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Genetic Variation of the Iranian *Sclerotinia sclerotiorum* Isolates by Standardizing DNA Polymorphic Fragments

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Abstract: The aim of this study was to increase the Random Amplified Polymorphic DNA (RAPD) reproducibility by standardization of RAPD bands before 0 and 1 score and study of genetic variation of the Iranian *Sclerotinia sclerotiorum* isolates, which collected from Canola plant's fields. For this aim, twelve isolates are provided from different province of Iran which contains various growth degrees on Potato Dextrose Agar (PDA). The CTAB method used for genomic DNA extraction from *Sclerotia* powdered. The RAPD studied by 18 random primers. By using of three random primers (Ar0R2, Ar081 and Ar173) in arbitrarily primed (AP-) PCR a total of 284 RAPD products were compared in a sample of 12 individuals which contain highly polymorphism. Cluster analysis for PCR products of these three primers performed by UPGMA (Unweighted pair grouped method by arithmetic average) method. We found that RAPD profiles markedly differed between *S. sclerotiorum* isolates. Therefore, it is recommend that if the polymorphism of the isolates bands were standardized by migration of marker bands, RAPD technique not only could separate the isolates in multiple groups but also is an effective, rapid, reliable technique to study genetic variability between fungal isolates. Moreover, standardization RAPD polymorphic bands were used in the similar molecular techniques. RAPD markers may be a useful tool for investigation of the genetic variation within *S. sclerotiorum* isolates but these genetic variations were not significantly related with geographical positions of isolates.

Key words: *Sclerotinia sclerotiorum*, RAPD, standardization, genetic diversity

INTRODUCTION

Sclerotinia sclerotiorum is a necrotrophic, phytopathogenic, filamentous ascomycete. It is recognized as a broad-host-range, omnivorous plant pathogen with worldwide distribution. Over 400 species of plants are susceptible to this pathogen (Boland and Hall, 1994; Tu, 1997).

Since, *S. sclerotiorum* is one of economic importance pathogen, genetic diversity research will lead to more effective disease management. The success of any disease management strategy will be influenced by understanding genetic structure of pathogen population which it is designed to control. Genetic diversity of variable pathogen population is of importance when devising disease management and resistance-screening strategies (Sexton and Howlett, 2004). It may be necessary to target different pathogen populations with appropriate strains of the biocontrol agent. Therefore, in conjunction with developing a biological control agent for

S. sclerotiorum, we have surveyed the genetic composition of *S. sclerotiorum* populations from the Iran.

In the last few years, in spite of controversies, the use of molecular techniques has contributed to the resolution of many systematic and phylogenetic problems (Patterson *et al.*, 1993; Jones *et al.*, 2004; Moritz and Hillis, 1996). Recently, several techniques such as Restriction Fragment Length Polymorphism (RFLP), DNA-DNA hybridization, Randomly Amplified Polymorphic DNA (RAPD), Arbitrary Fragment Length Polymorphism (AFLP) and DNA sequencing, have been used with success to clarify relationships at different phylogenetic levels (Crawford, 1990; Masters, 1995; Weising *et al.*, 1995; Hillis *et al.*, 2006).

The apparent advantage of the RAPD analysis over other techniques include no requires to foreknowledge about any particular gene in a target taxon, simplicity and rapidity cause to many researcher prefer to use this method to determination of taxonomic identities, detection

of interspecific gene flow, assessment of kinship relationships, analysis of mixed genome samples and production of specific probes (Hadrys *et al.*, 1992; Masters, 1995; Fouly and Wilkinson, 1999; Hsiang and Wu, 2000; Echeverrigaray *et al.*, 2001; Doherty *et al.*, 2003; Kanaan-Atallah, 2003; Moraes *et al.*, 2006; Arbaoui *et al.*, 2008).

Since, use of molecular markers is especially interesting for the genetic study of the *S. sclerotiorum*, whose morphologic and physiologic variability prevents the knowledge of his populational structure. Therefore, in present study, we used DNA polymorphism to determine the level of genetic variation present between Iran's *S. sclerotiorum* populations, using eighteen random primers in Arbitrarily Primed (AP-) PCR.

MATERIALS AND METHODS

Source of isolates: Twelve *Sclerotinia sclerotiorum* isolates collected from four geographically distinct of the Canola plant's fields from Mazandaran, Golestan, Tehran and Kurdistan Provinces in 2006-2007 year (Table 1). Isolation performed from Sclerotia of the *S. sclerotiorum*. Sclerotia were surface sterilized by washing in 50% ethanol, 6.75% sodium hypochlorite for 3 min, then rinsed 3 times in sterile water. They were then cut in half and placed on Potato Dextrose Agar (PDA) with chloramphenicol 0.05% [w/v], with the cut surface contacting the PDA. Cultures were incubated at 20°C in the dark.

Isolation of fungal DNA: All isolates were subcultured on PDA supplemented with chloramphenicol 0.05% [w/v] and incubated at 20°C for 10 days. For DNA extraction, surface sterilized Sclerotia in a mortar and the cells were grounded with a pestle. Genomic DNA extraction was improved by modifying some of the steps in the original CTAB-DNA isolation protocol (Doyle and Doyle, 1987, 1990). In brief, the 0.25 to 0.05 g Sclerotia powdered was transferred to an Eppendorf tubes and 500 µL of lysis buffer (50 mM Tris-HCl, pH 8.0; 1 mM EDTA; 150 mM NaCl) were added. Each sample was mixed for 1 h at 37°C and then 60 µL of 1% Sodium Dodecyl Sulfate (SDS) were added. After gently mix, sample incubated

for 20 min at 65°C and so, 130 µL of 10% [w/v] cetyltrimethyl ammonium bromide (CTAB; Sigma) and 150 µL of 5 M NaCl were added and incubation continued for a further 20 min at 65°C. The suspensions divided to two Eppendorf tubes and then extracted with 375 µL of phenolchloroform-isoamyl alcohol (25:24:1[v/v]) and centrifuged at 12,000 g for 10 min. For clean up of DNA, this procedure was repeated three times. The DNA was precipitated with 2.5 volumes of ice-cold pure ethanol at -20°C for 20 min and centrifuged at 12,000 g for 8-10 min. DNA was washed with 70% ethanol, pelleting by centrifugation, air dried. Finally, the DNA was resuspended in 100 µL TE buffer. The RNA was removed by the addition of 1 µL RNAase A (Fermentas: 10 mg mL⁻¹) per 100 µL DNA solution and incubate at 37°C for 15-30 min. The 0.2 mg of proteinase K was then added and the solution incubated at 50°C for 20 min. The fractions were pooled and the DNA was phenol extracted and centrifuged as before. The DNA was ethanol precipitated in a 1/10 volume of 3 M sodium acetate for 1 h at -20°C. After ethanol 70% washing, the DNA was centrifuged and finally resuspended in 50 µL sterile double distilled water double distilled water. DNA concentration was estimated by measuring the optical density at 260 nm.

Primers: Oligonucleotides (decamers) of arbitrary sequence were used as single primers in the RAPD experiments (Table 2). The primers tested comprised the series Ar (Ar079, Ar081, Ar082, Ar171, Ar173, Ar174 and ArPU1-ArPU3) and OP (OPE12, OPE14, OPE20, OPX22, OPX25, OPY18 and OPS16). These primers were used for *Ascochyta rabiei* isolates in our earlier study (Hosseinzadeh and Barzegar, 2008). Three primers (Ar081, Ar173 and Ar0R2) were selected for the analysis

Table 1: The 12 isolates used for RAPD analysis

Canola's					
Name	Province	field	Name	Province	Canola's field
S01	Mazandaran	Juybar	S07	Kurdistan	Marivan
S02	Mazandaran	Juybar	S08	Golestan	Kalaleh
S03	Mazandaran	Sari	S09	Tehran	Varamin
S04	Kurdistan	Marivan	S10	Mazandaran	Dasht-e-Naz
S05	Mazandaran	Sari	S11	Mazandaran	Dasht-e-Naz
S06	Golestan	Gonbad	S12	Mazandaran	Sari

Table 2: Number of RAPD fragments and polymorphic bands for each RAPD primer

Primer	5'-d[decamers]-3'	No. of RAPD fragments	No. of polymorphic bands
Ar079	5'-GAAAGAGCGG-3'	6	4
Ar081	5'-ACGGTCTTGG-3'	15	10
Ar082	5'-ACGATCGCGG-3'	8	5
Ar171	5'-GAAACAGCGG-3'	6	4
Ar173	5'-GGAGGGTGT-3'	13	9
Ar174	5'-ACGATCGCCG-3'	4	2
ArPU1	5'-ACTGGGACTC-3'	5	4
ArPU2	5'-AGATGCAGCC-3'	7	3
ArPU3	5'-ACTGCGACTC-3'	5	1
Ar0R1	5'-CGGCCACCCCT-3'	5	2
Ar0R2	5'-CGCGTGCCAG-3'	11	9
OPE12	5'-GTGACATGCC-3'	4	2
OPE14	5'-CACCAGGTGA-3'	3	1
OPE20	5'-AGGGGTCTTG-3'	6	3
OPX22	5'-CTGGGCACGA-3'	4	2
OPS16	5'-AAAGGGGTCC-3'	5	2
OPY18	5'-AGTCGCCCTT-3'	4	1
OPX25	5'-GAGCGAGGCT-3'	5	2

Primers with prefix Ar obtained from primer bulk of *Ascochyta rabiei* (Hosseinzadeh and Barzegar, 2008)

of genetic diversity observed on the basis of the polymorphic DNA standard obtained after the RAPD assays.

PCR conditions: For the optimization of RAPD reaction, various concentrations of the target DNA and $MgCl_2$, oligonucleotide primers were used for amplification to standardize the PCR conditions. Reactions without DNA were used as negative controls. Amplification reactions were performed in total volumes of 25 μL which contained about 20 ng of template DNA, 1 X PCR buffer (10 mM Tris HCl pH 8.8; 50 mM KCl, 0.8% Nonidet P40), 0.2 mM dNTP Mix, 0.5 μM of single primer, 0.2 U of Taq DNA polymerase. Without genomic DNA, other PCR reactants prepared from Fermentas Co.

Amplification was performed in a DNA thermocycler (Master cycler Gradient, Eppendorf) as follows: one initial cycle for 5 min at 94°C, followed by 32 cycles comprising denaturation (90 sec at 94°C), annealing (90 sec at 28°C) and extension (90 sec at 72°C) and then a final extension for 6 min at 72°C.

Agarose technique: Amplification fragments were separated by standard electrophoresis methods on 1% (w/v) agarose gels in 1x TBE in a Paya Pajooesh (Paya Pajooesh Laboratories Inc., Iran, www.payapajooesh.com) submarine system at 50 V for 3 h and then visualized by staining with ethidium bromide (0.5 $\mu g mL^{-1}$). Gel image was captured under UV-illumination by a CCD camera.

Statistical analysis: After PCR, all PCR products have been electrophoresis on 1% agarose gel in same conditions. The gels photographed by UV transilluminator after ethidium bromide staining. Bands migration rate applied for statistical analyze of RAPD products. The migration rate of each marker bands estimated by collies in 0.1 mm accuracy and then migration logarithm matrix in all marker bands calculated. One of the gels was selected and named as standard gel, for example in this research marker bands migration of Ar173 primer considered as standard. Since, DNA running in agarose gel following the $LogY = A + LogX$ equation (Sambrook and Russell, 2001) linear logarithmic equation of standard gel marker calculated with other gels marker. Standardized migration of each gels calculated with insertion the bands migration of any gels in above equation and so migration of other isolates band has been standardized. Since, marker bands migration in all gels were not identical, for uniform these, low and high limit defined for each marker bands. For this purpose, first the mean (m) and variance (δ^2) of each corresponding bands calculated and then low

and high limits of each bands calculated by using of normal distribution function and corresponding equation [$X = m \pm Z\delta$]. Distance between two large letters indicated with small letters. Present and non present of RAPD bands were scored with 0 and 1, respectively. By using of 0 and 1 matrix table, analogical matrix created according to Nei (1972) analogical coefficient. This matrix converted to distance matrix and then hierarchically clustering of zero and one polymorphism matrix has been drawn by UPGMA (Unweighted pair grouped method by arithmetic average) method.

RESULTS

Fungal DNA isolation: DNA extraction was improved by modifying some of the steps in the original CTAB protocol (Doyle and Doyle, 1987, 1990) from surface sterilized *Sclerotia*. Quality of extract DNA has been determined by using 0.1% agarose gel electrophoresis stained by ethidiumbromide (Sambrook and Russell, 2001). The high intensities of DNA bands with minor smears indicate the high molecular weight of extracted genomic DNA with high purity for RAPD analysis. The extracted DNA was of high purity as it showed a reading of between 1.6-1.9 after calculating the 260/280 nm absorbance. The ratios at 260/280 nm were mostly 1.8 and this is an acceptable ratio for further analysis (Sambrook and Russell, 2001; Weising *et al.*, 1995). The DNA yield obtained ranged from 5 to 10 $\mu g \mu L^{-1}$. DNA isolated by this method yielded strong and reliable amplification products showing its compatibility for RAPD using random decamer primers.

DNA polymorphism: Almost all the tested parameters for RAPD like the concentration of template DNA, primer, $MgCl_2$, Taq polymerase, dNTPs, temperature and time intervals during denaturation, annealing and elongation were also optimized which also had an effect on amplification, banding patterns and reproducibility. The size of the amplified fragments ranged from 831-4, 268 bp (Fig. 1a-c).

Statistical analysis: Because RAPD products of the verify isolates were loaded in different gels, at first gels were standardized according to above methods by a common marker that can be in all gels. After calculated the upper and lower limit one of the bands, by above method, RAPD bands scored by 0 and 1, then cluster analysis of RAPD products in 12 isolates investigated with these three primers (Ar081, Ar173 and Ar0R2). According to zero and one matrix, number of RAPD

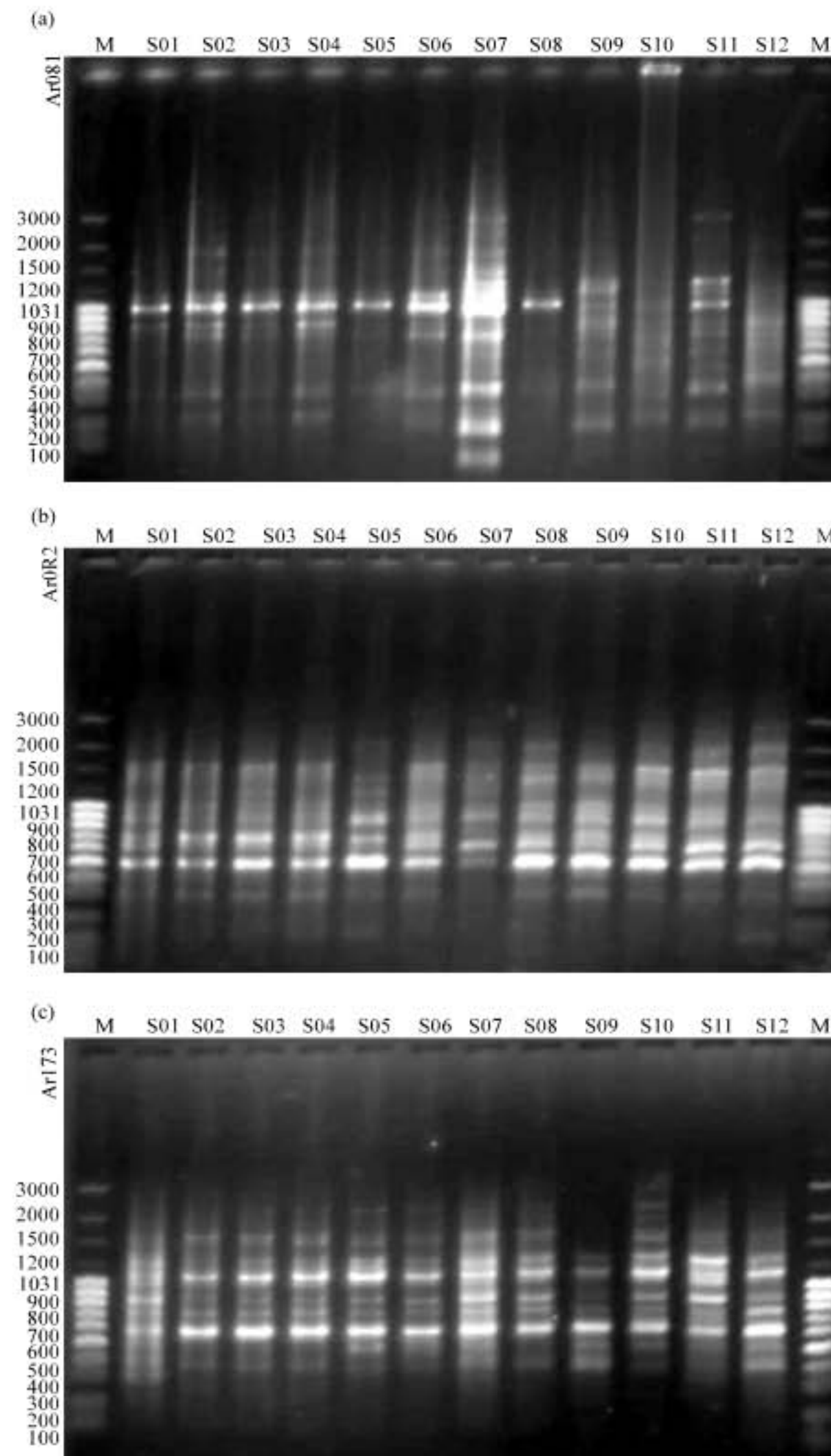


Fig. 1: Ethidium-bromide-stained agarose gel of a RAPD reaction with (a) Ar081, (b) Ar173 and (c) Ar0R2 primers of the *Sclerotinia sclerotiorum* isolates: Electrophoresis of the RAPD products was done in a 1% agarose gel

products from Ar081, Ar173 and Ar0R2 primers was 92, 106 and 96 bands, respectively. Cluster analysis of RAPD products from 12 isolates with Ar081 and Ar173 primers in 10 cut-off and Ar0R2 primer in 6.5 cut-off, divided them to 5, 4 and 4 groups, respectively. For comparison of DNA polymorphisms from RAPD products, dendrogram diagram was drawn between all three primers by

UPGMA method. By analyzing the dendrogram diagram from Ar081 and Ar0R2, primers, all isolates divided to 5 and 4 groups, respectively.

At the end for comparison of RAPD products from Ar081, Ar0R2 and Ar173 primers, combinational dendrogram between RAPD products has been drawn by UPGMA method. This concluded dendrogram

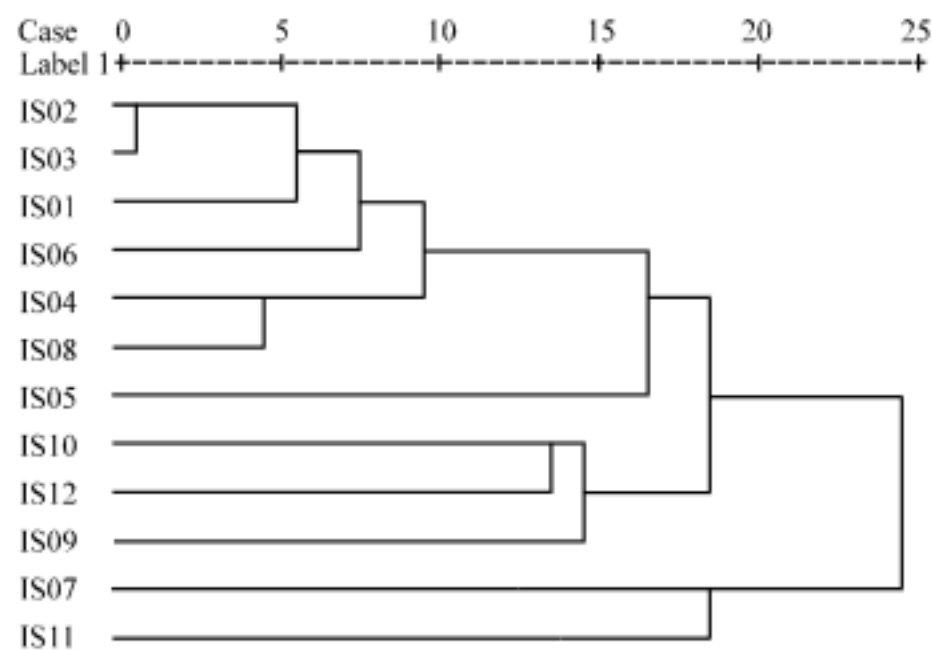


Fig. 2: Dendrogram showing *S. sclerotiorum* isolates based on Nie's genetic distance using UPGMA method and 284 RAPD replicated bands

individuated most isolates in differed groups, suggesting that high genetic diversity between *S. sclerotiorum* isolates (Fig. 2).

DISCUSSION

Recently progress in molecular biology techniques provided the basis for revealing virtually unlimited numbers of DNA markers. The utility of DNA-based markers is generally determined by the technology that is used to reveal DNA-based polymorphism. At present, RAPD and microsatellite markers have found a wide range of applications in gene mapping, population genetics, molecular evolutionary genetics and plant and animal breeding (Scott and Straus, 2000; Hemmati *et al.*, 2009). This is mainly due to the speed, easy, cheap, cost and efficiency of the RAPD technique to generate large numbers of markers in a short period compared with other methods (Voigt *et al.*, 1995). The RAPD is considered as an efficient tool for species identification (Hillis *et al.*, 2006; Hosseinzadeh and Barzegar, 2008) and despite controversies, it has been applied successfully in the evaluation of interspecific relationships of many different organisms, including *S. sclerotiorum*. Moreover, RAPD offers the opportunity to access information about a large portion of the genome.

In present study, genetic diversity of *S. sclerotiorum* isolates has been evaluated by RAPD method and then statistically analyzed. Eighteen primers selected for RAPD-PCR on the basis of the number, intensity and distribution of bands generated that were able to clearly distinguish among *S. Sclerotiorum* isolates. Three primers (Ar081, Ar173 and Ar0R2) were selected for the analysis of genetic diversity observed on the basis of the polymorphic DNA standard obtained after the RAPD

assays. By using of these three aleatory decamer primers to amplify genomic DNA from 12 isolates of the *S. sclerotiorum* species a total of 284 bands were scored that contained ranging in size from 80 to 3000 bp with 1-10 polymorphic bands for each primer. An example of the patterns obtained by RAPD analysis of *S. sclerotiorum* species is shown in Fig. 1. We compared RAPDs of *S. sclerotiorum* isolates from four distinct geographical sites by statistical analysis and found differentiation among the *S. Sclerotiorum* isolates. The cluster analysis based on RAPD-PCR was clear enough to allow discrimination between local isolates. This cluster analysis technique demonstrated the diverse populations of *S. Sclerotiorum* isolates. Present results showed that dendrogram analysis was not significantly related with geographical positions of isolates. Hemmati *et al.* (2009) showed 276 Iranian isolates from four geographic populations of *S. sclerotiorum* by used of the microsatellite loci classified to high, moderate genotypic diversity levels and a number of shared haplotypes among populations and showed genotypic diversities did not differ significantly among populations.

This is may be due to high genetic diversity and so high percent of DNA mutations in *S. sclerotiorum* isolates. This finding was comparative with study of Cervone *et al.* (1977) and Kohn *et al.* (1991).

CONCLUSIONS

The RAPD method is the best suited for identification of species and for differentiating among nonspecific populations, particularly in cases where the morphological characters do not permit an unambiguous or a rapid identification of species. The present optimized protocol for DNA isolation and RAPD technique may serve as an efficient tool for further molecular studies. The RAPD markers could be seen as a reliable method for both species identification and the evaluation of genetic relationships among the *S. sclerotiorum* isolates. But dendrogram analysis of *S. sclerotiorum* isolates were not significantly related with geographical positions of isolates. We comment that integrating RAPD and microsatellite markers might be a good source of information about the coloration between genetic diversity and geographical positions of isolates.

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