Phytophthora boehmeriae Revealed as the Dominant Pathogen Responsible for Severe Foliar Blight of *Capsicum annuum* in South India

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Abstract

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Prior to 2011, foliar blight was not reported as a serious threat to hot pepper cultivation in India. During the June-to-January cropping season of 2011 and 2012, severe foliar blight epidemics were observed in Karnataka and Tamil Nadu states of India. In all, 52 *Phytophthora* isolates, recovered from blight-affected leaf tissues of hot pepper from different localities in Karnataka and Tamil Nadu states between 2011 and 2012, were identified: 43 isolates as *P. boehmeriae* and 9 isolates as *P. capsici*, based on morphology, a similarity search of internal transcribed spacer sequences at GenBank, polymerase chain reaction (PCR) restriction fragment length polymorphism patterns, and species-

specific PCR using PC1/PC2 and PB1/PB2 primer pairs. The isolates were further assessed for metalaxyl sensitivity and aggressiveness on hot pepper. All isolates of *P. boehmeriae* were metalaxyl sensitive while *P. capsici* isolates were intermediate in sensitivity. *P. boehmeriae* isolates were highly aggressive and produced significantly ($P < 0.01$) larger lesion than those of *P. capsici* isolates. Thus, emergence of *P. boehmeriae* was responsible for severe leaf blight epidemics on hot pepper in South India, although it is not serious pathogen on any crop in any part of the world. These results have epidemiological and management implications for the production of hot pepper in India.

Chili (*Capsicum annuum* L.) is one of the most widely cultivated vegetable crops worldwide, consumed in various forms such as fresh, dry powder, paste, and sauce (2). It enhances food palatability and also is rich in vitamin C (2). Chili is grown on 3.7 million hectares worldwide, with an annual production of 33.4 million tons, and on 0.9 million hectares in India, with an annual production of 1.6 million tons (23). India is the world's single largest producer and exporter of chilies (22) and its cultivation is mainly concentrated in Andhra Pradesh, Karnataka, Maharashtra, Gujarat, Tamil Nadu, and Orissa (46). *Phytophthora* blight caused by *Phytophthora capsici* Leonian is a major threat to pepper production (6,7) and is ranked as the second most devastating disease worldwide (53). *P. capsici* causes a root and crown rot, as well as an aerial blight of leaves, fruit, and stems, on bell pepper (40). Other species of *Phytophthora* such as *P. cactorum*, *P. cryptogea*, *P. drechsleri*, *P. infestans*, *P. citrophthora*, *P. megasperma*, *P. nicotianae* (*P. parasitica*), and *P. palmivora* have been reported to infect chili fruit after artificial inoculation (21), but natural disease incidence caused by these pathogens is not known.

P. boehmeriae, first isolated from leaf spot of *Boehmeria nivea* in Taiwan in 1927, was named by Sawada (42) and has subsequently been found to cause brown rot of citrus fruit, root rot of *Pinus* spp., cotton boll blight, basal rot of Geraldton wax plant (*Chamaelaucium uncinatum* Schauer.), and gummosis on black wattle (*Acacia mearnsii*) (4,19,21,25,52). *P. boehmeriae* has been reported only in Argentina, Australia, Brazil, China, Greece, Japan, and Papua New Guinea (4,19,21,25,52). However, there are no previous published reports of *P. boehmeriae* causing serious disease epidemics on any crop. In India, the occurrence of *Phytophthora* blight caused by *P. capsici* was first observed on bell pepper in 2006 (13), but no major outbreaks occurred until 2011. During the southwest monsoon period (June to September) of 2011, in which prevailing wet weather conditions are ideal for

disease development, severe leaf blight epidemics occurred in the hot-pepper-growing areas of Karnataka and Tamil Nadu states of India. The objective of the current study was to understand the phenotypic and genotypic characteristics of the *Phytophthora* isolates responsible for severe leaf blight of hot pepper in India.

Materials and Methods

Sample collection and isolate isolation. Isolates were obtained from naturally infected foliage and fruit samples of hot and bell pepper in different districts of Karnataka, Coimbatore, and Ooty in Tamil Nadu (Fig. 1), where severe disease epidemics occurred, during the southwest monsoon period (June to September) of 2011 and 2012. When all the chili fields in these localities were infected, leaves or fruit with single lesions were collected. Each sample consisted of three infected leaves or fruit from each of five plants within a disease focus. The locality of the sample collection and the isolates used in this study are shown in Table 1. To obtain isolates, a small piece (5 by 5 mm) of foliage and fruit tissue taken from the margin of the lesion was washed in sterile distilled water, surface sterilized in 70% ethanol for 30 s, rinsed twice in sterile distilled water, and placed on carrot agar medium (CA). After incubation at $25 \pm 1^\circ\text{C}$ for 5 days, the isolates were obtained by transferring a hyphal tip to fresh CA. Isolates were maintained on CA slants at 4°C by periodical (2 to 3 months) subculturing.

Morphological characterization. Colony morphology and growth rate were determined on 7-day-old cultures grown on CA at $25 \pm 1^\circ\text{C}$. Sporangia were obtained by flooding 7-day-old CA culture with water. The length and breadth of sporangia of each isolate were measured under a Zeiss light microscope Axioscope A1 (approximately $\times 400$) using Axio vision software. The length and breadth of 50 sporangia/isolate were obtained to calculate an average. Other morphological characteristics, including the shape of sporangia and the presence of papillae, were studied (Table 2).

Mating type determination. The mating types of all *P. capsici* isolates were determined by pairing with known A1 (98-75) or A2 (98-01) reference *P. capsici* isolates that were obtained from the Indian Institute of Spices Research, Calicut. Mycelial plugs (5 mm in diameter) on CA from the advancing margin of 5-day-old cultures were placed together, 2 cm apart, on clarified V8 juice agar, incubated at 24°C in darkness for 21 days (39), then examined microscopically for the presence of oospores where the two colo-

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nies overlapped. The diameters of 50 oospores/isolate were measured using Axio vision software at $\times 400$ magnification by taking a measurement of the length and width at the widest point.

Chlamydospore production. Chlamydospore production was assessed using the submerged culture method (26). A single 5-mm-diameter plug from the advancing margin of each isolate grown on CA was transferred to 25 ml of carrot broth in a 150-ml conical flask, incubated in the dark at $25 \pm 2^\circ\text{C}$ for 24 h, shaken vigorously for 5 min, and then incubated for 5 days in the dark at $25 \pm 2^\circ\text{C}$. The carrot broth was removed from the conical flask and was replaced with 50 ml of sterile distilled water. Cultures were incubated in a BOD incubator at $18 \pm 2^\circ\text{C}$ for 21 days in darkness and checked for the presence of chlamydospores under $\times 400$ magnification according to the method of Granke et al. (26).

Growth at 35°C . To examine growth of the isolates at 35°C (21), a 5-mm-diameter CA plug from the advancing margin of a 5-day-old culture of each isolate was inoculated to the center of six 80-mm-diameter CA plates. The plates were sealed with parafilm and incubated in the dark in a BOD incubator at 35°C . Three independent experiments were conducted for each isolate. Each experiment contained three replicates. Each replicate consisted of six petri plates.

Metalaxyl sensitivity. Sensitivity to metalaxyl was determined on V8 juice agar plates amended with metalaxyl at 0, 5, or 100

$\mu\text{g/ml}$ and 25°C . Metalaxyl was added to V8 juice agar medium at 50°C just prior to dispensing it into petri dishes. Agar blocks (5 mm in diameter) with active mycelia were taken from the colony margin of each isolate and transferred to the center of three replicate plates of metalaxyl-amended V8 juice agar. The same isolates grown on V8 juice agar plates without metalaxyl served as controls. Radial colony growth of each isolate was measured after 7 days of incubation, and the mean of the three replicates was calculated. The mean percent growth of each isolate on metalaxyl-amended agar compared with growth on unamended agar control plates was calculated. Isolates were classified as sensitive, intermediate, and resistant based on the parameters of Silvar et al. (45). Isolates were considered resistant if the colony diameter was $<40\%$ of the nonamended control at 5 and 100 $\mu\text{g/ml}$, isolates with a colony diameter $>40\%$ of the nonamended control at 5 $\mu\text{g/ml}$ but $<40\%$ of the nonamended control at 100 $\mu\text{g/ml}$ were classified as intermediate, and isolates with a colony diameter $>40\%$ of the nonamended control at both 5 and 100 $\mu\text{g/ml}$ were classified as metalaxyl resistant.

Aggressiveness. To measure the aggressiveness of the isolates, lesion size was recorded on detached leaflets of chili according to the method described by Ristaino (39). Chili 'Arka lohit' plants were grown for 6 weeks under greenhouse conditions. The fully expanded third or fourth leaves from the crown were detached and

Fig. 1. Locations in South India where *Phytophthora capsici* and *P. boehmeriae* isolates were collected.

placed on wet filter paper in 90-mm petri dishes (two per dish). Each leaf was then inoculated with a 10 µl droplet of sporangial suspension adjusted to a concentration of 2×10^4 sporangia/ml on the ventral side (abaxial) of the main vein. Leaves were incubated at $25 \pm 1^\circ\text{C}$ (16 h of light per day) and, after 7 days of incubation,

lesions were measured. Three independent experiments were conducted for each isolate. Each experiment contained three replicates. Each replicate consisted of 12 leaves.

DNA extraction. To extract DNA, isolates were grown in carrot broth for 7 days at $25 \pm 1^\circ\text{C}$. Mycelia were dried on filter paper

Table 1. Geographical origin of *Phytophthora capsici* and *P. boehmeriae* isolates^z

Isolate	Location	District	Crop	Source	Metalaxyl sensitivity	GenBank accession	NCBI identity
OCPC1	IIHR	Bangalore Rural	Chili	Stem	Intermediate	KC677803	<i>P. capsici</i>
OCPC2	Rajankunte	Bangalore Rural	Chili	Stem	Intermediate	KC677804	<i>P. capsici</i>
OCPC3	Rajankunte	Bangalore Rural	Bell pepper	Stem	Intermediate	KC677805	<i>P. capsici</i>
OCPC4	Kurubarahalli	Bangalore Rural	Chili	Fruit	Sensitive	KC677806	<i>P. boehmeriae</i>
OCPC5	Navillae	Shivamogga	Chili	Leaf	Sensitive	KC677807	<i>P. boehmeriae</i>
OCPC6	Nurahonae	Shivamogga	Chili	Fruit	Sensitive	KC677808	<i>P. boehmeriae</i>
OCPC7	Bekannahalli	Shivamogga	Chili	Leaf	Sensitive	KC677809	<i>P. boehmeriae</i>
OCPC8	Kumbaenahalli	Davanegere	Chili	Leaf	Sensitive	KC677810	<i>P. boehmeriae</i>
OCPC9	Savalanya	Shivamogga	Chili	Leaf	Sensitive	KC677811	<i>P. boehmeriae</i>
OCPC10	Abbigere	Bangalore Rural	Chili	Leaf	Sensitive	KC677812	<i>P. boehmeriae</i>
OCPC11	Kodekoppa	Shivamogga	Chili	Leaf	Sensitive	KC677813	<i>P. boehmeriae</i>
OCPC12	Nurahonae	Shivamogga	Chili	Leaf	Sensitive	KC677814	<i>P. boehmeriae</i>
OCPC13	Bellary	Bellary	Bell pepper	Stem	Intermediate	KC677815	<i>P. capsici</i>
OCPC14	Bellary	Bellary	Bell pepper	Stem	Intermediate	KC677816	<i>P. capsici</i>
OCPC15	Coimbatore	Coimbatore	Bell pepper	Stem	Intermediate	KC677817	<i>P. capsici</i>
OCPC16	Mysore	Mysore	Bell pepper	Stem	Intermediate	KC677818	<i>P. capsici</i>
OCPC17	Honavara	Bangalore Rural	Chili	Fruit	Sensitive	KC677819	<i>P. boehmeriae</i>
OCPC18	Vadarahalli	Shivamogga	Chili	Leaf	Sensitive	KC677820	<i>P. boehmeriae</i>
OCPC19	Mathkur	Bangalore Rural	Chili	Leaf	Sensitive	KC677821	<i>P. boehmeriae</i>
OCPC20	Andrali	Kolar	Bell pepper	Leaf	Sensitive	KC677822	<i>P. boehmeriae</i>
OCPC21	Thotadagere	Bangalore Rural	Chili	Leaf	Sensitive	KC677823	<i>P. boehmeriae</i>
OCPC22	H.Cross	Kolar	Chili	Leaf	Sensitive	KC677824	<i>P. boehmeriae</i>
OCPC23	Manchaenahalli	Bangalore Rural	Chili	Fruit	Sensitive	KC677825	<i>P. boehmeriae</i>
OCPC24	Balepura	Kolar	Bell pepper	Leaf	Sensitive	KC677826	<i>P. boehmeriae</i>
OCPC25	Hungenthi	Kolar	Chili	Fruit	Sensitive	KC677827	<i>P. boehmeriae</i>
OCPC26	Dodathimanahalli	Bangalore Rural	Chili	Fruit	Sensitive	KC677828	<i>P. boehmeriae</i>
OCPC27	Balenahalli	Kolar	Bell pepper	Leaf	Sensitive	KC677829	<i>P. boehmeriae</i>
OCPC28	Hungenalli	Kolar	Chili	Leaf	Sensitive	KC677830	<i>P. boehmeriae</i>
OCPC29	Thurnahalli	Kolar	Bell pepper	Leaf	Sensitive	KC677831	<i>P. boehmeriae</i>
OCPC30	Thuruvanahalli	Bangalore Rural	Chili	Fruit	Sensitive	KC677832	<i>P. boehmeriae</i>
OCPC31	Balepura	Kolar	Bell pepper	Fruit	Sensitive	KC677833	<i>P. boehmeriae</i>
OCPC32	Mangasandra	Kolar	Chili	Leaf	Sensitive	KC677834	<i>P. boehmeriae</i>
OCPC33	Eragasandra	Kolar	Chili	Leaf	Sensitive	KC677835	<i>P. boehmeriae</i>
OCPC34	Odagur	Kolar	Bell pepper	Leaf	Sensitive	KC677836	<i>P. boehmeriae</i>
OCPC35	Hubanahalli	Chikmagalur	Chili	Leaf	Sensitive	KC677837	<i>P. boehmeriae</i>
OCPC36	Guddahalli	Bangalore Rural	Chili	Leaf	Sensitive	KC677838	<i>P. boehmeriae</i>
OCPC37	Golahalli	Bangalore Rural	Bell pepper	Leaf	Sensitive	KC677839	<i>P. boehmeriae</i>
OCPC38	Shivakote	Bangalore Rural	Chili	Leaf	Intermediate	KC677840	<i>P. capsici</i>
OCPC39	Rajankunte	Bangalore Rural	Chili	Fruit	Sensitive	...	<i>P. boehmeriae</i>
OCPC40	Devihalli	Hassan	Bell pepper	Leaf	Sensitive	KC677841	<i>P. boehmeriae</i>
OCPC41	Jaldihalli	Chikmagalur	Chili	Leaf	Sensitive	KC677842	<i>P. boehmeriae</i>
OCPC42	T-Agrahara	Kolar	Bell pepper	Leaf	Sensitive	KC677843	<i>P. boehmeriae</i>
OCPC43	Ajampura	Chikmagalur	Chili	Leaf	Sensitive	KC677844	<i>P. boehmeriae</i>
OCPC44	Sulibele	Kolar	Bell pepper	Leaf	Sensitive	KC677845	<i>P. boehmeriae</i>
OCPC45	Yeliyuru	Kolar	Bell pepper	Leaf	Sensitive	KC677846	<i>P. boehmeriae</i>
OCPC46	Siddapura	Chikmagalur	Chili	Leaf	Sensitive	KC677847	<i>P. boehmeriae</i>
OCPC47	Valalu	Hassan	Bell pepper	Leaf	Sensitive	KC677848	<i>P. boehmeriae</i>
OCPC48	Kumci	Chikmagalur	Bell pepper	Leaf	Sensitive	KC677849	<i>P. boehmeriae</i>
OCPC49	Harnahalli	Chikmagalur	Chili	Leaf	Sensitive	KC677850	<i>P. boehmeriae</i>
OCPC50	Malenahalli	Chikmagalur	Chili	Leaf	Sensitive	KC677851	<i>P. boehmeriae</i>
OCPC51	Rajankunte	Bangalore Rural	Chili	Leaf	Sensitive	KC677852	<i>P. boehmeriae</i>
OCPC52	Ooty	Ooty	Chili	Leaf	Intermediate	KC677853	<i>P. capsici</i>

^z Data shown include isolate number, location, host, source, metalaxyl sensitivity, GenBank accession number, and National Center for Biotechnology Information (NCBI) identity.

Table 2. Morphological characteristics of *Phytophthora boehmeriae* and *P. capsici* isolates^z

Species	Growth rate at 24°C (mm/day)	Sporangial morphology	Length (µm)	Breadth (µm)	L/B ratio	Stalk length	Mating type	Oospore diameter
<i>P. boehmeriae</i> (43 isolates)	7.61 ± 0.055 (6.79–8.02)	Globose	35.33 ± 4.77 (32.76–38.80)	20.49 ± 3.42 (18.21–28.57)	1.74 ± 0.20 (1.22–1.96)	4.13 ± 0.58 (3.02–4.8)	H	22.48 ± 3.92 (21.9–25.1)
<i>P. capsici</i> (9 isolates)	7.62 ± 0.061 (7.1–8.2)	Lemoniform	43.57 ± 6.67 (24.26–43.31)	28.04 ± 3.24 (17.63–23.52)	1.55 ± 0.08 (1.29–1.95)	51.33 ± 15.61 (42.55–75.58)	A1	32.25 ± 3.28 (31.64–34.10)

^z Mean of isolates, range in parentheses. L/B = length/breadth. H = homothallic.

and ground in a mortar and pestle using liquid nitrogen. DNA was isolated from the powdered mycelia according to the procedure described by Raeder and Broda (38) and slightly modified by Chowdappa et al. (12) and stored at -20°C until use.

Amplification of internal transcribed spacer region of ribosomal DNA, cloning, and sequencing. The ribosomal DNA (rDNA) containing the partial 18S and 28S ribosomal region along with the internal transcribed spacer (ITS)1, ITS2, and 5.8S rRNA genes was amplified from all the isolates with ITS1 (5'-TCC GTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTAT TGATATGC-3') primers (51). The polymerase chain reaction (PCR) reaction mixture consisted of 5 μl of 10 $\times$ PCR buffer, 40.75 μl of sterile distilled water, 1 μl of 2.0 mM dNTPs, 1 μl each of 50 pM primers ITS1 and ITS4, 0.25 μl of Taq polymerase (5 U/ μl) (Merck Bio Sciences), and 1 μl of genomic DNA for a total volume of 50 μl . Amplification conditions were as follows: 95°C for 3 min; followed by 35 cycles 95°C for 30 s, 55°C for 45 s, and 72°C for 1 min; followed by a final extension of 72°C for 10 min. PCR products were analyzed by electrophoresis in 2% (wt/vol) agarose gel in 1 $\times$ Tris borate-EDTA buffer, stained with ethidium bromide (5 $\mu\text{g}/\text{ml}$), and visualized by Alpha imager EP (Alpha Innotech Corporation). The resulting PCR product was ligated into pTZ57R/T vector and transformed into *Escherichia coli* DH5 α . The plasmid was purified from *E. coli* and the cloned DNA fragment was sequenced to identify the isolates with previously reported rDNA sequences of *Phytophthora* spp.

PCR restriction fragment length polymorphism analysis. The ITS PCR product of all the isolates, generated by primer pair ITS6

(5'-GAAGGTGAAGTCGTAACAAGG-3') and ITS4 as described earlier (18), were digested with different restriction enzymes (*AluI*, *MspI*, and *TaqI*), as per the manufacturer's recommendations. Further, a genus-specific amplicon obtained using forward primer A2 (5'-ACTTCCACGTGAACCGTTTCAA-3') in combination with reverse primer I2 (5'-GATATCAGGTCCAATTGAGATGC-3') (20), was also subjected to three different restriction enzymes (*MspI*, *RsaI*, and *TaqI*). The digested products were electrophoresed on a 3% NuSieve agarose gel in 1 $\times$ Tris borate-EDTA buffer, stained with ethidium bromide (5 $\mu\text{g}/\text{ml}$), and visualized by Alpha imager EP (Alpha Innotech Corporation).

Fig. 3. Polymerase chain reaction amplification products of *Phytophthora capsici* using specific PC-1 and PC-2 primers. Lane M, 100-bp ladder; lane 1, OCPC1; lane 2, OCPC2; lane 3, OCPC3; lane 4, OCPC13; lane 5, OCPC14; lane 6, OCPC15; lane 7, OCPC 16; lane 8, OCPC 38; lane 9, OCPC 52; lane 10, OCPC 4; lane 11, OCPC 5; lane 12, OCPC 6; lane 13, OCPC 7; lane 14, OCPC 8.

Fig. 2. Colony, sporangial, and oogonial morphology of *Phytophthora boehmeriae* and *P. capsici* isolates. **A–C**, Colony morphology of *P. capsici*; **D**, colony morphology of *P. boehmeriae*; **E–G**, sporangial morphology of *P. capsici*; **H**, sporangial morphology of *P. boehmeriae*; **I–K**, oogonial morphology of *P. capsici*; **L**, oogonial morphology of *P. boehmeriae*.

Amplification with specific primer. All isolates were amplified using the *P. capsici*-specific primers PC-1 (5'-GTCTTG TACCCTATCATGGCG-3') and PC-2 (5'-CGCCACAGCAGG AAAAGCATT-3') (54) and *P. boehmeriae*-specific primers PB1 (5'-CGGCTTTTCGGGCTGCTGC-3') and PB2 (5'-ATACCCGAA GGCAAAGCGC-3') (43). PCR reaction mixture consisted of 5 µl of 10× PCR buffer, 40.75 µl of sterile distilled water, 1 µl of 2.0 mM dNTPs, 1 µl each of 50 pM primers PC1/PC-2 or PB1/PB2, 0.25 µl of Taq polymerase (5 U/µl) (Merck Bio Sciences), and 1 µl of genomic DNA for a total volume of 50 µl. Amplification conditions for PC1/PC2 were 95°C for 3 min; followed by 35 cycles of 95°C for 30 s, 55°C for 45 s, and 72°C for 1 min; followed by a final extension of 72°C for 10 min. Thermal cycling parameters for PB1/PB2 were initial denaturation at 94°C for 5 min; followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 62°C for 40 s and, extension at 72°C for 1 min; followed by a final extension of 72°C for 8 min.

Phylogenetic analysis. Phylogenetic relationships among all the sequences were inferred by maximum parsimony (MP) analysis using MEGA5.0 software (47). The sequences of *P. boehmeriae*, *P. kernoviae*, *P. gallica*, *P. morindae* (clade 10), *P. capsici*, and *P. tropicalis* (clade 4) were also included in the phylogenetic analysis. The analysis was bootstrapped with 1,000 replicates to determine the support for various clades of the trees. In total, 10 replicates were used for the random addition of samples to construct the tree. The MP tree was obtained using the close-neighbor-interchange algorithm (34) with search level 1, in which the initial trees were obtained with the random addition of sequences (10 replicates). The tree was drawn to scale, with branch lengths calculated using the average pathway method and, in units, of the number of changes over the whole sequence.

Results

In all, 52 isolates of *Phytophthora* spp. were obtained from leaf blight infected chili samples. Of these, 43 isolates (83%) fit the description of *P. boehmeriae* (25) and 9 isolates (17%) fit the description of *P. capsici* (26) (Table 2).

Morphological identification. Cultures of *P. boehmeriae* exhibited no distinct colony pattern (Fig. 2), with a growth rate of 6.79 to 8.02 mm/day (Table 2). In contrast, isolates of *P. capsici* had either cottony or petaloid growth patterns (Fig. 2), with a growth rate of 7 mm/day. Sporangia of *P. boehmeriae* were borne on simple sporangiophores. They were caducous and papillate. Most of the sporangia were globose, ovoid to ellipsoid, or nearly spherical or obpyriform to obpyriform (Fig. 2). The length of the sporangia was 32.76 to 38.80 µm and breadth varied from 18.21 to 28.57 µm, with a length-to-breadth (L:B) ratio of 1.22 to 1.96. The pedicel length of the isolates was less than <5µm. Sporangia of *P. capsici* isolates were also caducous and papillate. Sporangia were mostly lemoniform or ellipsoid with tapered base and some were obpyriform (Fig. 2). They carried a long stalk, varying from 42.55 to 75.58 µm. The sizes of the sporangia of *P. capsici* were 24.26 to 43.31 by 17.63 to 23.52 58 µm, with an L:B ratio of 1.29 to 1.95.

All 43 isolates of *P. boehmeriae* were homothallic and readily produced oospores on carrot agar plates. All nine isolates of *P. capsici* were heterothallic and belonged to the A1 mating type. All of the oospores examined in both species had amphigynous antheridia. The mean diameter of the oospores of *P. capsici* isolates was 31.64 to 34.10 µm, greater than those of *P. boehmeriae* (21.9 to 25.1µm). Of the nine isolates of *P. capsici*, two isolates produced

chlamydospores. Among the 43 isolates of *P. boehmeriae*, 27 isolates produced chlamydospores. All *P. capsici* and *P. boehmeriae* failed to grow at 35°C. All *P. boehmeriae* isolates were sensitive to metalaxyl whereas *P. capsici* isolates were intermediate in sensitivity.

Molecular identification. Amplification of DNA from nine *P. capsici* isolates with primers PC1 and PC2 produced PCR product of 560 bp (Fig. 3), as expected for *P. capsici*, and no amplification was detected from the DNA of 43 isolates of *P. boehmeriae*. Similarly, PCR amplification of DNA of *P. boehmeriae* using primers PB1 and PB2 yielded a single PCR product of 750 bp but no amplification was achieved from *P. capsici* isolates (data not shown). The digestion of an ITS-PCR amplicon with *AluI*, *MspI*, and *TaqI* resulted in characteristic fingerprints specific to *P. capsici* and *P. boehmeriae* (Table 3; Fig. 4). The restriction digestion profiles generated by *MspI*, *RsaI*, and *TaqI* on the resulting amplicon obtained using the genus-specific primers A2 and I2 also separated *P. capsici* and *P. boehmeriae* (Table 4; Fig. 5).

Phylogenetic analysis. The MP analysis of ITS gene sequences resulted in detection of two main clades (Fig. 6). Clade I consisted of 43 *P. boehmeriae* isolates characterized by globose sporangia with short pedicels that were homothallic. Clade 2 comprised nine isolates of *P. capsici* and had lemoniform sporangia with long pedicels that were heterothallic. Comparisons with sequences of *P. boehmeriae*, *P. capsici*, and *P. tropicalis* isolates downloaded from GenBank also revealed a clear separation. *P. boehmeriae* isolates formed two subgroups in clade 1. All isolates of *P. boehmeriae* examined in this study formed as subgroup I, along with isolates CBS29129 (National Center for Biotechnology Information accession number HQ 643149) from Japan and P6950 (HQ 2261504) from Taiwan downloaded from GenBank; and isolates from Argentina, South Africa, and Papua New Guinea formed subgroup II. Our *P. capsici* isolates clustered with ITS sequences of *P. capsici* isolates downloaded from GenBank under subgroup I of clade 2 while the *P. tropicalis* isolate from GenBank formed as subgroup II under clade 2.

Aggressiveness. The wound drop inoculation on detached leaves of *Capsicum annuum* Arka lohit with the *P. boehmeriae* and *P. capsici* isolates produced typical symptoms with white mycelia growth and sporangia after 6 days of inoculation. They differed significantly in lesion sizes (Table 5). *P. boehmeriae* isolates were highly aggressive and produced significantly ($P < 0.01$) larger lesion than those of *P. capsici* isolates.

Fig. 4. Restriction fragment length polymorphism patterns of internal transcribed spacer (ITS) region after polymerase chain reaction using ITS6 and ITS4 primer pair and restriction digestion with restriction enzymes *MspI*. Lane M, 50-bp ladder; lane 1, OCPC 1; lane 2, OCPC 2; lane 3, OCPC 3; lane 4, OCPC 13; lane 5, OCPC 14; lane 6, OCPC 15; lane 7, OCPC 16; lane 8, OCPC 38; lane 9, OCPC 52; lane 10, OCPC 4; lane 11, OCPC 5; lane 12, OCPC 6; lane 13, OCPC 7; lane 14, OCPC 8.

Table 3. Size (bp) of restriction fragments for *Phytophthora* spp. amplified with primers internal transcribed spacer (ITS)6 and ITS4 and digested with the restriction enzymes^a

<i>Phytophthora</i> sp.	PCR amplicon (bp)	<i>AluI</i>	<i>MspI</i>	<i>TaqI</i>
<i>P. boehmeriae</i>	900	565, 210, 170	570, 387	300, 270, 197, 89, 59
<i>P. capsici</i>	860	535, 172, 155	292, 221, 204, 145	262, 149, 148, 101, 90, 59, 46

^a Amplified products were digested with the indicated restriction enzyme and separated in a 3% agarose gel. PCR = polymerase chain reaction.

Discussion

Proper identification of *Phytophthora* spp. responsible for foliar blights in chili is crucial for developing effective disease management strategies and also for the selection of the appropriate pathogen isolates to use in resistance screening programs. A combined application of morphological characters, molecular diagnostic tools, and pathogenicity led to identification of *P. capsici* and *P. boehmeriae* responsible for severe outbreaks of leaf blight in chili in South India. This is the first report of the involvement of *P. boehmeriae* in the causation of foliar blight of chili in India. *P. boehmeriae* was first reported from Taiwan on *B. niver* in 1927 (42) and has been known to infect only *B. niver*, *Broussonetia papyraria*, *Gossypium* spp., citrus, *Pinus* spp., and *A. mearnsi* in Australia, Argentina, Brazil, China, Greece, Japan, Papua New Guinea, Taiwan, and South Africa (19,21,25,36,43). *P. capsici* is distributed worldwide (21) and is a serious pathogen of vegetable crops in the families Cucurbitaceae, Solanaceae, and Fabaceae (27) and on nonvegetable hosts such as cacao (*Theobroma cacao* L.), rubber (*Hevea brasiliensis* Mull. Arg.), macadamia (*Macadamia integrifolia* Maiden & Betcher), papaya (*Carica papaya* L.), and black pepper (*Piper nigrum* L.) (21).

Previously, sporangium morphology (including pedicel length and presence or absence of chlamydospores), growth at 35°C, oospore diameters (5,26), species-specific PCR (44), PCR amplification and sequencing (8,37), and restriction enzyme digestion patterns (8,33) have been used to identify, characterize, and separate *P. capsici* from closely related species. In the present study, *P. capsici* sporangia were caducous and papillate, with long pedicels of 43 to 75 µm in length, in agreement with other studies that reported pedicel lengths of sporangia of 24 to 177 µm (5,26,28,33,39). In contrast, sporangia for isolates *P. boehmeriae* were also caducous and papillate, with short pedicels of <5 µm, similar to the descriptions reported in previous studies (21,25,52). In this study, the mean sporangial length and breadth for each *P. capsici* isolate was 24 to 43 µm long and 17 to 23 µm wide, with an L:B ratio (1.29 to 1.95) which falls within the range of 38 to 60 µm long and 23 to 35 µm wide and an L:B ratio (1.34 to 2.07) that were reported in a previous study by Granke et al. (26) using worldwide collection of *P. capsici* isolates. Prior to 2011, many studies have also shown that similar mean sporangial length varied from 33 to 66 µm long and 17 to 43 µm wide (5,28,33,39). Ristaino (39) reported that sporangia of *P. capsici* isolates from pepper (39 to 60 µm long) were shorter than isolates from cucurbit hosts (56 to 63 µm long). *P. boehmeriae* isolates had sporangia that were 32 to 38 µm long and 18 to 28 µm wide, with an L:B ratio of 1.22 to 1.96 in our study. These sporangial measurements falls within the range reported for *P. boehmeriae* in earlier studies (25,36,52).

In the present study, when cultures were incubated in liquid media, two of nine (22%) *P. capsici* isolates and 27 of 43 (51%) *P. boehmeriae* isolates produced chlamydospores. In their study, Granke et al. (26) reported that *P. capsici* isolates from hosts in the families Cucurbitaceae, Fabaceae, and Solanaceae produced chlamydospores, and isolates retrieved from nonvegetable hosts produced no chlamydospores. In contrast, *P. capsici* isolates from solanaceous or cucurbitaceous hosts did not produce chlamydospores (39) but chlamydospores were produced by isolates from nonvegetable hosts (3,50). Mchau and Coffey (33) reported that 16 of 29 (55%) CAP2 (*P. tropicalis*) isolates produced chlamydospores, but none of the CAP1 isolates that likely represent *P. capsici* sensu stricto isolates produced chlamydospores. Aragaki and Uchida (5) showed that no *P. capsici* isolates and most (46 of 53,

87%) *P. tropicalis* isolates produced chlamydospores. Thus, chlamydospore production is not reliable character for distinction of *P. capsici* from *P. tropicalis*. Previous studies have reported chlamydospore production by some isolates of *P. boehmeriae* (25,36,52). *P. capsici* isolates were heterothallic and isolates belong to A1 mating type whereas *P. boehmeriae* isolates were homothallic, in agreement with previous studies (25,26). Although no information is available on mating types of *P. capsici* isolates from pepper, the presence of both mating types in *P. capsici* isolates from cocoa has been recorded in India (14). Oospore diameters for *P. capsici* isolates were 31.64 to 34.10 µm in our study, which is in agreement with earlier studies (26,28,33,39). In contrast, oospore diameters of *P. boehmeriae* are smaller (21.9 to 25.1 µm) than those of *P. capsici* and sizes fall within the ranges reported for *P. boehmeriae* (25,36,52). Earlier studies have demonstrated that growth at 35°C is a robust characteristic to separate *P. capsici* from *P. tropicalis* and other *Phytophthora* spp. (5,8,33). In this study, *P. capsici* and *P. boehmeriae* isolates failed to grow at 35°C. The maximum growth temperature for *P. boehmeriae* isolates was found to be 32.5°C (25). A previous study by Bowers et al. (8) showed that only temperate isolates of *P. capsici* grew at 35°C and tropical isolates did not.

In addition to morphological approaches, restriction fragment length polymorphism analysis of PCR-amplified ITS regions (11,17,18,41,49) and amplicons obtained using the genus-specific primers A2 and I2 (20) have been used for discrimination of *Phytophthora* spp. In this study, restriction profiles of the ITS region of the rDNA gene cluster and amplicons generated by the genus-specific primers separated the *P. capsici* isolates from *P. boehmeriae* isolates. The identities of *P. capsici* and *P. boehmeriae* isolates were further confirmed by species-specific PCR (43,54) and ITS sequence matches against those in GenBank. The ITS-based phylogenetic inference has been found to be highly robust in differentiating *Phytophthora* spp. (16). Our ITS sequence data clearly distinguished *P. boehmeriae* and *P. capsici* isolates. *P. boehmeriae* has been assigned to clade 10 within genus *Phytophthora* (31) along with other three homothallic species: *P. gallica* on oak and reed in Germany and France (29), *P. kernoviae* on beech in the United Kingdom (9), and *P. morindae* on noni in Hawaii (35). Comparison of ITS sequence data of *P. boehmeriae* isolates used in this study against sequences deposited in the GenBank showed that ITS se-

Fig. 5. Restriction fragment length polymorphism patterns of the amplicon after polymerase chain reaction using *Phytophthora* genus-specific primer pair A2-I2 and restriction digestion with *RsaI*. Lane M, 50-bp ladder; lane 1, OCPC 1; lane 2, OCPC 2; lane 3, OCPC 3; lane 4, OCPC 13; lane 5, OCPC 14; lane 6, OCPC 15; lane 7, OCPC 16; lane 8, OCPC 38; lane 9, OCPC 52; lane 10, OCPC 4; lane 11, OCPC 5; lane 12, OCPC 6; lane 13, OCPC 7; lane 14, OCPC 8.

Table 4. Size (bp) of restriction fragments for *Phytophthora* spp. amplified with primers A2 and I2 and digested with the restriction enzymes^z

<i>Phytophthora</i> sp.	PCR amplicon size (bp)	<i>MspI</i>	<i>RsaI</i>	<i>TaqI</i>
<i>P. boehmeriae</i>	828	515, 313	351, 303, 174	256, 227, 197, 89, 59
<i>P. capsici</i>	752	281, 250, 221	318, 274, 105	194, 194, 149, 90, 60

^z PCR = polymerase chain reaction.

quences HQ 643149, FJ801955, HQ 261504, and GU 259127 generated from voucher CBS29129, clone WPC 6950A251, voucher P6950, and WPC 1387A15 of *P. boehmeriae*, respectively, clustered in the same subgroup I of group I in the parsimonious tree.

However, ITS sequences (deposited in the GenBank) GQ166763, FJ801479, and GU 963637 derived from isolates ph 698P0701 of *P. morindae*, ICMP14761 of *P. kernoviae*, and WPC 16827D2028 of *P. gallica*, respectively, clustered in subgroup II of group I. Simi-

Fig. 6. Phylogenetic tree generated using maximum parsimony analysis of internal transcribed spacer gene sequences. Values above branching nodes represent percentage bootstrap support calculated from 1,000 replicates. Branch lengths are proportional to the numbers of nucleotide substitutions and are measured by scale bars (bar = 10% sequence divergence).

larly, nine *P. capsici* isolates were clustered in group II along with ITS sequence GU 259193 generated from isolate WPC 1091D396 of *P. capsici* and sequence GU 259391 derived from *P. tropicalis* isolate WPC 10379B1476 formed as separate subgroup under group II.

Variations in sensitivity to metalaxyl have been reported within and between *Phytophthora* spp. (15,30,48). In this study, isolates of *P. boehmeriae* collected in different locations in India from pep-

per plants were sensitive to metalaxyl whereas *P. capsici* isolates were intermediate in sensitivity. Isolates of *P. capsici* that were resistant or intermediate in sensitivity to mefenoxam have been found widely within the United States (24), southern Italy (1), and Spain (45). In *P. capsici*, a single, incompletely dominant gene regulates insensitivity to mefenoxam (32). However, no information in the literature is available on sensitivity of *P. boehmeriae* to metalaxyl. Some studies on *P. capsici*, *P. infestans*, and *P. nicotianae* have demonstrated that metalaxyl-resistant isolates were more aggressive than their sensitive counterparts (10,30,48). In the present study, even though *P. boehmeriae* isolates are metalaxyl sensitive, they are highly aggressive compared with *P. capsici* isolates.

Pathogenicity assays with *P. boehmeriae* and *P. capsici* isolates derived from infected chili leaves showed that *P. boehmeriae* isolates were more aggressive than those of the *P. capsici* isolates. Plant breeders and pathologists should note the potential of *P. boehmeriae* to be a major pathogen and develop chili cultivars for resistance to foliar blight and management strategies accordingly. This study is the first that showed that *P. boehmeriae* was responsible for severe leaf blight epidemics in hot pepper production areas of India, and it is not clear whether it is migrated or evolved in India through sexual recombination or any other mechanism because it is not serious pathogen on any crop in any part of the world. Whole-genome sequence analysis and studies of the pathogen population structure over time will provide information on its evolutionary history which can help in the effective deployment of host resistance and fungicides for disease management. From this study, it is not possible to understand the extent of spread of *P. boehmeriae*, and a large-scale survey and population analysis of *Phytophthora* spp. across chili-growing regions of India is warranted. Integrated management strategies, including the use of pathogen-free transplants, elimination of sources of inoculum, and use of fungicides on foliage, will help chili growers to reduce crop losses caused by this emerged disease.

Acknowledgments

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Table 5. Aggressiveness of *Phytophthora capsici* and *P. boehmeriae* isolates on 'Arka Lohit' chili

Isolates	Lesion size (cm ²) ^y
<i>P. capsici</i>	
OCPC 1	2.21 ± 0.23 m
OCPC 2	2.33 ± 0.23 m
OCPC 3	2.39 ± 0.12 m
OCPC 13	2.32 ± 0.14 m
OCPC 14	2.37 ± 0.11 m
OCPC 15	2.50 ± 0.19 m
OCPC 16	2.65 ± 0.20 m
OCPC 38	2.32 ± 0.12 m
OCPC 52	2.24 ± 0.15 m
<i>P. boehmeriae</i>	
OCPC 4	3.44 ± 0.22 j
OCPC 5	3.90 ± 0.18 a
OCPC 6	3.63 ± 0.16 g
OCPC 7	3.85 ± 0.23 b
OCPC 8	3.45 ± 0.20 j
OCPC 9	4.02 ± 0.24 a
OCPC 10	3.85 ± 0.10 b
OCPC 11	3.53 ± 0.30 h
OCPC 12	3.69 ± 0.25 e
OCPC 17	3.64 ± 0.30 g
OCPC 18	3.91 ± 0.14 a
OCPC 19	3.77 ± 0.29 d
OCPC 20	3.69 ± 0.18 g
OCPC 21	3.47 ± 0.19 i
OCPC 22	3.80 ± 0.19 d
OCPC 23	4.16 ± 0.11 a
OCPC 24	3.66 ± 0.21 g
OCPC 25	4.23 ± 0.16 a
OCPC 26	4.14 ± 0.16 a
OCPC 27	3.76 ± 0.27 d
OCPC 28	3.63 ± 0.18 g
OCPC 29	4.17 ± 0.13 a
OCPC 30	3.66 ± 0.22 g
OCPC 31	3.97 ± 0.28 a
OCPC 32	3.67 ± 0.28 f
OCPC 33	3.56 ± 0.18 h
OCPC 34	3.37 ± 0.26 l
OCPC 35	3.99 ± 0.27 a
OCPC 36	3.71 ± 0.25 f
OCPC 37	3.86 ± 0.19 b
OCPC 39	3.99 ± 0.15 a
OCPC 40	3.83 ± 0.12 d
OCPC 41	3.61 ± 0.31 g
OCPC 42	3.97 ± 0.27 a
OCPC 43	4.06 ± 0.14 a
OCPC 44	3.33 ± 0.10 l
OCPC 45	3.60 ± 0.18 g
OCPC 46	3.82 ± 0.17 c
OCPC 47	3.41 ± 0.19 k
OCPC 48	3.49 ± 0.33 i
OCPC 49	3.89 ± 0.17 b
OCPC 50	3.97 ± 0.12 a
OCPC 51	3.88 ± 0.10 b
CD1% ^z	0.43

^y Lesion areas (cm²) measured 7 days after inoculation on detached leaves of chili leaflets (Arka lohit) with isolates of *P. capsici* and *P. boehmeriae*. Data are the means and standard deviation of three independent experiments. Each experiment contained three replicates. Each replicate contained 12 detached leaves. For each row, values followed by a different lowercase letter are significantly different at *P* < 0.01, according to Fisher's least significant difference test.

^z CD denotes critical difference at 1% level.

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