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Phenotypic Characterization and Molecular Phylogenetic Relationship of *Erysiphe necator* Infecting Grapes (*Vitis vinifera)*

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Authors' contributions

This work was carried out in collaboration among all authors. Authors AK, VGM, VP, US and MK designed the study, supervised and facilitated the research and wrote the first draft of the manuscript. Author NG managed the literature searches. Author MK performed the experiments and analyzed the results obtained in the study. All authors read and approved the final manuscript.

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ABSTRACT

Grapes powdery mildew is caused by the most destructive pathogen *Erysiphe necator* leading to severe yield losses around the world. In order to study the phenotypic and molecular characters, the powdery mildew infected leaf samples were collected from eight different places in Coimbatore and Theni districts in the state of Tamil Nadu India. The identity of the pathogen as *E. necator* was established by microscopic studies and the isolates were further confirmed molecularly by amplification of Internal transcribed spacer (ITS) and Cytochrome b gene (Cyt b). Further molecular

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confirmation was obtained by characterizing Cytochrome b. An amplicon size of ~ 367 and ~ 470 bp were obtained from amplification with Uncin144 and Uncin511 and Cyt b F and Cyt b R gene primers respectively. The identity for cyt b gene segment was 96 to 98%, similarity with *E. necator* isolates deposited in NCBI genbank (KY418048.1, KY418049.1).
A phylogenetic tree was constructed on the basis of nucleotide sequence of cytochrome b gene of the study isolates as well as *E. necator* and other *Erysiphe* species in NCBI database. From the tree it was evident that the study isolates from Tamil Nadu, India were very distinct from other *E. necator* isolates deposited in NCBI genbank database.

Keywords: Grapes; powdery mildew; E. necator; ITS; cytochrome b region; PCR; detection; phylogenetic analysis; sequence identity matrix.

1. INTRODUCTION

Grapes (*Vitis vinifera)* powdery mildew is a world devastating disease caused by *Erysiphe necator* (Schw.) Burr. (synonym Uncinula necator), an obligative biotrophic ascomycetes fungi [1,2]. The fungus infects all green tissue of the grapevine including, leaves, stems flowers and fruits. The disease symptoms are due to presence of sign of the pathogen including superficial mycelial structures consisting of conidiophores and conidia of the fungi on the infected host organ. Epidemic outbreak of grapes powdery mildew in Europe from 1847-1854 was recognized as the first report of severe economic yield loss [3].

Symptoms of powdery mildew vary throughout the growing season both in grapevine and different developmental stages in *E. necator*. All growing fresh tissues shows high compatible reaction with *E. necator*. Initially the lesions appear as small discolored area followed by the formation of white, thin powdery layer of fungal structures [4]. The infection also reduces the net photosynthesis and degrades wine quality and finally causes dramatic yield losses [5].

The detection of plant pathogen through PCR has been well developed currently in order to identify the fungi, bacteria and viruses [6,7].

Mostly detection of fungal species depends on amplification and sequencing of internal transcriber spacer (ITS). High variability in ITS region among the populations of the same species has been reported [8]. Cytochrome b (cytb) region play a significant role in studying the phylogenetic relation in higher fungi and Oomycota [9]. Specifically, the cyt b gene is known to harbor broad variation at intra-specific level which leads to molecular identification of species in a fine manner and in its taxonomy and also genetic studies in population level.

2. MATERIALS AND METHODS

2.1 Collection of *Erysiphe necator* Infected Powdery Mildew Leaf Samples from Different Places

The powdery mildew infected leaf samples were collected from different plantations in different villages in Coimbatore and Theni district, Tamil Nadu, India. In Coimbatore, India the samples were collected at Tamil Nadu Agricultural University orchard and the village Mathypalayam. In Theni the infected samples were collected at Grape research station (Anamalayathanpatty), Kamayagoundanpatti**,** Rayappanpatty, Anaipatty, Surulipatty, and Cumbum. The conidial spore mass was collected and stored in -20ºC for further use.

2.2 Microscopic Observation of *E. necator* Infected Leaf Sample

Grapes powdery mildew infected leaf samples expressing the typical symptom of white powdery growth of fungus consisting of conidiophores and conidia of the fungus was sectioned, and observed under light microscope (Labomed – IVU 5100). The image was photographed using a Labomed camera model LX400 with an image analyzer - pixel pro programme. For every location fifty conidia were observed for its morphometric analysis (Fig. 2).

2.3 Genomic DNA Extraction from *E. necator* Isolates

The DNA was isolated from the conidia of *E. necator* following the protocol [10] with some modification. About 200 mg of conidia were collected from powdery mildew infected leaves using a camel hair brush and transferred to a Micro centrifuge tube containing 500 µl of CTAB extraction buffer (50 mM Tris- HCl, PH 8.0; 0.7 M NaCl and 1% CTAB (w/v) vortexed for 30 s and

incubated at 60ºC for 1 h. After incubation, the mixture was centrifuged at 13,000 x g for 10 min and the supernatant was collected and extracted twice with an equal volume of Chloroform: isoamylalcohol (24:1). The aqueous phase was transferred to a 1.5 ml Micro centrifuge tube and the DNA was precipitated by addition of an equal volume of cold isopropanol and incubation at -20ºC for 1 h. The DNA was collected by centrifugation at 13, 000 x g at 4ºC for 10 min. the pellet was washed twice with cold 70% ethanol, air dried and resuspended in 50 µl of Tris- EDTA buffer (10mM Tris- HCl and 1 mM EDTA, pH 8.0). The genomic DNA was checked by agarose gel electrophoresis and the concentrations of the DNA were determined using a Nanodrop ND-3300 Fluoro spectrometer (NanoDrop products, Thermo Scientific, Wilmington, DE, USA).

2.3.1 Specific primer designing for *E. necator*

PCR amplification of ITS region was performed with Uncin144 (5-CCGCCAGAGACCTCATCCAA-3) and Uncin511 (5- TGGCTGATCACGAGCGTCAC-3) primers. The nucleotide sequence data of *Cytochrome b (cytb)* gene of the powdery mildew isolate from NCBI available under, Accession number KY418049.1 **(**https://www.ncbi.nlm.nih.gov/nuccore/KY418049.1**)** were used for designing primers which was

Fig 1. Symptoms of grapes powdery mildew observed under field condition

Disease symptoms caused by E. necator on grapes A) symptoms of the disease mostly visible adaxial leaf surface. b) Development of individual spots at initial stage of infection b) spots cover the entire leaf at later stage of infection c) White mycelial growth on berries at early stage d) At matured stage fungal growth covers entire fruit and splitting of berries occurs (white arrow)

Fig. 2. Phenotypic structures of *E. necator* under light microscope at 40X magnification

A) Formation of septate hyaline conidiophores white arrow indicates the septation in conidiophores B) Single celled hyaline conidia at the tip of conidiophores white arrow indicates conidiophores, red arrow indicates single celled conidia C) Formation of oval, hyaline and single celled group of conidia

specific to *E. necator*. The set of Cyt PM F and Cyt PM R primer sequences were designed through NCBI online primer designing tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and the primer sequences are: forward primer cyt b F (TGTTGTAATATTTATTTTAATG) and cyt b R reverse primer (TGGGTTAGCCATAATATAA). PCR reactions were set up in 20µl mixture containing ~50 ng of total DNA, 10 µl of TaKaRa master mix (2X concentration) and 20 pmol each of forward primer and reverse primers. The reaction was carried out in thermocycler (Eppendorf master cycle). The PCR program for the amplification of Uncin144 and Uncin511 region consisted of an initial denaturation of 3 min at 94ºC followed by 40 cycles of denaturation at 94ºC for 30 s, 1 min of annealing at 68ºC, 90 s of extension at 72ºC and a final extension for 7 min at 72ºC. For cyt b F and cyt b R the cycle conditions are initial denaturation of 3 min at 94ºC followed by 40 cycles of denaturation at 94ºC for 30 s, 1 min of annealing at 42ºC, 90 s of extension at 72ºC and a final extension for 7 min at 72ºC. Finally Amplified products were separated by electrophoresis in 1.0% agarose containing ethidium bromide (give concentration) at 80 V for 2-3 h and documented in an gel documentation unit (Alpha Imager EC (USA)).

2.3.2 Sequencing and phylogenetic tree construction

The amplified PCR products were sequenced by Sanger's dideoxy sequencing method and the obtained sequences were edited and aligned. In order to study the variability, the sequence of collected *E. necator* isolates were compared with those of *E. necator* and other species like *E. alphoides*, *E. polygoni* available in NCBI database. The sequence identity matrix was constructed between the isolates with bioedit software version 7.2. Further the phylogenetic tree was developed by using Mega 7.0 software [11] including the sequences of *E. alphitoides* (JN981011.1) *E. polygoni* (KF925326.1) which were retrieved from NCBI database by Maximum neighbor joining method.

3. RESULTS AND DISCUSSION

3.1 Symptoms and Phenotypic Characterization

The grapes leaf samples expressing typical symptoms of whitish dull powdery growth were collected from Coimbatore and Theni district (Fig. 1).The collected isolates were examined for various phenotypic characters by thin sectioning of infected leaf. Fungal structures were observed under light microscope. The conidial size of different isolates was measured at 40 X magnification. The average size of the conidia varies from 31.74 – 36.43 µm in length and 12.85- 15.45 µm in breadth. Largest size of conidia was observed in Rayappanpatty with an average size of 36.43µm in length and 15.45 µm in breadth (Table 1). The observed results are similar to observations of Calonnec et al., 2004 [12] and Stummer et al., 2005 [13] who have studied the morphological of characters of Genus Oidium and symptoms of grapes powdery mildew caused by *E. necator.*

3.2 PCR amplification of *E. necator* through ITS and Specific Primer

The *E. necator* isolates collected from different places were initially amplified with ITS specific primer (Uncin144 and Uncin511) to *E. necator* developed, An amplicon size of ~367 bp with ITS primers confirming the test samples to be *E. necator* isolates (Fig. 3). The species specific primers targeting cyt b gene designed in this study when used in PCR specific amplicon of 470 bp were obtained (Fig. 4). The characteristic amplicon was observed in eight out of eight grapes powdery mildew infected leaf samples collected from different places. The amplicons from eight locations were sequenced and sequences are available in NCBI database under accession numbers MK637521.1 , MK685859.1, MK693024.1, MK693023.1, MK637520.1, MK704508.1, MK704509.1, MN116456 (Table 2).

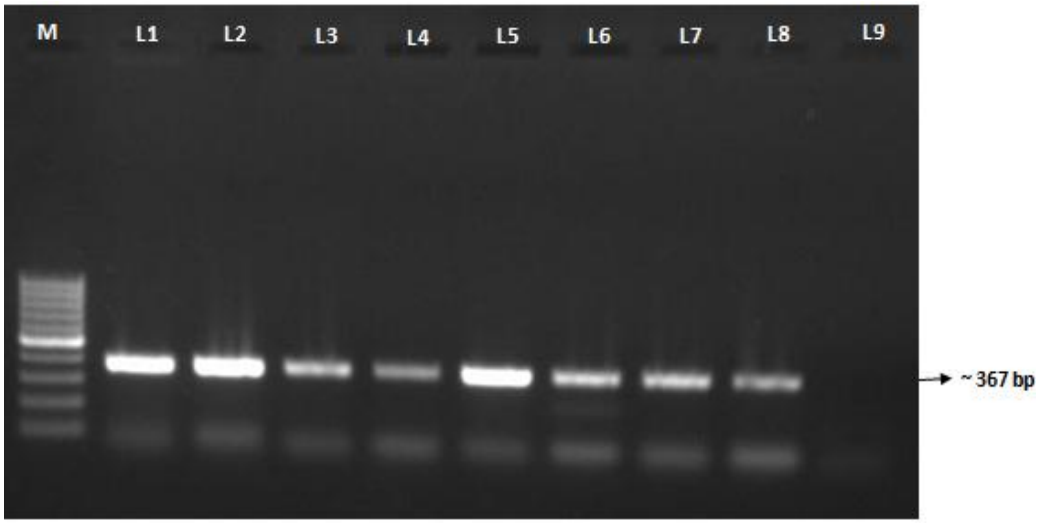

Fig. 3. PCR amplification of Uncin144 and Uncin511 region in *E. necator*

Primer sequences used were forward Cyt b (TGTTGTAATATTTATTTTAATG) and reverse cyt b (TGGGTTAGCCATAATATAA). M – 100 bp ladder, Total DNA isolated from fungal propagules on leaves used as DNA template following are the locations : L1- TNAU, L2- Mathipatty, L3- Kamayagoundanpatti, L4- Anaipatty, L5- Cumbum, L7- GRS AMP, L8- Rayappanpatty, L9- Negative control (Nuclease free water).

Table 1. Phenotypic character of conidial size measurement of *E. necator* at 40X Magnification

S. No	Place	District	Length (µm)	Breadth (µm)
1.	TNAU	Coimbatore	32.46	13.05
2.	Mathipatty	Coimbatore	34.89	15.07
3.	Grape research station (Anamalayapatty)	Theni	33.26	13.78
4.	Kamayagoundanpatti	Theni	31.74	12.85
5.	Rayappanpatty	Theni	36.43	15.45
6.	Anaipatty	Theni	35.59	15.21
7.	Surulipatty	Theni	34.76	14.94
8.	Cumbum	Theni	34.91	15.14

Table 2. Accession number of nucleotide sequence of Cyt b gene of study isolates of *E. necator*

S. No	Location	*E. necator* isolates	Accession Number
1	TNAU	TNAU	MK637521.1
2	Mathipatty	MP	MK685859.1
3	Grape research station (Anamalayapatty)	GRS AMP	MK693024.1
4	Kamayagoundanpatti	KP	MK693023.1
5	Rayappanpatty	RP	MK637520.1
6	Anaipatty	ANP	MK704508.1
7	Surulipatty	SP	MK704509.1
8	Cumbum	CM	MN116456

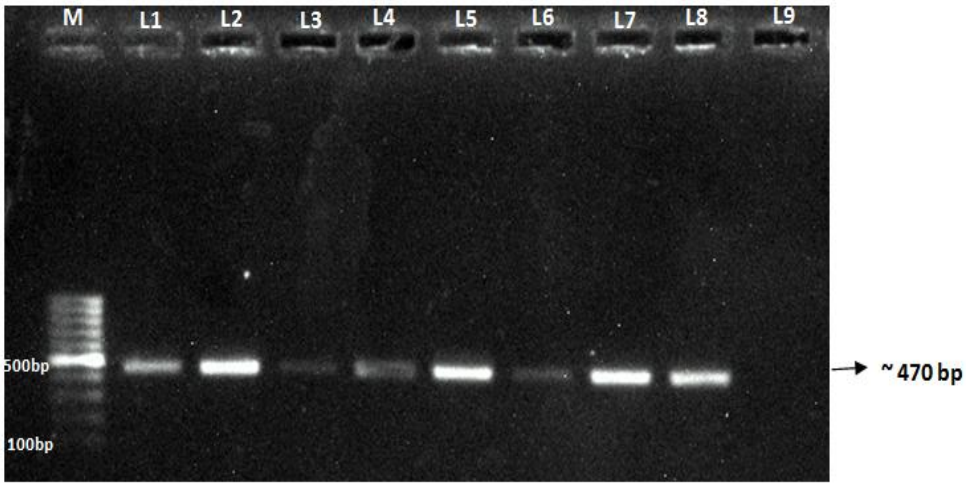

Fig. 4. PCR amplification of Cytb gene in *E. necator* isolates

Primer sequences used were forward cyt b (TGTTGTAATATTTATTTTAATG) and reverse cyt b (TGGGTTAGCCATAATATAA). Total DNA isolated from fungal propagules on leaves used as DNA template. M – 100 bp ladder, L1- TNAU, L2- Mathipatty, L3- Kamayagoundanpatti, L4- Anaipatty, L5- Cumbum, L7- GRS AMP, L8- Rayappanpatty, L9- Negative control (Nuclease free water)

3.2.1 Phylogenetic tree construction and sequence identity matrix

The amplicons obtained with study isolates were sequenced and the matching similarity was performed through NCBI BLAST analysis which shows 96 to 98 % similarity with *E. necator* isolates KY418049.1 (SAA2) and KY418048.1 (HP1), But the similarity was less with other isolates JN981011.1 *Erysiphe alphitoides* (clone 1) and KF925326.1 *Erysiphe polygoni* (H101) (Fig. 5). Results clearly established the primer specificity to distinguish *E. necator* isolates from other isolates. Identity matrix generated on the basis of nucleotide sequence of cyt b gene (Table 3) clearly revealed the difference among the isolates of *E. necator*. The study isolates shared nearly 99 to 100% identity between them.

Interestingly, when identity with one isolate from *E. necator* (Genbank no KY418049) was as high as 98-99%, it was less (95-96%) with another isolate (Genbank no KY418048). All isolates of *E. necator* shared only 89-94% identity with other two species, *E. alphitoides* and *E. plolygoni*. Phylogenetic tree constructed on the basis of nucleotide sequence revealed the relationship between the isolates. All study isolates clustered together well separated from two isolates *E. necator* (Genbank no KY418049) and *E. necator* (Genbank no KY418048) from *E. alphitoides* (Genbank no JN981011.1) *E. polygoni* (Genbank no KF925326.1). However within the study isolates the isolate from Cumbum (Genbank no MN116456) was found to be distinct. As such *E. necator* isolates occupied separate clade well separated from *E. alpitoides* and *E. polygoni*.

The use of molecular characters specifically ITS region provide a promising results for determination of species in some powdery mildews [14,15,16,17]. The molecular confirmation of ITS region in Erysiphales should provide an accurate information about anamorphic stage of the fungi. These primers also appear to be suitable for detection of most if not all isolates of *E. necator* because they successfully amplified numerous *E. necator* isolates from diverse geographic regions. The Uncin144 and Uncin511 specific primers are highly suitable for detection of most isolates of *E. necator* from diverse geographic regions. In the present communication attempts were made to develop more specific PCR diagnostic marker for detection of mildew pathogen. Therefore the cytochrome b gene was selected, which detected the pathogen in 100 % of samples tested. Cytochrome b gene is encoded by the mitochondrial genome [18]; and DNA of mitochondria exists as large copies per cell [19,20,21] which will help in easy detection of the pathogen. Miles et al., 2012 [22] utilized cytochrome b (CYTB) gene based conventional PCR assay for the detection of the presence or absence of the G143A mutation because which is responsible for Strobilurin (QoI) resistance in *E. necator*. Similar attempts have been made by Fernandez et al., 2018 [23] applied specific primer F3cytb-Px and R3cytb-Px targeting Cytochrome b gene for the detection of *Podosphaera xanthii* in zucchini. The primer sequences were designed by Kears et al. 2012 [24] On the basis of sequences based on sequence data, contigs c364 and c24909 retrived from haustorial and epiphytic transcriptome of *P. xanthii*. Sandra Mosquera, (2019) [25] reported G143A substitution in within field populations of *Leveillula taurica* in tomato which confer high level of resistance against Q_oIs in several fungi based on *cytb* gene.

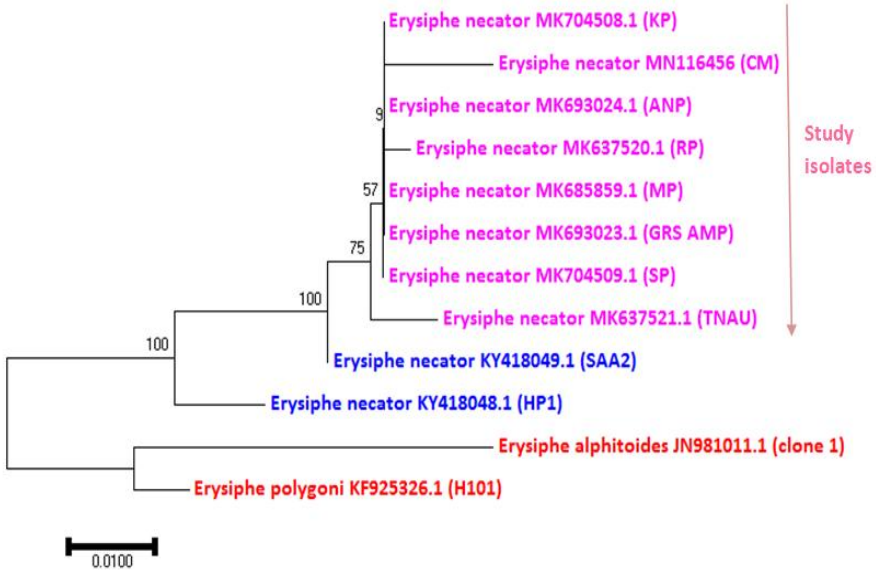

Fig. 5. The Phylogenetic tree constructed based on the cytochrome b (cytb) gene nucleotide sequences of *E. necator* and other *Erysiphe* species retrieved from NCBI database. The tree was generated through Neighbor joining Tree method in mega 7.0 with 1000 boot strap replications

Table 3. Nucleotide identities compared on the basis of pairwise allighment of *E. necator* isolates and other Erysiphe species

Seq->	**MK637521.1** ***E. necator***	**MK685859.1** ***E. necator***	**MK693024.1** ***E. necator***	**MK693023.1** ***E. necator***	**MK704508.1** ***E. necator***	**MK637520.1** ***E. necator***	**MK704509.1** ***E. necator***	**MN116456** ***E. necator***	**KY418049.1** ***E. necator***	**KY418048.1** ***E. necator***	**JN981011.1** ***E. alphitoides***	**KF925326.1** ***E. polygoni***
MK637521.1 *E. necator*	ID	99.00%	99.00%	99.00%	99.00%	98.70%	99.00%	97.80%	98.40%	96.20%	90.40%	93.40%
MK685859.1 *E. necator*	99.00%	ID	100.00%	100.00%	100.00%	99.60%	100.00%	98.70%	99.30%	96.50%	90.70%	93.80%
MK693024.1 *E. necator*	99.00%	100.00%	ID	100.00%	100.00%	99.60%	100.00%	98.70%	99.30%	96.50%	90.70%	93.80%
MK693023.1 *E. necator*	99.00%	100.00%	100.00%	ID	100.00%	99.60%	100.00%	98.70%	99.30%	96.50%	90.70%	93.80%
MK704508.1 *E. necator*	99.00%	100.00%	100.00%	100.00%	ID	99.60%	100.00%	98.70%	99.30%	96.50%	90.70%	93.80%
MK637520.1 *E. necator*	98.70%	99.60%	99.60%	99.60%	99.60%	ID	99.60%	98.40%	99.00%	96.20%	90.40%	93.40%
MK704509.1 *E. necator*	99.00%	100.00%	100.00%	100.00%	100.00%	99.60%	ID	98.70%	99.30%	96.50%	90.70%	93.80%
MN116456 *E. necator*	97.80%	98.70%	98.70%	98.70%	98.70%	98.40%	98.70%	ID	98.10%	95.30%	89.40%	92.50%
KY418049.1 *E. necator*	98.40%	99.30%	99.30%	99.30%	99.30%	99.00%	99.30%	98.10%	ID	97.20%	91.30%	94.40%
KY418048.1 *E. necator*	96.20%	96.50%	96.50%	96.50%	96.50%	96.20%	96.50%	95.30%	97.20%	ID	91.60%	95.30%
JN981011.1 *E. alphitoides*	90.40%	90.70%	90.70%	90.70%	90.70%	90.40%	90.70%	89.40%	91.30%	91.60%	ID	95.30%
KF925326.1 *E. polygoni*	93.40%	93.80%	93.80%	93.80%	93.80%	93.40%	93.80%	92.50%	94.40%	95.30%	95.30%	ID

4. CONCLUSION

The PCR protocol developed in this research work will help in early detection of *E. necator* and pave way for timely and minimal number of spraying of fungicide and thereby help in mitigation of the disease.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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