DNA BARCODES FOR IDENTIFICATION OF *PHYTOPHTHORA* SPP. INFECTING NATURAL RUBBER TREES IN INDIA

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Abnormal leaf fall (ALF) disease caused by *Phytophthora* spp. is one of the most destructive diseases of rubber causing extensive defoliation leading to a reduction in the yield of natural rubber. Shoot rot and die back occur on young rubber plants due to infection with *Phytophthora* spp. Panel diseases *viz.* black stripe and patch canker are also caused by *Phytophthora* spp. on mature rubber trees. As a molecular tool for species identification, DNA barcodes offer significant potential using a short, unique, standardized portion of the DNA. This study aimed at presenting a molecular phylogeny of the four major *Phytophthora* species *viz. P. meadii, P. botryosa, P. colocasiae* and *P. citrophthora* predominantly infecting rubber trees in India. Two nuclear DNA regions: Internal Transcribed Spacer region of the nuclear ribosomal DNA and the microtubule constituent protein β-tubulin, and the mitochondrial gene cytochrome c oxidase subunit II were used in this study to get a more resolved phylogeny of the four *Phytophthora* species of rubber, providing better interpretation of the overall evolutionary history of the genus. Sequence information suggests that *P. meadii* has a hybrid origin with *P. colocasiae* as a parent and it is also possible that *P. citrophthora* is an asexual derivative of *P. colocasiae.* The study also demonstrates significant diversity in the population of *Phytophthora* spp. isolated from rubber plantations in India.

Key words: β-tubulin, Cytochrome c oxidase, DNA barcoding, Internal Transcribed Spacer, Molecular phylogeny, *Phytophthora* spp., Species identification.

INTRODUCTION

DNA sequences are a major source of information for understanding any species. DNA barcoding is an important species identification technique, holding promise for reliable, quick and accurate identification of fungal pathogens. As a unique and standard species identification tool, it offers a great potential by using various conserved universally accepted DNA barcode regions. DNA barcoding was first proposed by Hebert *et al*. (2003a) who used standardized 500 to 800 bp cytochrome c oxidase I sequences to identify species of all eukaryotic kingdoms with primers applicable for the broadest possible taxonomic group. DNA barcoding is being used for accurate, efficient and high-throughput assignment and discrimination of numerous animal, plant and fungal taxa (Kress and Erickson, 2007; Seifert *et al*., 2007; Chen *et al*., 2010; Massimiliano *et al*., 2010).

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Some of the reported genetic markers as potential regions for fungal barcodes are elongation factor 1-α (Geiser *et al*., 2004), β-tubulin (Samson *et al*., 2004), calmodulin (Hong *et al*., 2008), nuclear small and large ribosomal subunits (Seifert, 2009), largest and second largest subunit of RNA polymerase II (Balajee *et al.,* 2009), small actin (Roe *et al*., 2010), cytochrome c oxidase subunit I (Robideau *et al*., 2011), heat shock protein 90 (Zhao *et al*., 2011), internal transcribed spacer region (Schoch *et al*., 2012) and chitin synthase (Zeng *et al*., 2012). However, there are a lot of discrepancies relating to the variation in locus length, intraspecific/ interspecific variations, intron interference and appropriate barcoding gaps (Krüger *et al*., 2009; Begerow *et al*., 2010). The ITS region has been proposed as the core, most powerful and standard barcode region (Seifert, 2009). However, there is a lot of criticism on its limitations, including the methodological dimension of revealing intraspecific variation in ITS region (Schoch *et al*., 2012).

A good DNA barcoding marker should be simple (easy to PCR-amplify and sequence), universal (effective for a wide range of lineages) and should have high resolving power (high interspecific and low intraspecific variations). Therefore, an ideal DNA barcoding marker is a relatively short and reasonably variable gene fragment for species discrimination flanked by highly conserved sequences for primer design. The pioneering DNA barcoding work used mitochondrial cytochrome c oxidase 1 (*cox* 1) to identify animal species (Hebert *et al*., 2003a; 2003b). The 28S and 18S ribosomal subunits were also utilized for taxonomic identification above the genus. In a few fungal groups where these ribosomal regions were clearly shown to provide inadequate resolution, one or two additional housekeeping gene loci such as translation elongation factor 1a (EF-1), cytochrome c oxidase subunit I and II (*cox* I and II), NADH dehydrogenase subunit 1 (*nadh*1) and β-tubulin were also used in combination.

The genus *Phytophthora* contains a large diversity of devastating plant pathogens which occur in both natural and agricultural systems. Abnormal leaf fall disease caused by *Phytophthora* spp. is an annually recurring destructive disease of rubber trees in India causing significant loss to rubber production. This study aimed at presenting a genus-wide phylogeny for the four major *Phytophthora* species *viz. P. meadii*, *P. botryosa*, *P. colocasiae* and *P. citrophthora* reported to infect rubber trees in India.

MATERIALS AND METHODS

Isolate selection and DNA isolation

A total of 115 isolates from six species of *Phytophthora* were analyzed in this study (Table 1). All these isolates were hosted at the World Phytophthora and Genetic Resource Collection (WPGRC), USA. Of these 45 belonged to *P. citrophthora* (collected from USA, Brazil, Indonesia, Taiwan, Argentina, Japan, France and Poland affecting *Theobroma cacao, Fragaria, Colocasia esculenta* and *Ficus carica*), 27 belonged to *P. meadii* (collected from India and Sri Lanka affecting *Hevea brasiliensis, Elettaria cardamomum*, *Areca catechu* and *Citrus*), 19 belonged to *P. colocasiae* (collected from India, Indonesia, Hawaii, Philippines, China, American Samoa affecting *Colocasia* spp.) and nine belonged to *P. botryosa* (collected from Malaysia and Thailand affecting *H. brasiliensis*). Nine isolates of *P. capsici* and six isolates of *P. palmivora* were used as outgroups. In addition, thirty one isolates of *Phytophthora* collected from India affecting rubber trees were also used to study their evolutionary relationship. Isolation of *Phytophthora* spp. from typical symptomatic portions of petioles and leaves of rubber plants were made by plating surface-sterilized pieces of diseased tissue on laboratory made potato

Table 1. **Isolates used for species delineation of *Phytophthora meadii, P. botryosa, P. colocasiae* and *P. citrophthora* affecting rubber trees in India**

No.	*Phytophthora* species	Isolate details[1]				
		Isolate identification		Isolate origin		
		Local[2]	International	Host	Country	Year
1	*P. botryosa*	P6714	ATTC 66634	*Hevea brasiliensis*	Malaysia	
2	*P. botryosa*	P6715		*Hevea brasiliensis*	Malaysia	
3	*P. botryosa* (T)	P3425	IMI 136915	*Hevea brasiliensis*	Malaysia	1998
4	*P. botryosa*	P1044	ATTC 52221	*Hevea brasiliensis*	Malaysia	
5	*P. botryosa*	P10603	MYA 4059	*Hevea brasiliensis*	Thailand	
6	*P. botryosa*	P6944	ATCC 64862	*Hevea brasiliensis*	Vietnam	
7	*P. botryosa*	P6945	IMI 130422	*Hevea brasiliensis*	Malaysia	1990
8	*P. botryosa*	P6716		*Hevea brasiliensis*	Malaysia	
9	*P. botryosa*	P6213	ATCC 26483	*Hevea brasiliensis*		
10	*P. citrophthora*	P0479	ATCC 52231	*Citrus*	California	
11	*P. citrophthora*	P1324	ATCC 64854	*Citrus*	California	1977
12	*P. citrophthora*	P1200	ATCC 64812	*Theobroma cacao*	Brazil	1980
13	*P. citrophthora*	P7627	ATCC 76180	*Colocasia esculenta*	Taiwan	
14	*P. citrophthora*	P10368		Soil	Argentina	2001
15	*P. citrophthora*	P10370		Strawberry	Argentina	2001
16	*P. citrophthora*	P11353		*Pieris*	Poland	2004
17	*P. citrophthora*	P11452		*Syringa vulgaris*	Poland	2004
18	*P. citrophthora*	P11473		*Buxus sempervirens*	Poland	2004
19	*P. citrophthora*	P0318	ATCC 64851	*Citrus*	Australia	
20	*P. citrophthora*	P10208		*Taxus*	Massachusetts	2003
21	*P. citrophthora*	P10785				
22	*P. citrophthora*	P10786				
23	*P. citrophthora*	P10787				
24	*P. citrophthora*	P10861				
25	*P. citrophthora*	P10867				
26	*P. citrophthora*	P10868				
27	*P. citrophthora*	P10871				
28	*P. citrophthora*	P10873				
29	*P. citrophthora*	P10878				
30	*P. citrophthora*	P10938				
31	*P. citrophthora*	P11055				
32	*P. citrophthora*	P11498				

[1] Complete details of certain isolates are not available
[2] Identification number registered with World Phytophthora and Genetic Resource Collection, USA

33	*P. citrophthora*	P6310		*Theobroma cacao*	Indonesia	1989
34	*P. citrophthora*	P10205				2003
35	*P. citrophthora*	P10207		*Taxus*	Massachusetts	2003
36	*P. citrophthora*	P10788				
37	*P. citrophthora*	P10860				
38	*P. citrophthora*	P10870				
39	*P. citrophthora*	P1212	ATCC 64858	*Theobroma cacao*	Brazil	1979
40	*P. citrophthora*	P1323		*Citrus*	California	1975
41	*P. citrophthora*	P10142		*Citrus*	California	2002
42	*P. citrophthora*	P10144		Bark mix	South Carolina	1997
43	*P. citrophthora*	P6626		*Fragaria*	Taiwan	
44	*P. citrophthora*	P10866				
45	*P. citrophthora*	P10872				
46	*P. citrophthora*	P10875				
47	*P. citrophthora*	P10876				
48	*P. citrophthora*	P10877				
49	*P. citrophthora*	P11098				
50	*P. citrophthora*	P11285				
51	*P. citrophthora*	P1213		*Theobroma cacao*	Brazil	1979
52	*P. citrophthora*	P1324	ATCC 64854	*Citrus*	California	1977
53	*P. citrophthora*	P10206	ATCC MYA4158			2003
54	*P. citrophthora*	P0776	ATCC 46720	*Theobroma cacao*	Brazil	1971
55	*P. colocasiae*	P6102		*Colocasia esculenta*	India	
56	*P. colocasiae*	P10273		*Colocasia esculenta*	American Samoa	2003
57	*P. colocasiae*	P6396		*Colocasia esculenta*	Indonesia	
58	*P. colocasiae*	P8604		*Colocasia esculenta*	China	
59	*P. colocasiae*	P7538		*Colocasia esculenta*	Philippines	
60	*P. colocasiae*	P8170			India	
61	*P. colocasiae*	P8605		*Colocasia esculenta*	China	
62	*P. colocasiae*	P10701		*Colocasia esculenta*	American Samoa	1993
63	*P. colocasiae*	P3774		*Colocasia esculenta*	Indonesia	1988
64	*P. colocasiae*	P3775		*Colocasia esculenta*	Indonesia	1988
65	*P. colocasiae*	P6315		*Colocasia esculenta*	Indonesia	
66	*P. colocasiae*	P6316		*Colocasia esculenta*	Indonesia	
67	*P. colocasiae*	P6318		*Colocasia esculenta*	Indonesia	
68	*P. colocasiae*	P6320		*Colocasia esculenta*	Indonesia	
69	*P. colocasiae*	P7174		*Colocasia esculenta*	Hawaii	1990
70	*P. colocasiae*	P7177		*Colocasia esculenta*	Hawaii	1990

71	*P. colocasiae*	P7464		*Colocasia antiquorum*	India	
72	*P. colocasiae*	P6290		*Colocasia esculenta*	Indonesia	1989
73	*P. colocasiae*	P6317		*Colocasia esculenta*	Indonesia	1989
74	*P. meadii*	P6128	IMI ICRI-240	*Elettaria cardamomum*	India	1989
75	*P. meadii*	P6947		*Dioscorea*	Malaysia	1986
76	*P. meadii*	P6263		*Hevea brasiliensis*	India	
77	*P. meadii*	P7494	IMI 335651	*Hevea brasiliensis*	India	1988
78	*P. meadii*	P7849	IMI 352316	*Areca catecha*	India	1990
79	*P. meadii*	P7981		*Hevea brasiliensis*	India	
80	*P. meadii*	P10191	ATCC MYA4043	*Citrus*	India	2003
81	*P. meadii*	P3433	IMI 129185	*Hevea brasiliensis*	India	
82	*P. meadii*	P3501	IMI 80030	*Hevea brasiliensis*	Sri Lanka	
83	*P. meadii*	P3950	IMI 129185	*Hevea brasiliensis*	India	1968
84	*P. meadii*	P6256		*Hevea brasiliensis*	India	1989
85	*P. meadii*	P6257		*Hevea brasiliensis*	India	
86	*P. meadii*	P6258		*Hevea brasiliensis*	India	1989
87	*P. meadii*	P6259		*Hevea brasiliensis*	India	
88	*P. meadii*	P6261		*Hevea brasiliensis*	India	
89	*P. meadii*	P6264		*Hevea brasiliensis*	India	
90	*P. meadii*	P6265		*Hevea brasiliensis*	India	
91	*P. meadii*	P6504		*Hevea brasiliensis*	India	1989
92	*P. meadii*	P6505		*Hevea brasiliensis*	India	1989
93	*P. meadii*	P6506		*Hevea brasiliensis*	India	1989
94	*P. meadii*	P7493	IMI 335648	*Hevea brasiliensis*	India	1988
95	*P. meadii*	P10190		*Citrus*	India	2003
96	*P. meadii*	P6262		*Hevea brasiliensis*	India	1989
97	*P. meadii*	P6865	ATCC 66771	*Hevea brasiliensis*	Sri Lanka	
98	*P. meadii*	P0643	IMI 99687			
99	*P. meadii*	P6030	IMI 131374	*Hevea brasiliensis*	Sri Lanka	1968
100	*P. meadii*	P3500	IMI 131374	*Hevea brasiliensis*	Sri Lanka	
List of isolates used as outgroups in the study						
101	*P. capsici*	P0622	IMI 207158	*Theobroma cacao*	Brazil	1969
102	*P. capsici*	P15127				
103	*P. capsici*	P15129				
104	*P. capsici*	P0253	ATCC 46012	*Theobroma cacao*	Mexico	1964
105	*P. capsici*	P10452		Irrigation water	California	2002
106	*P. capsici*	P10386		*Cucumis sativus*	Michigan	1997

107	*P. capsici*	P10735		*Piper nigrum*	China	2003
108	*P. capsici*	P1314		*Capsicum annuum*	California	1982
109	*P. capsici*	P1319	ATCC 64808	*Capsicum annuum*	California	1983
110	*P. palmivora*	P10422		*Theobroma cacao*	Costa Rica	
111	*P. palmivora*	P6375		*Durio zibethinus*	Malaysia	1989
112	*P. palmivora*	P10272		*Theobroma cacao*	American Samoa	2003
113	*P. palmivora*	P6220	ATCC 26484	*Hevea brasiliensis*		1989
114	*P. palmivora*	P6509		*Hevea brasiliensis*	India	1989
115	*P. palmivora*	P8484		*Hevea brasiliensis*	Malaysia	

dextrose agar (PDA) media. All isolates were purified and stored at room temperature. All isolates were maintained at the World Phytophthora Genetic Resources Collection (WPGRC; http://phytophthora.ucr.edu) at the University of California, Riverside, where accessions are preserved cryogenically under liquid nitrogen. Working cultures were maintained on V8 agar. At all stages of growth, cultures were checked for bacterial contamination by incubation for 24 h in Luria-Bertani broth. For DNA extraction, actively growing cultures were produced in V8 broth and harvested after 4-10 days. Approximately 200 mg of mycelium was rinsed with ultrapure water, placed in a 1.7 ml microcentrifuge tube and frozen by immersion in liquid nitrogen. DNA was extracted from frozen tissue using the Fast DNA kit and Fast Prep FP 120 instrument (Thermo Savant, France). DNA concentration was determined using spectro photometrically (260/280 ratio) and the DNA samples were stored in ultrapure water at -86^{o}C.

Marker selection and DNA amplification

Two nuclear DNA regions (Internal Transcribed Spacer region of the nuclear ribosomal DNA and the microtubule

Table 2. **Primers used for amplifying the Internal Transcribed Spacer region, β-tubulin and cytochrome oxidase II genes**

No. Primer name	Sequence (5′– 3′)	Position	Amplicon length	Reference
1. Internal Transcribed Spacer region				
ITS 4	TCCTCCGCTTATTGATATGC	+39 to +59 of 26S rRNA	754-834	White *et al.*
ITS 5	GGAAGTAAAAGTCGTAACAAGG	-54 to -32 from ITS 1 region	nucleotides	(1990)
2. β-tubulin				
BTUB F1	GCCAAGTTCTGGGAGGTCATC	52-72 bp	1228	Kroon *et al.*
BTUB R1	CCTGGTACTGCTGGTACTCAG	1259-1279 bp	nucleotides	(2004)
3. Cytochrome oxidase II				
FM 35	CAGAACCTTGGCAATTAGG	+50 to +68 of *cox* II		Martin
FMPhy-10b	GCAAAAGCACTAAAAATT		1040-1061	(2000)
	AAATATAA	+73 to +99 of *cox* II	nucleotides	Martin *et al.*
				(2004)

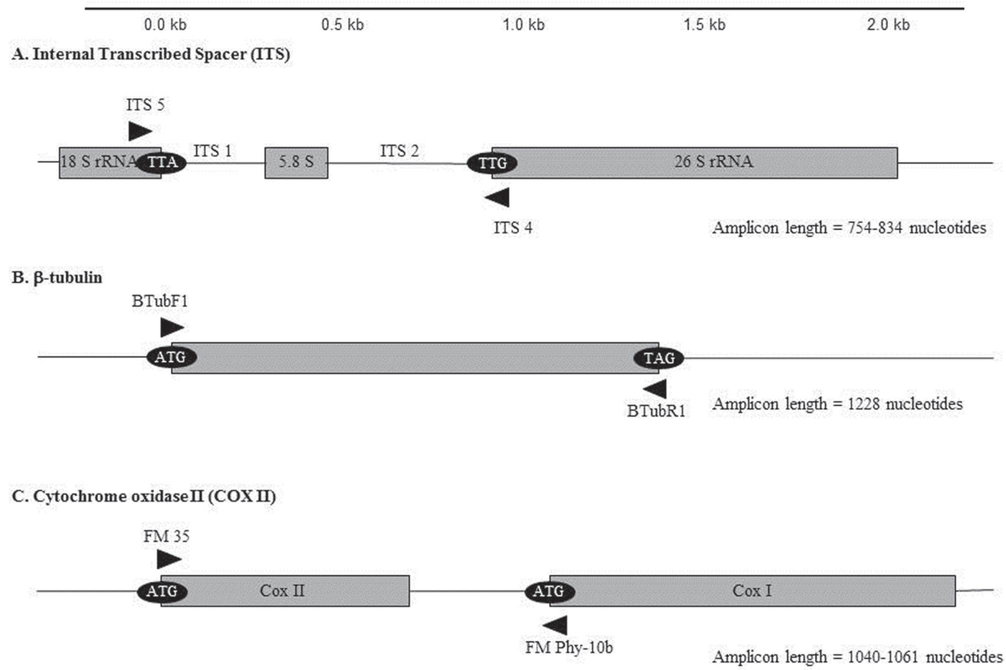

Fig. 1. Schematic diagram showing location of primers designed for amplification of ITS (A), β-tubulin (B) and *cox* II (C) barcoding markers along with their expected amplicon length

constituent protein β-tubulin) and one mitochondrial gene (cytochrome c oxidase subunit II) were selected as markers in this study to get a more resolved phylogeny of *Phytophthora* species affecting rubber. The primers employed for amplifying the three regions are indicated (Table 2) and the regions amplified are shown in Figure 1. Standard PCR conditions were applicable for all the three markers. Reaction mixtures were prepared using 5 ng template DNA, 200 mM dNTPs, 1 U *Taq* DNA Polymerase, 2.5 mM $MgCl_2$ and 1 mM of each primer in a reaction volume of 20 µl. Amplifications were performed with a MJ Research PTC-100 thermal cycler using the following cycling protocol: an initial denaturing step of 94°C for 2 minutes; 35 cycles of 94°C for 30 seconds, the locus-specific annealing temperature for 30 seconds, 72°C extension for 1 minute and final extension of 5 minutes at 72°C. A touchdown PCR program was used when the standard amplification protocol was unsuccessful: 10 cycles of 94°C for 30 seconds, 65°C for 30 seconds (reduced by one degree each cycle to 56°C), 72°C extension for two minutes, 30 cycles of 94°C for 30 seconds, 56°C for 30 seconds, 72°C extension for two minutes and final extension of five minutes at 72°C. All PCR products were evaluated for successful amplification through agarose gel electrophoresis.

Sequencing and phylogenetic analyses

PCR products were prepared for sequencing using an enzymatic purification system following the manufacturer's instructions

(Exo SAP-IT, USB Corporation, Cleveland, OH). Cycle sequencing reactions were performed at the Pennsylvania State University's Huck Institute Nucleic Acid Facility using the Big Dye system (version 3.1 dye terminators; Applied Biosystems, Foster City, CA) and run on an ABI 3730XL DNA Analyzer, using the ABI Data Collection Program (version 2.0) and ABI Sequencing Analysis software. ABI trace files were analyzed using Sequencher version 4.6 (Gene Codes, Ann Arbor, MI); heterozygous or ambiguous sites were labelled using the IUPAC code and consensus sequences were exported for phylogenetic analysis. Sequence data were deposited in the Phytophthora Database (http://www.PhytophthoraDB.org). Sequences were aligned using ClustalX. Alignments were visually inspected and edited manually for small indels. Preliminary phylogenetic trees were constructed for each marker using neighbor-joining with a Kimura two parameter nucleotide substitution model (Kimura, 1980) as implemented in MEGA version 3.1. All three markers were used for rigorous analysis as taxonomic representation across the genus was complete, or nearly so, for these loci. Neighbor-joining trees were constructed for each dataset and bootstrap analyses were performed with 1000 replicates.

RESULTS AND DISCUSSION

DNA barcoding using short DNA sequences derived from genes that evolve rapidly has revolutionized the identification of individual organisms and species providing clear differences between them as they evolve (Ali *et al.,* 2014). Thus, DNA barcodes serve a dual purpose as a new tool in the taxonomists' tool box supplementing his knowledge as well as being an innovative device for non-experts who need to make a quick identification. This study is the first attempt to assess utility of a nucleotide diagnostic approach for species delineation of four species of *Phytophthora* pathogen *viz*. *P. meadii*, *P. botryosa*, *P. colocasiae* and *P. citrophthora* of rubber trees in India.

Internal Transcribed Spacer (ITS) region of the nuclear ribosomal DNA consisting of ITS1 - 5.8S rDNA - ITS2, was earlier considered as the ideal DNA barcode as they have stable priming regions, their variable regions provide specificity and the size of single-pass sequencing run is about 700 bp making sequence analysis easier. Moreover, many ITS sequences already exist in the GenBank enabling easier comparison. In the present study, while this locus was easily amplified containing a large number of variable sites, the quality of the multiple sequence alignment of ITS data degraded as evolutionary distance increased. Hence, it was observed that this region was not fully suitable for phylogenetic studies.

Previous molecular studies have explored the relationships among *Phytophthora* species using one or a few genetic loci, predominantly the ITS region of the nuclear ribosomal DNA (Lee and Taylor, 1992; Crawford *et al*., 1996; Cooke *et al*., 2000) and cytochrome c oxidase I and II of the mitochondrion (Martin and Tooley, 2003a). More recent studies have used multiple loci from both the nuclear and mitochondrial genomes (Martin and Tooley, 2003b; Ivors *et al*., 2004; Kroon *et al*., 2004; Donahoo *et al*., 2006; Villa *et al*., 2006). Blair *et al*. (2008) used genome and other large sequence databases to identify over 225 potential genetic markers for phylogenetic analyses of *Phytophthora* spp. and a genus-wide phylogeny for 82 *Phytophthora* spp. was constructed using seven of the most informative loci (covering approximately 8700 bp). The alignments of seven loci were concatenated and analyzed revealing the

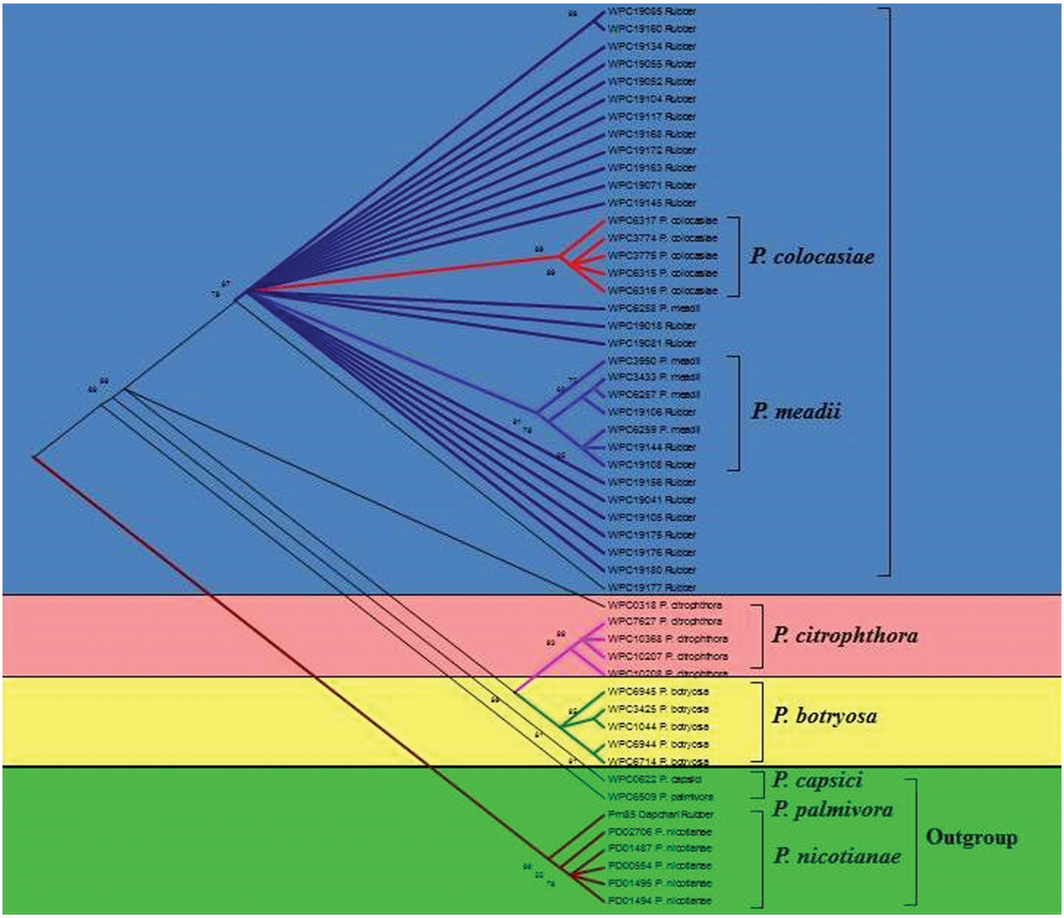

Fig. 2. Phylogenetic tree constructed using ITS sequences (831 bases) of four species of *Phytophthora* infecting rubber. Greater homology of rubber isolates collected from India with *P. colocasiae* and *P. meadii* was observed. Isolate name is indicated as deposited in the World Phytophthora Genetic Resource Collection, USA

division of the genus into 10 well-supported clades.

In this study, a combination of two nuclear DNA regions (Internal Transcribed Spacer region of the nuclear ribosomal DNA and the microtubule constituent protein β-tubulin) and one mitochondrial gene (*cox* II) were used for understanding phylogeny of these four *Phytophthora* species affecting rubber. Over 800 sequences were generated from the three markers used in the study. The proportion of informative sites and therefore the amount of phylogenetic signal present in each locus was variable. These individual loci were able to resolve relationships among these closely related species. The ITS region was consistently amplified across most of the isolates but the quality of multiple sequence alignment did not give a clear picture of evolutionary history. Although rDNA has been widely used in phylogenetic studies, it was found

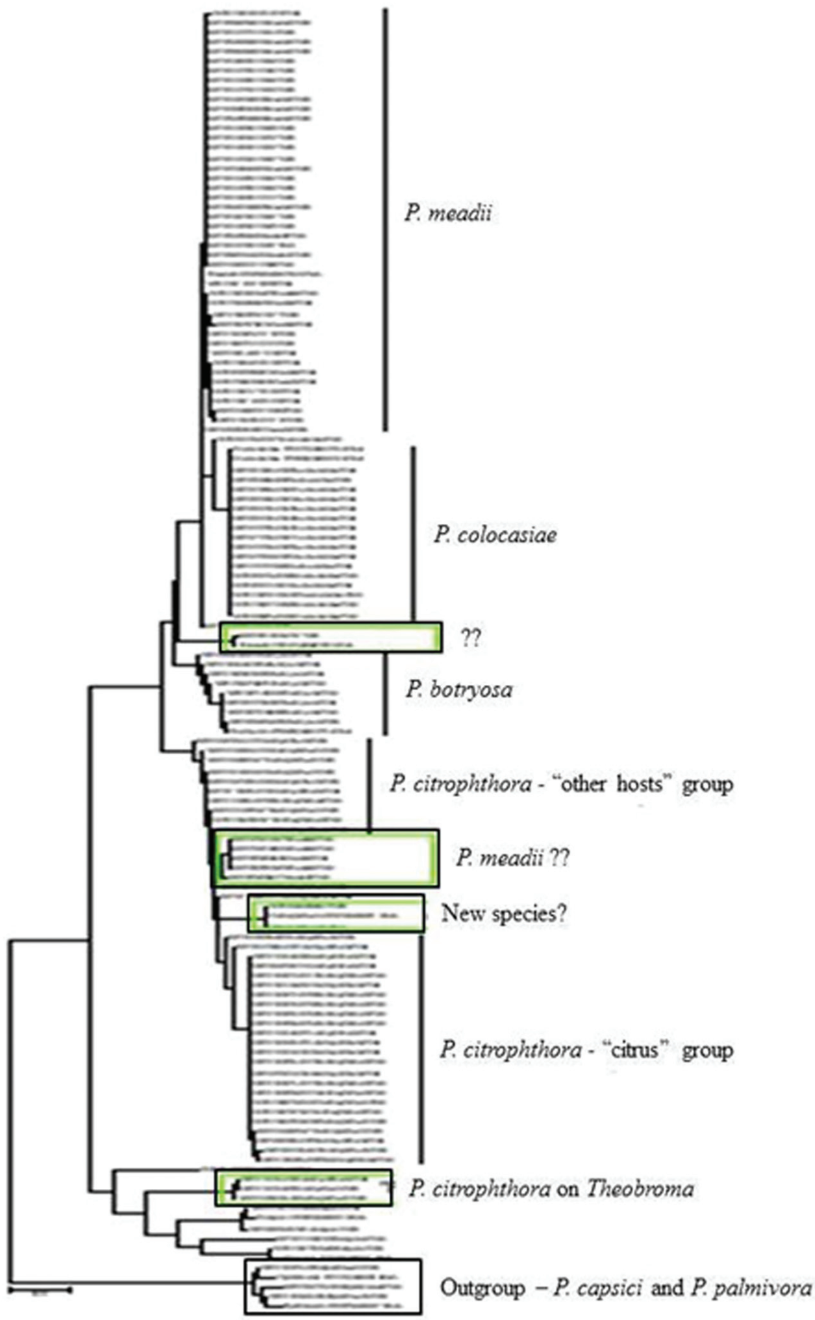

Fig. 3. UPGMA phylogram constructed based on β-tubulin barcode region for four *Phytophthora* spp. affecting rubber: *P. meadii*, *P. colocasiae*, *P. botryosa* and *P. citrophthora*

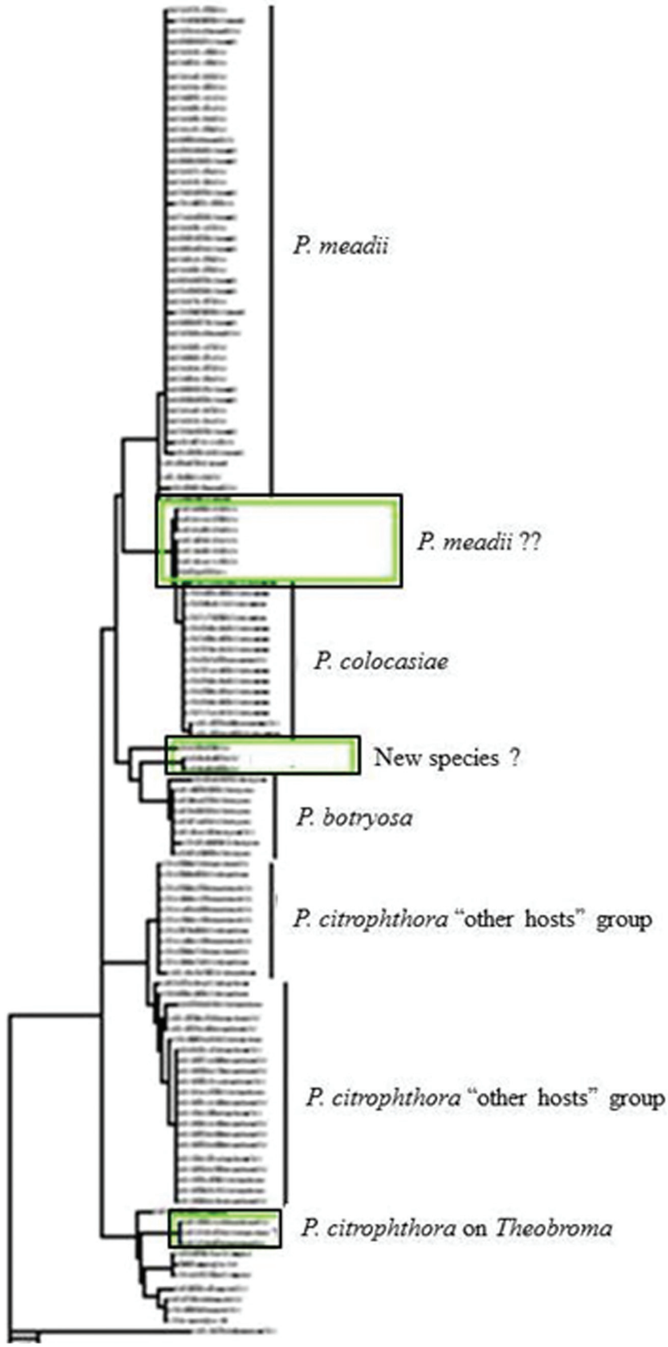

Fig. 4. UPGMA phylogram constructed based on *cox* II barcode region for four *Phytophthora* spp. affecting rubber: *P. meadii*, *P. colocasiae*, *P. botryosa* and *P. citrophthora*

that the evolution of one gene may not represent the evolution of the entire genome (Shen, 2001). Therefore, it was necessary to separately sample as many additional independent genes as possible and compare the phylogenies derived from these genes to see whether they support or contradict each other. Genes coding for metabolic and structural proteins such as cytochrome c oxidase II and β-tubulin, respectively, were found to be conserved and the alignment of their sequences was less ambiguous compared to rDNA-ITS region. The phylogenetic tree constructed with ITS sequences of 831 bases for the four species of *Phytophthora* grouped all rubber isolates collected from Indian rubber plantation with *P. meadii* and *P. colocasiae*. All *P. citrophthora* and *P. botryosa* isolates fell under two separate clades. The outgroups *P. capsici*, *P. palmivora* and *P. nicotianae* fell under different groups revealing distinct variability in their nucleotide sequence (Fig. 2).

Beta-tubulin gene codes for one of the two conserved families of tubulins, the building

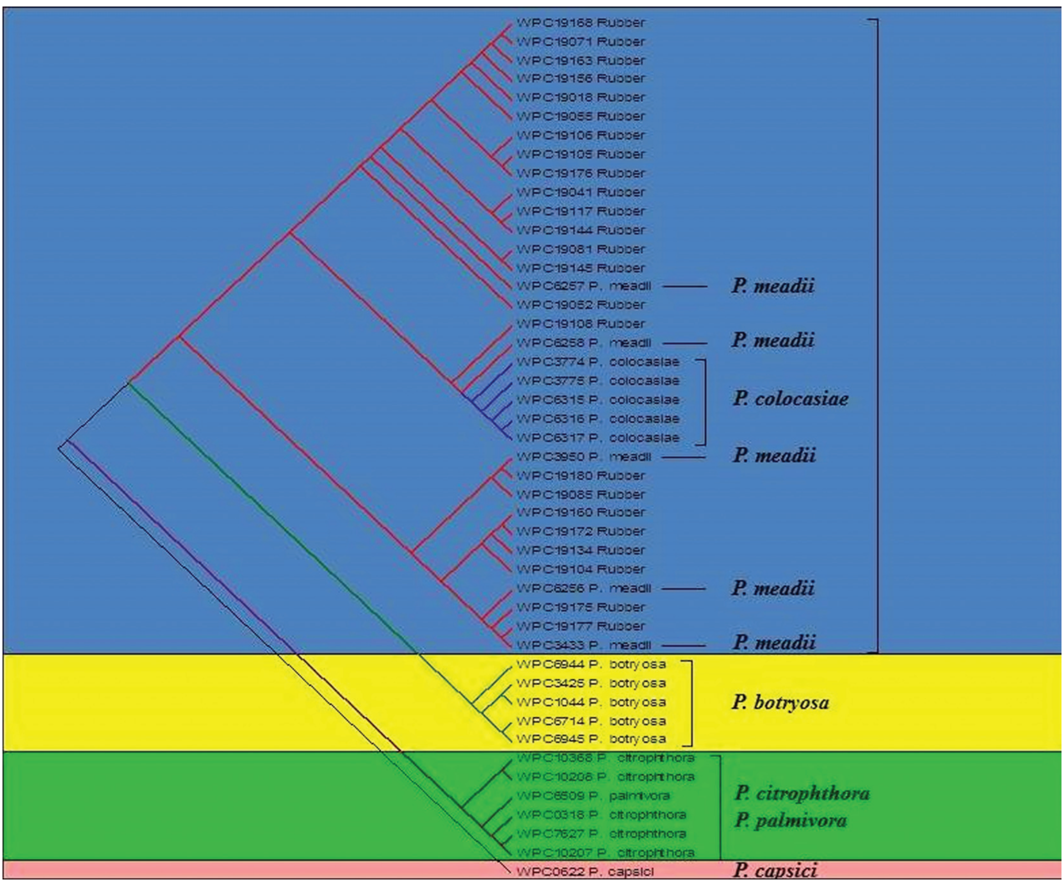

Fig. 5. Phylogenetic tree constructed using β-tubulin sequence (1228 bases) of four species of *Phytophthora* infecting rubber. Greater homology of rubber isolates collected from India with *P. meadii* and *P. colocasiae* was observed. Isolate name is indicated as deposited in the World Phytophthora Genetic Resource Collection, USA

blocks of microtubules which make up the cytoskeleton, mitotic spindles and flagella of eukaryotic cells. It has been found useful in reconstructing the phylogenetic relationships among fungi at all levels (Thon and Royse, 1999). In our study, a larger portion of the β-tubulin gene was used (1228 bp) and this locus provided the highest level of phylogenetic signal across the genus. However in the β-tubulin tree, there were a group of *P. meadii* isolates that showed up within the *P. citrophthora* clade (Fig. 3). But, these were placed with the *P. meadii* group in the mitochondrial tree (Fig. 4) indicating hybridization between *P. meadii* and *P. colocasiae* as well as between *P. meadii* and *P. citrophthora*. Dendrogram constructed using β-tubulin sequence (1228 bp) to understand genetic relationship among the four species of *Phytophthora* infecting rubber revealed greater homology of the rubber isolates collected from within Indian rubber plantation with *P. meadii* and *P. colocasiae*. All *P. botryosa* and *P. citrophthora* isolates fell under two separate clusters. The outgroup *P. capsici* was uniquely different. However, *P. palmivora* showed greater homology with *P. citrophthora* isolates (Fig. 5).

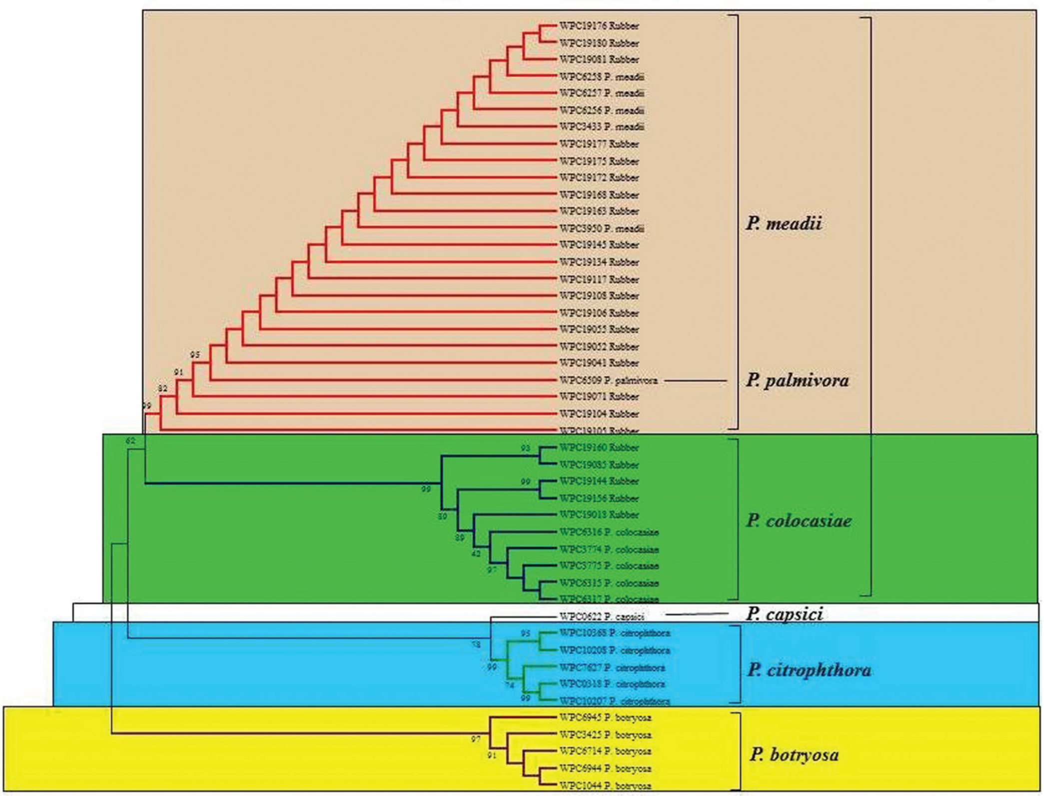

Fig. 6. Phylogenetic tree constructed using *cox* II sequence (1040 bases) of four species of *Phytophthora* infecting rubber. Greater homology of rubber isolates collected from India with *P. meadii* and *P. colocasiae* was observed. Isolate name is indicated as deposited in the World Phytophthora Genetic Resource Collection, USA

The cytochrome c oxidase II (*cox* II) gene codes for the enzyme that catalyzes the terminal step in the electron transport chain, the transfer of an electron from cytochrome c to oxygen. Hence, unlike the ITS region, it is mitochondrially encoded and so it is considered generally to be more variable than nuclear DNA. It has also proven useful for exploring relationships especially at the subgeneric or lower levels of various taxa (Villa *et al*., 2006). In this study, unlike the ITS region, gaps were not observed both in the β-tubulin and *cox* II sequence alignments. The highly conserved mitochondrial gene *cox* II revealed a set of *P. meadii* isolates that showed a closer relationship to *P. colocasiae* (Fig. 4). The *cox* II phylogenetic tree constructed with four species of *Phytophthora* infecting rubber showed greater homology of most rubber isolates with *P. meadii* and only five isolates showed homology with *P. colocasiae*. *P. botryosa* was a distinctly different cluster showing maximum variation. *P. citrophthora* was also a separate cluster. However, *P. capsici*, the outgroup used in the study, showed greater homology with *P. citrophthora* (Fig. 6).

Results of the present phylogenetic analyses based on the sequences of two

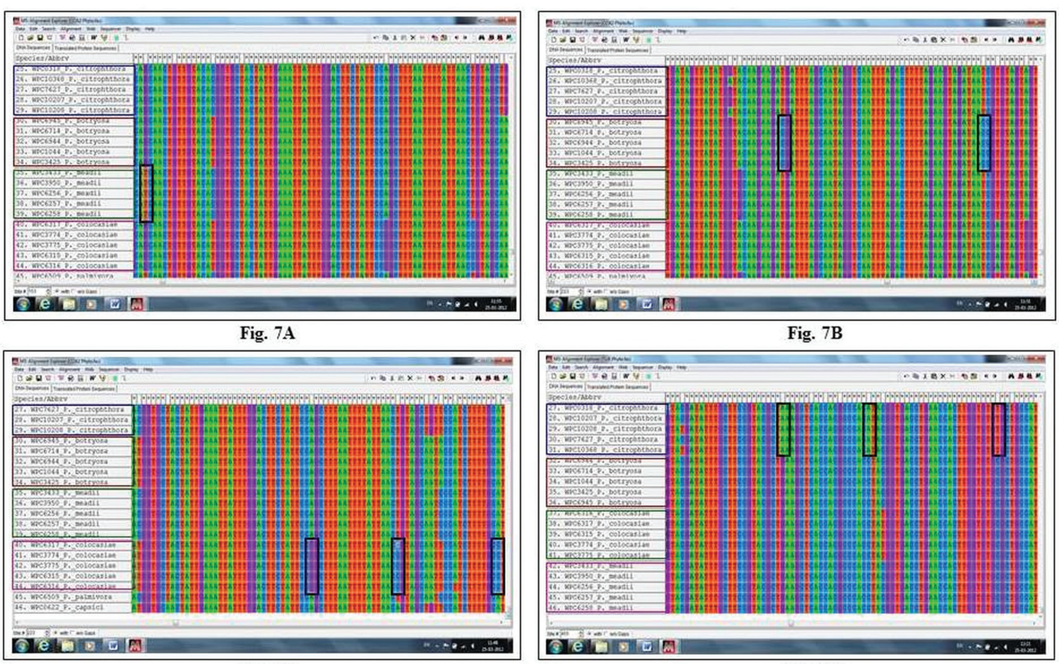

Fig. 7A Fig. 7B Fig. 7C Fig. 7D

Fig. 7. A snap shot of sequence alignment comparing four species of *Phytophthora* affecting rubber. Unique sequences/ DNA barcodes for each species were identified. (A) *P. meadii* at base position 155 in *cox* II sequence alignment (1040 bases); (B) *P. botryosa* at base positions 391 and 433 in *cox* II sequence alignment (1040 bases); (C) *P. colocasiae* at base positions 205, 223 and 244 in *cox* II sequence alignment (1040 bases) and (D) *P. citrophthora* at base positions 479, 497 and 524 in β-Tubulin sequence alignment (996 bases). Isolate name is indicated as deposited in the World Phytophthora Genetic Resource Collection, University of California, Riverside, USA

nuclear DNA regions (rDNA-ITS region and b-tubulin gene) and one mitochondrial gene (*cox* II) indicate a robust phylogenetic framework for interpreting the evolutionary history of these four species. Sequence information suggests that *P. meadii* has a hybrid origin with *P. colocasiae* as a parent and it is also possible that *P. citrophthora* is an asexual derivative of *P. colocasiae.* Phylogeny of the genus *Phytophthora* presented here represents a significant advancement in our knowledge on the four species of *Phytophthora* infecting rubber. A thorough understanding of the nucleotide variability and relationships among *Phytophthora* species allowing for better validation of diagnostic methods are becoming increasingly important for quarantine issues and disease monitoring.

Unique sequences/ DNA barcodes for each species were identified through this study. A snap shot of the sequence alignment comparing the four species of *Phytophthora* affecting rubber tree is shown in Fig. 7. The isolate names are indicated in the figure as deposited in the World Phytophthora Genetic Resource Collection, University of California, Riverside, USA. With the *cox* II sequence alignment consisting of 599 bases, unique barcodes were detected for *P. meadii* at base position 155 (Fig. 7A); for *P. botryosa* at positions 391 and 433 (Fig. 7B) and for *P. colocasiae* at base positions 205, 223 and 244 (Fig. 7C). With the β-Tubulin sequence alignment of 996 bases, unique DNA sequence for *P. citrophthora* was observed at base positions 479, 497 and 524 (Fig. 7D).

Successful use of different genes as barcodes described here is an initial step to provide the *Phytophthora* community with suitable loci for species identification, population and clade-level phylogenetic analyses, and targets for future multi-locus sequence typing diagnostic methods. Further, the data provide an essential database that permits development of new diagnostic methods based on specific oligonucleotides or other molecular based probes.

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