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Genetic diversity of *Erysiphe necator* (minus mating type) causing powdery mildew of grape in Tamilnadu-India

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Abstract: The fungus causing powdery mildew (*Erysiphe necator*) was collected from nine commercial belts of grape in Tamilnadu, India. Genomic DNA was isolated from conidia and used as template for random polymorphic DNA analysis (RAPD) with OPA1-20 primers. In PCR, 192 different sets of bands were obtained in which 33% were polymorphic. The size of amplicons ranged between 0.2 to 2.0 kb. The hypothesis of Délye was found correct while analysing the presence of (-) mating type of fungus.
Keywords: Random Amplified Polymorphic DNA, Grape, Powdery mildew, *Erysiphe necator*.

Introduction

According to historical evidences, Grape (*Vitis vinifera*) cultivation was introduced by Moghul emperors in northern part of India and the cultivation was spread to peninsular region by French priests. The annual production of grape in India accounts to approximately 1,235,000 metric tons and mainly loss in production (50%) is due to powdery mildew disease caused by *Erysiphe necator* belongs to Phylum Ascomycota and Family Erysiphaceae. It exhibit two life cycles, both sexual (*Erysiphe necator* Schw) and non-sexual stage (*Oidium tuckeri*)[1]. The symptoms of the disease were characterized by white patches of growth on the surface of leaf which produce millions of conidia that spread to fields causing secondary infections and severe yield loss[2].

The genetic variability of a pathogen need to be analysed for effective disease and pest management in agriculture. For assessing genetic variability of fungi various techniques such as RAPD, RFLP, AFLP, SSR, isoenzymes, EST and transposon PCR are available[3]. Nonavailability of complete genome sequence of this fungus makes RAPD a better and reliable choice to assess its genetic variability. Very little work has been carried out in India on *E. necator*. In this paper authors mainly aim to determine the genetic diversity existing in (-) mating type available in Tamilnadu state and also indicating a increased possibility of development of incompatibility for sexual reproduction in this ascomycete in South India[4].

Materials and Methods

Materials

Powdery mildew infected leaves were collected from (Fig. 1) Dindigul (S1, 10.270161, 77.938185), Theni (S2, 10.04619100000, 77.445885), Sempatty (S3, 9.514164, 78.132005), K.K. Patty (S4, 9.744461, 77.308981), N. Patty (S5, 9.711447, 77.315336), Chinamanoor (S6, 9.84029, 77.375396), Royappanpatty (S7, 9.778254, 77.32825), Paalayam (S8, 9.864349, 77.284698) and Appachi pannai (S9, 9.656896, 77.32825). For

RAPD analysis, purchased OPA1-20 operon decamer primers from Operon technologies (USA); PCR buffer, Taq polymerase and dNTPs from New England Bio Labs (USA).

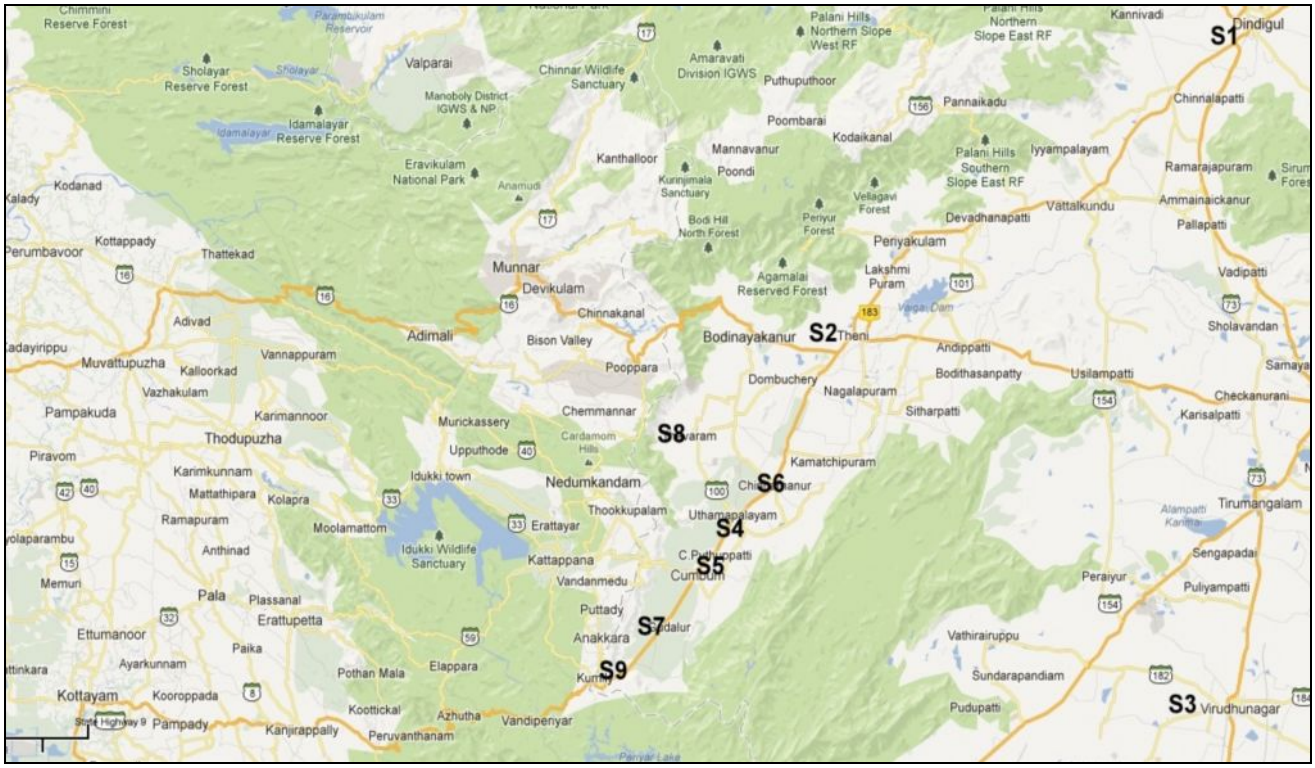


Fig. 1-Map represent the major grape growing regions in the state Tamilnadu. The samples were selected from nine location as shown (S1-S9). S1 (Dindigul; 10.270161, 77.938185), S2 (Theni; 10.046191, 77.445885), S3 (Sempatty; 9.514164, 78.132005), S4 (K.K patty; 9.744461, 77.308981), S5 (N.patty; 9.711447, 77.315336), S6 (Chinamanoor; 9.84029, 77.375396), S7 (Royappanpettai; 9.778254, 77.32825), S8 (Paalayam; 9.864349, 77.284698), S9 (Appachi pannai; 9.656896, 77.32825). (latitude and longitude are given in parenthesis along the name of place).

Sample collection

Collected leaf samples were transported in air tight sterile polythene bags to the laboratory. Since organic farming was practiced in locality S9, to asses the genetic variability, from another 3 km distance (S7) samples were collected. In addition samples were also collected from a locality where it is kept under heavy chemical control (S1).

Microscopic analysis of plant samples

The infected leaves were dried by placing in between Whatmann No. 3 filter paper for 48 hours. SEM images of dried infected leaf samples were taken to confirm the presence of mycelium[5]. The dried leaves were mounted on a clean cylindrical stub. Since the samples were not taken for compositional analysis, after mounting, the samples were coated with a layer of gold (50 nm). After fixation of coated samples, a series of images at different magnifications (500 to 5000x) were taken. The working distance and accelerating voltage were fixed at 8.2 mm and 3 kV respectively in all sessions.

DNA extraction

The conidial DNA was extracted within 48 hours after collection of infected leaves[6]. The reproducibility and robustness of the RAPD analysis depends on the purity of template genomic DNA. Hence two methods of extraction (microwave treatment and modified CTAB method) were executed. In both methods, the fungal conidia were collected using a cut piece (1 x 1cm^2) of office tape by gently jotting on the infected part of the leaf to collect maximum amount of conidia and to avoid extraction of genomic DNA of grape plant.

Modified CTAB method

In this method, to the eppendorfs containing office tapes with conidia, 500 µl of TES (100 mM Tris HCl, pH 8.0; 10 mM EDTA, pH 8.0 and 2% SDS) buffer[7] was added. Then, 1 µl of freshly prepared proteinase K (100 µg/ml) was added and incubated at 60ºC for one hour. After incubation, 140 µl of 5 M NaCl and 64 µl of 10% CTAB was added and incubated for 65ºC for 30 minutes. After that into this mixture, equal volumes of chloroform: isoamyl alcohol (24:1) was added and centrifuged at 12,000 rpm for 10 minutes. The aqueous layer was transferred to a new eppendorf tube then ice cold isopropanol (0.6v) and 3M sodium acetate (0.1v) were added to precipitate DNA, 70% ethanol wash was done twice and the resultant DNA was suspended in TE buffer having RNAse. The quality and quantity was analyzed in 1% agarose gel having 0.5 µg/ml EtBr electrophoresed at 5 v/cm for an hour.

Microwave treatment

The eppendorfs, containing office tape with conidia and 500 µl of TES buffer, was subjected to microwave treatment for 15 seconds, followed by an incubation at 60 ºC for 30 minutes. After that sample was centrifuged at 3000 rpm for 5 minutes. Precipitation of DNA was done by adding ice cold isopropanol (0.6v) to the supernatant and DNA was suspended in TE buffer having RNAse after washing in 70% ethanol. The quality and quantity was analyzed as above.

PCR analysis

Different concentrations of the template DNA (1, 3, 5 and 10 ng) were used in the PCR reactions[8]. The reaction volume (20 µl) was made using 5x PCR buffer, 10 mM dNTPs, 1 unit Taq polymerase, 10 pM primer and template DNA. The PCR conditions: step.1 initial denaturation (95ºC - 3 minutes), step.2 denaturation (92ºC – 1 minute), step.3 annealing (38ºC - 1 minute) and step.4 extension (72ºC - 1 minute) for 32 cycles with a step.5 final extension at 72ºC for 4 minutes. From reaction volume, 15 µl were loaded onto 1.6% agarose gels containing 0.5 µg/ml EtBr. Electrophoresis was done in 1x TAE buffer at 3 V/cm for 3 hours. The gels were photographed using Uvitech Cambridge Fire Reader gel documentation system (UK). These photographs were used for data analysis later.

RAPD analysis

Reproducible bands were considered for data analysis[9]. The repeatability of the bands for every individual primer were calculated using the formula $R_b = 2b_{12} /(b_1 + b_2)$, where b_{12} is the total number of individuals possessing the band 'b' in both the replicates while b_1 and b_2 are the number of individuals that possess the band in the first and second replicate respectively. The values for band repeatability generally range from 0 to 1. Values closer to 1 reflect on the ease of repeatability of the bands.

The genetic dissimilarity (D) between isolates were calculated using the Jaccard[10] and Sorensen-Dice coefficient (also called as Nei and Li coefficient)[11]. In the Jaccard coefficient, the similarity values are calculated using the formula, S = a/(a+b+c), where 'a' represents the bands that are present in both the isolates, 'b' and 'c' represents the total number of bands present in the two isolates respectively. In the Sorensen-Dice coefficient, the similarity values were calculated using the formula, S = (2a/(2a+b+c) where 'a' represents the bands that are present in both the isolates, b and c represents the total number of bands present in the two isolates respectively. In both case, the distance values were calculated using the formula D = 1-S, where S corresponds to the similarity value and D represents the distance value. The RAPD data matrix was analyzed using FREETREE[12] to generate distance matrix and 1000 bootstrap resamplings were done. The reference tree saved in PHYLIP format was viewed using TREEVIEW[13].

Determination of mating type

Delye et al[4] suggested that an amplification of 1000 bp band using primer OPE7 show the presence of plus (+) mating type and absence would reflect to minus (–) mating type. Mating type was determined using 5 ng of genomic template.

Results

RAPD analysis

Conidia produced from the mycelium on grape leaf surface (Fig. 2) was used for genomic DNA extraction and through CTAB method good quality DNA was obtained than microwave method[14]. For RAPD analysis, PCR amplicons were obtained using varying concentration of template DNA such as 5, 3 and 1 ng (Fig. 3). Among OPA1-20; bands from OPA6 and OPA17 were not reproducible and OPA15 did not amplify any DNA region.

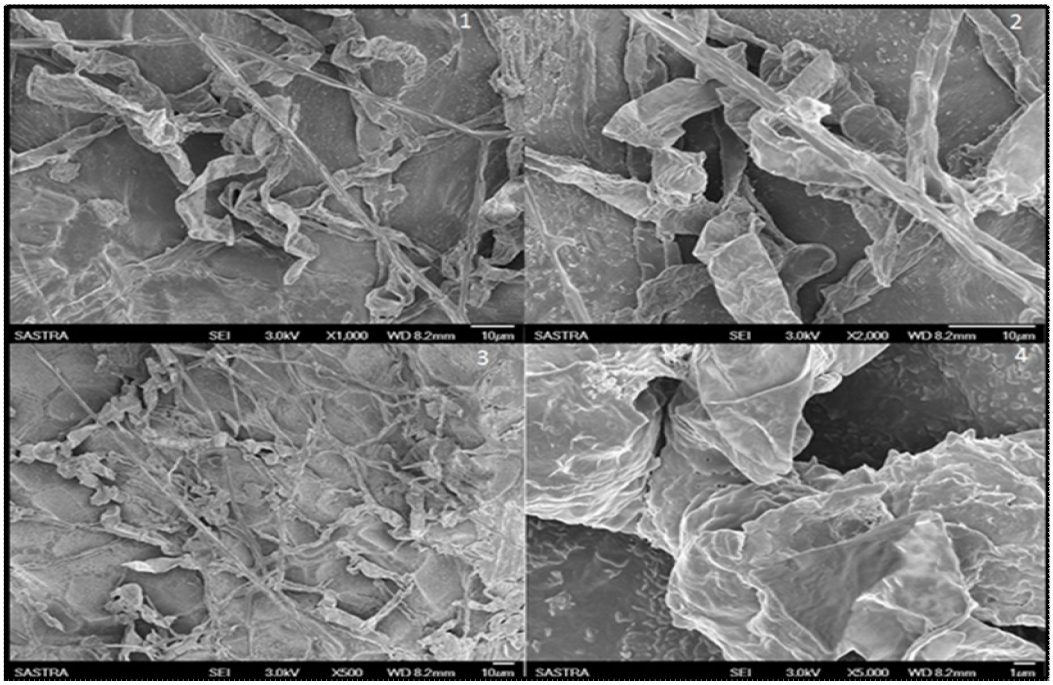
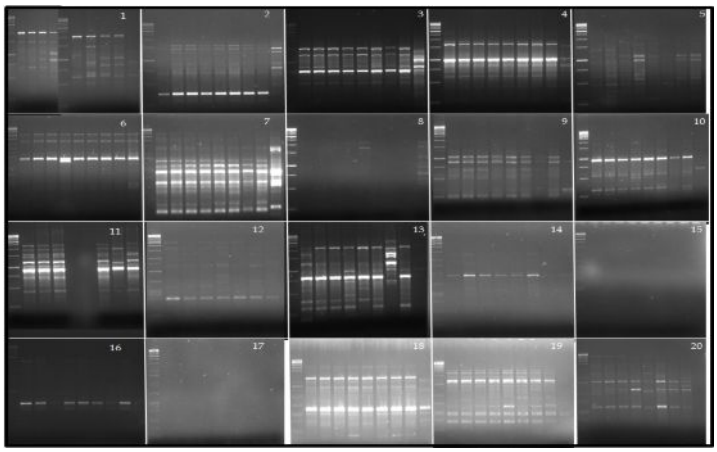
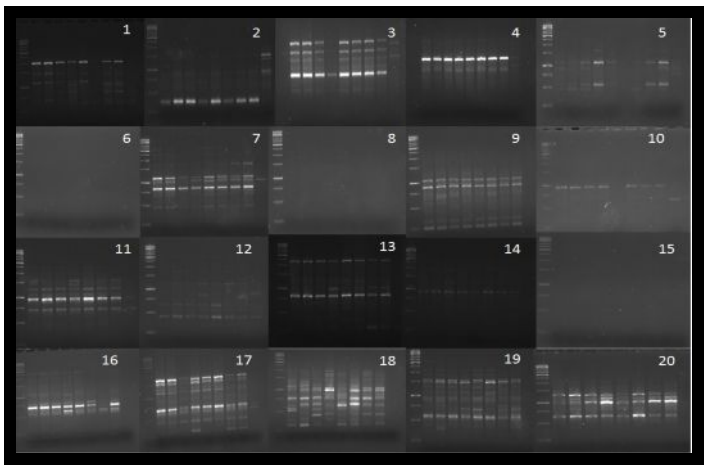


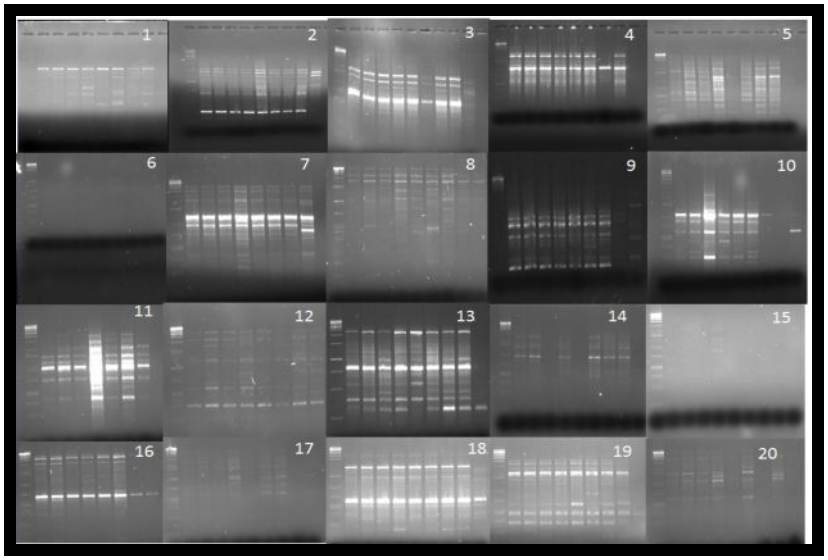
Fig. 2 – SEM images of dried grape leaf samples infected with powdery mildew. Dried mycelium could be observed with ease on visual inspection. In the plate, top left (1) is 1000x; top right (2) is 2000x; bottom left (3) is 500x; bottom right (4) is 5000x. The working distance (WD) was fixed at 8.2mm and accelerating voltage at 3kV in all the cases.



A



B



C

Fig. 3-Electrophoretic separation of PCR amplicons obtained with operon primers at three different concentrations of template DNA using 1.6% agarose gel having 0.50µg/ml EtBr in 1x TAE buffer. The gels were run at 3v/cm for 3 hours. From reaction volume 15µl were loaded onto each wells. Lane 1-10 represents, DNA molecular weight marker, S7, S9, S4, S5, S8, S2, S6, S3, S1 in the order.. The number on each gel present at the top left portion represents every individual primer. A. 5 ng; B. 3 ng; C. 1 ng.

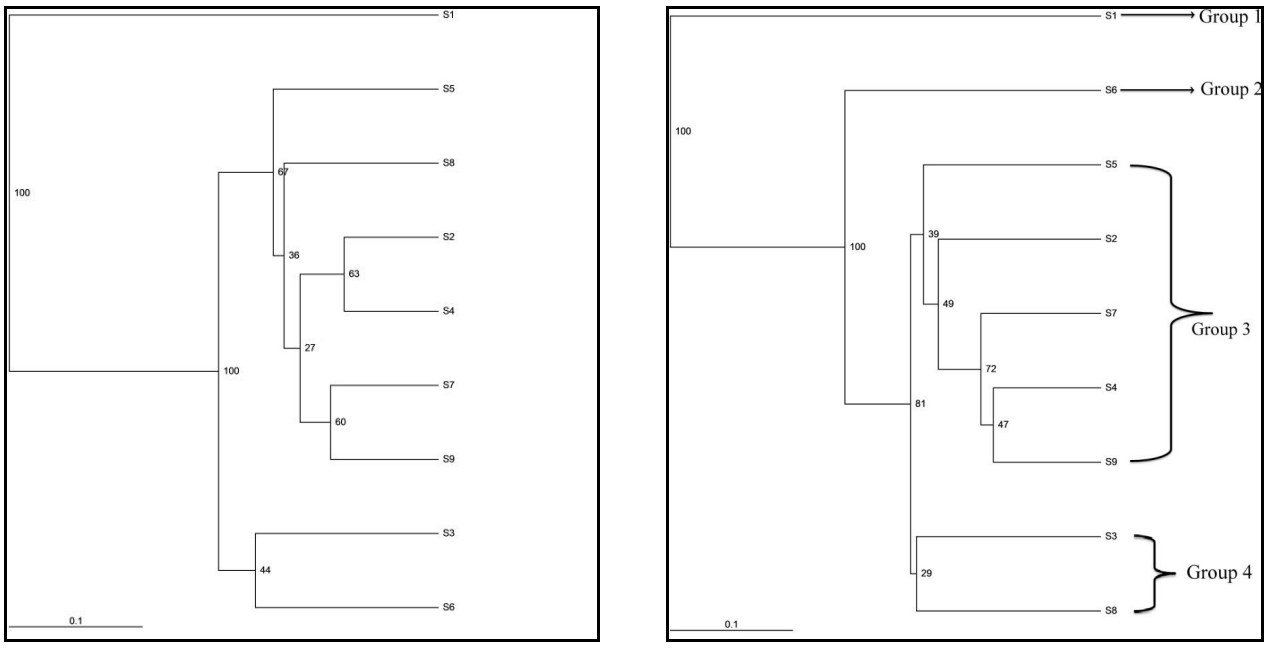


Fig-4 Dendogram generated with Sorenson – Dice coefficient (A) and Jaccard coefficient (B).

The phylogenetic tree (Fig. 4) was constructed with Jaccard and Sorensen-Dice coefficients. The band repeatability values (Table 1) suggested fair amount of band repeatability. The similarity matrix generated using both the coefficients (Table 2 A & B) show fair amount of genetic similarity between the isolates present in the location. In the similarity matrix generated using Sorensen-Dice coefficient, the lowest value of similarity points to 0.36686 while the highest value being 0.90377. In the similarity matrix generated using Jaccard coefficient, the lowest value of similarity is 0.22464 and the highest value being 0.82443. Out of twenty, 17 decamer primers primed total 192 RAPD bands. In this 63 (33%) were polymorphic bands and 13 bands were unique (Table 3).

The cluster analysis showed the extent of variability among the isolates in the region. The isolate S1 is far from other isolates. However, the isolates S4 and S9 formed a subgroup inside the group containing members S7, S2 and S5. The isolate S6 was also placed separately as a group. The isolates S3 and S8 took to another group being very close in genetic distance.

Table 1-Band repeatability value for each operon primer. The band repeatability values were calculated for each primer using the formula $R_b = (2b_{12})/(b_1+b_2)$ where b_{12} = number of individuals possessing band b, b_1 and b_2 are the numbers of individuals possessing that band in first and second replicate.

Primer	Band Repeatability value
OPA 1	0.747
OPA 2	0.830
OPA 3	0.912
OPA 4	0.890
OPA 5	0.742
OPA 6	0
OPA 7	0.784
OPA 8	0.878
OPA 9	0.786
OPA 10	0.749
OPA 11	0.826

OPA 12	0.740
OPA 13	0.844
OPA 14	0.625
OPA 15	0
OPA 16	0.905
OPA 17	0
OPA 18	0.945
OPA 19	0.915
OPA 20	0.723

Table 2A-Lower triangular matrix of similarity values generated using Sorenson – Dice coefficient. The values of similarity were calculated using the formula, S = (2a/(2a+b+c)) where S = Similarity value, a = present in both samples (1,1),b = present in sample i, absent in sample j (1,0),c = present in sample j, absent in sample i (0,1).

S1	0							
S2	0.46316							
S3	0.47059	0.81328						
S4	0.48045	0.87554	0.80870					
S5	0.43655	0.82869	0.82258	0.85833				
S6	0.36686	0.73543	0.80000	0.74528	0.73913			
S7	0.48913	0.82353	0.82553	0.88106	0.82449	0.71889		
S8	0.47778	0.82906	0.82251	0.83408	0.80498	0.69484	0.82456	
S9	0.45918	0.84000	0.79352	0.90377	0.80934	0.70742	0.90164	0.8000

Table 2B-Lower triangular matrix of similarity values generated using Jaccard coefficient. The values of similarity were calculated using the formula, S = (a/(a+b+c)) where S = Similarity value, a = present in both samples (1,1),b = present in sample i, absent in sample j (1,0),c = present in sample j, absent in sample i (0,1).

S1								
S2	0.30137							
S3	0.30769	0.68531						
S4	0.31618	0.77863	0.67883					
S5	0.27922	0.70748	0.69863	0.75182				
S6	0.22464	0.58156	0.6667	0.59398	0.58621			
S7	0.32374	0.70000	0.70290	0.78740	0.70139	0.56115		
S8	0.31384	0.70803	0.69853	0.71538	0.67361	0.53237	0.70149	
S9	0.29801	0.72414	0.65772	0.82443	0.67974	0.54730	0.82090	0.6667

Table 3-Observed number of unique bands in the RAPD – PCR analysis. Thirteen unique bands were observed in the 192 unique bands and these bands can be used for SCAR marker development.

Sample name	Primer	Band size (base pairs)
S1	OPA 4	1600
	OPA 5	260
	OPA 9	1800
	OPA 10	550
	OPA 13	1000

S2	OPA 1	600
S4	OPA 7	1600
S5	OPA 5	980
	OPA 8	1500
	OPA 10	300
	OPA 11	1700
S7	OPA 13	180 & 600

Determination of mating type

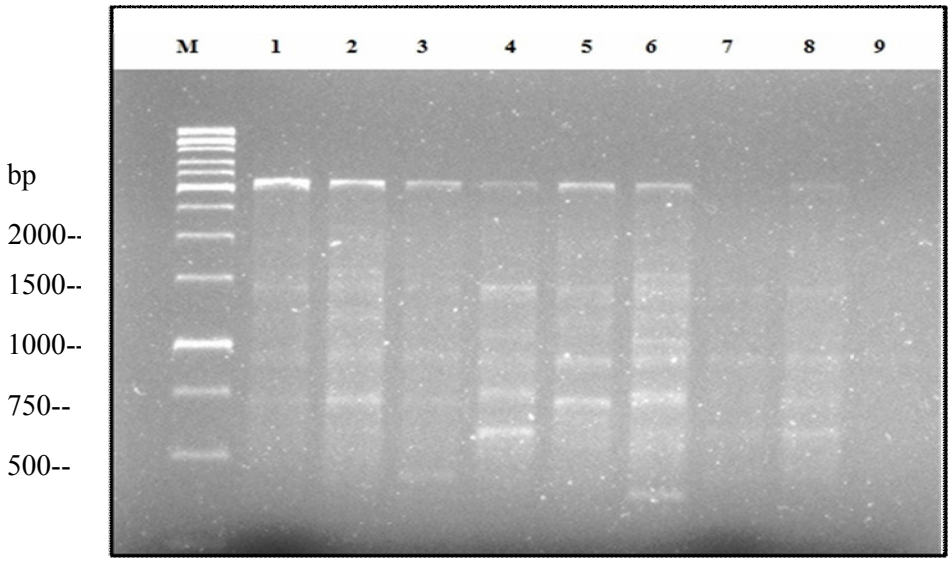


Fig. 5-Determination of mating type in *E. necator*. The samples were subjected to PCR amplification using OPE 7 primer as suggested by Delye and no band was observed at 1kb, indicating the absence of + mating type in the sampling region

The RAPD marker for + mating type, a 1000bp fragment was not observed in any of the isolates (Fig. 5) supporting the hypothesis of Delye that there is no sexual life cycle for *E. necator* in South India.

Discussion

The genetic diversity directly reflects the pathogens ability to adapt to surrounding environment. Thus, the extent of diversity observed in a pathogen could help in understanding the extent of adaptability. In our study we observed a fair amount of genetic diversity in *E. necator* which leads to the assumption that the pathogen has acquired its ability to adapt to both the macro and micro environment to which it is subjected to in the vine yards.

A new method of fungal DNA extraction using microwave reported in *M. grisea* was not successful in our experiment. Delye et al[4] used 95 primers with a polymorphic band generation of 4% while Miazzi et al[15] took up 23 primers of which 2% were polymorphic. In our study, the selection of primers were unbiased and 33% polymorphism was calculated from minus mating type.

The decamer primer OPA15 which did not amplify any DNA region can be used in combination with other primers to distinguish *E. necator* from fields. Upon amplification with OPA1-20, except OPA6 &17, if there is presence of amplified products then it could indicate the possibility of the sample not being *E. necator*. In our study, it was observed that the fungal pathogen stood apart in 4 closely knit groups, group 1 through 4 comprising a sub group in group 3. In group 4, the isolates S3 and S8 stood circumferent, though they are geographically apart. This might be due to exchange of plant cuttings between vineyards present in both the areas and not through wind. The wind factor can be ruled out due to the fact that, in recent studies, the dissemination of fungal spores of *E. necator* through wind for higher distances has been proved not possible[16].

In a phylogeographic analysis conducted in Southern France by Jean Pierre Person et al[17] using RAPD analysis and NAS PCR technique, a total of 101 isolates were used. In the study, seven primers were used which generated 20 polymorphic bands and the isolates clustered into two different groups. Rahman Yousefi[1] conducted a survey on genetic variation in Iran using RAPD analysis with 24 isolates and 11 primers were employed which yielded 97 amplicons. Of the 97 amplicons, 91 amplicons were polymorphic and clustered in seven distinct groups. Delye[4] conducted RAPD analysis of *E. necator* with 96 isolates collected from various parts of Europe and India. In the study, 46 primers were used which yielded 414 amplicons. Out of the 414 amplicons, 216 were polymorphic and clustered into three different groups. The heterothallism existing in *E. necator* can lead to low disease incidence if more variability is observing in a single mating type[18].

In yet another population study on *E. necator* carried out by Miazzi et al[15] in Italy using RAPD analysis, 374 isolates from 31 vineyards were collected. 23 decamer primers were used in the study from which 137 amplicons were obtained among that 55 amplicons were polymorphic and clustered in two groups with 7 subgroups. In a similar study carried out on Australian isolates using RFLP probes by Stummer[16], the isolates clustered into two groups with respect to the mating type and 90% polymorphism was observed with all the four probes used.

In our study, the isolates stood in four different groups. On comparison with the aforementioned works, the way the isolates have clustered seems to be varied with every location. An extremely wider area of sampling in Delye's experiment, the clustering seems to produce three different groups while in Yousefi's[1] + mating type produce four different groups. Since only the presence of – mating type was observed in the fields, it could be deciphered that only asexual production is present is these parts. When powdery mildew was introduced from North America to Europe, for the first 4 decades, no instances of cleistothecia were recorded. Gaduory and Pearson stated that this was due to the delayed compatibility[19] between the two isolates. There have been no reports of cleistothecia appearance in regions of Tamilnadu till now. A concrete conclusion on the account of cleistothecia cannot be brought about until there is an appearance of the same in the vine yards of Tamilnadu which may lead to more delayed compatibility.

Owing to the fact that there is no dormancy of grape plants in the South India[20] the pathogen overwinters in either the bud or the shoots, giving it ample time and opportunity to disseminate through air to the nearby regions. Thus the deposition and reach of the secondary inoculum to the fresh leaves and healthy plant parts would be high leading to increase in the spread of the disease. On a note of conclusion it could be seen that 1) the genetic distance between isolates seems varied across the samples of Tamilnadu. 2) Organic and modern methods of farming does not seem to cause much diversity in the pathogen, pointing to a need in both the change of control practices and a detailed study on the pathogen in the near future. 3) The genetic distance observed is not due to the sexual reproduction as there is a rarity of + mating types in the populace of *E.necator* present in Tamilnadu region. The climatic conditions might have not allowed the growth of + mating type in India. Disease severity is maily due to the genetic chages caused by overwintering. It is necessary to conduct further experiment by crossing –mating type existing in India with + mating type.

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