Use of ITS Region for Molecular Differentiation of *Exobasidium vexans* Causing Blister Blight in Tea

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Abstract

Blister blight caused by biotrophic fungus *Exobasidium vexans* Massee is the most problematic leaf disease in tea *(Camellia sinensis (L.) O. Kuntze)* However, there is little information available on the genetic variation of *E. vexans* in Sri Lanka. Understanding the molecular make-up of the pathogen and pathogen population will be helpful for identifying virulent strains, developing and deploying cultivars with durable resistance and development of appropriate disease control strategies. This study was carried out to develop a method to collect pathogen materials for DNA analysis and to test the feasibility of using Basidiomycetes specific ITS-1F and ITS-4B primers for studying molecular diversity of *E. vexans.* DNA was extracted using DNeasy® mini extraction kit from Basidiospores of *E. vexans* collected by spore fall technique and pure cultures of *Pestolotia theae, Cladosporium sp.* (Ascomycetes) and *Poria hypolateritia* (Basidiomycetes) for comparisons. PCR assays were carried out using ITS-F and ITS-4B primer pairs. The primer pair preferentially amplified a prominent and reproducible region (700 bp) for both of *E. vexans* and *P. hypolateritia* but not the two ascomycetes fungi tested. The excised bands of *E. vexans* collected from Talawakelle and *P. hypolateritia* were then used as templates in a second PCR, using the same primers and the re-PCRed product was sequenced" The DNA sequences of both species showed higher similarity (>80%) with the DNA sequences of Basidiomycetes fungi in the Genbank. ITS sequences of *E. vexans* showed 77-89% homology with other *Exobasidium* spp. when subjected to BLAST analysis confirming the accuracy of the amplified region. The results of this study suggest that ITS-1F and ITS-4B primer pair can be successfully used to discriminate *E. vexans* from mixed templates and to study the genetic diversity of this pathogen in Sri Lanka.

Key words: Basidiomycetes, Blister blight, DNA sequencing, *E. vexans*, Tea

Introduction

Exobasidium vexans is a destructive pathogen that causes Blister blight in tea in almost all tea-growing areas of Asia. As infection is directly to harvestable shoots used for manufacturing of black tea, crop loss caused by this pathogen is enormous especially under misty and rainy weather conditions besides adversely affect the quality of made tea. The pathogen also attacks young shoots that emerge after pruning, badly affecting the recovery of the pruned bushes. The symptoms start with small yellowish translucent spots then infected area slowly become sunken into a shallow depression on the upper side of the leaf and on the corresponding under-side they become convex, forming the typical blister lesion. Blistered tissues turn into necrotic spots after releasing spores (Gadd and Loos, 1949).

Tea is grown under different climatic conditions and varieties of recommended tea cultivars are available in Sri Lanka. Blister blight seems to infect tea in almost all tea growing areas and a cultivar showing total resistant to blister blight has not been identified so far. Disease control is virtually impossible, if the high variability exists within the pathogen population. Despite its wide distribution and seriousness, information on genetic variability of *E. vexans* is limited in Sri Lanka. One of the reasons attributed for this is *E. vexans* is an obligate pathogen hence cannot be cultured in pure form in the laboratory and large-scale multiplication is therefore, not possible. Therefore, it is required special methods to collect sufficient materials for extracting DNA for subsequent molecular analysis.

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The preliminary investigation of genetic diversity of *E. vexans* using RAPD markers showed high variation with respect to different locations and different cultivars (Kumari *et al.,* 2010). The major drawback of the above method is poor repeatability due to the nature of the primers used.

The Internal Transcribed Spacer (ITS) regions are widely engaged in fungal classification. ITS are sequences located in eukaryotic rDNA repeat between the small subunit, 5.8S, and the large subunit coding regions. Studies on restriction site variation in the rDNA in populations have shown that, while coding regions are conserved, spacer regions are variable (Grades and Burns, 1993). They are useful for phylogenetic analyses among related species and/or among populations within a species.

In this study it was aimed at developing a methodology for collection of sufficient basidiospores of *E. vexans* and to test the feasibility of using basidomycetes specific ITS primers ITS-1F and ITS-4B for molecular differentiation of *E. vexans.*

Materials and Methods

Fungal material

Basidiospores were collected from mature blisters by modifying spore fall technique described by Sharma and Chakraborty (2004). Shoots with well-developed sporulating lesions collected from Talawakelle, Nuwar Eliya, Passara and Ratnapura were selected and dipped in conical flasks containing sterile 2% sucrose solution. The leaves with the sporulating blisters were placed horizontally (ventral side) with the support of a sterile microscopic slide over a wide mouth sterile glass bottles (50 ml capacity) containing 2 ml of 6% sterile sucrose solution in the way allowing spores to be fall into the sucrose solution. Similarly the spores were collected in to several bottles. The whole setup was covered by a moisture chamber made by glass bell jar and incubated at Room Temperature (23 °C) for collection of basidiospores. After 24–48 h of incubation, the basidiospores deposited on the sucrose solution were collected into 1.5 ml eppendorf tubes by centrifugation at 4000 g for 5 minutes. The collected spores were stored at -80 °C until use.

Besides, a culture of *Cladosporium* sp. and *P. theae* were isolated on potato dextrose agar (PDA) from necrotic tissues following blister blight infection and *P. hypolateritia* from red root disease lesion following surface sterilization with 1% NaOCl for 1 min. Seven day old monosporic cultures grown on PDA at 27±2 °C were used for DNA extraction for comparison purposes.

DNA extraction and PCR

DNA was extracted from the collected spores of *E. vexans* and pure cultures of *Cladosporium* sp. and *P. theae* and *P. hypolateritia* using DNeasy® plant mini DNA extraction kit (QIAGEN) following manufactures' protocol. PCR was carried out in 25 µl reaction volumes each containing 2 µM ITS-1F and ITS-4B primers, 2 mM $MgCl_2$, 2 mM dNTP-mix, 1X PCR buffer (Promega) and 2 units Taq DNA Polymerase (Promega). Cycling parameters were 1 cycle of 95 °C for 5 min of initial denaturation followed by 30 cycles of denaturation at 94 °C for 4 min, primer annealing at 55 °C for 1 min, extension at 72 °C for 90 sec and final extension for 7 min. PCR product was separated and visualized on silver stained PAGE with 100 bp molecular weight marker. A prominent band at 700 bp of *E. vexans* and *P. hypolateritia* was excised using sterile sharp blade and re-PCRed. The re-PCRed products were sequenced using AB3500DX genetic analyzer. The obtained sequences were subjected to BLAST analysis. A phylogenetic tree was constructed by using DNADist Neighbor-Joining method in BioEdit(version 3.6a2.1).

Results and Discussion

E. vexans as an obligate pathogen, the main body of the fungus is within the infected plant parts. Basidiospores are produced in tremendous numbers in a distinct hymenium that appeared as a white, felt-like layer. A mature blister lesion can produce about 2 million spores in 24 h (Huysman, 1952). Though several authors time to time reported culturing this pathogen on artificial media, success and repeatability of those methods remain questionable. Hence, basidiospores are the only reliable fungal structures that could be collected for *in vitro* studies. Sharma and Chakraborty (2004) described a method for collecting basidiospores from mature blister for inoculation purpose. Here, we made modifications to the method described in Sharma and Chakraborty (2004) in order to get enough fungal material for extraction of DNA.

The modified spore fall technique yielded germinating basidiospores in the sucrose solution which is free of plant DNA. Spore palette of 2-5 mm height from the bottom of 1.5 µl eppendorf tube was enough for extracting DNA from *E. vexans* using DNeasy® DNA extraction kit and yielded good quality DNA. Generally, monoconidial cultures are used for molecular studies of fungi in order to maintain uniformity and purity. In the absence of such purification, there are chances of contamination with other leaf surface inhabiting fungi and bacteria in the collected basidiospores. Here, we used Basidomycetes specific ITS-1F and ITS-4B (Grades and Burns, 1993) primers to amplify *E. vexans* together with possible phylopllane contaminants *Cladosporium* sp. and *P. theae* belong to Asocomycetes and a Basidomycetes tea root pathogen, *P. hypolateritia*, as a positive control to test the specificity of the primer pair.

In order to optimize PCR conditions, a series of incrementally higher annealing temperatures and series of DNA quantities were tested. Annealing at 55°C gave the cleanest results when about 25 ng of DNA of *E. vexans* was used. However, the primer pair showed higher sensitivity to *P. hypolateritia* and even 3 ng of DNA was sufficient. The primer pair efficiently amplified the Basidiomycetes, *E. vexans* and *P. hypolateritia*. Amplification of DNA of *Cladosporium* sp. and *P. theae* resulted in either no product or extremely faint product. The results showed that the Basidiomycetes specific ITS-1F and ITS-4B primer pair is suitable for amplifying *E. vexans* from mixed template total DNA sample which may contain possible contamination of phylloplane Ascomycetes.

To identify the target region of the rDNA, specific bands of of *E. vexans* and *P. hypolateria* were sequence characterized. *E. vexans* yielded a sequence of 650 bp while *P. hypolateria* resulted a sequence of 780 bp. All the BLAST hits were Basidiomycotina when query sequence was *E. vexans* and *P. hypolateria* separately (for *E. vexans*: total hits 95 hits, 53 organisms, similarity 90-82%; E value, 0 to111e-1, coverage 98-58%, and for *P. hypolateria*: total 78 hits, 34 organism 95-84% similarity E value 0, coverage 97-79%).

Pair wise comparison of *E. vexans* with neucleotide sequences of 26 *Exobasidium* spp. available in Genbank showed 87-77% similarity. Similarity with *E. camellia* known to cause disease in other *Camellia* sp. and *E. rhododendri* known to cause blister blight in Rhododendron in Sri Lanka showed 77% and 80%, respectively confirming accuracy of the region amplified. *E. vexans* sequence obtained from this study (TRI F Ev) and Genbank sequence of this species (KC442794.1) clusterd separately from other *Exobasidium* spp. (Figure 1) while *P. hypolateritia* formed an out-group.

Conclusion

In conclusion, the developed spore fall technique is easy and suitable for collecting basidiospores of E. *vexans* from naturally infected tissues for DNA extraction using DNeasy® extraction kit. ITS-1F/ITS-4B preferential amplification of *E. vexans* in a possible mixed template showed its suitability for the use in molecular differentiation of this serious pathogen of tea.

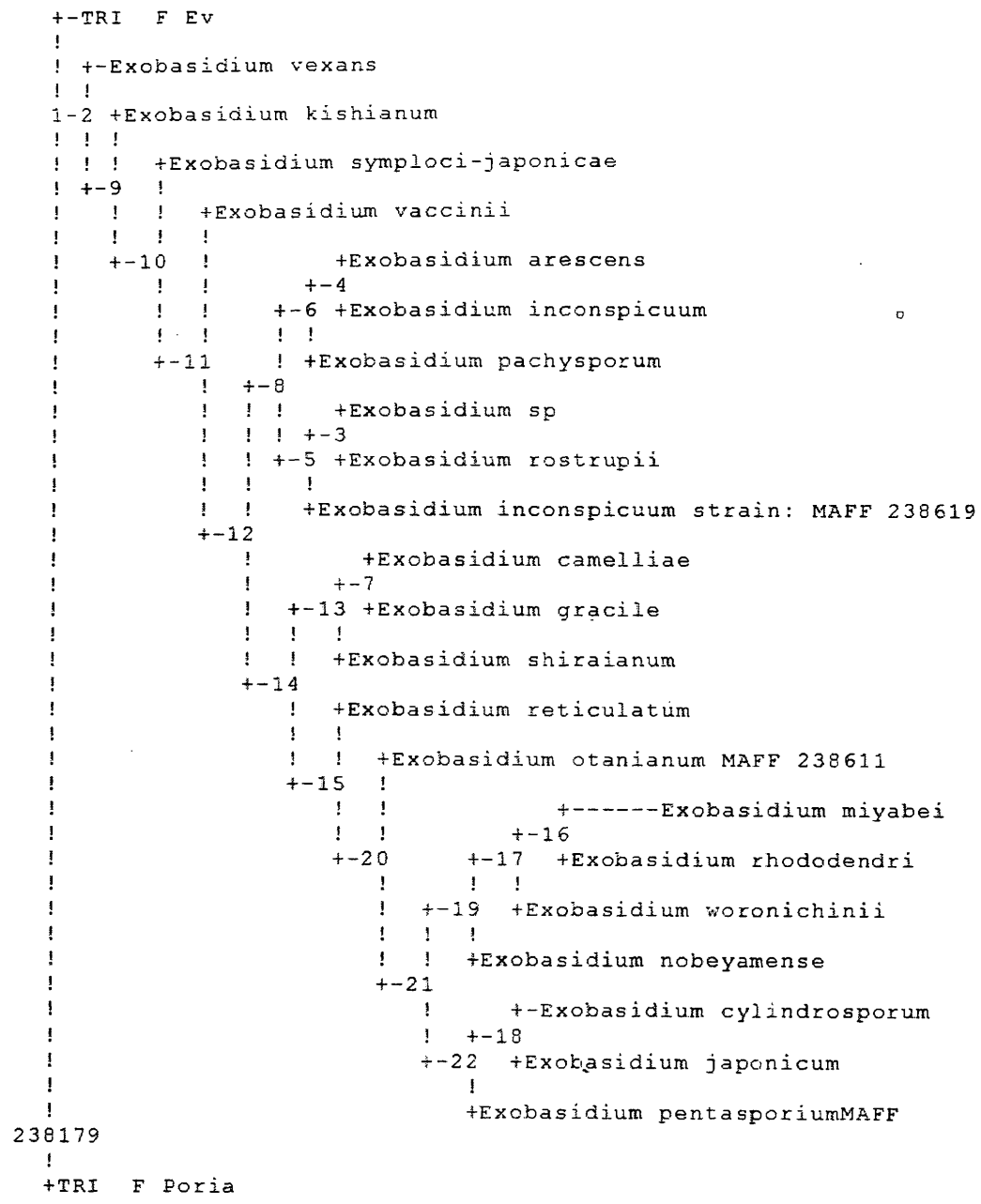

Figure 1. Unrooted neighbour joining phylogenetic tree for the obtained *E. vexans* (Tri F Ev), *P. hypolateritia* (TRI F Poria) and other *Exobasidium* spp. sequences available in Genbank.

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