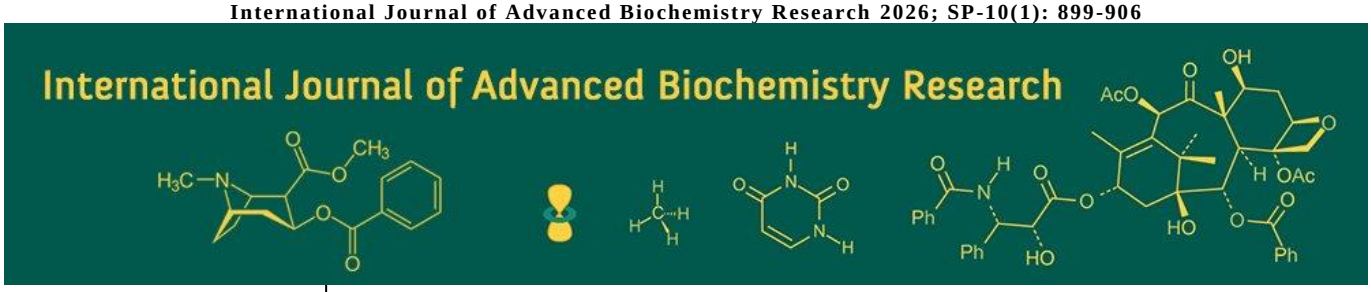

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Molecular diversity assessment of *Trichoderma* isolates collected from copper mining areas of Uttarakhand

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Abstract
Forty nine *Trichoderma* isolates purified from the soil samples collected from the copper mining locations of Uttarakhand on Trichoderma specific medium. The isolates were classified using the primers ITS 1 and ITS 4. There was no inter or intra species length diversity was noticed, indicating the highly conserved nature of 5.8S r RNA gene in the genome. Similar band pattern of all the isolates confirmed that the isolates were belonged to the genus *Trichoderma.* Molecular diversity of 49 *Trichoderma* isolates was performed using eight ISSR markers. Based on UPGMA cluster analysis, 49 *Trichoderma* isolates were grouped into cluster I and II at 30 per cent similarity. Cluster-I comprise of 28 isolates, was further subdivided into three sub clusters, each of which was further subdivided. Cluster II comprising of 21 isolates, further subdivide into two sub clusters 2a, 2b. Sub cluster 2a was subdivided into two clusters 2a', 2b'; 2a' comprised of 15 isolates while 2b' contain 3 isolates. Sub cluster 2b had only one group of three isolates. Among all the isolates maximum similarity (minimum diversity) was found between TCMS 46 and TCMS 71 (0.67), while minimum genetic similarity (highest diversity) was observed between TCMS 1 and TCMS 79 (0.294). Results showed high diversity among the isolates of *Trichoderma.* The potential strains may further be tested for antagonism and may use for sustainable crop disease management.

Keywords: Biological control, taxonomy, molecular markers, diversity, sustainability

Introduction
Trichoderma sp., are prevalent in all kinds of soil and habitats. It belongs to Deuteromycotina (sub-division), Hyphomycetes (Class), Moniliales (Order), and Moniliaceae (Family). This genus has hundreds of species, but important ones are *T. harzianum*, *T. atroviride*, *T. hamatum*, *T. viride*, *T. asperellum*, *T. koningii*, and *T. virens* which are mainly acts as bio-control agents against plant diseases. *Trichoderma* spp. enhances plant growth and development (Mukhopadhyay and Mukherjee, 1996; Hjeljord and Tronsmo, 1998; Singh *et al*., 2006) [19, 28]. These species alone or in combination with compatible chemical fungicides have been used for to manage several diseases like root rots, wilts, damping off, white rot, collar rots etc. in a wide variety of crops (Samuels, 1996).
Antagonism potential of *Trichoderma* spp. depends on strain of *Trichoderma*, genera of pathogenic fungi, environmental conditions, soil type, soil pH, soil nutrient availability (Harman, 2000) [18]. Keeping the importance of *Trichoderma* species and its diversity, a research study was carried out on "Molecular diversity assessment of *Trichoderma* isolates collected from copper mining areas of Uttarakhand".

Review of Literature
Trichoderma colonies typically grow quickly, smooth and watery white. As they grow, they become floccose or tightly tufted and change colour to different hues of green. Hyaline, septate, highly branched, smooth-walled hyphae make up the mycelium. The phialospores are hyaline or yellowish green to dark green, sub-globose, and have smooth or slightly rough walls. (Bisset, 1991a) [4]. Persoon was the first to establish the genus *Trichoderma* (Cook and Baker, 1983; Bisset, 1991a) [9, 4]. Reports revealed that they commonly produce asexual spores (conidia), the genus *Trichoderma* has historically been categorized under Fungi Imperfectii

(Singh et al., 2006) [28]. Many of the species have been reclassified as belonging to the genus *Hypocrea* of the class *Ascomycetes* due to recent developments in molecular taxonomy (Gams and Bisset, 1998; Druzhinina *et al*., 2005) [14, 11]. Under *Ascomycotina*, teleomorph name of *Trichoderma* is *Hypocrea* Fr. (Rifai and Webster, 1966a) [29].

Rifai (1969) outlined the speciation concept with in genus *Trichoderma* and given nine species aggregates. Samuels *et al.,* found that Rifai was unable to define limits of individual biological species. Bisset (1992) [5] elevated Rifai's species aggregates to species level and recognized two to several species within each of five sections of the genus. *Trichoderma* strains were isolated using serial dilution and pour plate technique on *Trichoderma* specific medium (TSM) (Gupta *et al.,* 2016) [16] and further classified on the morphological characteristics and molecular characterization. Currently, speciation within aggregates and the relationship between anamorphs and teliomorphs are resolved using biochemical and molecular data (Kuhls et al., 1997; Kindermann et al., 1998; Samuels et al., 1998) [25, 22].

The first step in comprehending the population structure has been to assess the genetic diversity among microbial isolates. Morphology by itself has not produced a satisfactory characterization for *Trichoderma* (Samuels, 1996). Researchers are attempting to resolve the ambiguity using information obtained from nucleic acid analysis. reclassified some *Trichoderma* aggregate species using DNA finger printing. Patent strains of *T. harzianum* were successfully identified using RAPD fingerprinting. Several researchers employed internal transcribed spacer (ITS) sequences, RAPD, and PCR-fingerprinting to characterize various strains of *Trichoderma* spp. (Lieckfeldt et al., 1999) [26].

For identifying microbial species and evaluating genetic variation within collections and populations, molecular techniques have proven to be dependable and excellent tools (Sundravadana *et al.,* 2011) [30]. The taxonomic usage of von Arx and Bissett was supported by the results of rDNA analysis (28S rDNA) and its internal transcribed spacer (ITS) sequences, which demonstrated unequivocally that *T. virens* is phylogenetically different from the type species of *Gliocladium penicillioides*, and is close to *T. harzianum* (Samuels, 1996). Based on morphological characteristics, *T. reesei* and *T. longibrachiatum* were regarded as synonyms in the same study. The phylogenetic relationships between the aforementioned species were examined by Kuhls et al. (1997) [25] using teleomorph groups and rDNA internal transcribed spacer sequences.

Turner found that *T. longibrachiatum* and *T. citrinoviride* overlapped across geographic ranges using ribotyping of ITS-1 and ITS-2 regions of various isolates of *T. longibrachiatum* from various biogeographic regions. *T. longibrachiatum* is common in Africa and India but not in Central Asia, whereas *T. citrinoviride* is common in southeast Asia and not vice versa. Gams and Meyer (1998) [15] redescribed *T. harzianum* after it was neotypified by an isolate from the type specimens from the concerned region/s. Dodd *et al.* (2002) [10] demonstrated that *H. pulvinata* and *H. sulphurea* form two separate subclades of a strongly supported but phylogenetically unresolved clade using the ITS1-5.8S-ITS2 rDNA (ITS) region. The phylogenetic indistinguishability of sects. *Hypocreanum* and *Pachybasium* was not concluded by these authors. The conclusion of Kubicek *et al.* (2002) [24] that section *Pachybasium* is paraphyletic was corroborated by the findings of Dodd *et al.* (2002) [10] and Chaverri *et al.* (2003) [8]. To determine the phylogenetic boundaries of *Trichoderma* sect. *Hypocreanum*, the seven species are compared to a subset of species examined by Overton *et al.* (2006) [31].

According to Samuels *et al.* (2002) [10], PCR amplification of the 5.8S rRNA gene's ITS region produced an ITS fragment that was 600-650 bp long in every isolate of *Trichoderma*. ITS length diversity was not found within or between species. This is because the 5.8s rRNA gene is known to be highly conserved at the genus level, which only served to confirm that each isolate belonged to a single genus. Using RAPD and ITS-PCR, Chakraborty et al. (2010) [6] investigated 19 isolates of *T. viride* and *T. harzianum* from rhizosphere soil of plantation crops, forest soil, and agricultural fields in the North Bengal region. Six random primers were used to examine the genetic relatedness between eight isolates of *T. harzianum* and eleven isolates of *T. viride*. The isolates' genetic diversity was revealed by RAPD profiles, which formed eight clusters. Dendrogram analysis showed that the similarity coefficient varied between 0.67 and 0.95. A DNA oligonucleotide barcode was created by Druzhinina *et al.* (2005) [11] as a quick way to identify *Hypocrea* and *Trichoderma* species. The technique is integrated into TrichOKey v. 1.0, an application with an intuitive web interface built on a collection of brief, nearly constant, and species-specific oligonucleotide sequences from the ITS 1 and 2 loci. A genus-specific primer pair that amplifies the ITS 1, 5.8S rDNA, and ITS2 regions of *Trichoderma* species from all clades described was created and tested by Hagn *et al.* (2007) [17].

Inter simple sequence repeats (ISSR)

Another useful technique for characterizing genetic variability is inter-simple sequence repeats (ISSR). Compared to RAPD, there is a higher chance of discovering polymorphism because the evolutionary rate within ISSR is significantly higher than that of other DNA types (Charlesworth *et al.,* 1994) [7]. Only tiny amounts of DNA are needed for direct PCR and gel electrophoresis analysis when using the microsatellite-primed polymerase chain reaction (MP-PCR), which creates DNA fingerprint profiles using single primers (Ellegren, 2004) [13]. Its high reproducibility, ease of scoring, high throughput, co-dominance, and high polymorphism of band profiles even among related species and genera make it a straight forward and effective fingerprinting tool when compared to other marker techniques like AFLP, RAPD, and RFLP (Powell *et al.,* 1996; Yamamoto *et al.,* 2001) [32, 33]. Additionally, SSRs are useful for analyzing large populations in various laboratories because they are amenable to PCR amplification and relatively simple to score, which is not possible with RAPDs or AFLPs (Zane *et al.,* 2002) [34].

A primer made up of a microsatellite sequence and a anchor sequence, which is not arbitrary, but rather intended to anneal to nucleotides flanking the microsatellite repeat is used in the random amplified microsatellites (RAMS) DNA technique. Microsatellite primer PCR (MP-PCR) and inter simple sequence repeat (ISSR) are other names for RAMS (Wunsch and Hormaza, 2002) [35]. Fungi can be identified at the genus and species level using this molecular method. In crop breeding programs, marker-assisted selection to

increase disease resistance is commonly facilitated by the use of simple sequence repeats markers. For intra-species research, it is an easy, fast, accurate, and efficient method (Billotte, 2001) [3]. Using RAPD, identified 15 strains of *T. harzianum* infecting for edible mushrooms in the United States and England. These strains were referred to as "*T. harzianum* group 4" and showed a high degree of homogeneity. The isolates of *T. harzianum* from group-4 differed from those of group-2, the causative agent of the green mold epidemic in industrial mushrooms in England, according to a comparison of their molecular data. In order to differentiate *Sinocalycanthus chinensis* from its closely related species, Qian *et al.* (2006) [36] employed the ISSR method to identify particular fragments among sample species and converted the ISSR fragment into a SCAR marker. Using a PCR-based method, Hongyan *et al.* (2008) [20] developed a straight-forward and dependable strain diagnostic system for *Flammulina velutipes*, the most common edible mushroom.

Materials and Methods

Isolation, purification, and maintenance Trichoderma spp.

Soil samples were collected in various agricultural settings from Uttarakhand's copper mining districts, including Nainital, Bageshwar, and Tehri Garhwal. Soil sampling was conducted at accessible locations of copper mining sites. Rhizospheric soil was collected and generally healthy plants were chosen from a location's standing crop. Samples of soil were allowed to air dry for four hours. The serial dilution technique was used for isolation. *Trichoderma* was isolated using Trichoderma selective medium (Elad *et al.*, 1981) [12]. The pour plate method of was used to purify the *Trichoderma* isolates. A Petri dish seeded with Trichoderma Specific Medium was inoculated with the small mycelial disc. The single new colony's growing front was used for subculturing. In the end, the culture was preserved and utilized for further research. Based on the location and soil sample serial number, 49 isolates of *Trichoderma* were identified as Trichoderma from Copper Mining Site (TCMS series).

Trichoderma isolates' molecular characterization using ITS and ISSR markers

DNA extraction (CTAB method) from mycelium

With a small modification, polyvinyl pyrrolidone (PVP) was removed from the process because fungi do not contain interfering amounts of phenolic compounds. The CTAB method is based on the technique used to isolate genomic DNA from the fungus. In order to effectively disrupt the cell wall and cell membrane, liquid nitrogen was added to the mycelial mat during grinding, or cover slips were sterilized after being dipped in ethanol.

Preparation of mycelium

The isolates were cultivated in potato dextrose broth (PDB). Each of the 250 ml conical flasks holding 100 ml of PDB was inoculated with one milliliter of the isolates' spore suspension. The flasks were incubated at 25°C in a shaker with 120 rpm. A vacuum pump was used to filter the liquid culture after 72 hours, and the fungal mycelium was collected on the filter paper in a Buchner funnel. After being cleaned with distilled water, the mycelium was frozen and kept at -20°C until it was needed for extraction.

Extraction of DNA

1. In a mortar and pestle, one gram of recently harvested mycelium was ground into an extremely fine powder using liquid nitrogen.
2. Ten milliliters of DNA extraction buffer was used to suspend the powder.
3. One milliliter of 10% SDS was added following adequate shaking.
4. For one hour, the mixture was gently shaken at 37°C.
5. After adding 1.5 ml of 5 M NaCl, the mixture was thoroughly mixed.
6. After adding and thoroughly mixing 1.25 ml of CTAB/NaCl solution, the mixture was incubated for 20 minutes at 65°C in an incubator shaker set at 60 revolutions per minute.
7. To extract DNA, an equal volume of Chloroform: Isoamyl Alcohol (24:1, v/v) was added, thoroughly mixed, and centrifuged at 10000 rpm for 12 minutes at 10°C.
8. Aqueous viscous supernatant was removed to a fresh tube and precipitated with 0.6 volumes of ice-cold isopropanol and 0.1 volume sodium acetate and left overnight in the freezer at -20°C.
9. The mixture was centrifuged at 10000 rpm for 10 min at 10°C.
10. Pellet was washed with 70% ethanol and then dried completely.
11. After that, the pellet was dissolved in the TE buffer.

DNA Quantification

Using a UV-VIS spectrophotometer (*Spectronic Thermolabsystem*), the absorbance of DNA samples dissolved in TE buffer was measured against TE buffer as a blank. The following formula was used to determine the concentration of DNA based on optical density:

$$\text{Concentration of DNA in } \mu g/\mu l = \frac{OD_{260} \text{ x } 50 \text{ x Dilution factor}}{1000}$$

PCR amplification

PCR reaction mix:

Components and concentration	Final concentration	Single tube (µl)
1. template DNA (50 ng/ µl)	50 ng / µl	1
2.dNTPs (10 mM each)	800 µM	2
3.Taq DNA polymerase (3U / µl)	1.5 U	0.3
4.Reaction buffer (10X)	1X	2
5. Primer (20 ng / µl)	-	1
6.$MgCl_2$ (0.8 µl)	(0.8 µl)	0.8
7. Deionized water	18	12.9
Total		20

Confirmation of Trichoderma by using ITS markers

The two primers, ITS-1 and ITS-4, which were created based on conserved regions of the eukaryotic rRNA gene, were used to amplify the internal transcribed spacer (ITS) regions of the rDNA repeat from the 3' end of the 18S gene and the 5' end of the 28S gene (White *et al.*, 1990) [37]. A 50.0 µl mixture comprising 50.0 mM KCl, 20.0 mM Tris HCl (pH 8.4), 2.0 mM $MgCl_2$, 200.0 µM of each of the four deoxynucleotide triphosphates (dNTPs), 0.2M of each primer, 40g/µl of template, and 2.5 U of Taq polymerase was used for the PCR-amplification reactions. The cycle parameters included an initial denaturation at 94°C for five minutes, forty cycles of denaturation at 94°C for one minute, primer annealing at 55°C for two minutes, primer extension at 72°C for three minutes, and a final extension at 72°C for ten minutes. The amplified products were separated on a 2.0% agarose gel in TAE buffer, pre-stained with 1.0 µg/ml ethidium bromide, and electrophoresed in TAE buffer for three hours at 60 volts. A single Kb + ladder (MBI, Fermentas) served as the marker. The gel was examined under ultraviolet light in a trans-illuminator.

Molecular Characterization of Trichoderma Isolates using ISSR markers

The isolates' genomic DNA was amplified using eight primers at various annealing temperatures. The eight anchored ISSR primers, 5'(AG)8AT3', 5'(AG)8AC3', 5'(GA)8AC3', 5'(GA)9T3', 5'(GA)9AC3', 5'BDB(GA)8 T3', 5'YT(CA)7T3', and 5'YC(TG)7T3', provided adequate amplification and band resolution. 25.0 ng of genomic DNA, 2.5 mM MgCl2, 1 U Taq DNA polymerase, 1 X PCR buffer without $MgCl_2$, 1.0 µM ISSR primer, and 0.2 mM dNTP mix were used for the PCR amplification. The volume was increased to 25.0 microliters. In a Perkin Elmer Gene-Amp 9600 thermocycler, PCR reactions were conducted under the following conditions: denaturation at 94°C for 7 minutes; 30 cycles of denaturation at 94°C for 30 seconds; primer annealing at temperature specific to each primer for 45 seconds; primer extension at 72°C for 2 minutes; and final extension step at 72°C for 7 minutes. Random amplified polymorphic DNA (RAPD) and ISSR PCR products were separated on 1.4% agarose gel in 1 X TBE buffer pre-stained with ethidium bromide (1.0 µg/ml). Electrophoresis was performed at 90 volts for 1.5 hours, then 70 volts for 2.0 hours, and the results were seen under UV light using a UV-Transilluminator. A Gel documentation system was used to take pictures of the gel.

Primer No.	Sequence (5'-3')	Annealing temp (°C)
1	$(AG)_8$ AC	52
2	$(AG)_8$ AT	52
3	$(GA)_8$ AC	51
4	$(GA)_9$ T	53
5	$(GA)_9$ AC	53
6	BDB $(GA)_8$ T	52
7	YT $(CA)_7$ T	55
8	YC $(TG)_7$ T	52

Data Analysis

The ISSR amplification products were given a score of "1" for presence, "0" for absence, and "9" for missing data. By computing the Jaccard's similarity coefficient for pairwise comparisons based on the percentage of shared bands generated by the primers, the genetic relationships between isolates were assessed. The similarity matrix was subjected to cluster analysis by unweighted pair group method for arithmetic mean (UPGMA) and a dendrogram was generated. The computations were performed using the program NTSYS - PC version (Rohlf, 1997).

Principal component analysis was performed on the Jaccard's similarity matrix. The multidimensional solution of the observed relationships is used in this coordination method. PCA reduces complicated relationships to a function of fewer, more straightforward variables. The distances (or similarities) between the operational taxonomic units are used in this method to create the data matrix. The (principal) axes through the hyper-ellipsoid are computed in order to make the description of these "clouds" of points simpler. The largest, second-largest, etc. amount of variation is explained by the successive principal axes, which stand for the first major axis, the second axis, etc.

Results and Discussion

Molecular identification of various Trichoderma spp. isolates using ITS markers

The study's goal was to precisely identify 49 isolates of *Trichoderma* spp. that were obtained from rhizosphere soil that was gathered from various Uttarakhand locations. This study made use of all 49 DNA samples from *Trichoderma* isolates. The internal transcribed spacer region of ribosomal DNA, which includes the 5.8S rRNA gene and both ITS-1 and ITS-4 regions, was amplified using the polymerase chain reaction (PCR) primers ITS-1 and ITS-4 created by White *et al.* (1990) [37]. In every isolate of *Trichoderma*, PCR amplification of the 5.8S rRNA gene's ITS region produced an ITS fragment that was 600 base pairs (bp) long. ITS length diversity was not found within or between species. This may be because the 5.8s rRNA gene, which is known to be highly conserved at the genus level, only verified that every isolate belonged to the single genus *Trichoderma*, as shown in plate 1.

There are numerous reports on the use of ITS markers to identify *Trichoderma* isolates. The method is dependable because the ITS region is the most conserved part of the fungal genome. Samuls *et al.* (2002) reported similar findings: in every isolate of *Trichoderma*, PCR amplification of the ITS region of the 5.8S rRNA gene produced an ITS fragment of 600 bp length. Several researchers used internal transcribed spacer (ITS) sequences, RAPD, and PCR-fingerprinting to characterize various strains of *Trichoderma* spp. (Arisan-Atac *et al.*, 1995; Zimand *et al.*, 1996; Kindermann *et al.*, *1998*; Ospina Giraldo *et al.*, 1999; Lieckfeldt *et al.*, *1999*) [2, 27, 22, 26]. For identifying microbial species and evaluating genetic variation within collections and populations, molecular techniques have proven to be dependable and excellent tools (Sundravadana *et al.*, 2011) [30].

ITS and intergenic region domains, on the other hand, are far more variable, making them more valuable for phylogenetic analyses (White *et al.*, 1990) [37]. Kuhls *et al.* (1997) [25] examined the phylogenetic relationships between various *Trichoderma* species using teleomorph groups and rDNA internal transcribed spacer sequences. Dodd *et al.* (2002) [10] demonstrated that *Hypocrecea pulvinata* and *Hypocrecea sulphurea* form two separate subclades of a strongly supported but phylogenetically unresolved clade using the ITS1-5.8S-ITS2 rDNA (ITS) region. To determine the phylogenetic boundaries of the *Trichoderma* sect, the seven species are compared to a subset of species examined by Overton *et al.* (2006) [31]. A genus-specific primer pair that amplifies the ITS-1, 5.8S rDNA, and ITS-2 regions of *Trichoderma* species from all clades described was created and tested by Hagn *et al.* (2007) [17]. Regardless of

cultivability, this set can be used for environmental sample detection, monitoring, and quantification. For the identification of *Trichoderma* spp., multigene sequencing based on the ITS region, TEF-1-α, and RPB-2 genes was found to be highly appropriate (Hoyos-Carvajal *et al.*, 2009) [21]. *Trichoderma guizhouense* was identified by ITS, TEF-1-α, and RPB-2 sequencing (Korkom and Yıldız, 2024) [23]. Using molecular techniques, Sana Sharma *et al.* (2025) [38] identified the locally accessible *Trichoderma* species by focusing on the internal transcribed spacer (ITS) region, translation elongation factor 1-alpha (TEF-1-α), and RNA polymerase B subunit II (RPB-2).

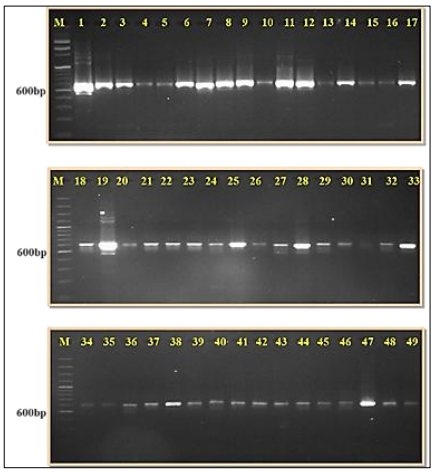

Plate 1: DNA amplification of TCMS series (1-49) *Trichoderma* spp. with ITS-1 and ITS-4 primers.

Index: 1: TCMS 1, 2: TCMS 2, 3: TCMS 3, 4: TCMS 4, 5: TCMS 5, 6: TCMS 6, 7: TCMS 7, 8: TCMS 9, 9: TCMS 10, 10: TCMS 11, 11: TCMS 12, 12: TCMS 13, 13: TCMS 14a, 14: TCMS 14b, 15: TCMS 15, 16: TCMS 16, 17: TCMS 17, 18: TCMS 20, 19: TCMS 22, 20: TCMS 24, 21: TCMS 25, 22: TCMS 26, 23: TCMS 31, 24: TCMS 32, 25: TCMS 34, 26: TCMS 35, 27: TCMS 36, 28: TCMS 37, 29: TCMS 43, 30: TCMS 46, 31: TCMS 51, 32: TCMS 60, 33: TCMS 62, 34: TCMS 64, 35: TCMS 65, 36: TCMS 66, 37: TCMS 71, 38: TCMS 72, 39: TCMS 73, 40: TCMS 74, 41: TCMS 77, 42: TCMS 79, 43: TCMS 82, 44: TCMS 85, 45: TCMS 90, 46: TCMS 92, 47: TCMS 93, 48: TCMS 95, 49: TCMS 103.

Analysis of molecular variability of different isolates of Trichoderma spp. using ISSR marker

Table 1, Figure 1, and Plates 2.1 and 2.2 display the molecular variability results. The DNA of *Trichoderma* isolates could be amplified using all of the primers. A total of 2068 bands were produced by amplifying genomic DNA from 49 isolates of *Trichoderma* spp. using ISSR primers; 132 of these bands were discovered to be polymorphic. Six (primer 6) to twenty-two (primer 7) bands were amplified. The amplicon size was between 1500 and 3000 base pairs. The average number of bands and polymorphic bands was 16.5 and 17.13, respectively. With an average polymorphism of 89.58% across all isolates, the percentage of polymorphism varied from 16.66% (primer 6) to 100% (primer 1 to 8 except 6).

Table 1: ISSR loci generated by eight primers in 49 *Trichoderma* isolates

Primer Code	Total no. of loci	Amplified product range (bp)	ISSR loci				PIC
			Mono bands	Poly bands	% Polymorphism	Unique bands	
Primer 1	21	150-2000bp	0	21	100	0	0.860
Primer 2	19	150-2000bp	0	19	100	0	0.877
Primer 3	19	200-3000bp	0	19	100	0	0.945
Primer 4	15	200-2000bp	0	15	100	0	0.738
Primer 5	18	170-3000bp	0	18	100	0	0.871
Primer 6	6	200-900bp	5	1	16.66	0	0.000
Primer 7	22	160-2000bp	0	22	100	0	0.922
Primer 8	17	250-2000bp	0	17	100	0	0.863

- **Primer 1:** For this primer, 21 ISSR loci with 100% polymorphism were scored. None were distinct and monomorphic. The size of the amplicon varied from 0.150 to 2.00 kb. The primer's PIC value was 0.860.
- **Primer 2:** The primer with 100% polymorphism scored 19 ISSR loci in total. None were distinct and monomorphic. The size of the amplicon varied from 0.150 to 2.00 kb. The primer's PIC value was 0.877.
- **Primer 3:** For this primer, 19 ISSR loci with 100% polymorphism were scored. None were distinct and monomorphic. The size of the amplicon varied from 0.200 to 3.00 kb. The primer's PIC value was 0.945.
- **Primer 4:** The primer with 100% polymorphism scored 15 ISSR loci in total. None were distinct and monomorphic. The size of the amplicon varied from 0.300 to 2.00 kb. The primer's PIC value was 0.738.
- **Primer 5:** For this primer, 18 ISSR loci with 100% polymorphism were scored. None were distinct and monomorphic. The size of the amplicon varied from 0.170 to 3.00 kb. The primer's PIC value was 0.871.
- **Primer 6:** With five monomorphic and one polymorphic band, a total of six ISSR loci were scored for this primer, yielding only 16.66 percent polymorphism. The size of the amplicon varied from 0.200 to 0.900 kb. The primer's PIC value was 0.00.
- **Primer 7:** The primer with 100% polymorphism scored 22 ISSR loci in total. None were distinct and monomorphic. The size of the amplicon varied from 0.160 to 2.00 kb. The primer's PIC value was 0.922.
- **Primer 8:** For this primer, 17 ISSR loci with 100% polymorphism were scored. None were distinct and monomorphic. The size of the amplicon varied from 0.250 to 2.00 kb. The primer's PIC value was 0.863.

Genetic variability based on ISSR markers

Data recorded from 49 isolates of *Trichoderma spp.* with eight ISSR primers was used to generate similarity

coefficients Dendrogram shows the pair wise similarities among 49 fungal isolates. Jaccard's similarity coefficient between different isolates obtained from different crops ranged from 0.27-0.70. Among all the isolates highest genetic similarity (minimum diversity) was observed between isolates TCMS 46 and TCMS 71 (0.67), while minimum genetic similarity (highest diversity) was observed between TCMS 1 and TCMS 79 (0.294). A UPGMA (unweighted pair group method with arithmetic mean) dendrogram was created using the NTSYS program, and cluster analysis was carried out using the jaccard similarity coefficient of ISSR marker data produced on isolates of *Trichoderma*. Cluster analysis grouped 49 isolates into two main clusters *viz*., cluster I and cluster II at 30% similarity level.

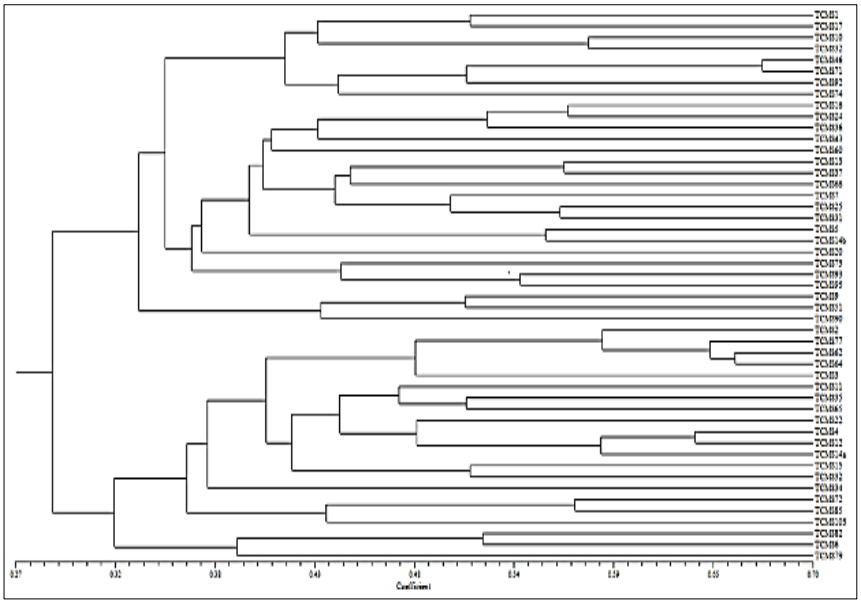

Fig 1: Dendrogram depicting diversity of the 49 Trichoderma cultures (UPGMA method) based on ISSR marker. The scale at the bottom gives Jaccard' coefficient of genetic similarity.

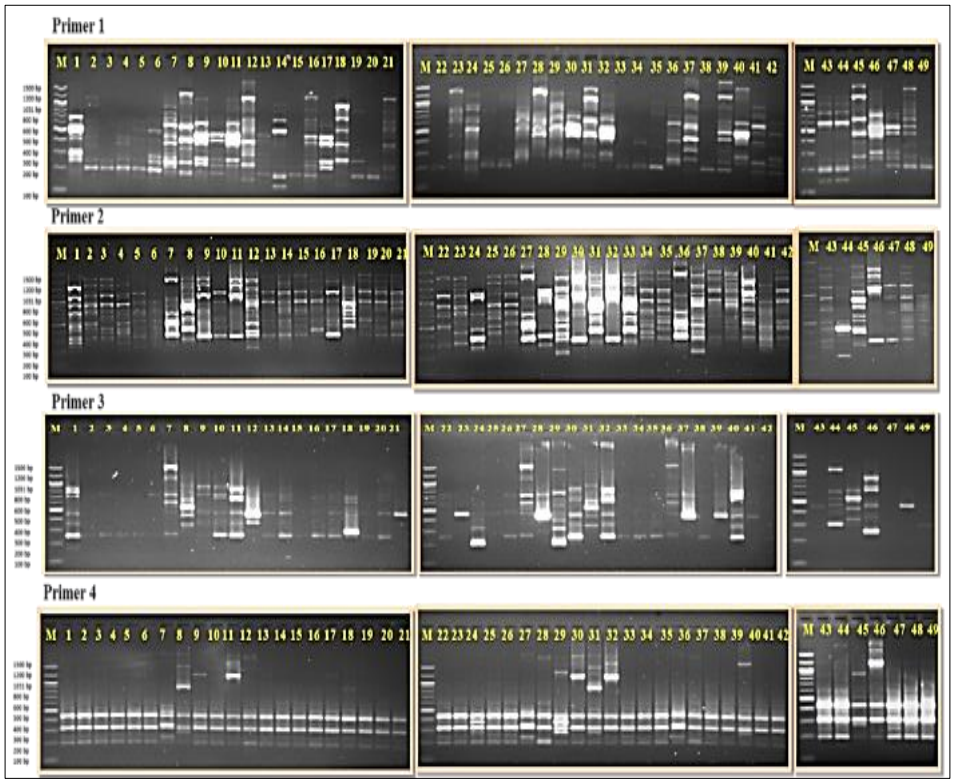

Plate 2.1: DNA amplification of 49 TCMS series *Trichoderma* isolates with ISSR primer 1-4

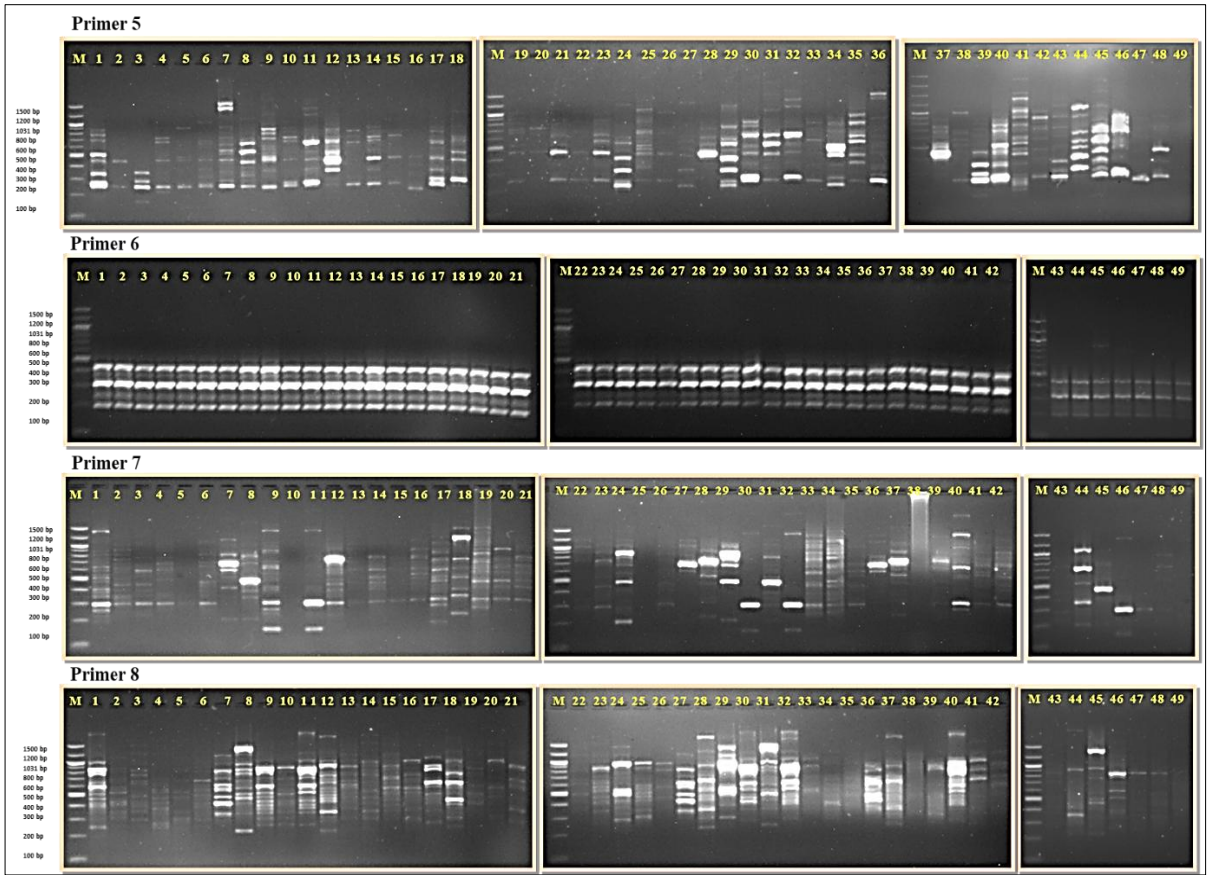

Plate 2.2: DNA amplification of 49 TCMS series *Trichoderma* isolates with ISSR primer 5-8

Cluster I comprising of 28 isolates, was further subdivide into 3 subclusters, each of which was further subdivided. Maximum diversity was found between TCMS 90 and TCMS 1 while minimum between TCMS 46 and TCMS 71. Cluster II comprised of 21 isolates and was further subdivided into 2 subclusters 2a and 2b. Subcluster 2a was divided into two subsubclusters 2a', 2b'and 2a' comprising of 15 isolates while 2b' contained three isolates. Subcluster 2b had only three isolates. Results revealed high diversity among *Trichoderma* isolates.

Inter-simple sequence repeats (ISSR) are another helpful method for describing genetic variability, because of its high reproducibility, ease of scoring, high throughput, codominance, and high polymorphism of band profiles even among related species and genera, it is a fairly simple and effective fingerprinting tool when compared to other marker techniques like AFLP, RAPD, and RFLP (Powell *et al.*, 1996; Yamamoto *et al.*, 2001) [32, 33]. Additionally, SSRs are useful for analyzing large populations due to their ease of scoring and suitability for PCR amplification (Zane *et al.*, 2002) [34]. Fungi can be identified at the genus and species level using this molecular method (Wunsch and Hormaza, 2002) [35]. *Sinocalycanthus chinensis* was distinguished from its closely related species by Qian *et al.* (2006) [36] using the ISSR method to identify specific fragments among sample species. The ISSR fragment was then converted into a SCAR marker.

Conclusion

The study unveiled the diversity of *Trichoderma* isolates within the copper mining areas of Uttarakhand. The study also helps in characterization of the *Trichoderma* spp. This study deepens our understanding about the existence of diversity among the Trichoderma isolates and helpful for future research on ecological roles of *Trichoderma* spp. and their role in sustainable agricultural practices. These efforts reveal a plethora of new species, some of which might not have been known before. These recently identified taxa contribute to future developments in this field because they have great potential for a variety of biological control, biotechnological, and bioremediation applications. The molecular techniques in combination with other biochemical and serological methods can be successfully used for the accurate identification of the *Trichoderma* spp. as well as their diversity assessment. The diversity assessment data may further used for the identification of new species or specific characters in the *Trichoderma* spp.

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