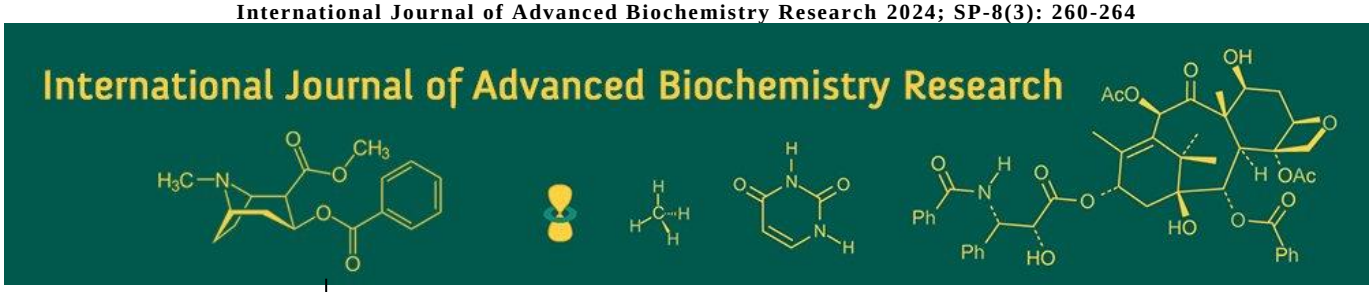


ISSN Print: 2617-4693
ISSN Online: 2617-4707
IJABR 2024; SP-8(3): 260-264
www.biochemjournal.com
Received: 01-12-2023
Accepted: 09-01-2024

Swapnil Srivastava
Department of Agricultural Biotechnology, Sardar Vallabhbhai Patel University of Agriculture and Technology, Meerut, Uttar Pradesh, India

Mukesh Kumar
Department of Agricultural Biotechnology, Sardar Vallabhbhai Patel University of Agriculture and Technology, Meerut, Uttar Pradesh, India

Pushpendra Kumar
Department of Agricultural Biotechnology, Sardar Vallabhbhai Patel University of Agriculture and Technology, Meerut, Uttar Pradesh, India

Shiv Kumar Singh
Department of Genetics and Plant Breeding, Sardar Vallabhbhai Patel University of Agriculture and Technology, Meerut, Uttar Pradesh, India

Aniruddh Yadav
Department of Agricultural Biotechnology, Sardar Vallabhbhai Patel University of Agriculture and Technology, Meerut, Uttar Pradesh, India

Garima Sharma
Department of Agricultural Biotechnology, Sardar Vallabhbhai Patel University of Agriculture and Technology, Meerut, Uttar Pradesh, India

Corresponding Author:
Swapnil Srivastava
Department of Agricultural Biotechnology, Sardar Vallabhbhai Patel University of Agriculture and Technology, Meerut, Uttar Pradesh, India

Genetic diversity analysis of *Trichoderma* isolates using SSR marker

Swapnil Srivastava, Mukesh Kumar, Pushpendra Kumar, Shiv Kumar Singh, Aniruddh Yadav and Garima Sharma

DOI: https://doi.org/10.33545/26174693.2024.v8.i3Sd.719

Abstract
Trichoderma is a genus of fungi in the family Hypocreaceae that is found in all types of soils and is the most common culturable fungus. They occur worldwide and can be easily isolated from soil, plant organic matter and decaying wood etc. It is a well-known aggressive biocontrol agent and has a higher potential for reproduction and colonization. As the diversity and characteristics of *Trichoderma* species are difficult to determine using morphological methods, henceforth molecular tools are crucial. This study utilized Simple Sequence Repeat (SSR) technique to investigate the genetic diversity of *Trichoderma* isolates. In this study, the genetic diversity among fourteen *Trichoderma* isolates (TBT- 01 to TBT- 14) was evaluated using ten SSR markers. Rhizospheric soil samples from various locations of Uttar Pradesh were collected and subjected to serial dilution and plating on a *Trichoderma* Selective Media. The DNA was isolated from the isolates and subjected to PCR amplification using ten SSR primers. The study revealed, a total of fifteen alleles obtained across the ten primers of which thirteen alleles were polymorphic and two monomorphic. The Jaccard's similarity coefficient calculated ranged from 0.429 to 0.929. The dendrogram constructed using the NTSYS software divided the isolates into major and minor clusters. The minor cluster comprised only one isolate TBT- 05 while the major cluster comprised of the other thirteen isolates. The study revealed a significant level of genetic diversity among the isolates.

Keywords: Biocontrol, dendrogram, diversity, microsatellite, polymorphism, SSR, *Trichoderma*

Introduction
Fungal species belonging to the genus *Trichoderma* occur worldwide and can be easily isolated from soil, plant organic matter and decaying wood etc. Numerous species in this genus fit into the category of opportunistic, non-pathogenic plant symbionts. This relates to different *Trichoderma* species' capacity to develop mutualistic endophytic partnerships with various plant types (Bae *et al.,* 2011) [1]. *Trichoderma spp.* is characterized its rapid growth ability to form colonies yielding tufted or postulate, repeatedly branched conidiophores with phialides and hyaline conidia borne in the head**.** Primarily, the mode of biocontrol mechanisms of Trichoderma spp. includes (1) mycoparasitism; (2) production of secondary metabolites; (3) competition for nutrients and space; and (4) induction of plant defence responses (Mukhopadhyay and Kumar, 2020) [12].
Genus *Trichoderma* comprises of a total of 405 reported and recognised species as of July 2021 (Bustamante *et al.,* 2021, Rodríguez *et al.,* 2021) [15]. Some of the agriculturally important species include *T. asperellum, T. atroviride, T. harzianum, and T. viride.* Trichoderma can be found and is widespread throughout several ecological niches, which has played a role in the evolution of the species and genetic and metabolic diversity. Understanding the richness of Trichoderma species in the agricultural rhizosphere is important given their enormous ecological relevance and plant interaction (Jambhulkar *et al.* 2023) [3].
Determining this species' genetic makeup is essential given its importance and impact on food supply. Despite the high relevance of this species, its complexity prevents it from having well-defined taxonomic boundaries. Trichoderma is a complex genus with a great capacity for adapting to a variety of settings, according to research on the genetic and phylogenic diversity of the species conducted across the globe (Danesh *et al.* 2022) [14]. *Trichoderma* species are difficult to differentiate morphologically, so to characterize a new

species, a combination of molecular, morphological, genomic, and physiological studies is used (Badaluddin *et al.*, 2018) [17]. Recently molecular methods are being used extensively for the characterization of *Trichoderma* isolates, which includes techniques of DNA sequencing (Apple and Gordon, 1996), SSR (Simple Sequence Repeats) analysis, RAPD (Random Amplification of Polymorphic DNA) analysis, ITS (Internal Transcribed Sequences) of the ribosomal DNA analysis (rDNA-ITS1), and UP-PCR (Universally Primed Polymerase Chain Reaction), etc. (Cumagun *et al.*, 2012) [4]. The characterization of fungal species using traditional approaches is not as definite as the method of genotyping. Hence, the polyphasic approach, the inclusive outcome of various techniques, such as molecular, morphological, genomic, and physiological analysis, are used to discover the characterization of a new species.

Microsatellites, also referred to as simple sequence repeats (SSRs), consist of tandem repeats of small, 2–6 base pair DNA sequences (Litt and Lutty, 1989) [9] and they have abundant and random distribution throughout the genome. They can be easily automated for high throughput screening, transferred between laboratories, and analyzed using a quick, technically simple, and inexpensive PCR-based test. They also require a small amount of DNA. SSR variation or polymorphism is thought to be caused by polymerase slippage during DNA replication or uneven crossing-over (Levinson and Gutman, 1987) [8].

The following experiment aimed to evaluate the genetic diversity among the *Trichoderma* strains isolated from the rhizospheric soil samples collected from various districts of Uttar Pradesh using ten SSR markers.

Materials and Methods

Trichoderma isolation from soil: Fourteen *Trichoderma* isolates were isolated from the soil samples collected from various districts of Uttar Pradesh by Serial Dilution method to a dilution of 10^{-5} and plating the diluted solution on to Petri plates containing *Trichoderma* Selective Media and incubating it for 5-7 days at 27 ºC in an incubator. The pure colonies obtained were further recultured on Petri plates containing Potato Dextrose Agar medium and incubated at 27 ºC for 5-7 days and the growth rates and colony characteristics were observed. The isolates were further recultured in Potato Dextrose Broth for mycelial growth.

DNA isolation and confirmation of *Trichoderma* isolates

The mycelium obtained in the PDB was filtered, washed using a Whattman filter paper no. 1 and allowed to air dry. The DNA was then extracted using the established CTAB method (Doyle *et al*., 1987) [5] with some modifications and quantified by 0.8% agarose gel electrophoresis. The isolates were confirmed to be *Trichoderma* species by amplification of their Internal Transcribed Spacers (ITS) regions using ITS-1 and ITS-4 markers.

SSR marker amplification

Ten SSR markers (SSR-01, SSR- 06, SSR- 08, SSR- 10, SSR- 11, SSR-13, SSR- 14, SSR-15, SSR- 18, and SSR- 20) were selected for the study. The SSR amplification reactions were conducted in a total volume of 25µL; DNA template (2 µL), GeNei PCR Mastermix (7 µL), Nuclease free water (5 µL), and 1 µL each forward and reverse primers (10 pmol/µL) were mixed to make the reaction mixture. The Prima-Duo thermocycler was programmed with an Initial Denaturation of 94 ºC for 4 minutes, followed by Denaturation of 1 minute at 94 °C for 35 cycles of; Annealing at 43 °C to 55 °C depending on the primer for 1 minute; Extension for 2 minutes at 74 °C, and a final extension for 8 minutes at 74 °C.

Gel Electrophoresis

The PCR amplified products was mixed with loading buffer (1 µ1) containing 0.25% Bromophenol Blue, 40% (w/v) sucrose in water and was analyzed on 2% agarose gel in 1X TBE buffer (for 50X buffer- Tris base- 242gm, Glacial Acetic acid- 57.1 ml, EDTA- 100ml) at 70 V for 45 minutes with a 100 bp DNA ladder as a size marker. The gel was stained with ethidium bromide (2 µg/mL) and visualized under a gel documentation system.

Results and Discussion

Radial Growth

All the fourteen isolates were cultured on PDA plates by inoculating a five mm disc of fresh culture in the centre of Petriplate and incubating at 27 ºC. Radial growth was recorded from 24 hours and thereafter every 48 hours. Fastest The fastest growth was observed in the ate TBT- 01 while the slowest in isolate was TBT- 12. Isolate TBT- 01 covered the whole petri-plate with its growth within 5 days of inoculation while the other isolates nearly covered the plates completely. After 24 hours of incubation, maximum average radial growth of 12.52 mm was recorded in isolate TBT- 01 while the minimum average radial growth of 8.02 mm was recorded in isolate TBT-12. However, after prolonged incubation of 72 and 120 hours, the growth pattern remained same with isolate TBT- 01 with a maximum radial growth of 80.00 mm and TBT- 12 with the minimum of 72.82mm.

Table 1: Colony Colour and Reverse colour

Isolates	Colony Colour	Reverse colour	Growth rate (cm day^{-1})
TBT-01	Dark green	Creamish	1.34 ± 0.11
TBT-02	Dark green	Creamish	1.32 ± 0.14
TBT-03	White to Green	Light yellow	1.32 ± 0.15
TBT-04	Dark green	Creamish	1.33 ± 0.13
TBT-05	Light green	Creamish	1.30 ± 0.15
TBT-06	Yellowish green	Light yellow	1.32 ± 0.09
TBT-07	Dark green	Creamish	1.26 ± 0.15
TBT-08	Dark green	Creamish	1.34 ± 0.12
TBT-09	Light green	Light yellow	1.33 ± 0.08
TBT-10	Yellow to green	Light yellow	1.31 ± 0.16
TBT-11	Dark green	Colourless	1.23 ± 0.06
TBT-12	Yellow to green	Light yellow	1.03 ± 0.14
TBT-13	Dark green	Creamish	1.32 ± 0.1
TBT-14	Light green	Light yellow	1.16 ± 0.3

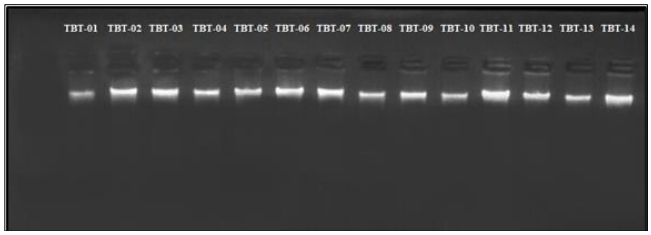
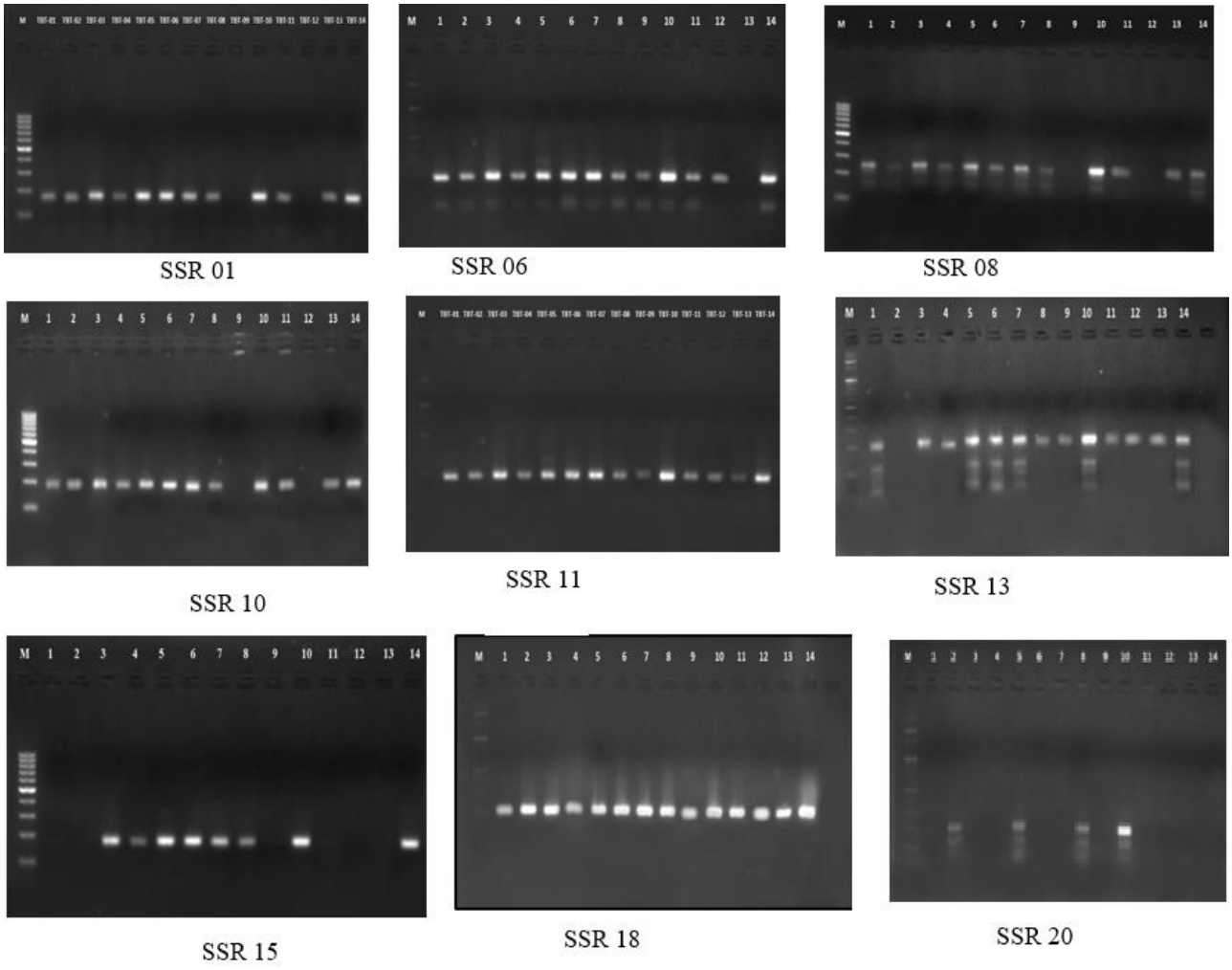


Fig 1: Genomic DNA isolated from Trichoderma isolates on 0.8% Agarose gel

SSR Marker Analysis

The PCR amplified products were analysed on a 2% agarose gel and visualized in a Gel Documentation System. Across the ten primers used a total of 15 alleles were obtained of which 13 were polymorphic and 2 monomorphic. The number of polymorphic bands for each strain varied from 1 to 3 among the fourteen isolates. A total of 80% polymorphism was observed indicating a high level of genetic diversity among the 14 *Trichoderma* isolates studied.



Primer analysis

Calculation of the Polymorphic Information Content (PIC) value of the primers revealed that the PIC value ranged between 0.132 (for primers SSR- 01 and SSR- 15) to 0.334 (for primer SSR- 18). The Average PIC value of the 10 primers was reported to be 0.178.

The Heterozygosity index (H) varied from a lowest value of 0.132 (SSR- 01, SSR- 15) to a highest value of 0.374 (SSR- 18), with an average heterozygosity index of 0.192 among all primers.

The Effective Multiplex Ratio (EMR) value was recorded to be 1.000 across all the 10 primers, while the Marker index (MI), ranged from 0.132 (SSR- 01, SSR- 15) to a highest value of 0.374 (SSR- 18), with an average MI of 0.192 among all primers.

The Distinguishing Power (DP), of the primer SSR- 18 was reported to be the highest 0.079, while it was lowest for the primers SSR- 01 and SSR- 15 with a value of 0.00. The average distinguishing power of all the 10 primers was calculated to be 0.031.

Binary similarity matrix: The binary similarity matrix (Jaccard's similarity coefficient) of the combined data from amplicons produced by ten SSR primers of *Trichoderma* isolates was prepared by scoring the presence or absence of bands in the gel. The molecular weight was assumed to be identical. the similarity coefficient value ranged between 0.429 to 0.929 across the *Trichoderma* isolates indicating a high degree of polymorphism with respect to genetic similarity. The minimum similarity or highest variability according to the Jaccard's similarity index (0.429) was observed among the isolates TBT- 03 and TBT-12 and also TBT- 05 and TBT- 12, while a maximum similarity or lowest variability (0.929) was recorded amongst the isolates TBT- 01 and TBT- 02, TBT- 01 and TBT- 08, TBT- 02 and TBT- 08, and isolates TBT- 13 and TBT- 14.

Table 2: Monomorphic alleles, Polymorphic alleles and Percent Polymorphism

Primer	Monomorphic alleles	Polymorphic alleles	Percent Polymorphism	H	PIC	EMR	MI	DP
SSR 01	1	0	0	0.142	0.132	1.000	0.142	0.000
SSR 06	0	1	100	0.144	0.137	1.000	0.144	0.028
SSR 08	0	2	100	0.145	0.139	1.000	0.145	0.038
SSR 10	0	1	100	0.143	0.136	1.000	0.143	0.020
SSR 11	0	1	100	0.143	0.134	1.000	0.143	0.011
SSR 13	0	2	100	0.270	0.250	1.000	0.270	0.061
SSR 14	0	1	100	0.272	0.255	1.000	0.272	0.039
SSR 15	1	0	0	0.142	0.132	1.000	0.142	0.000
SSR 18	0	4	100	0.374	0.334	1.000	0.374	0.079
SSR 20	0	1	100	0.145	0.140	1.000	0.145	0.041
Total	2	13	-	1.92	1.789	14	1.92	0.317
Average			80	0.192	0.178	1.4	0.192	0.031

Genetic diversity among *Trichoderma* isolates

A genetic similarity estimate (Jaccard's coefficient) based on the SSR banding pattern was used for cluster analysis to establish the genetic relationship in the form of a dendrogram. Analysis of the dendrogram constructed by the NTSYS software revealed that the highest and lowest value of similarity coefficient for the isolates were 0.929 and 0.500 respectively while the isolates were grouped into two clusters A and B. The minor cluster B comprised only one isolate TBT- 05 which indicates that the isolate has the least similarity or the highest diversity among the studied isolates while the major cluster A comprised of all the other thirteen isolates. Within the major cluster A, sub-clustering was observed based on different similarity coefficients. The major cluster A was divided into two sub-clusters A1 and A2 of which sub-cluster A1 comprised of nine isolates i.e., TBT- 01, TBT- 02, TBT- 03, TBT- 04, TBT-06, TBT- 07, TBT- 08, TBT- 09, TBT- 10 while sub-cluster A2 consisted of four isolates i.e. TBT- 11, TBT-12, TBT- 13 and TBT-14. Within the two sub-clusters A1 and A2 further sub-clustering was observed at an individual level. Isolates TBT-01, TBT- 02 and TBT- 08 formed a single cluster, revealing their relatedness to each other while isolates TBT- 13 and TBT- 14 grouped together into one cluster indicating that they were closely related to each other. The relatedness or clustering of the isolates into a single cluster presumably may be attributed to the closely situated geographical locations from where the soil samples for the isolation of *Trichoderma* isolates were collected. This can be explained by the high genetic variation observed among the isolates of *Trichoderma* combined with the fact that many of these isolates are the result of mutagenesis by ultraviolet light (Belludi *et al*. 2022) [2].

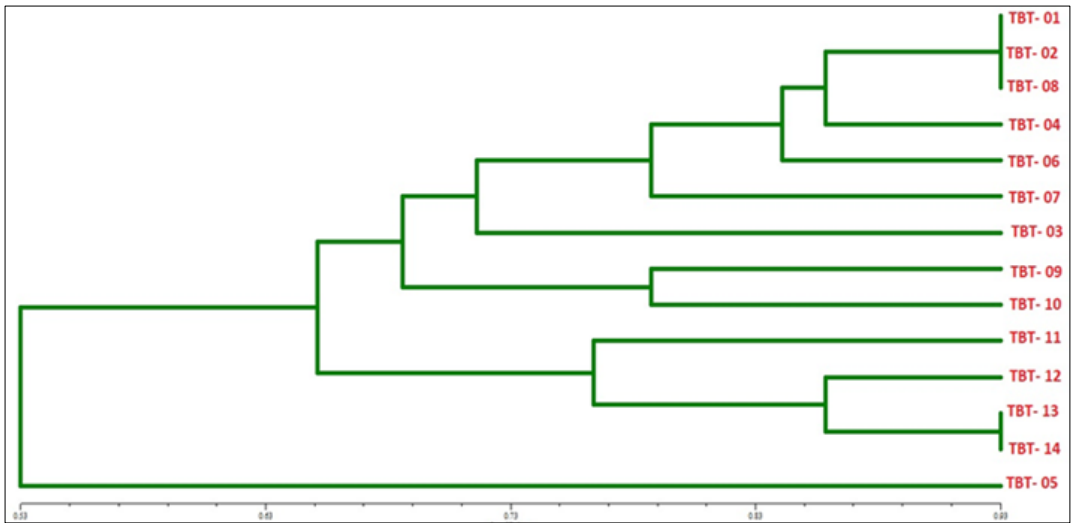


Fig 2: Coefficient

The research and studies conducted by several scientists and researchers aligned with the results obtained in the following study. Simple Sequence Repeat (SSR) markers proved to be an important tool in analysis of the genetic diversity among *Trichoderma* species. In a similar study, Shahid *et al.* (2013) [16] reported a 77% polymorphism and a high genetic diversity among seven *Trichoderma* isolates studied. Geistlinger *et al.* (2015) [6], Mahfooz *et al.* (2017) [11], Rai *et al.* (2019) [13], and Maheshwary *et al.* (2022) [10] also obtained similar results and reported a significant level of genetic diversity among the *Trichoderma* isolates studied using SSR marker.

Conclusion

Genetic diversity among the *Trichoderma* isolates was studied using a set of ten SSR primers which produced a total of fifteen alleles of which thirteen bands were polymorphic while two were monomorphic.Analysis of the SSR markers based on Jaccard's similarity coefficient revealed two major clusters in the isolates. Cluster A consisting of thirteen isolates divided into two sub-clusters A1 (TBT- 01, TBT- 02, TBT- 03, TBT- 04, TBT-06, TBT-07, TBT- 08, TBT- 09, TBT- 10) and A2 (TBT- 11, TBT-12, TBT- 13 and TBT- 14) while major cluster comprising of only one isolate TBT- 05.The Jaccard's similarity

coefficient value ranged from 0.429 to 0.929 among the fourteen isolates. Primer SSR-18 was found to be the most effective among the 10 primers based on the primer indexing results. Genetic diversity analysis using SSR marker showed that the average level of polymorphism was 80%.

Future scope

Trichoderma species offer huge scope for further studies. The following study can be further extended by Sequencing the ITS region and Species-level identification of the isolates. The isolates can be further tested for their Bio-control efficiency against several pathogens. A cumulative result of all the parameters would help in the identification and selection of the most effective isolates. The best isolate identified can be used as an efficient bio-control agent to enhance agricultural productivity.

Conflict of Interest: Authors have declared that no competing interests exist.

Acknowledgement

I extend my sincere thanks to Dr. Mukesh Kumar (major advisor) and to my advisory committee members for giving me proper guidance throughout the course of study.

References

1. Bae H, Roberts DP, Lim HS, Strem MD, Park SC, Ryu CM, *et al*. Endophytic Trichoderma isolates from tropical environments delay disease onset and induce resistance against Phytophthora capsici in hot pepper using multiple mechanisms. Mol Plant Microbe Interact. 2011;24(3):336-51.
2. Belludi R, Sandhu PS, Sharma P, Sekhon AS. Diversity analysis of aflatoxigenic isolates of Aspergillus sp. from major groundnut growing states of India. Indian Phytopathology. 2022;75(3):681-90.
3. Bustamante DE, Calderon MS, Leiva S, Mendoza JE, Arce M, Oliva M. Three new species of Trichoderma in the Harzianum and Longibrachiatum lineages from Peruvian cacao crop soils based on an integrative approach. Mycologia. 2021;113(5):1056-72.
4. Cumagun CJR. Managing plant diseases and promoting sustainability and productivity with Trichoderma: The Philippine experience. J Agric Sci Technol. 2012;14(4):699-714.
5. Doyle JJ, Doyle JL. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem Bull. 1987;19(1):11-5.
6. Geistlinger J, Zwanzig J, Heckendorff S, Schellenberg I. SSR markers for Trichoderma virens: their evaluation and application to identify and quantify root-endophytic strains. Diversity. 2015;7(4):360-84.
7. Jambhulkar PP, Singh B, Raja M, Ismaiel A, Lakshman DK, Tomar M, *et al*. Genetic diversity and antagonistic properties of Trichoderma strains from the crop rhizospheres in southern Rajasthan, India.
8. Levinson G, Gutman GA. Slipped-strand mispairing: a major mechanism for DNA sequence evolution. Mol Biol Evol. 1987;4(3):203-21.
9. Litt M, Luty JA. A hypervariable microsatellite revealed by *in vitro* amplification of a dinucleotide repeat within the cardiac muscle actin gene. Am J Hum Genet. 1989;44(3):397.
10. Maheshwary NP, Naik BG, Chittaragi A, Naik MK, Satish KM, Nandish MS, *et al*. Morpho-molecular characterization, diversity analysis and antagonistic activity of Trichoderma isolates against predominant soil borne pathogens. Indian Phytopathology. 2022:1-12.
11. Mahfooz S, Singh SP, Mishra N, Mishra A. A comparison of microsatellites in phytopathogenic Aspergillus species in order to develop markers for the assessment of genetic diversity among its isolates. Front Microbiol. 2017;8:1774.
12. Mukhopadhyay R, Pan S. Effect of biopriming of radish (Raphanus sativus) seed with some antagonistic isolates of Trichoderma. J Bot Soc Bengal. 2012;66(2):157-60.
13. Rai S, Ramteke PW, Sagar A, Dhusia K, Kesari SK. Diversity Assessment of Antagonistic Trichoderma Species by Comparative Analysis of Microsatellites in Plant Growth Promoting Rhizobacteria (PGPR). In: Prospects for Sustainable Agriculture. 2019:233-54.
14. Rezaee Danesh Y, Pellegrini M, Kariman K, Boyno G, Djebaili R, Farda B, *et al*. Genetic Diversity of Trichoderma harzianum Isolates in Sunflower Rhizosphere: The Application of the URP Molecular Marker. Sustainability. 2022;14(22):15111.
15. Rodriguez del C HM, Evans HC, Abreu de L M, Macedo de D M, Ndacnou M K, Bekele K B, *et al*. New species and records of Trichoderma isolated as mycoparasites and endophytes from cultivated and wild coffee in Africa. Sci Rep. 2021;11(1):1-30.
16. Shahid M, Srivastava M, Sharma A, Kumar V, Pandey S, Singh A. Morphological, molecular identification and SSR marker analysis of a potential strain of Trichoderma/Hypocrea for production of a bioformulation. J Plant Pathol Microbiol. 2013;4(10):1.
17. Zin NA, Badaluddin NA. Biological functions of Trichoderma spp. for agriculture applications. Ann Agric Sci. 2020;65(2):168-78.