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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 (*JuglansregiaL.*) 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 (*JuglansnigraL.*) 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 of polymerase chain reaction technology to identification of plant pathogenic fungi. Such methods offer theadvantage of reducing or eliminating the need for lengthy culturing and difficult morphological identification procedures [17,24].PCR-based genomic fingerprinting is a good alternative tomethods that rely on specifically targeted primers [17]. RAPD<sup>1</sup> and ISSR<sup>2</sup> or MSPPCR<sup>3</sup> 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 10base 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. Belisarioet al. (1992) surveyed on genetic variation of 176Italian O. leptostyla isolates using PCR-RFLPs of ITS and 18S rDNA and found no polymorphism [4]. Salahiet 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 regions of nuclear ribosomal DNA in their studies on Gnomoniaceae phylogeny [9,15]. Sogonovet al. (2008) studied four genetic regions sequencing like tef1, 28S rDNA, β-tubulin, and RNA Polymerase II and 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 Juglansregia (CBS 110136, CBS 844.79 and CBS 110136). There were two closely related subclades 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 IranianO. 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) and sodium hypochlorite 1% (for 60 s), then washed fourtimes with sterile

<sup>&</sup>lt;sup>1</sup> Random Amplified Polymorphic DNA

<sup>&</sup>lt;sup>2</sup> Inter Simple Sequence Repeats

<sup>&</sup>lt;sup>3</sup> 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 were used for DNA extraction.

Collection site   Province   Latitude   Longitude   Antimude     Abk   Abhar, Shanat   Zanjan   49' 16G   36' 13   1645     Ahr   AzarbaijaneSharghi   45' 53G   37' 28   1341     Ajb   Ajabshir, Bonalu   AzarbaijaneSharghi   45' 53G   37' 28   1341     Ajd   Ajabshir, Bonalu   AzarbaijaneSharghi   45' 50   37' 29   1375     Ajd   Ajabshir, Bonalu   AzarbaijaneSharghi   45' 50   37' 29   1333     Ard   Ardebil   Ardebil   Ardebil   47' 17   38' 14   1500     Arf   Ardebil, Oskanlu   Ardebil   47' 17   38' 16   1432     Asa   Abhar, Sharifabad   Zanjan   49' 12   38' 16   1322     Asa   Abhar, Sharifabad   ZanjajaneSharghi   45' 58   37' 47   43     Azar   AzarsajaneSharghi   46' 31   34' 06   1514     Firm   Fuman   Gilan   46' 31   34' 06   1514     Firm <th>T1-4- A</th> <th colspan="8">Sampling Information</th>	T1-4- A	Sampling Information							
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EagEslamabad'egharbKermanshahan $46^{\circ}$ $31^{\circ}$ $34^{\circ}$ $06^{\circ}$ $1514$ FmnFumanGilan $49^{\circ}$ $17^{\circ}$ $37^{\circ}$ $13^{\circ}$ $50$ GgtGogan, TaimurluAzarbaijaneSharghi $45^{\circ}$ $54^{\circ}$ $37^{\circ}$ $46^{\circ}$ $1961$ GlvGilvanGilan $49^{\circ}$ $25^{\circ}$ $36^{\circ}$ $44^{\circ}$ $439$ GnjGanjehGilan $48^{\circ}$ $28^{\circ}$ $39^{\circ}$ $00^{\circ}$ $993$ HmdHamedanHamedan $48^{\circ}$ $21^{\circ}$ $37^{\circ}$ $47^{\circ}$ $1660$ HeIlam, EywanIlam $47^{\circ}$ $05^{\circ}$ $37^{\circ}$ $47^{\circ}$ $1660$ IleIlam, EywanIlam $46^{\circ}$ $19^{\circ}$ $33^{\circ}$ $48^{\circ}$ $1645$ IlmIlamIlam $44^{\circ}$ $55^{\circ}$ $33^{\circ}$ $38^{\circ}$ $1759$ JzzJolfa, ZaviyeAzarbaijaneSharghi $45^{\circ}$ $45^{\circ}$ $38^{\circ}$ $53^{\circ}$ $879$ JolJolfaAzarbaijaneGharbi $44^{\circ}$ $49^{\circ}$ $38^{\circ}$ $43^{\circ}$ $1309$ KhdKhorramdarrehZanjan $49^{\circ}$ $11^{\circ}$ $35^{\circ}$ $42^{\circ}$ $1681$ KrpPavehKermanshah $46^{\circ}$ $21^{\circ}$ $35^{\circ}$ $42^{\circ}$ $1681$ KrpPavehKermanshah $46^{\circ}$ $21^{\circ}$ $35^{\circ}$ $12^{\circ}$ $2210$ KasKhosroshahrAzarbaijaneGharbi $44^{\circ}$ $90^{\circ}$ $37^{\circ}$ $75^{\circ}$ $1357$ LhjLahijanGilan $50^{\circ}$ $37^{\circ}$ $36^{\circ}$ $57^{\circ}$ $1292$ LhrTaleghan, LahranTehran	Did	Divan darreh	Kordestan	47° 02 □	35° 54□	1821			
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$\begin{array}{c c c c c c c c c c c c c c c c c c c $	Eag	Eslamabad'egharb	Kermanshahan	46° 31 □	34° 06□	1514			
Giv Gilvan Gilan 49° 25 36° 44 439   Gnj Ganjeh Gilan 48° 25 36° 51 804   Grm Germi Ardebil 48° 05 39° 00 993   Hmd Hamedan Haredan 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   Jol Jolfa AzarbaijaneSharghi 45° 38 38° 56 70   Jol Jolfa AzarbaijaneSharghi 45° 38 38° 56 70   Khd Khoramdarreh Zanjan 49° 11 36° 11 1655   Khf Khoy, Mortezagholi AzarbaijaneGharbi 44° 49 38° 43 1309   Krk Karaj, Kamalabad Tehran 51° 38 35° 49 1681   Krp Paveh <	Fmn		Gilan	49° 17□	37° 13□	50			
Gnj Ganjeh Gilan 48° 28 36° 51 804   Grm Germi Ardebil 48° 21 34° 11 2150   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   Jlm Ilam Ilam 46° 25 33° 38 1759   Jlz Jolfa, Zaviye AzarbaijaneSharghi 45° 38 38° 56 720   Khd Khorramdarreh Zanjan 49° 11 36° 11 1655   Khf Khoy, Firuragh AzarbaijaneGharbi 44° 49 38° 32 1309   Khr Karaj, Kamalabad Tehran 51° 38 35° 49 1681   Krp Paveh Kermanshah 46° 21 35° 02 2210   Kss Khosroshahr AzarbaijaneSharghi 46° 00 37° 57 1357   Lhj <t< td=""><td>Ggt</td><td>Gogan, Taimurlu</td><td>AzarbaijaneSharghi</td><td>45° 54□</td><td>37° 46□</td><td>1961</td></t<>	Ggt	Gogan, Taimurlu	AzarbaijaneSharghi	45° 54□	37° 46□	1961			
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IlmIlamIlamIlam $46^{\circ} 25^{\circ}$ $33^{\circ} 38^{\circ}$ $1759^{\circ}$ JzJolfa, ZaviyeAzarbaijaneSharghi $45^{\circ} 40^{\circ}$ $38^{\circ} 53^{\circ}$ $879^{\circ}$ JolJolfaAzarbaijaneSharghi $45^{\circ} 38^{\circ}$ $38^{\circ} 56^{\circ}$ $720^{\circ}$ KhdKhorramdarrehZanjan $49^{\circ}$ $11^{\circ}$ $36^{\circ} 11^{\circ}$ $1655^{\circ}$ KhfKhoy, FiruraghAzarbaijaneGharbi $44^{\circ} 49^{\circ}$ $38^{\circ} 32^{\circ}$ $1369^{\circ}$ KhmKhoy, MortezagholiAzarbaijaneGharbi $44^{\circ} 57^{\circ}$ $38^{\circ} 32^{\circ}$ $136^{\circ}$ KrkKaraj, KamalabadTehran $51^{\circ} 38^{\circ} 32^{\circ}$ $2210^{\circ}$ KssKhosroshahrAzarbaijaneSharghi $46^{\circ} 21^{\circ}$ $37^{\circ} 57^{\circ}$ $1357^{\circ}$ LhjLahijanGilan $50^{\circ} 00^{\circ}$ $37^{\circ} 12^{\circ}$ $29^{\circ}$ MdbMiandoabAzarbaijaneGharbi $48^{\circ} 90^{\circ}$ $36^{\circ} 57^{\circ}$ $1292^{\circ}$ MddMarand, DizajolyaAzarbaijaneSharghi $45^{\circ} 43^{\circ}$ $37^{\circ} 23^{\circ}$ $1326^{\circ}$ MdoMarand, OrdakluAzarbaijaneSharghi $45^{\circ} 45^{\circ}$ $37^{\circ} 36^{\circ}$ $1292^{\circ}$ MidMiyaneh, BalesinAzarbaijaneSharghi $45^{\circ} 35^{\circ}$ $37^{\circ} 38^{\circ}$ $1237^{\circ}$ MijMiyaneh, BalesinAzarbaijaneSharghi $45^{\circ} 35^{\circ}$ $37^{\circ} 36^{\circ}$ $1237^{\circ}$ MijMiyaneh, BalesinAzarbaijaneSharghi $45^{\circ} 35^{\circ}$ $36^{\circ} 26^{\circ}$ $1294^{\circ}$ MirTaleghan, Mir	Ile	Ilam, Eywan		46° 19□	33° 48□	1645			
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KhdKhorramdarrehZanjan $49^{\circ}$ 11 $36^{\circ}$ 11 $1655$ KhfKhoy, FiruraghAzarbaijaneGharbi $44^{\circ}$ 49 $38^{\circ}$ 43 $1309$ KhmKhoy, MortezagholiAzarbaijaneGharbi $44^{\circ}$ 57 $38^{\circ}$ 32 $1136$ KrkKaraj, KamalabadTehran $51^{\circ}$ 38 $35^{\circ}$ 49 $1681$ KrpPavehKermanshah $46^{\circ}$ 21 $35^{\circ}$ 022210KssKhosroshahrAzarbaijaneSharghi $46^{\circ}$ 02 $37^{\circ}$ 57 $1357$ LhjLahijanGilan $50^{\circ}$ 00 $37^{\circ}$ 57 $1292$ MdbMiandoabAzarbaijaneGharbi $48^{\circ}$ 90 $36^{\circ}$ 57 $1292$ MddMarand, DizajolyaAzarbaijaneSharghi $45^{\circ}$ 37 $38^{\circ}$ 27 $1442$ MdkMarand, OrdakluAzarbaijaneSharghi $45^{\circ}$ 37 $38^{\circ}$ 27 $1442$ MdoMarand, AghkandAzarbaijaneSharghi $45^{\circ}$ 35 $37^{\circ}$ 38 $1237$ MiaMiyaneh, AghkandAzarbaijaneSharghi $45^{\circ}$ 35 $37^{\circ}$ 38 $1237$ MijMiyaneh, BalesinAzarbaijaneSharghi $45^{\circ}$ 35 $37^{\circ}$ 38 $1237$ MijMiyaneh, BalujehAzarbaijaneSharghi $45^{\circ}$ 35 $36^{\circ}$ 26 $1294$ MrdMarandAzarbaijaneSharghi $45^{\circ}$ 35 $36^{\circ}$ 26 $1294$ MrdMarandAzarbaijaneSharghi $45^{\circ}$ 33 $1503$ MirTaleghan, MirTehran $50^{\circ}$ 33 $35^{\circ}$ 15 $1753$ <tr< td=""><td>Jlz</td><td>Jolfa, Zaviye</td><td>AzarbaijaneSharghi</td><td><math>45^{\circ} 40</math></td><td>38° 53□</td><td>879</td></tr<>	Jlz	Jolfa, Zaviye	AzarbaijaneSharghi	$45^{\circ} 40$	38° 53□	879			
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KhmKhoy, MortezagholiAzarbaijaneGharbi $44^\circ$ 57 $38^\circ$ 32 $1136$ KrkKaraj, KamalabadTehran $51^\circ$ 38 $35^\circ$ 49 $1681$ KrpPavehKermanshah $46^\circ$ 21 $35^\circ$ 022210KssKhosroshahrAzarbaijaneSharghi $46^\circ$ 02 $37^\circ$ 57 $1357$ LhjLahijanGilan $50^\circ$ 00 $37^\circ$ 1229LhrTaleghan, LahranTehran $50^\circ$ 37 $36^\circ$ 11 $1893$ MdbMiandoabAzarbaijaneGharbi $48^\circ$ 90 $36^\circ$ 57 $1292$ MddMarand, DizajolyaAzarbaijaneSharghi $45^\circ$ 37 $38^\circ$ 27 $1442$ MdkMarand, AtanlojAzarbaijaneSharghi $45^\circ$ 43 $37^\circ$ 23 $1326$ MdoMarand, OrdakluAzarbaijaneSharghi $45^\circ$ 43 $37^\circ$ 14 $1747$ MiaMiyaneh, AghkandAzarbaijaneSharghi $45^\circ$ 35 $37^\circ$ 38 $1237$ MijMiyaneh, BalesinAzarbaijaneSharghi $45^\circ$ 55 $36^\circ$ 26 $1294$ MrMarandAzarbaijaneSharghi $45^\circ$ 55 $36^\circ$ 26 $1294$ MrdMarandAzarbaijaneSharghi $45^\circ$ 16 $38^\circ$ 25 $1334$ MrgMaraghehAzarbaijaneSharghi $45^\circ$ 16 $38^\circ$ 25 $1334$ MrgMaraghehAzarbaijaneSharghi $45^\circ$ 16 $38^\circ$ 25 $1334$ MrgMaraghehAzarbaijaneSharghi $45^\circ$ 16 $38^\circ$ 23 $1452$ MrdMarandKordestan $46^\circ$	Khd	Khorramdarreh	Zanjan	49° 11□	36° 11□	1655			
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KrpPavehKermanshah $46^{\circ} 21$ $35^{\circ} 02$ $2210$ KssKhosroshahrAzarbaijaneSharghi $46^{\circ} 02$ $37^{\circ} 57$ $1357$ LhjLahijanGilan $50^{\circ} 00$ $37^{\circ} 12$ $29$ LhrTaleghan, LahranTehran $50^{\circ} 37^{\circ}$ $36^{\circ} 11^{\circ}$ $1893$ MdbMiandoabAzarbaijaneGharbi $48^{\circ} 90^{\circ}$ $36^{\circ} 57^{\circ}$ $1292$ MddMarand, DizajolyaAzarbaijaneSharghi $45^{\circ} 37^{\circ}$ $38^{\circ} 27^{\circ}$ $1442$ MdkMarand, CrdakluAzarbaijaneSharghi $45^{\circ} 43^{\circ}$ $37^{\circ} 23^{\circ}$ $1326$ MdoMarand, OrdakluAzarbaijaneSharghi $45^{\circ} 41^{\circ}$ $37^{\circ} 24^{\circ}$ $1353$ MiaMiyaneh, AghkandAzarbaijaneSharghi $45^{\circ} 35^{\circ}$ $37^{\circ} 38^{\circ}$ $1237$ MijMiyaneh, BalesinAzarbaijaneSharghi $45^{\circ} 35^{\circ}$ $37^{\circ} 36^{\circ}$ $1503$ MirTaleghan, MirTehran $50^{\circ} 33^{\circ}$ $35^{\circ} 15^{\circ}$ $1753$ MlkMalekanAzarbaijaneSharghi $45^{\circ} 55^{\circ}$ $36^{\circ} 26^{\circ}$ $1294$ MrdMarandAzarbaijaneSharghi $45^{\circ} 46^{\circ}$ $37^{\circ} 23^{\circ}$ $1334$ MrgMaraghehAzarbaijaneSharghi $45^{\circ} 46^{\circ}$ $37^{\circ} 23^{\circ}$ $1449$ MrsMarivan, SeyfKordestan $46^{\circ} 16^{\circ}$ $35^{\circ} 31^{\circ}$ $1543$ MshMeshkinshahrArdebil $47^{\circ} 40^{\circ}$ $38^{\circ} 23^{\circ}$ $1452$ MslMasu	Krk	Karaj, Kamalabad	Tehran	51° 38□	35° 49□	1681			
KssKhosroshahrAzarbaijaneSharghi $46^{\circ} 02^{\circ}$ $37^{\circ} 57^{\circ}$ $1357^{\circ}$ LhjLahijanGilan $50^{\circ} 00^{\circ}$ $37^{\circ} 12^{\circ}$ $29^{\circ}$ LhrTaleghan, LahranTehran $50^{\circ} 37^{\circ}$ $36^{\circ} 11^{\circ}$ $1893^{\circ}$ MdbMiandoabAzarbaijaneGharbi $48^{\circ} 90^{\circ}$ $36^{\circ} 57^{\circ}$ $1292^{\circ}$ MddMarand, DizajolyaAzarbaijaneSharghi $45^{\circ} 37^{\circ}$ $38^{\circ} 27^{\circ}$ $1442^{\circ}$ MdkMarand, KandlojAzarbaijaneSharghi $45^{\circ} 43^{\circ}$ $37^{\circ} 23^{\circ}$ $1326^{\circ}$ MdoMarand, OrdakluAzarbaijaneSharghi $45^{\circ} 41^{\circ}$ $37^{\circ} 24^{\circ}$ $1353^{\circ}$ MiaMiyaneh, AghkandAzarbaijaneSharghi $45^{\circ} 35^{\circ}$ $37^{\circ} 38^{\circ}$ $1237^{\circ}$ MibMiyaneh, BalesinAzarbaijaneSharghi $45^{\circ} 35^{\circ}$ $37^{\circ} 36^{\circ}$ $1503^{\circ}$ MirTaleghan, MirTehran $50^{\circ} 33^{\circ}$ $35^{\circ} 15^{\circ}$ $1753^{\circ}$ MlkMalekanAzarbaijaneSharghi $45^{\circ} 46^{\circ}$ $38^{\circ} 25^{\circ}$ $1294^{\circ}$ MrdMarandAzarbaijaneSharghi $45^{\circ} 46^{\circ}$ $38^{\circ} 25^{\circ}$ $1334^{\circ}$ MrgMaraghehAzarbaijaneSharghi $45^{\circ} 46^{\circ}$ $38^{\circ} 23^{\circ}$ $1449^{\circ}$ MrsMarivan, SeyfKordestan $46^{\circ} 16^{\circ}$ $38^{\circ} 23^{\circ}$ $1449^{\circ}$ MshMeshkinshahrArdebil $47^{\circ} 40^{\circ}$ $38^{\circ} 31^{\circ}$ $1543^{\circ}$ MslMasulehGilan $48^{\circ}$	Krp		Kermanshah	46° 21 □	35° 02□	2210			
$ \begin{array}{cccccccccccccccccccccccccccccccccccc$	Kss	Khosroshahr	AzarbaijaneSharghi		37° 57□	1357			
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MdkMarand, KandlojAzarbaijaneSharghi $45^{\circ} 43^{\circ}$ $37^{\circ} 23^{\circ}$ $1326$ MdoMarand, OrdakluAzarbaijaneSharghi $45^{\circ} 41^{\circ}$ $37^{\circ} 24^{\circ}$ $1353^{\circ}$ MiaMiyaneh, AghkandAzarbaijaneSharghi $48^{\circ} 04^{\circ}$ $37^{\circ} 14^{\circ}$ $1747^{\circ}$ MibMiyaneh, BalesinAzarbaijaneSharghi $48^{\circ} 04^{\circ}$ $37^{\circ} 38^{\circ}$ $1237^{\circ}$ MijMiyaneh, BalujehAzarbaijaneSharghi $45^{\circ} 35^{\circ}$ $37^{\circ} 36^{\circ}$ $1503^{\circ}$ MirTaleghan, MirTehran $50^{\circ} 33^{\circ}$ $35^{\circ} 15^{\circ}$ $1753^{\circ}$ MlkMalekanAzarbaijaneSharghi $45^{\circ} 55^{\circ}$ $36^{\circ} 26^{\circ}$ $1294^{\circ}$ MrdMarandAzarbaijaneSharghi $45^{\circ} 55^{\circ}$ $36^{\circ} 25^{\circ}$ $1334^{\circ}$ MrgMaraghehAzarbaijaneSharghi $46^{\circ} 14^{\circ}$ $37^{\circ} 23^{\circ}$ $1449^{\circ}$ MrsMarivan, SeyfKordestan $46^{\circ} 09^{\circ}$ $35^{\circ} 31^{\circ}$ $1563^{\circ}$ MrvMarivanKordestan $46^{\circ} 09^{\circ}$ $35^{\circ} 31^{\circ}$ $1543^{\circ}$ MshMeshkinshahrArdebil $47^{\circ} 40^{\circ}$ $38^{\circ} 23^{\circ}$ $1452^{\circ}$ MslMasulehGilan $48^{\circ} 59^{\circ}$ $37^{\circ} 09^{\circ}$ $1050^{\circ}$ MyrMohammadyarAzarbaijaneGharbi $45^{\circ} 31^{\circ}$ $36^{\circ} 57^{\circ}$ $1360^{\circ}$	Mdd	Marand, Dizajolya		45° 37□	38° 27 □	1442			
MiaMiyaneh, AghkandAzarbaijaneSharghi48° 04 $37° 14$ $1747$ MibMiyaneh, BalesinAzarbaijaneSharghi $45° 35$ $37° 38$ $1237$ MijMiyaneh, BalujehAzarbaijaneSharghi $45° 35$ $37° 36$ $1503$ MirTaleghan, MirTehran $50° 33$ $35° 15$ $1753$ MlkMalekanAzarbaijaneSharghi $45° 55$ $36° 26$ $1294$ MrdMarandAzarbaijaneSharghi $45° 46$ $38° 25$ $1334$ MrgMaraghehAzarbaijaneSharghi $46° 14$ $37° 23$ $1449$ MrsMarivan, SeyfKordestan $46° 16$ $35° 33$ $1563$ MrvMarivanKordestan $46° 09$ $35° 31$ $1543$ MshMeshkinshahrArdebil $47° 40$ $38° 23$ $1452$ MslMasulehGilan $48° 59$ $37° 09$ $1050$ MyrMohammadyarAzarbaijaneGharbi $45° 31$ $36° 57$ $1360$	Mdk			45° 43□	37° 23□	1326			
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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	U								
Msh   Meshkinshahr   Ardebil   47° 40 □   38° 23 □   1452     Msl   Masuleh   Gilan   48° 59 □   37° 09 □   1050     Myr   Mohammadyar   AzarbaijaneGharbi   45° 31 □   36° 57 □   1360									
Msl   Masuleh   Gilan   48° 59□   37° 09□   1050     Myr   Mohammadyar   AzarbaijaneGharbi   45° 31□   36° 57□   1360									
Myr Mohammadyar AzarbaijaneGharbi 45° 31 36° 57 1360									
	Ngd	Naghadeh	AzarbaijaneGharbi	45° 23□	36° 57⊡	1383			

#### Table 1- Geographical characterization of studied O. leptostyla isolates

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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 adjusted to 25 ng/ $\mu$ l.

#### 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)<sub>5</sub> [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 TaqDNA 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)<sub>5</sub> and 44°C for M13/50 s, 72°C/2 min (35×) and 72°C/10 min.PCR amplified products 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)<sub>5</sub>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).

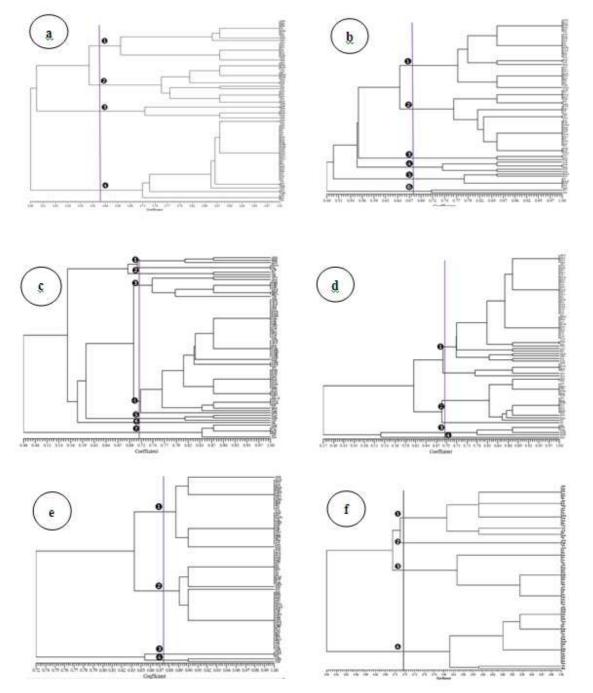


Figure 1 – Dendrogram of *O. leptostyla* Iranian isolates based on RAPD and ISSRs primers. a) RAPID211, b) RAPD1, c) RAPD19, d) RAPD12, e) (GTG)<sub>5</sub>,and f) M13.

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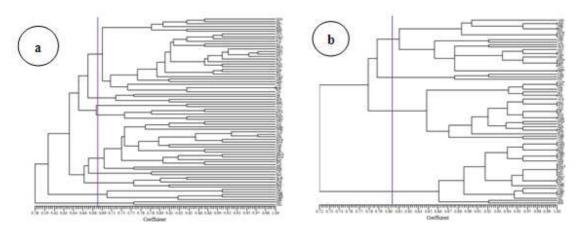


Figure 2 – Dendrogram of O. leptostyla Iranian isolates based on all RAPDs (a) and all ISSRs (b) primers.

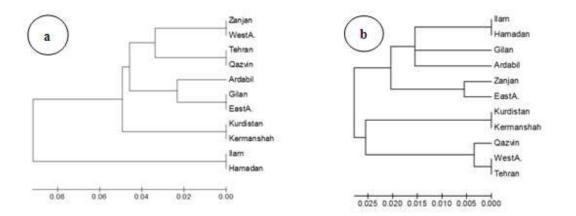


Figure 3 – Population analysis of *O. leptostyla* Iranian isolates based on all RAPDs (a) and all ISSRs (b) primers by neighbor-joining method. (each province was considered as population located on clades)

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