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Genetic diversity of Iranian *Ophiognomonia leptostyla* (Fr.) populations using RAPD and ISSR markers

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ABSTRACT

Walnut anthracnose is the most important fungal disease in Persian walnut in Iran and all over the world caused by Ophiognomonia leptostyla. Limited attempts to genetic variation uncovering in this species were not successful by now. In this study, genetic diversity of 75 Ophiognomonia leptostyla isolates collected from the northwest of Iran was studied by RAPD and ISSR primers. RAPDs revealed more polymorphism in studied isolates than ISSRs, but fewer bands were produced. There were slight correlations with obtained RAPD dendrograms with homothallism and collection site, but no correlation with phenotypic traits in ISSRs. Population analysis identified genetic relationship between neighbor provinces but isolates from Tehran and West Azarbaijan with reasonable distance were relative according to trees were obtained by RAPD and ISSR fingerprinting. Using both markers were useful in genetic variation understanding of the species unlike ITS and LSU RFLP techniques were used in previous researches.

Keywords: *Gnomonialeptostyla*, walnut leaf blotch, biodiversity, microsatellite.

INTRODUCTION

Iran takes the third position of walnut production in the world, 270,300tonnes in 2010, after China and USA [8]. Persian or European walnut (*Juglansregia*L.) is commonly present in most parts of Iran except the Persian Gulf coasts [20]. Walnut anthracnose or black spot/blotch isone of the most important fungal diseases on black walnut (*Juglansnigra*L.) in the North and South America and onPersian walnut in Europe and Asia [3]. It is widespread in Iran and has been reported from the north, west, northwest,and northeast of the country [2]. Anthracnose epidemics might be very destructive in rainy and cool seasons in walnut [2,3,21]. Thecausal agent fungus is *Ophiognomonia leptostyla* (Fr.) Sogonov 2008, with *Marssoniellajuglandis*(Lib.) Höhn 1916 as itsanamorph [22].

There has been increasing interest in the application ofpolymerase chain reaction technology to identificationof plant pathogenic fungi. Such methods offer theadvantage of reducing or eliminating the need for lengthy culturingand difficult morphological identification procedures [17,24].PCR-based genomic fingerprinting is a good alternative tomethods that rely on specifically targeted primers [17]. RAPD[1] and ISSR[2] or MSPPCR[3]fingerprintingsare two capable techniques to detect polymorphism in populations in total genome level and to discriminate among taxa [19,23]. These techniques,which analyze the whole genome, have been shown tobe relatively robust and discriminatory [13]. RAPD markers are generated via PCRusing short 10-base primers of an arbitrary sequence and a lower annealing temperature than standard PCR reactions [1]. Microsatellites(tandem repeats of 1-5 base pairs) are ubiquitous componentsof eukaryotic genomes along with minisatellites [26]. MSP-PCR uses single primersto generate DNA fingerprints that are useful for discriminatingbetween fungal species and strains [25,24,14]. Genetic diversity of *O. leptostyla* has been poorly studied. Belisario*et al*. (1992) surveyed on genetic variation of 176Italian *O. leptostyla* isolates using PCR-RFLPs of ITS and 18S rDNA and found no polymorphism [4]. Salahi*et al*.(2007) used the same technique with 30 Iranian isolates collected from East Azarbaijan province and the results werethe same with no polymorphism [21]. Mejia *et al*. (2002) and Green &Castlebury (2007) amplified ITS1 and ITS2 regionsof nuclear ribosomal DNA in their studies on*Gnomoniaceae*phylogeny [9,15]. Sogonov*et al*. (2008) studied four genetic regions sequencing like *tef1*, 28S rDNA, β-tubulin, and RNA Polymerase IIand offered a new concept of *Gnomonia* and *Ophiognomonia*, and subsequently introduced the new combination which is currently used as *Ophiognomonia leptostyla* [22]. Jamshidi and Zare (2010) investigated on 16 *O. leptostyla* Iranianisolates by ITS sequencing and showed that 13 Iranian isolates are in the same clade with *G.leptostyla* CBS strains isolated from *Juglans*regia (CBS 110136, CBS 844.79 and CBS 110136). There were two closely related sub-clades with low bootstrap support on Iranian's. Three isolates located on the same sub-clade were isolated from wild walnut trees in woodland area, considering the passible different variety of the species on these plants [11]. In previous researches, ITS and LSU nrDNA RFLP-PCR were not useful and outstanding tools to find genetic diversity in Italian and Iranian*O. leptostyla* isolates. They only cover a very limited part of the genome, nrDNA. Due to RAPD and ISSR are two simple and quick techniques tools which are scanning the total genome, they might be efficient in this fungus genetic polymorphism investigations. Therefore, the aim of the present study was to evaluate RAPD and ISSR-PCR assays asmolecular marker for clarifying genetic variation in Iranian isolates of *O. leptostyla*.

MATERIELS AND METHODS

Fungal materials, isolation and mycelial mass production

Seventy-five Persian walnut anthracnose-infected leaf samples were collected from 11 provinces in the northwest of Iran during 2006-08 (Table 1) and the causal agents wereidentified as *O. leptostyla* according to identification key presented by Sogonov*et al*. (2008) [22].

Three leaf discs (6 mm in diameter) bearingacervuliwere superficially sterilized using ethanol 75% (for 30 s) andsodium hypochlorite 1% (for 60 s), then washed fourtimes with sterile

[1] Random Amplified Polymorphic DNA
[2] Inter Simple Sequence Repeats
[3] Microsatellite-Primed Polymerase Chain Reaction

distilled water. Macroconidia were releasedby crushing leaf samples and picked off small leaf pieces in1.5 ml Eppendorph tubes containing 1 ml sterile distilledwater and vortexed for 20 s. Then, 100 ml of conidialsuspension were transferred into 2% water agar (Agar-Agar, Merck,Germany) and incubated at 21◦C, 50% relative humidity, and dark condition.Germinating macroconidia were transferred to 39% PDA(potato dextrose agar, Merck, Germany) added 7 gr/L oatmealand incubated at 21◦C, 50% relative humidity and 12:12 alternativephotoperiod for 10 days. Obtained 10-day old mycelia wereused for DNA extraction.

Table 1– Geographical characterization of studied *O. leptostyla* isolates

IsolateAcronym	Sampling Information				
	Collection site	Province	Latitude	Longitude	Altitude
Abk	AbharKahrizak	Zanjan	49˚ 04□	36˚ 21□	1673
Abs	Abhar, Shanat	Zanjan	49˚ 16□	36˚ 13□	1645
Ahr	Ahar	AzarbaijaneSharghi	47˚ 03□	38˚ 28□	1341
Ajb	Ajabshir, Bonalu	AzarbaijaneSharghi	45˚ 53□	37˚ 28□	1423
Ajd	Ajabshir, Danalu	AzarbaijaneSharghi	45˚ 50□	37˚ 29□	1375
Ajm	Ajabshir, Mehmandar	AzarbaijaneSharghi	45˚ 51□	37˚ 25□	1333
Ard	Ardebil	Ardebil	48˚ 17□	38˚ 14□	1500
Arf	Ardebil, Fuladlu	Ardebil	47˚ 17□	38˚ 15□	1530
Aro	Ardebil, Oskanlu	Ardebil	47˚ 19□	38˚ 16□	1432
Asa	Abhar, Sharifabad	Zanjan	49˚ 12□	38˚ 08□	1713
Asl	Asalem	Gilan	48˚ 57□	37˚ 47□	43
Azr	Azarshahr	AzarbaijaneSharghi	45˚ 58□	37˚ 45□	1390
Bnb	Bonab	AzarbaijaneSharghi	46˚ 03□	37˚ 20□	1300
Did	Divan darreh	Kordestan	47˚ 02□	35˚ 54□	1821
Dlh	Dalahu	Kermanshahan	46˚ 07□	34˚ 17□	2058
Eag	Eslamabad'egharb	Kermanshahan	46˚ 31□	34˚ 06□	1514
Fmn	Fuman	Gilan	49˚ 17□	37˚ 13□	50
Ggt	Gogan, Taimurlu	AzarbaijaneSharghi	45˚ 54□	37˚ 46□	1961
Glv	Gilvan	Gilan	49˚ 25□	36˚ 44□	439
Gnj	Ganjeh	Gilan	48˚ 28□	36˚ 51□	804
Grm	Germi	Ardebil	48˚ 05□	39˚ 00□	993
Hmd	Hamedan	Hamedan	48˚ 21□	34˚ 11□	2150
Hsh	Hashtrud	AzarbaijaneSharghi	47˚ 05□	37˚ 47□	1660
Ile	Ilam, Eywan	Ilam	46˚ 19□	33˚ 48□	1645
Ilk	Ilkhchi	AzarbaijaneSharghi	45˚ 58□	37˚ 57□	1352
Ilm	Ilam	Ilam	46˚ 25□	33˚ 38□	1759
Jlz	Jolfa, Zaviye	AzarbaijaneSharghi	45˚ 40□	38˚ 53□	879
Jol	Jolfa	AzarbaijaneSharghi	45˚ 38□	38˚ 56□	720
Khd	Khorramdarreh	Zanjan	49˚ 11□	36˚ 11□	1655
Khf	Khoy, Firuragh	AzarbaijaneGharbi	44˚ 49□	38˚ 43□	1309
Khm	Khoy, Mortezagholi	AzarbaijaneGharbi	44˚ 57□	38˚ 32□	1136
Krk	Karaj, Kamalabad	Tehran	51˚ 38□	35˚ 49□	1681
Krp	Paveh	Kermanshah	46˚ 21□	35˚ 02□	2210
Kss	Khosroshahr	AzarbaijaneSharghi	46˚ 02□	37˚ 57□	1357
Lhj	Lahijan	Gilan	50˚ 00□	37˚ 12□	29
Lhr	Taleghan, Lahran	Tehran	50˚ 37□	36˚ 11□	1893
Mdb	Miandoab	AzarbaijaneGharbi	48˚ 90□	36˚ 57□	1292
Mdd	Marand, Dizajolya	AzarbaijaneSharghi	45˚ 37□	38˚ 27□	1442
Mdk	Marand, Kandloj	AzarbaijaneSharghi	45˚ 43□	37˚ 23□	1326
Mdo	Marand, Ordaklu	AzarbaijaneSharghi	45˚ 41□	37˚ 24□	1353
Mia	Miyaneh, Aghkand	AzarbaijaneSharghi	48˚ 04□	37˚ 14□	1747
Mib	Miyaneh, Balesin	AzarbaijaneSharghi	45˚ 35□	37˚ 38□	1237
Mij	Miyaneh, Balujeh	AzarbaijaneSharghi	47˚ 46□	37˚ 36□	1503
Mir	Taleghan, Mir	Tehran	50˚ 33□	35˚ 15□	1753
Mlk	Malekan	AzarbaijaneSharghi	45˚ 55□	36˚ 26□	1294
Mrd	Marand	AzarbaijaneSharghi	45˚ 46□	38˚ 25□	1334
Mrg	Maragheh	AzarbaijaneSharghi	46˚ 14□	37˚ 23□	1449
Mrs	Marivan, Seyf	Kordestan	46˚ 16□	35˚ 33□	1563
Mrv	Marivan	Kordestan	46˚ 09□	35˚ 31□	1543
Msh	Meshkinshahr	Ardebil	47˚ 40□	38˚ 23□	1452
Msl	Masuleh	Gilan	48˚ 59□	37˚ 09□	1050
Myr	Mohammadyar	AzarbaijaneGharbi	45˚ 31□	36˚ 57□	1360
Ngd	Naghadeh	AzarbaijaneGharbi	45˚ 23□	36˚ 57□	1383

IsolateAcronym	Sampling Information				
	Collection site	Province	Latitude	Longitude	Altitude
Nhv	Nahavand	Hamedan	48˚ 30□	34˚ 35□	2916
Osk	Osku	AzarbaijaneSharghi	46˚ 04□	37˚ 55□	1579
Pir	Piranshahr	AzarbaijaneGharbi	45˚ 07□	36˚ 41□	2505
Prs	Pars abad	Ardebil	47˚ 55□	39˚ 38□	46
Qza	Abyek	Qazvin	50˚ 31□	36˚ 02□	1366
Qzm	Qazvin	Qazvin	49˚ 59□	36˚ 16□	1390
Qzn	Nezamabad	Qazvin	49˚ 50□	36˚ 16□	1369
Qzs	Sharifabad	Qazvin	50˚ 07□	36˚ 11□	1264
Rst	Rasht	Gilan	49˚ 35□	37˚ 16□	2
Shd	Shahindezh	AzarbaijaneGharbi	46˚ 33□	36˚ 40□	1838
Shn	Taleghan, Sohan	Tehran	50˚ 38□	36˚ 12□	1895
Shs	Taleghan, Shahrazar	Tehran	50˚ 39□	36˚ 14□	2330
Sms	Somee-Sara	Gilan	48˚ 18□	37˚ 17□	13
Sof	Sofyan	AzarbaijaneSharghi	45˚ 58□	38˚ 16□	1505
Thl	Lavasanat	Terhan	46˚ 27□	35˚ 25□	1781
Tls	Talesh	Gilan	48˚ 54□	37˚ 48□	72
Tof	Toyeserkan, Falakeh	Hamedan	48˚ 30□	34˚ 47□	1873
Toy	Toyserkan	Hamedan	48˚ 26□	34˚ 50□	2060
Tst	Takestan	Qazvin	49˚ 42□	36˚ 30□	1323
Zia	Zia abad	Qazvin	49˚ 26□	35˚ 59□	1423
Znj	Zanjan	Zanjan	48˚ 29□	36˚ 39□	1959
Znk	Kushkan	Zanjan	48˚ 27□	36˚ 41□	1706

DNA extraction

DNA extraction was carried out according to slightly modified Liu *et al*. (2000) method. The quality and quantity of DNA were assessed by spectrophotometer (Jenway 6305 UV/Visible, USA). The final DNA concentration of each sample was adjustedto 25 ng/µl.

PCR amplificationand gel electrophoresis

Thirty random RAPD primers obtained from CinnaGen, Tehran were preliminarily screened based on their DNA bands polymorphism creating on 1.2 gr/L agarose gel and four RAPD primers (RAPD1 [5′-ccg gcctta g], RAPD12 [5´-cct gggcct c], RAPD211 [5´-gaagcgcgat], and RAPD213 [5´-cagcgaacta]) were selected for main evaluation. ISSRprimers [1] (CinnaGen, Tehran) being used in this study were $(GTG)_5$ [5'-gtg gtggtggtggtg] and M13 [5'-cac aggaaa cag ctatga cc]. The PCR reaction (25 µl) contained 50 ng of genomic DNA, 12.5 pmol of each primer, 0.3 mMdNTPs and 1× PCR buffer containing 2 mM MgCl2, 1.5 U *Taq*DNA polymerase (CinnaGen, Tehran). PCR amplification was carried out using Apollo (ATC. 401, ver. 4/88, CLP, Inc. USA) PCR machine. The PCR program for RAPD markers was 95°C/3 min (initial denaturation), 95°C/30 s, annealing temperature/50 s, 72°C/2 min (40×) and 72°C/10 min (final extension). Also, PCR program for MSP markers was 94°C/3 min, 94°C/30 s, 50°C for $(GTG)_5$ and 44°C for M13/50 s, 72°C/2 min (35×) and 72°C/10 min.PCR amplifiedproducts of RAPD and ISSR primers were subjected tohorizontal gel electrophoresis using 1.2% agarose gel in 1X TBE buffer at 95 V for 30 mins using HU-150 and HU-70 standardgel electrophoresis units (Padideh Pars Co., Iran). As size marker we useda DNA ladder (GeneRuler 1kb DNA, Fermentas, SM0313 100bp,Germany) and the ethidium bromide stained gels weredocumented using Gel document (ULIdoc Inc. UK).

Data analysis

Molecular data were initially entered to Microsoft Excel 2010 and transferred to NTedit 1.07c and cluster analysis was performed by NTSYSpc ver. 2.02e software [10]. Similarity matrix was made by simple matching and dendrogramwas drawn using UPGMA method. Cut-off line was determined by mean of similarity matrix calculation [10] and coephenetic coefficient correlation

for each cluster was obtained by similarity and coephenetic correlation calculation by NTSYSpc software. For dendrogramscomparison and for population analysis, we used Mantel test and GenALex 6.41 and MEGA 5 software.

RESULTS AND DISCUSSION

For qualitative and quantitative DNA extraction, using fresh mycelium up to 20 days old was necessary. The best result was obtained with 10-day mycelia. Only four primers out of 30 could produce considerable bands with sufficient polymorphism, comprising RAPD1, RAPD12, RAPD211, and RAPD213. On the whole, 243 (up to 9 bands per fungal isolate) electrophoretic bands were produced by RAPD211, ranged 750-5000 bp in length. Based on RAPD211, isolates were grouped in four clades in which all homothallic isolates were located in the second clade. However, there were other non-homothallic isolates in this clade. On the other hand, Mir, Mrs and Shs as homothallic isolates were in the other categories (Figure 1-a). RAPD1 generated 250 bands, up to seven bands for each isolate ranged 300-1400 bp (Figure 1-b). Isolates were placed in sixclades with no correlation with geographic and morphologic traits. RAPD19produced 249 bands, up to eight bands for each isolate ranged 400-1500 bp. Isolates were placed in sevenclades (Figure 1-c). Resulted dendrogram was slightly correlated to sampling place. For example, in the sixth clade, all isolated were from north of Iran (Gilan province) but Zia and Fmn were from Fuman as a northern isolate were in the first group. Also, isolates from Marand and Qazvin and Jolfa were placed in the same sub-clade, separately. Using RAPD12,the least 145 bands were obtained ranged 800-10,000 bp. Isolates were in four groups (Figure 1-d). This primer produced the heaviest DNA but the least polymorphism in *O. leptostyla*.

M13 ISSR primer produced considerably the most bands (694) averagely 15 bands per isolate ranged 250-2000 bp. It could also produce the smallest DNA fragments comparing other primers and four groups were gained using the primer (Figure 1-e). Thus lower polymorphism with no correlation with morphological and geographic data observed. Also, $(GTG)_5$produced high number of bands too (657) with 10 bands per isolate ranged 300-600 bp. Four groupsof isolates distinguished using this primer (Figure 1-f). In the second group, there were only two isolates. The primer could not have suitable efficacy for genetic polymorphism study in this fungal species. There was no correlation between obtained groups with this primer with fungus morphology, too.

O. leptostyla has morphologically considerable variation in Iran [11,21], the variation might justify by versatility in ecological features and climates and supernatural diversity in Persian walnut genotypes resulting from propagation by seeds and heterogamous pollinationin Iran. This might be resulted in high genetically variation in the species. Morphological characteristics like homothallic were slightly correlated with RAPD12 marker and there were some correlations between RAPD29 marker and sampling place, promising finding specific primers. Considering deficiency of ITS RFLP and LSU of nrDNA [4,21] and finding genetic diversity using RAPD and ISSR technique, it is suggested to use total genome techniques for genetic polymorphism studies in this species. However, RAPD was more efficient than ISSR in this regard.

Dendrogram which is drawn with all RAPDs resulted in nine groups, while four groups resulting from ISSRs. Therefore, RAPD was more effective for *O. leptostyla* genetic polymorphism study

(Figure 2 a,b). Populations' analysis based RAPD showed there are two main groups between isolates and Ilam and Hamedan Isolates are in the same group and the others in the other one. In this group, Kurdestan and Kermanshah isolates was in the same group. On the whole there is a correlation between province of sample collection and RAPDs (Figure 3a).

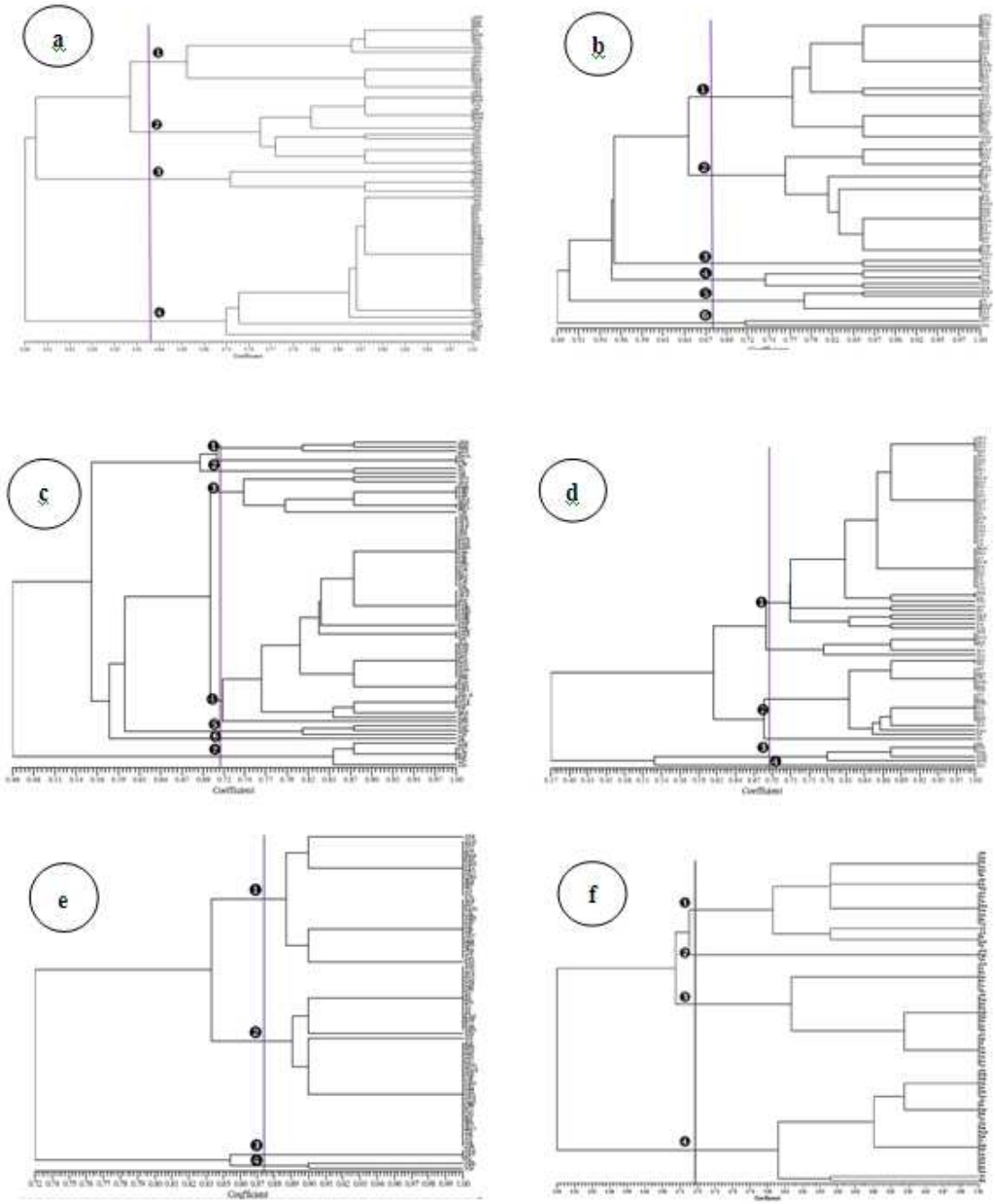

Figure 1 – Dendrogram of *O. leptostyla* Iranian isolates based on RAPD and ISSRs primers. a) RAPID211, b) RAPD1, c) RAPD19, d) RAPD12, e) $(GTG)_5$,and f) M13.

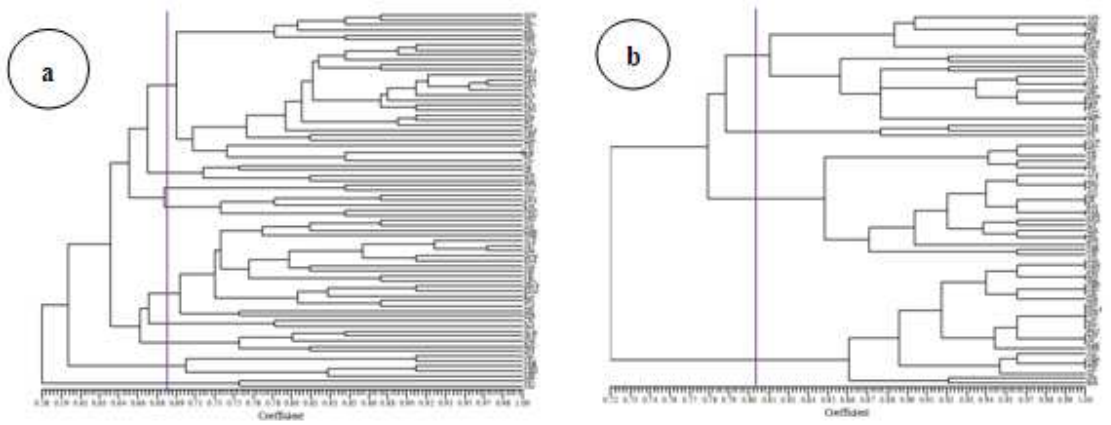

Figure 2 – Dendrogram of *O. leptostyla* Iranian isolates based on all RAPDs (a) and all ISSRs (b) primers.

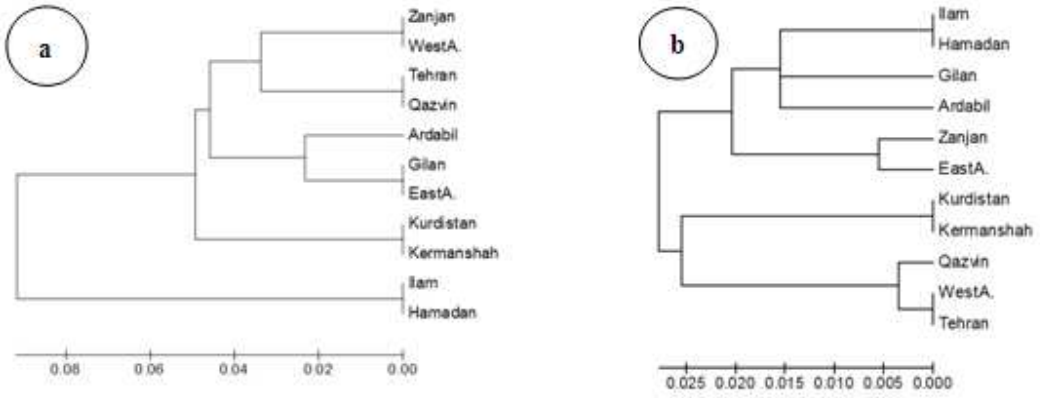

Figure 3 – Population analysis of *O. leptostyla* Iranian isolates based on all RAPDs (a) and all ISSRs (b) primers by neighbor-joining method. (each province was considered as population located on clades)

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