BIOLOGY INSIGHTS

MIDITERRANEAN JOURNALS

Original Research

DOI: 10.55085/bi.2022.625

Diversity and Phylogenetic Analysis of Phytophthora Species Infecting Coorg Mandarin

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ABSTRACT

Coorg mandarin is a particular type of mandarin grown in Western Ghats' high humid tropical region. The crop is cultivated in multiple cropping systems of pepper and coffee plantations in Karnataka, Tamil Nadu, and the Kerala states of India. The crop is attacked by a number of diseases. Among them, Phytophthora is a century-old disease causing a major threat to Coorg mandarin cultivation and production in Southern states of India. A systematic random survey was conducted to collect Phytophthora infected samples from the seeds and grafted Coorg mandarin plants in 184 orchards located in different parts of Karnataka. A total of 111 Phytophthora isolates were isolated and characterized from infected plant roots (59) and soil (52) infected Coorg Mandarin samples collected in different orchards. Based on the pathogenicity parameter, only 45 out of 111 Phytophthora isolates were selected, and the ITS region was amplified by PCR using ITS primers and sequenced. Based on the ITS sequence pairwise identity score, 45 Phytophthora isolates were assembled into three groups. The type I group of *P. palmivora* isolates showed the highest nucleotide identity of 88.5 to 90.8% with *P.palmivora* (JX198562). In contrast, the type II group of *P. palmivora* isolates showed nucleotide identity of 93.6 to 99 % with *P.palmivora* (KF010299) infecting different citrus species, and the type III group of *P.nicotianae* isolates showed maximum nucleotide identity of more than 93% with *P.nicotianae* (KJ549640) infecting different citrus species.

Keywords: Coorg Mandarin, *Phytophthora*, Polymerase Reaction, Phylogenetic analysis, Genetic Diversity.

Received: 10 Jan 2022
Revised: 28 Feb 2022
Accepted: 17 Mar 2022
Published: 06 Apr 2022

Academic Editor:
Raveendran Muthurajan

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Cite this article as: Sonavane P Venkataravanappa V, Reddy MK. Diversity and Phylogenetic Analysis of Phytophthora Species Infecting Coorg Mandarin. Biol Insights. 2022;1:625. [https://doi.org/10.55085/bi.2022.625]

Authors' contributions
The participation of each author corresponds to the criteria of authorship and contributorship emphasized in the Recommendations for the Conduct, Reporting, Editing, and Publication of Scholarly work in Medical Journals of the International Committee of Medical Journal Editors. Indeed, all the authors have actively participated in the redaction, the revision of the manuscript, and provided approval for this final revised version.

Acknowledgments
The authors are grateful to the Director, Indian Institute of Horticultural Research, Bengaluru for providing research facilities and their keen interest in this study.

Conflict of interest
The authors declare that there is no conflict of interest regarding the publication of this article.

INTRODUCTION

Coorg mandarin is a special type of mandarin cultivated in the Kodagu, Hassan, and Chikmagalur districts of Karnataka (India) in coffee and pepper plantations. The region resides in the cradle of the Western Ghats, with heavy rainfall and high humidity in the months of May-October. Though the ecotype of mandarin is grown in the region hundreds of years ago, their production is drastically reduced due to the attack of different Phytophthora spp[8]. Decades of losses in the Coorg mandarin crop due to disease have led to the shifting of cropping patterns to other spices, coffee, and other exotic fruits. Phytophthora spp. Both soil and water-borne fungi cause disease in citrus species[6,7,10,11]. The *Phytophthora spp* infects all the plant parts and causes significant loss to the citrus industry[16]. Three species of Phytophthora, namely *Phytophthora nicotianae*, *Phytophthora palmivora*, and *Phytophthora citrophthora*, have been recorded to cause the disease on Citrus spp. Worldwide. In India, *Phytophthora nicotianae* and *Phytophthora palmivora* were found to be predominant species causing the decline and root rot of citrus. The extensive surveys were carried out by various researchers in mandarin growing areas in Southern states in India indicate that mandarin was severely affected by *Phytophthora palmivora* and *Phytophthora nicotianae* [3,18]. *P. palmivora* affecting Citrus mandarin was reported in Maharashtra and Karnataka [15]. The spread and pathogenicity of *P. palmivora* are more aggressive in infecting citrus roots than *P. nicotianae* and *P. citrophthora* [21,22]. Thus, the current study aims (i) to identify and study the population of the *Phytophthora* species causing root rot and gummosis in Coorg mandarin.

MATERIAL AND METHODS

Collection of Phytophthora infected Coorg mandarin disease samples

The survey was conducted during 2015-2016 in the diverse location of Karnataka state (Madikeri, Chikamagaluru, Hassan) of India to collect the *Phytophthora* infected samples (Plant tissues and soil) from the plants raised from true seeds and grafted plants. A total of 111 *Phytophthora* infected samples of plant tissues (59) and soil (52) were collected from the 184 Coorg mandarin fields surveyed. The pure culture of the pathogen was isolated on PARPH selective media as described by Sonavane et al. [18], was inoculated on six-month-old six-month-old Coorg mandarin seedlings to prove the isolates' pathogenicity re-isolated. The pure culture of the pathogen was maintained as agar plugs in sterile distilled water at room temperature till further use [5].

DNA isolation and polymerase chain reaction

The pure cultures of the forty-five *Phytophthora* isolates were grown on a PDB medium for ten days. After ten days, the mycelium was harvested and washed with sterile distilled. Two grams of mycelium of different *Phytophthora* isolates were used for total DNA isolation using the CTAB method [4]. To confirm the identity of the pathogen the DNA of different *Phytophthora* isolates was subjected to PCR amplification using universal ITS1 and ITS4 primers [20]. The amplified PCR products were sequenced.

Sequence analysis

The Internal Transcribed Spacer Region (ITS) sequences obtained from forty-five *Phytophthora* isolates were compared with Genbank isolates at NCBI BLASTn. The ITS sequences of forty-five *Phytophthora* isolates showed maximum identity score with *Phytophthora* isolates infecting different crops were retrieved from Genbank used for analysis. The sequence analysis was carried out using the Sea view program [9]. The pair-wise identity matrixes between *Phytophthora* isolates obtained in the present study and other *Phytophthora* isolates were generated using the Sequence Demarcation Tool [13]. The evolutionary relationship between *Phytophthora* isolates was generated using the neighbor-joining method by using MEGA X software with 1000 bootstrapped replications [14].

RESULTS

Survey, collection and isolation of Phytophthora from the Coorg mandarin samples

A survey conducted in different places of Karnataka revealed that the incidence of *Phytophthora* is prevailing in all the surveyed locations and disease incidence ranged from 12 to 55%. The highest incidence was observed in Madikeri followed by Chikamagaluru districts and the least incidence was observed in Hassan district. A total of 111 *Phytophthora* infected (59 plant tissues) Coorg mandarin samples and 52 soil samples were collected from 184 surveyed orchards. The pure culture pathogen was isolated on PARPH selective media as described by Sonavane et al. [18]. Based on pathogencity only 45 out of 111 *Phytophthora* isolates were selected for further characterization [18].

PCR detection of Phytophthora isolates

The total nucleic acid of 45 *Phytophthora* isolates was amplified by PCR using universal ITS primers. The resulting amplicon of 550 bp size close to the ITS region of *Phytophthora* was amplified in all the samples. The amplified PCR product was cloned and sequenced. The obtained ITS sequences of 45 *Phytophthora* isolates were analyzed using different bioinformatics programs. The similarity of ITS sequences of forty five *Phytophthora* isolates was checked at the NCBI database using Blastn. The analysis indicates that the *Phytophthora* isolates isolated from the Coorg mandarin plants belong to two species, *Phytophthora palmivora*, and *Phytophthora nicotianae*. Thirty seven *Phytophthora* isolates out of 45 belong to *P. palmivora*, and eight *Phytophthora* isolates belong to *Phytophthora nicotianae*. The consensus sequences of *Phytophthora* isolates were submitted to Genbank.

Comparison of ITS region of Phytophthora isolates infecting Coorg mandarin.

Comparison of ITS nucleotide sequences of thirty-seven *P. palmivora* and isolates were among themselves using SDT. The analysis showed that thirty-seven *P. palmivora* isolates sheared maximum nucleotide identity of 87.0 to 97.8% among themselves. Further, the analysis within species of *P. palmivora* isolates revealed that the thirty-seven isolates were grouped into three clusters (Type I, Type II, and Type III). Type I contains twenty five *P. palmivora* isolates (Pph1, Pph2, Pph3, Pph4, Pph5, Pph6, Pph7, Pph8, Pph9, Pph10, Pph11, Pph12, Pph13, Pph26, Pph27, Pph28, Pph29, Pph30, Pph31, Pph32, Pph33, Pph34, Pph35, Pph36, Pph37) are showing the maximum nucleotide identity of more than 95% among themselves (Fig 1), and showed less than 87.0 nucleotide identity with type II category contains twelve *P. palmivora* isolates (Pph14, Pph15, Pph16, Pph17, Pph18, Pph19, Pph20, Pph21, Pph22, Pph23, Pph24 and Pph25). Similar analysis showed that all eight *P. nicotianae* isolates are sheared highest nucleotide identity of 99.7 to 100%

among themselves (Data not shown) and less than 88% with type II and type II category of isolates.

The ITS sequences of Type I and Type II category of *P. palmivora* isolates were compared, the result revealed that the isolates of both the category are sheared maximum nucleotide identity of 87.1 to 94.6% between the two groups. Similarly, the nucleotide identity between Type I and Type III groups of *Phytophthora* isolates is less than 80%. The nucleotide identity between Type II of *P. palmivora* and Type III category *P. nicotianae* isolates showed less than 88%. These results were supported by Sequence Demarcation Tool, in which *P. palmivora and P. nicotianae* isolates were clearly differentiated (Fig 1).

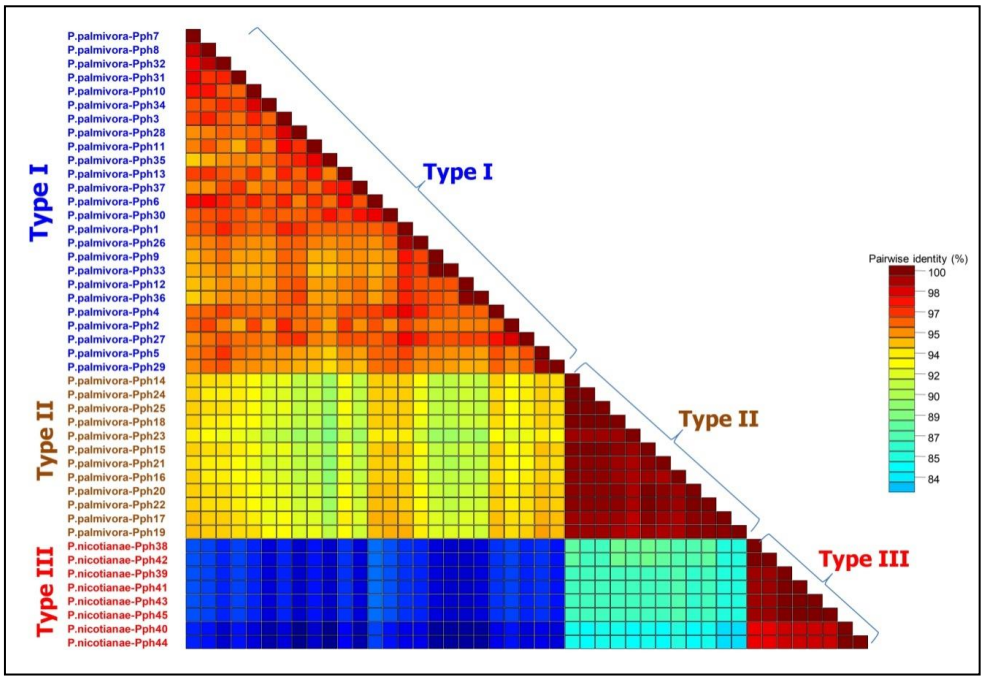

Figure 1: Two-dimensional color-coded matrix of pairwise identity scores of the *Phytophthora palmivora* and *Phytophthora nicotianae* infecting Coorg mandarin were generated by using the Sequence demarcation tool.

Comparison of ITS region *P. palmivora* isolates with other Phytophthora isolates infecting different citrus species

ITS sequences of Type I, Type II category of *P. palmivora*, and Type III category of *P. nicotianae* isolates were compared with ITS sequences of 62 Phytophthora isolates [*P. palmivora* (20), *P. nicotianae* (36), *P. parasitica* (1), and *P. citrophthora* (5)]. The analysis showed that the Type I category of *P. palmivora* isolates showed a maximum nucleotide identity of 88.5 to 90.8% with *P. palmivora* (JX198562). The sequence is available in NCBI Genbank, whereas the Type II group of *P. palmivora* isolates showed nucleotide identity of 93.6 to 99 % with *P. palmivora* (KF010299, JX198562, and KP183963) infecting different citrus species, in which the sequence is available in the NCBI database. Further Type III group of P. nicotianae isolates showed maximum nucleotide identity of more than 93% with *P. nicotianae* (KJ549640, KJ549641, KJ494919, KJ494917, KJ494914, KJ494913, KJ494911, JF792535, JF792536, JF792537, JF792538, JF792539, JX965375, and JX965375) infecting different citrus species in which the sequence are available in NCBI database (Table 1, Fig 2).

These results were supported by Sequence Demarcation Tool, in which *P. palmivora* and *P. nicotianae* isolates were clearly differentiated (Fig 2).

Phylogenetic analysis

The ITS sequences of forty-five *Phytophthora* isolates belonging to Type I, Type II category of *P. palmivora*, and Type III category of *P. nicotianae* isolates were compared with ITS sequences of 62 *Phytophthora* isolates infecting different citrus species and 74 *Phytophthora* isolates infecting diverse crops in the world. The analysis showed that all forty-five *Phytophthora* isolates infecting Coorg mandarin were formed into three groups/clusters. Majority of the *P. palmivora* isolates belong to the Type I category (Pph1, Pph2, Pph3, Pph4, Pph5, Pph6, Pph7, Pph8, Pph9, Pph10, Pph11, Pph12, Pph13, Pph26, Pph27, Pph28, Pph29, Pph30, Pph31, Pph32, Pph33, Pph34, Pph35, Pph36, Pph37) showing highest nucleotide identity of 88.5 to 90.8% with *P. palmivora* (JX198562) and grouping in

Table 1: Highest identity matrix of Phytophthora isolates with Gen bank Phytophthora isolates.

Types	Phytophthora species	Isolates codes	Highest identity with isolates in gen bank	Per cent identity
Type I	*P.palmivora*	Pph1	*P.palmivora*.[JX198562]	90.3
	P.palmivora	Pph2	*P.palmivora*.[JX198562]	89.7
	P.palmivora	Pph3	*P.palmivora*.[JX198562]	89.3
	P.palmivora	Pph4	*P.palmivora*[JX198562]	90.2
	P.palmivora	Pph5	*P.palmivora*.[JX198562]	90.4
	P.palmivora	Pph6	*P.palmivora*.[JX198562]	90.3
	P.palmivora	Pph7	*P.palmivora*.[JX198562]	90.8
	P.palmivora	Pph8	*P.palmivora*.[JX198562]	90.4
	P.palmivora	Pph9	*P.palmivora*.[JX198562]	88.7
	P.palmivora	Pph10	*P.palmivora*.[JX198562]	89.6
	P.palmivora	Pph11	*P.palmivora*.[JX198562]	88.9
	P.palmivora	Pph12	*P.palmivora*.[JX198562]	88.8
	P.palmivora	Pph13	*P.palmivora*.[JX198562]	89.4
	P.palmivora	Pph26	*P.palmivora*.[JX198562]	89.6
	P.palmivora	Pph27	*P.palmivora*.[JX198562]	89.8
	P.palmivora	Pph28	*P.palmivora*.[JX198562]	88.8
	P.palmivora	Pph29	*P.palmivora*.[JX198562]	89.7
	P.palmivora	Pph30	*P.palmivora*.[JX198562]	89.8
	P.palmivora	Pph31	*P.palmivora*.[JX198562]	90.1
	P.palmivora	Pph32	*P.palmivora*.[JX198562]	89.4
	P.palmivora	Pph33	*P.palmivora*.[JX198562]	88.6
	P.palmivora	Pph34	*P.palmivora*.[JX198562]	89.0
	P.palmivora	Pph35	*P.palmivora*.[JX198562]	88.5
	P.palmivora	Pph36	*P.palmivora*.[JX198562]	88.8
	P.palmivora	Pph37	*P.palmivora*.[JX198562]	89.1
Type II	*P.palmivora*	Pph14	*P.palmivora*-NRCPh-100.[KF010299]	98.6
	P.palmivora	Pph15	*P.palmivora*[KP183963]	98.7
	P.palmivora	Pph16	*P.palmivora*-NRCPh-100[KF010299]	98.2
	P.palmivora	Pph17	*P.palmivora*-NRCPh-100.[KF010299]	92.9
	P.palmivora	Pph18	*P.palmivora*.[KP183963	98.5
	P.palmivora	Pph19	*P.palmivora*-NRCPh-100.[KF010299]	93.6
	P.palmivora	Pph20	*P.palmivora*.[KP183963]	99.0
	P.palmivora	Pph21	*P.palmivora*.[KP183963]	98.6
	P.palmivora	Pph22	*P.palmivora*.[KP183963]	98.8
	P.palmivora	Pph23	*P.palmivora*.[KP183963]	98.1
	P.palmivora	Pph24	*P.palmivora*.[JX198562]	95.0
	P.palmivora	Pph25	*P.palmivora*.[JX198562]	95.2
Type II	*P.nicotianae*	Pph38	*P.nicotianae*-NRCPh-61.[JX965375]	97.2
	P.nicotianae	Pph39	*P.nicotianae*-NRCPh-61.[JX965375]	97.6
	P.nicotianae	Pph40	*P.nicotianae*-NRCPh-61.[JX965375]	96.7
	P.nicotianae	Pph41	*P.nicotianae*-NRCPh-61.[JX965375]	97.6
	P. nicotianae	Pph42	*P.nicotianae*-NRCPh-61.[JX965375]	97.2
	P. nicotianae	Pph43	*P.nicotianae*-NRCPh-61.[JX965375]	97.6
	P. nicotianae	Pph44	*P.nicotianae*-NRCPh-61.[JX965375]	96.7
	P. nicotianae	Pph45	*P.nicotianae*-NRCPh-61.[JX965375]	97.6

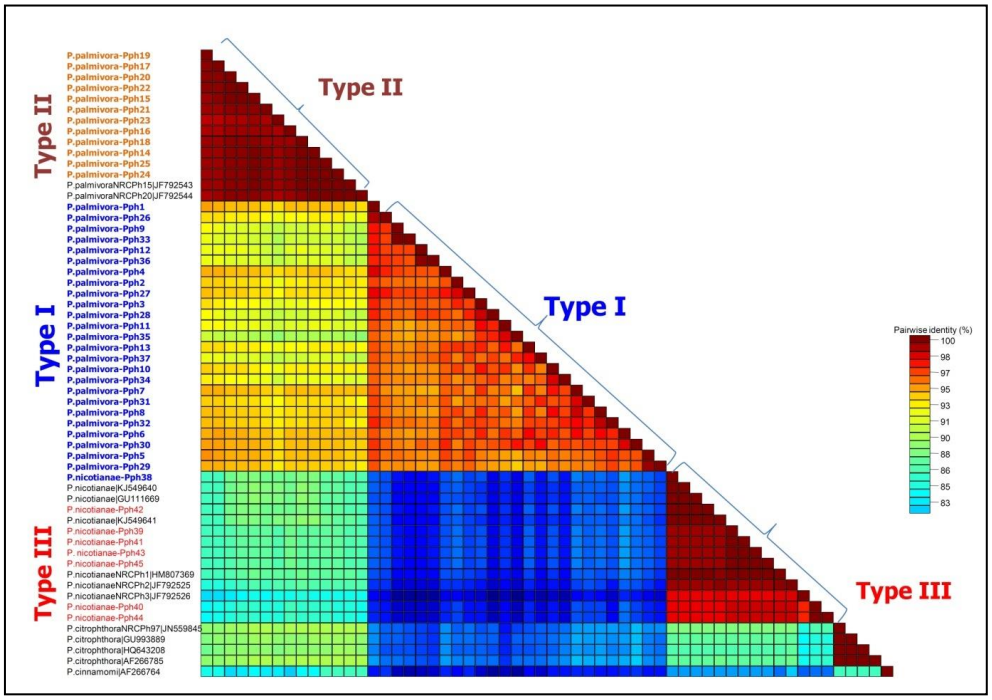

Figure 2: The pairwise identity scores were calculated for *Phytophthora palmivora* and *Phytophthora nicotianae* in the present study with other *Phytophthora* sp. available in the NCBI database using the Sequence demarcation tool.

a separate cluster. The type II category contains twelve *P. palmivora* isolates (Pph14, Pph15, Pph16, Pph17, Pph18, Pph19, Pph20, Pph21, Pph22, Pph23, Pph24 and Pph25) showing maximum nucleotide identity of 93.6 to 99 % and closely clusters with *P. palmivora* (AF266780, JF792543 and JF792544). The type III category contains eight *P. nicotianae* isolates (Pph38, Pph39, Pph40, Pph41, Pph42, Pph43, Pph44, Pph45) shearing maximum nucleotide identity of more than 93% with *P. nicotianae* and closely clusters with *P. nicotianae* isolates (JF792526, GU111669, HM807369, KJ549640, AF266776, and JF792525) infecting different citrus species which are available in the NCBI database (Fig 3).

The study clearly showed that there is an existence of variability in *P. palmivora* isolates infecting Coorg mandarin in different places of Karnataka. The literature survey also showed similar variability in *P. palmivora* and *P. nicotianae* isolates, affecting different citrus species in India. Further, they also reported that *P. nicotianae* is the dominant species infecting different citrus species in India, followed by *P. palmivora* and *P. citrophthora* [3]. On the contrary, our study showed that *P. palmivora* is a more predominant species, followed by *P. nicotianae* infecting Coorg mandarin in different places of Karnataka. The *P. palmivora* fungus was first reported from central India [15, 3] later in Florida, USA [21], and they say that *P. palmivora* is highly pathogenic to fibrous and large roots of citrus as compared to *P. nicotianae* and *P. citrophthora* [22,16]. The literature survey also indicated that *P. palmivora* and its distribution was limited to the state of Maharashtra and not detected in other citrus-growing states [3], but in our study, we have detected more citrus samples infected with *P. palmivora* than the other two species, such as *P. nicotianae* and *P. citrophthora*. As per nature and spread of different *Phytophthora* species is concerned, the *P. palmivora* may spread faster than other two species, such as *P. nicotianae* and *P. citrophthora*, due to its deciduous nature sporangia that are widely disseminated, and its outcompeting parasitic ability [12,16]. Similar results were also showed that *P. palmivora* infects several horticultural crops in India, such as palms, cocoa, black pepper, and cassava, besides citrus [1]. The *P. nicotianae* is a destructive pathogen widely distributed in tropical and warm temperate regions. Similarly, in the present study, we have detected a few numbers of samples having *P. nicotianae* in different places of Karnataka. Earlier several workers recorded *P. nicotianae* causes citrus decline in different citrus growing regions of India [15,19,172] mainly based on their morphological characteristics. But our study showed that *P. palmivora* was the predominant species in citrus plantations, followed by *P. nicotianae* in different places of Karnataka and none of the Coorg mandarin samples were showed positive for *P. citrophthora*. The study also clearly showed that there is more variation in the ITS region of the P. *palmivora* isolates infecting Coorg mandarin than *P. nicotianae* infecting Coorg mandarin.

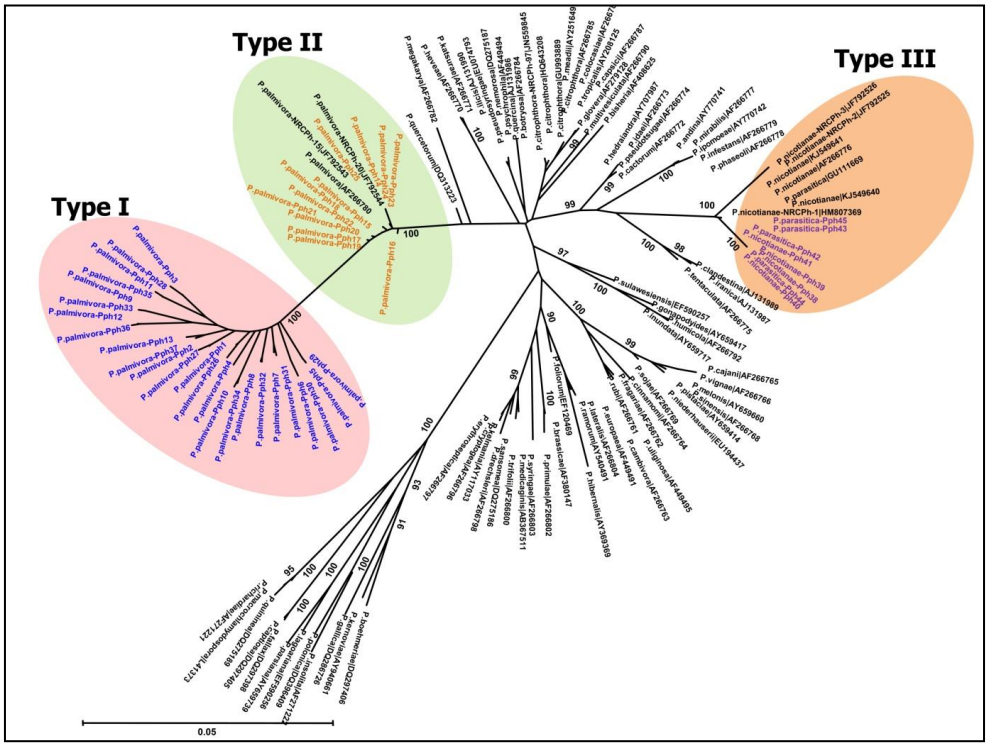

Figure 3: The phylogenetic tree shows the relationships of the *Phytophthora palmivora* and *Phytophthora nicotianae*, an understudy with selected *Phytophthora* sp. infecting different crops available in the NCBI database. The phylogenetic trees were constructed employing the MEGAX tool, using the neighbor-joining method with 1000 bootstrap replicates.

CONCLUSION

Coorg mandarin is a special type of mandarin cultivated in the Kodagu, Hassan, and Chikmagalur districts of Karnataka (India) in coffee and pepper plantations. Phytophthora causes root rot, and gummosis is a bottleneck for the cultivation of Coorg mandarin in high humid tropical regions of Western Ghats. The pathogen causes a significant loss of yield every year. In the present study survey, pathogenicity and ITS region characterization showed that the Coorg mandarin is infected by two types of Phytophthora species viz; *P. palmivora* and *P. nicotianae* in different places of Karnataka. Further, it was also shown that *P. palmivora* is the most predominant species in citrus plantations, followed by P. nicotianae in different places of Karnataka.

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