

## Phylogenetic Analysis of *Alternaria* species Associated with Citrus Black Rot in Iran

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### Abstract

Citrus black rot caused by *Alternaria* spp. is significant post harvest problem in Iran. The causal agent of black rot was originally identified as *A. citri* by Ellis and Pierce. The genus *Alternaria* circumscribes considerable morphological diversity and citrus black rot may be caused by more than one morphospecies in addition to *A. citri*. Morphological identification of small-spored *Alternaria* spp. is difficult. Phylogenetic analysis was performed on 7 small-spored *Alternaria* isolates from black rotted citrus fruit in Iran using sequence data from an anonymous locus, OPA10-2. Samples were collected from navel oranges in Mazandaran province in the north of Iran. All isolates caused black rot in a fruit inoculation assay with Navel and Valencia oranges and significant differences were observed between cultivars and among isolates. No association was found between morphological classification and phylogenetic clade. We identified *A. tenuissima* (ALT 6) as the causal agents of citrus black rot. Two isolates (ALT8 and ALT9) grouped together, separately from other clades in most parsimonious trees. It suggested that both of them belong to different species. This is the first report of molecular characterization of citrus-associated *Alternaria* species in Iran and *A. tenuissima* is reported as the causal agent of citrus black rot in Iran for the first time.

**Keywords:** Citrus; *Alternaria* spp.; Black rot; Phylogenetic analysis; Anonymous locus

### Introduction

Citrus black rot is a well known post harvest disease which produces internal decay of citrus fruit. The disease is caused by several small-spored *Alternaria* species [1-4]. *Alternaria* species cause other diseases of citrus including, brown spot of tangerine, leaf and fruit spot of rough lemon and Rangpur lime, and *Mancha foliar de los citricos* affecting Mexican lime [2,5].

*Alternaria* black rot of citrus may occur in the field before harvest. It infected most commonly citrus, navel oranges (*Citrus sinensis* (L.) Osbeck) in the field and their hybrids in the storage. The pathogen can infect all citrus fruits under suitable condition [2,6]. The disease is a common problem in Mediterranean climates due to cool, moist winters and hot dry summers [7]. Black rot isolates do not require host specific toxins for pathogenicity [1,5]. Wounds or natural opening in the stylar facilitate the infection process. *Alternaria* spp. may induce dormant infections on calyx and disk and invade the columella of matured fruits resulting in black rot [2,6,8].

The causal agent of black rot was first described in early 1900s as *Alternaria citri* by Ellis & Pierce [2,9,10]. However, other species of *Alternaria* may cause citrus black rot, too [11-13]. Historically the causal agent of black rot, brown spot of tangerine and leaf spot of rough lemon named as *A. citri* just for their association with *Citrus* spp. hosts [6,7,14-16]. But morphological identification or DNA analysis was not used to identify these fungi. 10 morphological species were described within brown spot and rough lemon leaf spot isolates which collected worldwide [2,17]. Peever et al. [18] revealed that many of the morphospecies which described by Simons [4,17] were both paraphyletic and polyphyletic. Peever et al. [18] studied citrus-associated species of *Alternaria* using mitochondrial large subunit (mtLSU) and  $\beta$ -tubulin sequence data. All small and catenulate-spored species formed monophyletic lineage. This lineage included causal agent of brown spot of tangerine, leaf spot of rough lemon and saprophytic isolates associated with citrus and other plants.

Peever et al. [2] showed that phylogenetically distinct lineage of *A. alternata* is caused citrus black rot. Citrus associated isolates included isolates which cause brown spot of tangerine and their hybrids, leaf spot of rough lemon, isolate of healthy citrus leaf tissue and black rotted fruit were assigned in eight clades in a combined endo-PG, OPA1-3 and OPA1-2 phylogeny [2]. Isolates which collected from black rotted fruit were distributed in six combined clades clustered with isolates which collected from other ecological niches on citrus and other plant host including peanut, tomato and carnation. These isolates were able to cause black rot, too [2].

Rang et al. [12] studied morphologically the isolates of black-rotted Minneola Tangelo, Mandarin and Navel Orange in South Africa. They assigned the isolates to the *A. alternata*, *A. pellucida*, *A. citri*, *A. tenuissima* and *A. arborescens* morphospecies. They used ITS and histone 3 sequences for phylogenetic analysis. The isolates divided to four clades. Researchers [19-22] concluded the isolates which cause brown spot of tangerine and leaf spot of rough lemon were morphologically identical and conspecific with causal agent of black rot [2]. These comparisons were based on morphological data which were insufficient for taxonomic differentiation of small-spored *Alternaria* [13,17]. Peever et al. [2] showed that *A. citri* sensu Simons include paraphyletic or polyphyletic fungi.

There is no phylogenetic or pathological evidence to demonstrate that *A. citri* is distinct taxonomic category that causes black rot.

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Peever et al. [18] suggested that citrus associated small-spored species should be referred to *A. alternata* until more genetic or physiological data achieved for their differentiation [23]. Sequence data from mitochondrial large ribosomal subunit,  $\beta$ -tubulin and other regions of genome such as region that encoding chitin synthase, calmodulin, actin, 1,3,8-trihydroxy naphthalene reductase unable to differentiate 10 morphological species and also unable to detect causal agent of black rot from isolates which recovered from healthy citrus tissue [1,24]. Only endo-polygalacturonase and two anonymous loci enable to differentiate members of the *Alternaria* specie group [18].

There is a considerable morphological diversity in the genus *Alternaria*. Many researchers attempt to organize taxa to sub group based on similar morphological characters [25-27]. Simmons [13] proposed 14 morphological groups to describe morphospecies. Then molecular studies confirmed many of these groups as monophyletic and assigned as "species-group" [28-30]. Other researchers differentiate species of small-spored *Alternaria* using metabolic profiling, morphology of the colony on standardized media and pattern of the conidial branching [31-41].

Stewart [42] used *endoPG*, OPA1-3, OPA2-1 and two microsatellite flanking regions for phylogenetic analysis of citrus brown spot worldwide. Pathogenic forms of *A. alternata* appear to have radiated from a recent common saprophytic ancestor [43]. With the divergence of the citrus 2 lineage, Stewart [42] suggested that a more complex evolution history is possible. Radiation may have occurred with the brown spot pathogen, two lineages of the citrus brown spot pathogen exist. Based on the coalescent analyses and GCPSR results; lineage citrus 2 appears to be derived from citrus 1. Loci Flank-F3 and OPA1-3 revealed strong evidence for a history of recombination among lineages. Andrew et al. [23] estimated phylogenies using *endoPG*, OPA1-3, and OPA10-2, and showed that *A. arborescens* formed a monophyletic sister group to *A. alternata* in the OPA1-3 and 10-2 phylogenies, but not in the *endoPG* phylogeny. This and the putative recombination observed in evolutionary history of OPA1-3 suggest that *A. arborescens* and *A. alternata* are recently diverged closely-related species. For future studies, a more distant relative of *A. alternata* may be more appropriate as an out group [42].

Kakvan et al. [44] showed high genetic diversity of *A. alternata* isolates which were collected from citrus hybrids of Iran using RAPD-PCR. There is no report on phylogenetic study of citrus black rot from Iran. The object of this study is phylogenetic classification of causal agent of citrus black rot in Iran and complementary studies are in progress.

## Materials and Methods

### Isolate sampling

Twenty single-conidial isolates of small and catenulate spored citrus-associated *Alternaria* spp. were isolated from citrus with symptoms of black rot from North of Iran, including, Sari, Jouybar, Ghaemshahr and Ramsar in Mazandaran province (Table 1). Also, we used some NCBI accessions for comparison (Table 1).

### Preparing conidial inoculation

Single-conidial isolates were grown on Potato Carrot Agar (PCA) medium incubated at 27°C, under 16 hours photoperiod for 3 to 5 days to promote conidial production. Conidia were collected by washing with water sterile from the plates. Conidia concentration were adjusted to  $5 \times 10^4$  conidia/ml using haemocytometer.

### Pathogenicity test

Navel and Valencia oranges were obtained from the field and disinfested with 70% ethanol. Calyces were removed from fruits before inoculation. Inoculation was performed by injecting 100  $\mu$ l of conidial suspension ( $5 \times 10^4$  conidia/ml) into columella with syringe. For each isolates, six fruits were inoculated and incubated at 23 to 25°C for 21 days. Fruits were scored on a scale of 0 to 3 [2].

### Morphological assay

Isolates were grown on PCA medium under 16 hour photoperiod at room temperature for 5-7 days. Morphological classification was performed by comparing colony morphology, sporulation branching patterns and conidial size using Simmons identification key [45].

### Fungal culture and DNA extraction

Isolates were grown in PDB (Potato Dextrose Broth) medium for 5 days and incubated on shaker with 135 rpm at 25°C. Mycelium was collected under vacuum. DNA was extracted by Safaie et al. [46] procedure. DNA concentration was estimated visually in 0.8% agarose gel.

### PCR and sequencing

An anonymous region of *Alternaria* genome, OPA10-2 [23], was amplified and sequenced as described by Andrew et al. [23]. An approximately 800 bp amplicon was obtained with OPA10-2 primers. Amplification products were visualized in 1.2% agarose gel. PCR products were direct sequenced. Sequencing was performed by the Kawsar Biotech Company (www.kawsar.ir).

### Phylogenetic analysis

OPA10-2 sequences were aligned using Mega 4.0 software. Maximum parsimony was used in Mega 4.0 to infer the phylogeny. Support for clades was estimated using non-parametric bootstrapping with 100 pseudo-replicated datasets in Mega. Consensus tree was produced by Mega 4.0.

## Results and Discussion

### Isolates sampling and morphological identification

Twenty isolates were collected from infected citrus fruit (Figure 1A) and studied morphologically using Simmons' [45] identification key. Seven isolates were selected for phylogenetic analysis (Table 1).

Isolates were cultured on PCA medium and incubated under 16 hours photoperiod at room temperature for 5-7 days (Figure 1B).

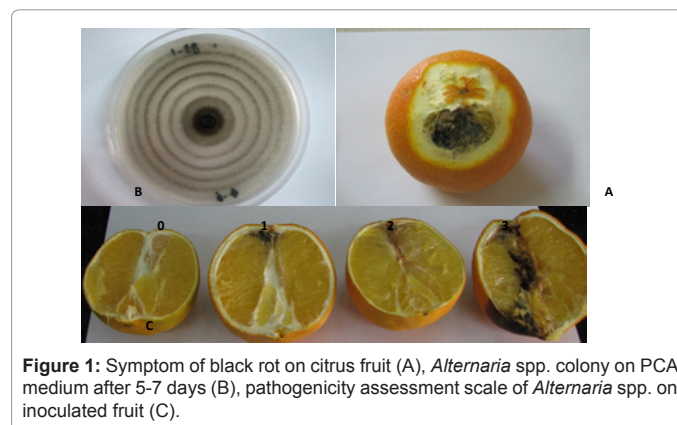


Figure 1: Symptom of black rot on citrus fruit (A), *Alternaria* spp. colony on PCA medium after 5-7 days (B), pathogenicity assessment scale of *Alternaria* spp. on inoculated fruit (C).

Name	Host	Location
Alt 2	Orange	Sari
Alt 4	Orange	Sari
Alt 6	Orange	Ghaemshahr
Alt 8	Orange	Ghaemshahr
Alt 9	Orange	Jouybar
Alt 10	Orange	Sari
Alt 20	Kiwi fruit	Ramsar
gi- 154433690	-	Andrew et al. [23]
gi- 154433692	-	Andrew et al. [23]
gi- 154433695	-	Andrew et al. [23]
gi- 154433697	-	Andrew et al. [23]
gi- 154433698	-	Andrew et al. [23]
gi- 154433700	-	Andrew et al. [23]
gi- 154433701	-	Andrew et al. [23]
gi- 154433745	-	Andrew et al. [23]
gi- 154433886	-	Andrew et al. [23]

Table 1: List of *Alternaria* spp. isolates used for phylogenetic analysis.

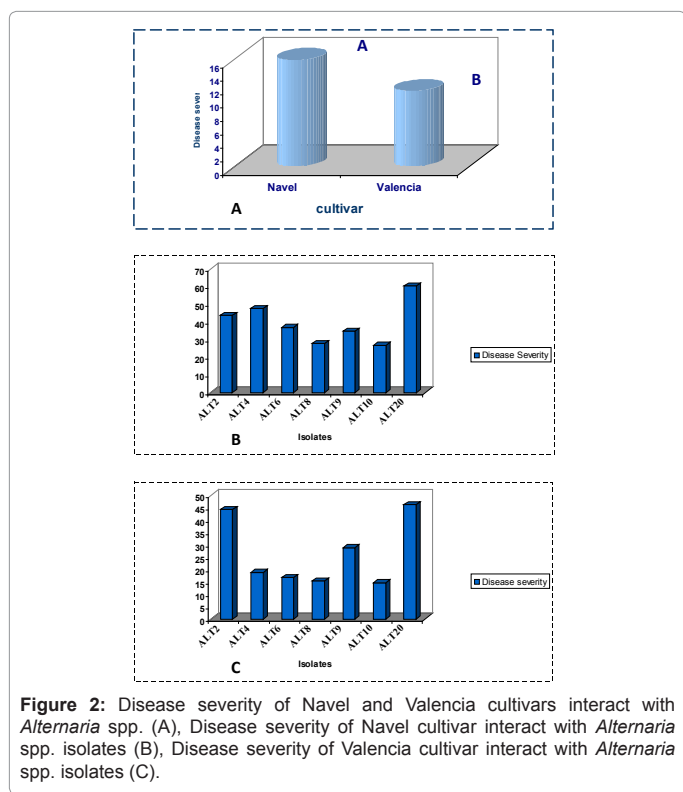


Figure 2: Disease severity of Navel and Valencia cultivars interact with *Alternaria* spp. (A), Disease severity of Navel cultivar interact with *Alternaria* spp. isolates (B), Disease severity of Valencia cultivar interact with *Alternaria* spp. isolates (C).

Morphological characters including colony morphology, conidial size and branching patterns could not separate species. Our result corresponding to other researchers, that few morphological characters can differentiate small spored species within *Alternaria* species-group [18,23].

### Pathogenicity test

Seven isolates from black-rotted fruit were inoculated to Navel and Valencia cultivars in order to test pathogenicity. Twenty-one days post-inoculation disease severity was recorded according to 0 to 3 scale described by Peever et al. [2]. Significant differences in mean disease rating were observed between cultivars (Figure 2). Disease severity was higher on Navel compared to Valencia. However, no significant

isolate by cultivar interaction was observed. ANOVA of cultivar-isolate interactions and different isolates and cultivars are shown in table 2.

Our results agree with those of Brown and Eckert [7]. Isolates Alt20 and Alt10 had highest and lowest disease severity on Navel (26.58 and 60.14%) and Valencia (14.61 and 46.02%), respectively.

### Phylogenetic analysis

Approximately 800 bp of an anonymous, non-coding region was sequenced for 7 isolates from black-rotted fruit in Iran. Sequenced data were analyzed by Mega 4.0 software and a consensus tree estimated. The consensus trees estimated for OPA10-2 locus was showed at figure 3.

Morphological characters were not a reliable predictor of phylogenetic lineage, a result similar to that observed by Andrew et al. [23]. No association was found between morphological and phylogenetic data. We identified *A. tenuissima* as the causal agent of citrus black rot. *A. tenuissima* introduce for the first time as causal agent of citrus black rot from Iran.

The results showed that (Figure 3), ALT2, ALT4 and ALT10 grouped with *A. gaisen* and *A. limoniasperae* but they were not supported well and were non stable in different trees. Also, ALT20 placed in the clade of *A. citrimacularis* and *A. citriarbasti*. Its situation was changed in different trees. ALT6 was designated as *A. tenuissima* in the most parsimonious trees. The isolates ALT8 and ALT9 grouped together, separately from other clades in most parsimonious trees. It suggested that both of them belong to different species.

Morphological distinguish within small-spored *Alternaria* species is a taxonomic problem and Rapid identification need to determine species name. Meanwhile, molecular and morphological characters should be considered together [23]. Phylogenetic classification of small-spored *Alternaria* species associated with citrus black rot in Iran

Source	Degrees of Freedom	Sum of Squares	Mean Squares	F Value
Isolate	1	0.082	0.082	4.9631*
Cultivar	6	0.206	0.034	2.08
Cultivar-Isolate	6	0.030	0.005	0.30
Error	70	1.153	0.016	
<b>Total</b>	<b>83</b>	<b>1.471</b>		

\* P ≤ 0.05, Coefficient of variation=14.57%

Table 2: ANOVA of disease severity in cultivars (Navel and Valencia) interact with seven genotypes of *Alternaria* spp.

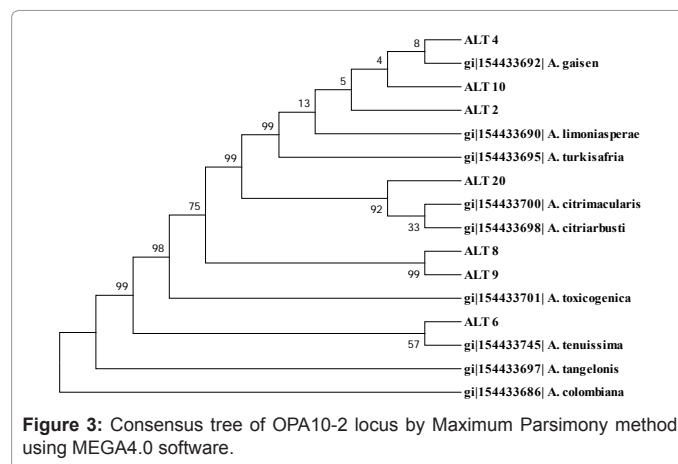


Figure 3: Consensus tree of OPA10-2 locus by Maximum Parsimony method using MEGA4.0 software.



needs to complementary studies with more isolates and more sequence data from other genome loci.

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