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Polyphasic taxonomic characterization of nine *Penicillium* species from soil of different parts of India

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Abstract

Aim: Morpho-molecular analyses for taxonomic characterization of nine predominant *Penicillium* species present in the soil of different parts of India.

Methodology: Fifteen *Penicillium* isolates were isolated from the soil samples collected from the experimental field of ICAR-Indian Agricultural Research Institute (IARI), New Delhi. Another twenty-six isolates were procured from Indian Type Culture Collection (ITCC), Division of Plant Pathology, ICAR-IARI, New Delhi which were isolated from the soil of different parts of India. Total 41 isolates were characterized following distinct macroscopic (colony texture, colony colour exudate production; soluble pigmentation; reverse coloration and mycelial growth) and microscopic observations (type of penicillus; shape of phialides; conidial shape, size and pigmentation). Molecular characterization was done using partial β -tubulin gene sequence which is considered an excellent marker in differentiating *Penicillium* species.

Results: The morphological data in species description perfectly matched with molecular data generated using β -tubulin gene marker and nine different species viz., *P. aethiopicum*, *P. chrysogenum*, *P. crustosum*, *P. janthinellum*, *P. mononematosum*, *P. oxalicum*, *P. polonicum*, *P. singorense* and *Talaromyces pinophilus* (Syn. *P. pinophilum*) were confirmed.

Interpretation: Above nine *Penicillium* species are found predominantly in the soil collected from different parts of India. The β -tubulin gene can be considered as an excellent marker to differentiate *Penicillium* species as confirmed from this study. The combined morpho- molecular analyses can be further utilized to delineate morphologically similar *Penicillium* species and also helpful to establish new species of *Penicillium*.

Key words: β -tubulin gene, Morpho-molecular approach, *Penicillium* species

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Introduction

The genus *Penicillium* is an ascomycetous fungus that belongs to family *Trichomaceae*. The name *Penicillium* is derived from its reproductive structure called penicillus, which means "little brush" (Link, 1809). The shape of penicillus may be monoverticillate, biverticillate, terverticillate or quarterverticillate. It contains fertile hyphae, rami, stripes, metulae, ramuli, phialides and conidia. Phialides shapes are ampulliform (flask-shaped) and acerose (cylindrical) with long and short collula. The conidia of *Penicillium* are ornamented, pigmented, green or blue in colour and variable in shape and size. Morphological characters (radial growth and colony characters) on specific media are essential for identification of fungal species. Malt Extract agar (MEA) and Czapek Yeast Autolysate agar (CYA) are considered as two standard media recommended for the excellent growth of *Penicillium* and morphological characterization. Species concept describes not only according to the morphological and genetic similarity of a species but also their interbreeding within the populations. In *Penicillium*, the species concept was first described by Thom (1954) for systematically standardizing different morphological characters to delineate species and later, was further emphasized by Pitt (1979) and Okuda *et al.* (2000). Due to large number of species, the genus *Penicillium* is further divided into different subgenera viz., *Furcatum*, *Biverticillium*, *Aspergilloides* and *Penicillium* and further into sections and series. Although the genus *Penicillium* is well known, however, their identification is still problematic due to overlapping of macro- and micro-morphological characters. Many workers have suggested the challenge to resolve closely related *Penicillium* species based on morphological characters alone (Banke *et al.*, 1997; Frisvad and Samson, 2004; Henk and Fisher, 2011). Thus, alternative methods are essential for accurate identification of species of this genus. Most of the names mentioned in the research papers are invalid names due to misidentification, giving a false impression regarding species identification (Visagie *et al.*, 2014).

So there is a need to find an alternative method for accurate identification of *Penicillium* species. Various molecular markers have been used to characterize *Penicillium* at species level for precise identification. Recently, few workers have reported phylogenetic analysis using single gene sequences of different *Penicillium* species using ribosomal genes and protein-coding genes (Samson *et al.*, 2014; Wang *et al.*, 2017; Su and Niu, 2018; Varriale *et al.*, 2018). However, the molecular characters should be integrated with morphological characters as an integrated approach for authentic identification of *Penicillium* species. Therefore, for species delineation, this study was carried out for morphological and molecular characterization of *Penicillium* species from Indian soils.

Materials and Methods

Morphological examination of *Penicillium* isolates: The soil samples were collected from the experimental field of ICAR-Indian Agricultural Research Institute (IARI), New Delhi and

were screened for the presence of *Penicillium* fungus through serial dilution method. Fifteen isolates were identified as *Penicillium* from the collected soil samples based on the macroscopic examination (colony colour, exudate of soluble pigment, reverse coloration, mycelial growth and colony texture) and microscopic observations (type and shape of penicillus; shape of phialides; conidial shape, size and pigmentation) (Pitt, 1979; Visagie *et al.*, 2014) (Table 1).

Twenty-six more isolates were procured from Indian Type Culture Collection (ITCC), Plant Pathology Division, ICAR-IARI, New Delhi (Table 1) which were collected from the soil of different parts of India. The morphological characters of all isolates were recorded in 7-days-old cultures on Czapek Yeast Autolysate agar (CYA). Further, the isolates were subjected to microscopic examination by prepared slides. The prepared slides were observed under 10X, 40X and 100 X magnifications. Observations on phialide branching pattern, size and shape of phialides and spores were recorded by Olympus Microscope (BX51) with image analyses software (Progres 2.7 version, Jenoptik, USA). Ten random observations were recorded while measuring the spore characteristics of different isolates.

DNA extraction, PCR and sequencing: Genomic DNA of all 41 isolates was isolated using CTAB (Cetyltrimethyl Ammonium Bromide) method (Culling, 1992). PCR was performed using β -tubulin genes specific forward Bt2a primer (5'-GGTAACCAAATCGGTGCTGCTTTC-3') and reverse Bt2b primer (5'-ACCCTCAGTGTAGTGACCCTTGGC-3') (Glass and Donaldson, 1995). Total 25 μ l reaction mixture were conducted in 0.2ml PCR tubes measuring 1 μ l of each forward and reverse primers, 12.5 μ l of Dream TaqThermo Scientific master mixture (2X) (containing Taq DNA polymerase, buffer, dNTP's, MgCl₂ and loading dye), 1 μ l (100ng) of DNA template and remaining 9.5 μ l added sterile nuclease free double distil water to make the final volume. Thermocycler was programmed as follows initial heating at 94°C for 5 min, 35 cycles of denaturation at 94°C for 1min, annealing at 61°C for 1min, extension at 72°C for 2 min and final extension at 72°C for 5 min. Electrophoresis of the amplified (550bp) PCR products separated and visualized on agarose gel (1.2%) using intercalating agent ethidium bromide. Purification of distinct bands and sequencing of all the samples were performed through Eurofins Scientific, Bangalore.

Sequence alignment and phylogenetic analysis: All the 41 sequences were subjected to alignment using algorithm clustalW of MEGA 6.0 (<http://www.megasoftware.net>) software (Thompson *et al.*, 1994). To analyze the phylogenetic relationship among taxa unaligned sequence of β -tubulin gene was removed from both the end borders. These conserved sequences were evaluated using NCBI BLAST (<http://blast.ncbi.nlm.nih.gov>) and CBS-KNAW database to identify all 41 isolates. To construct a phylogenetic tree, the maximum parsimony (MP) algorithm was adopted in MEGA6.0. One thousand bootstrap values were evaluated for parsimonious tree.

Results and Discussion

A total of forty-one isolates were examined for characters associated with macro-and micro-morphology on CYA media. The dissimilarities of key phenotypic characteristics were taken into consideration for discrimination of all the isolates. For species determination of *Penicillium* reported taxonomical keys were adopted. Based on the similarities and dissimilarities of crucial cultural and phenotypic parameters, the isolates were grouped into nine different species of *Penicillium* viz., *P. aethiopicum*, *P. chrysogenum*, *P. crustosum*, *P. janthinellum*, *P.*

mononematosum, *P. oxalicum*, *P. polonicum*, *P. singorense* and *Talaromyces pinophilus* (Syn. *Penicillium pinophilum*) (Table1, Fig. 1). In this study, seventeen isolates viz., ITCC 2410, ITCC 3211, ITCC 4254, ITCC 4482, S1, S2, S3, S9, S11, S12, S13, S16, S18, S19, S20, S21 and S22 were identified as *P. aethiopicum*, seven isolates viz., S7, ITCC 1601, ITCC 1495, ITCC 2168, ITCC 3122, ITCC 5080 and ITCC 5082 as *P. chrysogenum*, six isolates viz., ITCC 5235, ITCC 5285, ITCC 5287, ITCC 5292, ITCC 5298 and ITCC 6171 as *P. polonicum*, five isolates viz., ITCC 3146, ITCC 3212, ITCC 4276, ITCC 5251 and ITCC 6755 as *P. mononematosum*, two isolates ITCC 6093 and S15 as

Table1: List of S series isolates of *Penicillium*, their ITCC and gene bank accession numbers

Isolates/ITCC no.	<i>Penicillium</i> species	Location	β tubulin (Accession number)
S1(8077)	<i>P. aethiopicum</i>	New Delhi	MT681750
S2 (8078)	<i>P. aethiopicum</i>	New Delhi	MH766361
S3 (8079)	<i>P. aethiopicum</i>	New Delhi	MH766362
S9 (8080)	<i>P. aethiopicum</i>	New Delhi	MH766363
S11 (8081)	<i>P. aethiopicum</i>	New Delhi	MH766364
S12 (8082)	<i>P. aethiopicum</i>	New Delhi	MH766365
S13 (8083)	<i>P. aethiopicum</i>	New Delhi	MH766366
S16 (8084)	<i>P. aethiopicum</i>	New Delhi	MH766367
S18 (8085)	<i>P. aethiopicum</i>	New Delhi	MH766368
S19 (8086)	<i>P. aethiopicum</i>	New Delhi	MH766369
S20 (8087)	<i>P. aethiopicum</i>	New Delhi	MH766370
S21 (8088)	<i>P. aethiopicum</i>	New Delhi	MH766371
S22 (8089)	<i>P. aethiopicum</i>	New Delhi	MH766372
2410	<i>P. aethiopicum</i>	Nainital, Uttarakhand	MH699755
3211	<i>P. aethiopicum</i>	Darjeeling, West Bengal	KY271163
4254	<i>P. aethiopicum</i>	Karnal, Haryana	MH699773
4482	<i>P. aethiopicum</i>	Jammu, Jammu & Kashmir	MH699768
S7 (8090)	<i>P. chrysogenum</i>	New Delhi	MH766376
1495	<i>P. chrysogenum</i>	West Bengal	MH699774
1601	<i>P. chrysogenum</i>	New Delhi	MH699767
2168	<i>P. chrysogenum</i>	Assam	MH699779
3122	<i>P. chrysogenum</i>	New Delhi	MH699759
5080	<i>P. chrysogenum</i>	Hyderabad, Telangana	MH699775
5082	<i>P. chrysogenum</i>	Hyderabad, Telangana	MH699776
4255	<i>P. crustosum</i>	Karnal, Haryana	MH699778
5297	<i>P. janthinellum</i>	New Delhi	MH699765
3146	<i>P. mononematosum</i>	Burdwan, West Bengal	MH699761
3212	<i>P. mononematosum</i>	Lucknow, Uttar Pradesh	MH699777
4276	<i>P. mononematosum</i>	Jammu, Jammu & Kashmir	KY271156
5251	<i>P. mononematosum</i>	Chaphra, Bihar	MH699763
6755	<i>P. mononematosum</i>	New Delhi	KY271162
6374	<i>P. oxalicum</i>	Roorkee, Uttarakhand	MH699770
5235	<i>P. polonicum</i>	Abohar, Punjab	MH699771
5285	<i>P. polonicum</i>	Calcutta, West Bengal	MH699760
5287	<i>P. polonicum</i>	Almora, Uttarakhand	MH699769
5292	<i>P. polonicum</i>	Srinagar, Jammu & Kashmir	MH699762
5298	<i>P. polonicum</i>	New Delhi	MH699766
6171	<i>P. polonicum</i>	Sriganganagar, Rajasthan	MH699772
4603	<i>P. singorense</i>	Gwalior, Madhya Pradesh	KY271164
S15 (8091)	<i>Talaromyces pinophilus</i>	New Delhi	MH790890
6093	<i>Talaromyces pinophilus</i>	Madurai, Tamil Nadu	KY271165

Fig. 1: Morphological description: a, b- Colonies on CYA; c- phialides on 100X; d- spore on 100X; A- *P. aethiopicum*; B- *P. chrysogenum*; C- *P. crustosum*; D- *P. janthinellum*; E- *P. mononematosum*; F- *P. oxalicum*; G- *P. polonicum*; H- *P. singorense* and I- *Talaromyces pinophilus*.

Talaromyces pinophilus and one isolate each ITCC 4255, ITCC5297, ITCC 6374 and ITCC 4603 as *P. crustosum*, *P. janthinellum*, *P. oxalicum* and *P. singorense*, respectively.

Further, molecular confirmation the 550bp amplified PCR products of the β -tubulin gene were sequenced and each generated sequences were analyzed with GenBank database through Blast analysis (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>).

All the sequences showed 99-100% similarity with NCBI voucher accession of different *Penicillium* species. The morphological data in species description is perfectly matched with the molecular data generated using β -tubulin gene marker. These β -tubulin gene sequences of *Penicillium* species were deposited in NCBI, and their accession numbers were obtained (Tables 1). Maximum parsimony (MP) based phylogenetic tree was constructed from generated DNA sequences of β -tubulin gene from all 41 isolates

Fig. 2: Maximum parsimonious phylogenetic tree based on β -tubulin gene of different *Penicillium* isolates segregating into nine *Penicillium* species.

(Fig. 2). The tree exhibited seven clusters and each cluster distinctly separated from each other, and *P. aethiopicum*, *P. mononematosum*, *P. chrysogenum*, *Talaromyces pinophilus* and *P. oxalicum* exhibited as distinctly separated clusters as Cluster I, Cluster II, Cluster III, Cluster VI and Cluster VII respectively whereas *P. polonicum* and *P. crustosum* were found within Cluster IV, similarly *P. singorensis* and *P. janthinellum* in Cluster IV and V

showed relatively close *Penicillium* species which were difficult to separate in different cluster based on single gene phylogeny. Therefore, these closely related species of *Penicillium* viz., *P. polonicum*, *P. crustosum*, *P. singorensis* and *P. janthinellum* were confirmed by matching the molecular identification based on NCBI Blast and available morphological distinct characters. *Penicillium* is an anamorph genus that currently contains 483 accepted

species. Previously, *Penicillium* taxonomy was based only on morphology, where incorrect identification was probably due to overlapping characters of different species (Houbreken et al., 2020), therefore, the molecular phylogeny based characterization have started using ribosomal genes and protein-coding genes sequences of several *Penicillium* species. Therefore, an integrated approach was used considering both morphology, and molecular data was used to establish *Penicillium* species. Firstly, 41 isolates were segregated into different *Penicillium* species based on the morphological characters, i.e., conidiophore branching pattern and taxonomically important morphological characters (colony, margin, and reverse colour; colony texture; exudates and soluble pigments production, shape, size and texture of conidia, phialide, metulae, rami and stipe). These conidiophore branching patterns and other important microscopic characters were used by different workers for species delineation of genus *Penicillium* (Frisvad and Samson, 2004). Later all the 41 isolates were identified through NCBI Blast and phylogenetic analysis based on β -tubulin gene. Even though Internal transcribed spacer (ITS) region is considered as a universal barcode for fungal species differentiation (Visagie et al., 2014a), but β -tubulin gene was considered as a putative species-specific marker for phylogenetic analysis in genus *Penicillium* by several researchers (Geiser et al., 1998; Barbosa et al., 2018; Mincuzzi et al., 2020; Ghuffar et al., 2021).

All the morphological data in species description perfectly matches with molecular data generated using β -tubulin gene marker. In phylogenetic analysis, β -tubulin could successfully differentiate five *Penicillium* species in different clusters. Four species of *Penicillium* were clustered in two different clusters. This result reveals that the relatively close *Penicillium* species are difficult to separate among themselves by using single-gene phylogeny. These species can be differentiated by using multi-gene phylogenetic analysis. This finding is in line with the previous reports of Visagie et al. (2014) and Houbreken et al. (2020). In conclusion, nine predominant *Penicillium* species were identified through morpho-molecular approach and it was found that *P. aethiopicum*, *P. chrysogenum*, *P. mononematosum*, *P. polonicum* and *Talaromyces pinophilus* were common in the soil of different parts of India. Further, β -tubulin gene proved as a good molecular marker to differentiate morphological similar closely related *Penicillium* species. This study shows that combined morpho- molecular study can help resolve more morphological aberration of different *Penicillium* species and can also be helpful in establishing new species of *Penicillium*.

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Add-on Information

Authors' contribution: R. Debbarma: Planning and execution of Research work; T. Prameeladevi: Facilitation and guidance for

lab experiments; A. Tyagi: Guidance for molecular experiment and analysis; D. Kamil: Mentor.

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