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Genetic diversity among *Brenneria nigrifluens* strains in Iran

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Abstract To investigate the variability of *Brenneria nigrifluens*, the casual agent of shallow bark canker of Persian walnut (*Juglans regia* L.), a collection of 24 strains isolated from five geographic regions, was analyzed by means of three marker systems, repetitive polymerase chain reaction (rep-PCR), insertion sequence (IS50)-PCR and random amplified polymorphic DNA (RAPD). Cluster analysis was performed using UPGMA. Strains were differentiated into 6 groups at about 80% similarity according to geographic regions. This is possibly due to cultivation of Persian walnut being mainly based on the ecotype and/or local seedlings that have become adapted to particular environments and so have allowed selection of different *B. nigrifluens* populations. The results of this study showed that the four rep-PCR primers produced 75 products of which 73.3% were polymorphic, eight RAPD primers produced 146 fragments of which 74.6% were polymorphic and IS50 produced 32 fragments of which 93.75% were polymorphic. The usefulness of each system was examined in terms of polymorphism information content (PIC) and marker index (MI). The highest MI was observed for IS50-PCR (21.11) followed by RAPD (7.85), and rep-PCR (6.92). The Mantel test identified significant correlation between the similarity coefficients calculated from them. Among the molecular markers tested, IS50-PCR appears to be a more suitable marker for fingerprinting and assessing genetic relationships among *B. nigrifluens* strains. This is the first study on genetic diversity of *B. nigrifluens*. The results can have a bearing on the choice of disease management strategies.

Keywords Persian walnut · RAPD · rep-PCR · IS50-PCR · Shallow bark canker

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Introduction

Brenneria nigrifluens (Wilson et al. 1957; Hauben et al. 1998) is the casual agent of bark canker or the shallow bark canker of Persian walnut (*Juglans regia* L.) trees. This disease is one of the most prevalent diseases of walnut (Wilson et al. 1957) that leads to slow decline in growth and productivity. The disease affects the bark of trunk and scaffold branches of young nursery plants (Saccardi et al. 1998) and adult trees (Piccirillo 2003). Initially, small circular spots develop in cortical tissues and by the extension and

coalescence of a large number of lesions, extensive irregular cankers are formed. A brownish watery exudate oozes out the cankers of the bark. The cankers are restricted to the outer bark, however, in severe cases, they can deepen and encompass the entire depth of the bark resulting in cracking and rotting of the bark tissues and eventual girdling, decline and occasionally death of the affected scaffold branches or the entire tree (Harighi and Rahimian 1997; Rahimian 1989).

Decades after the occurrence of the disease in California (Wilson et al. 1957), it was recorded in Iran (Rahimian 1989), Spain (Lopez et al. 1994), Italy (Saccardi et al. 1998) and France (Menard et al. 2004). In Iran, the shallow bark canker of walnut has been reported from different regions including Mazandaran (Harighi and Rahimian 1997), Kerman (Baradaran and Ghasemi 2004), Kurdistan (Harighi 2006), Guilan and Golestan (Jamalzade et al. 2009) and Fars and Kohgiluyeh-va-Boyerahmad (Yousefi Kopaei et al. 2007).

The assessment of the genetic variability of a micro-organism can facilitate investigation on its taxonomy, epidemiology and detection (Milgroom and Fry 1997) and is of prime importance in breeding programs in the search for tolerant or resistant germplasms (Norelli et al. 1984). Little is known about the possible heterogeneity and genetic relationship among isolates of *B. nigrifluens*; the work of Harighi and Rahimian (1997) has demonstrated the existence of some variation in electrophoretic profile of cell proteins of strains isolated from two northern provinces of Iran.

Naturally occurring interspersed repetitive DNA elements, found in many bacteria, can serve as primer sites for genomic DNA amplification (Versalovic et al. 1991, 1994). Three families of repetitive sequences including repetitive extragenic palindromic (REP) sequence, enterobacterial repetitive intergenic consensus (ERIC) sequence, and BOX element (Versalovic et al. 1994) have been identified and studied in some detail. Genome organization is thought to be shaped by selection, and thus the dispersion of the REP, ERIC and BOX sequences may be indicative of the structure and evolution of the bacterial genome (Louws et al. 1994). These sequences appear to be located in distinct, intergenic positions all around the chromosome and the use of the respective primer(s) in PCR could lead to the selective amplification of distinct genomic regions located between REP, ERIC or BOX sequences.

Insertion sequence (IS) elements of various families, as movable DNA sequences carrying only transposition functions, are widespread in genomes of bacteria and other organisms (Berg and Howe 1989). These IS elements have been used as DNA markers to study the genetic variation, genomic rearrangement and genome plasticity of bacteria (Mahillon and Chandler 1998). IS50 from the transposon *Tn*5 has been used as primer for the assessment of genetic diversity in several bacterial genera including Pseudomonads (Weingart and Volksch 1997; Noble et al. 2006).

Random amplified polymorphic DNA (RAPD), is a semi-quantitative method which has been used widely in genetic mapping, taxonomy and phylogeny (Welsh and McClelland 1990; Bassam et al. 1992). It has served as the marker of choice for evaluation of genetic variability in almost any group of organisms especially in cases where information on DNA sequence have been lacking (Welsh and McClelland 1990). These markers have technical differences in cost and speed differ in the degree of polymorphism, precision of genetic distance estimates and the inherent statistical power of tests provided.

The purposes of this study were to examine genetic diversity among *B. nigrifluens* strains by rep-PCR, IS50-PCR and RAPD analysis and to compare the discriminative power of these markers in elucidating the degree of heterogeneity in the population of *B. nigrifluens*.

Materials and methods

Bacterial strains

The strains of *B. nigrifluens* used in this study were isolated from infected samples collected from different geographic regions of Iran including Kerman, Mazandaran and Golestan provinces. Eleven strains previously isolated from walnut trees in Fars and Kohgiluyeh-va-Boyerahmad provinces (Yousefi Kopaei et al. 2007) were received from S. M. Taghavi (College of Agriculture, Shiraz university) (Table 1).

The strains were identified as putative *Brenneria nigrifluens* using conventional phenotypic tests (Schaad et al. 2001; Wilson et al. 1957). All strains were Gram-negative, oxidase negative and had a fermentative type of metabolism. The strains caused water-soaked cankers around the inoculation wounds on

Table 1 Name, source and year of isolation of *Brenneria nigrifluens* strains utilized in this study

Strains	Source	Year isolated
K1	Kerman-Baft	2008
K2	Kerman- Baft	2008
K3	Kerman- Baft	2008
K6	Kerman- Baft	2008
K9	Kerman- Baft	2008
M1	Mazandaran- Neka	2008
M2	Mazandaran- Badeleh	2008
M4	Mazandaran- Badeleh	2008
M11	Mazandaran- Rostam Kola	2008
G2	Golestan- Gorgan	2008
G7	Golestan- Minoodasht	2008
G14	Golestan- Daland	2008
G16	Golestan- Daland	2008
F1	Fars- Bovanat	2001
F2	Fars- Bovanat	2001
F3	Fars- Eqlid	2001
F4	Fars- Eqlid	2001
F5	Fars- Eqlid	2001
F6	Fars- Dashtak	2001
F7	Fars- Dashtak	2001
F8	Fars- Hesarak	2001
F9	Fars- Hesarak	2001
F10	Fars- Mansour-Abad	2001
KO	Kohgiluyeh-va-Boyerahmad- Yasuj	2001

6–12 month old Persisan walnut seedlings. The identity of strains as *B. nigrifluens* was confirmed by subjecting them to PCR amplification. Strains generating a 255 bp fragment in PCR using primer pairs for *B. nigrifluens* (Loreti et al. 2008) were selected and used for analysis of genetic variation. All bacterial strains were cultured on nutrient agar at 28°C for 48 h and stored in glycerol (40% final concentration) at −70°C.

DNA extraction and rep-PCR, IS50-PCR and RAPD analysis

Genomic DNA was extracted using the AccPrep® Genomic DNA Extraction Kit (Bioneer, Korea) following the manufacturer's instructions. PCR was performed in a final volume of 20 µl containing 160 µM deoxynucleoside triphosphate (dNTPs), 2.5 mM $MgCl_2$, 30 pmol of each primers for rep-PCR and IS50 (Table 2), 2 µl of 10 × PCR buffer (100 mM Tris-HCl, 500 mM KCl (pH=8.4)), 2.5 U *Taq* polymerase (CinnaGen, Iran) and 60 ng of template DNA. The reaction was performed in a thermocycler (Mastercycler® gradient, Eppendorf, Hamburg, Germany).

Rep-PCR (Versalovic et al. 1991, 1994) was performed under the following conditions: initial denaturation at 94°C for 7 min; 35 cycles of denaturing at 94°C for 1 min, annealing at 43–51°C for different primers (Table 2), for 1 min and

Table 2 Primer names and sequences, annealing temperature, number of generated bands and degree of polymorphism of amplified DNA for each primer using rep-PCR, IS and RAPD analysis of *Brenneria nigrifluens* strains

Marker	Primer	Sequence	Annealing temperature	No. bands generated	% polymorphism
rep-PCR	REP-1R	5′- IIIICgICgICATCIggC -3′	43°C	22	77
	ERIC-1R	5′-ATGTAAGCTCCTGG GGATTCAC-3′	47°C	21	95
	ERIC-2I	5′ -AAGTAAGTGACTGG GGTGAGCG -3′	47°C	15	53
	BOX- A1R	5′ -CTACGGCAAGGCG ACGCTGACG -3′	51°C	17	58
IS50	IS50	5′ -CAGGACGCTACTTGTGT -3′	36.1°C	32	93
RAPD	OPA-03	5′-AGGTGACCGT -3′	33.3°C	11	73
	OPA-09	5′-CACGCCCTTC -3′	36.5°C	18	50
	OPA-11	5′-TGGACCGGTG -3′	34.5°C	10	60
	OPA-12	5′-GAGAGCCAAC-3′	34.5°C	19	84
	203	5′-CACGGCGAGT-3′	45.4°C	22	90
	211	5 -GAAGCGCGAT-3	38.7°C	23	74
	230	5′-CGTCGCCCAT-3′	36.6°C	20	85
	232	5′-CGGTGACATC-3′	38.7°C	23	70

extension at 70°C for 3 min. Final extension was performed at 72°C for 10 min and the reactions products were held at 4°C until used.

The PCR thermal profile for IS50 (Ullrich et al. 1993) consisted of an initial denaturation at 94°C for 4 min, followed by 30 cycles of denaturing at 94°C for 1 min, annealing at 36.1°C for 1 min and extension at 72°C for 2.5 min. The samples were subjected to a final extension at 72°C for 10 min.

Randomly amplified polymorphic DNA (RAPD) was performed by using eight 10-mer oligonucleotides which were selected from a set of 27 primers on the basis of their capability to produce polymorphic bands in a preliminaly evaluation. PCR was performed in a final volume of 20 μl containing 160 μM dNTPs, 2 mM $MgCl_2$, 15 pmol of each primers (Table 2), 2 μl of 10 × PCR buffer (100 mM Tris-HCl, 500 mM KCl (pH=8.4)), 1.25 U *Taq* polymerase (CinnaGen, Iran) and 60 ng of template DNA. PCR amplification was performed under the following conditions: an initial denaturation at 94°C for 5 min, followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 33.3–45.4°C for different primers (Table 2), for 1 min, and extension of 72°C for 2 min and a final elongation step for 10 min at 72°C.

PCR products were separated by electrophoresis on 1.3–1.5% agarose gel in 1 × TBE buffer (100 mM Tris, 500 mM boric acid and 1 mM EDTA), at 85 V for 45 min and stained with ethidium bromide. A 1 Kb GeneRuler™ ladder (Fermantas, Lithuania) was used as a size marker. All PCR assays were repeated at least twice.

Data analysis

DNA bands obtained on the gel were scored as present (1) or absent (0) and the readings were processed as a binary matrix. The similarity of all pair-wise combinations was determined using Jaccard's coefficient (Jaccard 1908) and clustering were performed by Unweighted Pair Group Method with Arithmetic Mean (UPGMA) using MVSP software. Bootstrap values were calculated using the WINBOOT software.

The Mantel test (Mantel 1967) was used to analyze the correlation existing among similarity matrices calculated by different methods. The test that assesses by means of product-moment correlation (r) was performed using the NTSYS-pc package (Rohlf 2000).

To determine the utility of each of the marker systems, polymorphism information content (PIC) (Lynch and Walsh 1998), effective multiplex ratio (EMR) and marker index (MI) were calculated according to Powell et al. (1996).

Result

Rep-PCR analysis

ERIC, REP and Box primers gave a total of 75 well-resolved bands as reproducible genomic PCR profiles. The number of fragments varied from 15 to 22 and 200–5,000 bp in number and size, respectively (Table 2). Patterns generated by ERIC and Box primers were more discriminative than those of REP-PCR in differentiating *B. nigrifluens* strains (Fig. 1b). The dendrogram obtained from the combined data for all primers is shown in Fig. 2a. UPGMA analysis indicated that *B. nigrifluens* strains could be differentiated into 7 groups at the 81% similarity level according to geographic regions. Clusters I and IV was comprised of three and two strains from Kerman, respectively, clusters II and III each comprised two strains from Golestan, cluster V were formed of four strains from Mazandaran, cluster VI encompassed five strains from Fars and one strain from Kohgiluyeh-va-Boyerahmad, and cluster VII contained five strains from Fars.

IS50 analysis

IS50-PCR generated 32 distinct DNA fragments ranging from approximately 250–3,500 bp in size (Fig. 1a; Table 2). *B. nigrifluens* strains were differentiated into four clusters at 56% similarity level (Fig. 2b). Cluster I included three strains from Golestan, cluster II comprised of one strain from Mazandaran, cluster III contained three strains from Mazandaran and four strains from Kerman and cluster IV was composed of 10 strains from Fars and one strain from Kohgiluyeh-va-Boyerahmad.

RAPD analysis

PCR amplification using eight primers resulted in the production of 146 different RAPD fragments, 10 to 23 fragments in each profile ranging from 150 to 4,000 bp in size (Table 2; Fig. 1c and d). Analysis of RAPD data differentiated *B. nigrifluens* strains into 6

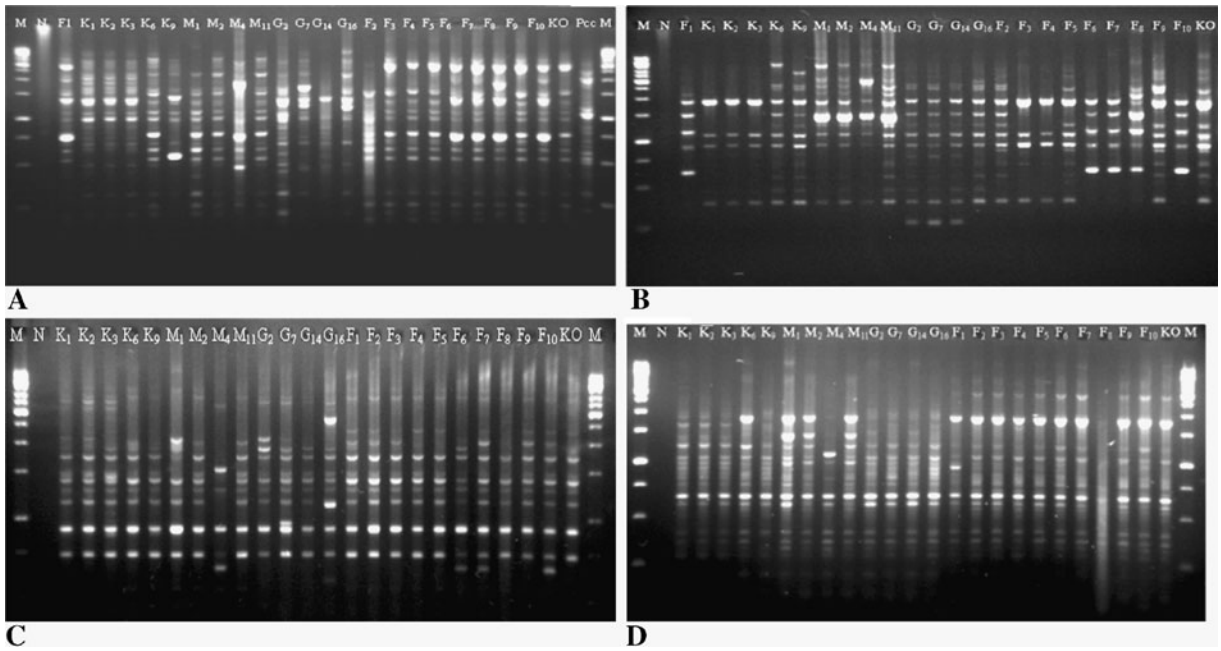

Fig. 1 PCR fingerprintig patterns of genomic DNA of *Brenneria nigrifluens* strains from different geographic regions of Iran generated by IS50 (**a**), ERIC-2I (**b**), 211 (**c**) and 230 (**d**) primers. *N* Negative control; *K* Kerman; *M* Mazandaran; *G* Golestan; *F* Fars; *KO* Kohgiluyeh-va-Boyerahmad; *M* molecular marker GeneRuler™ 1 kb DNA ladder (Fermantas, Lithuania)

groups at the 79% similarity level (Fig. 2c). Clusters I and IV included two and three strains from Kerman, respectively, the four strains collected from Golestan formed separately clusters II and III, each comprised two strains, cluster V comprised of 10 strains from Fars and one strain from Kohgiluyeh-va-Boyerahmad, and cluster VI included four strains from Mazandaran.

Combination of rep-PCR, IS50 and RAPD analysis

The dendrogram generated with combination of rep-PCR, IS50-PCR and RAPD fingerprints demonstrated that *B. nigrifluens* strains could be differentiated into 6 clades at 78% similarity which correlates well with their geographical distribution (Fig. 2d). Clade I and clade IV contained three and two strains from Kerman, respectively, clade II and clade III each contained two strains from Golestan, clade V had all the four strains collected from Mazandaran, and clade VI contained 10 strains from Fars province and one strain from the neighboring Kohgiluyeh-va-Boyerahmad province; among which five strains from Fars shared 83% similarity. *B. nigrifluens* strains isolated from the same locality clustered together in the combination of rep-PCR, IS50 and RAPD markers.

Comparison of different markers and primers

All three PCR assays reliably discriminated strains of *B. nigrifluens.* However, each of the PCR techniques differed in exploiting the degree of polymorphism existent. In the rep-PCR analysis, a total of 75 discrete amplified products were obtained, of which 55 (73.3%) were polymorphic. With RAPD assay, 109 out of 146 amplification products (74.6%) were polymorphic, whereas, in IS50-PCR the majority (30 out of 32, i. e., 93.75%) of the amplified fragments was polymorphic.

Marker index (MI), the product of the polymorphism information content (PIC) and effective multiplex ratio (EMR) was used as an estimate of the utility of each marker. The highest level of polymorphism as measured by PIC (0.75) was detected using RAPD and IS50-PCR, whereas in rep-PCR the value was 0.65. The average EMR was highest for IS50 (28.125), followed by rep-PCR (10.58) and RAPD (10.46). MI was highest (21.11) in IS50-PCR, low to

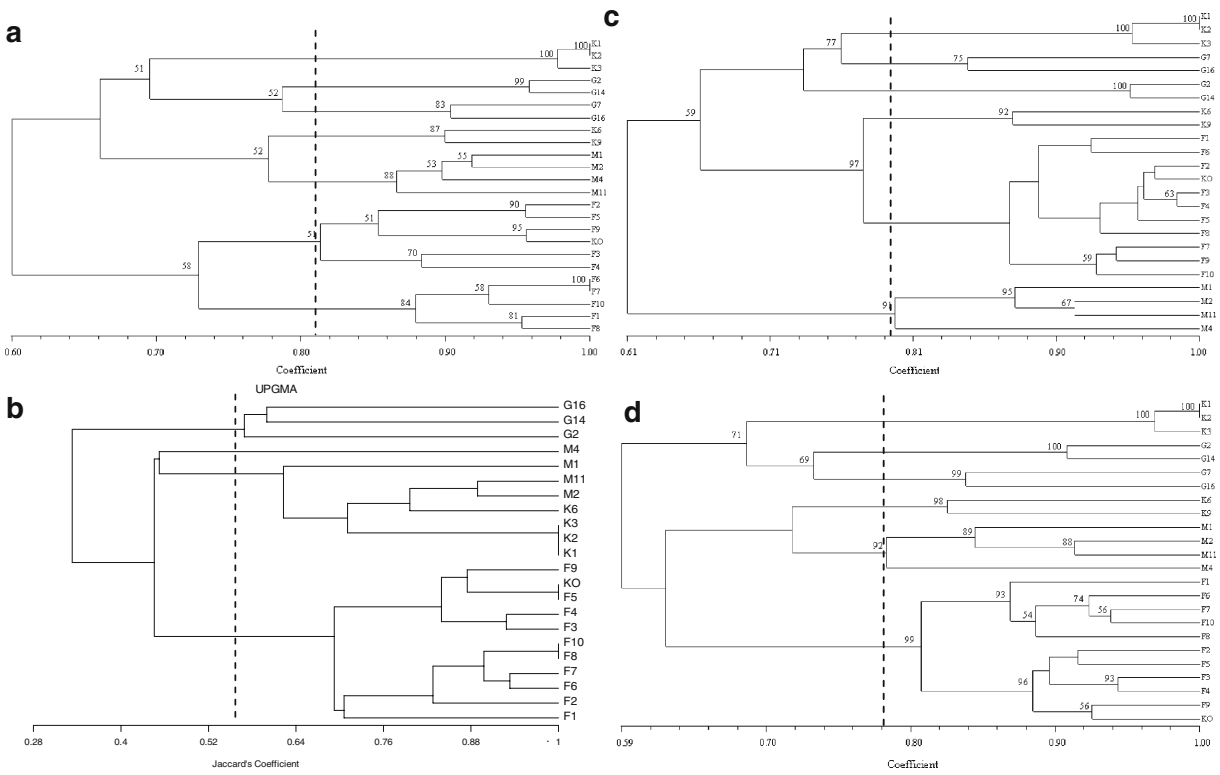

Fig. 2 Dendrograms illustrating the relationship between 24 *Brenneria nigrifluens* strains by UPGMA clustering. **a** Relationship based on rep-PCR; **b** Relationship based on IS50; **c** Relationship based on RAPD **d** Relationship based on combination of rep-PCR, RAPD and IS50 markers. K, M, G, F, and KO strand for strains from Kerman, Mazandaran, Golestan, Fars, and Kohgiluyeh-va-Boyerahmad provinces, respectively

medium (7.85) in RAPD and low (6.92) in rep-PCR. However, among the rep-PCR primers, ERIC-2I acted more differentiate with an MI of 11.94.

Correlations between rep-PCR, IS50-PCR and RAPD

The separate dendrograms obtained from each primer had cophenetic correlation efficient of 0.956, 0.965, 0.998 and 0.913 for ERIC-1R, ERIC-2I, REP-1R and BOX-A1R dendrograms, respectively. The cophenetic values for the combined similarity matrices of rep-PCR, RAPD and IS50 fingerprints were 0.825, 0.937 and 0.906, respectively.

Discussion

Rep-PCR fingerprinting revealed a considerable level of heterogenity among the populations of *B. nigrifluens* existing in different geographic areas of Iran. Selection for a specialised niche could affect the distribution of repititive sequences, leading to fingerprints unique to specific strains present in a given area (Louws et al. 1994). The evolution of bacterial genomes is commonly considered to be linked with repeated DNA elements (Sharples and Lloyd 1990). In this contex, the relativly limited degree of heterogenity of strains collected from Fars and Kohgiluyeh-va-Boyerahmad provinces, could reflect more recent introduction and limited evolution of strains in these regions. The present study also confirmed that the rep-PCR technique is a reliable, reproducible, and highly discriminative in assessment of the diversity of *B. nigrifluens* strains.

Insertion sequences (IS) which are ubiquitously distributed within bacterial genomes and have been successfully applied to diversity analysis (Mahillon and Chandler 1998; Nelson et al. 1994) were also found to be valuable in the analysis of heterogeneity of *B. nigrifluens* strains. IS50-PCR appeared to be

the most discriminating technique, among the three fingerprinting methods employed in the present study. Abundance of IS elements in the *B. nigrifluens* genome can potentially lead to genome fluidity by inducing a variety of mutations including gene inactivation, modification of gene expression, activation of cryptic genes, and genome rearrangements (Lee et al. 2005; Ardales et al. 1996; Hu et al. 2007).

Although the RAPD technique is widely used, problems of reliability and low reproducibility with this marker exist. Many factors such as concentration of reaction component, DNA purity, thermal cycler and the source of *Taq* polymerase affect amplification. To avoid these variables, different PCR amplifications were performed using the same thermocycler (Mastercycler® gradient, Eppendorf, Germany) and the same source of material. Moreover, when a large number of RAPD bands are generated to study the genetic diversity, the non-homology of errors of RAPD is usually random and has little effect on the relative similarities (Adams and Rieseberg 1998). Taking into account these considerations, RAPD marker did reliably discriminate the *B. nigrifluens* strains based on geographic regions in Iran. Besides, differentiation by RAPD marker was supported by rep-PCR and IS50-PCR too.

Overall the Mantel tests demonstrated existence of significant, positive correlations between the similarity matrices generated by the three markers used. The dendrograms also show the remarkable similarity in genetic structure of populations of *B. nigrifluens*, as reflected by different molecular typing methods.

As *B. nigrifluens* strains from the same geographical areas cluster together, they appear to share close genetic relationships. Whether this is a reflection of coevolution of the strains adapted in one locality or that distinct clonal populations of *B. nigrifluens* have been estabilished in a particular geographical region is yet to be determined. Moreover, different walnut ecotypes cultivated in different regions, could have selected different *B. nigrifluens* populations. The genetic diversity found in *B. nigrifluens* populations might also be related to the relatively mild aggressiveness of the pathogen. This pathogen causes damage to the trunk and scaffold branches but the affected trees frequently survive. This also may favor the selection of different genotypes of the bacterium co-infecting the same tree.

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