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Taxonomic position of *Bipolaris oryzae* among other *Cochliobolus* species using Ribosomal region and some Protein Coding genes

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Abstract

Using of gene sequence data to clarify evolutionary relationships and determine taxonomic status of organisms, including fungi, is common nowadays. This study investigates taxonomic position of Bipolaris oryzae among the genus Cochliobolus. For this purpose ITS rDNA and partial sequences of translation elongation factor (TEF1-α) and second largest subunit of RNA polymerase II gene (RPB2) were analyzed with Neighbor joining methods. Cochliobolus can be segregated into two groups as previously proposed and B. oryzae placed with C. sativus and C. heterostrophus. Generally, the results of all three markers were the same and in congruent with previous studies. It seems that, this part of TEF gene wasn't able to break up species of Cochliobolus correctly but, RPB2 is a good marker for determination of taxonomic position of ambiguous species of this genus.

Keywords: *Cochliobolus miyabeanus, bipolaris oryzae,* Phylogeny, ITS, RPB2, EF1-α.

Introduction

Cochliobolus miyabeanus[1] is the telemorph of a fungus that causes brown spot disease in rice. This disease was the causal agent of the Bengal famine in 1943 [2]. At first the causal agent of brown spot disease was named *Helminthosporium oryzae* by Breda de Haan[3]. Nowadays, the graminicolous *Helminthosporium* species were divided into three genera based on colony, conidiophore and conidial morphology, type of conidial germination and the type of hilum structure: *Bipolaris, Drechslera, Exserohilum*[4]. Anamorph of this fungus is named *Bipolaris oryzae* nowadays. *Bipolaris oryzae*[5] was classified in the subdivision Deuteromycotina (imperfect fungi), class Deuteromycetes, order Moniliales, and family Dematiaceae[6].

The genus *Cochliobolus* (anamorphs *Bipolaris, Curvularia*) includes 55 species which comprises many destructive plant pathogens that cause severe crop losses worldwide. The taxonomy of *Cochliobolus* is confused as frequent nomenclatural changes have occurred in the sexual and asexual states of species over the past 50 years [7]. A natural classification of this pathogenic system and correct identification of species are important for disease control, plant breeding and establishment of phytosanitary measures[8].

Increasingly, molecular biology techniques have been used to explore genetic variability in fungi[9].

Cochliobolus can be segregated broadly into two groups as previously proposed[10]. In this investigation we tried to determine the taxonomic position of this important pathogenic fungus among other *Cochliobolus* species, using ITS rDNA region and a part of second largest subunit of RNA polymerase II genes (RPB2). A part of translation elongation factor gene (TEF) was used for this purpose too.

Material and Methods

Isolates, Media and Growth conditions: Six anamorphic forms of *Cochliobolus* species which were isolated from various geographical origin of Iran were selected for this study. In addition, thirty four sequences of two genes (RPB2 and TEF1-α) and rDNA region (ITS and 5.8s) were downloaded from GenBank.

Species names and accession numbers of the isolates in this study are listed in table 1.

DNA extraction: Genomic DNA was extracted from fresh fungal mycelia following a protocol as outlined by Cai L, Jeewon R et al[11].

Briefly, mycelia were scraped off from the surface of the plate. The mycelia were ground with 200 mg of sterilized quartz sand and 600 mL of 23 CTAB extraction buffer (2% w/v CTAB, 100 mM Tris-HCL, 1.4 M NaCl, 20 mM EDTA, pH 8) in a 1.5 mL Eppendorf tube. The contents were incubated at 60° C in a water bath 40 min with gentle swirling every 10 min. The solution was extracted three times with an equal volume of phenol: chloroform (1:1) at 13000 Rpm in 30 min until no interface was visible. The upper aqueous phase containing the DNA was precipitated by addition of 2.5 volumes of absolute ethanol and kept at -20° C overnight. The precipitated DNA was washed two times with 70% ethanol, dried under vacuum and suspended in TE buffer (1 mM EDTA, 10 mM Tris-HCl, pH 8.0) and treated with RNase (1 mg/mL) before DNA amplification.

Table-1
Fungal species and GenBank accession number for taxa used in phylogenetic analyses

Species	GenBank Accession number		
	ITS	**EF1-α**	**RPB2**
Curvularia inaequalis	JQ585672	JQ965132	JQ585693
Curvularia trifolii	JN093302	-	-
Curvularia brachyspora	JN006761	-	AF107803
Curvularia lunata	JQ585674	JQ965130	-
Curvularia pallescens	JQ585675	JQ965131	-
Curvularia tuberculata	-	JN601004	-
Cochliobolus cynodontis	JQ517486	-	-
Cochliobolus heterostrophus	GU480772	DQ497603	DQ247790
Cochliobolus sativus	JQ936305	-	DQ677939
Cochliobolus victoriae	JN601027	JN601005	-
Cochliobolus graminicola	JN192376	JN601008	-
Cochliobolus ovariicola	JN601031	JN601020	-
Bipolaris hawaiiensis	JQ517484	JN601010	-
Bipolaris pereginensis	JN601034	JN601022	-
Bipolaris periotidis	-	JN601021	-
Bipolaris setariae	GU073108	-	-
Bipolaris portulacae	AY004779	-	-
Bipolaris zea	AF081452	-	-
Bipolaris indica	AF081449	-	-
Bipolaris sorghicola	JQ517488	-	-
Bipolaris sorokiniana	JN128888	-	-
Bipolaris specifera	JQ585669	-	JQ585694
Bipolaris oryzae	**JQ585685**	**JQ965127**	**JQ585692**
Bipolaris australiensis	JQ585665	JQ965129	JQ585695
Alternaria alternata	JQ585677	JQ965134	JQ585696

Primers and PCR amplification: PCR primers used in the amplification reactions for Internal transcribed spacers and 5.8S region were ITS4 (TCCTCCgCTTATTgATATgC) and ITS5 (ggAAgTAAAAgTCgTAACAAgg)[12].

FRPB2-5f (gAYgAYMgWgATCAYTTYgg) and FRPB2-7CR (CCCATRgCTTgYTTRCCCAT) primers were used to amplify RNA polymerase II, subunit 2 and Efdf (AAggAYggNCARACYCgNgARCAYgC) and EF1-1567R (ACHgTRCCRATACCACCRATCTT) were used for amplification and sequencing of approximately 400 nucleotides in the middle of elongation factor gene[13].

Total DNA from each isolate was subjected to polymerase chain reaction in PCR thermal cycler (What man-Biometra, Goettingen, Germany) programmed as follows: initial denaturation 4 min at 94 c; 35 cycles of: denaturation, 1 min at 94 c; annealing 1 min (at a temperature corresponding to the Tm of the primers used); extension 2 min at 74c; and final extension, 5 min at 74c. Each reaction was performed in microfuge tubes 0.2 ml in a volume of 25 µl, including 2.5 µl of 10x PCR buffer (10 mM Tris-HCl, pH 9.0 at 25 x, 1.5 mM MgCl2, 50mM KCl, 0.1% Triton X-100), 1 µl of MgCl2, 1µl of each primer (Final concentration 10 pmol/µl), 1µl DNTP, 2 units Thermophilic Taq DNA Polymerase (5.0 units mlx1; Promega, Madison, WI), 50 ng of template DNA and sterile ultrapure water. The reaction products were resolved on a 1.5 % Agarose gel in 1xTBE buffer. A1 kb DNA ladder (SMO) was included as DNA size marker.

DNA sequencing, data analysis and phylogenetic relationships: After the amplification of ITS region of the rDNA and the three genes, each product was purified using the QIA quick PCR Purification Kit using manufacturer protocol (Qiagen Inc., Valencia, CA, USA). The purified PCR product was send by overnight mail to DNA Sequencing Facility at BioNeer in Korea where amplifications were sequenced.

Sequences were aligned using BioEdit[14]. Phylogenetic analysis were performed by neighbor joining and Maximum parsimony methods (The bootstrap settings were 1,000 replicates and retaining groups with frequency >50 %) and Phylogenetic trees were constructed using MEGA 5. Gaps were treated as missing data and uninformative characters were excluded from the analysis.

Results and Discussion

PCR amplification of studied regions was done for collected species of *Cochliobolus* including: *Curvularia inaequalis*, *Curvularia lunata*, *Curvularia pallescens*, *Bipolaris specifera*, *Bipolaris australiensis*, *Bipolaris oryzae*, *Alternaria alternata*

and in all cases amplified a single product. Although the neighbor joining and the parsimony trees shared similar topology, the neighbor joining tree offered a better resolution, since the parsimony analysis was limited by fewer numbers of informative sites in all studied regions. For this reason, only the neighbor joining trees are shown. Both methods of phylogenetic analysis divided the taxa into two major clusters (A and B).

ITS: In ITS region among 559 aligned nucleotide characters 369 were conserved, 184 were variable and 125 were parsimony informative. Designated out group was *Alternaria alternata*. Neighbor joining analysis of the aligned ITS sequences have shown in figure 1.

RPB2: In this study middle part of the second largest sub unite of RNA polymerase II was amplified, sequenced and used for phylogenetic analysis. There wasn't many data for this region in GenBank. Among 1173 aligned nucleotide characters, 886 were conserved, 305 were variable and 197 were parsimony informative. *Alternaria alternata* used as an out group. Neighbor joining analyses of the aligned RPB2 sequences have shown in figure 2.

TEF: About 380 bp of middle part of translation elongation factor gene was used for phylogenetic study of this group. In between, 262 nucleotides were conserved, 112 were variable and 61 were parsimony informative. *Alternaria alternata* shared a lot of similarities with *Cochliobolus* species in this part of TEF gene. So, *Fusarium proliferatum* was used as out group. Neighbor joining analyses of the aligned TEF sequences have shown in figure 3.

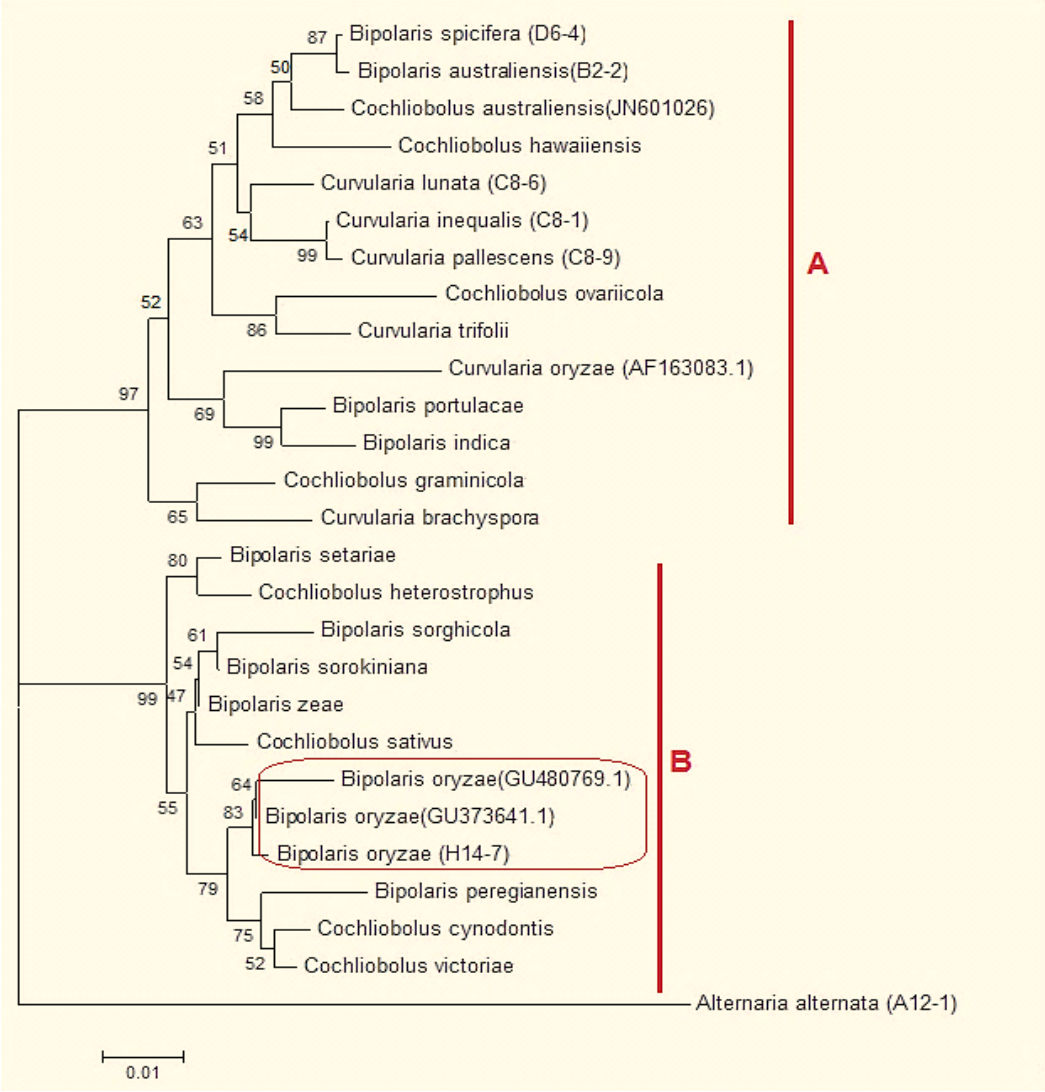

Figure-1
Neighbor joining tree generated based on ITS sequence analysis of ITS dataset. Bootstrap support values above 50% shown at nodes are based on 1000 replicates. *Alternaria alternata* used as an out group

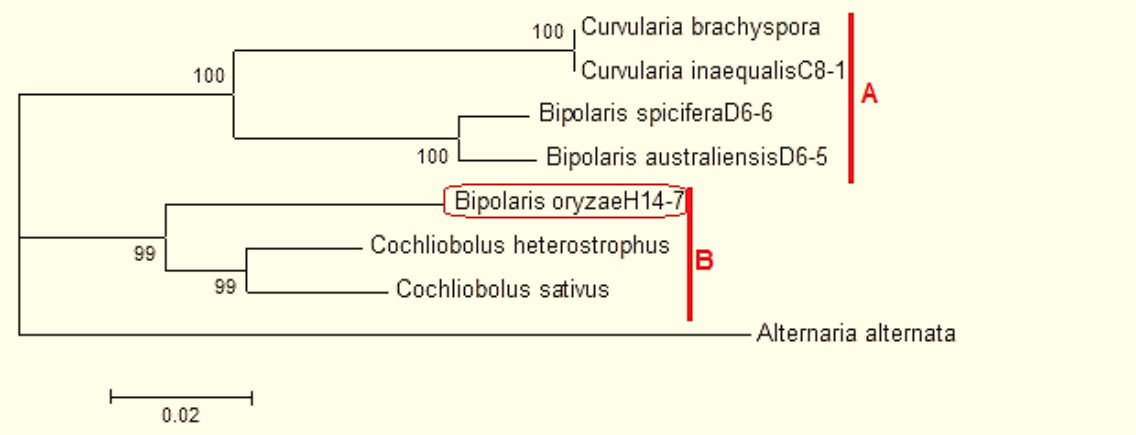

Figure- 2

Neighbor joining tree generated based on analysis of RPB2 dataset. *Alternaria alternata* was rooted as outgroup in the analysis. Values associated with branches indicate the degree of bootstrap support expressed as percentage of 1000 bootstrapped trees in which the corresponding clades are present

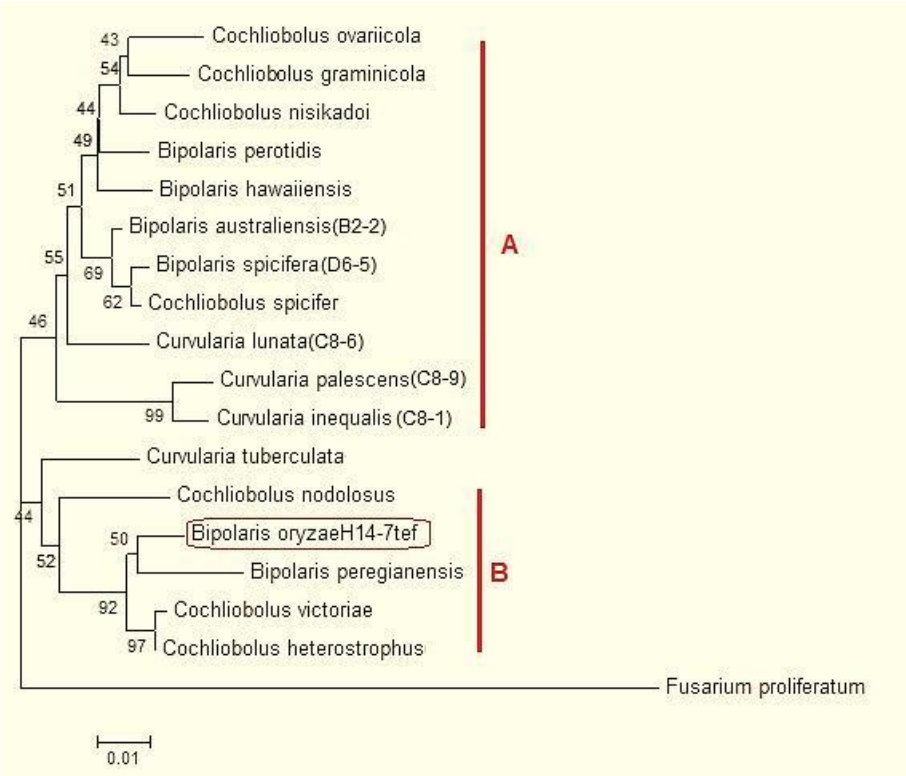

Figure-3

Neighbor joining tree generated based on TEF sequence analysis, Bootstrap support values above 40% shown at nodes are based on 1000 replicates, *Fusarium proliferatum* used as an out group

The genus *Cochliobolus* (anamorphs *Bipolaris,Curvularia*) comprises many destructive plant pathogens that cause severe crop losses worldwide. The taxonomy of *Cochliobolus* is confused as frequent nomenclatural changes have occurred in the sexual and asexual states of species over the past 50 years [7]. In an investigation which was done in order to assess the relationship between *Cochliobolus* species and species of *Curvularia* and *Bipolaris* using ITS 1, ITS 2 and 5.8S rDNA sequences and a 600 bp fragment of the housekeeping gene *gpd*, coding for glyceraldehyde-3-phosphate dehydrogenase[10] and other study which was performed based upon sequence analyses of partial 28S rDNA [15] divided this

genus into two groups. Our study showed the same results in dividing *Cochliobolus* species into two groups (A and B) by using ITS 4, ITS5 sequences and protein coding genes (RPB2 and TEF). Our A group matches with second group of Berbee et al. (1999) study and includes both *Curvularia* and *Bipolaris* species. Bootstrap support value for this cluster in ITS and RPB2 analysis was 97 % and 100% respectively. But, in TEF analysis was low (46%). Group B was comply with members of group 1 in Berbee et al. (1999) investigation including *C. sativus, Bipolaris oryzae* and *C. heterostrophus*. Only *Bipolaris* states were associated with Group 1 *Cochliobolus* species in ITS and RPB2 data analysis which were in congruent with Berbee et al. (1999) study.
But in TEF dendrogram *Curvularia tuberculata* placed in this group.

Conclusion

Although the results of all tree markers were the same, it seems that, this part of TEF gene wasn't able to break up species of *Cochliobolus* correctly.

According to expressed subject results obtained from analysis of these three regions were the same and in congruent with other investigations which was done using different gene regions. Since using of multi gene data analysis which had the same results confirm the correct evolutionary location of a taxon we can say the results of this study indicate the correct taxonomic status of *Bipolaris oryzae*. On the other hand, despite the low number of data sets used for phylogenetic analysis of RPB2 gene, it seems that, this protein coding gene have high differentiation ability for the species of *Cochliobolus* and is capable to determine proper taxonomic position of ambiguous species of this genus. So, RPB2 is a good marker for determination of taxonomic status of the species that are vague.

References

1. Drechsler ex Dastur, *Cochliobolus miyabeanus, Indian Journal of Agricultural Research* **12**, 733 **(1942)**

2. Scheffer RP, The nature of disease in plants, *Cambridge University Press***, (1997)**

3. Gangopadhyay S. & Padmanabhan S. Y., Breeding for disease resistance in rice, 340 *Oxford & IHB Publishing Co. Calcatta*, **(1987)**

4. Sivanesan A., Graminicolous Species of *Bipolaris, Curvularia, Drechslera, Exserohilum* and their teleomorphs, *Mycologia* **158**, 1-261 **(1987)**

5. Shoemaker R.A., *Bipolaris oryzae*, *Can. J. Bot.*, **37**, 883 **(1959)**

6. Barron L. George in *The genera of Hyphomycetes from soil* 363 P, Robert E. Krieger publishing co., INC. Malabar, Florida, (**1971)**

7. Manamgoda D.S., Cai L., Bahkali A.H. and Chukeatirote E. and Hyde K.D., Cochliobolus, an overview and current status of species, *fugal diversity*, **53,** 3-40, doi:10.1007/s13225-011-0139-4 **(2011)**

8. Hyde K.D. *et al.,* A case for reinventory of Australia's plant pathogens, *persoonia* **45**, 50-61, doi:10.3767/003158510 X548668 **(2010)**

9. Caligiorne R.B., Resende M.A., Paiva E. and Azevedo V., Use of RAPD (random amplified polymorphic DNA) to analyse genetic diversity of dematiaceous fungal pathogens, *Can. J. Microbiol.,* **45**, 408-412 **(1999)**

10. Berbee M.L., Pirseyedi M. and Hubbard S., *Cochliobolus* Phylogenetics and the origin of known, highly virulent pathogens, inferred from ITS and glyceraldehyde-3-phosphate dehydrogenase gene sequences, *Mycologia*, **91**, 964-977 **(1999)**

11. Cai L., Jeewon R. and K.D., H. Phylogenetic evaluation and taxonomic revision of *Schizothecium* based on ribosomal DNA and protein coding genes, *Fung Divers*, **19**, 1–21 **(2005)**

12. White T.J., Bruns T., Lee S. and Taylor J.W., Amplificatin and Direct Sequencing of Fungal Ribosomal RNA Genes for Phylogenetics, *Academic press, INC*, 315-323 **(1990)**

13. Vilgalys R., Hopple J.S., JR and Hibbett DS Phylogenetic Implications of Generic Concepts in Fungal Taxonomy, The impact of molecular systematic studies, *mycological helvetica* **6**, 73-91 **(1994)**

14. Hall, T. BioEdit v.7.0.1. *Isis Pharmaceuticals,* **(2004)**

15. Kodsueb R. *et al,* The family Pleosporaceae: intergeneric relationships and phylogenetic perspectives based on sequence analyses of partial 28S rDNA, *Mycologia* **98(4)**, 571–583 **(2006)**