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Phylogenetic relationships of some anamorphic Pleosporalean genera based on the analysis of ITS rDNA and *RPB2*

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Abstract: Pleosporaceae is an important Dothideomycetes family. To elucidate relationships among some selected anamorphic pleosporalean taxa, their Internal Transcribed Spacer (ITS) and RNA polymerase second largest subunit (*RPB2*) were sequenced and compared. Phylogenetic analyses of both ITS and *RPB2* regions were almost similar and generally congruent with previously described phylogenies and morphology based classification schemes. ITS was inefficient to show the taxonomic placement of some species, especially *Alternaria* species; but *RPB2* was appropriate for this purpose.

Key words: *Alternaria*, *Cochliobolus*, *Bipolaris*, Phylogeny

INTRODUCTION

Pleosporaceae is the largest family within the Pleosporales. Its species are parasites or saprobes on wood and dead herbaceous stems or leaves (Sivanesan 1984).

Anamorphic forms of Pleosporaceae had been previously placed in Deutromycota. However, after molecular revolution in fungal taxonomy which commenced in the early 1990s, with analyses of PCR-amplified ribosomal RNA genes (White et al. 1990), anamorphic forms of Ascomycota and Basidiomycota were mostly placed in the orders and families that owned their teleomorphs (Hibbett et al. 2007). Despite efforts to clarify phylogenetic relationships of this group, there is still uncertainty about taxonomic position of some genera. This investigation was performed in order to clarify the issue, and also due to the importance of a natural classification and correct species identification in disease control, plant breeding and establishment of phytosanitary measures (Cai et al. 2009, Hyde et al. 2010).

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MATERIALS AND METHODS

We collected abundant anamorphic species of *Cochliobolus* (*Bipolaris* and *Curvularia*) and some *Alternaria* species from various hosts and different geographical regions of Iran. After morphological and molecular identification, their ITS rDNA regions and *RPB2* sequences were compared to each other, and to some other sequences downloaded from GenBank (www.ncbi.nlm.nih.gov/Taxonomy) to verify the placement of these genera and assess which marker is able to determine their taxonomic position.

Fresh specimens were collected from soil and host tissues from different provinces of Iran during 2010 to 2011. Strains were initially identified according to the criteria used by Ellis (1971), and were later verified by molecular identification.

Twenty five isolates from different hosts and geographic origins were selected for molecular analysis. Total genomic DNA was extracted using modified CTAB protocol (Ashktorab et al. 1992). PCR was performed using the primer pairs ITS4 (5′-TCCTCCgCTTATTgATATgC-3′) and ITS5 (5′-ggAAgTAAAAgTCgTAACAAgg-3′) (White et al. 1990) to amplify Internal Transcribed Spacers (ITS) and 5.8S region FRPB2-5f (5′-gAY gAYMgWgATCAYTTYgg-3′) and FRPB2-7CR (5′-CCCATRgCTTgYTTRCCCAT-3′) (Hall lab, fungal specific) to amplify RNA polymerase II, subunit 2. Phylogenetic analysis was performed by using Neighbor Joining and Maximum Likelihood methods. The bootstrap settings were 1000 replicates and retaining groups are those with frequency of more than 50 %. Then, phylogenetic trees were constructed using MEGA 5.

RESULTS AND DISCUSSION

The ITS 4 and ITS 5 primers directed the amplification of a single product. DNA sequencing revealed that these fragments ranged in size from 530 to 569 bp (including primer sequences). Among 558 significant sites, 311 (55.73 %) were conserved and 247 were variable. Phylogram constructed by Neighbor Joining method is shown in Figure 1. On the other hand, applying the primers RPB2-5F and RPB2-7CR resulted in amplification of a single fragment, ranging from 1178 to 1184 bp (including primer sequences). Phylogenetic tree was constructed by using neighbor joining (Fig. 1). Among significant

sites, 601 (50.67 %) were conserved and 585 were variable.

Phylogeny of *Cochliobolus* (clades D1 and D2): RPB2 results were almost similar to ITS. In all the phylograms *Curvularia* was closely related to *Bipolaris*. Similar to the findings of a study by Kodsueb et al. (2006) in which *Cochliobolus* was segregated into two groups as a result of phylogenetic analysis of 28S rDNA region, two groups (D1 and D2) were obtained as a result of analysis of both ITS and *RPB2* regions in this study. Clade D1 includes *Bipolaris oryzae*, *B. sorokiniana*, *Cochliobolus sativus* and *C. heterostrophus*. Conidium morphology is almost similar in members of this clade (morphology of their anamorphs were considered for *C. sativus* and *C. heterostrophus*). They have (6-12) pseudosepta and size of their conidim is (30-120 × 14-20) µm. All of them produce a hilum with length of 3-4 µm (Ellis 1971). It should be mentioned that members of this clade have big spore and are highly virulent pathogens (Manamgoda et al. 2011) comparing clade D2. Taxonomic position of *Bipolaris oryzae* in both ITS and *RPB2* analyses was the same (D1). It was placed in a separate branch with *C. hetrostrophus* and *C. sativus* in *RPB2* analysis, and with *B. sorokiniana* in ITS analysis.

Bipolaris australiensis, *B. spicifera*, *B. hawaiiensis*, *Curvularia inaequalis*, *C. pallescens*, *C. lunata*, *C. brachyspora* and *C. geniculata* in clade D2 represent a distinct monophyletic group (similar to Group 2, as defined by Berbee et al. 1999). This clade comprises mild pathogens with both *Bipolaris* and *Curvularia* asexual states. The *Pseudocochliobolus* species were clustered in this group. They have also small spores (18-40 × 6-17 µm) comparing clade D1.

Results indicate that classification derived from both ITS and *RPB2* are consistent with morphology of the spore. Although *RPB2* analysis had a similar result to ITS, *RPB2* segregated small-spore containing species better. As observed in Fig. 2, clade D2 was divided into 2 subclades and *Curvularia* species were placed in D2-1 with a bootstrap value of 100%. *RPB2* seems to be a suitable marker for differentiation of *Curvularia* and *Bipolaris*.

Phylogeny of *Alternaria* and *Ulocladium*: In this investigation, *Embellisia*, *Alternaria* and *Ulocladium* were placed together in a large clade with a bootstrap value of 100%, which is similar to the results of the study by Pryor and Gilbertson (2000). Within this clade, the following distinct species-clades were revealed: A (A1, A2, A3), B and C. Section A1: *Ulocladium* species were composed of a monophyletic group with 99% and 100% bootstrap values in ITS and *RPB2* analyses, respectively. Section A2: *Alternaria brassicicola* was placed in section A2 with *Ulocladium botrytis*, and lonely in this section in ITS and *RPB2* analyses, respectively (Fig. 1).

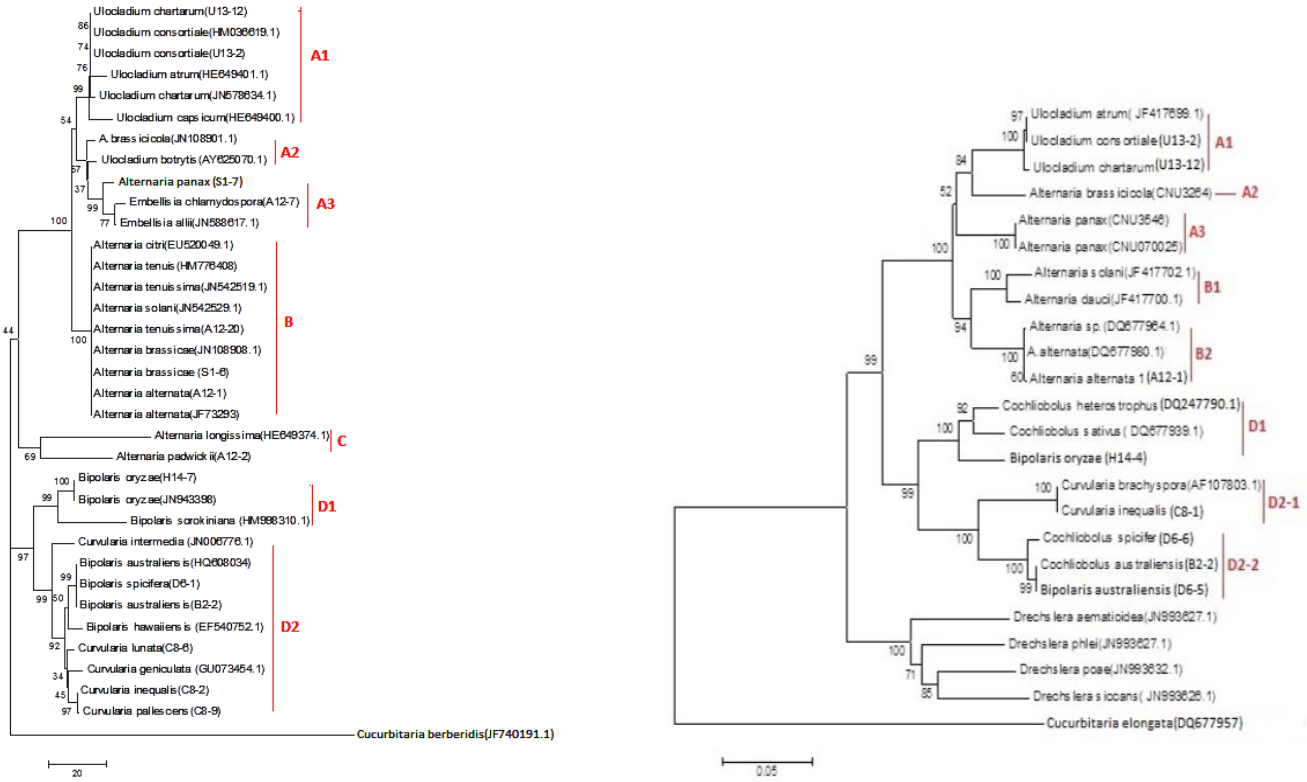

Fig. 1. Phylogram generated from the neighbor joining analysis based on ITS **(right)** and RPB2 sequence **(left)** of the selected isolates. Bootstrap support values are shown below or above the branches.

Since the result obtained from *RPB2* is consistent with morphological characters described by Lawrence et al. (2013) for section *brassicicola* of *Alternaria* species, RPB2 is considered as a suitable marker for segregation of this group.

Section A3: *Alternaria panax* was placed in A3, which formed a sister group with *Embellisia chlamydospora* and *Embellisia allii* in ITS analysis (Fig. 1) and was placed singly in analysis of *RPB2*. These two genera of *Embellisia* belong to section *Embellisia* described by Simmons (1971), whereas *A. panax* is a type species of section *panax* characterized by Lawrence et al. (2013), recently. This result also matches with morphology of this genus. Section B: *Alternaria tenuis*, *A. tenuissima*, *A. citri*, *A. solani*, and *A. alternata* which have different morphological specifications were placed in a single clade according to ITS results, but *RPB2* presented accurate results. As shown in Fig. 1, *A. solani* and *A. dauci* were placed in section B1. These two species share some similarities. For example, they both have broadly ovoid, obclavate, ellipsoid, subcylindrical or obovoid (medium) large conidia, disto- and euseptate, solitary or in short to moderately long chains, with a simple or branched, long to filamentous beak (Woudenberg et al. 2013).

Alternaria alternata was placed in section B2. This is a small-spore containing *Alternaria* species, which was properly isolated from other species by *RPB2*. The mentioned species had been put in section B, using ITS analysis.

Alternaria longissima and *A. padwickii* which are big-spore containing *Alternaria* species were placed in clade C in ITS analysis. So, ITS segregation result was similar to the morphology of these two genera. It seems that *RPB2* is a suitable marker for investigating phylogenetic relationships of the selected genera.

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ارتباط فیلوژنتیکی برخی گونه های تیره Pleosporaceae براساس تجزیه و تحلیل ناحیه ITS rDNA و ژن *RPB2*

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چکیده: تیره Pleosporaceae یکی از تیره های مهم آسکومیست ها است. به منظور روشن نمودن روابط بین برخی گونه های مهم این تیره، ناحیه ITS از rDNA هسته ای و ژن *RPB2* آنها توالی یابی و با یکدیگر مقایسه گردید. آنالیز فیلوژنتیکی هر دو ناحیه مورد بررسی تایید کننده فیلوژنی توصیف شده قدیمی و طبقه بندی صورت گرفته بر اساس خصوصیات مورفولوژیکی است. این بررسی نشان می دهد که ناحیه ITS برای نشان دادن جایگاه تاکسونومیکی برخی گونه ها به ویژه گونه های *Alternaria* مناسب نمی باشد، در حالیکه ژن *RPB2* برای این منظور مناسب است.

کلمات کلیدی: *Alternaria*، *Cochliobolus*، *Bipolaris* و فیلوژنی

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