

4 **Phenotypic characterization and Molecular phylogenetic relationship of *Erysiphe necator***
5 **infecting grapes**

6
7 **Abstract**

8 Grapes powdery mildew is caused by the most destructive pathogen *Erysiphe necator*
9 leading to severe yield losses around the world. In order to study the phenotypic and molecular
10 characters, the powdery mildew infected leaf samples were collected from eight different places
11 in Coimbatore and Theni districts in the state of Tamil Nadu India. The identity of the pathogen
12 as *E. necator* was established by microscopic studies and the isolates were further confirmed
13 molecularly by amplification of Internal transcribed spacer (ITS) and Cytochrome b gene (Cyt
14 b). Further molecular confirmation was obtained by characterizing Cytochrome b. An amplicon
15 size of ~ 367 and ~ 470 bp were obtained from amplification with Uncin144 and Uncin511 and
16 Cyt b F and Cyt b R gene primers respectively. The identity for cyt b gene segment was 96 to 98
17 %, similarity with *E. necator* isolates deposited in NCBI genbank (KY418048.1, KY418049.1).
18 A phylogenetic tree was constructed on the basis of nucleotide sequence of cytochrome b gene
19 of the study isolates as well as *E. necator* and other *Erysiphe* species in NCBI database. From
20 the tree it was evident that the study isolates from Tamil Nadu, India were very distinct from
21 other *E. necator* isolates deposited in NCBI genbank database.

22 **Keywords:**

23 *Grapes, Powdery mildew, E. necator, ITS, Cytochrome b region, PCR, detection, Phylogenetic*
24 *analysis, Sequence identity Matrix.*

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29 **1. Introduction**

30 Grapes powdery mildew is a world devastating disease caused by *Erysiphe necator*
31 (Schw.) Burr. (synonym *Uncinula necator*), an obligative biotrophic ascomycetes fungi (Deliere
32 et al., 2010; Dufour et al., 2011). The fungus infects all green tissue of the grapevine including,
33 leaves, stems flowers and fruits. The disease symptoms are due to presence of sign of the
34 pathogen including superficial mycelial structures consisting of conidiophores and conidia of the
35 fungi on the infected host organ. Epidemic outbreak of grapes powdery mildew in Europe from
36 1847-1854 was recognized as the first report of severe economic yield loss (Yarwood, 1978).

37 Symptoms of powdery mildew vary throughout the growing season both in grapevine and
38 different developmental stages in *E. necator*. All growing fresh tissues shows high compatible
39 reaction with *E. necator*. Initially the lesions appear as small discolored area followed by the
40 formation of white, thin powdery layer of fungal structures (Falacy et al., 2007). The infection
41 also reduces the net photosynthesis and degrades wine quality and finally causes dramatic yield
42 losses (Gadoury et al., 2007).

43 The detection of plant pathogen through PCR has been well developed currently in order
44 to identify the fungi, bacteria and viruses (Venkateswaran *et al.*, 1997; Williams *et al.*, 2001;
45 Kong *et al.*, 2003).

46 Mostly detection of fungal species depends on amplification and sequencing of internal
47 transcriber spacer (ITS). High variability in ITS region among the populations of the same
48 species has been reported (Mior et al., 2017). Cytochrome b (cytb) region play a significant role

49 in studying the phylogenetic relation in higher fungi and Oomycota (Schena and Cooke, 2007).
50 Specifically, the cyt b gene is known to harbor broad variation at intra-specific level which leads
51 to molecular identification of species in a fine manner and in its taxonomy and also genetic
52 studies in population level.

53 **2. Materials and methods**

54 **2.1 Collection of *Erysiphe necator* infected powdery mildew leaf samples from different** 55 **places**

56 The powdery mildew infected leaf samples were collected from different plantations in
57 different villages in Coimbatore and Theni district. In Coimbatore the samples were collected at
58 Tamil Nadu Agricultural University orchard and the village Mathyalayam. In Theni the infected
59 samples were collected at Grape research station (Anamalayathanpatty), Kamayagoundanpatti,
60 Rayappanpatty, Anaipatty, Surulipatty, and Cumbum. The conidial spore mass was collected and
61 stored in -20 °C for further use.

62 **2.2 Microscopic observation of *E. necator* infected leaf sample**

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

68 **2.3 Genomic DNA extraction from *E. necator* isolates**

69 The DNA was isolated from the conidia of *E. necator* following the method of McDermott
70 et al. (1994) with some modification. About 200 mg of conidia were collected from powdery
71 mildew infected leaves using a camel hair brush and transferred to a microcentrifuge tube

72 containing 500 µl of CTAB extraction buffer (50 mM Tris- HCl, PH 8.0; 0.7 M NaCl and 1 %
73 CTAB (w/v) vortexed for 30 s and incubated at 60° C for 1 h. After incubation, the mixture was
74 centrifuged at 13,000 x g for 10 min and the supernatant was collected and extracted twice with
75 an equal volume of chloroform:isoamylalcohol (24:1). The aqueous phase was transferred to a
76 1.5 ml microcentrifuge tube and the DNA was precipitated by addition of an equal volume of
77 cold isopropanol and incubation at -20 °C for 1 h. The DNA was collected by centrifugation at
78 13, 000 x g at 4 °C for 10 min. the pellet was washed twice with cold 70 % ethanol, air dried and
79 resuspended in 50 µl of Tris- EDTA buffer (10mM Tris- HCl and 1 mM EDTA, pH 8.0). The
80 genomic DNA was checked by agarose gel electrophoresis and the concentrations of the DNA
81 were determined using a Nanodrop ND-3300 Fluorospectrometer (NanoDrop products, Thermo
82 Scientific, Wilmington, DE, USA).

83 **2.3.1 Specific primer designing for *E. necator***

84 PCR amplification of ITS region was performed with Uncin144 (5-
85 CCGCCAGAGACCTCATCCAA-3) and Uncin511 (5- TGGCTGATCACGAGCGTCAC-3)
86 primers (Falacy *et al.*, (2007). The nucleotide sequence data of *Cytochrome b (cytb)* gene of the
87 powdery mildew isolate from NCBI available under, Accession number KY418049.1
88 (<https://www.ncbi.nlm.nih.gov/nuccore/KY418049.1>) were used for designing primers which
89 was specific to *E. necator*. The set of Cyt PM F and Cyt PM R primer sequences were designed
90 through NCBI online primer designing tool ([https://www.ncbi.nlm.nih.gov/tools/primer-
92 blast/](https://www.ncbi.nlm.nih.gov/tools/primer-
91 blast/))and the primer sequences are: forward primer cyt b F
92 (TGTTGTAATATTTATTTAATG) and cyt b R reverse primer
93 (TGGGTTAGCCATAATATAA). PCR reactions were set up in 20µl mixture containing ~50
94 ng of total DNA, 10 µl of TaKaRa master mix (2X concentration) and 20 pmol each of forward

95 primer and reverse primers. The reaction was carried out in thermocycler (Eppendorf master
96 cycle). The PCR program for the amplification of Uncin144 and Uncin511 region consisted of
97 an initial denaturation of 3 min at 94°C followed by 40 cycles of denaturation at 94°C for 30 s,
98 1 min of annealing at 68°C, 90 s of extension at 72°C and a final extension for 7 min at 72°C.
99 For cyt b F and cyt b R the cycle conditions are initial denaturation of 3 min at 94°C followed
100 by 40 cycles of denaturation at 94°C for 30 s, 1 min of annealing at 42°C, 90 s of extension at
101 72°C and a final extension for 7 min at 72°C. Finally Amplified products were separated by
102 electrophoresis in 1.0 % agarose containing ethidium bromide (give concentration) at 80 V for
103 2-3 h and documented in an gel documentation unit (Alpha Imager EC (USA)).

104 **2.3.2 Sequencing and phylogenetic tree construction**

105 The amplified PCR products were sequenced by Sanger's dideoxy sequencing method
106 and sequences were edited and aligned. In order to study the variability, the sequence of
107 collected *E. necator* isolates were compared with those of *E. necator* and other species like *E.*
108 *alphoides*, *E. polygoni* available in NCBI database. The sequence identity matrix was
109 constructed between the isolates with bioedit software version 7.2. Further the phylogenetic tree
110 was developed by using Mega 7.0 software (Kumar *et al.*, 2016) including the sequences of *E.*
111 *alphitoides* (JN981011.1) *E. polygoni* (KF925326.1) which were retrieved from NCBI database
112 by Maximum neighbor joining method.

113 **Results and Discussion**

114 **2.4 Symptoms and phenotypic characterization**

115 The grapes leaf samples expressing typical symptoms of whitish dull powdery growth
116 were collected from Coimbatore and Theni district (Fig. 1).The collected isolates were examined
117 for various phenotypic characters by thin sectioning of infected leaf. Fungal structures were

118 observed under light microscope. The conidial size of different isolates was measured at 40 X
119 magnification. The average size of the conidia varies from 31.74 – 36.43 µm in length and
120 12.85- 15.45 µm in breadth. Largest size of conidia was observed in Rayappanpatty with an
121 average size of 36.43µm in length and 15.45 µm in breadth (Table 1). The observed results are
122 similar to observations of Calonnec *et al.* , 2004; Stummer *et al.*, 2005, who have studied the
123 morphological of characters of Genus Oidium and symptoms of grapes powdery mildew caused
124 by *E. necator*.

125 **2.5 PCR amplification of *E. necator* through ITS and specific primer**

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

135 **2.5.1 Phylogenetic tree construction and sequence identity matrix**

136 The amplicons obtained with study isolates were sequenced and the matching similarity
137 was performed through NCBI BLAST analysis which shows 96 to 98 % similarity with *E.*
138 *necator* isolates KY418049.1 (SAA2) and KY418048.1 (HP1), But the similarity was less with
139 other isolates JN981011.1 *Erysiphe alphitoides* (clone 1) and KF925326.1 *Erysiphe polygoni*
140 (H101) (Fig 5). Results clearly established the primer specificity to distinguish *E. necator*

141 isolates from other isolates. Identity matrix generated on the basis of nucleotide sequence of cyt
142 b gene (Table 3) clearly revealed the difference among the isolates of *E. necator*. The study
143 isolates shared nearly 99 to 100% identity between them. Interestingly, when identity with one
144 isolate from *E. necator* (Genbank no KY418049) was as high as 98-99%, it was less (95-96%)
145 with another isolate (Genbank no KY418048). All isolates of *E. necator* shared only 89-94%
146 identity with other two species, *E. alphitoides* and *E. ploygoni*. Phylogenetic tree constructed on
147 the basis of nucleotide sequence revealed the relationship between the isolates. All study isolates
148 clustered together well separated from two isolates *E. necator* (Genbank no KY418049) and *E.*
149 *necator* (Genbank no KY418048) from *E. alphitoides* (Genbank no JN981011.1) *E. ploygoni*
150 (Genbank no KF925326.1). However within the study isolates the isolate from Cumbum
151 (Genbank no MN116456) was found to be distinct. As such *E. necator* isolates occupied separate
152 clade well separated from *E. alpitoides* and *E. polygoni*.

153 The use of molecular characters specifically ITS region provide an promising results for
154 determination of species in some powdery mildews (Almeida *et al.*, 2008; Braun and Takamatsu
155 2000; Cunnington *et al.*, 2003; Takamatsu *et al.*, 2002). The molecular confirmation of ITS
156 region in Erysiphales should provide an accurate information about anamorphic stage of the
157 fungi (Cunnington., 2003). These primers also appear to be suitable for detection of most if not
158 all isolates of *E. necator* because they successfully amplified numerous *E. necator* isolates from
159 diverse geographic regions (Falacy *et al.*, 2007). The Uncin144 and Uncin511 specific primers
160 are highly suitable for detection of most isolates of *E. necator* from diverse geographic regions.
161 In the present communication attempts were made to develop more specific PCR diagnostic
162 marker for detection of mildew pathogen. Therefore the cytochrome b gene was selected, which
163 detected the pathogen in 100 % of samples tested. Cytochrome b gene is encoded by the

164 mitochondrial genome (Zheng and Koller 1997); and DNA of mitochondria exists as large copies
165 per cell (Lesemann et al. 2006; Villani and Cox 2014; Hashimoto et al., 2015), which will help in
166 easy detection of the pathogen. Miles et al., 2012 utilized cytochrome b (CYTB) gene based
167 conventional PCR assay for the detection of the presence or absence of the G143A mutation
168 because which is responsible for Strobilurin (QoI) resistance in *E. necator*. Similar attempts have
169 been made by Fernandez et al., 2018 applied specific primer F3cytb-Px and R3cytb-Px
170 targeting Cytochrome b gene for the detection of *Podosphaera xanthii* in zucchini. The primer
171 sequences were designed by Kears et al. 2012 On the basis of sequences based on sequence data,
172 contigs c364 and c24909 retrieved from haustorial and epiphytic transcriptome of *P. xanthii*
173 (Seoane et al., unpublished data). Sandra Mosquera, (2019) reported G143A substitution in
174 within field populations of *Leveillula taurica* in tomato which confer high level of resistance
175 against Q_oIs in several fungi based on *cytb* gene .

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

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



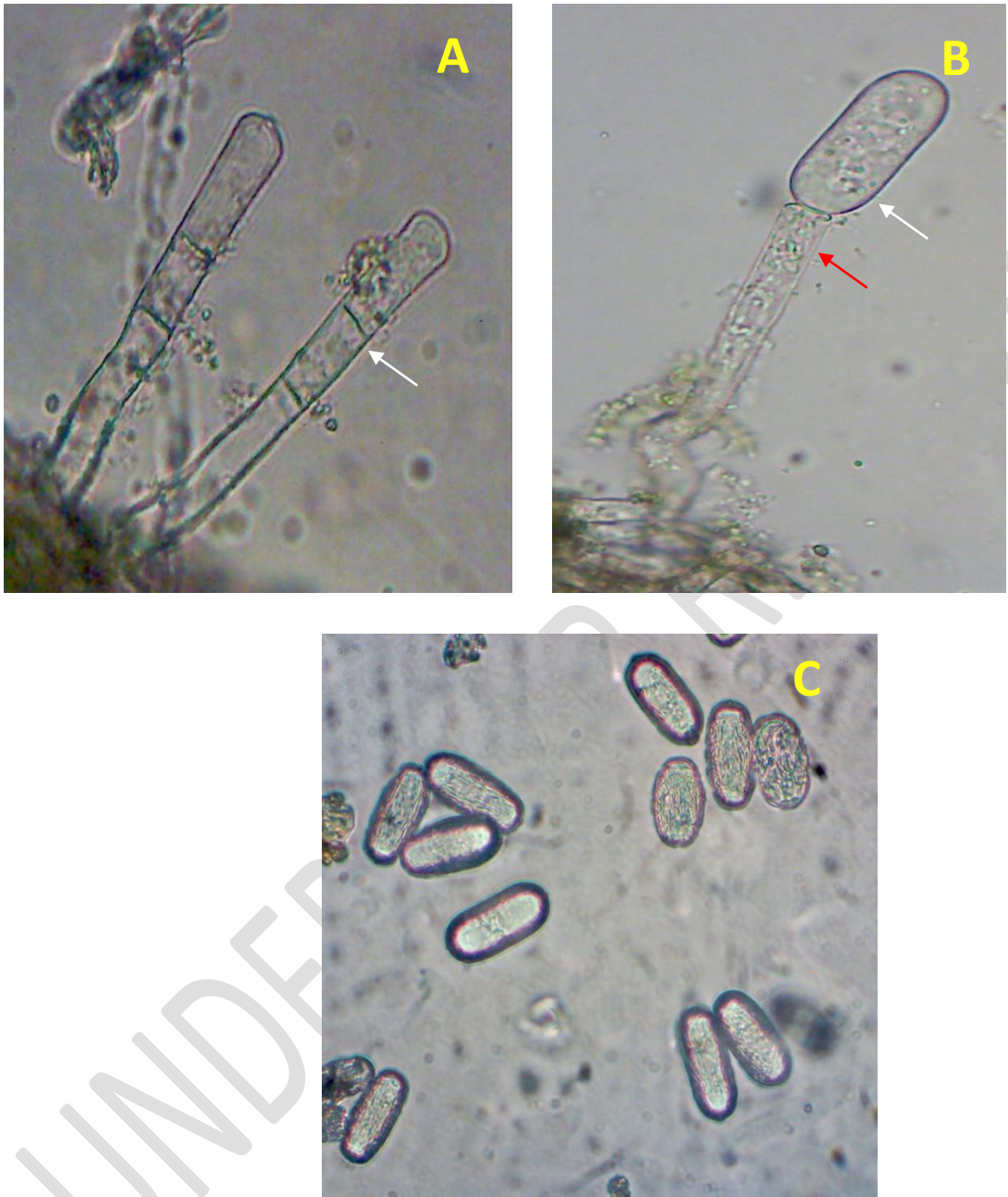
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260 **Disease symptoms caused by *E. necator* on grapes A) symptoms of the**
 261 **disease mostly visible adaxial leaf surface. b) Development of individual spots at initial**
 262 **stage of infection b) spots cover the entire leaf at later stage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

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



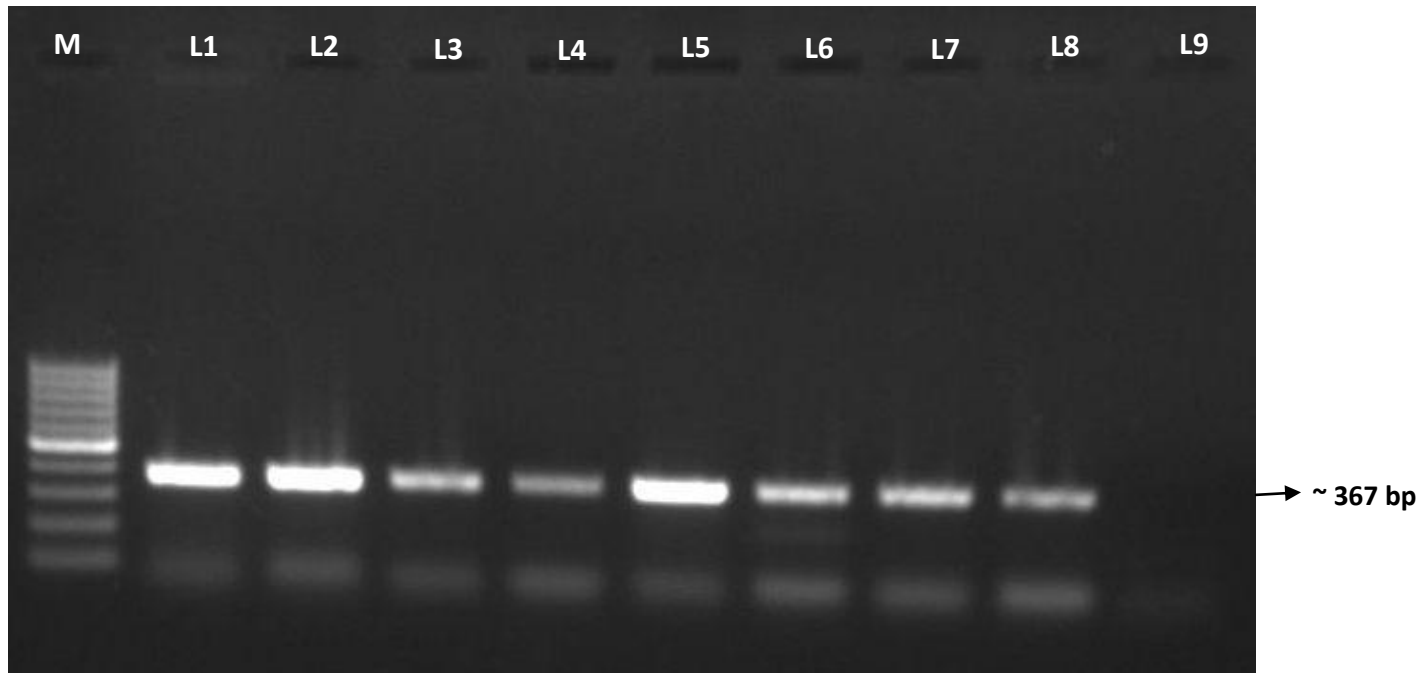
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268 **A) Formation of septate hyaline conidiophores white arrow indicates the septation in**
269 **conidiophores B) Single celled hyaline conidia at the tip of conidiophores white arrow**
270 **indicates conidiophores, red arrow indicates single celled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

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

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

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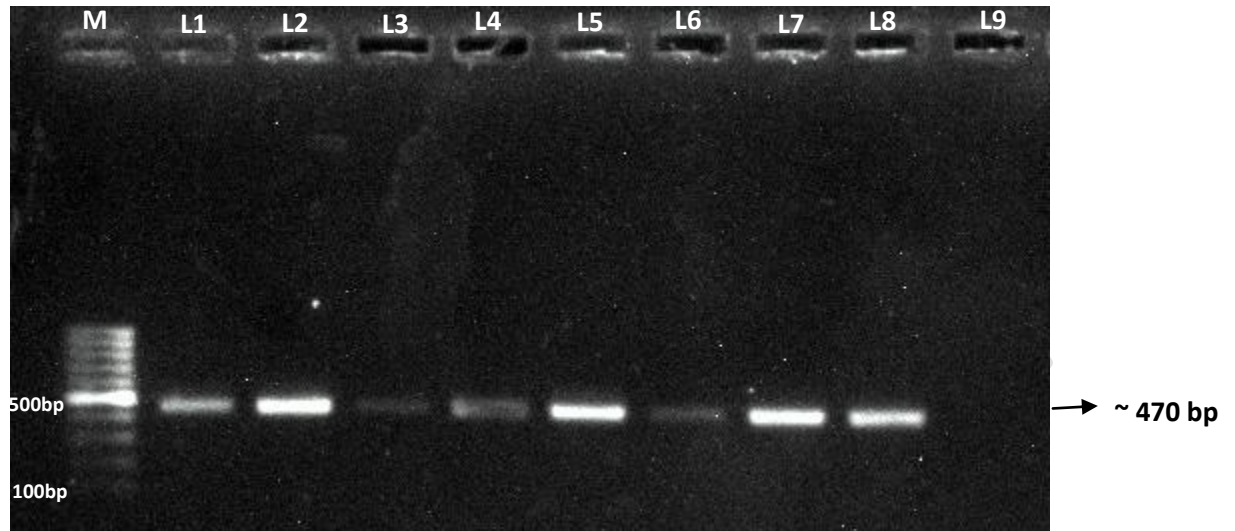
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288 **Fig 4 PCR amplification of Cytb gene in *E. necator* isolates**



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

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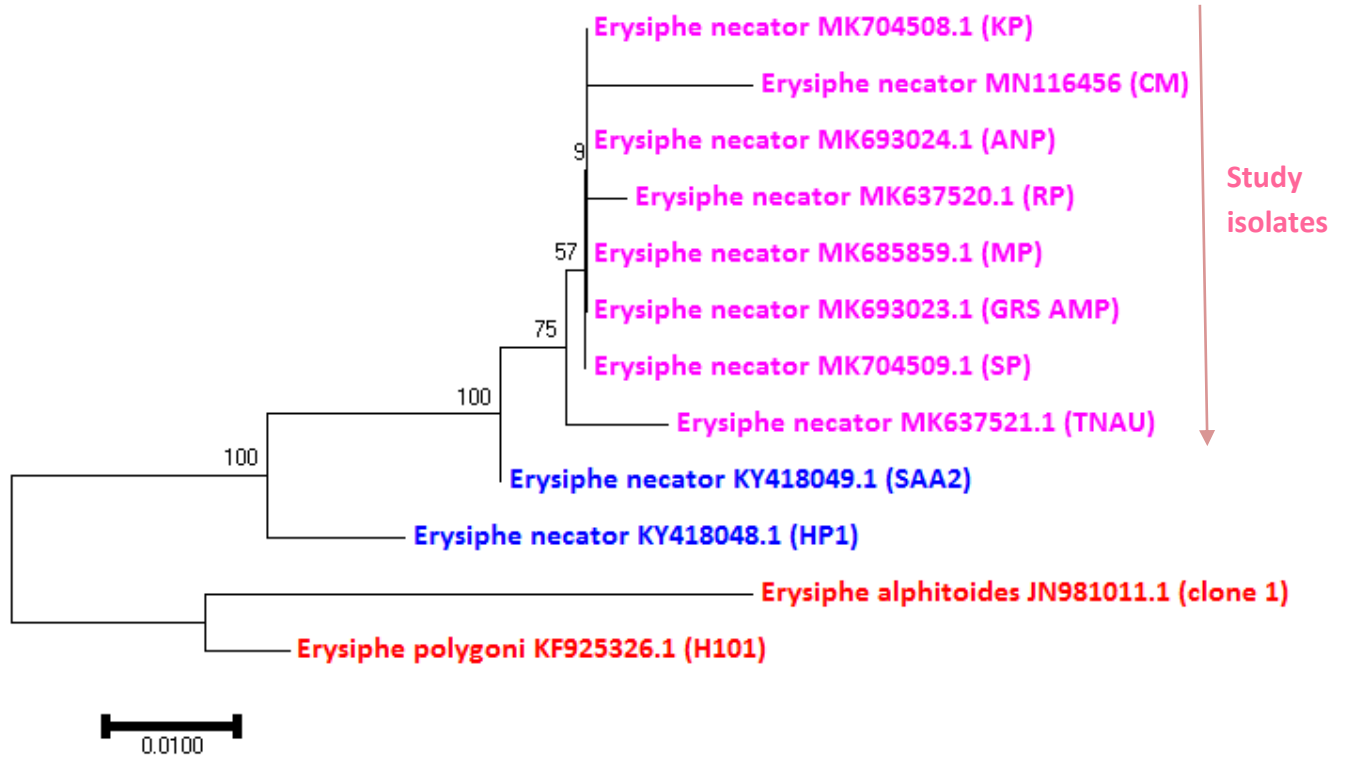
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310 **Fig. 5** The Phylogenetic tree constructed based on the cytochrome b (cytb) gene nucleotide
311 sequences of *E. necator* and other *Erysiphe* species retrieved from NCBI database. The tree
312 was generated through Neighbor joining Tree method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



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323 **Table 1. Phenotypic character of conidial size measurement of *E. necator* at 40X**324 **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

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326 **Table 2. Accession number of nucleotide sequence of Cyt b gene of study isolates of *E.***327 ***necator***

S. No	Location	<i>E. necator</i> 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

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UNDER PEER REVIEW

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

Seq->	MK637521.1 <i>E. necator</i>	MK685859.1 <i>E. necator</i>	MK693024.1 <i>E. necator</i>	MK693023.1 <i>E. necator</i>	MK704508.1 <i>E. necator</i>	MK637520.1 <i>E. necator</i>	MK704509.1 <i>E. necator</i>	MN116456 <i>E. necator</i>	KY418049.1 <i>E. necator</i>	KY418048.1 <i>E. necator</i>	JN981011.1 <i>E. alphitoides</i>	KF925326.1 <i>E. polygona</i>
MK637521.1 <i>E. necator</i>	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 <i>E. necator</i>	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 <i>E. necator</i>	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 <i>E. necator</i>	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 <i>E. necator</i>	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 <i>E. necator</i>	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 <i>E. necator</i>	99.00%	100.00%	100.00%	100.00%	100.00%	99.60%	ID	98.70%	99.30%	96.50%	90.70%	93.80%
MN116456 <i>E. necator</i>	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 <i>E. necator</i>	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 <i>E. necator</i>	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 <i>E. alphitoides</i>	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 <i>E. polygona</i>	93.40%	93.80%	93.80%	93.80%	93.80%	93.40%	93.80%	92.50%	94.40%	95.30%	95.30%	ID

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UNDER PEER REVIEW