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ORIGINAL ARTICLE

Phytophthora chrysanthemi sp. nov., a new species causing root rot of chrysanthemum in Japan

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Abstract A new species of *Phytophthora* was isolated from stem and root rot of chrysanthemum in the Gifu and Toyama prefectures of Japan. The species differs from other *Phytophthora* species morphologically, and is characterized by nonpapillate, noncaducous sporangia with internal proliferation, formation of both hyphal swellings and chlamydospores, homothallic nature, distinctive intercalary antheridia, and funnel-shaped oogonia. The new species can grow even at 35°C, with an optimum growth temperature of 30°C in V8 juice agar medium. In phylogenetic analyses based on five nuclear regions (LSU rDNA; genes for translation elongation factor 1α, β-tubulin, 60 S ribosomal protein L10, and heat shock protein 90), the isolates formed a monophyletic clade. Although the rDNA ITS region shows a high resolution and has proven particularly useful for the separation of *Phytophthora* species, it was difficult to align the sequences for phylogenetic analysis. Therefore, ITS region analysis using related species as defined by the multigene phylogeny was performed, and the topology of the resulting tree also revealed a monophyletic clade formed by the isolates of the species. The morphological characteristics and phylogenetic relationships indicate that the isolates represent a new species, *Phytophthora chrysanthemi* sp. nov. In pathogenicity tests, chrysanthemum plants inoculated with the isolates developed lesions on stems and roots within 3 days, and the symptoms resembled the ones originally observed. Finally, the pathogen's identity was confirmed by re-isolation from lesions of infected plants.

Keywords High temperature · Chrysanthemum · Phylogeny · *Phytophthora* · Root rot

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Introduction

The genus *Phytophthora* consists of more than 120 species (Phytophthora Database, http://www.phytophthoradb.org/index.php), many of which cause important diseases in a wide range of ornamentally and agriculturally valuable plants and forest trees (Erwin and Ribeiro 1996). *Phytophthora* belongs to the oomycetes, a diverse group of fungus-like eukaryotes that was recently placed in the new Kingdom Straminipila including the diatoms, brown algae and golden-brown algae (Beakes and Sekimoto 2009).

Morphological features are variable (Erwin and Ribeiro 1996) and subtle (Gardes and Bruns 1993) in *Phytophthora* which makes species identification difficult and time-consuming. Identification of new species has been facilitated by advanced molecular methods which allowed major steps beyond the morphological classification into groups I–VI by Waterhouse (1963). Recent molecular analyses of *Phytophthora* species provide a reliable phylogenetic framework for taxonomists and plant pathologists, and have led to the identification of new taxa. Cooke et al. (2000) presented a molecular phylogeny based on the sequences of the internal transcribed spacer region (ITS) of nuclear ribosomal DNA (rDNA), suggesting the presence of 10 clades within the genus. This interpretation was supported by subsequent phylogenetic studies (Blair et al. 2008; Donahoo et al. 2006; Ivors et al. 2004; Kroon et al. 2004; Martin and Tooley 2003a, b; Villa et al. 2006).

Chrysanthemum is cultivated all over Japan mainly for ornamental use, and the production yield is the highest among the cultivated flowering plants in Japan (Tsukiboshi et al. 2007). In 1998, an apparently new *Phytophthora* species caused stem and root rot disease in cut chrysanthemum in Toyama prefecture (Chikuo et al. 2007). In 2003, similar root and stem rot symptoms were observed in hydroponically grown potted chrysanthemum in an ebb and flow mat irrigation system in Gifu (Watanabe et al. 2007). Morphological features of isolates from both outbreaks were similar and differed from known *Phytophthora* species. In this study, we analyzed these isolates morphologically and phylogenetically and describe them here as a new *Phytophthora* species.

Materials and methods

Isolation

Gifu isolates were collected from rotten stems of chrysanthemum grown in ebb and flow mat irrigation systems in 2003. The infected stems were washed under running tap water, and excess water was removed with filter paper. The stems were cut into 5-mm segments and disinfected with 1% sodium hypochlorite solution for 3 min, followed by rinsing three times with sterile distilled water. Stem segments were dried on sterilized filter paper and then placed on corn-meal agar (CMA) with 100 mg/L streptomycin. After 5 days incubation at 20°C in darkness, pure cultures were established by transferring hyphal tips to CMA medium slants. Toyama isolates were obtained in 1998 from infected roots of chrysanthemum using a selective medium, BNPRA-HMI (20 mg benomyl, 25 mg nystatin, 25 mg PCNB, 10 mg ampicillin, and 25 mg 3-hydroxy-5methylisoxazole per liter of potato-dextrose agar; Masago et al. 1977). The cultures were maintained in V8 juice agar (V8A) medium.

Morphology and growth rate

Asexual and sexual structures were produced in grass leaf blade culture (Waterhouse 1967). Autoclaved blades were placed on V8A medium inoculated with an isolate. After 7 days incubation at 25°C, the colonized blades were transferred to autoclaved pond water (pond water:distilled water=1:2) and were incubated at 25°C. Growth patterns were observed on V8A medium after 30 days incubation at 25°C in the dark.

Mycelial growth was measured on three replicate plates of V8A medium. Each plate was inoculated with a 6-mm mycelial disk from the margin of a colony on V8A medium, and incubated at temperatures ranging from 5 to 40°C at 5°C intervals. Colony sizes were measured after 7 and 14 days.

DNA extraction and PCR

For DNA extraction, the isolates were incubated for 7–10 days in V8 broth medium at 25°C. A small amount of mycelium was collected on sterilized filter paper. Then the mycelium was transferred to PrepMan Ultra Reagent (Applied Biosystems, California, USA) and heated to 100°C for 10 min. After 2 min incubation at room temperature, the sample was centrifuged at 18,000*g* for 3 min and the supernatant (DNA solution) was transferred into a new tube and diluted 10 times with TE buffer (10 mM Tris-HCl, pH 7.5, and 0.1 mM EDTA) for PCR.

The ITS rDNA region, the large subunit of rDNA (LSU rDNA), the translation elongation factor 1α gene (EF-1α), and the genes for β-tubulin, 60 S ribosomal protein L10, and heat shock protein 90 were amplified using the primer sets and PCR condition listed in Table 1. The 25-μL reaction mixture used contained 1 μM of each primer (0.2 μM for LSU), 0.625 units of rTaq DNA polymerase (TaKaRa Bio, Shiga, Japan), 0.2 mM dNTPs mixture, and PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl and 1.5 mM $MgCl_2$). The PCR reaction was carried out with a 2700 DNA Thermal Cycler (Applied Biosystems). The reaction sequence consisted of 5 min at 94°C (2 min for 60 S ribosomal protein L10 and heat shock protein 90) followed by 35 cycles of 30 sec at 94°C, annealing at appropriate temperatures (see Table 1 for details), and extension for 1 min at 72°C (2 min for 60 S ribosomal protein L10 and heat shock protein 90), and finally one cycle of extension 10 min at 72°C (5 min for 60 S ribosomal protein L10 and heat shock protein 90).

KOD-plus-DNA polymerase Taq (Toyobo, Osaka, Japan) was used when amplification with rTaq DNA

Table 1 Primers used in this study for DNA amplification and sequencing

Locus	Primer name	Primer sequence (5′–3′)	Genomic record	Primer location[a]	Annealing temp. (°C)	References
ITS	ITS1 ITS4	TCCGTAGGTGAACCTGCGG TCCTCCGCTTATTGATATGC			55 (30)[b]	White et al., 1990
28 S Ribosomal DNA	NL1 NL4	GCATATCAATAAGCGGAGGAAAAG GGTCCGTGTTTCAAGACGG			55 (60)	O'Donnell, 1993
Beta-tubulin	TUBUF2_for TUBUR1_rev	CGGTAACAACTGGGCCAAGG CCTGGTACTGCTGGTACTCAG	U22050[c]	570–589 1,538–1,558	60 (30)	Kroon et al. (2004)
Elongation Factor1 alpha	ELONGF1_for ELONGR1_rev	TCACGATCGACATTGCCCTG ACGGCTCGAGGATGACCATG	AJ249839[c]	180–199 1,132–1,151	60 (30)	Kroon et al. (2004)
60 S Ribosomal Protein L10	60SL10_for 60SL10_rev	GCTAAGTGTTACCGTTTCCAG ACTTCTTGGAGCCCAGCAC	72378[d]	16–36 512–493	53 (30)	Blair et al. (2008)
Heat shock Protein 90	HSP90_F1 HSP90_F1int[e]	GCTGGACACGGACAAGAACC CAAGGTGATCCCGGACAAGGC	71510[d]	171–190 198–218	62 (30)	Blair et al. (2008)
	HSP90_F3[e]	ACGCCTCGTTCTACAAGTCG		848–867		
	HSP90_F2[e]	ATGGACAACTGCGAGGAGC		1,039–1,057		
	HSP90_R1[e]	ACACCCTTGACRAACGACAG		1,091–1,072		
	HSP90_R2	CGTGTCGTACAGCAGCCAGA		1,935–1,916		

[a] Reference to the location of the primer within the original DNA sequence

[b] Values in parentheses are the time (s) of annealing

[c] Reference to the DNA database accession number containing the DNA sequence, on which the primer is based

[d] Reference recorded from the *Phytophthora ramorum* genome project v1.1 (Joint Genome Institute, http://genome.jgi-psf.org/Phyra1_1/Phyra1_1.home.html)

[e] Primers used for sequencing only

polymerase was unsuccessful, and this generally was required for the genes encoding heat shock protein 90 from *P. richardiae*, *P. kernoviae*, and *P. parsiana*, and the 60 S ribosomal protein L10 and β-tublin from *P. richardiae*. The 25 μL of the KOD-plus reaction mixture contained 0.3 μM of each primer, 0.5 units of KOD-Plus-DNA polymerase, 0.2 mM dNTPs mixture, 1.0 mM $MgSO_4$, and 1×PCR buffer for KOD-Plus. The complete reaction sequence consisted of 2 min at 94°C, followed by 35 cycles of denaturing 15 s at 94°C, annealing under appropriate conditions (for details, see Table 1), and extension for 1 min at 68°C.

Sequencing

The PCR products were purified using the GenElute PCR Clean-up Kit (Sigma-Aldrich, Missouri, USA) following the manufacturer's instructions. For the heat shock protein 90 gene, sequencing primers HSP90_F1int, HSP90_F2, HSP90_F3, and HSP90_R1 (Table 1) were used. Sequencing was then performed using the BigDye Terminator version 3.1 Cycle Sequencing Reaction Kit (Applied Biosystems) with the same primers as in the initial PCR. After purifying the sequencing reaction mixture through ethanol precipitation, it was analyzed on an ABI 3100 DNA Sequencer (Applied Biosystems). The obtained sequences were assembled with ChromasPro version 1.33 (Technolysium Pty Ltd., Queensland, Australia), and the consensus sequences were used for alignment analysis. The nucleotide sequence data obtained have been deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession numbers listed in Table 2.

Phylogenetic analyses

The sequence datasets were combined data of five genes including LSU rDNA, translation elongation factor 1α (EF-1α), β-tubulin, 60 S ribosomal protein L10, and heat shock protein 90, from 76 taxa. A phylogenetic analysis of the ITS rDNA was conducted to establish the lineage relationship of phylogenetically related species. The dataset for this analysis consisted of 16 taxa that had been identified as closely related to the new species in the tree constructed on the basis of the five genes, and *Phythophthora foliorum* as an outgroup. The nucleotide sequence data used in the phylogenetic analyses were obtained from DDBJ DNA database; accession numbers are listed in online resource (Electronic supplementary material, Table 1). Sequences were aligned using MAFFT version 6.240 with E-INS-i option (Katoh et al. 2002, 2005). Sequences were truncated at the 5' and 3' ends.

Table 2 List of the isolates sequenced for molecular phylogenetic analysis in this study

Species	Isolate no.		DNA database accession no.					
	Local	International[a]	ITS rDNA	β-tubulin	Elongation factor 1α	LSU rDNA	Heat shock protein 90	Ribosomal protein L10
P. chrysanthemum	GF749	NBRC 104917,	AB437135	AB511995	AB511925	AB465349	AB511987	AB511926
P. chrysanthemi	GF753	CBS 123163	AB511826	AB511996	AB511927	AB511313	AB511988	AB511934
P. chrysanthemi	Chr3	NBRC 104918	AB437136	AB511997	AB511928	AB465508	AB511989	AB511935
P. chrysanthemi	Chr5		AB511827	AB511998	AB511929	AB511314	AB511990	AB511936
P. kernoviae	P1571	IMI 393170	AY940661[b]	AB511992	AB511931	AB511831	AB511984	AB511940
P. parsiana	SUC25	IMI 395329	AY659739[b]	AY659746[b]	AY659753[b]	AB511830	AB511983	AB511939
P. polonica	P131445		AB511828	AB511991	AB511930	AB511829	AB511982	AB511938
P. richardiae	P7789		AB367498[b]	AB511993	AB511932	AB511832	AB511985	AB511941
Pythium helicoides	H5sz1C14	CBS 286.31	AB108025	AB511994	AB511933	AB511833	AB511986	AB511937

[a] *IMI* International Mycological Institute, *CBS* Centraalbureau voor Schimelcultures, Utrecht, the Netherlands, *NBRC* NITE Biological Resource Center, Japan

[b] Accession numbers obtained from DDBJ nucleotide sequence databases

When ambiguous sites and gaps were found out, they were manually removed. The alignment has been deposited in TreeBASE under the accession number SN4874. Phylogenetic trees were obtained using maximum-parsimony (MP), maximum likelihood (ML), and Bayesian phylogenetic analyses. The best-fit evolutionary model was determined for each dataset by comparing different evolutionary models via the corrected Akaike information criterion (AICc; Akaike 1974; Sugiura 1978) for MP and ML analyses, and the Bayesian Information Criterion (BIC; Schwarz 1978) for the Bayesian analysis. Kakusan3 (Tanabe 2007) and PAUP* version 4.0β10 (Swofford 2002) were used for likelihood calculations.

MP analysis with the selected evolutionary model was done with PAUP*. MP analysis was performed for 1,000 replications with different random starting points using the step-wise addition option to increase the likelihood of finding the most parsimonious tree. Alignment gaps were treated as missing data, and all characters were unordered and had equal weight. The branch-swapping algorithm was tree bisection and reconstruction (TBR). Branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. The best tree topology of MP trees was established using the Kishino–Hasegawa likelihood test (Kishino and Hasegawa 1989) on PAUP*. Tree length (TL), consistency index (CI), retention index (RI), homoplasy index (HI), and rescaled consistency index (RC) were calculated.

The ML analysis was performed by the likelihood ratchet method (Vos 2003). For the ML tree search, 1,000 sets of 25% site-upweighted data were created using the pgresampleseq command in Phylogears 1.0.2009.07.17 (Tanabe 2007), and the ML trees with the upweighted data were estimated using Treefinder (June 2008 version; Jobb et al. 2004) with application of the best-fit model.

The strength of the internal branches from the resulting tree was tested by bootstrap (BS) analysis (Felsenstein 1985) using 1,000 replications in both MP and ML analysis. Moreover, decay indices (DI; Bremer 1988, 1994; Donoghue et al. 1992) were calculated from MP trees and the dataset using AutoDecay v. 5.04 for Perl (http://www.bergianska.se/).

Bayesian phylogenetic analysis with the selected evolutionary model was done using MrBayes5d v. 3.1.2.2008.10.20 (Ronquist and Huelsenbeck 2003, http://www.fifthdimension.jp/products/mrbayes5d/). It was launched with random starting trees for 7×10^6 (in the combined data) and 3×10^6 (in the ITS regions) generations, and the Markov chains were sampled every 100 generations. To ensure that the Markov chains did not become trapped in local optima, we used the Metropolis-coupled Markov chain Monte Carlo algorithm, performing the estimation with four incrementally heated Markov chains. Trace files generated after each run were evaluated using Tracer v. 1.4.1 (http://tree.bio.ed.ac.uk/). In each of these analyses, the first 10,000 trees in ITS and 50,000 trees in the combined data generations were discarded as burn-in. The remaining trees were combined into a 50% majority-rule consensus tree, yielding the probabilities of each clade for being monophyletic.

Pathogenicity

Pathogenicity of chrysanthemum isolates (NBRC 104917) was tested on three cultivars of potting chrysanthemum (Dark Bizarria, Miramar and Lompoc).

Chrysanthemum seedlings were grown in a greenhouse for 1 month. About ten 5-mm mycelial disks, cut from the growing edge of 2-week-old V8A cultures (grown at 25°C in darkness) were placed around the bases of the seedling shoots. The plants were misted with water, placed in sealed plastic bags, and kept at 30°C. Uninfected agar disks were used as a control. Stem rot extension was examined from the 3rd to the 9th day after inoculation. The pathogen was re-isolated from symptomic stems on *Phytophthora*-selective medium as described above. Treatments consisted of three replicate pots, and the experiments were repeated three times.

Results

Taxonomy

Phytophthora chrysanthemi Naher M, Watanabe, H., Chikuo, Y., & Kageyama K. sp. nov.

Etymology

'Chrysanthemum', referring to the original host plant of this pathogen

Latin description

Hypha sympodice ramosa, usque ad 7.3 μm diam.; dilatatione hyphae plerumque laterali, intercalari, copiosa in agaro aquaque, elongata, irregulari et globosa, usque ad 67 μm diam.; chlamydospora globosa, terminali, laterali vel intercalari, interdum abunda in mycelio, formata in dies 10–15 in agaro aquaque; pariete paulo tenui (1–2 μm crasso), 27–46 μm (in medio 39.3± 4.9 μm) diam.; sporangio pauculo in agaro, plurimo in aqua, fere terminali, saepe sporangiophoro sympodiali, non caduco, non papillari ellipsoideo, ovato vel pyriformi, 24–59 μm (in medio 42.2±8 μm) longo, 16–43 μm (in medio 27.9±6.1 μm) lato, interim atque interim prolifero cum sporangiis 3–8, cum exitu zoosporarum 6–14 μm lato; homothallica; oogonio terminali, saepe laterali et intercalari, laevi, fere globoso vel subgloboso, basaliter infundibuliformi, 26–46 μm (in medio 38.6±3.4 μm) diam., fulvo; oospora aplerotica, globosa, 19–35 μm (in medio 29.4±3.98 μm) diam., pariete 2–5 μm (in medio 3.4±0.6 μm) crasso, fulva; antheridio fere terminali, raro intercalari, fere paragyno, saepe amphigyno, doliiformi vel clavato, 12–24 μm (in medio 19±5.9 μm) longo, 7–19 μm (in medio 12.1± 3.9 μm) lato.

Holotype

Japan, Gifu, Minokamo (36°N, 137°E), from root rot of potting chrysanthemum (*Chrysanthemum* X *morifolium*), 2000, collector H. Watanabe. Isolate NBRC 104917-holotypus (deep-freezing). Ex-type CBS 123163. DDBJ accession numbers of the ITS rDNA region, LSU rDNA, the β-tubulin, translation elongation factor 1α gene (EF-1α), Heat shock protein 90 and 60 S Ribosomal protein L10 sequences are AB437135, AB465349, AB511995, AB511925, AB511987, and AB511926, respectively.

Morphological characteristics

Hypha is sympodially branched and main hypha is up to 7.3 μm in diameter. Mostly lateral, intercalary hyphal swelling is abundantly produced both in agar medium and in water culture. Hyphal swellings are elongated, irregular to globose (Fig. 1b, c), and usually large (up to 67 μm). Spherical chlamydospore is terminal, lateral or intercalary, and sometimes abundant on mycelium (Fig. 1d, e), formed within 10–15 days in V8A medium or water culture. The thin-walled (1–2 μm) chlamydospore measures 27–46 μm (average 39.3±4.9 μm) in diameter. Sporangium is formed rarely on solid agar, but profuse in water culture when mycelial plug from V8A medium is incubated for 24 h. Sporangium is mainly terminal (Fig. 1f–k), and sympodial sporangiophore is occasionally formed in water culture (Fig. 1h). Sporangium is noncaducous, nonpapillate, usually ellipsoid, ovoid or pyriform (Fig. 1f–j). Average length and width of sporangia is $42.2 \pm 8 \times 27.9 \pm 6.1\,\mu$m (overall range 24–59×16–43 μm), and the length/width ratio is 1.4. Sporangium proliferates extended and nested or internally with 3–8 sporangia (Fig. 1j, k). Zoospores are discharged through an exit pore 6–14 μm wide. The isolate is homothallic; oogonia are abundantly produced even in a single-zoospore isolate on V8A medium after 10 days. Oogonium is terminal, often laterally sessile and intercalary (Fig. 2a–c) with smooth walls; mostly spherical or nearly spherical, and occasionally possessing a distinct funnel-shaped stalk (Fig. 2d, e); oogonium is 26–46 μm in diameter (average 38.6±3.4 μm) and acquires a golden brown color with aging. Oospore is aplerotic, spherical and average 29.4±3.98 μm in diameter, ranging from 19 to 35 μm. The thick oospore wall is about 2–5 μm (average 3.4±0.6 μm), often turning golden brown with age. Antheridium is mostly terminal and occasionally intercalary; predominantly paragynous but sometimes amphigynous (Fig. 2g–i). It is barrel-or club-shaped to irregular, and dimensions range from 12–24×7–19 μm (average $19 \pm 5.9 \times 12.1 \pm 3.9\,\mu$m).

Colony grows moderately slow in V8A medium. Radial growth averages 4.1 mm per day at the optimum temperature of 30°C on V8A. The maximum temperature is 35°C and no growth occurs at 5°C (Fig. 3). Colony is radiate with cottony aerial mycelia on PDA at 20°C in darkness. On V8A, colony is submerged, radiate and exhibit slightly mosaic patterns (Fig. 1a).

Phylogenetic analysis

In the combined dataset of LSU rDNA, translation elongation factor 1α (EF-1α), β-tubulin, 60 S Ribosomal protein L10, and heat shock protein 90, the aligned data matrix of 76 taxa consisted of 2,991 characters, of which 964 were variable and 703 were phylogenetically informative for parsimony analysis. The MP analyses using PAUP* generated 18 equally parsimonious trees with 3,656 steps (CI=0.3714, RI=0.6286, HI=0.6286 and RC=0.2631). Although slight differences in the branching pattern of the terminal branches and in branch length were observed, tree topologies were generally consistent among all 18 trees (data not shown). The topology of the tree generated by the Bayesian phylogenetic analysis (Fig. 4) was similar to that of the MP and ML trees. As shown in Fig. 4, the 76 taxa were divided into 10 clades.

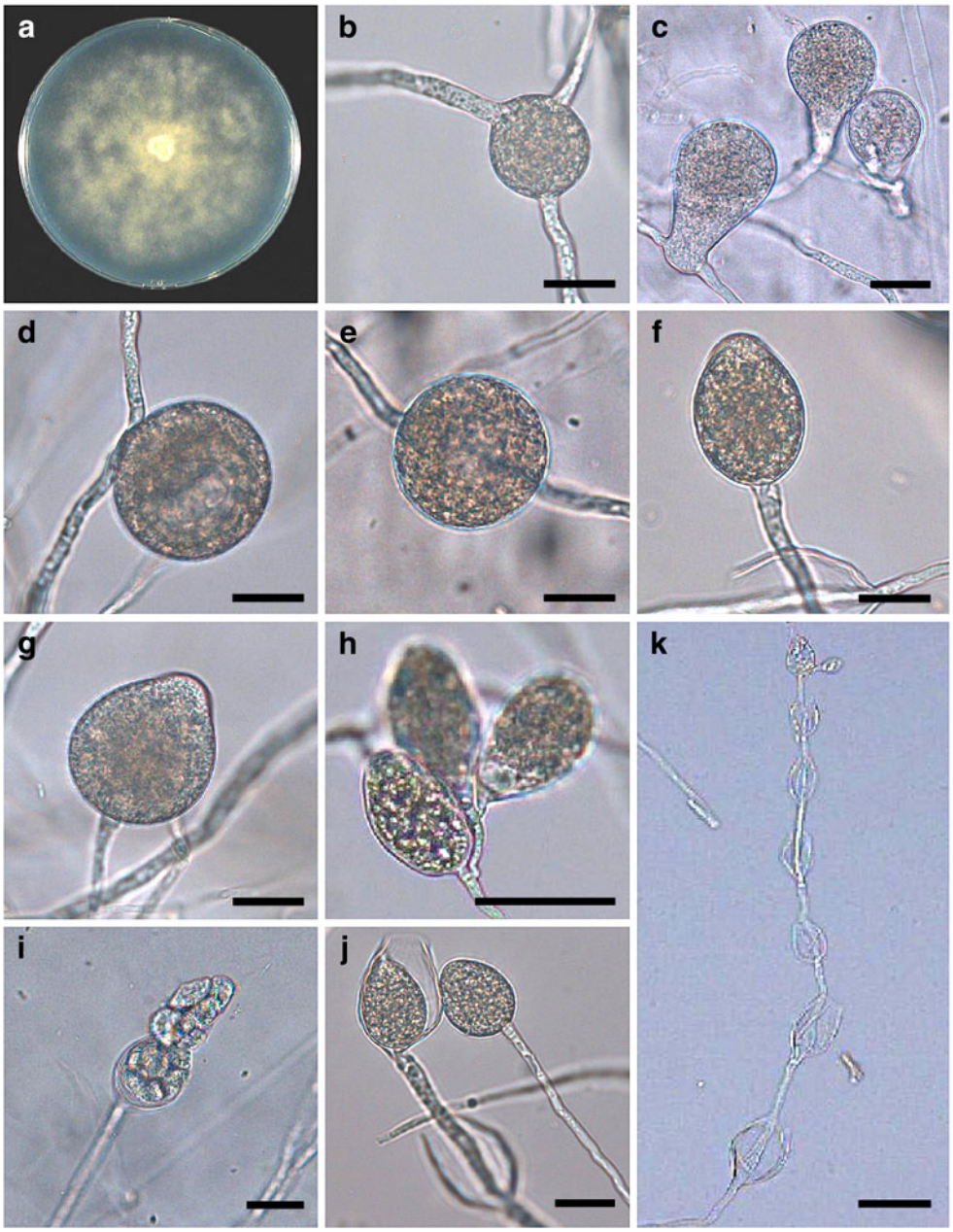

Fig. 1 Colony pattern and morphology of asexual structures of chrysanthemum isolate NBRC 104917. **a** Colony pattern on V8 juice agar medium. **b** Globose hyphal swelling in water. **c** Elongated to irregular hyphal swelling. **d,e** Lateral and intercalary chlamydospores. **f,g** Ellipsoid and pyriform nonpapillate sporangia. **h** Simple sympodial sporangiophore branching with ovate sporangia. **i** Zoospore discharge. **j** Extended and nested proliferation of ovoid sporangium. **k** Internal proliferation of sporangia. *Bars* 20 μm

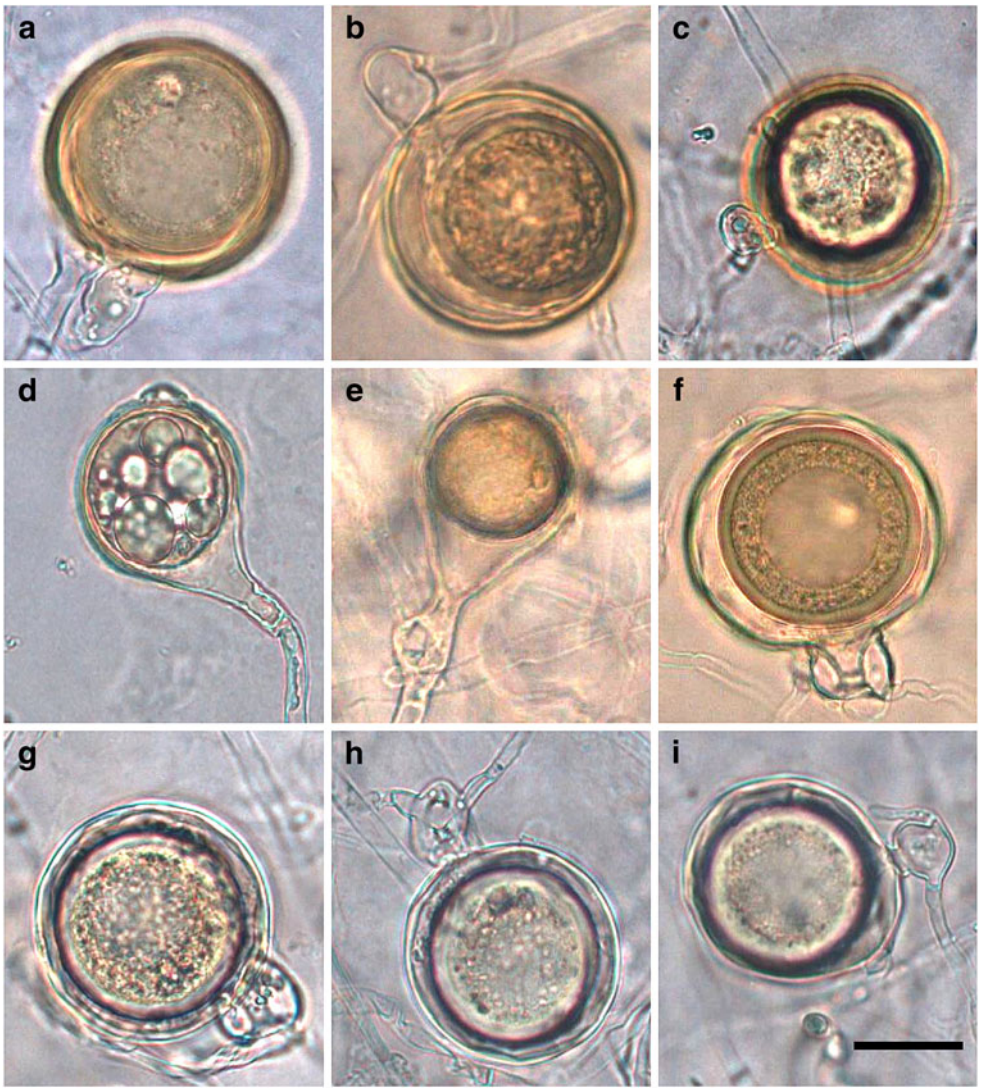

Fig. 2 Morphology of sexual structures in chrysanthemum isolate NBRC 104917. **a–i** Spherical smooth oogonium and aplerotic oospore. **a–c** Terminal, lateral and intercalary spherical oogonium with paragynous antheridia. **d,e** Funnel-shaped oogonia. **f–i** Amphigynous antheridia with clearly showing aplerotic oospore. **i** Intercalary paragynous antheridia. *Bars* 20 μm

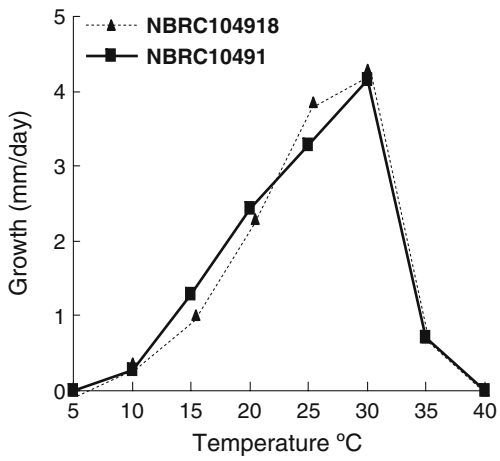

Fig. 3 Growth rates at different temperature of the chrysanthemum isolates, Gifu (*NBRC 104917*) and Toyama (*NBRC 104918*), on V8 juice agar (n=3)

In this study, the tree topology obtained by the Bayesian method resembled that reported by Blair et al. (2008) with respect to clades 1 to 8. However, clade 9 as classified by Blair et al. (2008) was divided into two clades in our tree. The Gifu (NBRC 104917, GF753) and Toyama (NBRC 104918, Chr5) isolates formed a monophyletic clade which was well supported with 99.8–100% in BS, 1.00 in the Bayesian posterior probabilities (PP), and 59 in DI. Moreover, this monophyletic clade is closely related to *P. parsiana* (Mostowfizadeh-Ghalamfarsa et al. 2008).

For the ITS dataset, the aligned data matrix of 16 taxa belonging to clade 9 and 10 consisted of 426 characters, of which 153 characters were variable and 110 characters were phylogenetically informative for parsimony analysis. The MP analyses using PAUP* generated only one parsimonious tree with 291 steps (CI=0.7010, RI=0.7723, HI=0.2990 and RC=0.3926). The tree topology in the Bayesian tree was similar to the ML and MP trees

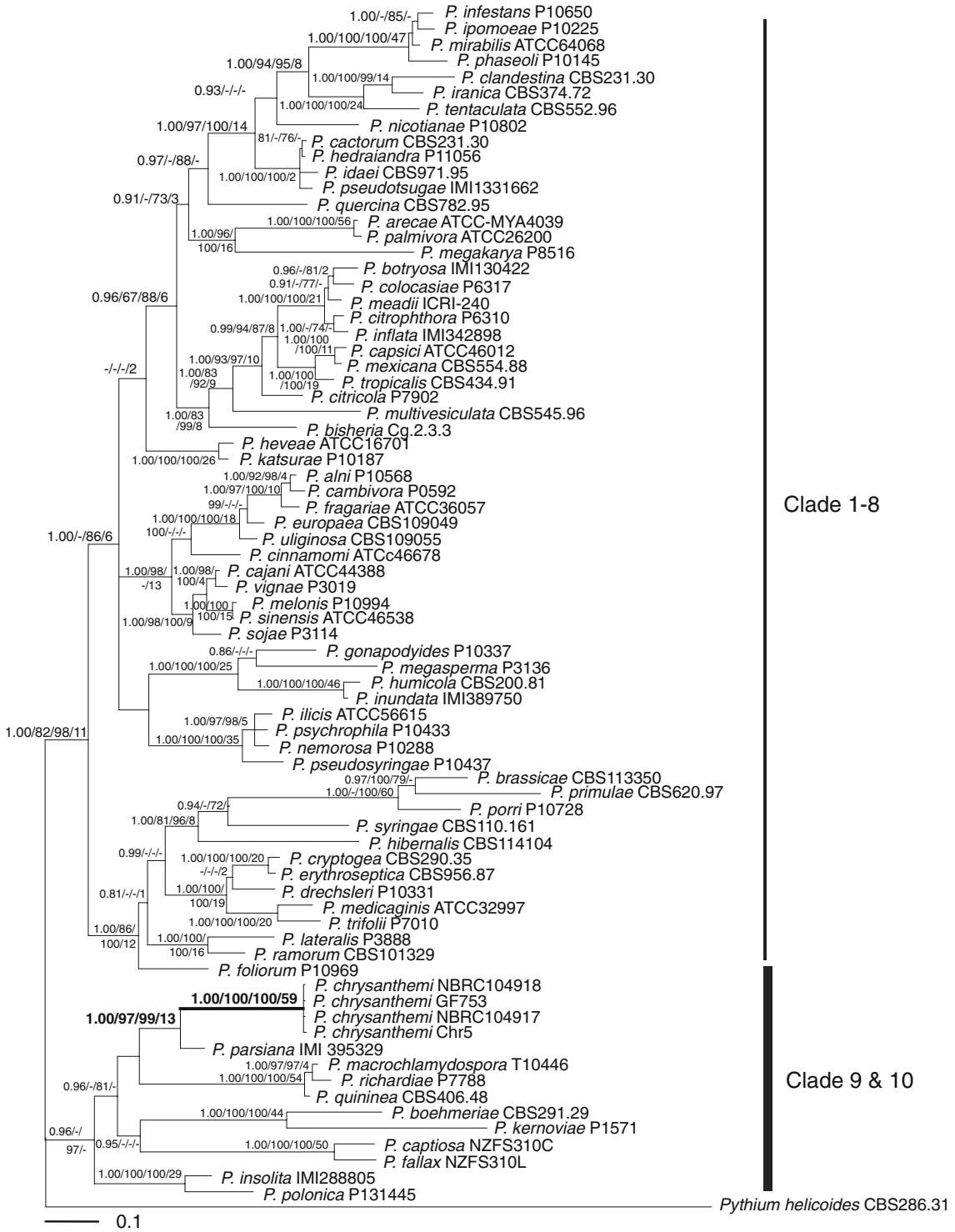

Fig. 4 Phylogenetic tree showing the relationship of *Phytophthora chrysanthemi* within the genus *Phytophthora* based on five nuclear sequences (LSU rDNA, translation elongation factor 1α , β-tubulin, 60 S ribosomal protein L10, heat shock protein 90) inferred by Bayesian analysis. Nodes of support values are shown above and below. Node support given for >0.80 Bayesian posterior probabilities, >70 maximum-parsimony bootstrap values and maximum livelihood bootstrap values, and >2 decay index

(Fig. 5). The isolates of NBRC 104917, GF753, NBRC 104918, and Chr5 were closely related to *P. parsiana* in trees produced by all methods and were members of a monophyletic clade. This monophyletic clade was well supported with 99.1–100% in BS, 1.00 in the Bayesian PP, and 13 in the DI. The final alignments and trees were

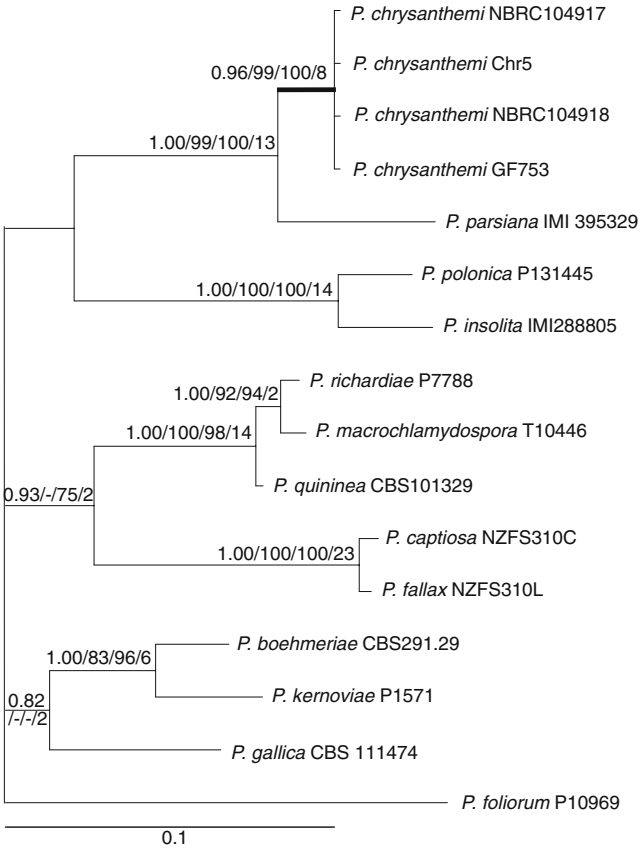

Fig. 5 Phylogenetic tree inferred by Bayesian analysis based on the ITS region of rDNA (Cooke et al. 2000) of the taxa in the *Phytophthora* major clades 9 and 10 of the phylogenetic analysis based on the 5 nuclear regions. Nodes of support values are shown above and below. Node support given for >0.80 Bayesian posterior probabilities, >70 maximum-parsimony bootstrap values and maximum livelihood bootstrap values, and >2 decay index

deposited in TreeBASE (http://www.treebase.org) and are available under the study accession number SN4874.

Pathogenicity test

Chrysanthemum seedlings inoculated with the isolate NBRC 104917 developed lesions on stems and roots at 3 days after inoculation, and the symptoms resembled the ones originally reported. Leaves were classified as diseased when rotting of the basal and central part of the stem reached to the petioles. Infected portions became black and wilted, followed by death of the plants. However, virulence differed between chrysanthemum cultivars. On day 6, chrysanthemum cv. Lompoc developed more severe symptoms (stem rot extended up to the petioles of 4.0 leaves on average) than cv. Dark Bizarria and Miramar (stem rot extended up to the petioles of 1.7 and 2.0 leaves on average; Fig. 6). Similarly, on day 9, severe symptoms extended to the petioles of 10.0 leaves in cv. Lompoc, whereas only 7.0 and 4.7 leaves were affected in cv. Dark Bizarria and Miramar, respectively. The isolate was re-isolated from lesions of infected plants, satisfying Koch's postulates. Control plant inoculated with uninfested V8A disks remained healthy.

Discussion

The new species isolated from stem and root rot of chrysanthemum is morphologically distinct and described here as *Phytophthora chrysanthemi*. Furthermore, chrysanthemum isolates from Gifu and Toyama prefectures were identical, although the isolation procedures were different. As the isolates are homothallic and form nonpapillate, noncaducous sporangia with internal proliferation and paragynous antheridia, they belong to group V of the morphological classification by Waterhouse (1963). The species is characterized by the formation of hyphal swellings and chlamydospores, internal proliferation with 8–9 sporangia, distinctive intercalary antheridia, and funnel-shaped oogonia. It is a high temperature species as the isolates can grow even at 35°C.

Phylogenetic relationships in the genus *Phytophthora* were investigated using MP, ML, and Bayesian phylogenetic analyses. Trees constructed based on five nuclear regions (LSU rDNA, EF-1α, β-tubulin, 60 S ribosomal protein L10 and heat shock protein 90) validated the results of morphological comparison and strongly supported the chrysanthemum isolates as a new species, as they formed a monophyletic clade.

In the multi-locus phylogenetic tree, the chrysanthemum isolates were placed together with *P. parsiana* in the basal clade 9 as designated by Blair et al. (2008). Many newly described *Phytophthora* species have been placed in these

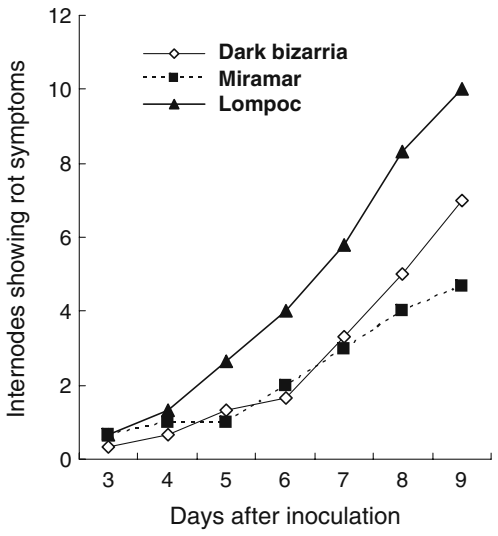

Fig. 6 Development of symptoms caused by chrysanthemum isolate NBRC 104917 in three chrysanthemum cultivars as determined by the extension of stem rot

unresolved, basal clades that appear to be outside the main radiation of the genus (Belbahri et al. 2006; Brasier et al. 2005; Dick et al. 2006). Cooke et al. (2000) reported only three species in clade 9 and 10 (*P. macrochlamydospora, P. richardiae* and *P. insolita*); since then, 10 new *Phytophthora* species have been added to these clades (Blair et al. 2008).

The rDNA ITS region shows a high resolution and has proven particularly useful for the separation of fungal taxa at the species level (Bruns et al. 1991; Lee and Taylor 1992) and of intra-isolate variation (Matsumoto et al. 2000, Kageyama et al. 2007). However, it was difficult to align the sequences for the phylogenetic analysis, since this region has variable lengths and sites. As we used only species from the closely related clades 9 and 10, we achieved a reproducible and reliable alignment. In conclusion, our analysis revealed the monophyletic clade of *P. chrysanthemi* within clade 9.

Phytophthora chrysanthemi is a distinct species morphologically related to the high temperature species in Waterhouse's group V, *P. insolita*, *P. humicola*, and *P. polonica*. *Phytophthora chrysanthemi* is quite different from *P. insolita* which forms oogonia without any antheridia (Ann and Ko 1980). *Phytophthora chrysanthemi* forms chlamydospores, while *P. humicola* (Ko and Ann 1985) does not. Although we initially considered the isolates morphologically similar to the recently described *P. polonica* (Belbahri et al. 2006) in group V, this interpretation was eliminated because *P. polonica* does not produce sympodial sporangiophores, external proliferation of sporangia, and intercalary paragynous antheridia. Moreover, *P. polonica* forms only a few sporangia in gelatin solution, while *P. chrysanthemi* forms numerous sporangia within a few days in water culture. The phylogenetically related *P. parsiana* is high temperature tolerant like *P. chrysanthemum*, and also forms nonpapillate, noncaducous, and proliferating sporangia.

The existence of 10 clades within the genus *Phytophthora* is supported by several phylogenetic studies (Cooke et al. 2000; Blair et al. 2008; Donahoo et al. 2006; Ivors et al. 2004; Kroon et al. 2004; Martin and Tooley 2003a, b). In this study, we analyzed the isolates from chrysanthemum phylogenetically using six nuclear regions each of which formed a similar phylogenetic tree exhibiting the 10 clades (data not shown). However, the genes for the 60 S ribosomal protein L10 and heat shock protein 90 were very difficult to amplify in some species because of the lack of universal primer sets, while rDNA ITS, LSU rDNA, EF-1α, and β-tubulin proved highly suitable for amplification and sequencing. Further studies are necessary to select target genes for an even more detailed phylogenetic analysis.

In pathogenicity tests, three cultivars of chrysanthemum inoculated with the isolate developed lesions on stems and roots within 3 days after inoculation, and these symptoms were similar to the original ones. Moreover, re-isolation from lesions of infected plants satisfied Koch's postulates. In Japan, a chrysanthemum disease caused by *P. cactorum* was first reported in Tokyo in 1972 (Kagiwata 1972). *P. cactorum* causes stem rot and blight in potting chrysanthemum, whereas *P. chrysanthemi* causes stem and root rot in cutting and potting chrysanthemum. Optimum growth temperatures are 25 and 30°C in *P. cactorum* and *P. chrysanthemi*, respectively. *P. cactorum*-induced symptoms occur mainly in May, June, September and October, while *P. chrysanthemi* effects show in July and August. There is no report of chrysanthemum diseases caused by *Phytophthora* species at high temperatures in chrysanthemums grown hydroponically in ebb and flow irrigation systems.

The primary inoculum source of root rot caused by *P. chrysanthemi* is still unknown, but there are two obvious possibilities. In Japan, chrysanthemum plants are commonly grown in peat moss medium which is imported from other countries. The pathogen may have been introduced with this medium and, being adapted to high temperatures, may have thrived in the warm Japanese climate. Another possibility is that the species is indigenous and lives as a saprophyte in the natural environment. Once the species enters hydrophonic culture systems with recycling nutrient solution, it will encounter ideal conditions for mass proliferation and enhanced virulence and transmission rates, which ultimately will result in widespread occurrences of the disease symptoms. Further research is needed to identify the primary source and transmission mechanisms of *P. chrysanthemi*.

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