

Molecular Phylogeny of *Ophiognomonia leptostyla* Isolates Collected from Iran Based on ITS nrDNA Sequences

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Abstract—Anthracnose is the most important fungal disease on Persian walnut in Iran. The current study was performed on 16 *Ophiognomonia leptostyla* isolates collected from northwest region of Iran. ITS amplification was performed by using ITS1 and ITS4 primers and the sequences were analyzed. Phylogenetic trees are made based on ITS sequences which showed that all Iranian isolates are in the same branch in a clade with *Ophiognomonia leptostyla* from CBS isolated from *Juglans regia*. The Iranian isolates are divided into two closely related sub-clades with low bootstrap support, the smaller sub-clade comprised of only three isolates were collected from wild walnut trees located in woodland areas.

Index Terms—*Gnomonia leptostyla*, ITS sequencing, phylogenetic tree, walnut leaf blotch.

I. INTRODUCTION

Iran takes the third position in walnut production in the world (FAO, 2009). Persian or European walnut (*Juglans regia* L.) is common in most parts of Iran except the Persian Gulf coasts [1]. Walnut anthracnose or black spot/blotch is one of the most important fungal diseases on black walnut (*Juglans nigra* L.) in the North and South America and on Persian walnut in Europe and Asia [2]. It is widespread in Iran and has been reported from the north, west, northwest and northeast of the country [3]. Disease epidemics might be very destructive in rainy and cool seasons [2]-[4]. The causal agent is *Ophiognomonia leptostyla* (Fr.) Sogonov 2008, with *Marssoniiella juglandis* (Lib.) Hahn 1916 as its anamorph [5].

Genetic diversity of *O. leptostyla* has been poorly studied. Belisario *et al.* (1992) surveyed on genetic variation of 176 Italian *O. leptostyla* isolates using PCR-RFLPs of ITS and 18S rDNA and found no polymorphism [6]. Salahiet *al.* (2007) used the same technique with 30 Iranian isolates collected from East Azarbaijan province and the results were the same with no polymorphism [4]. Mejia *et al.* (2002) and Green & Castlebury (2007) amplified ITS1 and ITS2 regions of nuclear ribosomal DNA in their studies about *Gnomoniaceae* phylogeny [5], [7]. Sogonov *et al.* (2008) studied four genes sequencing (tef 1, 28S rDNA, β -tubulin and RNA Polymerase II) in this genus and offered a new concept of *Gnomonia* and *Ophiognomonia*, and subsequently introduced the new combination which is currently used [8], [9]. ITS sequencing is one of the main tools in phylogenetic

studies. In previous researches, ITS and LSU nrDNA RFLP-PCR was not useful for finding genetic diversity in Italian and Iranian isolates. The restriction enzymes which have been used might not cut the informative sites in ITS sequences. Therefore, in order to evaluate the ITS region sequences in evolutionary studies of this species among Iranian isolates, we sequenced the ITS region of 16 isolates and compared them with those available at GenBank. Another aim of the study was to find genetic diversity among Iranian isolates.

II. MATERIAL AND METHODS

A. Fungus Isolation and Mycelial Mass Production

Sixteen Persian walnut leaf samples having anthracnose infection were collected from the northwest of Iran (Table 1) in spring and summer of 2006-08 and the causal agent were identified as *O. leptostyla* according to Sogonov *et al.* (2008). Three leaf discs (each 6 mm-diameter) containing acervuli were surface sterilized using ethanol 75% (30 sec) and sodium hypochlorite 1% (60 sec), then were washed four times with sterile distilled water. Macroconidia were released by crushing leaf samples and picked off small leaf pieces in 1.5 ml Eppendorf tubes containing 1 ml sterile distilled water and vortexed for 20 seconds. Then, 100 ml of conidial suspension were transferred into 2% WA (water agar, Merck, Germany) and incubated at 21°C, 50% RH, dark condition. Germinating macroconidia were transferred to 39% PDA (potato dextrose agar, Merck, Germany) + 7 gr/L oatmeal and incubated at 21°C, 50% RH and 12/12 alternative photoperiod for 10 days. Obtained 10-day old mycelia were used for DNA extraction.

B. DNA Extraction and Amplification

DNA extraction was carried out according to slightly modified Liu *et al.* (2000). The ITS region (ITS1-5.8S-ITS2) was amplified using primers ITS1 [5'-tcc gtagtgaaacctgcg g] and ITS4 [5'-tcc tccgt tat tga tat gc] (White *et al.*, 1990). The PCR reaction (25 μ l) contained 50 ng of genomic DNA, 12.5 pmol of each primer, 0.3 mM dNTPs (CinnaGen, Tehran) and 1 \times PCR buffer containing 2 mM MgCl₂, 1.5 U *Taq* DNA polymerase (Cinna Gen, Tehran). PCR amplification was carried out using Apollo (ATC. 401, ver. 4/88, CLP, Inc. USA) PCR machine. The PCR program for ITS region amplification was 94 °C/5 min (initial denaturation), 94 °C/40 s, 50 °C/50 s, 72 °C/2 min (35 \times) and 72 °C/7 min (final extension). The PCR products were purified using a Genomic DNA Purification Kit, K0514, Fermentas). The purified DNA samples were then submitted to a capillary sequencing machine (Eurofins MWG Operon, Ebersburg, Germany).

Manuscript received July 15, 2010; revised October 20, 2012.

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TABLE I: STUDIED ISOLATES.

Acronym	Sampling Information		
	Location (Province)	Climate	Altitude
Abs	Abhar - Shanat (Zanjan)	semi-arid cold	1645 ^b
Aro	Ardebil - Oskanlou (Ardebil)	semi-humid cold	1432
Asa	Abhar - Sharif Abad (Zanjan)	semi-arid cold	1713
Fmn	Fuman (Gilan)	humid moderate	50
Ggn	Goovgan - Taimorlou (EA ^a)	arid cold	1961
Glv	Gilvan (Gilan)	humid moderate	493
Ile	Ilam - Eivan (Ilam)	semi-arid moderate	1645
Mib	Miynah - Balesin (E.A)	arid cold	1237
Mij	Miyaneh - Balojeh (EA)	arid cold	1503
Mir	Taleghan - Mir (Tehran)	humid moderate	1752
Mlk	Malekan (EA)	arid cold	1294
Mrg	Maragheh (EA)	semi-arid cold	1449
Mrs	Marivan - Seyf (Kurdistan)	semi-humid cold	1563
Msl	Masouleh (Gilan)	humid cold	1050
Qza	Qazvin - Abyek (Qazvin)	semi-arid cold	1366
Shn	Taleghan - Sohan (Tehran)	humid moderate	1895

a. East Azarbaijan; b. in meter

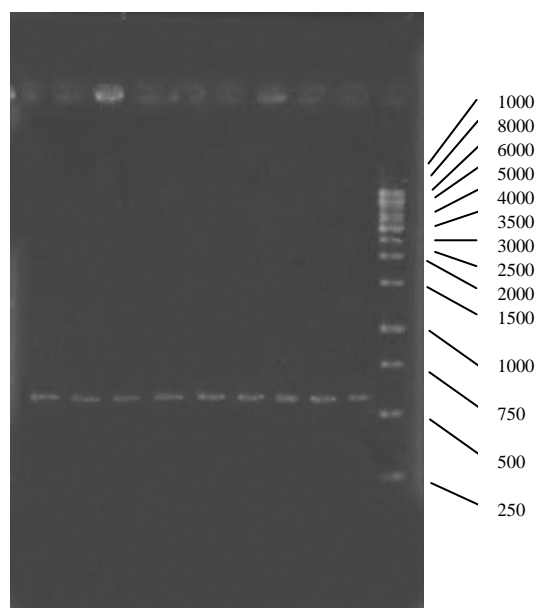


Fig. 1. Electrophoresis of ITS-PCR products on 1% agarose gel.

C. Figures phylogenetic Analysis

The programs EditSeq and SeqMan, parts of the DNA*Lasergene (DNASTar, Madison, WI, USA) software package, were used to assemble and edit the sequence files. The alignments were initially obtained using the Pairwise Alignment option in GeneDoc (Nicholas and Nicholas, 1997). Sequences of the ITS region were analyzed using MEGA4 (Tamura *et al.*, 2007). The phylogeny was inferred using the Distance (NJ) and Maximum Parsimony (MP) methods. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.*, 2004). All positions containing alignment gaps, and missing data were eliminated only in pairwise sequence comparisons (Pairwise

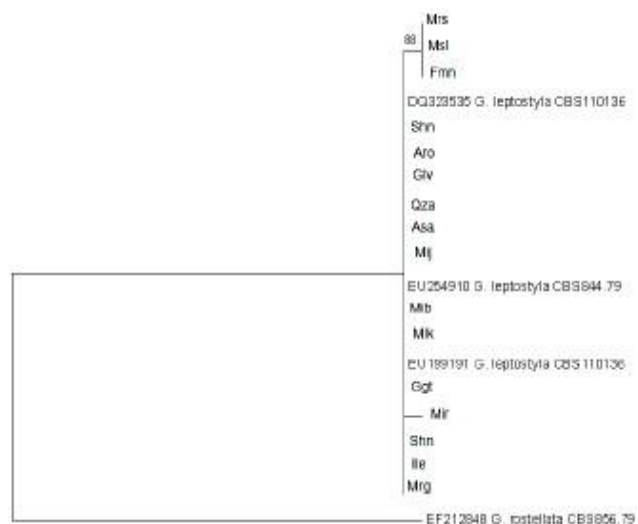
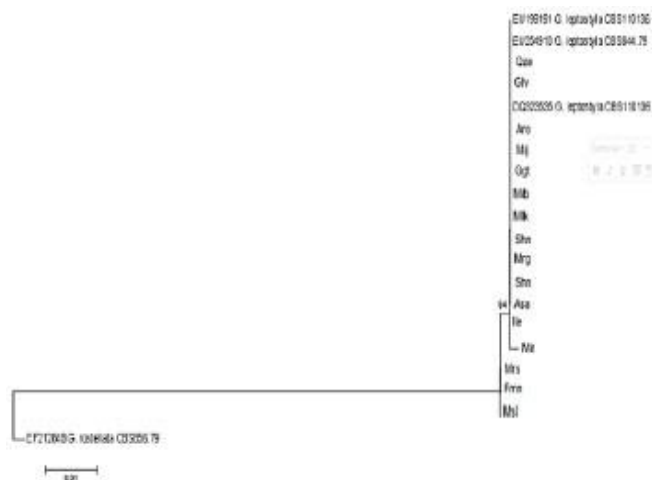
deletion option). Relevant sequences from Genbank were included in the analyses.

III. RESULTS AND DISCUSSION

The ITS region has been amplified and a single band (about 500 bp) was obtained as measured by agarose gel (Fig. 1). Similar ITS sequences were searched in BLAST Genbank and *Gnomoniarostellata* strain CBS 856.79 isolated from strawberry was found the closest submitted ITS sequence to *O. leptostyla*, thus it was chosen as out-group.

IV. UNITS

Phylogenetic trees reconstructed based on ITS sequences using parsimony and neighbor-joining methods showed that 13 Iranian isolates are in the same clade with *Ophiogmonomonia leptostyla* from 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. These two groups were very similar with only one informative site in ITS sequences. The results of parsimony agreed with those of neighbor-joining (Fig. 2 and Fig. 3).

Fig. 2. Phylogenetic tree based on ITS sequences of Iranian and other isolates of *Ophiogmonomonia leptostyla* using NJ method.Fig. 3. Phylogenetic tree based on ITS sequences of Iranian and other isolates of *Ophiogmonomonia leptostyla* using parsimony method.

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. It will be useful to use other gene sequences, such as *tef* and β -tubulin, to investigate the relationship of the three isolates from wild walnuts.

ACKNOWLEDGMENT

Special thanks to Dr. H. R. Zamanizadeh and Dr. S. Rezaee for their kind support. We are also grateful to Mr. M. Mokhtari and Mr. M. Mianaji for their assistance in Biotechnology Laboratory of IAU, Miyaneh Branch regarding this work.

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