VH6R (Stiller and Hall, 1997; Matheny *et al*., 2002) and *rpb2*-5F and *rpb2*-7cR (Liu *et al*., 1999), respectively.

Reaction conditions were the same as those used by Bischoff *et al*. (2009). PCR product size was examined and confirmed by agarose gel electrophoresis. After measuring the DNA quality using nanodrops, the strength of the resulting DNA band was compared with the bands obtained from a standard molecular weight marker using electrophoresis.

2.4. Polymerase chain reaction (PCR)

The primers used in the phylogenetic studies were as described in Table 1, prepared by Takapouzist® company.

Table 1. Primers used to perform PCR reaction.

Locus	Primers	Sequence (5' → 3')	Nucleotide NO.	Reference
rpb1	*rpb1*Af	GAGTGTCCAGGTCATTTTGG	20	Modified from Matheny *et al*. 2002
	*rpb1*A_VH6R	ATGACCCATCATGGATTCCTTGTG	24	Modified from Stiller and Hall 1997
rpb2	*rpb2*_5F	GACGACAGAGACCACTTTGG	20	Modified from Liu *et al*., 1999
	PB2A_7cR	CCCATCGCTTGTTTACCCAT	20	Modified from Liu *et al*., 1999

The PCR program consisted of an initial cycle of denaturation at 94 °C for 5 min; followed by 35 cycles of denaturation at 94 °C for 30 s, 45 s at 50 °C (*rpb2*), and 56 °C (*rpb1*), 1 min at 72 °C and a final elongation step at 72 °C for 5 min. The reaction mixture contained 1 reaction buffer (500 mM KCl; 100 mM Tris–HCl, pH 9.20 at 25 °C; 1% Triton X-100 without magnesium); 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.3 lM each primer; 1U of Taq DNA Polymerase (Zist Fanavaran® RENA-Iran) and 50 ng of template DNA per reaction, in a final volume of 15 lL. Amplification products were resolved by electrophoresis on a 1% w/v agarose gel supplemented with a safe stain (100 ng ml^{-1} final concentration).

2.5. PCR-RFLP molecular marker data (polymerase chain reaction-based restriction fragment length polymorphism)

Image of electrophoresis gels from primers that produced polymorphic bands with appropriate resolution, after placement of DNA product amplified with *rpb1* and *rpb2* primers using Hind III restriction enzyme (Takapouzist®-Iran) at 37°C was placed for one hour and then placed at 80 °C for 20 minutes to inactivate the enzyme. This enzyme cuts two strands of DNA from the A↓AGCTT site. Based on the presence of n, the number of these loci along the amplified strand will be n + 1. After amplification of DNA fragments using *rpb1* and *rpb2* primers, PCR products were cut with a restriction enzyme, electrophoresis was performed and gel images were photographed using a gel-document. Then, the presence or absence of a band in each sample was scored. The scoring of strips and scoring was done in the presence (1) and non-presence (0) in comparison with the leader (DNA marker) for each strip. The presence of polymorphisms in the composition of different primers used in all isolates was investigated. The stripe pattern of polymorphic strips in agarose gels was scored 1 and 0 using version 3.22 MVSP (MultiVariate Statistical Package - Kovach) software. Finally, the obtained data matrix was ranked on 12 isolates and the obtained data were analyzed using this software. Simpson's Coefficient was used based on the UPGMA clustering method. In the study of genetic diversity, the main criteria for selecting markers are their degree of polymorphism, reproducibility, and degree of validity.

DNA markers show differences in the level of DNA molecules and have high polymorphism, random distribution in the genome, the ineffectiveness of environmental factors, phenotypic neutrality, high frequency, independent dependence on growth stage, tissue, and organ, and can be evaluated at any stage and in the laboratory. Today, a wide range of DNA markers are available that must be selected based on criteria such as their genomic location (coding, non-coding), and their relative distribution in the genome (in the case of markers with a specific genomic location). In the study of genetic diversity, the main criteria for the selection of markers are their degree of polymorphism, reproducibility, and degree of validity.

2.6. Cluster analysis

Cluster analysis is one of the most common multivariate methods in the study of genetic diversity and grouping of individuals and populations. This method is used in cases where there is no previous pattern for the studied genetic material. Several algorithms have been proposed for cluster analysis. These algorithms are divided into two main groups: (1) distance-based methods in which distance or similarity matrices of two individuals or populations are used for grouping, and (2) Model-based methods applications in which it is assumed that individuals within each cluster are random observations of some parametric models and statistical inferences related to the parameters of each cluster and determination of membership in each cluster using standard statistical methods such as maximum proofreading is done (Pritchard *et al*., 2000).

3. Results and discussion

3.1. Quality and quantity of extracted DNA

Several factors can affect the quality and quantity of extracted DNA. These factors are present in the sample collection stages up to the final stages of DNA extraction. To evaluate the DNA extraction protocol as well as the quality and concentration of DNA in agarose gel and nanoprop, the extracted DNAs were loaded. The results of the observations of the agarose gel made and the nanodrop device indicated that the quality of the extracted DNA samples were desirable for the continuation of the DNA-Homology application process.

3.2. Amplifying and cutting of the isolates' DNA

For grouping of *B. bassiana* isolates, the DNA of each isolate was amplified by primers of the *rpb1* based on RFLP-*rpb1* and RFLP-*rpb2* primers, respectively, then the fragments of 12 isolates cut with the help of Hind III restriction enzyme. It showed a variety of band patterns as shown in Figure 1.

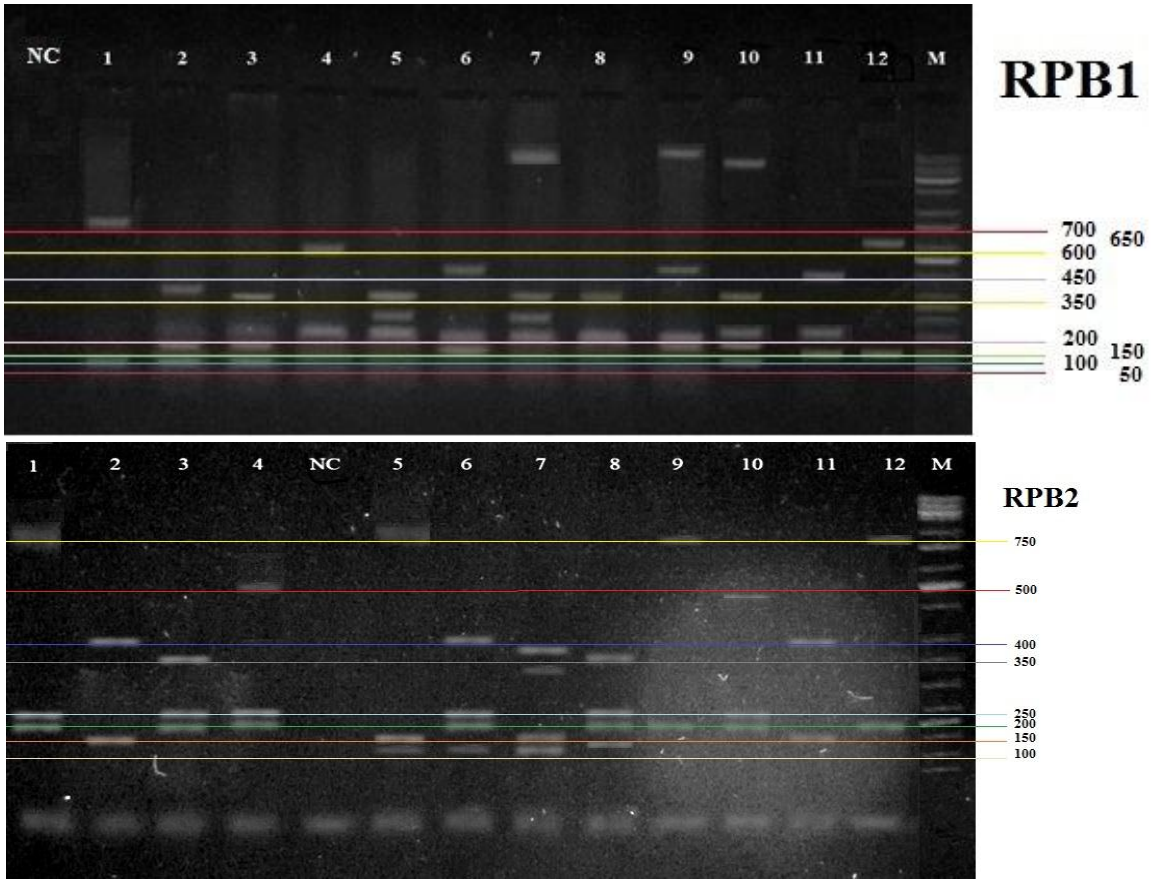
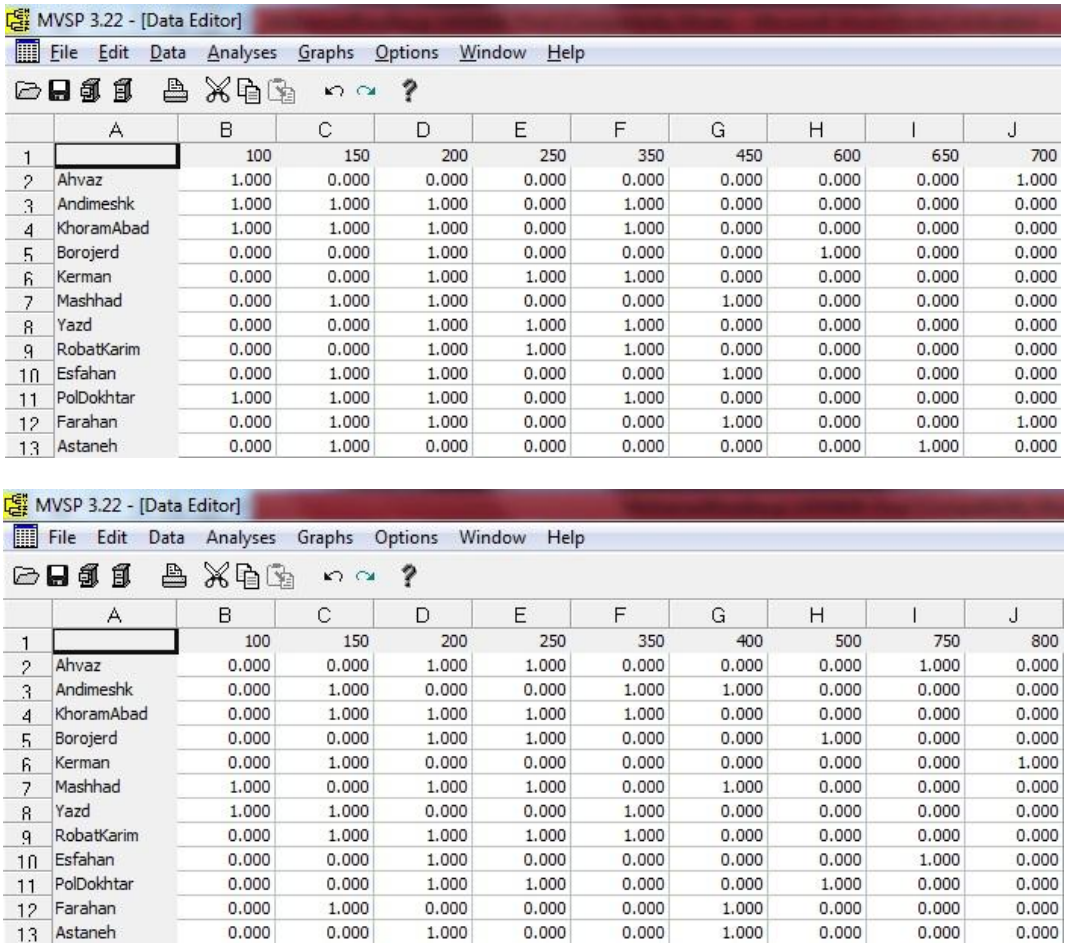


Figure 1. Band pattern obtained from electrophoresis of amplified DNA of Beauveria isolates with *rpb1* (up) and *rpb2* (down) primer pair in 1.5% agarose gel after cutting with Hind III enzyme. M: 100 bp molecule marker, NC: control

The matrix obtained from the amplified and cutting *rpb1* and *rpb2* based on the size of the fragments of 12 isolates via MVSP software, respectively is shown in Figure 2.



MVSP 3.22 - [Data Editor]

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	A	B	C	D	E	F	G	H	I	J
1		100	150	200	250	350	450	600	650	700
2	Ahvaz	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000
3	Andimeshk	1.000	1.000	1.000	0.000	1.000	0.000	0.000	0.000	0.000
4	KhoramAbad	1.000	1.000	1.000	0.000	1.000	0.000	0.000	0.000	0.000
5	Borojerd	0.000	0.000	1.000	0.000	0.000	0.000	1.000	0.000	0.000
6	Kerman	0.000	0.000	1.000	1.000	1.000	0.000	0.000	0.000	0.000
7	Mashhad	0.000	1.000	1.000	0.000	0.000	1.000	0.000	0.000	0.000
8	Yazd	0.000	0.000	1.000	1.000	1.000	0.000	0.000	0.000	0.000
9	RobatKarim	0.000	0.000	1.000	1.000	1.000	0.000	0.000	0.000	0.000
10	Esfahan	0.000	1.000	1.000	0.000	0.000	1.000	0.000	0.000	0.000
11	PolDokhtar	1.000	1.000	1.000	0.000	1.000	0.000	0.000	0.000	0.000
12	Farahan	0.000	1.000	1.000	0.000	0.000	1.000	0.000	0.000	1.000
13	Astaneh	0.000	1.000	0.000	0.000	0.000	0.000	0.000	1.000	0.000

MVSP 3.22 - [Data Editor]

File Edit Data Analyses Graphs Options Window Help

	A	B	C	D	E	F	G	H	I	J
1		100	150	200	250	350	400	500	750	800
2	Ahvaz	0.000	0.000	1.000	1.000	0.000	0.000	0.000	1.000	0.000
3	Andimeshk	0.000	1.000	0.000	0.000	1.000	1.000	0.000	0.000	0.000
4	KhoramAbad	0.000	1.000	1.000	1.000	1.000	0.000	0.000	0.000	0.000
5	Borojerd	0.000	0.000	1.000	1.000	0.000	0.000	1.000	0.000	0.000
6	Kerman	0.000	1.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000
7	Mashhad	1.000	0.000	1.000	1.000	0.000	1.000	0.000	0.000	0.000
8	Yazd	1.000	1.000	0.000	0.000	1.000	0.000	0.000	0.000	0.000
9	RobatKarim	0.000	1.000	1.000	1.000	1.000	0.000	0.000	0.000	0.000
10	Esfahan	0.000	0.000	1.000	0.000	0.000	0.000	0.000	1.000	0.000
11	PolDokhtar	0.000	0.000	1.000	1.000	0.000	0.000	1.000	0.000	0.000
12	Farahan	0.000	1.000	0.000	0.000	0.000	1.000	0.000	0.000	0.000
13	Astaneh	0.000	0.000	1.000	0.000	0.000	1.000	0.000	0.000	0.000

Figure 2. Display of the matrix obtained from cutting the amplified and cut parts of *rpb1* (Up) and *rpb2* (Down) on agarose gel in MVSP 3.22 software.

3.3. Clustering of *B. bassiana* isolates

The data matrix obtained from 12 isolates was analyzed using MVSP statistical software version 3.22. Based on the UPGMA clustering method and using Simpson's Coefficient: They showed a diverse band pattern. Grouping was performed based on PCR-RFLP of *rpb1* and *rpb2* genes. For *rpb1* the first group was: Astana, Farahan, Isfahan, and Mashhad, the second group was: Boroujerd, Robatkarim, Yazd, Kerman, Poldakhtar, Khorramabad, Andimeshk, and in the third group was just Ahvaz placed as showed in Figure 3 (up). The interesting point of this grouping was the placement of 4 isolates in the southeast of the country in one group 2. The isolate Ahvaz in this grouping was still determined as an independent group that was not related to other isolates. For PCR- RFLP *rpb2* two clusters were obtained from PCR-*rpb2* cluster analysis. The groups were: first group: Kerman, Yazd, Farahan, and Andimeshk, and the second group: Astana, Mashhad, Poldakhtar, Boroujerd, Robatkarim, Khorramabad, Isfahan, and Ahvaz. Yazd and Kerman were in the same group.

4. Conclusions

Although in this study, a pattern was not obtained under the geographical pattern, nevertheless, special results were obtained. For example, the isolates of Kerman and Yazd from

Hot and dry climates in both genes were placed next to each other and Boroujerd, Robatkarim, and Poldakhtar were placed in the same procedure.

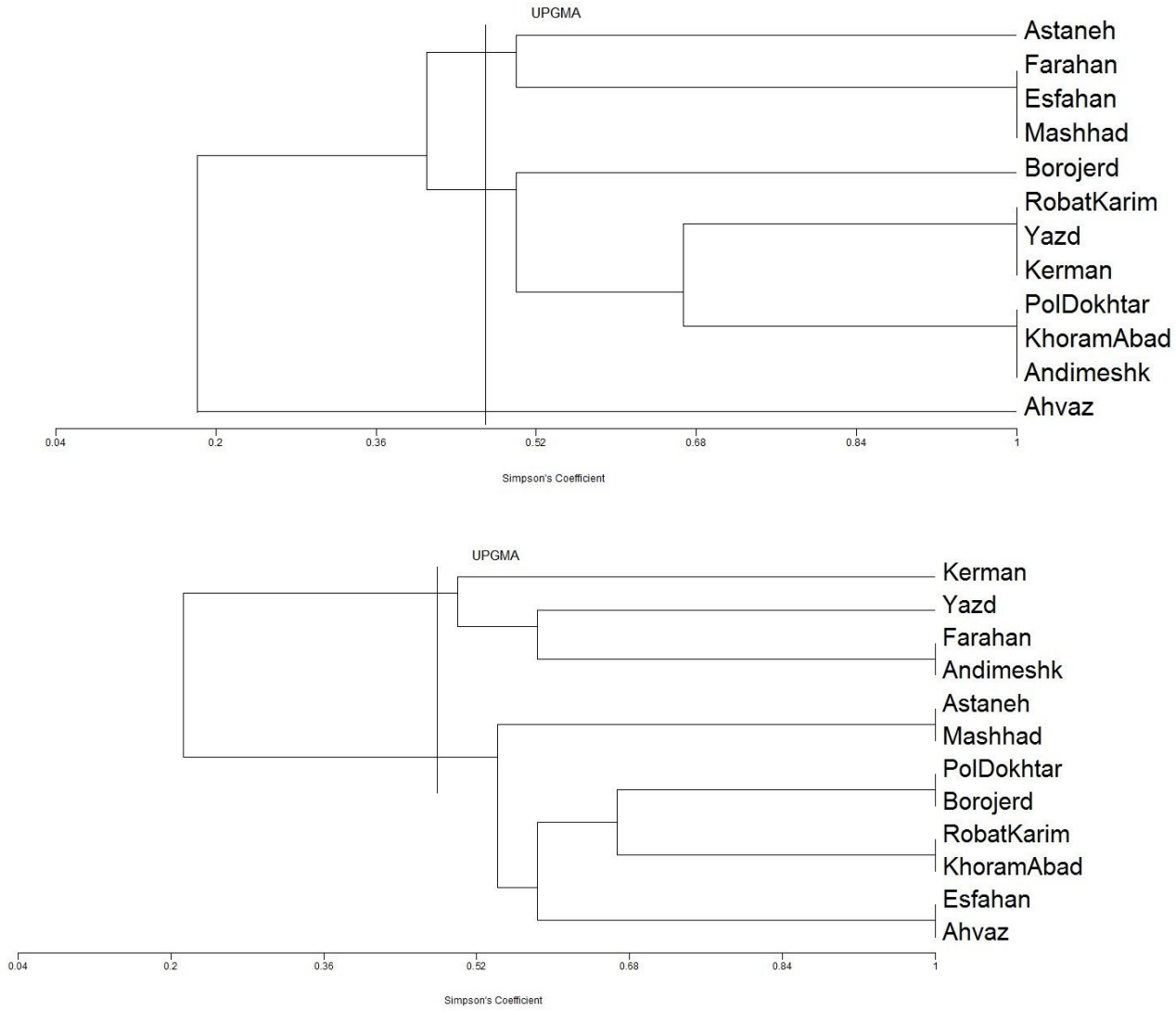

Figure 3. Clustering and plotting of tree sections from amplified parts of *rpb1* (Up) and rpb2 (Down) of *B. bassiana* isolates in MVSP 3.22 software by UPGMA clustering method using Simpson's Coefficient similarity coefficient and distance. The isolates were divided into 3 groups based on PCR-RFLP *rpb1*, and 2 groups based on PCR-RFLP *rpb2*.

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