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# Molecular Phylogeny of *Ophiognominia 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 using ITS1 and ITS4 primers and the sequences were analyzed. Phylogenetic trees made based on ITS sequences 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.**

***Keywords-Gnomonia leptostyla; walnut leaf blotch; phylogentic tree; ITS sequencing***

## 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 [6]. 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, 3, 9]. The causal agent is *Ophiognomonia leptostyla* (Fr.) Sogonov 2008, with *Marssoniella juglandis* (Lib.) Höhn 1916 as its anamorph [8].

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 [1]. Salahi *et al.* (2007) used the same technique with 30 Iranian isolates collected from East Azarbaijan province and the results were the same with no polymorphism [9]. Mejia *et al.* (2002) and Green & Castlebury (2007) amplified ITS1 and ITS2 regions of nuclear ribosomal DNA in their studies about *Gnomoniaceae* phylogeny [5, 8]. 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 [7]. 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 sequences 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. MATERRIAL 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 Eppendorph 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 gta ggt gaa cct gcg g] and ITS4 [5′-tcc tcc gct tat tga tat gc] (White *et al.*, 1990). The PCR reaction (25 µl) contained 50 ng of genomic DNA, 12.5 pmol of each primer, 0.3 mM dNTPs (CinnaGen, Tehran) and 1× PCR buffer containing 2 mM $MgCl_2$, 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 ITS region amplification was 94°C/5 min (initial denaturation), 94°C/40 s, 50°C/50 s, 72°C/2 min (35×) 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).

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 (Kurdestan) | 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

### C. *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 *Gnomonia rostellata* strain CBS 856.79 isolated from strawberry was found the closest submitted ITS sequence to *O. leptostyla*, thus it was chosen as outgroup.

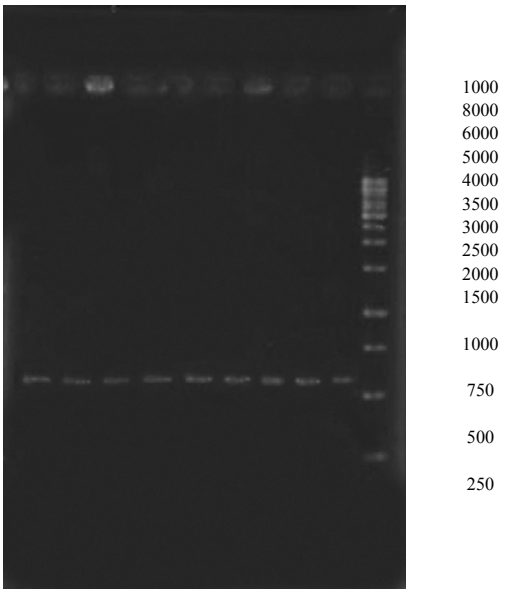


Figure 1. Electophoresis of ITS-PCR products on 1% agarose gel.

Phylogenetic trees reconstructed based on ITS sequences using parsimony and neighbor-joining methods showed that 13 Iranian isolates are in the same clade with *Ophiognomonia 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 (Figs 2 and 3).

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.

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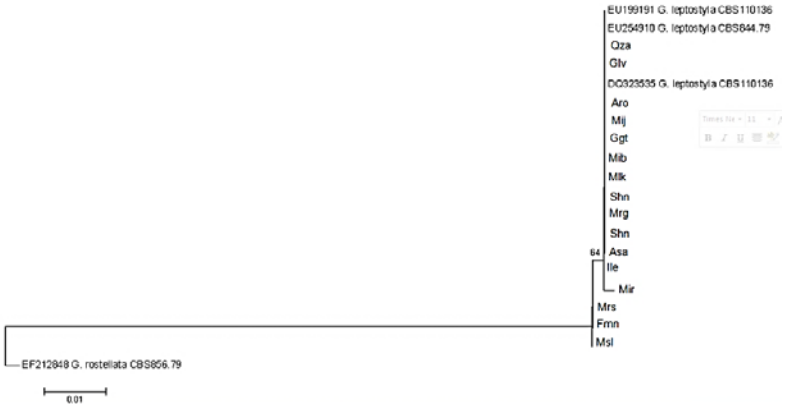


Figure 2. Phyogenetic tree based on ITS seqences of Iranian and other isolates of Ophiognomonia leptostyla using NJ method.

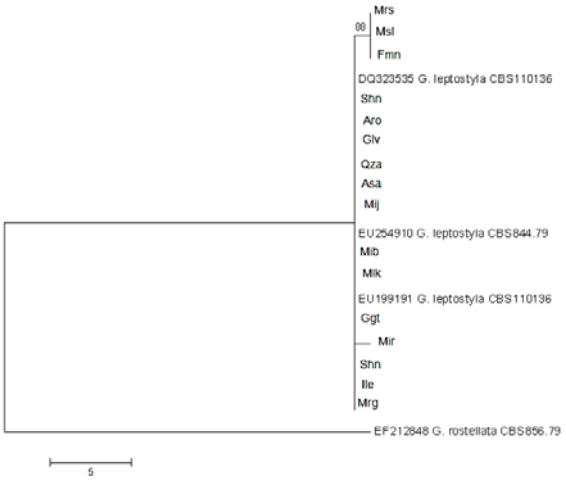


Figure 3. Phyogenetic tree based on ITS seqences of Iranian and other isolates of Ophiognomonia leptostyla using parsimony method.