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Natural Incidence and Genetic Variability of *Erysiphe pisi*, the Causal Agent of Powdery Mildew on Peas in the Nilgiris District, Tamil Nadu, India

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Authors' contributions

This work was carried out in collaboration between all authors. Authors MM, TR, DA and SH designed the study, supervised and facilitated the research and wrote the first draft of the manuscript. Author SP performed the experiments and analyzed the results obtained in the study. All authors read and approved the final manuscript.

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ABSTRACT

Erysiphe pisi, which causes pea powdery mildew, is an obligate biotrophic pathogen that can easily adapt to its host plant. Understanding the natural incidence and genetic variations of the pathogen is essential for disease control and for breeding resistant variety to pea powdery mildew. The incidence was studied in different locations of the Nilgiris district, and the disease recorded from 32.61 to 45.55 and 14.08 to 20.84 per cent in leaves and pods, respectively. A total of 10 isolates of *E. pisi* were obtained from the diseased samples to study the genetic variability if any, among them using internal transcribed spacer region amplification and inter-simple sequence repeats (ISSRs). PCR amplification of total genomic DNA with ISSR primers generated unique banding patterns depending upon primers and isolates. Twelve oligonucleotide primers were selected for the ISSR assays, which resulted in 742 bands for ten isolates of *E. pisi*. The number of bands obtained was entered into a PRIMER 7 to understand genetic relationship and Bray-Curtis

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coefficient for hierarchical cluster analysis. The results showed that the variability among the pathogen isolates was moderate. The above ten isolates were grouped into two major clusters and one single cluster had an average Polymorphic Information Content value (PIC) of 0.883 and Effective Multiplication Ratio (EMR) of 90. Hence, it is inferred that the *E. pisi* infecting peas in the Nilgiris consists of a single clonal lineage with a moderate level of genetic diversification.

Keywords: Bray-Curtis coefficient; molecular characterization; genetic diversity; ITS; ISSR; Pisum sativum.

1. INTRODUCTION

Pea (*Pisum sativum* L.) is an important commercial edible legume as vegetable and dry seeds. It has been grown worldwide for food and feed and is known for high protein content of vegetarian food. In addition, it is able to fix nitrogen and assist in improving soil nitrogen status. However, this crop is highly susceptible to powdery mildew caused by the obligate parasite *Erysiphe pisi* [1]. In favourable conditions, this pathogenic fungus infects the entire leaf surface, causing leaves to wither and resulting in severe yield losses. This disease affects biomass yield, plant height, number of nodes, number of pods per plant and number of seeds per pod [2], therefore 25-50 per cent of yield reduction was normally observed [3]. In severe epidemics, the Nilgiris district has recorded pea powdery mildew disease incidence up to 70 per cent [4]. Because of the geographically distinct climates in the Nilgiris, pathogens can over-summer in cool climates with high altitudes. The pathogen populations that over-summer on volunteer pea plants can serve as an infection source and can attack plants at adjacent attitudes and next season, potentially causing gene flow and reconstruction of the pathogen population in an environment. Pathogen population divergence may occur as a result of genetic drift and local adaptation to increase relative fitness in local environments. To understand the ecological adaptation and genetic diversity of pea's powdery mildew, reliable molecular evidence is needed. In particular, phenotypic and genetic characterization of the pathogenic variants of the plant pathogens prevalent in an area is required for efficient disease management [5].

DNA markers are in wide use for analyzing the dynamics of plant pathogen populations due to their high precision levels. The Internal Transcribed Spacer (ITS) region has been most useful for molecular characterization at the species level detection. Because of its high degree of variation than other generic regions of ribosomal deoxyribonucleic acid (rDNA), variation among individual rDNA repeats can sometimes be observed within both the ITS and ribosomal DNA intergenic spacer (IGS) regions. ISSRs have been demonstrated to be useful for analyzing the genetic diversity of a wide range of fungal species [6]. ISSR marker is a simple, easy, rapid and consistent technique requires no sequence information and using a single primer for detection and random amplification [7]. Only small amounts of DNA template are required and the results are clearly scorable demonstrated [8]. Perhaps the main reason for the success of ISSR analysis is the gain of numerous genetic markers that require small amounts of DNA without the requirement for cloning, sequencing or any other form of the molecular characterization of the genome of the species. ISSR marker has reported to have high efficiency due to high polymorphic, repetition motifs as well as reasonable cost compared to AFLP, RFLP and RAPD. ISSR markers are suggested to be the best markers in evaluation of genetic variability and polymorphism [9]. Moreover, at the molecular level, the genetic diversity of powdery mildew pathogen populations in India is not well known. The occurrence of *E. pisi* in the Nilgiris, Tamil Nadu, at 10 different locations was investigated and also characterized the genetic diversity by using ISSR marker. Here, the most updated population structure of pea powdery mildew pathogen and its distribution due epidemics in the Nilgiris are presented very elaborately.

2. MATERIALS AND METHODS

2.1 Collection and Isolation

Survey and collection of powdery mildew affected pea samples were done in ten different locations of the Nilgiris District, Tamil Nadu State *viz*., Anumapuram, Emarald, Indira Nagar, Thuneri, Iduhatty, Kenthorai, Bigatti, Palada, Coonoor and Kothagiri biospheres for assessing the incidence of powdery mildew of peas during September 2015 to January 2016 (Fig. 1). In each village

three farmer's fields were selected and in each farmer's field, at three places 20 plants were selected at random. The percentage of leaf area affected by powdery mildew was assessed visually following the 0-9 scale [10] and The disease data recorded based on scale was converted to per cent disease index (PDI) according to the formula [11] (Table 1). The powdery mildew infected pea plants were collected showing the presence of white mycelial powdery patches on the upper surface of the leaves initially. Further the greyish-white patches and premature defoliation of leaves was also observed in certain fields. To avoid contamination, each diseased leaf sample was placed in a clean paper bag.

2.2 Genomic DNA Extraction from *E. pisi* Isolates

Spore suspension of ten isolates of *E. pisi* was collected in Eppendorf tubes separately for DNA extraction by the CTAB method [12] with slight modifications. About 100 mg of fungal propagules was scrapped from surface of the leaves under sterile condition. The isolated propagules were then transferred into a test tube containing 5 mL extraction buffer (700 mM of NaCl, 50 mM of Tris HCl with pH 8.0, 10 mM of EDTA, 2.0 per cent CTAB, and 1.0 per cent mercapto ethanol). Samples were incubated for 1 h at 60ºC followed by two consecutive extractions with 5 mL chloroform-isoamyl alcohol (24:1). The emulsions were centrifuged at 3,500 rpm for 15 min and the aqueous phase was recovered and transferred to another tube. DNA was precipitated by adding ice cold isopropanol and chilled at -20ºC for at least 30 min. DNA was collected by centrifugation at 10,000 rpm at 4ºC for 15 min. The supernatant was disposed and the pellet was dehydrated at room temperature. The dried pellet was dissolved in 500 µL of TE buffer (10 mM of Tris HCl with pH 8.0, 1 mM of EDTA with pH 8.0) and treated with 5 µL of RNAse A (10 mg/mL) at 37ºC for 1h. The quality of genomic DNA was checked by 0.8 per cent of agarose which was dissolved in 100 ml of 1x TAE buffer. Two µL of DNA loaded with 2 µL of loading dye in electrophoresis unit contain 0.8 per cent agarose gel. Electrophoresis was carried out on 60V for 1 hour and then the genomic DNA visualized on UV transilluminator and documented using gel documentation system (Alpha Innotech Corporation, California). The quantification of DNA was done by using Nanodrop Spectrophotometer. Based on the nanodrop readings, DNA dilution was made in distilled water to make a final concentration of 50-60 µg/µL and stored in -20ºC for further use [13].

2.3 Confirmation of *E. pisi* Isolates Using Molecular Tool

The field collected isolates were confirmed as the genus *Erysiphe* using universal primers ITS-1 (5' - TCCGTAGGTGGACCTGCGG - 3') and ITS-4 (5'-TCCTCCGCTTATTGATATGC- 3') [14]. PCR amplification was carried out as 25 µL reaction mixture containing 2.5 µL of 10x Taq buffer, 0.5 µL of 25 mM $MgCl_2$, 2.0 µL of ITS-1 primer (0.6 picomolar/µL), 2.0 µL of ITS-4 primer (0.6 picomolar/µL), 0.5 µL of 100 mM dNTP mix, 0.125 µL of Taq polymerase (0.5 u/µL) and 14.375 µL of sterile PCR water (Genei, Bangalore) and 3 µL (50-60 ng) of DNA sample. Reactions were performed using an eppendorf PCR master cycler. PCR cycling conditions considered of 35 cycles, of which denaturation at 94℃ for 1 min, annealing at 56℃ for 1 min and extension at 72℃ for 1.5 min with initial denaturation at 94℃ for 4 min before cycling and final extension at 72℃ for 6 min after cycling. Amplified products of ITS regions were screened using 1.0 per cent agarose gel electrophoresis in 1.0 per cent TAE buffer, 5 µL of PCR product along with 2 µL of loading dye loaded on the agarose gel. 65 voltage was supplied for 1 hour and visualized under UV transilluminator with ethidium bromide staining. The size of the PCR product was estimated by comparison with known DNA marker of 100 bp DNA ladder. The banding profiles of ITS-PCR products were documented in gel documentation system (Alpha Innotech Corporation, California).

2.4 Genetic Diversity Analysis of *E. pisi* Isolates by ISSR Marker

Totally, ten ISSR primers were used for assessing the genetic diversity of *E. pisi.* The list of primers and conditions used are furnished in Table 2. For ISSR, the amplifications were run in a final volume of 20 µL containing the following reagents: 10 mmol L^{-1} Tris-HCl, pH 8.3, 50 mmol L^{-1} KCl, 2.4 mmol L^{-1} $MgCl_2$, 100 µmol L^{-1} of each dNTP, 0.4 µmol L^{-1} of oligonucleotide primers, 50 ng of genomic DNA, and 0.75 units of Taq DNA polymerase. Two µL of DNA aliquot was placed in each tube and the above ingredients were added. The amplified PCR products were tested on 1.5 per cent agarose gel. The gel was visualized with a UV transilluminator and photographed in the gel documentation.

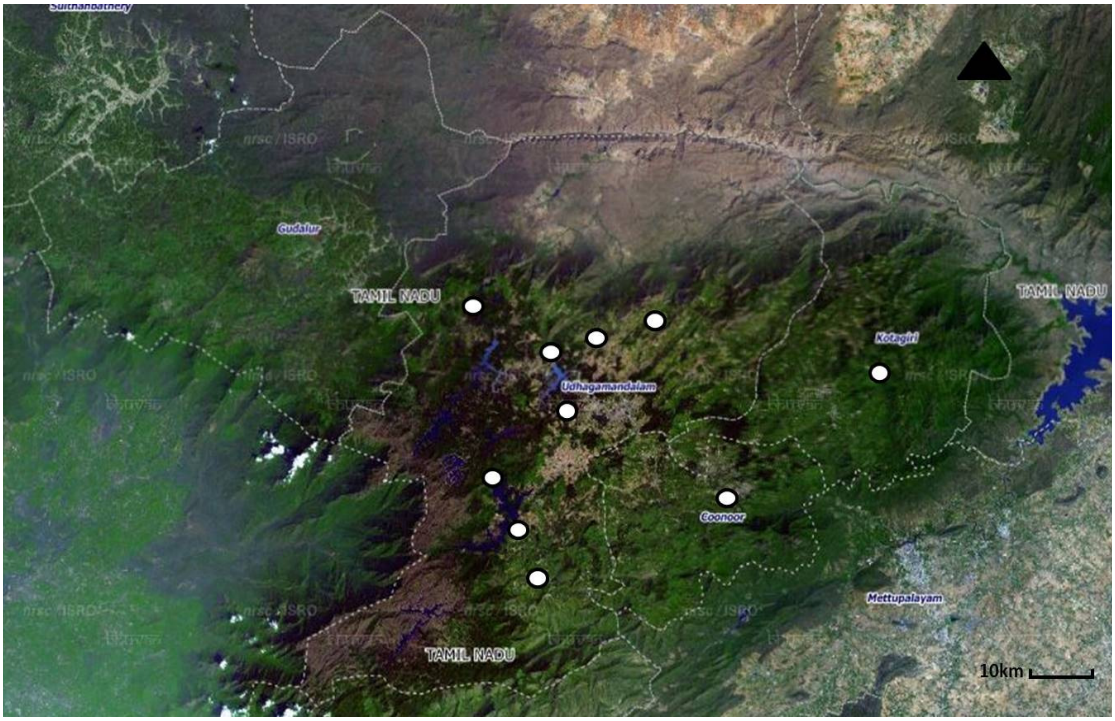


Fig. 1. Collection of pea powdery mildew samples from the different locations of Nilgiris district

Table 1. Geographical information of *E. pisi* isolates in the Nilgiris District

S. no.	Isolates	Name of the village	Geographical position		
			Latitude (N)	Longitude (E)	MSL (m)
1	P-ANU1	Anumapuram	11.46087915	76.54895781	2029
2	P-EMV1	Emerald Valley	11.32171175	76.62474628	1935
3	P-IDN1	Indira Nagar	11.34119423	76.60562749	2008
4	P-TNI1	Thuneri	11.45753389	76.74057484	1821
5	P-IHY1	Iduhatty	11.46173987	76.77370548	1887
6	P-KTR1	Kenthorai	11.44059366	76.73546791	2070
7	P-BGI1	Bigatti	11.28660566	76.63410187	1864
8	P-PLD1	Palada	11.37266034	76.66234016	2015
9	P-CNR1	Coonoor	11.34202375	76.78693055	1790
10	P-KGI1	Kotagiri	11.41424019	76.86432838	1903

2.5 Band Scoring and Data Analysis

The banding patterns were scored for ISSR primers in each *E. pisi* isolates starting from the large size fragment to small sized one. Presence and absence of each band in each isolate was coded as 1 and 0 respectively. The scores were used to create a data matrix to analyse genetic relationship using PRIMER 7 statistical software (Plymouth Routines in Multivariate Ecological Research, version 7.0.9; PRIMER-E, Plymouth, UK). A similarity matrix was constructed between each pair of samples using the Bray–Curtis coefficient [15] and analyzed by hierarchical cluster analysis (HCA). HCA was performed by a weighted, group average linkage agglomerative method and a dendrogram was constructed from the ranked similarities using PRIMER 7 software.

2.6 The Efficacy of ISSR Molecular Markers for Assessing the Genetic Diversity of *E. pisi* Isolates

The marker system ISSR was analyzed for their efficiency in assessing the genetic diversity of *E. pisi* isolates. The parameters *viz.*, total number of

Table 2. Primers used for ISSR analysis of *E. pisi* isolates

Nucleotide sequence 5' to 3'	PCR conditions		
1. $(CAG)_5$	Initial denaturation	95°C; 5 min.	30 cycles [30]
2. $(GTG)_5$	Denaturation	95°C; 30 sec.	
3. $(AGG)_5$	Annealing	60°C; 30 sec.	
	Extension	72°C; 1.5 min.	
	Final extension	72°C; 10 min.	
4. $(GACA)_4$	Initial denaturation	95°C; 5 min.	30 cycles [31,32]
5. $(GACAC)_3$	Denaturation	95°C; 30 sec.	
6. $(TGTC)_4$	Annealing	48°C; 30 sec.	
	Extension	72°C; 1.5 min.	
	Final extension	72°C; 10 min.	
7. $(CAG)_3$	Initial denaturation	95°C; 5 min.	40 cycles [33]
	Denaturation	94°C; 1 min.	
	Annealing	50°C; 1min.	
	Extension	72°C; 1 min.	
	Final extension	72°C; 10 min.	
8. $(CAC)_5$	Initial denaturation	95°C; 5 min.	40 cycles [26]
	Denaturation	94°C; 1 min.	
	Annealing	51°C; 1min.	
	Extension	72°C; 2 min.	
	Final extension	72°C; 10 min.	
9. $(AG)_8$ T	Initial denaturation	94°C; 2 min.	35 cycles [8]
10. $(AG)_8$ C	Denaturation	94°C; 1 min.	
11. $(GA)_8$ T	Annealing	52°C; 1min.	
12. $(GA)_8$ YG	Extension	72C; 1.5 min.	
	Final extension	72°C; 6 min.	

bands, the number of monomorphic and polymorphic bands, bands per primer, per cent polymorphism and monomorphism were documented. In addition, Polymorphic Information Content (PIC) was calculated using the formula [16].

$$PIC_j = 1 - \sum_{l=1 to L} P^2_{lj}$$

where P_{lj} is the relative frequency if the l^{th} allele for the locus j and is summed across all the alleles (L) over all lines. Marker Index (MI) was determined as the product of PIC and the number of polymorphic bands per assay unit and EMR (E) is the product of the fraction of polymorphic loci and the number of polymorphic loci for an individual assay [17].

$$EMR\ (E) = n_p\ (n_p/n)$$

where 'n_p' is the number of polymorphic loci and 'n' is the total number of loci.

2.7 Statistical Analysis

The data obtained were statistically analysed [18] and the disease incidence means were compared by Duncan' package used for analysis (IRRISTAT version 92) developed by the International Rice Research Institute, Biometrics Unit, The Philippines.

3. RESULTS AND DISCUSSION

3.1 Incidence of *E. pisi* in the Nilgiris District

Powdery mildew of pea caused by *E. pisi* is one of the most annihilate and widely predominant diseases occurring both on main and offseason crop in India. The disease occurs in epidemic proportion almost every year, as crop is grown around the year in different zones of Himalayan ranges [19,20]. Yield losses upto 45-70 per cent have been reported due to powdery mildew by different workers [4,21,22]. In addition to reduction in yield, it also reduces the quality of the marketable harvest significantly. In the present study, powdery mildew incidence caused by *E. pisi* isolates in the Nilgiris district was ranged from 32.61 to 45.55 and 14.08 to 20.84 per cent in leaves and pods respectively. Among the ten isolates tested in the present study, the isolate collected from Anumapuram (P-ANU1)

showed higher per cent disease incidence followed by Iduhatty (P-IHY1) and Emerald Valley (P-EMV1) isolates. The samples collected from Coonoor and Palada recorded low powdery mildew incidence (Table 3). The differences in the pathogen diversity and incidence during the growing seasons may be due to differences in the climatic conditions between regions. The higher altitude in Anumapuram region might have stimulated the growth and development compared with the lower altitude in Coonoor which could have mildly obstructed the disease development in the growing season.

3.2 Molecular Confirmation of *E. pisi* Isolates with ITS Primer

The ITS region of fungal DNA is highly useful for molecular systematics at the species level and even within species (e.g., to identify geographic races). Because of its prominent degree of variation than other regions of rDNA, variation among individual rDNA repeats can sometimes be observed within both the ITS and IGS regions. In the present study, ten isolates were amplified using ITS universal primers *viz*., ITS-1 and ITS-4 and the result indicated that the expected amplicon of 550 bp in all the isolates were observed (Fig. 2). These results confirmed the identity of all the isolates belonging to genus *Erysiphe*. Based on this result, the molecular ITS region could not establish any variation within *E. pisi* isolates instead grouped all the isolates into one line. The amplicon size of ITS primer on *E. pisi* agree with the nucleotide size of *E. pisi* collected from central and north east India [23]. It has been observed that the discrimination capacity of this technique decreases relatively when populations become distant in terms of location. It has also been observed that ITS method is more successful in genetic diversity analyses of more asunder populations [24].

3.3 Inter Simple Sequence Repeats (ISSR)

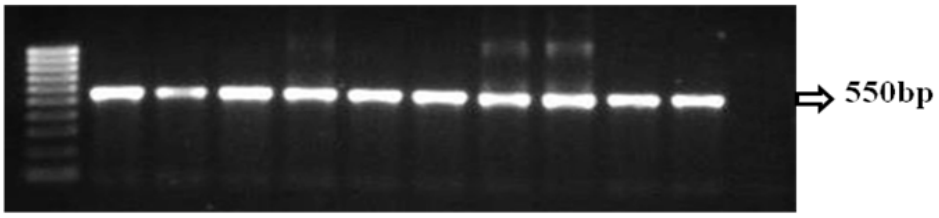
Inter simple sequence repeats (ISSR) is an alternative technique to study polymorphism based on the presence of microsatellites through-out genomes [25]. In the present study, ISSR markers were used to investigate genetic diversity among 10 isolates of *E. pisi* isolated from different regions of the Nilgiris district, Tamil Nadu. The primers amplified a total of 742 bands from 10 isolates tested. Of the twelve primers used in the study, most of the primers showed cent per cent polymorphism (Fig. 3). However, the primer $(CAC)_5$ yielded maximum bands of 109 and mostly polymorphic (Table 4).

Table 3. Incidence of pea powdery mildew disease in the different locations of Nilgiris district

S. no.	Isolates	Name of the village	Percent disease index (PDI)	
			Leaves	Pods
1	P-ANU1	Anumapuram	45.55[a] (42.43)	20.84[a] (27.04)
2	P-EMV1	Emerald Valley	40.21[b] (38.74)	19.06[b] (25.49)
3	P-IDN1	Indira Nagar	36.83[d] (36.48)	16.85[c] (23.71)
4	P-TNI1	Thuneri	34.12[e] (35.34)	16.17[e] (23.54)
5	P-IHY1	Iduhatty	45.08[a] (42.15)	19.72[ab] (26.26)
6	P-KTR1	Kenthorai	38.59[c] (38.35)	16.04[d] (23.50)
7	P-BGI1	Bigatti	39.76[b] (39.06)	16.65[c] (24.02)
8	P-PLD1	Palada	32.68[f] (34.81)	14.08[f] (22.02)
9	P-CNR1	Coonoor	32.61[f] (34.81)	14.89[e] (22.68)
10	P-KGI1	Kotagiri	34.85[e] (36.00)	16.53[c] (23.74)

Values are mean of three replications, Figures in parentheses represent arcsine transformation. Means in a column followed by same superscript letters are not significantly different according to DMRT at P ≤ 0.05

Hence, the primer $(CAC)_5$ was most efficient in assessing the diversity of *E. pisi* isolates. Two-dimensional Non-metric Multi-Dimensional Scaling (MDS) plot using the Bray-Curtis similarity index coordinating the data from ISSR marker fingerprints clearly discriminated each isolates of *E. pisi*. The different isolates of *E. pisi* were evenly distributed in the MDS plot with less Kruskal's stress (0.1). The isolate of *E. pisi* P-PLD1 is stackly and distinctly placed when compared to all other isolates of *E. pisi* in the MDS plot, which shared different ordination. Some isolates of *E. pisi viz*., P-ANU1, P-EMV1, P-IDN1, P-KGI1 and P-TIN1 shared the same line of similarity of coordination in the plot. The HCA-based clustering of strains and isolates showed 73-93 per cent similarity (according to Bray–Curtis similarity index) with reference to ISSR fingerprint. The isolates of *E. pisi viz*., P-ANU1 and P-EMV1 had 93 per cent similarity between them. A dendrogram resulting from a cluster analyses showed two main distinct groups, exhibiting overall genetic relationship among the isolates (Fig. 4). The isolate P-PLD1 was 73 per cent distinct genetically than the all other remaining isolates while grouping them in non-metric MDS (Fig. 5). The ten isolates studied were grouped into two major clusters and one single cluster had an average PIC value of 0.883 and EMR of 90. The ISSR marker profiling analysis showed similar resolutions and the ISSR bands appeared to be more informative. From the result of the study, it was concluded that the ISSR markers generally group the isolates according to their host but also grouped according to geographical locations. There was no significant association between these populations and the incidence of the disease. The results suggest that ISSR marker is efficient for the characterization of closely related populations versus distantly related populations. Hence, these markers can be used to assess the genetic diversity of *E. pisi* existed within single location. The results of the experiments are in line with findings of earlier workers [26,27].



Lane:

M- Marker 1. P-ANU1 2. P-EMV1 3. P-IDN1 4. P-TNI1 5. P-IHY1
6. P-KTR1 7. P-BGI1 8. P-PLD1 9. P-CNR1 10. P-KGI1 C. Control

Fig. 2. PCR amplification of rRNA-ITS gene region of *E. pisi*

Table 4. Genetic diversity of *E. pisi* isolates revealed by ISSR

S. no.	Primers	Total no. of bands	No. of polymorphic banding pattern	No. of monomorphic banding pattern	PIC value	EMR
1	$(AGG)_5$	52	7	1	0.926	7.53
2	$(AG)_8C$	35	5	1	0.915	5.38
3	$(AG)_8T$	47	6	0	0.767	6.00
4	$(CAC)_5$	109	11	1	0.851	11.68
5	$(CAG)_3$	51	6	1	0.969	6.73
6	$(CAG)_5$	89	9	1	0.585	9.62
7	$(GACA)_4$	82	11	1	0.858	11.68
8	$(GACAC)_3$	39	6	0	0.958	6.00
9	$(GTG)_5$	75	11	0	0.933	11.00
10	$(GA)_8T$	51	6	1	0.950	6.46
11	$(GA)_8YG$	39	5	0	0.962	5.00
12	$(TGTC)_4$	73	7	1	0.918	7.29
	Total	742	90	8	0.883	90

PIC-Polymorphic Information Content; EMR- Effective Multiplication Ratio

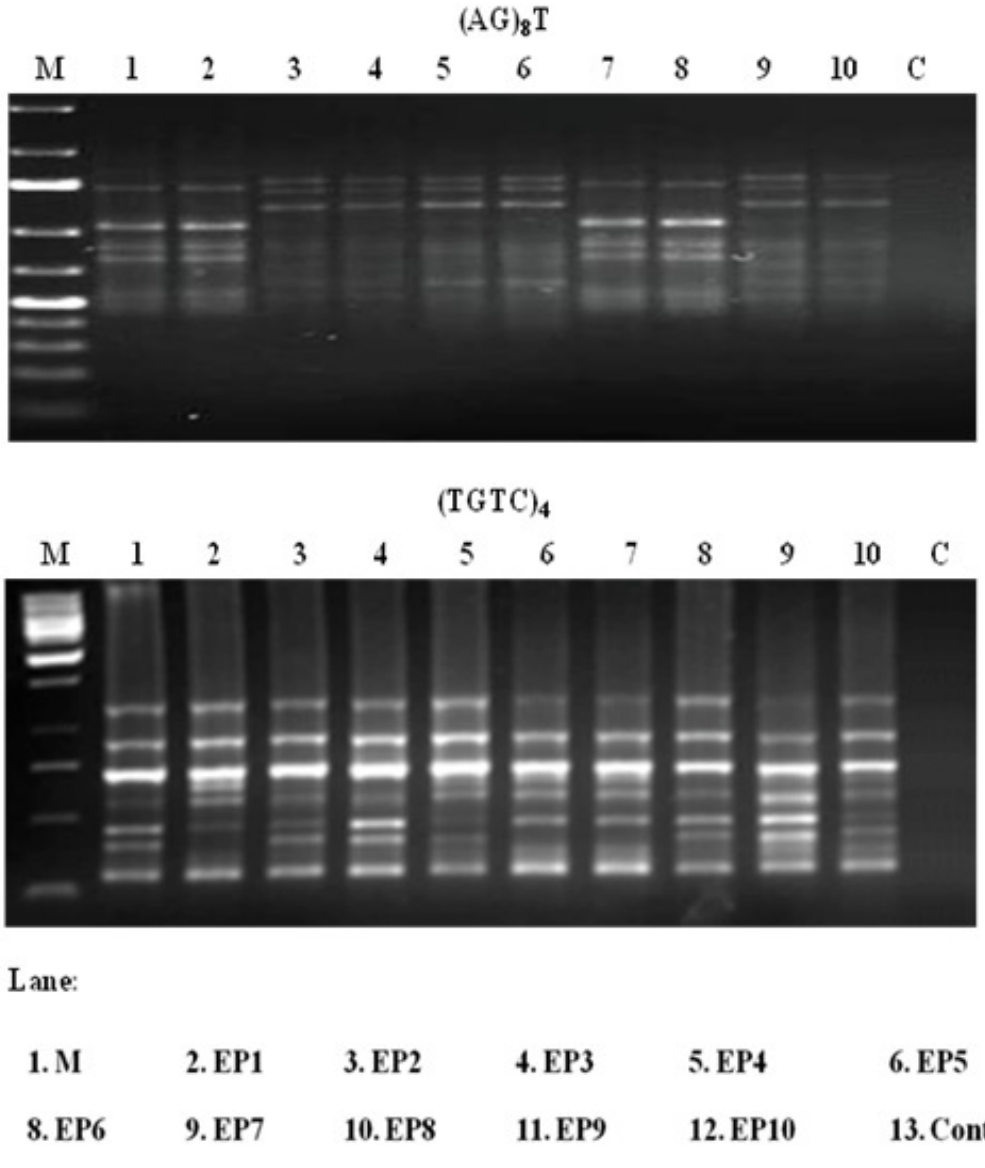


Fig. 3. PCR amplification of ISSR primers

The result of this study demonstrated that powdery mildew is widespread plant disease causing severe yield loss in crop plants. Geographical distribution of this disease is increasing rapidly in different parts of the Nilgiris District. This geographical expansion of *E. pisi* population in confined location has been related to global climate change because powdery mildew pathogen arouses rapidly, showing high rate of variation due to coexistence of anamorphic and teleomorphic stages their quick dissemination capacity [28]. The ITS and ISSR-PCR method is more suitable to study genetically highly diversified species among populations. In contrast to ISSR-PCR method, ITS method provided more clear data in regional genetic discrimination of populations. Consequently, it is proved that ITS method is more suitable for genetic diversity analyses of populations in wide geographic areas. The ITS regions have the highest probability for successful identification of the broadest range of fungi and ITS markers are a popular phylogenetic marker in certain groups [29]. But genetic diversity at the sub-specific level is best explored with a multi-locus approach such as fingerprinting techniques (ISSR, RAPD, etc.).

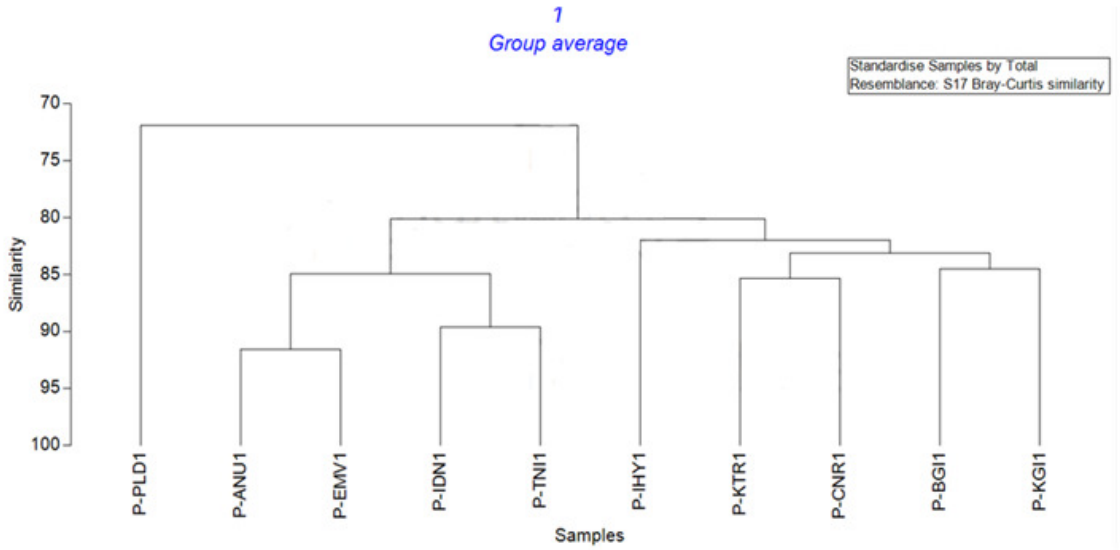


Fig. 4. ISSR - dendrogram showing the relationship between ten isolates of *E. pisi*

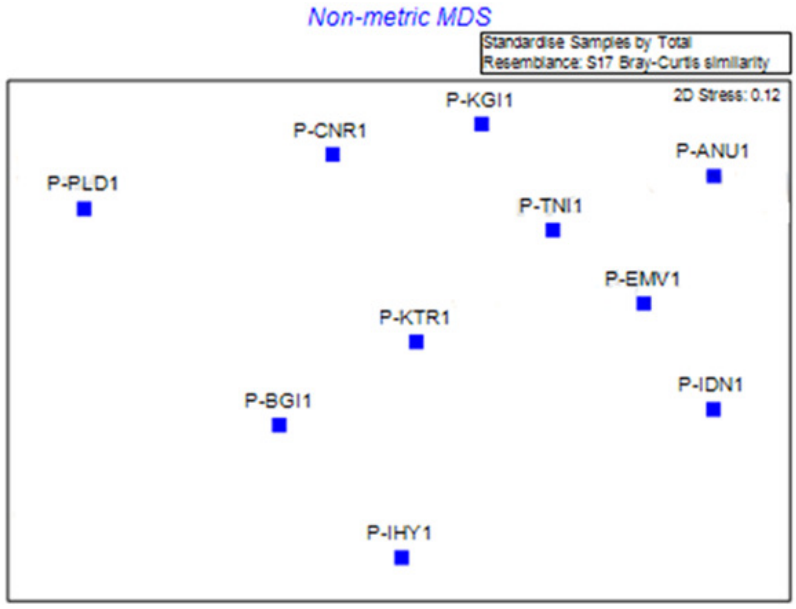


Fig. 5. Non-metric multidimensional scale of ten isolates of *E. pisi*

4. CONCLUSION

In the Nilgiris biosphere, pea is a major crop and is planted in large areas. The uniquely diverse geography and climate provide conditions that are favourable for pea's powdery mildew fungal growth, transference, and the resultant epidemics. This study highlights the potential threat of this disease and its expansion in major peas growing regions of India. The ISSR marker developed in this study were useful to comprehend the genetic diversity and population structure of isolates of *E. pisi,* which were collected from pea growing regions of Tamil Nadu. Understanding the genetic diversity of the pathogen is essential for the development of efficient disease control programs. The data regarding the virulence structure and population diversity of this pathogen that were presented in this study will support more focused efforts in the management of pea powdery mildew. It is known that ISSR is a useful method in genetic

discrimination of closely related populations in the same habitats.

DECLARATION

The Department of Plant Pathology, Centre for Plant Protection Studies, Tamil Nadu Agricultural University is an advisory agent for plant disease management and designated inspection authority for Tamil Nadu state. Therefore, no specific permissions are required for the plant disease survey, including the collection of diseased samples from these defined regions. This study does not involve any endangered or protected species.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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