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Genetic diversity and species pattern of *Trichoderma* and *Hypocrea* in Manipur using *in-silico* analysis

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Abstract:
We investigated the occurrence and genetic diversity of *Trichoderma* and *Hypocrea* in Manipur which lies in the Indo-Burma biodiversity hot spot region. 65 *Trichoderma* isolates were identified at species level by morphological as well as sequence based analysis of the internal transcribed spacer region 1 and 4. Altogether 22 different species of *Trichoderma* and *Hypocrea* were found, of which *Trichoderma harzianum* represent the dominant species. Phylogenetic analysis reveals a clear cut distinction of strains isolated from various collection sites which further hints the need for detail study of *Trichoderma* on molecular level.

Background:
The hypocreomycetidae genus *Trichoderma* was known for their rapid growth, capability of utilizing diverse substrates and resistance to noxious chemicals **[1].** They are often the predominant components of the mycoflora in soils of various ecosystems, such as agricultural fields, prairie, forest, salt marshes and deserts, in all climatic zones **[2].** Several *Trichoderma* species are significant biocontrol agents against fungal plant pathogens for nutrients, stimulators of plant health, or inducers of plant systemic resistance to pathogens **[3].** *Trichoderma* species produce a wide diversity of metabolites as well as the toxins and trichothecenes that display *in vitro* cytotoxicity **[4].**

Due to the ecological importance of *Trichoderma* and its application as a biocontrol agent in the field, it is important to understand its biodiversity and biogeography. However, accurate species identification based on morphology is difficult at best because of the paucity and similarity of useful morphological characters **[5, 6]** and increasing numbers of morphologically cryptic species that can be distinguish only through their DNA characters are being described **[7].** With the advent of molecular methods and identification tools, which are based on sequence analysis of multiple genes, it is now possible to identify every *Trichoderma* isolate and /or recognize it as a putative new species **[8].** Considering the environmental conditions as one of the important factors, the right selection of BCAs, which begins with a safe characterization of biocontrol strains in the new taxonomic schemes of *Trichoderma*, is equally important since the exact identification of strains to the species level is the first step in utilizing the full potential of fungi in specific applications **[9].** The current diversity of the holomorphic genus *Hypocrea*/*Trichoderma* is reflected in approximately 160 species, the majority of which have been recognized on the basis of DNA sequence analysis and molecular phylogeny of pure cultures and/or herbaria specimens **[8].** Manipur belongs to the rich Indo-Burma mega biodiversity hotspot region of the world which lies between 23°47'- 25°45' North latitude and 96°61'- 94°48' East longitude. This region is representing an active center of gene pool and having a diverse range of *Trichoderma* spp. with potential biocontrol activity.

Methodology:
Geography of sample sites
Sampling was done from nine different districts of Manipur comprising of four different agro-climatic zones *viz.* i. Subtropical plain zone, ii. Sub-tropical hill zone, iii. Temperate

sub-Alpine zone and iv. Mid tropical hill zone, which differ in their geographic location, altitude and climate.

Isolation of pure cultures

Trichoderma selective medium **[10]** was used as a selective medium for *Trichoderma*, using the soil dilution plating method. Putative *Trichoderma* colonies were purified by two rounds of subculturing on potato-dextrose agar (PDA).

Morphological analysis

For morphological analysis, strains were grown on PDA at 25ºC - 30°C. Microscopic observations, measurements were made from slide preparation by using trinocular microscope. Conidiophore structure and morphology were examined on macronematous conidiogenous pustules or from fascicles when conidia were maturing. Conidial morphology and sizes were recorded after 6-7 days of incubation.

DNA isolation

The genomic DNA of *Trichoderma* isolates were extracted from the pure culture of young and actively growing hyphae using NBAIM method. Mycelia were obtained by inoculating potato dextrose broth (PDB;Difco) with aerial mycelium from PDA plates, and after incubation at 24ºC for 48h on an orbital shaker (120 rpm). Mycelia were collected on filter paper in a Buchner funnel, washed with sterile water and grinded in a sterile motar and pastle **[11]** with minor modifications as described by **[12].**

DNA amplification

Trichoderma nuclear small-subunit rDNA sequence containing the Internal Transcribed Spacer (ITS) 1 and 4 regions and the 5.8S rRNA gene were amplified by Polymerase Chain Reaction (PCR) in an automated thermocycler using a combination of two specific primers ITS1 (TCCGTAGGTGAACCTGCGG) and ITS 4 (TCCTCCGCTTATTGATATGC) **[13].**

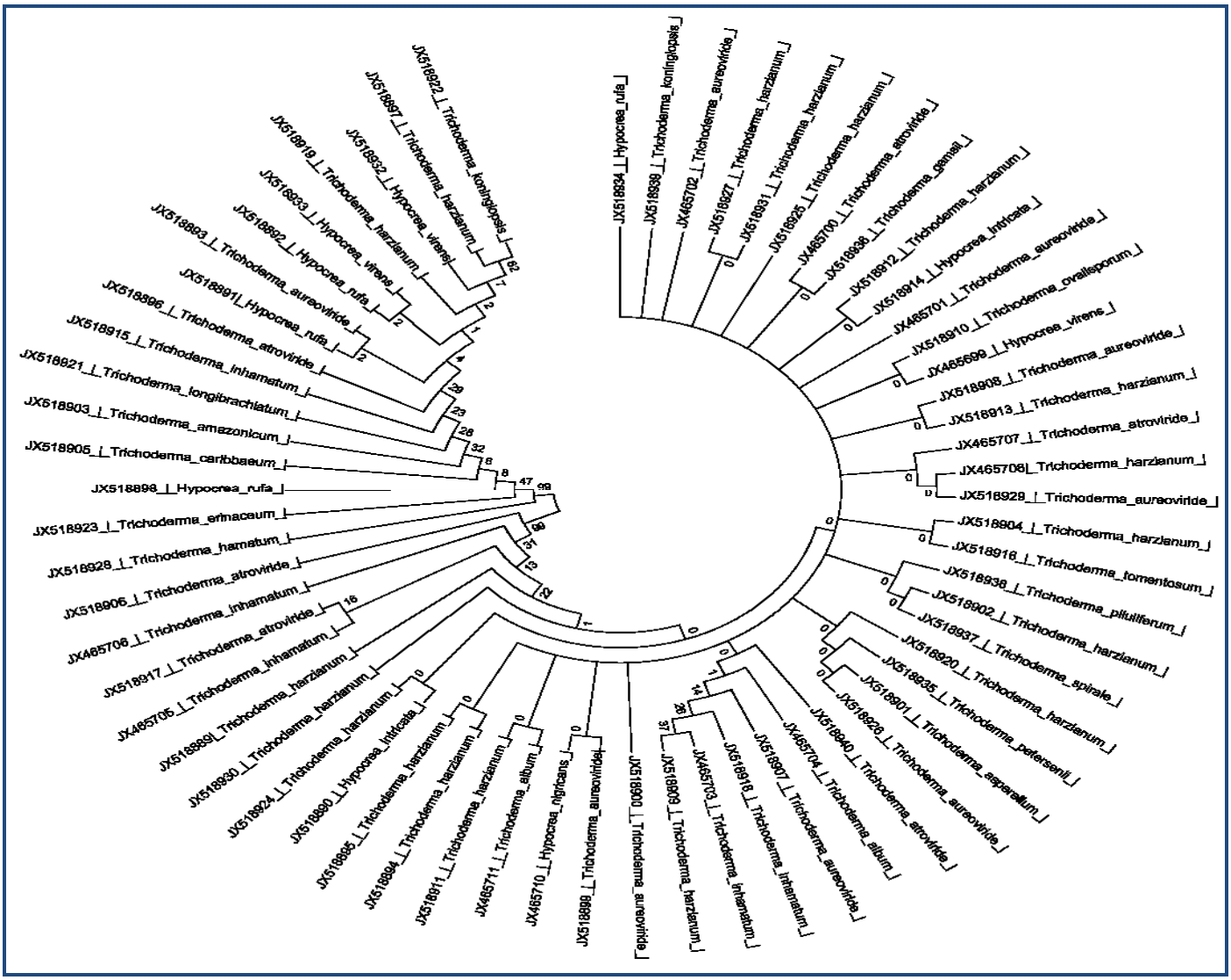

Figure 1: phylogenetic analysis of Trichoderma strains using MEGA 5.05 with 1000 number of replications and Neighbour-joining statistical method.

Sequence assembly and alignment

Amplification products obtained from PCR reactions with unlabeled ITS primers (ITS1 & ITS4) were sended to B'Genei for sequencing. DNA sequences obtained for each strain from each forward (ITS) and reverse (ITS4) primer were inspected individually for quality. Both strands of the DNA were then assembled to produce a consensus sequence for each strain using Gene Runner software and submitted to NCBI blast. Multiple sequence alignment was performed using ClustalW tool with default parameter in MEGA 5.05.

Phylogenetic Analysis

The phylogenetic analyses of the aligned sequence were performed using MEGA 5.05 **[14]** with 1000 number of bootstrap replicates and Neighbor-Joining method of statistical analysis.

Results:

A total of 193 isolates were obtained from the nine geographically diverse areas of Manipur. Out of the total isolates, 65 representative isolates were preliminarily identified at the species level by morphological characteristics. Later, 22 different *Trichoderma* spp. among the total 65 strains were identified by the analysis of their ITS1 and ITS4 sequences (amplicon sizes ranging from 560-600bp). *T. harzianum* was the most dominant species among the 22 *Trichoderma* spp. Sequence strains of the nearest accession numbers obtained from the Gene Bank along with the strain identity percentage and the result of the blast searches are listed in **Table 1 (see supplementary material).** Phylogenetic studies revealed considerable variations among the isolates collected from different districts of Manipur.

Discussion:

We have carried out a survey of the occurrence of *Trichoderma* and *Hypocrea* in Manipur which aimed to obtain a more complete picture of the biodiversity of these genuses in Manipur. A collection of 65 isolates obtained from 9 different districts of Manipur were identified by morphological observations and by analysis of the ITS sequence analysis. A wide diversity of *Trichoderma* isolates were found (22 species were identified among 65 isolates) in comparison with the studies on the biodiversity of *Trichoderma* in South-East Asia **[15]**, Austria **[16]**, South America **[5]**, China **[17],** Sardinia **[18]** and in Poland **[19].**

We found that the amplification products for the ITS region of 65 species of *Trichoderma* collected from nine different districts of Manipur ranges from 560-600bp. These results were in accordance with **[20]** and other several workers also observed the amplified rDNA fragment of approximately 500 to 600 bp by ITS-PCR in *Trichoderma* **[21].** *T. harzianum*, which was the most dominant species in this study, was reproducibly associated with all the types of soils of nine different districts **[22].** In previous studies that used cultivation-dependent methods to quantify *Hypocrea* / *Trichoderma* in various habitats, *T. harzianum sensu lato* represented the most dominant species **[18, 23, 6].** Among the total 675 strains belonging to *Trichoderma* and *Hypocrea* strains available in the International Subcommision on *Trichoderma* and *Hypocrea*, 6 strains from the present study namely *H. intricata, T. amazonicum, T. album, H. rufa, T. gamsii* and *H. nigricans* have not been reported in ISTH.

The results from this study stress the importance of the use of molecular identification tools to describe the biodiversity of *Trichoderma* in a natural habitat. This further corroborate from phylogenetic tree **(Figure 1).** The high number of species of *Trichoderma* and *Hypocrea* found in the nine different districts of Manipur confirms that this is one of india's biologically most diverse regions with a large portion of endemic species.

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Supplementary material:

Table 1: Isolated and Identified Trichoderma of Manipur with relationship to the strain found in NCBI and their percentage identity

Sl. No.	Manipur Strain	Accession No. of Manipur Strain	Closely related NCBI Accession No	NCBI Strain	Country of NCBI Strain	Host	Identity
1	T1	JX518889 (*T. harzianum*)	JX4654781	NBAII Th-9	India (Karnataka)	Rhizosphere soil	99
2	T2	JX518890 (*H. intricata*)	JX518890	IBSD-T2	India (Manipur)	Soil	100
3	T8	JX518891 (*H. rufa*)	AB374534	Tv-03	India (Lucknow)	Sugarcane	99
4	T9	JX518892 (*H. rufa*)	JX518892	IBSD-T9	India (Manipur)	Soil	100
5	T10	JX518893 (*T. aureoviride*)	JN811061	UOM-09	Malaysia	Asian Elephant Dung	95
			HM037926	Wxm8	China	River water	95
6	T11	JX518894 (*T. harzianum*)	JX465478	NBAII Th-9	India (Karnataka)	Rhizosphere soil	100
			AF443922	GJS.00-24	USA		99
			AF194011	NR6929	Germany		99
7	T12	JX518895 (*T. harzianum*)	JX465478	NBAII Th-9	India (Karnataka)	Rhizosphere soil	100
			JX262928	NBAII TN-19B	India (Karnataka)	Rhizosphere soil	100
8	T15	JX518896 (*T. atroviride*)	JX500739	AMAAS53	India (Mizoram)		99
			JQ580979	SAKUN 15-10	India (Tmil nadu)	Tea plantation soil	99
9	T17	JX518897 (*T. harzianum*)	JX465478	NBAII Th-9	India (Karnataka)	Rhizosphere soil	99
			JX262928	NBAII TN-19B	India (Karnataka)	Rhizosphere soil	99
10	T20	JX518898 (*H. rufa*)	GU067751	F53	Finland (Sweden)	Stump (*Picea abies*)	96
11	T21	JX465711 (*T. album*)	JX465711	IBSD-T21	Manipur (Imphal)	Soil	100
12	T22	JX465709 (*T. atroviride*)	JQ580979	SAKUN15-10	India (Tamil nadu)	Tea plantation soil	99
			HQ259983	T6	India (New Delhi)	Rhizosphere soil	99
13	T34	JX465706 (*T. inhamatum*)	JX465706	IBSD-T34	India (Manipur)	Soil	100
14	T35	JX518899 (*T. aureoviride*)	JX077037	PYL12-6	China	Wetland sediment	99
			JQ040330	XZNM5001	China		99
15	T36	JX518900 (*T. aureoviride*)	JX077037	PYL12-6	China	Wetland sediment	99
			JQ040330	XZNM5001	China		99
16	T37	JX465705 (*T. inhamatum*)	AM889227	RRLF-162	Jammu and Kashmir		94
17	T38	JX465703 (*T. inhamatum*)	AM889227	RRLF-162	India (Jammu & Kashmir)		94
18	T39	JX518901 (*T. asperellum*)	GU589845	Th-CARI3	India (Andaman		99
			JN108915	T16	&Nicobar)	Rhizosphere soil	98
			FJ004799	TNC52	India (New Delhi)	Cacao plantation	98
			FJ412053	CPK2722	Indonesia (Riau)	Coffee plant	99
					Ethiopia	rhizosphere soil	
19	T40	JX518902 (*T. harzianum*)	AF469188	GJS94-26	USA		99
			AF194011	NR6929	Germany		99
			AJ224016	2930	Spain		99
			AJ507133	MA3639	Austria	Soil	
20	T41	JX518903 (*T.amazonicum*)	HM142359	IB95	USA	Endophyte	92
			HM142358	IB50	USA	Endophyte	92
21	T47	JX518904 (*T. harzianum*)	AF443922	GJS	USA		99
			AF194011	NR6929	Germany		99
			AY154949	Ir.112	Iran	Soil	99
			AJ224016	IMI304056	Spain		99
22	T54	JX465708 (*T. harzianum*)	JX465478	NBAII Th-9	India (Karnataka)	Rhizosphere soil	99
			AF194011	NR6929	Germany		99
23	T61	JX465707 (*T. atroviride*)	JQ580979	SAKUN15-10	India (Tamil nadu)	Tea plantation soil	99
24	T62	JX518905 (*T. caribbaeum*)	DQ323436	Dis320c	USA	Stems	98
25	T66	JX518906 (*T. atroviride*)	JQ580979	SAKUN15-10	India (Tamil nadu)	Tea plantation soil	94
26	T68	JX518907 (*T. aureoviride*)	JX077037	PYL12-6	China	Wetland sediment	99
			JQ040330	XZNM5001	China		99
27	T69	JX518908 (*T. aureoviride*)	JX77037	PYL12-6	China	Wetland sediment	100
28	T70	JX518909 (*T. harzianum*)	JX465478	NBAII Th-9	India (Karnataka)	Rhizosphere soil	97
			JX262928	NBAII TN-19B	India (Karnataka)	Rhizosphere soil	97

29	T71	JX518910 (*T. ovalisporum*)	EU280118	DAOM229859	Canada		98
30	T72	JX465701 (*T. aureoviride*)	JX077037	PYL12-6	China	Wetland sediment	100
			JQ040330	XZNM5001	China		100
31	T74	JX518911 (*T. harzianum*)	AF443922	G.J.S.00-24	USA	Rhizosphere soil	96
			AF194011	NR6929	Germany		96
			JX465478	NBAIITh-9	India (Karnataka)		96
32	T75	JX518912 (*T. harzianum*)	AF194011	NR6929	Germany	Soil	99
			AY154949	Ir.112c	Iran		99
33	T77	JX518913 (*T. harzianum*)	JX465478	NBAII Th-9	India	Rhizosphere soil	98
			AF194011	NR6929	Germany		98
34	T78	JX518914 (*H. intricata*)	JX518914	IBSD-T78	India (Manipur)	Soil	100
35	T80	JX518915 (*T. inhamatum*)	GQ426033		India (Andaman & Nicobar Island)		97
36	T81	JX518916 (*T. tomentosum*)	FJ487916	ZH3-E1	China	Mangrove	99
37	T83	JX518917 (*T. atroviride*)	JX500739	AMAAS53	India (Mizoram)	Endophyte	98
			JQ580976	SAVAL30-08	India (Tamil nadu)	Tea plantation soil	98
38	T85	JX518918 (*T. inhamatum*)	HQ839779	F22	China	Rhizosphere soil	99
39	T86	JX518919 (*T. harzianum*)	JX262928	NBAII TN-19B	India (Karnataka)	Rhizosphere soil	92
40	T88	JX465700 (*T. atroviride*)	JQ580979	E	India (Tamil nadu)	Tea plantation soil	100
			JQ580975	SAVAN20-08	India (Tamil nadu)	Tea plantation soil	100
41	T89	JX518920 (*T. harzianum*)	JX465478	NBAII Th-9	India (Karnataka)	Rhizosphere soil	99
			AF194011	NR6929	Germany		99
			JX465708	IBSD-T54	India (Manipur)	Soil	99
42	T100	JX518924 (*T. harzianum*)	AJ224006	IMI 352940	Spain		99
43	T101	JX518925 (*T. harzianum*)	JX465478	NBAII Th-9	India (Karnataka)	Rhizosphere soil	99
			AF194011	NR6929	Germany		99
44	T105	JX518926 (*T. aureoviride*)	HQ596942	T59	China		99
			HQ596945	T77	China		99
45	T108	JX465710 (*H. nigricans*)	HE649469	TUT46	Saudi Arabia	Soil samples	100
			JN943370	NBRC 31289	USA		100
46	T110	JX518921 (*T. longibrachiatum*)	EU744190	T8-4	China		95
			JX213811	T2	India (Himachal)	Compost	95
			JN108926	T28	India (new Delhi)	Rhizosphere soil	95
47	T112	JX518927 (*T. harzianum*)	JX465478	NBAII Th-9	India (Karnataka)	Rhizosphere soil	100
			AY154949	Ir. 112 C	Iran	Soil	100
			AF194011	NR6929	Germany		100
48	T114	JX518928 (*T. hamatum*)	FJ411990	CPK 2676	Austria	Coffee plant	92
			JX160048	NFL2	UK	rhizosphere soil	92
			JQ040348	HNHK3007	China		92
			JN542526		India(Mao)		92
49	T116	JX518922 (*T. koningiopsis*)	JQ040366	CQSQ4004	China		95
50	T119	JX465702 (*T. aureoviride*)	JX077037	PYL12-6	China	Wetland sediment	100
			JQ040330	XZNM5001	China		100
51	T121	JX518929 (*T. aureoviride*)	JX077037	PYL12-6	China	Wetland sediment	100
			JQ040330	XZNM5001	China		100
52	T137	JX518930 (*T. harzianum*)	JX465478	NBAII Th-9	India	Rhizosphere soil	99
			AF443922	G.J.S. 00-24	USA		99
			AF194011	NR6929	Germany		99
			THU78881	Th1	USA		99
53	T142	JX518931 (*T. harzianum*)	AF443922	G.J.S. 00-24	USA		99
			AF194011	NR6929	Germany		99
			AJ224016	2930	Spain		99
			AF055215	TR112; TR108	New Zealand		99
54	T149	JX518932 (*H. virens*)	GU322025	T79	Vietnam	Forest soil	94
			JX174053	ATCC MYA-4894	USA		94
			JF501655		Italy		94
			JQ040400	T21 XZNM2007	China		94
55	T153	JX518923 (*T. erinaceum*)	GU479425	NBAII(N)TN	India (Karnataka)	Soil	88
			GQ249874	7-Te102/08	India (Rajasthan)		87
			DQ109534	DIS 7	USA	*Theobroma cacao*	87

56	T155	JX518933 (*H. virens*)	JX174053	ATCC MYA-4894	USA		92
			JF501655	T21	Italy		92
			JQ040400	XZNM2007	China		92
			HQ608079	TR039	Brazil		92
			HQ229950	T32	Taiwan	*Trachymyrmex septentrionalis* nest	92
57	T158	JX518934 (*H. rufa*)	JX518934	IBSD-T158	India (Manipur)	Soil	100
58	T161	JX465699 (*H. virens*)	JX174053	ATCC MYA-4894	USA		99
			JX173848	SZMC 20779	Hungary		99
			JQ040400	XZNM2007	China		99
59	T162	JX518935 (*T. petersenii*)	Z95923	tam35 (T)"	Germany		99
			DQ323426	GJS 98-139	USA	Decorticated wood	99
60	T168	JX465704 (*T. album*)	JX465711	IBSD-T21	India (Manipur)	Soil	95
61	T174	JX518936 (*T. gamsii*)	JX406518	CS11784	China	Roots of host grown in the field	99
			JX173876	SZMC 20783	Hungary		99
			HM534658	KUC1747	Korea		99
			GQ351597	ICC080	Spain		99
62	T176	JX518937 (*T. spirale*)	JQ040384	HNZZ1007	China	Stem endophyte	100
			EU718084	DMC 793a	Germany		100
			FJ442667	DIS 293F	USA		100
63	T179	JX518938 (*T. piluliferum*)	HM037966	wxm46	China	Freshwater	99
64	T184	JX518939 (*T. koningiopsis*)	JQ040366	CQSQ4004	China		100
65	T186	JX518940 (*T. atroviride*)	JF502439	1-24	China	*Lindera glauca*	99
			JN628163	Z116	China	leaf litters in a mid-subtrophic evergreen broad-leaved forest	99