Comparison of the multi gene regions for selection of potential barcode for *Bipolaris* spp

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Abstract
Bipolaris with Bipolaris maydis as type species is well known pathogen causing serious diseases i.e. Brown spot of rice [B. oryzae (Breda de Haan) Shoemaker], Brown stripe of sugarcane [B.stenospila (Drechsler) Shoemaker] and Southern leaf blight [B. maydis (Nisikado and Miyake) Shoemaker] of maize. Identification of organism is very difficult both by morphology and molecular. There have been no much studies to identify potential gene regions to group different Bipolaris species.

In present investigation, 24 Bipolaris isolates belonging to five different species were used for multi regions (ITS, tef-1, β-tubulin, LSU and SSU) phylogenetic analysis to know the potential region to cluster the same species. ITS was found to be the best in species discrimination followed by tef-1and β-tubulin. LSU and SSU were unable to segregate Bipolaris species.

Keywords: Multigene analysis, Phylogenetic analysis and translational elongation factor-1α.

Introduction
The genus *Bipolaris* with the type species *B. maydis* was established by Shoemaker[11] to accommodate the species germinating from both the polar cells. Subsequently some species of *Bipolaris* were segregated to *Exserohilum* and *Curvularia* - one having strongly protuberant hilum and the latter having curved conidia. There have been frequent nomenclatural changes in the sexual and asexual states of species over the past 50 years. Traditionally *Bipolaris* taxa have been identified microscopically after a period of incubation on culture media. Consequently, increased numbers of taxa have been introduced through morphological characterization.

Identification of species of *Bipolaris* using morphology alone is challenging. Most species have a wide range of overlapping conidial and conidiophore dimensions and the sexual state is rarely found in nature. A natural classification of this pathogenic system and correct identification of species are important for disease control, plant breeding and establishment of phytosanitary measures[2,5]. The refinement of character state homologies and the development of morphology-based classifications into a phylogenetic classification system are accelerating with the advent of molecular data. Studies in molecular phylogenetics typically deal with evolutionary relationships among deeper clades whereas those in population genetics target variation within and among populations of a single species.

The use of nucleotide sequence differences in a single gene to investigate evolutionary relationships was first widely applied by Woese and fox[16]. They recognized that sequence differences in a conserved gene, ribosomal RNA (rRNA), could be used to infer phylogenetic relationships. Sequence comparisons of rRNA from many different organisms led initially to recognition of the Archaea and subsequently to a redrawing of the tree of life. More recently, the polymerase chain reaction has allowed sequence diversity in any gene to be examined.

Genes that evolve slowly, like rRNA, often do not differ among closely related organisms but they are indispensable in recovering ancient relationships, providing insights as far back as the origin of cellular life[17]. Although there is a wide acceptance of the need for standardization of gene regions used in taxonomic studies[3], it has been proven that the particular gene region and sequence length are most informative[8,9].

Accurate species identification is the essence of any molecular diagnostic system[4] and potential genomic regions should be tested rigorously prior to adoption of a standardized system. On the other hand, genes that evolve rapidly may overwrite the traces of ancient affinities but regularly reveal divergences between closely related species. Both coding and non-coding genes have been employed in the detection and identification of fungal species. Fragments of 18S and 28S rRNA, internal transcribed spacer (ITS) region and elongation factor 1 alpha gene (EF-1 alpha) have been used as effective markers in the analysis of fungal communities.

Given the considerable taxonomic confusion, phylogenetic analyses of rDNA regions i.e. ITS, SSU, LSU and *tef-1* and β-tubulin sequence data were undertaken for understanding the relationships among different species of *Bipolaris* and to select the best candidate marker which helps in differentiating *Bipolaris* species in the present study.

Material and Methods
Isolation and purification of genomic DNA and PCR amplification: The genomic DNA was isolated from 24 *Bipolaris* species isolates by using standard CTAB method.

DNA was dried under a regular air flow for 20 min, resuspended in 70 µl TE buffer and stored at − 20 °C. Three subunits from the nuclear rRNA cistron were compared together with regions of two representative protein coding genes to know which one helps in proper segregation of different *Bipolaris* isolates into species. Three nuclear ribosomal regions (ITS, LSU, SSU) and two protein coding genes (*tef*-1 and β-tubulin) primers were used for amplification of these regions (Table 1). PCR mixture and PCR conditions for amplification of various genes are given in table 2 and 3. Agarose gel electrophoresis was performed to resolve the amplified product using 1.2 per cent agarose. 3 µl of PCR product was loaded to the agarose gel. Electrophoresis was carried at 70 V for 45 min. The gel was observed under UV light and documented using gel documentation unit.

Sequencing and Phylogenetic analysis: Sequencing was carried out by scigenome (Cochin, India) by an automated ABI 3100 Genetic Analyser (Applied Biosystems, USA). Sequences were submitted in NCBI and accession numbers were acquired (Table 4). Sequences were aligned using the clustral W version five. Maximum parsimony analysis was performed and the branches were supported by the bootstrap method (1000 replicates). *Drechslera tritici-repentis* was taken as out group. A dendrogram was generated based on the rDNA sequences by means of the Mega5 Software[12].

Results

Phylogenetic analysis was conducted based on five region's sequence data using maximum parsimony (MP) approach and evolutionary distance using Tamura-Nei model. MP was carried out using MEGA5.0.2. Alignment of DNA sequences was done using ClustralW program. Alignment gaps were treated as missing data. Statistical support for the internal branches was estimated by bootstrap analysis based on 1000 replications.

ITS region: Amplification of ITS region by ITS1 and ITS4 primers produced amplicons of 530 bp. Ampilifed products with 100bp marker are shown in fig. 1. A total of 24 isolates were sequenced and phylogenetic analysis was carried out by using *Drechslera tritici-repentis* as outgroup. Sequences blasted with NCBI sequences resulted in high similarity with the isolates. The isolates formed five different groups representing *B. sorokiniana, B. maydis, B. oryzae, B. hawaiiensis* and *B. spicifera* (Fig. 2).

Translation Elongation factor-1*α*: There was proper grouping of only three species i.e. *B. sorokiniana, B. oryzae* and *B. maydis*. But, *B. hawaiiensis, B. spicifera* were not grouped properly (Fig. 3). There was also some variation found between the isolates of the same species.

β-tubulin, LSU and SSU: Phylogenetic analysis of β-tubulin sequences revealed that there was no proper grouping in *B.hawaiiensis* and *B. spicifera.* There was high variability in the isolates of *B.sorokiniana, B. oryzae* and *B. maydis*. Phylogenetic analysis showed no proper grouping of *Bipolaris* species using LSU and SSU regions which failed as markers in identification of *Bipolaris* species.

Combined gene analysis using the sequences of five regions used in this study was carried out. The results were not satisfactory as it reduced the accuracy of grouping of various isolates.

To validate the ITS region as candidate marker for identification of various *Bipolaris* species, a combined phylogenetic analysis of these ITS sequences with NCBI sequences was carried out. The results obtained were similar to that of earlier studies (Fig. 4). ITS region was proved to be efficient in grouping of various species of *Bipolaris.*

Table 1
Nucleotide sequences of the primer used for PCR amplification of different gene/regions

Region	Primer sequence
ITS[15]	ITS-1 - 5'- TCCGTAGGTGAACCTGCGG-3' ITS-4 - 5'-TCCTCCGCTTATTGATATGC-3'
tef-1[10]	EF1- 983F - 5'- GCYCCYGGHCAYCGTGAYTTYAT-3' EF1-2218R - 5'- ATGACACCRACRGCRACRGTYTG-3'
β-tubulin[7]	B-Tubf1 - 5'- CAGCTCGAGCGTATGAACGTCTG-3' B-Tubr1 - 5'- AGTACCAATGCAAGAAAGCCTT-3'
LSU [14]	LR5 - 5'- TCCTGAGGGAAACTTCG-3' LROR - 5'- ACCCGCTGAACTTAAGC-3'
SSU [15]	NS1 - 5' - GTAGTCATATGCTTGTCTC -3' NS4 - 5' - CTTCCGTCAATTCCTTTAAG -3'

Table 2
PCR reaction mixture

Reaction mixture	Quantity
Template DNA (40 ng)	1 µl
Primer (5pM)	1 µl each
dNTP's (2 mM)	1 µl
Taq buffer A (10X)	5 µl
$MgCl_2$	0.5 µl
Taq DNA polymerase (3U/µl)	1 µl
Sterile water	19.5 µl
Total	25 µl

Table 3
PCR conditions for amplification

	ITS		*tef-1*		β-tubulin		LSU		SSU	
Step	T (°C)	D	T (°C)	D	T (°C)	D	T (°C)	D	T (°C)	D
Initial denaturation	95	3	95	3 min	94	1 min	95	3 min	95	3 min
Denaturation	94	30 sec	94	40 sec	94	15 sec	94	40 sec	94	40 sec
Annealing	52	30 sec	54	50 sec	54	20 sec	52	50 sec	52	50 sec
Extension	72	1	72	1 min	72	20 sec	72	1 min	72	1 min
Final extension	72	10 min	72	10 min	72	7 min	72	10 min	72	10 min
No. of Cycles Denaturation, Annealing, Extension			30 for all							
T: Temperature; D: Duration										

ITS region amplification

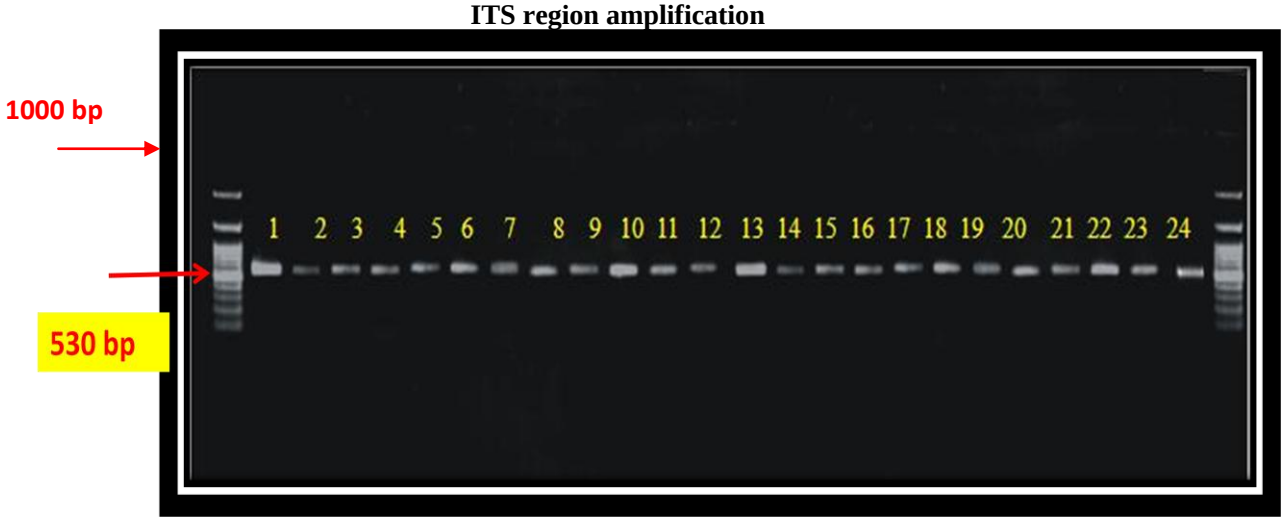


Fig. 1: ITS amplification of *Bipolaris* species (Lane 1-24, 24 isolates) Lanes M 100 bp at both the sides

Table 4
Accession numbers of different genes used in Multilocus analysis of *Bipolaris* species

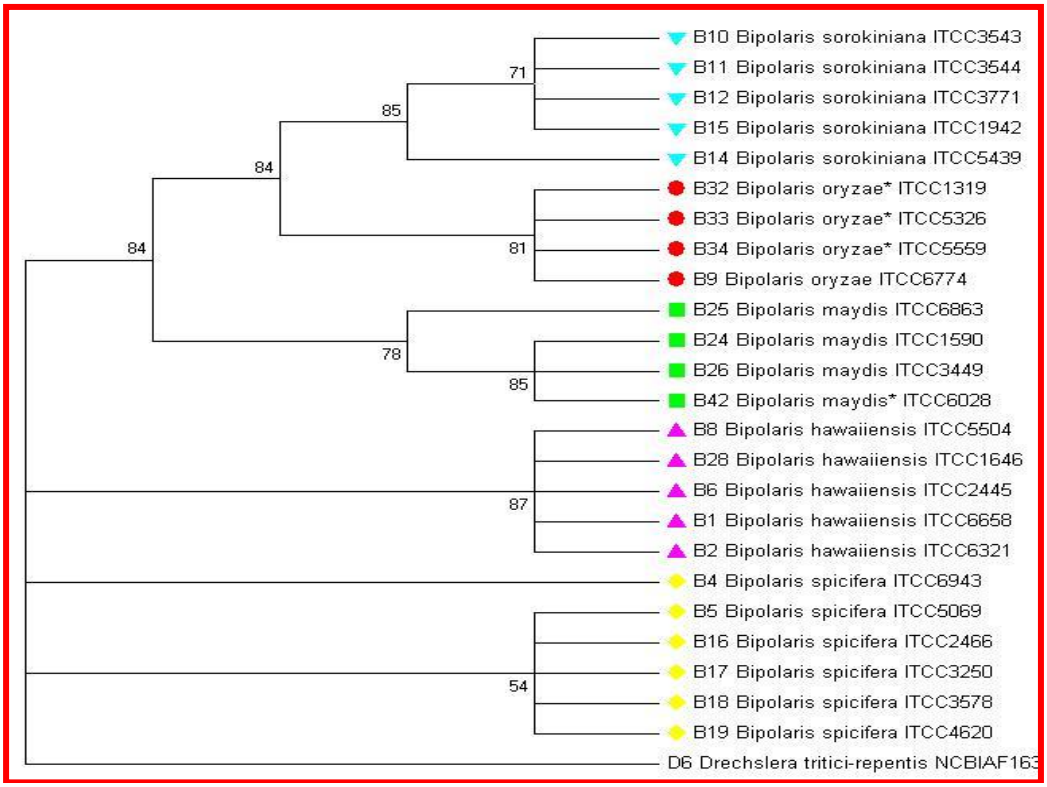
S. N.	Isolate No.	ITCC No.	Species	NCBI Accession No.				
				ITS	Tef-1	β-tubulin	LSU (Larger subunit)	SSU (Smaller subunit)
1	B1	6658	*B.hawaiiensis*	KF725776	KJ939495	KM062882	KM111218	KM111194
2	B2	6321	*B.hawaiiensis*	KF725777	KM062877	KM062883	KM111219	KM111195
3	B4	6943	*B.spicifera*	KF725779	KJ939496	KM062884	KM111220	KM111195
4	B5	5069	*B.spicifera*	KF725780	KJ939497	KM062882	KM111221	KM111197
5	B6	2445	*B.hawaiiensis*	KF725781	KJ939498	KM062882	KM111222	KM111198
6	B8	5504	*B.hawaiiensis*	KF725783	KJ939499	KM062882	KM111223	KM111199
7	B9	6774	*B.oryzae*	KF725784	KJ939500	KM062888	KM111224	KM111200
8	B10	3543	*B. sorokiniana*	KF725785	KM062878	KM062889	KM111225	KM111201
9	B11	3544	*B. sorokiniana*	KF725786	KJ939501	KM062890	KM111226	KM111202
10	B12	3771	*B. sorokiniana*	KF725787	KJ939502	KM062891	KM111227	KM111203
11	B14	5439	*B. sorokiniana*	KF725789	KJ939503	KM062892	KM111228	KM111204
12	B15	1942	*B. sorokiniana*	KF725790	KJ939504	KM062893	KM111229	KM111205
13	B16	2466	*B.spicifera*	KF725791	KM062878	KM062894	KM111230	KM111206
14	B17	3250	*B.spicifera*	KF725792	KM062879	KM062895	KM111231	KM111207
15	B18	3578	*B.spicifera*	KF725793	KM062880	KM062896	KM111232	KM111208
16	B19	4620	*B.spicifera*	KF725794	KJ939505	KM062897	KM111233	KM111209
17	B24	1590	*B. maydis*	KF725799	KJ939506	KM062898	KM111234	KM111210
18	B25	6863	*B. maydis*	KF725800	KJ939507	KM062899	KM111235	KM111211
19	B26	3449	*B. maydis*	KF725801	KJ939508	KM062900	KM111236	KM111212
20	B28	1646	*B.hawaiiensis*	KF725803	KJ939509	KM062901	KM111237	KM111213
21	B32	1319	*B.oryzae*	KF725807	KM062881	KM062902	KM111238	KM111214
22	B33	5326	*B.oryzae*	KF725808	KJ939510	KM062903	KM111239	KM111215
23	B34	5559	*B.oryzae*	KF725809	KJ939511	KM062904	KM111240	KM111216
24	B42	6028	*B. maydis*	KF725817	KJ939512	KM062905	KM111241	KM111217

Discussion

An investigation was done in order to assess the relationship between different species of *Bipolaris* using sequence analyses of ITS 1, ITS 2 and 5.8S rDNA sequences, *tef*-1, β-tubulin, LSU and SSU regions. It was found that ITS region was the best in segregating the genus *Bipolaris* into different species followed by *tef*-1, β-tubulin. LSU and SSU were unable to discriminate the species.

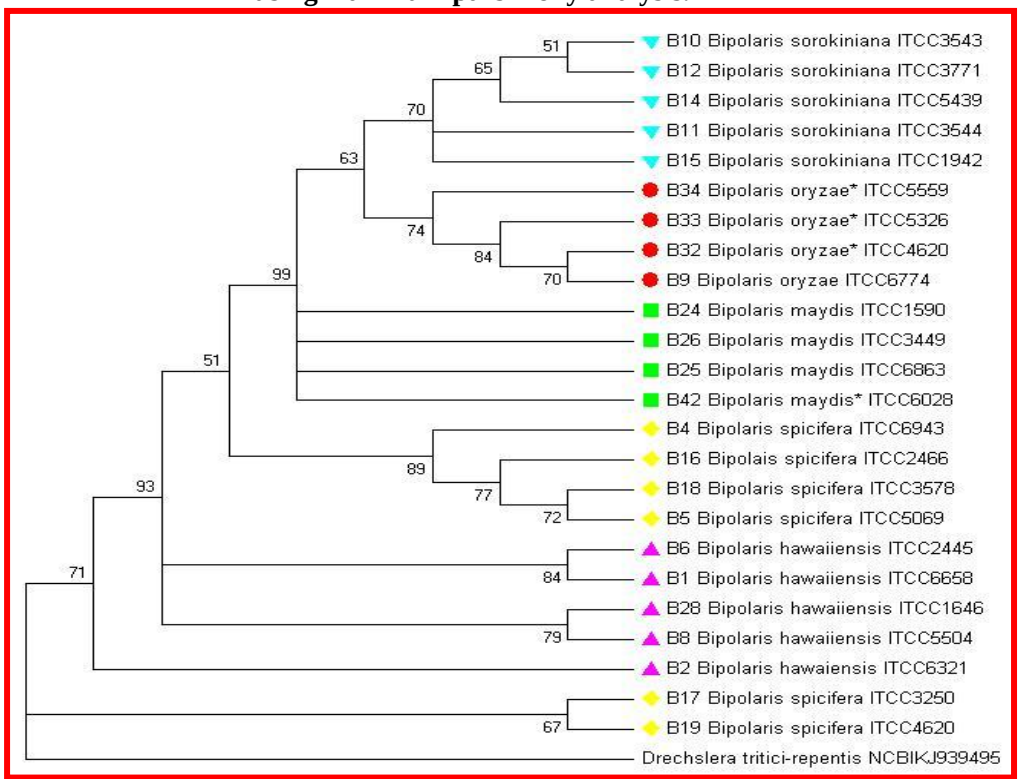
These results are in confirmation with the results obtained by Tazick et al[13] where the taxonomic position of *B. oryzae* was analyzed using ITS rDNA and partial sequences of *tef*-1 and second largest subunit of RPB2 with neighbor joining methods. *Cochliobolus* species were segregated into two groups and *B. oryzae* grouped with *C. sativus* and *C. heterostrophus*. It was found that *tef*-1 gene was unable to differentiate the species of *Cochliobolus*. Similar results were also observed by Berbee et al[1] using ITS1, ITS2, 5·8S rDNA and a portion of the glyceraldehyde-3-phosphate dehydrogenase (GPDH) sequences in which *Helminthosporium* species was segregated into three distinct clades: *Cochliobolus* (anamorphs *Bipolaris* and *Curvularia*); *Pyrenophora* (anamorph *Drechslera*); and *Setosphaeria* (anamorph *Exserohilum*).

Combined gene analysis of rDNA ITS, GPDH, LSU and EF1-α was carried out by Manamgoda et al[6] showing that *Bipolaris, Cochliobolus* and *Curvularia* complex divides into two groups. *Bipolaris* and *Cochliobolus* species clustered in Group 1 along with their type species whereas *Curvularia* species (including species named as *Bipolaris,Cochliobolus* and *Curvularia*) clustered in group 2 with its generic type.



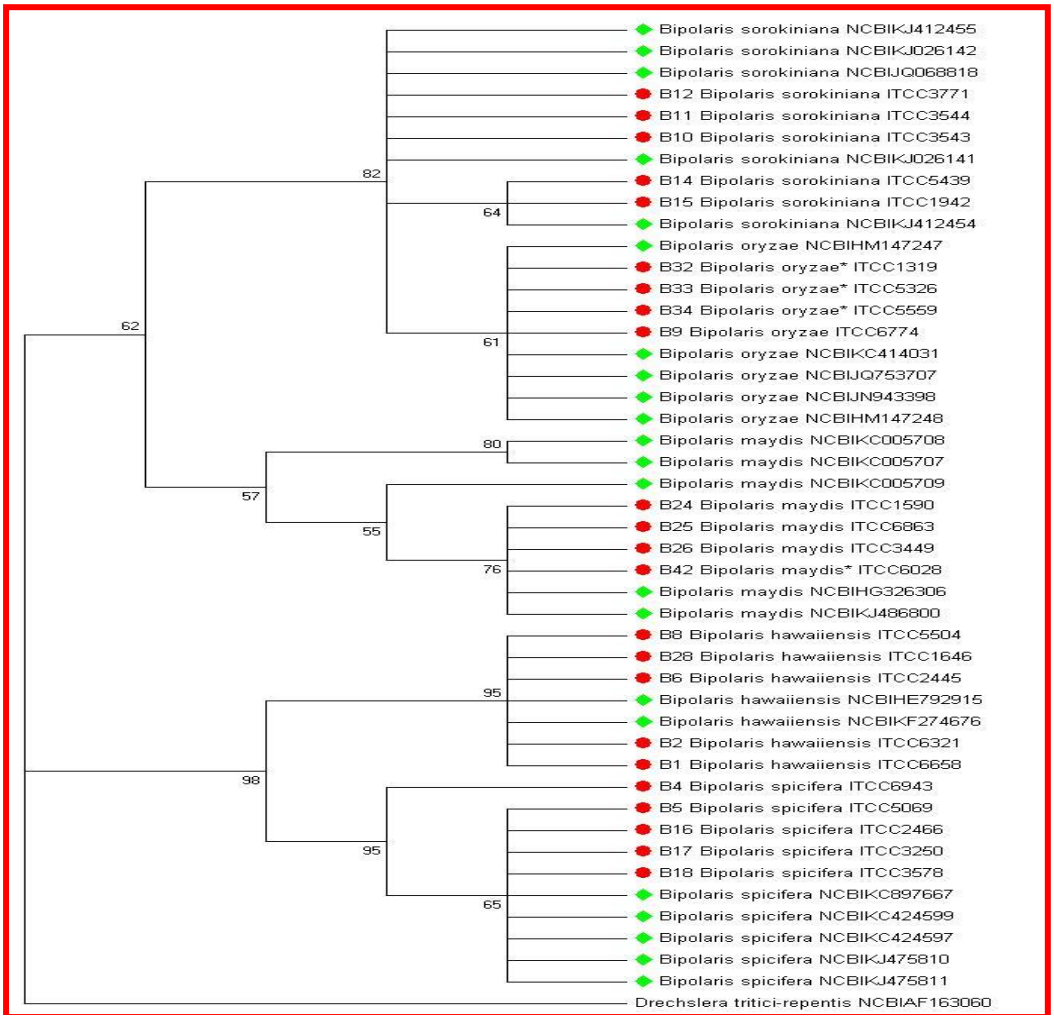
*Non-sporulating isolates

Fig. 2: Phylogenetic relationship of 24 isolates of *Bipolaris* inferred by ITS sequences obtained using maximum parsimony analysis.



*Non-sporulating isolates

Fig. 3: Phylogenetic relationship of 24 isolates of *Bipolaris* inferred by*Tef*-1gene sequences obtained using maximum parsimony analysis.



*Non-sporulating isolates

Fig. 4: Phylogenetic relationship of *Bipolaris* isolates of different species with sequences from NCBI inferred by ITS sequences obtained using maximum parsimony analysis.

Multigene analysis of *Bipolaris* species using ITS, *tef*-1, β-tubulin, LSU and SSU was carried out to select a potential barcode region. Results revealed that ITS region is the best for differentiating the species of *Bipolaris*. Translation Elongation factors 1α and β-tubulin were able to differentiate three species i.e. *B. sorokiniana*, B. *maydis* and *B. oryzae* but were unable to distinctly segregate *B. hawaiiensis* and *B. spicifera.* LSU and SSU could not separate the *Bipolaris* species. Therefore, ITS region can be proposed as potential candidate barcode for identification of *Bipolaris* species.

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