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Research Article

Development of a High-Resolution Multi-Locus Microsatellite Typing Method for *Colletotrichum gloeosporioides*

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Abstract *Colletotrichum gloeosporioides* is an economically important fungal pathogen causing substantial yield losses indifferent host plants. To understand the genetic diversity and molecular epidemiology of this fungus, we have developed a novel, high-resolution multi-locus microsatellite typing (MLMT) method**.** Bioinformatic analysis of *C. gloeosporioides* unannotated genome sequence yielded eight potential microsatellite loci, of which five, CG1 $(GT)_n$, CG2 $(GT1)_n$, CG3 $(TC)_n$, CG4 $(CT)_n$, and CG5 $(CT1)_n$ were selected for further study based on their universal amplification potential, reproducibility, and repeat number polymorphism. The selected microsatellites were used to analyze 31 strains of *C. gloeosporioides* isolated from 20 different host plants from India. All microsatellite loci were found to be polymorphic, and the approximate fragment sizes of microsatellite loci CG1, CG2, CG3, CG4, and CG5 were in ranges of 213–241, 197–227, 231–265, 209–275, and 132–188, respectively. Among the 31 isolates, 55 different genotypes were identified. The Simpson's index of diversity (D) values for the individual locus ranged from 0.79 to 0.92, with the D value of all combined five microsatellite loci being 0.99. Microsatellite data analysis revealed that isolates from *Ocimum sanctum*, *Capsicum annuum* (chili pepper), and *Mangifera indica* (mango) formed distinct clusters, therefore exhibited some level of correlation between certain genotypes and host. The developed MLMT method would be a powerful tool for studying the genetic diversity and any possible genotype-host correlation in *C. gloeosporioides*.

Keywords *Colletotrichum gloeosporioides*, Genotyping, Host-specificity, Microsatellites

Members of the genus *Colletotrichum* cause major losses to economically important crops, especially staple food, fruits, vegetables, and ornamentals [1]. They are particularly successful as a post-harvest pathogen; up to 100% of the stored fruit can be lost as a result of Colletotrichum disease [2]. Considering the scientific and economic importance, the genus was voted as the eighth most important group of plant pathogenic fungi in the world [3]. Among different species of the genus, *Colletotrichum gloeosporioides* causes substantial yield losses due to fruit decay and damage to vegetative parts in a variety of plant species [4]. Diseases caused by *C. gloeosporioides* include anthracnose, dieback, root rot, leaf spot, blossom rot and seedling blight on a wide range of fruit crops.

In India, *C. gloeosporioides* was shown to be one of the most important pathogens, which infects a variety of hosts with characteristic symptoms. Mango, Indian mulberry, papaya, grapes, Kokum (*Garcinia indica*), bell pepper, turmeric, vegetable crops, *Jasminum grandiflorum*, aloe vera, *Pisonia alba*, curry tree (*Murraya koenigii*), etc. have been found to be infected by *C. gloeosporioides* [5]. This pathogen was shown to exhibits considerable genetic and pathogenic diversity [6-10]. *Colletotrichum gloeosporioides* can infect variety of host plants; however, it is still not clear, whether there is any correlation between certain genotypes of this pathogen and host specificity. It becomes important to establish such a correlation between genotypes and host specificity, because it may be useful when breeding for resistance or monitoring the sensitivity of fungicides across

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a collection of well-defined isolates. Particularly, if the certain genotypes show preferential and exclusive association with certain host plants, it is pertinent to screen the isolates of that particular genotype to assess the sensitivity to antifungals being sprayed on that particular host plant for disease control. Likewise, if there is an evidence that certain genotypes are preferentially and exclusively association with certain host plants; it would be wise to use those particular isolates only for resistance breeding. Such correlation could not be established well for *C. gloeosporioides*and its host plants. The possible reason for this gap in the knowledge is the unavailability of an efficient and discriminatory method of genotyping.

Different genotyping methods such as random amplified polymorphic DNA, restriction fragment length polymorphism (RFLP), and amplified fragment length polymorphism fingerprinting, analysis of A-T-rich DNA sequences in mitochondrial DNA, PCR-RFLP of internal transcribed spacer of rDNA, and inter-simple-sequence repeat PCR have been used to study the genetic diversity of *C. gloeosporioides* with variable success [4, 7, 8, 11-14]. All those above mentioned methods are pattern-based typing techniques, which suffers from poor discriminatory power, poor inter-laboratory reproducibility and difficulty in exchanging the results obtained by these techniques [15]. Therefore, there has been a need to develop an efficient, reproducible, and discriminatory method of genotyping for *C. gloeosporioides.*

Microsatellite are tandemly arranged repeats of short DNA motifs (1–6 bp in length) found dispersed throughout all eukaryotic genomes and they exhibit variation in the number of repeats at locus [16, 17]. Microsatellites typing have become a valuable tool, which is widely used in population genetics, genetic diversity, and DNA fingerprinting studies in many organisms, from bacteria, fungi, plants, to mammalian, human. Microsatellite typing relies upon the size variation of microsatellite loci due to repeat number polymorphism. Such repeat number polymorphism can be assayed by amplifying the microsatellite loci and determining the variations in the sizes of microsatellite containing amplicons between different isolates of the same species. Determination of fragment sizes of amplicons can be done in an automated DNA sequencer using capillary electrophoresis (CE). The number of repeats can be extrapolated from the fragment sizes. Analysis of multiple microsatellite loci results in high degree of discrimination as compared to single microsatellite locus, therefore, the multi-locus microsatellite typing (MLMT) method has the potential to be a highly discriminatory typing method. The ideal MLMT scheme should amplify the same loci from all studied isolates and should demonstrate sufficient repeat number polymorphism to develop a discriminatory typing scheme [18]. One study showed that the MLMT method has high inter-laboratory reproducibility [19]. Considering the potential of this method, we describe the identification, characterization and evaluation of polymorphic microsatellite loci from the genome of *C. gloeosporioides*. Further, we have evaluated the MLMT with diverse *C. gloeosporioides* isolates from different host plants and differentiate them into different genotypes, so as to assess the genetic diversity and any possible correlation between certain genotypes and host specificity.

MATERIALS AND METHODS

Fungal strains. Thirty *C. gloeosporioides* strains isolated from diverse hosts from India and one reference strain CBS 953.97 were included in the study (Table 1). Nineteen strains were obtained from National Fungal Culture Collection of India (NFCCI) (WDCM-932), Agharkar Research Institute, Pune, India and twelve strains were obtained from Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh, India. The species identities of the isolates were confirmed by microscopic morphology analysis. The molecular identification of the isolates were done by sequencing internal transcribed spacer (ITS)-rDNA region following standard PCR protocol [20].

Identification of microsatellites and primer designing. The unannotated genome sequence of *C. gloeosporioides* was downloaded from data dryad website (http://datadryad.org) (When this work was started that time the annotated genome sequence of *C. gloeosporioides* was not available). The available genomic sequences were screened for microsatellites with the help of online bioinformatics tool Tandem Repeat Finder (http://tandem.bu.edu/trf/trf.html) [21]. The microsatellites were selected on the basis of loci with the highest repeat numbers and counter-selected on loci containing two or more repeat sequences within the boundaries of potential PCR primer regions. A number of primer pairs were designed to amplify different microsatellite loci ranging from 150 bp to 300 bp in size with help of an online tool "Primer 3.0" (Table 2) [22].

Validation of microsatellites and their stability. Genomic DNA was isolated from fungal colonies grown on Potato Dextrose Agar plates for a week, following a simple and rapid DNA extraction protocol [23] using FastPrep 24 tissue homogenizer (MP Biomedicals GmbH, Eschwege, Germany). Each individual microsatellite locus was amplified individually by PCR in a 25 µL reaction volume containing 16 µL PCR grade water (Sigma, St. Louis, MO, USA), 2.5 µL PCR buffer (10×), 2.5 µL of 10 mM dNTPs mix (Sigma-Aldrich), 1 µL of each primer (20 pmol/µL), 1 µL (5 U/µL) of Taq polymerase (Sigma-Aldrich) along with 20–50 ng of template DNA. Amplification was done using an Applied Biosystems ProFlex PCR System (Applied Biosystems, Waltham, MA, USA) with the following parameters: 5 min step at 95°C, followed by 30 cycles of 1 min at 95°C, 30 sec at 54°C, 1 min at 72°C, and a final 5 min extension step at 72°C. The amplified products were separated on 8% polyacrylamide gel (PAGE). The uniform presence and repeat number polymorphism of all microsatellite

Table 1. *Colletotrichum gloeosporioides* isolates used in the study, their strain numbers, ITS rDNA sequences Genebank accession numbers, source and place of isolation

No.	Strain No.	GeneBank accession No.	Source of isolation	Place of isolation (city/state)
1	NFCCI 3874	KX009766	*Paederia foetida* leaf	Imphal
2	NFCCI 3411	KX009767	*Capsicum annuum* (chili pepper)	Thiruvananthapuram
3	NFCCI 3412	KX009768	*Capsicum annuum* (chili pepper)	Thiruvananthapuram
4	NFCCI 2040	KX009769	Litter	Imphal
5	NFCCI AC 2184[a]	KX009770	*Oryza sativa* (rice) leaf	Ratnagiri
6	NFCCI 1034	KX009771	*Jasminus* sp.	Jaipur
7	NFCCI 1280	KX009772	*Vitis vinifera*	Pune
8	NFCCI 1547	-	*Catharanthus roseus*	Belgaum
9	NFCCI 3708	KX009773	*Cucumis sativus* (cucumber)	Thiruvananthapuram
10	NFCCI 3010	KX009774	*Vitex negundo* (Nirgudi) leaf	Pune
11	NFCCI 3012	KX009775	*Ocimum sanctum* (tulsi) stem	Pune
12	NFCCI 3061	KX066883	*Ocimum sanctum* (tulsi)	Maharashtra
13	NFCCI 3154	KX066884	*Areca catechu* (areca nut)	Calicut
14	NFCCI 3230	KX066885	*Glycine max* (soyabean)	Gwalior
15	NFCCI 3347	KX066886	Banana immature fruit	Bhubaneshwar
16	NFCCI 3471	KX066887	*Ocimum sanctum*	Amaravati
17	NFCCI 3808	KX066888	Cucumber leaf spot	Vellayani
18	NFCCI 3424	KX066889	*Camelia sinensis* leaf	Sonitpur
19	MTCC 10529	MG270074	Seeds of *Paeciloneuron indicum*	Chikmangalur
20	MTCC 8460	KX066891	Leaves of *Cassia fistula*	Chennai
21	MTCC 9343	KX066892	*Syzygium aromaticum*	Andaman
22	MTCC 9660	KX099742	Fruit of *Mangifera indica* (mango)	Goa
23	MTCC 9661	KX099743	Fruit of *Mangifera indica* (mango)	Goa
24	MTCC 9662	KX099744	Fruit of *Mangifera indica* (mango)	Goa
25	CBS 953.97	Standard strain	Necrotic spot on leaves	Calabria, Italy
26	MTCC 8479	KX099745	Leaves of *Hibiscus*	Assam
27	MTCC 10183	KX099746	*Asparagus* sp.	Lucknow
28	MTCC 4618	KX099747	*Mangifera indica* (mango)	Chandigarh
29	MTCC 8882	KX099748	*Mangifera indica* (mango) leaves	Andaman
30	MTCC 9664	KX099749	Fruit of *Carica papaya*	Goa
31	MTCC 10339	KX099750	Thallus of *Sargassum* sp.	Kodiyakkarai

ITS, internal transcribed spacer.
[a]The given strain number is a temporary number; a full accession number will be made available soon.

Table 2. Microsatellite loci and their respective repeat motif, oligonucleotide primers, their amplicon sizes range, number of genotypes and the Simpson's index of diversity (D) value

Loci	Repeat motif	Oligo name	Oligonucleotide sequence 5′ to 3′ with fluorescent reporter dye	Sizes (bp)	No. of genotypes	*D*-value
CG1	$(GT)_n$	gleo GT F	6-FAM-TCACTTGACCTGACTTGGGG	213–241	10	0.810
		gleo GT R	TACACACGCACTCATCTCGT			
CG2	$(GT1)_n$	gleo GT1F	6-FAM-GGGCCTTGCATTGTGTTTCT	197–227	8	0.800
		gleo GT1R	TCCCCGATCGACATGCAATA			
CG3	$(TC)_n$	gleo TC F	VIC-CTTTGGTTGAGTTGAGGCCG	231–265	11	0.791
		gleo TC R	CGTACGGGGTAGCGTTATCT			
CG4	$(CT)_n$	gleo CT F	VIC-GTGGTGGTGGTGGTAGAAGA	209–275	12	0.926
		gleo CT R	ACCGGAACTGTCAGTGTGAA			
CG5	$(CT1)_n$	gleo CT1 F	NED-GCAGAACGGTACATGATCCC	132–188	14	0.922
		gleo CT1 R	TGCCTCACGATATCTGCAAC			
Combined loci						0.998

loci were initially screened in 18 randomly selected *C. gloeosporioides* isolates by observing apparent size variation on PAGE. The microsatellite loci were subjected to DNA sequencing to confirm that the apparent size variation seen on PAGE was due to repeat numbers polymorphism. Sequencing reactions were performed with a BigDye terminator cycle

sequencing kit, ver. 3.1/1.1 (Applied Biosystems). All the sequencing reactions were purified and analyzed on an ABI Avant 3100 automated DNA sequencer (Applied Biosystems). The stability of microsatellites was evaluated by amplifying each microsatellite locus from the DNA preparations of three different isolates after each passage for up to fifteen passages.

Detection of microsatellite polymorphisms by capillary electrophoresis. The microsatellite loci, which met the criteria of uniform amplification, apparent size variation on PAGE and stability were further analyzed by fragment size analysis in an automated CE system. The selected microsatellite loci primers were custom labeled with 6-carboxyfluorescein (6-FAM)—a blue color dye, VIC (green) and NED (yellow) fluorescent dyes (Thermo Fisher Scientific, Waltham, MA, USA). The forward primer for amplification of CG1, CG3 and CG5 were labeled with 6-FAM, VIC, and NED and the forward primers for CG2 and CG4 were labeled with 6-FAM and VIC, respectively (Table 2). The selected microsatellite loci were amplified from 31 isolates of *C. gloeosporioides* by PCR using fluorescence labeled primer with the above mentioned PCR composition and cycling conditions. The samples were loaded in multiplex format, wherein, the fluorescent labeled products of CG1, CG3, and CG5 were mixed together, while the fluorescent labeled products of CG2 and CG4 were mixed together and subjected to capillary electrophoresis. Each fluorescent labeled PCR products 0.5 µL was added to 9.0 µL Hi-Di Formamide (Applied Biosystems) with 0.5 µL GenScan 500 (−250) LIZ Internal Size Standard (Applied Biosystems). This mixture was denatured at 95°C for 3 min and immediately chilled on ice before loading. The samples were then subjected to electrophoresis on the 3130 Genetic Analyzer (Applied Biosystems) using the FA_36_POP-7 run module and G5 dye set. The molecular sizes of the amplified microsatellite loci were analyzed using GENESCAN analysis software (ver. 2.1; Applied Biosystems). The 31 isolates were assigned to microsatellite genotypes based on one or more differences in amplicon sizes.

Data analysis and discriminatory power. Microsatellite typing data was imported into BioNumerics v5.1 (Applied Maths, Sint-Martens-Latem, Belgium) and was analyzed using the multistate categorical similarity coefficient. The discriminatory power of the microsatellite markers was calculated using Simpson's index of diversity (D). This index is a statistical measure that any two randomly chosen isolates are of the same genotype for a given (combination of) marker (s). A 'D' value of 1 indicates all isolates to be different whereas a 'D' value of 0 indicates all isolates to be identical.

RESULTS

Molecular identification of the fungal strains. The identities of all 31 *C. gloeosporioides* isolates used in the study were confirmed by microscopic morphology examination. Further, the identities of 29 *C. gloeosporioides* isolates were reconfirmed by ITS-rDNA sequence analysis. Their DNA sequences were deposited to Genebank and the accession numbers for ITS-rDNA sequences of 29 isolates are shown in Table 1. We could not generate the ITS sequence of NFCCI 1547 isolate.

***In-silico* identification of microsatellites and primers.** Computational analysis of *C. gloeosporioides* unannotated genome sequence yielded multiple microsatellite loci; however, the primers could be designed only for eight microsatellite loci, keeping in view the amplicon sizes, which could be resolved on capillary electrophoresis. The identified microsatellite loci were $(GAC)_n$, $(GT)_n$, $(TC)_n$, $(TCGTCTG)_n$, $(GT1)_n$, $(T)_n$, $(CT)_n$, and $(CT1)_n$.

Validation of microsatellites and their stability. Out of these eight microsatellite loci, five could be amplified uniformly from 18 randomly selected isolates of *C. gloeosporioides*. Therefore, these five microsatellite loci CG1 $(GT)_n$, CG2 $(GT1)_n$, CG3 $(TC)_n$, CG4 $(CT)_n$, CG5 $(CT1)_n$ were selected for analysis of 31 isolates of *C. gloeosporioides*. The nomenclature used for the microsatellite loci was CG for *C. gloeosporioides*, followed by a number. All five microsatellite loci were found to be stable, as they could produce the same fragment sized PCR products even after fifteen rounds of passages (Supplementary Fig. 1).

Microsatellite polymorphisms and discriminatory power. As a preliminary proof of repeat number polymorphism, the PCR products of CG1, CG2, CG3, CG4, and CG5 showed apparent size variation on PAGE (data not shown). Further, the accurate sizes of five selected microsatellite loci were determined by an automated CE system for thirty-one strains of *C. gloeosporioides* isolated from diverse host plants from India. The fragment sizes of CG1, CG2, CG3, CG4, and CG5 were found in ranges of 213–241, 197–227, 231–265, 209–275, and 132–188, respectively. The individual molecular sizes of each of these five microsatellite loci from 31 isolates are mentioned in Supplementary Table 1. All five microsatellite loci proved to be polymorphic displaying up to 14 genotypes per microsatellite marker (Table 2). The DNA sequence analysis confirmed the presence of the microsatellites and demonstrated polymorphism in the microsatellite repeat number. The sequence information for the various alleles of these five microsatellite loci were submitted to GeneBank with accession numbers given in Supplementary Table 2. The discriminatory power for the individual microsatellite locus ranged from 0.79 to 0.92. The panel of all five microsatellites combined yielded a 'D' value of 0.998 (Table 2).

High genotypic diversity and host specificity in *C. gloeosporioides*. Graphical representation of minimum spanning tree (Fig. 1) and dendrogram (Supplementary

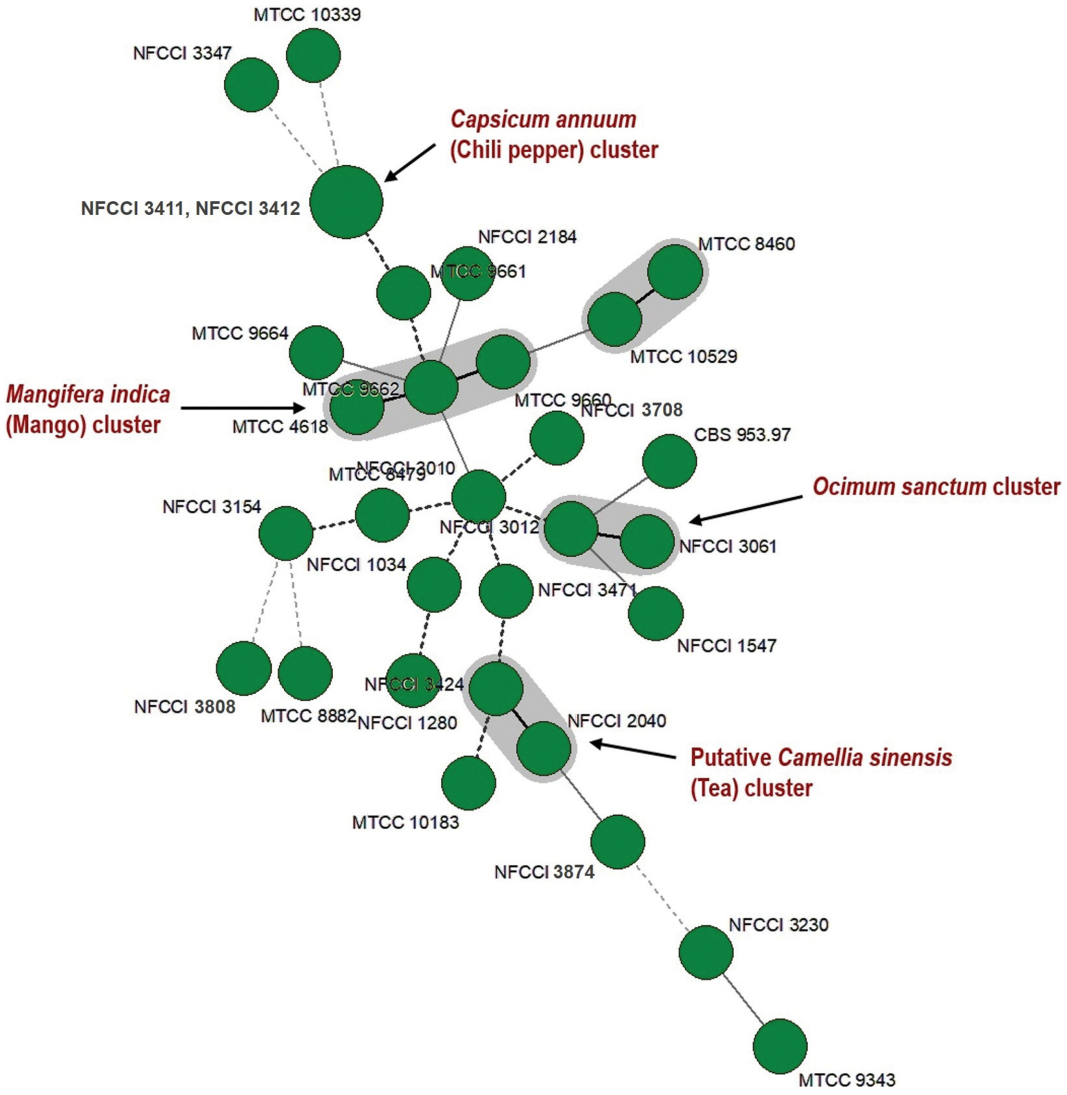

Fig. 1. Graphical representation of the results. Minimum spanning tree representing the genotypic diversity of 30 *Colletotrichum gloeosporioides* isolates and a reference strain (CBS 953.97) using microsatellite typing. The multi-locus microsatellite typing is based on a categorical analysis of the data. Each circle represents a unique genotype. The size of the circle corresponds to the number of isolates with the same genotype. The thickness and size of the connecting bars correspond to the number of different markers between linked genotypes. Genotypes with a shaded background contain 2 or more isolates with identical genotypes or contain genotypes that differ in only 1 microsatellite marker as the possible result of microevolutionary events and are likely to be clonally related.

Fig. 2) showed high genotypic diversity in 31 strains of *C. gloeosporioides* isolated from diverse host plants from India. In the collection of 31 pure culture isolates, 30 genotypes could be recognized. Among all genotypes, 29 genotypes were only found once. One genotype was shared between two isolates. The NFCCI isolates 3411 and 3412, which were isolated from chili found to be clustered together sharing the same genotypes. The another cluster consists of two NFCCI strains 3012 and 3061 isolated from *Ocimum sanctum*, wherein, the two strains shared same genotypes of four microsatellite markers and differ in only 1 microsatellite marker. Similarly, there is another clade of strains MTCC 9660, 9662, and 4618, which were isolated from Mango (*Mangifera indica*), this clade also contains strains with identical genotypes or contain genotypes that differ in only one or two microsatellite markers. One more cluster of strains NFCCI 2040 and NFCCI 3424 originally isolated from leaf litter and *Camelia sinensis* leaf respectively from North East States of India. We also observed one cluster of MTCC 8460 isolated from leaves of *Cassia fistula* and MTCC 10529 from seeds of *Paeciloneuron indicum*.

DISCUSSION

This study describes the development of a MLMT method for *C. gloeosporioides*. Whole-genome shotgun sequencing

of *C. gloeosporioides* Nara gc5, isolated from strawberry plants was reported in 2013 [24]. We had downloaded the *C. gloeosporioides* Nara_gc5_1. fasta file containing the genomic DNA sequences from Dryad Digital Repository (http://datadryad.org/resource/doi:10.5061/dryad.r4026). This had provided us the opportunity to search for potential microsatellite loci *in silico* by using Tandem Repeats Finder software in the available genomic sequence. We identified eight microsatellite loci, out of which only five microsatellite loci; CG1 $(GT)_n$, CG2 $(GT1)_n$, CG3 $(TC)_n$, CG4 $(CT)_n$, and CG5 $(CT1)_n$—fulfilled the criteria for an ideal microsatellite marker. The CG1 $(GT)_n$, CG2 $(GT1)_n$ are two different microsatellite loci having different genome locations [CG1$(GT)_n$–Nara_gc5_1_scaffold 223 and CG2 $(GT1)_n$–Nara_gc5_1_scaffold 289] but containing the same repeat motif. Likewise, CG4 $(CT)_n$ and CG5 $(CT1)_n$ are also two different microsatellite loci having different genome locations [CG4 $(CT)_n$–Nara_gc5_1_scaffold 419 and CG5 $(CT1)_n$–Nara_gc5_1_scaffold 403] but containing the same repeat motif. The five microsatellites described here were found to have reasonably good discriminatory power. The discriminatory power of the assay was greater when all five loci were used for analysis, compared with the discriminatory powers of individual loci. A combined discriminatory power of 0.998 was achieved, which is in good agreement with reports for typing of other fungi by microsatellite analysis. The MLMT methods have been developed for *Candida albicans*, *Aspergillus fumigatus*, *Aspergillus flavus*, *Rhizopus oryzae*, and *Lachancea thermotolerans* with the discriminatory power of 0.99, 0.99, 0.99, 0.98, 0.95, and 0.81, respectively [25-30].

Despite the fact that *C. gloeosporioides* can cause infection in variety of host plants, our current understanding of the extent that it exhibits host specificity is imperfect. This maybe due to a number of factors, one of such factor is that many studies on *Colletotrichum* are restricted to strains affecting single crop species, which, significantly reduce the extent of the gene pool being sampled [31-33]. Another reason being unavailiblity of a highly discrimiatory method for genotyping. We have evaluated the developed MLMT method with 31 isolates of *C. gloeosporioides* isolated from diverse host plants from diverse geographical locations of India. We tried to explore a correlation between genotypes and host specificity. The multistate categorical similarity coefficient analysis yielded very interesting results, which showed that the isolates from *Ocimum sanctum*, chili, and mango were significantly clustered together. We have observed that two strains NFCCI 2040 and NFCCI 3424 shared four microsatellite genotypes. These strains were originally isolated from leaf litter and *Camelia sinensis* (tea) leaf respectively from Tripura (Imphal) and Assam (Sonitpur) states of North East India. Interestingly, the isolate NFCCI 2040, which was isolated from liter and that liter, was from a tea garden, therefore, it is most likely that the fungal isolate must have infected the tea plant (*C. sinensis*), thereby showing the genotypic similarity with the other strain NFCCI 3424 isolated from a tea plant. We observed one cluster of MTCC 8460 isolated from leaves of *Cassia fistula* and MTCC 10529 from seeds of *Paeciloneuron indicum*, wherein, it is a possibility of a false association because, this fungus is known to be a very common post-harvest pathogen, therefore, it is quite likely that this strain would have been a passive contaminant on seeds surface (not a true infection within the seed). We have also observed that some isolates sharing one or more genotypes with the other isolates from different hosts, however, such sharing of genotype was not significant, therefore, did not get reflected as a significant cluster in our minimum spanning tree representing the genotypic diversity. Our results showed some level of correlation between certain genotypes with the host, however, we need to plan a future study, wherein, we shall be taking significantly more number of isolates from different hosts and analyze them by our developed MLMT method, so as to conclusively establish a true correlation between genotypes and hosts. This will be useful when breeding for resistance or monitoring the sensitivity of fungicides across a collection of well-defined isolates.

The ability to assign an identical genotype to the same isolate after multiple passages defines in vitro stability, which is another important criterion for evaluating a typing method [15]. For *Aspergillus* species, the stability of microsatellites has been determined for 14–30 passages [26, 34]. The reproducibility as well as the in vitro stability of the present MLMT method was found to be 100% till fifteen passages. For organisms with a short generation time, it may be necessary to assess the stability of the markers for up to several hundred generations, whereas for filamentous fungi, which have a longer generation time, 15–30 passages would be sufficient to test the stability and reproducibility [28]. The level of stability of the markers obtained in the current study may be sufficient to use them to answer various epidemiological questions concerning genetic diversity and genotype-host correlation.

Microsatellite markers have been used successfully for strain typing and population genetics studies of several fungal species because of their good discrimination power, ease of PCR amplification and interpretation, and potential use in automated assays. As the MLMT method is PCR based, it requires relatively small amounts (~30–40 ng per reaction) of template DNA compared with other methods. The multicolor fragment analysis approach used in this study helped in typing large number of isolates in short time; therefore, the developed method holds the potential to be fast and high throughput in nature. Using a standard panel of microsatellite loci, test isolates and allelic ladders, inter-laboratory comparisons and exchange of results are also feasible [19]. Another advantage of this approach would be a possibility of development of microsatellite databases for future comparison of data, e.g., a centrally held database (http://pmarneffei.multilocus.net/) provides a powerful epidemiological tool for analyzing the populations

of *Penicillium marneffei* [35]. In future, efforts should be directed towards development of a MLMT database for *C. gloeosporioides*, wherein the MLMT genotypes can be added to an online public database, which will allow an accrual and sharing of genetic information from a number of collaborating laboratories. In conclusion, we describe a highly discriminatory, reproducible MLMT method for *C. gloeosporioides*, which would be a powerful tool for studying the genetic diversity and any possible genotype-host correlation.

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ELECTRONIC SUPPLEMENTARY MATERIAL

Supplementary data including two tables and two figures can be found with this article online at http://www.mycobiology.or.kr/src/sm/mb-45-401-s001.pdf.

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