Genome-wide microsatellites and species specific markers in genus *Phytophthora* revealed through whole genome analysis

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Abstract

Genome wide microsatellite maps shall support *Phytophthora* systematics through the development of reliable markers, enabling species discrimination and variability analyses. Whole genome sequences of 17 *Phytophthora* accessions belonging to 14 species were retrieved from GenBank and the genome-wide microsatellites in each species were mined. A total of 51,200 microsatellites, including dinucleotide to decanucleotide motifs, have been identified across all the species and each one was characterized for uniqueness and repeat number. The *P. infestans* T30-4 genome had the highest (6873) and *P. multivora* 3378 had the lowest number of microsatellites (1802). Dinucleotide motifs (63.6%) followed by trinucleotide motifs (30.1%) were most abundant in all the genome. From 14 species, 250 microsatellites which are unique for the respective genomes are detailed along with their primer combinations and product sizes. *P. sojae* had the highest number of unique microsatellite motifs. Genome wide microsatellite maps for all the 14 *Phytophthora* species including the chromosome, position, motif, repeat number, forward and reverse primer sequences and expected PCR product size, for every microsatellite are presented. Markers based on the unique microsatellites could be used to identify each species, whereas the ones common to all species could be used to identify the genetic variability. Furthermore, to confirm the results, pure cultures of *P. capsici*, *P. nicotianae* and *P. palmivora* were procured from the *Phytophthora* Repository, DNA was isolated and the unique markers were screened across the species. The characteristic markers developed have confirmed the genome analysis results.

Keywords Fungal taxonomy · Genome annotation · Microsatellite markers · Next generation sequencing (NGS) data analysis · Species identification · Simple sequence repeats (SSR)

Introduction

Genus *Phytophthora* accommodates phytopathogenic oomycetes causing devastation in multiple crops. Even after describing more than 170 species, nearly 500 species are expected to be identified (Brasier 2009). Each species is found to have limited host range with characteristic symptoms. In the humid tropics, quick wilt of black pepper caused by *P. capsici*, bud rot of coconut caused by *P. palmivora*, late

blight of potato and tomato caused by *P. infestans*, abnormal leaf fall of rubber caused by *P. meadii* and root rot and die-back of many crops caused by *P. cinnamomi* and in temperate regions, bleeding stem lesions on forest trees caused by *P. kernoviae*, tree decline caused by *P. multivora*, cankers and leaf blight of forest trees caused by *P. nemorosa* are the major diseases caused by these oomycetes. The characters as well as criteria for species discrimination are yet to be defined, still the general features such as colony morphology, sporangial and oospore characteristics form the taxonomic base.

Decreasing costs of genome sequencing has led to the generation of great number of genome sequences, taking the gene finding and molecular marker based researches to an altogether higher level. Though the next generation sequence analysis still remains as a technically demanding step, the output will have extensive applications. One such application is the genome wide discovery of microsatellites,

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leading to the development of molecular markers for genes and other applications.

Microsatellites or simple sequence repeats (SSR) are tracts of nucleotide motifs of one to six base pairs repeated multiple times. SSRs are distributed throughout the genome of fungi, plants and animals. The DNA replication errors contribute to the variations in the number of repeats of these motifs associated with the different alleles of the genes (Levinson and Gutman 1987) and these variations are used to generate the codominant SSR marker system which will have numerous applications in biological systems including gene and QTL mapping, diversity assessment and fingerprinting.

The present system of allocation of this oomycetes to any specific species is based on molecular phylogeny proposed by Martin et al. (2014) and thus it is mandatory that a simple and reproducible molecular methodology has to be designed to confirm the *Phytophthora* species. The *Phytophthora* systematics has been so complicated that the causal species for foot rot of black pepper has been wrongly described as *P. nicotianae*, *P. palmivora* var. *piperina*, *P. palmivora*, *P. palmivora* MF4, *P. capsici* and *P. tropicalis*, on the basis of morphological characters (Muller 1937; Tsao 1991; Aragaki and Uchida 2001; Zhang et al. 2004).

Though works such as assessment of the evolutionary path (Martin et al. 2019; Arafa et al. 2020), host specialization analysis (Stroud et al. 2016), pathotype identification (Grijalba et al. 2020), population structure analysis (Göre et al. 2019; Zhang et al. 2019; Choi et al. 2020; Lindqvist-Kreuzer et al. 2020) and variability analyses (Runno-Paurson

et al. 2016; Arafa et al. 2018; Kiiker et al. 2019; Maurice et al. 2019) have been done in different species of *Phytophthora*, using limited number of described microsatellites, comprehensive microsatellite maps of these species are not available. This work was carried out with the objective to analyze the whole genome sequences of different *Phytophthora* species available at GenBank, to mine the microsatellites present in the genomes to identify the universal and unique microsatellites across the species and to develop and validate the molecular markers for each species.

Materials and methods

Sequence retrieval

Assembled genome sequences of 17 *Phytophthora* accessions belonging to 14 species were retrieved from the database of NCBI (Table 1). The genome assembly files consisted of scaffolds and contigs.

In silico identification of microsatellites

The complete panel of contigs for each species of *Phytophthora* was analysed using the software GMATo (Genome-wide Microsatellite Analysing Tool) (Wang et al. 2013), a tool for SSR mining in genomes of any size and freely available at <https://sourceforge.net/projects/gmato/files/>. A Perl script (Perl gmato.pl -r5 -m2 -x10 -s o -i) was run to

Table 1 *Phytophthora* genomes used in this study

Sl. no	<i>Phytophthora</i> species	Strain	Genome size (Mb)	GenBank accession	Disease caused
1	<i>P. kernoviae</i>	NZFS 3630	38.0	GCA_000785725.2	Leaf and stem necrosis
2	<i>P. kernoviae</i>	NZFS 2646	37.9	GCA_000785735.2	Leaf and stem necrosis
3	<i>P. agathidicida</i>	3772	38.2	GCA_001314435.1	Kauri dieback
4	<i>P. agathidicida</i>	3770	38.1	GCA_001314445.1	Kauri dieback
5	<i>P. cactorum</i>	10300	60.4	GCA_003287315.1	Root rots and crown rots of many genera
6	<i>P. cinnamomi</i>	NZFS 3750	55.3	GCA_001314505.1	Stripe canker on cinnamon, root rot or dieback
7	<i>P. capsici</i>	LT1534	57.8	GCA_000325885.1	Foot rot on black pepper
8	<i>P. infestans</i>	T30-4	231.9	GCA_000142945.1	Potato late blight
9	<i>P. multivora</i>	3378	40.9	GCA_001314345.1	Dieback
10	<i>P. multivora</i>	3448	41.1	GCA_001314355.1	Dieback
11	<i>P. pluvialis</i>	NZFS 3000	54.0	GCA_001314455.1	Infects tanoak-Douglas-fir forests of western Oregon
12	<i>P. ramorum</i>	CDFA1418886	61.0	GCA_002968915.1	Sudden oak death
13	<i>P. sojae</i>	P6497	87.3	GCA_000149755.2	Stem and root rot of soybean
14	<i>P. taxon totara</i>	NZFS 3727	56.3	GCA_001314925.1	Needle browning and needle loss in lower part of the crown of <i>Podocarpus totara</i>
15	<i>P. nicotianae</i>	race 0	83.7	GCA_001483015.1	Buckeye rot on tomato
16	<i>P. palmivora</i>	sbr112.9		GCA_002911725.1	Bud rot of palms, fruit rot of coconut and arecanut
17	<i>P. parasitica</i>	INRA-310	83.5	GCF_000247585.1	Dieback

search the microsatellite sequences in the genomes, with the FASTA file containing all the assembled sequences for each species used as input file. The parameters used were motif length range and minimum repeat times for a motif and had an option for highlighting microsatellites. Minimum length of the motif was fixed at two base pairs, repeated at least five times. Maximum length of the motif was fixed at ten base pairs.

Primer designing for the microsatellites

The BatchPrimer3 software was used to design forward and reverse primers flanking the SSR containing sequence (<https://wheat.pw.usda.gov/demos/BatchPrimer3/>) (You et al. 2008). Each sequence with SSR repeats was given as input and the parameter set were size of the PCR product 135–300 bp, primer length 18–23 nucleotides, GC content of primers 40–64% and melting temperature 40–62 °C.

Confirmation of unique microsatellites for species identification

To confirm the suitability of the unique microsatellites identified from this study, for species discrimination in *Phytophthora*, SSR marker patterns associated with these microsatellites were studied across different species.

Pure cultures of *P. capsici*, *P. nicotianae* and *P. palmivora* were procured from *Phytophthora* Repository at ICAR-Indian Institute of Spices Research, Kozhikode, Kerala State, India. Since the objective of this part of the study was to show the uniqueness of the identified primer sets, only the most devastating species in this part of the country were analysed using limited number of primer sets. Furthermore, the non-availability of pure cultures of other species was a limitation. These were sub-cultured on potato dextrose agar medium and maintained at Centre for Plant Biotechnology and Molecular Biology, Kerala Agricultural University and used for DNA isolation.

DNA isolation

Genomic DNA was isolated from *Phytophthora* subcultures following the Doyle and Doyle (1990) protocol. DNA quality and quantity were evaluated through electrophoresis on 0.8% agarose gel followed by A_{260}/A_{280} absorbance ratio in spectrophotometry and the working concentration was set at 50 ng/μL.

Thermal cycling for validation of unique markers

For validation of the unique markers, two primer pairs for *P. nicotianae* and one primer pair for *P. capsici* were used in PCR assay. The primer combination used for first

microsatellite (GGTAT)₁₉ of *P. nicotianae* was F 5'AAA TCAAACGAGAGAGAGAGAG3' and R 5'AATTTCCCT TCATATCCAAAG3' with product size 193 bp and annealing temperature 52 °C. The primer combination for second microsatellite (AACGAG)₈ of *P. nicotianae* was F 5'TCA TTACACGTTGGAGTGG3' and R 5'AATACAGACCTC GTTCACCTC3', with product size 139 bp and annealing temperature 51 °C. Primer combination for microsatellite (CGAGTT)₅ of *P. capsici* was F 5'GTCGAGTCAGTGGAA GTTGT3' and R 5'GTAAGTCCGGAAACAGCTC3' with product size 157 bp and annealing temperature 53 °C.

Thermal cycling was performed in 20.0 μL volumes containing 2.0 μL of template DNA, 2.0 μL of buffer, 1.8 μL dNTPs, 1.8 μL MgCl₂, 0.4 μL Taq Pol enzyme, 1.0 μL each of 10 μM primers, and 10.0 μL double distilled water. Cycling had initial denaturation at 95 °C for 2 min. followed by 30 cycles consisting of denaturation at 95 °C for 30 s., annealing at primer specific temperature for 30 s. and elongation at 72 °C for 1 min. followed by a final extension for 8 min. at 72 °C. Products were examined on 2% agarose gel. DNA marker 100 bp (3590A, Takara Biotech Co., Beijing) was used to determine the sizes of the PCR products.

Results

Identifying the genome-wide microsatellites

Assembled sequences of 17 *Phytophthora* accessions belonging to 14 species were downloaded and searched for microsatellite motifs. Depending on the length of the motif (2–10 bp) and number of repeats, the size of SSRs varied. Distribution of all the microsatellite motifs which have a minimum of two base pair length and repeated at least five times were documented. Total count of the microsatellites extracted through the computational methods and their comparison in each species are summarized in Table 2. Number of times each microsatellite motif is present in each genome is presented in Supplementary Table 1. SSRs loci were manually cross verified to avoid the duplications, e.g., the software has counted (AT)₆ at a particular locus also as (TA)₆. The loci finally obtained across all the genomes are presented in Supplementary Table 2. Maximum number of SSRs was found in *P. infestans* (6873) and minimum in *P. multivora* (1802). *P. cactorum* was rich in AG motifs and in this species, AG and CT motifs were repeated up to 49 times. *P. capsici* had comparatively more number of repeats for every motif. CA repeated up to 82 times, whereas longer motifs such as CCTAAAC, TTAGGG, TAAACCC have repeated up to 50, 53 and 48 times, respectively. *P. infestans* had TA repeated 497, 301, 206, 162 and 153 times in various regions of the genome. *P. sojae* had GATA motif repeated 151 times and in *P. palmivora*, TATC repeated 115 times,

Table 2 Details of SSR distribution in *Phytophthora* genome

Sl. no	Species	No. of SSRs identified	Motifs								
			di-	tri-	tetra-	penta-	hexa-	hepta-	octa-	nano-	deca-
1	<i>P. kernoviae</i> NZFS 3630	2150	1388	630	37	9	48	26	5	5	2
2	<i>P. kernoviae</i> NZFS 2646	2120	1382	64	33	7	44	19	6	2	3
3	<i>P. agathidicida</i> 3772	2264	1169	1021	46	5	19	2	1	1	0
4	<i>P. agathidicida</i> 3770	2257	1170	1018	43	3	18	2	2	1	0
5	<i>P. cactorum</i> 10300	2518	1799	581	75	14	22	17	7	3	0
6	<i>P. cinnamomi</i> NZFS 3750	4224	2383	1684	77	20	45	7	2	6	0
7	<i>P. capsici</i> LT1534	1982	1347	551	51	8	9	14	0	1	1
8	<i>P. infestans</i> T30-4	6873	4929	1488	259	10	29	150	0	5	3
9	<i>P. multivora</i> 3378	1802	1076	632	51	5	24	10	2	1	1
10	<i>P. multivora</i> 3448	1810	1092	632	44	5	22	10	4	1	0
11	<i>P. pluvialis</i> NZFS 3000	3619	2243	1284	40	9	20	15	1	6	1
12	<i>P. ramorum</i> CDF41418886	3264	2180	809	137	30	47	21	4	21	15
13	<i>P. sojae</i> P6497	5603	3116	2136	171	59	53	54	12	0	2
14	<i>P. taxon totara</i> NZFS 3727	2979	1841	967	54	12	69	21	7	7	1
15	<i>P. nicotianae</i> race 0	3389	2455	818	60	5	8	24	1	16	2
16	<i>P. palmivora</i> sbr112.9	2276	1502	624	89	2	18	27	8	7	1
17	<i>P. parasitica</i> INRA-310	2070	1505	498	45	5	8	1	3	5	0

whereas in *P. taxon totara*, maximum number of repeats for any motif was only 19.

On an average, di-nucleotide microsatellite motifs alone constituted 63.6% of the total microsatellites in *Phytophthora* genome, maximum and minimum numbers of di-nucleotide microsatellite motifs in *P. infestans* and *P. multivora*, respectively. Proportions of tri- and tetra-nucleotide motifs were 30.1 and 2.5%, respectively.

On an average, 21.3% of microsatellite motifs, mostly di-nucleotides, were common across the genomes, which could be used in diversity analyses. Unique microsatellites in each species, identified by comparing the genome wide microsatellites in each species are presented in Supplementary Table 3. Number of unique microsatellite motifs in *P. kernoviae* 3630 was 34, primer details and expected product sizes for each motif, except for those holding another microsatellite in either of its flanking regions, is presented in Supplementary Table 4. Since 13 of these unique microsatellite motifs had other microsatellites in their flanking regions, primers were not feasible and hence only 21 are presented. Similar details on *P. kernoviae* 2646, *P. agathidicida* 3772, *P. agathidicida* 3770, *P. cactorum*, *P. cinnamomi*, *P. capsici*, *P. multivora* 3378, *P. multivora* 3448, *P. sojae*, *P. palmivora*, *P. nicotianae*, *P. parasitica*, *P. taxon totara*, *P. infestans*, *P. pluvialis* and *P. ramorum* are presented in Supplementary Tables 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 and 20.

PCR primers for genome wide microsatellite markers were designed in each species. Sequence ID, genomic position of microsatellite motif, sequences of forward and

reverse primers, expected PCR product size, motif, motif sequence and motif length of each microsatellite in *P. kernoviae* 3630 genome is presented in Supplementary Table 21. As already mentioned, wherever either of the flanking regions of a motif was continued with another microsatellite, primer designing was not feasible and hence primers for 297 motifs are presented. Similar details for *P. kernoviae* 2646, *P. agathidicida* 3772, *P. agathidicida* 3770, *P. cactorum*, *P. cinnamomi*, *P. capsici*, *P. multivora* 3378, *P. multivora* 3448, *P. sojae*, *P. palmivora*, *P. nicotianae*, *P. parasitica*, *P. taxon totara*, *P. infestans*, *P. pluvialis* and *P. ramorum*, are presented in Supplementary Tables 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36 and 37.

Confirmation of unique markers for each species

To confirm the uniqueness of the species-specific microsatellites identified and their utility to identify the species of *Phytophthora*, DNA from three species, *P. nicotianae*, *P. capsici* and *P. palmivora* were screened using the primers for species-specific microsatellites. When the primers for microsatellite (GGTAT)₁₉ and (AACGAG)₈ from *P. nicotianae* were screened in *P. nicotianae*, *P. capsici* and *P. palmivora* genomes, both the primer combinations have generated markers at 193 and 139 bp, respectively in *P. nicotianae* only (Fig. 1). Similarly, when the primer combination for the microsatellite (CGAGTT)₅ from *P. capsici* was screened in *P. nicotianae*, *P. palmivora* and *P. capsici* genomes, marker at 157 bp was generated only in *P. capsici* (Fig. 2). Thus, it was proven that the microsatellites identified from this study

Fig. 1 *Phytophthora nicotianae* specific markers amplified using two primer combinations designed for the unique microsatellites identified through genome wide microsatellite mining

Fig. 2 *Phytophthora capsici* specific distinct marker amplified using primer combination designed for the unique microsatellites

are capable to resolve the species conflicts in *Phytophthora* systematics.

Discussion

EST sequences held in the *Phytophthora* Genome Consortium (PGC) database (Waugh et al. 2000) and *Phytophthora* Functional Genomics Database (PFGD) (Gajendran et al. 2006) were the initial source for gene and microsatellite

mining in this oomycete. Subsequently, whole genome sequencing has revolutionised the genomic studies. In 2004, United States Department of Energy Joint Genome Institute (JGI) completed the draft genome sequence of *P. sojae* and *P. ramorum* through whole genome shotgun approach (Tyler et al. 2006). Genome sequencing for the deadly pathogen *P. infestans* was completed in 2009 (Hass et al. 2009). Later, *P. capsici* (Lamour et al. 2012), *P. pluvialis*, *P. kernoviae*, *P. cinnamomi*, *P. agathidicida*, *P. multivora* and *P. taxon totara* (Studholme et al. 2016) were sequenced.

Comparative analysis of the sequences of *ITS*, *COI* or *CO2* is the presently accepted molecular methodology for the identification of *Phytophthora* species (Grünwald et al. 2011). DNA barcoding is a parallel and more precise strategy employing the similar loci. Similarly, sequence variations among 31 *Phytophthora* species at four intergenic regions of mtDNA, *ITS*, and ras-related protein *Ypt1* gene were also found useful in species identification (Schena and Cooke 2006). *ITS* sequences were also used to differentiate *P. capsici* and *P. tropicalis* (Zhang et al. 2004). All these methodologies suffer from the long protocol of PCR amplification, gel elution, purification of eluent and sequencing which is both time and money demanding. The species specific SSR markers will be the easiest methodology for species detection in a single step.

PCR based markers for differentiating the *Phytophthora* species was first done by Ersek et al. (1994) when they have designed PCR primers to amplify different bands from *P. parasitica* and *P. citrophthora*. A longer procedure of PCR amplification of 5.8S rDNA gene and *ITS* regions followed by restriction digestion of the product was employed to differentiate 14 species of *Phytophthora* (Ristaino et al. 1998). del Castillo-Múnera et al. (2013) have analyzed the *P. infestans*, *P. andina*, *P. sojae*, *P. cryptogea*, *P. nicotianae*, *P. capsici* and *P. cinnamomi* strains using 13 SSR markers and species specific alleles were amplified. Subsequently, the microsatellite markers have been extensively employed in *Phytophthora* population studies (Engelbrecht et al. 2017; Lurwanu et al. 2020), diversity analysis (Hieno et al. 2019; Chaves et al. 2020; Wu et al. 2020; Al-Kaabi et al. 2020), evolutionary studies (Dale et al. 2019) and to track the migration (Socorro Serrano et al. 2019; Afandi et al. 2019; Mizeriene et al. 2020; Gao et al. 2020). However, all these studies have employed only a limited number of microsatellite markers.

In the present study, we are reporting 250 microsatellites from 14 species which are unique for their respective genomes. With the primer combinations and product sizes detailed, they are the potential markers in species identification. Previous reports in this direction tried to identify the alleles unique to each species using established SSR loci, whereas the SSRs unique to the genome would be stronger strategy in systematics. Furthermore, we are reporting 5570

markers across the genomes and having wide variability in the repeat number in each species, along with their primer sets. QTL and gene mapping studies require genome wide microsatellite information with their precise positions. This extensive information has not been available in many of the *Phytophthora* species. Since this study presents the microsatellite map in different species with their respective primer combinations, it will be of extensive use in phylogenetic and mapping studies in this genus.

By analysing several ESTs and BAC clones from *Phytophthora infestans*, 100 microsatellite markers were developed previously and the polymorphic ones were shown useful to assess the genetic variation within this species (Leas et al. 2006). Identification of 205 SSR markers from 511 microsatellites obtained after removal of duplicates from a total of 6873 microsatellites, derived computationally in *P. infestans*, shows that whole genome based mining is the best strategy to design the SSR markers. A similar analysis of microsatellites from oomycete transcript sequences in *P. infestans*, *P. sojae* and *P. ramorum* had shown 1466, 4859 and 2554 microsatellites, respectively, of which > 60 per cent were the three nucleotide repeats (Garnica 2006). But none of these works seems to have attempted for removal of duplicates. Through comparative genome analysis in *P. sojae*, Cai et al. (2019) were successful to identify 157 informative markers, which is lower than the number of microsatellites identified in the present study.

Phytophthora Microsatellites Database [<https://bioinf.ibun.unal.edu.co/phytoossr>] and *Phytophthora* database [<https://www.phytophthoradb.org/>] are the leading databases on *Phytophthora* microsatellites in the world. *Phytophthora* Microsatellites Database currently offers primers for unique amplicons in three species *P. infestans*, *P. sojae* and *P. ramorum*. Additionally, the cross transferability of markers across these three species is also available. However, the unique microsatellites which act as fingerprints for each species is missing. *Phytophthora* database, a highly resource rich database, currently offers SSR loci search in *P. ramorum*.

To validate the results of in silico analyses, cultures of three most prevalent species in the humid tropics, *P. capsici*, *P. nicotianae* and *P. palmivora* were procured and thermal cycling was performed to analyze the marker pattern. The unique SSR markers designed for *P. capsici* have generated banding only in *P. capsici* and similarly, unique marker for *P. nicotianae* has generated banding only in that species. Identification of causal species is important, since there has been prevailing confusions even in case of major diseases such as quick wilt of black pepper. *P. palmivora* has been described as the causal agent for this disease (Holliday and Mowat 1963; Alconero et al. 1972; Zentmyer et al. 1977) until it was identified as *P. capsici* (Aragaki and Uchida 2001; Truong et al. 2008). Unique markers described in each species could be used for species identification, whereas

the universal markers or species transferable markers with variability in their repeat numbers could be used in diversity studies, development of fingerprints, gene tagging and QTL mapping.

Conclusion

Phytophthora genus accommodates 170 described phytopathogenic oomycetes causing enormous crop losses round the world. Even in the major diseases caused by *Phytophthora*, causal species identification has been challenging. Lack of genome wide markers and the time and money required for the genome sequencing are the major challenges for genetic analyses in this genus. Leading databases such as *Phytophthora* Microsatellites Database and *Phytophthora* database offer limited number of microsatellites in different species. Through whole genome analysis, we have identified genome wide microsatellites in 14 species of *Phytophthora*. Furthermore, unique microsatellites which could be specifically used for identifying each species and species transferable microsatellites which could be used in diversity analysis, phylogenetic studies, gene tagging and mapping, are described along with their primer combinations. As a proof to the in silico results, few unique microsatellite markers were used in thermal cycling to show their species specific marker generation. The exhaustive list of microsatellites along with the unique and universal microsatellites described could be used in future molecular applications in this phytopathogen.

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Availability of data and material The supplementary material relevant to this publication are available as online resources.

Compliance with ethical standards

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

Ethics approval Authors declare that no animals are used in this study and hence no ethical clearance is required.

Consent to participate and publish All the authors have read the manuscript completely and agree to participate in this publication.

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