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DNA Barcoding of *Bipolaris* species by using Genetic Markers for Precise Species Identification

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***Bipolaris* spp are the pathogens causing number of diseases in graminaceous crops. Taxonomic delimitation and precise species identification is difficult within this genus because it displays close resemblance with genus *Drechslera* and *Exserohilum* in morphological features. DNA barcoding is a novel technology of DNA sequences of standardized genetic markers for the simple and accurate identification of eukaryotic organisms. Here, we attempted to identify a barcode gene using 24 isolates belonging to five species of *Bipolaris* using five markers *i.e.* ITS, *tef*-1, ²-tubulin, LSU and SSU. ITS region was found as the best marker for species discrimination with highest PCR success rate for amplification and sequencing, with more clearly defined Barcode gap and with high probability of correct identification (PCI).**

Key words: Bipolaris, PCR success rate, Barcode gap and PCI .

Genus *Helminthosporium* was one large group of fungi when it was first named by Link in 1809. Then *Helminthosporium* has gone through frequent refinement in taxonomy over the past 50 years leading to establishment of new genera *Drechslera*[2], *Bipolaris*[14] and *Exserohilum*[9]. Literature defines that these genera were established based on very few and inadequate characters. For the casual observer these three genera are similar and therefore they have been used as synonyms frequently.

Morphology remains the cornerstone of taxonomic diagnosis and has enabled the description of an estimated 1.7 million species, a remarkable achievement. There are, however, limitations to relying on morphology in diagnosing life's diversity. Molecular methods have recently been introduced into fungal taxonomy. These techniques have been proven to be valuable tools in fungal taxonomy and their application has led to the reconsideration of several genera. The identification of species depends on the knowledge held by taxonomists whose work cannot cover all taxon identification requested by non-specialists.

The idea of a standardized molecular identification system emerged progressively during the 1990s with the development of PCR-based approaches for species identification. Several universal systems for molecular-based identification have been used for lower taxa (e.g. nematodes[4]) but were not successfully implemented for broader scopes. The Barcode of Life project soon after became that attempt, aiming to create a universal system for a eukaryotic species inventory based on a standard molecular approach. The barcode standard is administered by the Consortium for the Barcode of Life (CBOL).

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The direct benefits of DNA barcoding undoubtedly include: Relieve the enormous burden of identifications from taxonomists, so they can focus on more pertinent duties such as delimiting taxa, resolving their relationships and discovering and describing new species; Pair up various life stages of the same species (e.g. seedlings, larvae); Provide a bio-literacy tool for the general public.

The gene encoding the mitochondrial cytochrome c oxidase 1 (CO1, or *cox1*) was proposed as the DNA barcode region in animals[5,6] and then adopted by the Consortium for the Barcode of Life (CBOL) as the default barcode for all groups[12]. In plants, however, CO1 had limited use for differentiating species across a wide range of taxa and the a combination of *rbc*L + *mat*K was adopted for a two locus barcode system[8].This sets precedence for reconsideration of the default fungal barcode locus.

Keeping all these advantages of Barcoding in view, it was decided to carry out DNA barcoding of *Bipolaris* species. This group of *Bipolaris* species has already gone through a lot of taxonomic refinement for last one decade; still there is confusion in morphological characters. Therefore, there is a strong need to identify a potential barcode marker which will help in identification of *Bipolaris* species. So, five regions (ITS, *tef*-1, ²-tubulin, LSU and SSU) were tested to select a potential barcode marker for species discrimination in *Bipolaris*.

MATERIALS AND METHODS

DNA Isolation, Amplification, and Sequencing

The genomic DNA was isolated from 24 isolates of *Bipolaris* spp by following standard CTAB method. DNA was dried under a regular air flow for 20 min, resuspended in 70 ¼l TE buffer and stored at"20 °C.Three nuclear ribosomal regions (ITS, LSU, SSU) and two protein coding genes (*tef*-1 and ²-tubulin) were amplified by the respective primers (Table 1).

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 anddocumented using gel documentation unit.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.

Analysis for DNA barcoding

PCR success

Observations regarding success of PCR amplification and sequencing for the genes were recorded. Quality of PCR amplification and primer problems (PCR and sequencing) were also monitored. The genes were ranked for their ability to discriminate species.

Barcode gap

Sequences were aligned using ClustralW and, intra- and inter-specific pairwise distances were calculated using Kimura's two-parameter (K2P) model with MEGA 5.2.Barcode gap was calculated as difference between inter-specific distance and intra-specific distances. Region showing high barcode gap was considered as the best region.

Intra- and inter-specific variation, clustering with a given threshold, and the success rate of PCR and sequencing were used to evaluate the feasibility of the candidate markers[17]. Among them, intra- and inter-specific variation was treated as a very important criterion. The successful species identification of a DNA barcode requires a clear distinction between intra- and inter-specific divergences[5,6].

Probability of correct identification(PCI)

Two kinds of sequence alignment were calculated between every sample pair, namely a global alignment using the Needleman–Wunsch algorithm, which aligns the entire sequence length with penalties for gaps at the alignment ends and a semiglobal alignment using a variant Needleman–Wunsch algorithm that includes both ends of one sequence and finds the alignment with the highest score without penalizing end gaps in the other sequence[10]. Thus, the global alignment matches thewhole length of two sequences, and the semiglobal alignment matches on esequence to a subset of the other and then *vice versa*. Semiglobal alignment checks whether disparate sequence lengths degrade species identification; ifthey do not, global and semiglobal alignment should result in similar identifications. For the two types of alignment, the p-distance (the proportion of aligned

nucleotide pairs consisting of differing nucleotides) was calculated[13].

Based on three methods *i.e.* PCR success, Barcode gap and PCI potential barcode region was selected. Later, a project id (ITCCB) was developed in Barcode of life database (BOLD) website and all the specimen information and sequences were uploaded.

RESULTS

Success rate of PCR

Success rate of PCR amplification and sequencing influences directly the efficiency of DNA Barcoding application. If DNA barcoding is to be practical, the methodology must be accessible and easily carried out. Therefore, efficiency of PCR amplification is necessary. Observation revealed that out of 5 regions used for different amplifications, success rate of amplification was highest in ITS region. In the first attempt of sequencing *tef*-1 gave positive result for 19, ²-tubulin-20, LSU-17 and SSU-20. All the samples were retrieved with good sequence quality in ITS and SSU. In case of *tef-1* only 19 samples were of good quality (Table 2). Thus, percent success in sequence is 100 in ITS and SSU, followed by 87.5 (LSU), 83.34 (*²-tubulin*) and 79.16 (*tef-1*).

Barcode gap

To select an appropriate or ideal DNA barcode marker, comparison of intra- and inter-specific variations is treated as a very important criterion. Region showing high barcode gap is considered as the best region[16]. The comparisons of the five candidate gene markers for Barcoding gap is shown in Fig.4.4.1. The inter-specific variation in ²-tubulin and LSU was larger apparently than those of ITS and *tef*-1. But, intra specific variation within the isolates was also much higher in ²-tubulin and LSU. The maximum intra-specific variations were smaller than minimum inter-specific variation for all the species tested in ITS. The barcoding gap was also the highest in ITS as the intra-specific variation was very less compared to other genes (Fig. 1).

Probability of correct identification (PCI)

A species displays a barcode gap if its maximum intra-specific sequence distance is less than its minimum inter-specific sequence distance. The barcode gap PCI is the fraction of the species (with at least two samples) that display a barcode gap. With all the taxa considered, PCI of ITS was higher (0.095) compared to other genes. PCI in case of *tef*-1 was 0.065 followed by LSU (0.045) (Fig. 2).ITS consistently yielded high levels of species discrimination in all the species.

All the *Bipolaris* species sequences were submitted in BOLD website under the project ITCCB. Later, Barcodes were provided for *Bipolaris* species. BOLD pages with barcodes are shown below.

DISCUSSION

The ITS is the most frequently sequenced genetic marker for fungi[1] is widely used for species identification in many fungal lineages, and already functions as a *de facto* barcode[1,7,3 & 15]. Currently,

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

Region	Primer sequence	Reference
ITS	ITS-1 - 5'- TCCGTAGGTGAACCTGCGG-3' ITS-4 - 5'-TCCTCCGCTTATTGATATGC-3'	White *et al.*, 1990
tef-1	EF1- 983F - 5'- GCYCCYGGHCAYCGTGAYTTYAT-3' EF1-2218R - 5'- ATGACACCRACRGCRACRGTYTG-3'	Schoch *et al.*, 2009
β-tubulin	B-Tubf1 - 5'- CAGCTCGAGCGTATGAACGTCTG-3' B-Tubr1 - 5'- AGTACCAATGCAAGAAAGCCTT-3'	McKay and Cooke, 1992
LSU (Large subunit)	LR5 - 5'- TCCTGAGGGAAACTTCG-3' LROR - 5'- ACCCGCTGAACTTAAGC-3'	Vilgalys and Hester, 1990
SSU (Small subunit)	NS1 - 5' - GTAGTCATATGCTTGTCTC -3' NS4 - 5' - CTTCCGTCAATTCCTTTAAG -3'	White *et al.*, 1990

there are ~172,000 reasonably full-length fungal ITS sequences in GenBank. For a marker to be validated as a DNA Barcode three criteria i.e. success rate of PCR, Barcode gap and PCI are very important. ITS was found to be the best in species discrimination based on these three criteria. Success rate of PCR amplification and sequences in ITS gave cent percent result. There was very less difference within species isolates in case of ITS compared to other genes. Therefore, ITS can

Table 2. Success rates of PCR and sequencing of ITS, *tef*-1,²-tubulin, LSU, and SSU of 5 species of *Bipolaris*

Markers	ITS	*tef-1*	²-tubulin	LSU	SSU
No. of species/n	5	5	5	5	5
No. of samples/n	24	24	24	24	24
Amplification success	24	18	20	17	20
Success of sequencing/n	24	19	20	21	24
Success rate of sequencing %	100	79.16	83.34	87.5	100
Length/bp	530	950	1000	950	1000

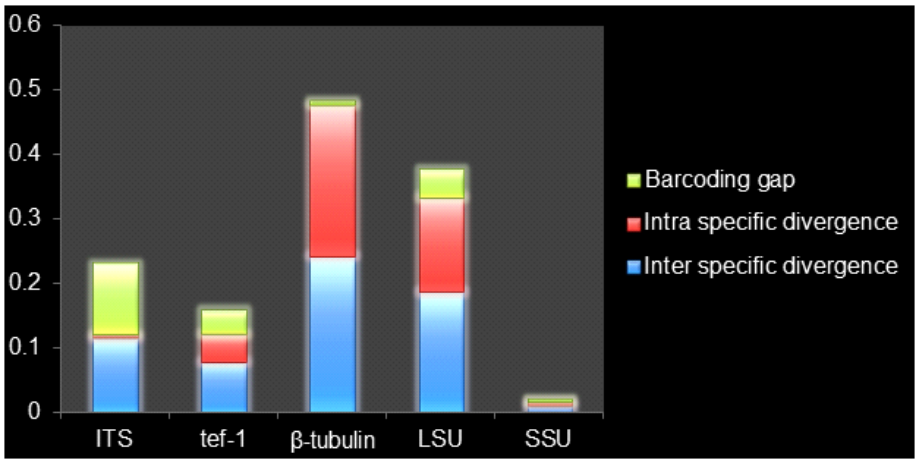

Fig. 1. Inter and Intra specific divergence and barcoding gap of candidate barcode regions

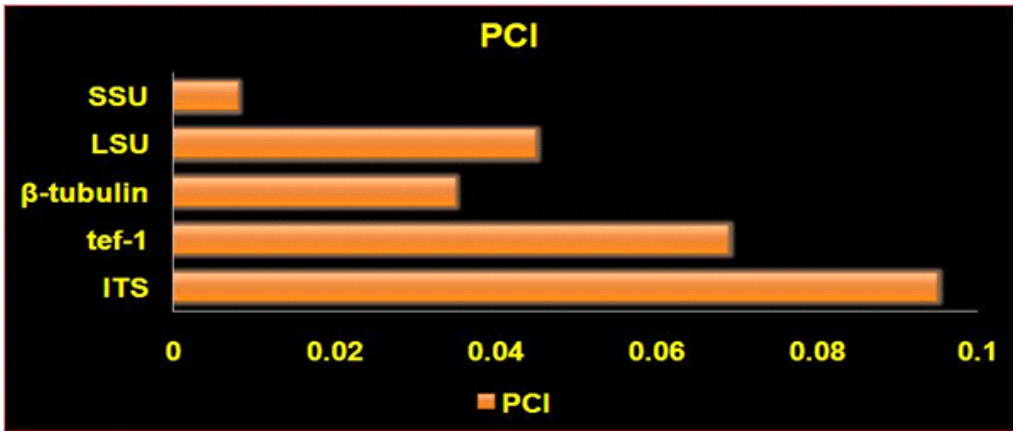

Fig. 2. Graph representing PCI of candidate barcode regions

be regarded as the best marker for *Bipolaris* species differentiation. The results are in accordance with those of Schoch *et al.*,(2012). Who carried out in different divisions of fungi having 206 species using six DNA regions for selection of universal barcode for fungi. The results marked ITS as the best barcode for fungi having highest probability of successful identification for the broadest range of fungi, with the most clearly defined barcode gap between inter- and intra-specific variation. Similar results were obtained by Suwannasai, *et al*.(2013), which states that Phylogenetic trees analysis discovered a cryptic species and strongly supported monophyletic clades for many *Annulohypoxylon* and *Hypoxylon* species, suggesting that ITS can contribute usefully to a barcode for these fungi.

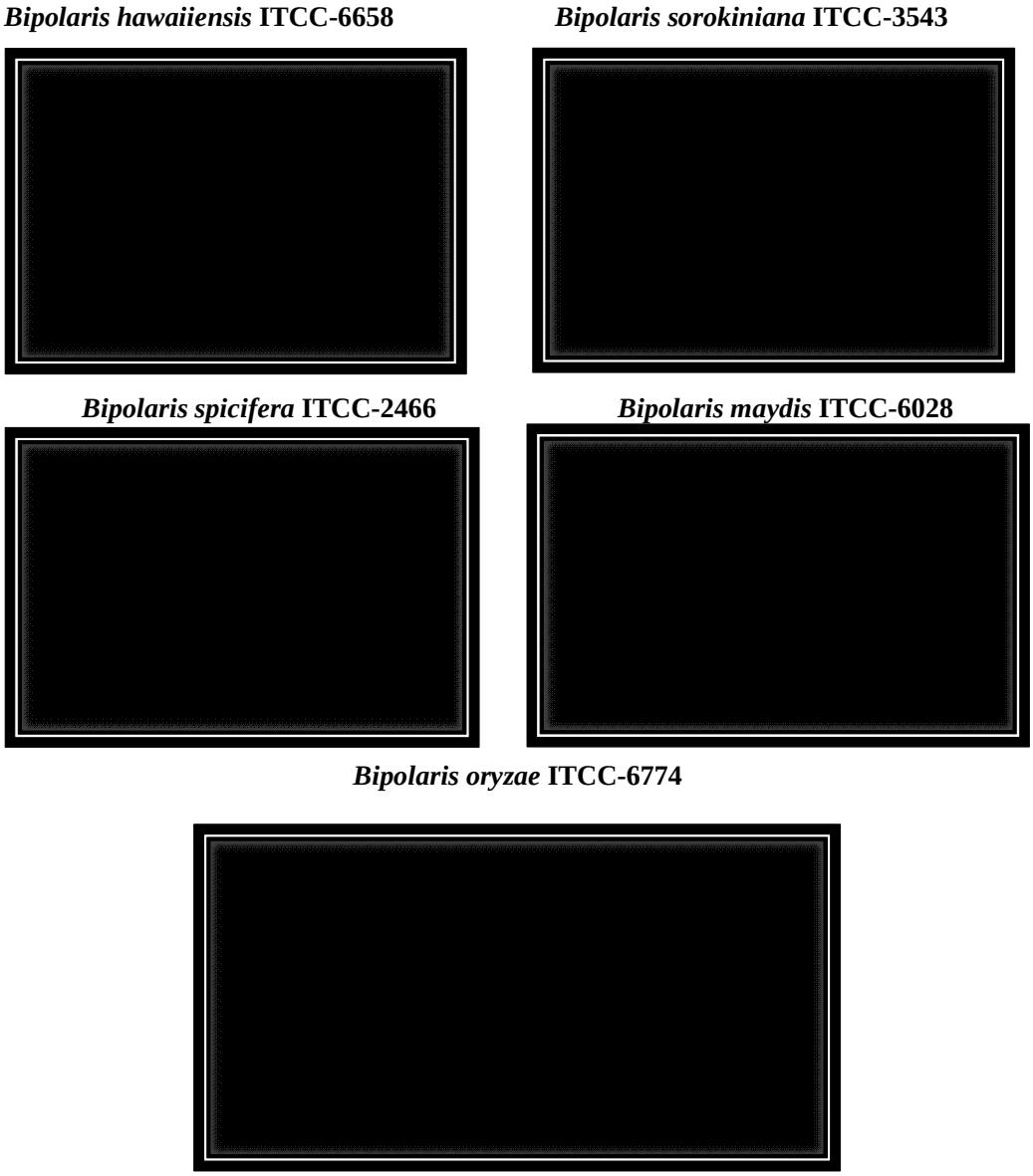

Fig. 3. DNA Barcodes of Five *Bipolaris* sp. from BOLD database

Two DNA barcode regions, the ITS nuclear rDNA region and the cytochrome oxidase subunit I (*CO1*)mitochondrial gene, were sequenced for identification of *Leohumicola* species. Single gene parsimony, dual-gene parsimony and dual-gene Bayesian inferencephylogenetic analyses support *L. levissima, L. atra, L. incrustata* as distinct phylogenetic species[11].

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